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CONTROL OF PHENOLIC OXIDATION IN THE IN VITRO CULTURE OF *Astronium urundeuva* (M. Allemão) Engl.

CONTROLE DA OXIDAÇÃO FENÓLICA NO CULTIVO IN VITRO DE Astronium urundeuva (M. Allemão) Engl.

CONTROL DE LA OXIDACIÓN FENÓLICA EN EL CULTIVO IN VITRO DE Astronium urundeuva (M. Allemão) Engl.Engl.

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PALABRAS CLAVE: Anacardiaceae; *Aroeira-do-sertão; Brotes juveniles; Antioxidante.*

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ABSTRACT

Astronium urundeuva (Aroeira-do-sertão) is a Brazilian native species with silvicultural potential for logging. In the in vitro cultivation of the species, difficulties such as phenolic oxidation have been observed, mainly from explants collected from adult matrix trees. Thus, the explants originating from seeds are an alternative for the propagation of the species to minimize oxidation. In this context, the study aimed to evaluate the action of ascorbic acid, activated charcoal, and polyvinylpyrrolidone in controlling phenolic oxidation in the in vitro culture of A. urundeuva. Apical shoots obtained from seedlings of A. urundeuva germinated in vitro were used as explants. They were cultivated in MS50% culture medium plus ascorbic acid (0.2; 0.4; 0.6, and 0.8 mg.L-1) and activated charcoal (3.0; 6.0; 9.0, and 12 g.L-1) and polyvinylpyrrolidone (0.5; 1.0; 1.5 and 2.0 g.L-1). After in vitro culture, ascorbic acid, activated charcoal, and PVP at concentrations of 0.2 mg.L-1, 12 g.L-1 and 1.5 g.L-1, respectively, were effective in controlling phenolic oxidation. At this concentration, activated charcoal completely regulated the phenolic oxidation of the seedlings. The results of this work demonstrate the feasibility of antioxidants in minimizing the effects of phenolic oxidation, especially with the use of activated charcoal, and open perspectives for further studies of micropropagation of A. urundeuva, both in juvenile and adult genetic materials, contributing to its conservation and assisting in the genetic improvement of the species.

RESUMO

Astronium urundeuva (aroeira-do-sertão) é uma das espécies nativas com potenciais silviculturais para exploração madeireira. No cultivo in vitro da espécie tem-se observado dificuldades como a oxidação fenólica, principalmente a partir de explantes coletados de árvores matrizes adultas. Dessa forma, a utilização de explantes originários de sementes é uma alternativa para a propagação da espécie, afim de minimizar a oxidação. Neste contexto, o objetivo do estudo foi avaliar a ação de ácido ascórbico, carvão ativado e polivinilpirrolidina no controle da oxidação fenólica no cultivo in vitro de A. urundeuva. Brotações apicais obtidas a partir de plântulas de A. urundeuva germinadas in vitro foram utilizadas como explantes. Estes foram subcultivados em meio de cultura MS50%, acrescido dos antioxidantes ácido ascórbico (0,2; 0,4; 0,6 e 0,8 mg.L^{-1}) carvão ativado (3,0; 6,0; 9,0 e 12 g.L^{-1}) e polivinilpirrolidona (0,5; 1,0; 1,5 e 2,0 g.L^{-1}). Apos o cultivo in vitro, o ácido ascórbico, carvão ativado e PVP nas concentrações de 0,2 mg.L^{-1},12 g.L^{-1} e 1,5 g.L^{-1} respectivamente, foram efetivos no controle de oxidação fenólica. O carvão ativado nesta concentração controlou totalmente a oxidação fenólica das plântulas. Os resultados deste trabalho demonstram a viabilidade dos antioxidantes na minimização dos efeitos da oxidação fenólica, especialmente com o uso do carvão ativado e abre perspectivas para maiores estudos de micropropagação de A.urundeuva, tanto de materiais genéticos juvenis quanto adultos, contribuindo para a sua conservação e auxiliando em trabalhos de melhoramento genético da espécie.

RESUMEN

Astronium urundeuva (aroeira-do-sertão) es una de las especies nativas con potencial silvícola para la tala. Se han observado dificultades en el cultivo in vitro de la especie, como la oxidación fenólica, principalmente a partir de explantes recolectados de árboles madre adultos. Así, el uso de explantes provenientes de semillas es una alternativa para la propagación de la especie, con el fin de minimizar la oxidación. En este contexto, el objetivo del estudio fue evaluar la acción del ácido ascórbico, carbón activado y polivinilpirrolidina en el control de la oxidación fenólica en el cultivo in vitro de A. urundeuva. Como explantes se utilizaron brotes apicales obtenidos de plántulas de A. urundeuva germinadas in vitro. Estos fueron subcultivados en medio de cultivo MS50%, más los antioxidantes ácido ascórbico (0,2; 0,4; 0,6 y 0,8 mg.L-1), carbón activado (3,0; 6,0; 9,0 y 12 g.L-1) y polivinilpirrolidona (0,5; 1,0 ;1.5 y 2.0 g.L-1). Después de cultivo in vitro, el ácido ascórbico, el carbón activado y la PVP en concentraciones de 0,2 mg.L-1,12 g.L-1 y 1,5 g.L-1, respectivamente, fueron efectivos en el control de la oxidación fenólica. El carbón activado a esta concentración controló completamente la oxidación fenólica de las plántulas. Los resultados de este trabajo demuestran la viabilidad de los antioxidantes para minimizar los efectos de la oxidación fenólica, especialmente con el uso de carbón activado, y abren perspectivas para futuros estudios de micropropagación de A. urundeuva, tanto a partir de materiales genéticos juveniles como adultos, contribuyendo a su conservación y asistencia en trabajos de mejoramiento genético de la especie.

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1. INTRODUCTION

The "*Aroeira-do-Sertão*" (*Astronium urundeuva* (M. Allemão) Engl.) is a Brazilian native tree species of the Anacardiaceae family, with geographical distribution in the North, Northeast, Midwest and South regions, being found in the Caatinga, Cerrado, Atlantic Forest, Pampa and Pantanal phytogeographic domains (Silva-Luz & Pirani, 2014). The *aroeira* is economically important due to its wood of great added value, pharmacological properties and also honey obtained from its flowering (Vieira et al., 2016; Demier, 2018). Despite of its multiple uses and the economic interest around the species, the exploitation still occurs in a solely extractive way, which resulted in a severe reduction in natural populations (Monteiro et al., 2006; Pacheco et al., 2006). As a result, *A. unrudeuva* was included in previous editions of the official Brazilian list of endangered species (Brasil, 2008). Thus, studies on its spread are necessary in order to ensure long-term conservation and sustainable exploitation strategies of this species.

The commercial production of seedlings of *A. unrudeuva* is carried out exclusively by seminiferous route, but the use of asexual propagation techniques is an important tool for the maintenance of desired genotypes and the obtaining of sound plants (Oliveira et al., 2013). In this context, micropropagation is a potential tool that would help and/or replace the propagation methods already used, since in vitro cultivation allows the production of seedlings (Hung et al., 2016; Isah, 2016). For micropropagation to be successful, it depends on factors inherent to plant tissue (Borges et al., 2012; Schuch et al., 2008;), as well as culture medium, growth regulators and in vitro microenvironment conditions (Bassan et al., 2006; Golle et al., 2012; Jardim et al., 2010). Therefore, for the development of micropropagation protocols for a given species, it is necessary to first establish in vitro cultures.

The introduction of in vitro cultures is often a challenging step in micropropagation, especially for native and woody species that present phenolic oxidation and fungal and/or bacterial contamination (Alfenas et al., 2009; Yoshiko & Teixeira, 2001). Phenolic compounds are substances that are part of the secondary metabolism of the plant and usually serve to protect plant tissues against injury, insects and animal attack (Vizzotto et al., 2010). By chemical definition, phenolic compounds are substances that have at least one aromatic ring in which at least one hydrogen is replaced by a hydroxyl group. These compounds are synthesized from the metabolic routes of chichemical acid and mevalonic acid, the latter being less significant (Vizzotto et al., 2010). In *in vitro* cultivation, the damage caused to cells during the excision of vegetative propagules stimulates the oxidation process of phenolic substances resulting in darkening of the culture medium and death of the explant (Ahmad et al., 2013; Cassells & Curry, 2010). Some methods to reduce phenolic oxidation can be used, including the use of antioxidant substances, the reduction of mechanical and chemical damage, the washing of vegetative propagules in running water, the use of more diluted basic media, and the removal of phenolic substances, among others (Xavier et al., 2009).

Antioxidants act on the inactivation of free radicals, in the complexation of metabolic ions or in the reduction of peroxides for products unable to form free radicals with the potential to

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oxidize (Araújo, 1985). In micropropagation, among the substances added to the culture medium with antioxidant function we have ascorbic acid, polyvinylpyrrolidone (PVP) and active charcoal (Lal et al., 2021). These substances may act by inhibiting the synthesis or activity of enzymes linked to the oxidation of polyphenols or acting as adsorbents of these substances.

In view of the above, this work aims to determine the best concentrations of activated carbon, ascorbic acid and PVP for the control of phenolic oxidation in the in vitro establishment phase of *A. unrudeuva*.

2. MATERIAL AND METHODS

2.1. PLANT MATERIAL

The experiment was conducted at the Laboratory of Forest Improvement (Laboratório de Melhoramento Florestal), located at the Center for Research in Agrarian Sciences (Centro de Pesquisa em Ciências Agrárias - CPCA) of the Institute of Agrarian Sciences of the Federal University of Minas Gerais (Instituto de Ciências Agrárias da Universidade Federal de Minas Gerais - ICA/UFMG), in the county of Montes Claros, Minas Gerais (latitude 16º40'59.7'' S, longitude 43º50'21,9'' W, altitude 680 m). The species was identified by means of comparison with sample of fallen plant in the “Herbário Norte Mineiro- MCCA”, registry number: MCCA 1517.

2.2. IN VITRO SEED DISINFESTATION AND GERMINATION

The diaspores of *A. unrudeuva* collected in September, from 21 matrices located in a cerrado fragment located in the county of Montes Claros/MG, were sent to the laboratory for manual processing, which consisted of the removal of the calyxes from the diaspores and the selection of those that did not visually present empty seed fruit (absence of tissues), physical damage and/or damage caused by insects. At asepsis, the selected diaspores were placed in running water for 10 minutes and then immersed in a solution of sodium hypochlorite (NaClO) at 2.0 - 2.5% (v/v) of active chlorine for 15 minutes. At the end of the asepsis, they were rinsed with deionized and autocled water three times and inoculated vertically in test tubes (2 x 10 cm), containing 5.0 mL of the MS culture medium (Murashige & Skoog, 1962) with half the concentration of salts (MS 50%), supplemented with sucrose (30 g L-1) and agar (6 g L-1). The pH of the culture medium was adjusted to 5.8 and its sterilization was chemically performed with polybac 7DC biocide (2 ml L-1). After inoculation, the explants were kept in a growth room under 16/8 hours photoperiod control (light and dark) and temperature of 27 ± 2 ° C.

2.3. PHENOLIC OXIDATION CONTROL

Seedlings at 25 days of age, counted from the emergence of the radicle, were used as a source of explants. The root was excised from the seedlings and only the aerial part was used as explant and inoculated in test tubes (2 x 10 cm), containing 5.0 mL of the MS culture medium with half the salt concentration, supplemented with sucrose (30 g L-1) and agar (6 g L-1). After inoculation, the explants were kept in a growth room under 16/8 hours photoperiod control (light and dark) and temperature of 27 ± 2 °C.

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Different concentrations of three antioxidant agents were added separately to the basic culture medium of in vitro multiplication of *A. unrudeuva* (Table 1).

Table 1. Description of the concentrations of antioxidants ascorbic acid (AA), activated carbon (AC) and polyvinylpyrrolilene (PVP) aiming at the control of phenolic oxidation in the in vitro multiplication of *A. unrudeuva*.

Treatment	Ascorbic Acid (mg/L)	Activated Carbon (g/L)	Polyvinylpyrrolide (g/L)
1	0.2	-	-
2	0.4	-	-
3	0.6	-	-
4	0.8	-	-
5	-	3.0	-
6	-	6.0	-
7	-	9.0	-
8	-	12.0	-
9	-	-	0.5
10	-	-	1.0
11	-	-	1.5
12	-	-	2.0

Source: Authors (2022).

30 days after the beginning of the experiment, the number of shoots and survival of the explants was evaluated. In the evaluation, surviving explants were considered those without any physiological disturbances and symptom necrosis of the aerial part.

The experiment was conducted in a completely randomized design, containing 12 treatments, each composed of 16 replications. The data measured were analyzed for their normality and homogeneity ($P > 0.05$), respectively, by the Shapiro-Wilk and Bartlett test. Then the data were submitted to a polynomial regression analysis ($P < 0.05$). Statistical analyses were performed using the package ExpDes.pt of the statistical software RStudio®, version 3.5.3.

3. RESULTS AND DISCUSSIONS

The micropropagation of forest species from mature material, collected from selected matrices trees is one of the means of propagation of superior genotypes for a given purpose, especially those derived from breeding programs (Silva et al., 2021). However, for native forest species there is a huge lack of studies on the in vitro propagation of adult genotypes.

Therefore, research within this theme is essential to overcome obstacles to the expansion of native forestry in Brazil. Initially preliminary studies were conducted with epiormic shoots from adult matrices of *A. unrudeuva*. In the in vitro cultivation of these shoots, there was intense oxidation of the medium and explants in MS medium half strength, with its death after 2 days of inoculation (Figure 1). In this context, due to the scarcity of information in the literature and the obstacles in the micropropagation of explants of adult material, it was decided to investigate the effect of antioxidants on the in vitro multiplication of seminal explants of *A. unrudeuva*.

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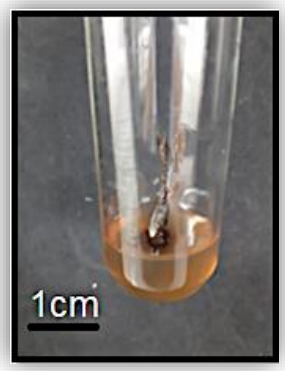
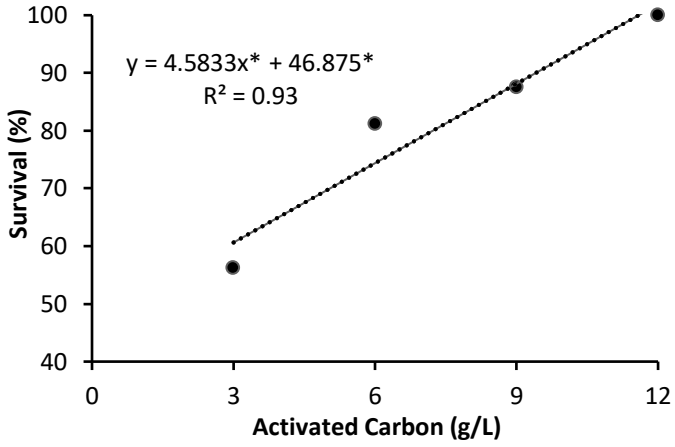
Figure 1. Phenolic oxidation of epiormic shoots of *A. unrudeuva* after 2 days of inoculation in MS 50% culture medium, supplemented with antioxidants. Bar = 1 cm.



Source: Authors (2022).

The use of activated charcoal as an antioxidant agent in the in vitro multiplication of *Astronium unrudeuva* was efficient in the control of phenolic oxidation. There was a drastic reduction in phenolic oxidation with the increase of activated carbon concentrations used, obtaining 100% survival of the explants at the concentration of 12 g L-1 (Figure 2). Moreover, the fact that explants are of seminal origin may have contributed to the minimization of phenolic oxidation. The metabolic route of chiquimate, responsible for the production of phenols in plants, is probably activated with greater intensity in mature tree tissues (Oliveira et al., 2013). In juvenile tissues, the cells have not yet undergone such a pronounced differentiation process, with gene expression responsible for the activation of the metabolic routes of phenol production and, consequently, will present minor problems regarding phenolic oxidation.

Figure 2. Percentage of surviving explants (%) after 30 days of in vitro cultivation in MS 50% culture medium, supplemented with different concentrations of activated carbon. *significant at the 5% level.



Source: Authors (2022).

The shoots of *A. unrudeuva* developed in the culture medium supplemented with activated charcoal showed adequate vegetative vigor, without apparent morphophysiological abnormalities (Figure 3), a result similar to that found for *Swietenia macrophylla* King (Lameira et al., 2001), *Sequoia sempervirens* (D. Don) Endl. (Meneguzzi et al., 2020) and *Khaya ivorensis* A. Chev. (Azevedo, 2018). On the other hand, despite the efficiency of activated charcoal in

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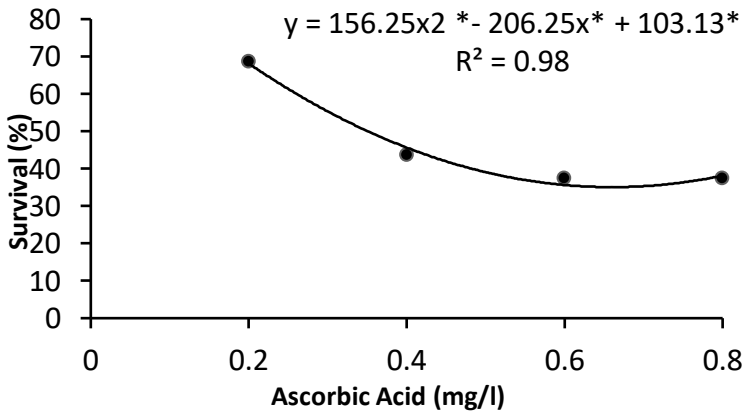
the control of oxidation, high concentrations of it in the culture medium may be a hindrance. These results came from the fact that activated charcoal acts as adsorbent in the culture medium, causing both phenolic substances and components in the medium to be adsorbed, such as nutrients and regulators (Lencina et al., 2018). As a result, in vitro cultivation may cause nutritional deficiencies in explants (Meneghetti, 2020). It is then recommended to subculture the shoots to a new culture medium in a maximum time of 30 days, in order to minimize possible effects on shoots with the use of high concentrations of activated carbon.

Figure 3. Visual aspect of sprouting of *A. unrudeuva* presenting adequate physiological vigor in the medium of culture MS half strength, supplemented with 12 g L-1 of activated carbon, after 30 days of in vitro cultivation. Bar = 1 cm.

Source: Authors (2022).

Ascorbic acid acts to control phenolic oxidation by inactivation of oxygen radicals. These radicals are released by the explants and potentiated in the presence of light. When cutting the segment, ascorbic acid acts in the inhibition of the radicals released in the caused injury (Verde et al., 2021). In the in vitro multiplication of *A. unrudeuva*, there was a decrease in the effectiveness of the phenolic oxidation control of shoots, as the concentration of ascorbic acid was increased (Figure 4).

Figure 4. Percentage of explants (%) survivors after 30 days of in vitro cultivation in MS 50% culture medium, supplemented with different concentrations of ascorbic acid. *significant at the 5% level



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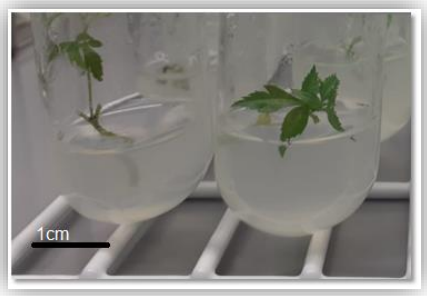
Ascorbic acid in the culture medium is absorbed by the plant and acts directly on the metabolic routes of phenol synthesis (Taiz & Zeiger, 2004). The result obtained for the in vitro multiplication of *A. unrudeuva* with the use of ascorbic acid may be linked to the fact that it is not effective as antioxidant agents of phenols released by shoots. It is also noteable that the inefficacy of ascorbic acid, high concentrations were also not efficient in Malus domestica Borkh (Erig & Schuch, 2003). *Astronium urundeuva* may be sensitive to ascorbic acid and the concentrations used may have exceeded the acceptable limit of the species, causing the death of the shoots. However, the surviving shoots at a concentration of 0.2 mg/L showed good physiological vigor (Figure 5). In addition, it is important to emphasize that shoots are of seminal origin and, therefore, present a genetic variability, providing different responses to the antioxidant and, consequently, to its survival.

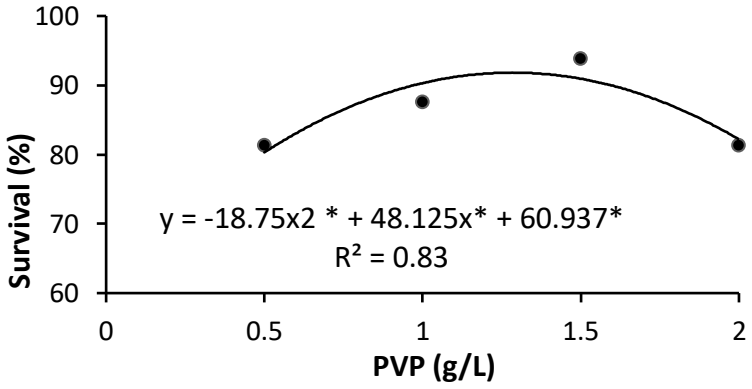
Figure 5. Visual aspect of sprouting of *A. unrudeuva* based on adequate physiological vigor in the medium of culture MS half strength, supplemented with 0.2 g L^{-1} of ascorbic acid, after 30 days of in vitro cultivation. Bar = 1 cm.



Source: Authors (2022).

PVP inhibits the release of phenolic compounds in the culture medium, acting on tissues in contact with it (Cyd &Teixeira, 2014). The phenolic oxidation of The Shoots of *A. unrudeuva* was reduced as pvp concentrations increased to the concentration of 1.5 g/L, from which there was a decrease in survival (Figure 6). The reduction of the survival of the shoots of *A. unrudeuva* with PVP at high dosages may be related to the reduction of nutrient absorption by explants, since the antioxidant forms a kind of protective and insulating layer of tissues in contact with the culture medium (Oliveira et al., 2018).

Figure 6. Percentage of surviving explants after 30 days of in vitro cultivation in medium MS half strength, supplemented with different concentrations of PVP. *significant at the 5% level



Source: Authors (2022).

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Thus, the addition of antioxidants to nodal segments favored the control of phenolic oxidation in *Astronium urundeuva* and establishing of the species in vitro, thus providing future studies with the other stages of micropropagation.

4. CONCLUSION

The antioxidants activated carbon, ascorbic acid and PVP at concentrations of 12 g/L, 0.2 mg/L and 1.5g/L, respectively, provided a greater in vitro establishment of *Astronium unrudeuva* shoots.

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