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Multiplication and *in vitro* rooting of *Handroanthus impetiginosus* (Mart. Ex DC.) Mattos

Multiplicação e enraizamento *in vitro* de *Handroanthus impetiginosus* (Mart. Ex DC.) Mattos

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Abstract

This study aimed to evaluate cytokinin influence during *in vitro* multiplication stage and activated charcoal in combination with indole-3-butyric acid (IBA) during *in vitro* rooting of *Handroanthus impetiginosus*. For *in vitro* multiplication, nodal segments of shoots obtained from *in vitro* seedlings establishment were inoculated in Woody Plant Medium (WPM), with and without the following concentrations of 6-benzylaminopurine (BAP), kinetin (KIN) and thidiazuron (TDZ): 0.5; 1.0; 2.0; 4.0 and 8.0 µmol L^{-1}. For *in vitro* rooting, shoots were inoculated in WPM containing combinations among IBA (1.0; 3.0; 6.0 and 9.0 µmol L^{-1}) and activated charcoal (1.0; 2.0 and 3.0 g L^{-1}), as well as their absence within culture medium. The highest number of shoots and buds was reached after using TDZ 8.0 µmol L^{-1}, where in the best rooting occurred after adding activated charcoal 2.0 g L^{-1}, regardless of IBA concentration. Our results show that using TDZ in a suitable level for *in vitro* multiplication stage followed by using 2.0 g L^{-1} of activated charcoal to obtain well rooted shoots is efficient for ipê-roxo micropropagation in a low cost-manner and quickly, besides providing knowledge about how to keep this threatened extinction species in an *in vitro* environment, which may help other conservation studies.

Keywords: *In vitro* propagation; Woody plant; Plant tissue culture; Ipê-roxo

Resumo

Este estudo objetivou avaliar a influência das citocininas durante o estágio de multiplicação *in vitro* e a combinação entre carvão ativado com o ácido indol-3-butírico (AIB) durante o enraizamento *in vitro* de *Handroanthus impetiginosus*. Para a multiplicação *in vitro*, os segmentos nodais dos brotos obtidos a partir do estabelecimento de plântulas *in vitro* foram inoculados em meio de cultura *Woody Plant Medium* (WPM), com e sem as seguintes concentrações de 6-benzilaminopurina (BAP), cinetina (KIN) e tidiazuron (TDZ): 0,5; 1,0; 2,0; 4,0 e 8,0 µmol L^{-1}. Para o enraizamento *in vitro*, brotos foram inoculados em meio WPM contendo combinações entre AIB (1,0; 3,0; 6,0 e 9,0 µmol L^{-1}) e carvão ativado (1,0; 2,0 e 3,0 g L^{-1}), bem como suas ausências no meio de cultura. O maior número de brotos e gemas foi alcançado após usar 8,0 µmol L^{-1} de TDZ, enquanto que o melhor enraizamento ocorreu após a adição de 2,0 g L^{-1} de carvão ativado, independente da concentração de AIB. Nossos resultados mostram que usar TDZ em níveis adequados para o estágio de multiplicação *in vitro* seguido pelo uso de 2,0 g L^{-1} de carvão ativado para obter brotos bem enraizados é eficiente para a micropropagação de ipê-roxo de maneira rápida e com baixo custo, além de fornecer conhecimento sobre como manter esta espécie ameaçada de extinção em um ambiente *in vitro* podendo ajudar outros estudos de conservação.

Palavras-chave: Propagação *in vitro*; Planta lenhosa; Cultura de tecidos vegetal; Ipê-roxo

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Introduction

Handroanthus impetiginosus (Mart. Ex DC.) Mattos, including the gender *Handroanthus* (GROSE; OLSMTEAD, 2007), popularly known as ipê-roxo, "Pau d'arco-roxo", "ipê-roxo-de-bola", among others, is a deciduous plant that belongs to Bignoniaceae family, with a tree size reaching heights between 8 and 20 m. Its occurrence extends from the state of Piauí to São Paulo, both in Atlantic Rainforest and in Semi-deciduous (LORENZI, 2002).Ipê-roxo trees are considered ornamental due to the beauty of its flowers and many places use it to landscaping in general. Its wood is used in carpentry, heavy construction and external structures, both civilian and naval (OLIVEIRA *et al*., 2008). As medicinal component, these trees have an active ingredient known as lapachol, present both in stem periderm and woody part, which has anti-inflammatory, analgesic, antibiotic and antineoplastic properties (FIORITO *et al*., 2014; HUSSAIN; GREEN, 2017). For all its uses, ipê-roxo is suffering with an intense exploitation, reducing a lot its population in native areas. Leading all these facts into account, ipê-roxo trees were inserted within the list of species for genetic *ex situ* conservation of the Forest Institute of São Paulo, in order to avoid the imminent extinction process (SIQUEIRA; NOGUEIRA, 1992).

Aiming to explore the potential of ipê-roxo species as well as to reduce its extinction risk, micropropagation has been used as a viable technique for multiplying several native species, helping in generation of homogenous plants and in production of healthy seedlings (MARTINS *et al*., 2011; LARRABURU *et al*., 2012). Among advantages of this technique may be highlighted the capability of largescale clonal plant multiplication (SOARES *et al*., 2011) to assist in degraded areas recovery (OLIVEIRA *et al*., 2008) by use propagated seedlings of native species. Some studies have already been conducted applying micropropagation technique for *Handroanthus impetiginosus* species as, for example, those carried out by Martins *et al*. (2011) and Larraburu *et al*. (2012).

Within the *in vitro* multiplication stage, cytokinins are essential growth regulators for induction of axillary bud proliferation. Type and concentration of this plant growth regulator (PGR) are some of factors that highly influence into *in vitro* propagation success of many species (BRONDANI *et al*., 2009). From number of buds per shoot as a time function, it is possible to determine the multiplication rates of the species, in order to perform experiments that provide, at the end of process, a greater number of seedlings in reduced time.

Formation of a well-developed root system is as important as multiplication stage for plants cultivated in *in vitro* environment. An *in vitro* rooting well established is essential both for shoot survival and plant growth, especially to next stages such as acclimatization and transplanting to field. For root formation, auxins are the most widely used regulators, and indole-3-butyric acid (IBA) is mostly used, since it allows a better rooting capacity and is less susceptible to biological degradation as compared to other synthetic auxins (INOCENTE; NIENOW; TRE, 2018). Activated charcoal has also been used as an alternative to induce root formation due to the dark physical environment suitable to rhizosphere (GANTAIT *et al*., 2009), in addition to adsorbing compounds from culture medium that can be harmful to explants (SÁENZ *et al*., 2010), such as phenolic compounds.

According to the context, the objective of this study was to evaluate influence of 6-benzylaminopurine (BAP), kinetin (KIN) and thidiazuron (TDZ) throughout the *in vitro* multiplication stage and auxin IBA combined with activated charcoal in *in vitro* rooting of ipê-roxo shoots.

Material and methods

In vitro establishment

Embryos extracted from ipê-roxo seeds collected in Alfenas, Minas Gerais, Brazil were used as explants. The asepsis of embryos was performed by immersion in sodium hypochlorite solution with 1.0 % ($w.v^{-1}$) NaClO for 10 minutes and in the next step they were washed with autoclaved distilled water for three times. After disinfection, explants were inoculated into 600 mL polyethylene terephthalate (PET) bottles containing 50 mL culture medium, as described by Martins *et al.* (2011), with modifications. The bottles were sealed with cotton plugs and adherent polyvinyl chloride (PVC) film. The plant material was kept in a growth room at 25 ± 2°C under continuous white-cold light and irradiance of 43 μmol m^{-2} s^{-1}.

In vitro multiplication stage

Nodal segments (around 1.0 cm high) were excised from plants at least 30 days-old previously established *in vitro* and inoculated into 24 x 150 mm test tubes containing 10 mL of the culture medium Woody Plant Medium (WPM) (LLOYD and MCCOWN, 1981) supplemented with 10 mg L^{-1} citric acid and different concentrations (0.5; 1.0; 2.0; 4.0 and 8.0 μmol L^{-1}) of BAP, KIN and TDZ, as well as their absence. The culture medium was gelified with 6.0 g L^{-1} agar and pH was adjusted to 5.8 before autoclaving at 120 °C and 1 atm for 20 minutes. After inoculation in a horizontal laminar flow chamber, the material was kept in a B. O. D. germination chamber at 25 ± 2 °C, irradiance of 43 μmol m^{-2} s^{-1} and 12 hours photoperiod.

The assessment was carried out 60 days after inoculation and the variables analyzed were the average number of shoots per explant, length of the largest shoot and the average number of buds. It was considered bud the present structures in axillary regions of shoots, and shoots are structures with, at least, expanded leaf primordia. The experiment was set in randomized blocks in a factorial scheme 3 x 6 (cytokinins x concentrations), consisting of four replicates per treatment, with each replicate consisting of six test tubes and one nodal segment per tube.

In vitro rooting

The best treatment from *in vitro* multiplication experiment was repeated and then the shoots with a size around 2.0 cm were used as explants. They were inoculated in WPM medium supplemented with 10.0 mg L^{-1} citric acid, containing combinations of IBA (1.0; 3.0; 6.0 and 9.0 μmol L^{-1}) and activated charcoal (1.0; 2.0 and 3.0 g L^{-1}), as well as their absence. After inoculation into culture medium, the material was kept in a growth room at 25 ± 2 °C under continuous light and irradiance of 43 μmol m^{-2} s^{-1}.

The evaluation was performed 40 days after inoculation and the variables analyzed were rooting percentage, number of primary and secondary roots and length of the longest primary root. The experiment was set in randomized blocks in a factorial scheme 5 x 4 (IBA x activated charcoal) and consisted of three replicates per treatment, with each repetition consisting of five test tubes containing one shoot per tube.

Statistical analysis

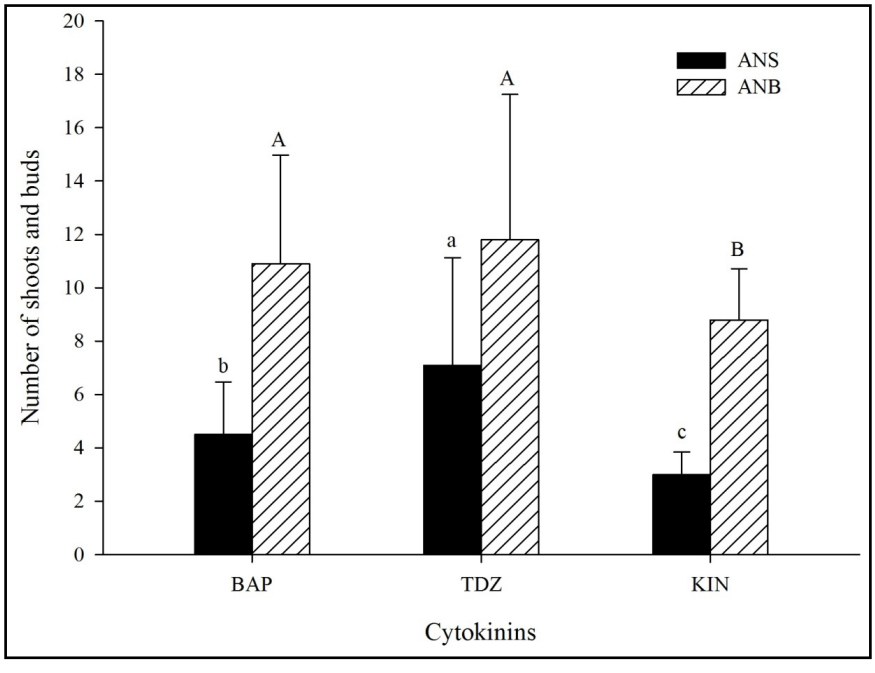
All statistical analyses were performed from data submission to analysis of variance (ANOVA), using the statistical software Sisvar (FERREIRA, 2014) and means were compared using Scott-Knott test at 5% significance for qualitative factors, and polynomial regression analysis for quantitative ones.

Results and discussion

Cytokinins used in culture medium led to an increase into amount of shoots and buds during *in vitro* culture of ipê-roxo. However, only for length of the largest shoot there was a significant interaction between type and cytokinin concentration ($p \leq 0.05$). A larger number of shoots was found in media containing TDZ, while the highest value for number of buds was determined both in media containing TDZ and those supplemented with BAP (Figure 1).

Figure 1 – Average number of shoots (ANS) and buds (ANB) of ipê-roxo nodal segments grown *in vitro* during 60 days in WPM medium containing cytokinins. Means followed by the same capital letter for ANB and small letter for ANS in columns are not different by the Scott-Knott test at 5% significance

Figura 1 – Número médio de brotos (ANS) e gemas (ANB) de segmentos nodais de ipê-roxo cultivados *in vitro* por 60 dias em meio de cultura WPM contendo diferentes citocininas. Médias seguidas pelas mesmas letras maiúsculas para ANB e minúsculas para ANS nas colunas não são diferentes pelo teste Scott-Knott a 5% de significância

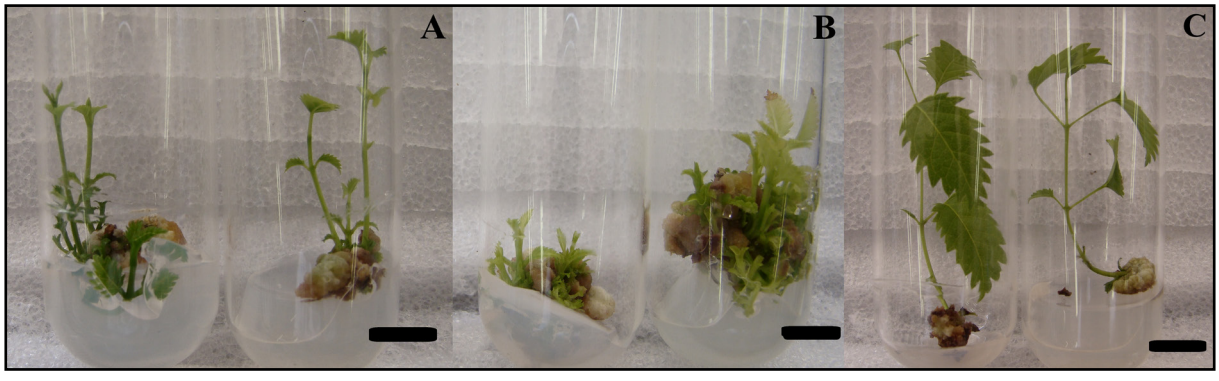


Source: Authors (2017)

Addition of cytokinins to culture medium may lead to gene expression control (GIGLI-BISCEGLIA *et al.*, 2018) in plant metabolism, promoting cell divisions and consequently influencing the differentiation of plant tissues (WYBOUW; RYBEL, 2019). Phenyl-urea derivatives such as thidiazuron are more effective than adenine derivatives like 6-benzylaminopurine and kinetin in bud induction (BANERJEE *et al.*, 2012). There are reports in literature showing that TDZ have ability to inhibit the cytokinin oxidase activity and thereby enhancing endogenous cytokinin levels (NISLER *et al.*, 2016). Moreover, TDZ causes changes in cellular signaling and endogenous levels of phytohormones (BANERJEE *et al.*, 2012), which could explain the high formation of new shoots and buds from ipê-roxo nodal segments as well as the difference on results obtained from other cytokinins used (Figure 2).

Figure 2 – Morphological aspect of ipê-roxo shoots cultivated under influence of different cytokinins within WPM medium after 60 days. Shoots were grown in presence of (A) BAP; (B) TDZ; and (C) KIN. Bar = 1.0 cm

Figura 2 – Aspecto morfológico de brotações de ipê-roxo cultivadas sob influência de diferentes citocininas no meio WPM após 60 dias. Brotos foram cultivados em presença de (A) BAP; (B) TDZ; e (C) KIN. Barra = 1,0 cm



Source: Authors (2017)

Increasing in the number of shoots and buds was proportional to rise of cytokinin concentrations regardless of cytokinin type used within WPM medium (Figure 3). Leitzke *et al.* (2010) also observed a higher number of shoots of blackberry 'Xavante' with an increase in cytokinin concentrations up to 15.0 µmol L^{-1}, similar to that found in our study, in which there was an increase in the number of shoots to the value of 8.0 µmol L^{-1}.

Cytokinin is a PGR class considered essential to induce proliferation of axillary buds, being type and cytokinin concentration some factors that most influence in success of *in vitro* multiplication stage (BRONDANI *et al.*, 2009). These plant regulators may interfere on expression of nitrate, potassium, sulfate and phosphate transporters (HOLUBOVÁ *et al.*, 2018), which are important macronutrient sources with structural and energy function, thus stimulating growth and plant development.

In relation to the length of the largest shoot, both 4.0 and 8.0 µmol L^{-1} KIN concentrations added to WPM led to the highest means as compared to others cytokinins used at the same levels (Table 1). Absence of PGR also led to shoot formation, but shoots were more etiolated and had few adventitious buds on stem extension.

Table 1 – Length of the largest shoot of ipê-roxo plants grown in *in vitro* environment during 60 days in WPM medium according to cytokinins and their concentrations

Tabela 1 – Comprimento do maior broto de plantas de ipê-roxo cultivadas em ambiente *in vitro* durante 60 dias em meio WPM de acordo com as citocininas e suas concentrações

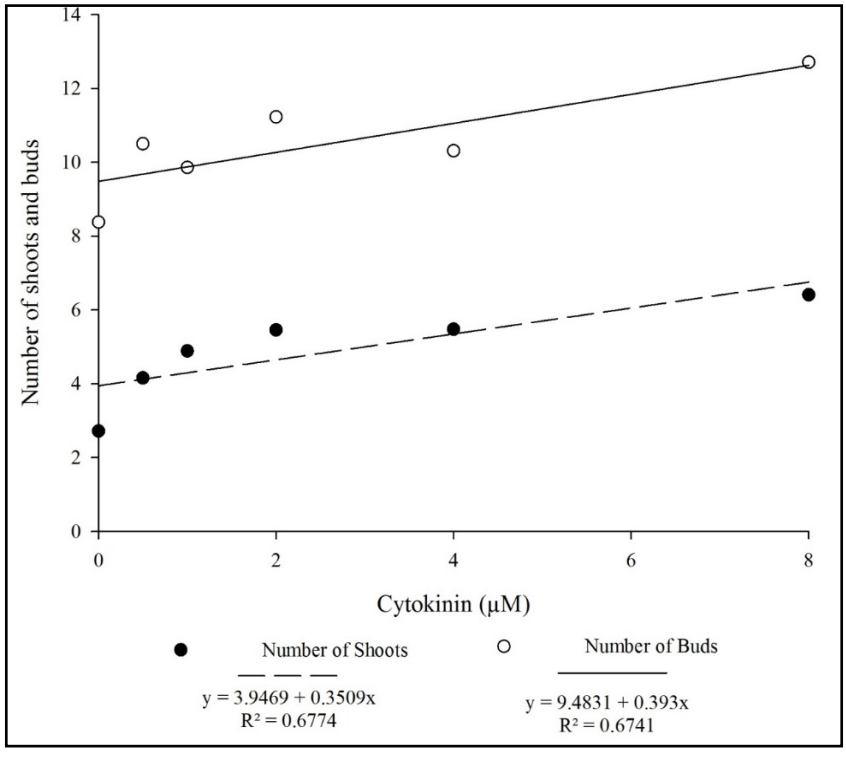
	Length of the largest shoot (cm)		
Concentration (µM)	BAP	TDZ	KIN
0.0	2.72 a	3.73 a	3.46 a
0.5	3.17 a	2.12 b	2.98 a
1.0	3.17 a	1.56 b	2.69 a
2.0	3.13 a	0.92 b	3.10 a
4.0	1.83 b	0.68 c	3.22 a
8.0	2.56 b	0.89 c	3.72 a

Source: Authors (2017)

On what: Means followed by the same letter in lines are not different by the Scott-Knott test at 5% significance.

Figure 3 – Average number of shoots and buds of ipê-roxo plants grown *in vitro* by 60 days under influence of cytokinin concentrations in WPM medium. Increasing in cytokinin concentration leads to a higher number of shoots and buds as shown in both behaviors

Figura 3 – Número médio de brotos e gemas de plantas de ipê-roxo cultivadas *in vitro* por 60 dias sob influência das concentrações de citocininas em meio WPM. O aumento na concentração das citocininas leva a um maior número de brotos e gemas como mostrado em ambos os comportamentos



Source: Authors (2017)

Although TDZ has ability to stimulate the induction of multiple shoots per explant, KIN was more efficient in producing more elongated shoots. This KIN effect may be related to its ability to cause cell division and to promote cell expansion (KATARIA; GURUPRASAD, 2018), which is better to elongation over production of new shoots.

High concentrations of cytokinins can reduce the shoot size and stimulate the occurrence of malformed buds, abnormal leaves and hyperhydricity, causing some toxicity to plant tissue (MAZRI, 2015). All these features were observed in explants cultivated at 8.0 µmol L^{-1} TDZ added to culture medium. Transfer shoots to the culture medium without any cytokinin in a timely-manner could be a solution (VASCONCELOS *et al.*, 2012) to avoid such problems and therefore allow new shoots emergence or leaves formation with morphology and anatomy similar to that found in normal plants. Giri and Tamta (2011) working with *Hedychium spicatum* (white ginger lily), also found that the use of TDZ was more efficient in inducing a higher number of shoots, even though reduced in size, while KIN led to the formation of shoots with greater length, similar to results obtained in our study.

Most explants after 60 days of culture on media containing cytokinin showed callus formation at the stem base. It also occurred with explants inoculated on media without any cytokinin. Calli generated on explant base cultivated on media without these PGR showed smaller size and had a lighter appearance as compared to those from media containing different types of cytokinin. At culture media with cytokinin the calli were larger and relatively darker on

the explant contact region with culture medium.

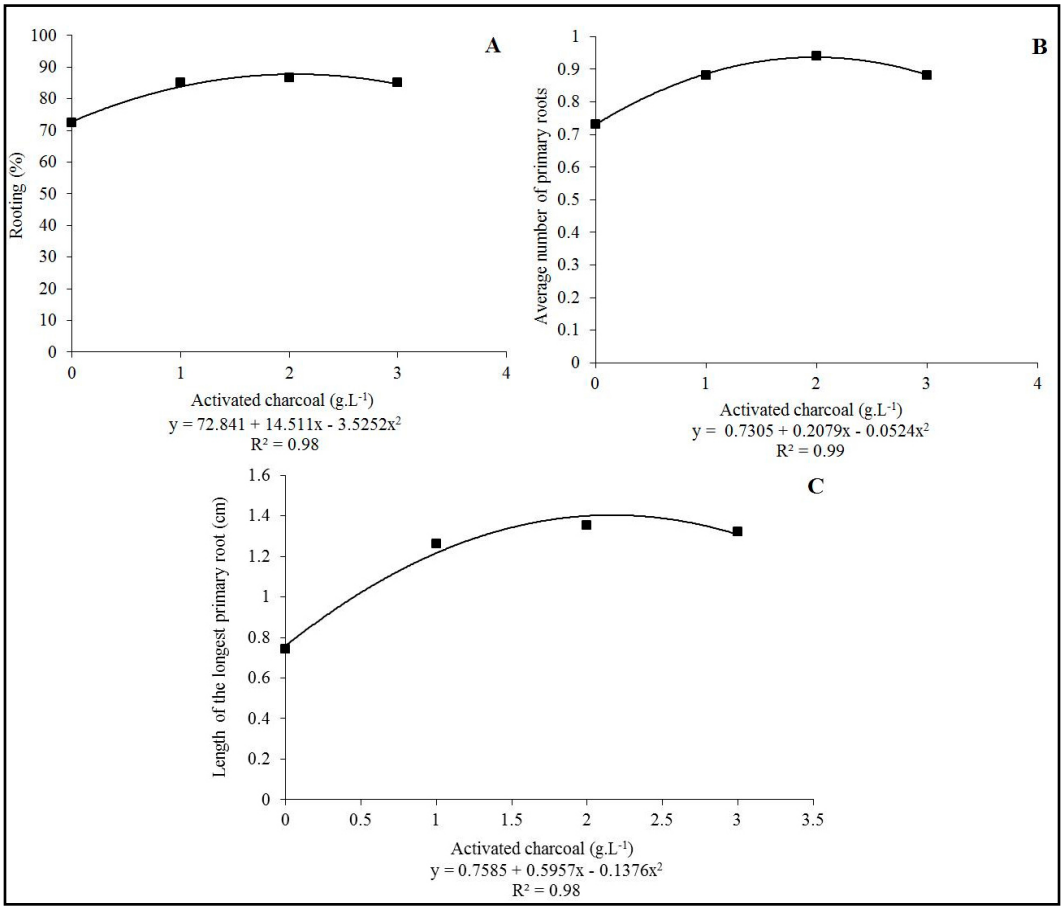
Callus formation at the explant base is common in woody species, but it is considered unfavorable to micropropagation process since its formation in rooting zone may affect the root quality, especially in regard to vascular connections, root formation and consequently the nutrient uptake (SEKELI *et al.*, 2013). However, the findings of our study suggest that shoot rhizogenesis was not completely affected with callus occurrence, since the direct induction of roots was verified at shoot base of some explants.

For the *in vitro* rooting stage, there was no significant interaction between IBA and activated charcoal for any analyzed variable. Treatments containing activated charcoal presented statistical differences ($p<0.05$) for all variables, except for the number of secondary roots. On the other hand, IBA treatments were statistically different only for the presence of roots.

Increasing activated charcoal concentrations within culture medium enhanced percentage of roots formed in explants during *in vitro* rooting up to concentration of 2.0 g L^{-1}. The highest percentage of formed roots was around 88%, with a slight decrease after using the higher concentration (Figure 4).

Figure 4 – Influence of activated charcoal (A) on root percentage; (B) average number of primary roots; and (C) length of the longest primary root of ipê-roxo shoots cultivated after 40 days *in vitro* in WPM containing IBA

Figura 4 – Influência do carvão ativado sobre (A) a percentagem de enraizamento; (B) número médio de raízes primárias; e (C) comprimento da maior raiz primária de brotos de ipê-roxo cultivados *in vitro* após 40 dias em meio WPM contendo AIB

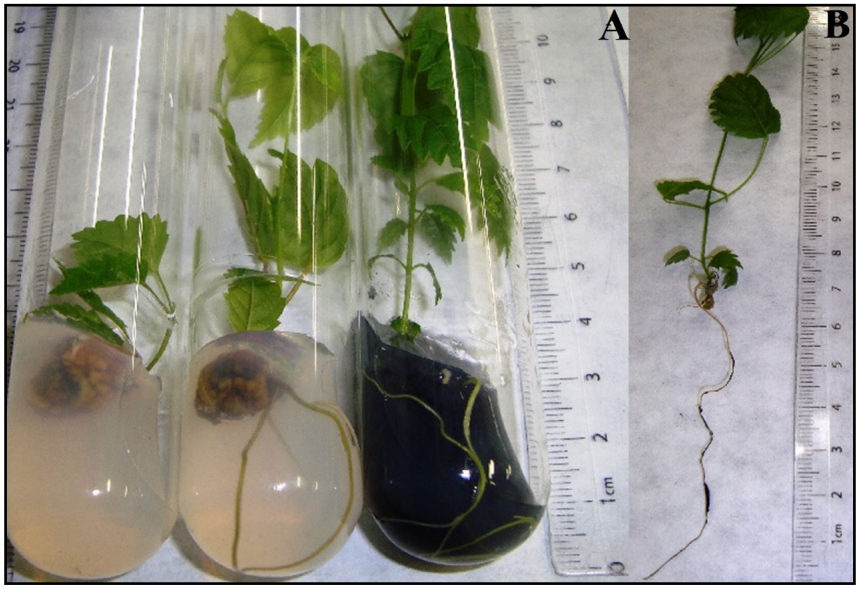


Source: Authors (2017)

Both number of primary roots and the length of the longest primary root increased as activated charcoal concentrations up to estimated values of 1.98 and 2.15 g L^{-1}, respectively. Then a decrease was observed in values of both variables from the concentration higher than those cited (Figure 4). Shoots inoculated onto media containing activated charcoal showed their stem base slightly etiolated, up to the first or second node. However, this did not affect the normal development of explants or root formation in shoots. Other aspect observed was a low growth of shoots inoculated onto medium with neither IBA nor activated charcoal likely related to low rate or none root formation on explant base, thus could be decreasing the nutrient absorption level of shoots from culture medium (Figure 5).

Figure 5 – Ipê-roxo rooted shoots under IBA and activated charcoal influence after 40 days of *in vitro* culture. (A) From left to right: shoot grown in WPM with neither IBA nor activated charcoal (*first tube*); just containing IBA (*second tube*); or containing activated charcoal only (*third tube*), respectively. (B) Primary root length of a shoot grown in 2.0 g L^{-1} of activated charcoal

Figura 5 – Brotos enraizados de ipê-roxo sob influência de AIB e carvão ativado após 40 dias de cultivo *in vitro*. (A) Da esquerda para a direita: brotos cultivados em WPM sem AIB e sem carvão ativado (primeiro tubo); contendo apenas AIB (segundo tubo); ou contendo apenas carvão ativado (terceiro tubo), respectivamente. (B) Comprimento da raiz primária de um broto cultivado em 2,0 g L^{-1} de carvão ativado



Source: Authors (2017)

Results observed in the treatments containing activated charcoal show the potential of this compound into *in vitro* ipê-roxo rooting. It may be related to its ability to change the culture medium pH to an optimum level during morphogenesis as well as allow a low incidence of light on roots growing inside the flasks, which is considered a favorable physical environment for rhizogenesis (GANTAIT *et al.*, 2009). Furthermore, positive effect of a dark environment on rooting frequency may be involved in the peroxidase-activity reduction, and consequently delaying the PGR degradation (VIEITEZ *et al.*, 2009). A lower degradation of plant regulators will allow that these compounds may be consumed for a longer time, since the explants will can absorb and transport them from culture medium to perform their biological functions in specific regions of shoots.

Treatments with IBA showed statistical differences between their concentrations only for the variable rooting percentage. Since the data did not fit to polynomial regression curves, the Scott-Knott test at 5% significance was applied to demonstrate the difference between the means (Table 2).

Table 2 – Rooting of ipê-roxo shoots according to IBA concentrations added to WPM after 40 days of *in vitro* cultivation

Tabela 2 – Enraizamento de brotos de ipê-roxo de acordo com as concentrações de AIB adicionadas ao meio WPM após 40 dias de cultivo *in vitro*

IBA (µM)	Rooting (%)
0.0	75.96 b
1.0	82.84 a
3.0	85.54 a
6.0	78.40 b
9.0	88.79 a

Source: Authors (2017)

On what: Means followed by the same letter in column are not different by the Scott-Knott test at 5% significance.

Regarding *in vitro* root formation, auxins are the main regulators involved in the morphogenetic path and indole-3-butyric acid, the most commonly used (INOCENTE; NIENOW; TRE, 2018). Synthetic auxins absorbed by basal shoot portion must be redistributed within the explant and transported back to the base through the basipetal transport system. Therefore, IBA exogenously added into culture medium may have been transported to shoot leaves, causing a stimulus for the production of indole acetic acid (IAA) in a peroxisome-dependent reaction inside leaf, with subsequent transport of this phytohormone to root initiation zone (BERTONI, 2011).

The fact that concentrations of IBA did not differ among themselves for the number of roots is similar to the results from studies applied to other tropical wood species, such as *Stereospermum suaveolens* (BAUL *et al*., 2009). The lack of IBA effect in root induction may be due to the presence of activated charcoal which has the ability to adsorb components from culture medium (SÁENZ *et al*., 2010) and thus may have reduced the availability of this regulator inside the medium to explants. It could have interfered with the appropriate level of IBA to be absorbed by shoots to lead to a higher number of roots. Furthermore, although auxins are important regulators during induction and root initiation, they may have inhibitory effects in root elongation (GRATTAPAGLIA; MACHADO, 1998) or not offer conditions for rooting (OLIVEIRA *et al*., 2015), which justify the absence of IBA effect on the length of ipê-roxo roots, as observed in our results.

Conclusion

For the *in vitro* multiplication stage of ipê-roxo, the use of TDZ 8.0 µmol L^{-1} is recommended, with subsequent transfer of shoots to a medium without cytokinin in order to avoid abnormal shoots production.

Use of 2.0 g L^{-1} activated charcoal leads to the *in vitro* root development and growth in *Handroanthus impetiginosus*, not requiring IBA for rhizogenesis, which results in cost savings for not use auxins.

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