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In vitro organogenesis as an efficient method for the propagation of *Dalbergia nigra*

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Abstract – The objective of this work was to establish an efficient protocol for the in vitro organogenesis of *Dalbergia nigra*. For this purpose, 30-day-old seedlings were sectioned at their cotyledonary nodes and nodal segments. These materials were cultivated in a medium with different combinations of the 6-benzylaminopurine and thidiazuron cytokinins. After 60 days in a growth chamber set at 27°C and a 16-hour photoperiod, growth characteristics were analyzed. Explants from the cotyledonary nodes show a greater morphogenetic potential, regardless of the addition of cytokinins. Cotyledonary nodes in the medium supplemented with 2.22 µmol L^{-1} BAP show the best combination for the in vitro propagation of *D. nigra*.

Index terms: *Dalbergia nigra*, Brazilian rosewood, explants, in vitro culture, 6-benzylaminopurine, thidiazuron.

Organogênese in vitro como método eficiente para a propagação de *Dalbergia nigra*

Resumo – O objetivo deste trabalho foi estabelecer um protocolo eficiente para a organogênese in vitro de *Dalbergia nigra*. Para isso, plântulas com 30 dias de idade foram seccionadas em seus nós cotiledonares e segmentos nodais. Esses materiais foram cultivados em meio com diferentes combinações das citocininas 6-benzilaminopurina e tidiazuron. Após 60 dias em sala de crescimento mantida a 27°C e submetida a fotoperíodo de 16 horas, foram analisadas as características de crescimento. Explantes dos nós cotiledonares apresentam maior potencial morfogenético, independentemente da adição de citocininas. Nós cotiledonares em meio suplementado com 2.22 µmol L^{-1} de BAP são a melhor combinação para a propagação in vitro de *D. nigra*.

Termos para indexação: *Dalbergia nigra*, jacarandá-da-Bahia, explante, cultivo in vitro, 6-benzilaminopurina, tidiazuron.

The Atlantic Forest neotropical hotspot comprises several endemic tree species that are threatened of extinction due to overexploitation of wood. *Dalbergia nigra* (Vell.) Allem. ex. Benth. (Fabaceae) is one of them. It propagates from seed. This way, the viability is subjected to insect attacks, such as that by *Troezon championi* beetle, to supra-annual fruiting, and to difficult vegetative propagation (Carvalho et al., 2003; Brasil, 2014; Santos et al., 2020).

Plant tissue culture allows of a large-scale production of plantlets with high phytosanitary quality. This technique is particularly beneficial to endangered species. When carried out for the seminiferous pathway, it allows of a high genetic variability in the final amount of the produced

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seedlings, contributing to reduce the vulnerability to diseases and promoting a greater adaptation to climatic conditions (Hoffmann & Sgrò, 2011).

The most commonly used growth regulators during the organogenesis induction are auxins and cytokinins (Bielach et al., 2012). In plants, cytokinins induce the stem branching from newly formed adventitious shoots by inhibiting the auxin-regulated apical dominance (Domagalska & Leyser, 2011). These cytokinins can be divided into adenine derivatives, such as the naturally occurring BAP, and phenylurea derivatives, such as the synthetic compound 1-phenyl-3-(thiadiazol-5-yl)-urea (thidiazuron; TDZ) (Flores et al., 2009).

BAP is one of the most used cytokinins, as plant tissues metabolize it more efficiently than other regulators. However, it shows a low success in the regeneration of woody plants (Abbasi et al., 2013). In turn, TDZ is widely used in woody plants and it is highly efficient even at low concentrations. This efficiency is attributed to its high stability toward the oxidase/dehydrogenase enzyme that degrades cytokinins (Nisler et al., 2021). Therefore, combining these cytokinins has allowed of the successful multiplication and regeneration of shoots (Vinoth & Ravindhran, 2018).

The objective of this work was to establish an efficient protocol for the in vitro organogenesis of *D. nigra*.

Seed of *D. nigra* (registered at the Brazilian Ministry of Agriculture, Livestock, and Supply) were obtained from the Sociedade de Pesquisa Floresta of the Universidade Federal de Viçosa, in the municipality of Viçosa (20°45'35"S, 42°52'06"W), in the state of Minas Gerais, Brazil.

Seed for propagation were kept at 4°C until the experiment was set up. When the experiment was installed, seed moisture was 8.30%. Using a flow hood chamber, seed were immersed in 70% (v/v) ethanol for 60 s, then immersed in solution at 2% sodium hypochlorite for 15 min, finally, seed were subjected to a triple wash with distilled and autoclaved water. Seed were then placed in 25×150 mm test tubes containing 10 mL woody plant medium - L449 (WPM) (Lloyd & McCown, 1980). WPM was composed of 2.41 g L^{-1} of L449 powder formulation with vitamins (Phytotechnology), 30 g L^{-1} sucrose (Dinâmica, Indaiatuba, SP, Brazil), 0.1 g L^{-1} myo-inositol (Sigma), and 6 g L^{-1} agar (Kasvi). pH was adjusted to 5.7±0.1 and autoclaved (Phoenix Luferco, Araraquara, SP, Brazil) for 20 min at 121°C and 1 atm. The seed-containing tubes were sealed with plastic film and maintained in a growth chamber for 30 days at 27±2°C, and subjected to 16-hour photoperiod and 50 µmol m^{-2} s^{-1} radiance provided by white 20 W LED lamps (Empalux).

Thirty days after the treatments, in vitro-raised seedlings were sectioned into two types of explants: those from cotyledonary nodes, and those from nodes containing two axillary buds. The explants were cultivated in glass flasks (12.5 cm height × 5 cm diameter, 350 mL) containing 30 mL WPM. Six combinations of BAP + TDZ were used: 2.22 + 0 µmol L^{-1}; 2.22 + 0.002 µmol L^{-1}; 2.22 + 0.004 µmol L^{-1}; 4.44 + 0 µmol L^{-1}; 4.44 + 0.002 µmol L^{-1}; and 4.44 + 0.004 µmol L^{-1}. The glass flasks were sealed with plastic film and kept in a growth chamber for 60 days. After this period, the number of shoots, number of leaves, shoot length (cm), callus diameter (mm), number of roots, root length (cm), shoot dry mass (mg), and root dry mass (mg) were evaluated.

A completely randomized experimental design was carried out as a 2×6 factorial arrangement (explants × cytokinins combinations), with four replicates of nine explants each. Data were submitted to analysis of variance, F test, Tukey's test, and Pearson's correlation at 5% probability after verifying the multicollinearity between the variables and the independence, normality and homogeneity of the variances of the scatter plot errors, tests by Lilliefors and Bartlett, respectively. The detection of multicollinearity was verified using the variance inflation factor (VIF). All statistics were performed with the software Genes (Cruz, 2016) and R version 4.2.0 (R Core Team, 2022).

The in vitro germination of *D. nigra* from aseptic seed can be used to generate juvenile propagules via

organogenesis (Figure 1 A–C). After 60 days of in vitro growth, plantlets originating from cotyledonary node segments showed a greater density and length of shoots and roots (Figure 1 E) than plantlets from nodal segments with two buds. The latter showed a limited root development (Figure 1 G).

In general, the cotyledonary nodes from explants were better than those from nodal segments with two buds. The former showed longer buds, as well as a greater number of leaves and shoots and roots, a high value of root dry mass, and longer roots. However, the mean number of shoots in the cytokine treatments was statistically higher for the nodal segment explants. Regardless of the explant used, the number of shoots was statistically equal, with superior results for the following concentrations of BAP + TDZ: 2.22 + 0 µmol L^{-1}; 2.22 + 0.002 µmol L^{-1}; 2.22 + 0.004 µmol L^{-1}; 4.44 + 0 µmol L^{-1} (Figure 2 Aa). Although the lack of cytokinins in the medium have resulted in fewer shoots (1.66 and 1.38 for cotyledonary and two-bud nodal segments, respectively), in comparison with different combinations of cytokinins (Figure 2 Aa), it markedly increased the shoot length in the cotyledonary node explants (2.81 cm) (Figure 2 Ab).

The largest callus diameter was obtained by incubating the cotyledonary node explant in the medium supplemented with 2.22 µmol L^{-1} BAP + 0.004 µmol L^{-1} TDZ, or the two-bud nodal segment in the medium containing 2.22 µmol L^{-1} BAP + 0.002 µmol L^{-1} TDZ (Figure 2 Ae). The number, length, and dry mass of roots were higher for the cotyledonary node segments than for the nodal segments, regardless of cytokinin treatment (Fig. 2 A). At 2.22 µmol L^{-1} BAP, the cotyledonary nodes displayed the average of 2.10 roots, whereas the nodal segments produced only 0.2 roots. This fact confirms the low morphogenetic activity of these explants for rooting in the presence of exogenous cytokinin (Figure 2 Af).

Based on correlation analysis of different morphological variables (Figure 2 Ba, b), the shoot length and the number of leaves displayed a positive correlation with the other variables, except for the number of shoots and the callus diameters. In contrast, a negative correlation emerged between callus diameter and root length or root number (Figure 2 Ba,

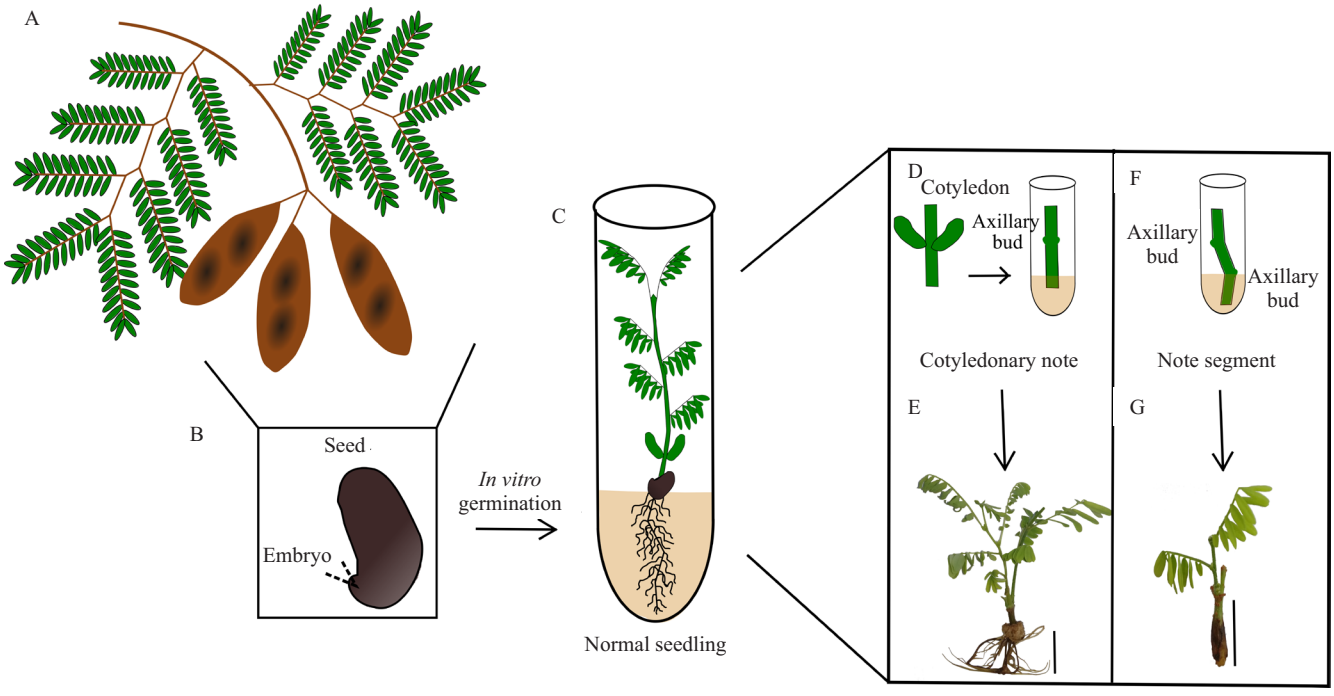

Figure 1. Schematic representation of *Dalberia nigra* morphology and experimental setup: A and B, branch with fruit and seed; C, in vitro germination in woody plant medium (WPM) leading to the formation of a normal seedling; D–G, sectioning of explants from a cotyledonary node (D, E), or from a node segment (F, G). Illustration by Caroline Palacio de Araujo

b), whereby a wider callus resulted in fewer and shorter roots (Figure 2 Ae-h), particularly at 2.22 µmol L^{-1} BAP + 0.002 µmol L^{-1} TDZ.

Overall, cytokinin-free treatment showed superior results for all traits related to the root system, such as root length and dry mass, followed by the treatment with the lowest cytokinin concentration (2.22 µmol L^{-1} BAP). Explants cultivated in the cytokinin-free medium resulted in fewer shoots; however, it showed more roots. These results suggest the possibility of a higher

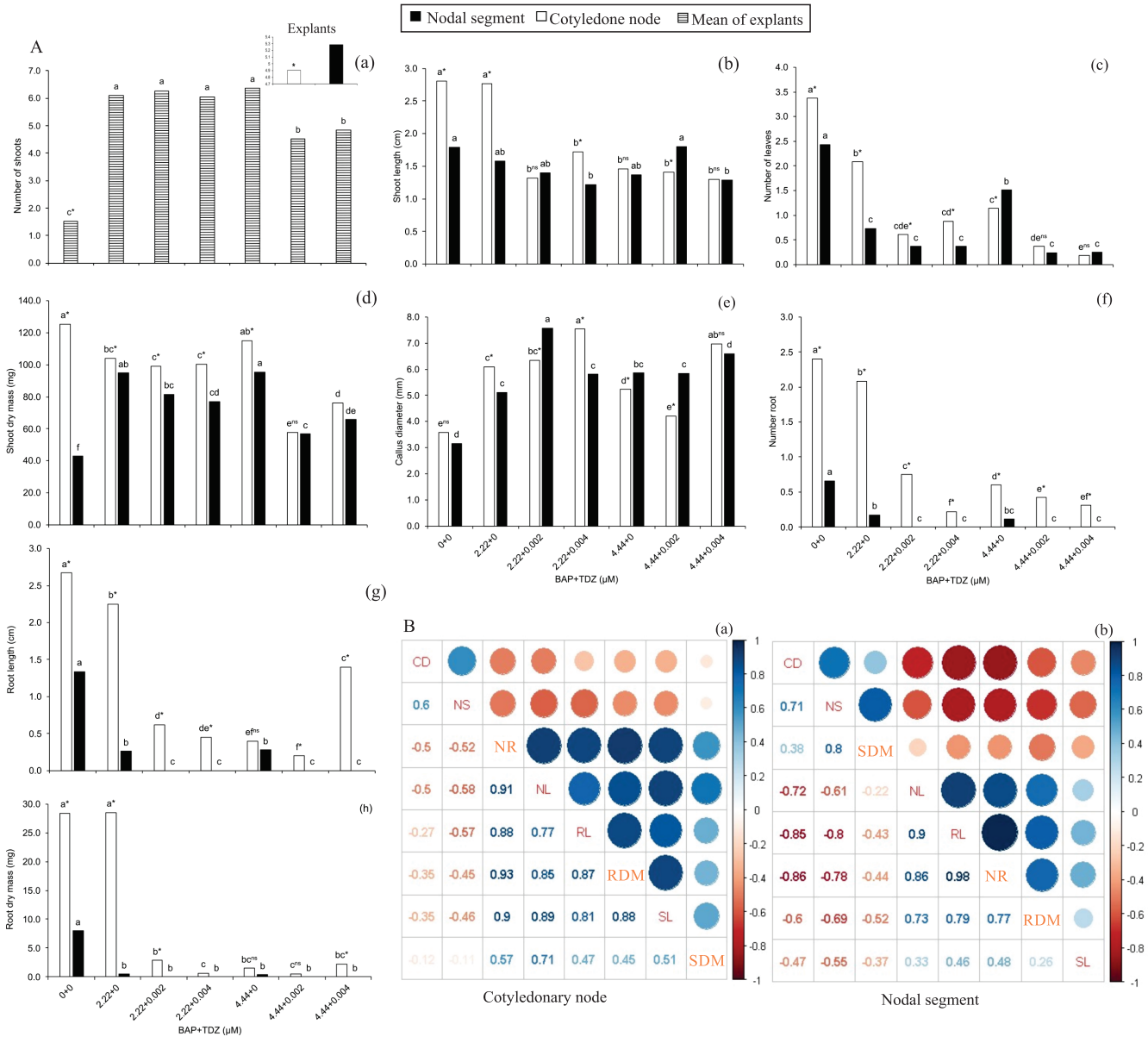

Figure 2. In vitro morphogenesis of juvenile explants of *D. nigra:* A, (a) number of shoots, (b) shoot length (cm), (c) number of leaves, (d) shoot dry mass (mg), (e) callus diameter (mm), (f) number of roots, (g) root length (cm), and (h) root dry mass (mg). [(1)]Means followed by equal lowercase letters between cytokinin combinations within each explant do not differ from each other, by the Tukey's test, at 5% probability. *Significant between the two types of explants, by the he F-test, at 5% probability. [ns]Nonsignificant. B, Pearson's correlation between the different morphological variables, for explants from cotyledonary nodes (a) and nodal segments (a), for the following parameters: NS, number of shoots; SL, shoot length; NL, number of leaves; SDM, shoot dry mass; CD, callus diameter; NR, number of roots; RL, root length; and RDM, root dry mass. Correlation values greater than 0.48 are significant, by the t-test, at 1% probability.

concentration of auxin in these tissues, as its role in the plant is to promote rooting and growth through cell division and elongation (Xiao & Zhang, 2020).

The results indicate that the concentration of BAP was not sufficient to cause an imbalance in the auxin / cytokinin ratio that is necessary for shoot growth and rooting. The use of the cotyledonary node and the supplementation of WPM medium with 2.22 µmol L^{-1} BAP led to the highest shoot and root production.

When a nodal segment with two buds was used as explant, the reduction by 42% of shoot length and 91% of the number of roots were observed even in the presence of cytokinins. This fact suggests that nodal segments above the cotyledonary nodes have a lower amount of endogenous auxins than those in the cotyledonary nodes.

BAP is necessary to obtain a higher shoot dry mass, when using nodal segments. The axillary nodes showed active centers, whose shoot growth potential was similar to the potential of the apical meristem. However, activity in these centers resumes from the region closest to the cotyledonary node (Long & Barton, 2000; Silva et al., 2020). The basipetal movement of auxin produced in the apical meristem and transported to the roots results in different hormone levels throughout the plant. Therefore, some regions may need more supplementation with plant growth regulators to activate the morphogenetic activity of the tissue (Long & Barton, 2000).

In some species, excessive callus formation may favor the rhizogenesis in the explants. In others, the opposite result may be obtained by decreasing the quality of the root system formed (affecting the vascular connection between plant/root) and, even, by inhibiting the rhizogenic process (Hartmann et al., 2011).

The in vitro growth without the addition of cytokinins resulted in superior root length and dry mass. The next best outcome came with the use of 2.22 µmol L^{-1} BAP. A possible explanation for this response is the low concentration of cytokinin in the tissue and, consequently, a usual high auxin / cytokinin ratio inside the plant. When 2.22 µmol L^{-1} BAP was added, the ratio decreased, lowering the number and length of roots (Figure 3 b, c). The auxin / cytokinin ratio determines the differentiation into roots or buds / shoots in tissues cultivated in vitro; and high ratios stimulate the root formation (Skoog & Miller, 1957; Cheng et al., 2013).

The cotyledonary nodes of *D. nigra* show stronger morphogenetic activity than the nodal segments with two buds, except for the number of shoots. BAP (at 2.22 µmol L^{-1}) is recommended for the in vitro morphogenesis of juvenile explants of *D. nigra*. However, when associated with TDZ, it interferes negatively with the in vitro rhizogenesis of juvenile explants, especially if both cytokinins are abundant.

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