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Benzyladenine Effects On Polyamine Contents And Proteomic Profiles During *In Vitro* Shoot Development And On *Ex Vitro* Rooting In *Dalbergia Nigra* (Vell.) Allemão Ex Benth. (Fabaceae)

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Research Article

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Abstract

Dalbergia nigra is an endangered species from the Brazilian Atlantic Rainforest, and *in vitro* propagation can be applied for the conservation of this species. The aim of this study was to establish *in vitro* propagation and ex vitro rooting in D. *nigra* and evaluate the alterations in polyamines (PAs) and protein profiles during shoot development. The effect of MS and WPM culture media on *in vitro* germination was tested (%). For shoot induction, explants of apical and cotyledonary nodal segments from 45-day-old seedlings were inoculated in WPM culture medium supplemented with benzyladenine (BA; 0, 2.5 and 5 µM). Shoots obtained *in vitro* without and with 2.5 µM BA were rooted *ex vitro* with different concentrations (0, 100 and 500 µM) of indole-3-butyric acid (IBA). The best growth of seedlings was obtained in WPM culture medium. Treatment with 2.5 µM BA significantly increased the length of shoots by increasing free putrescine contents and the accumulation of proteins associated with shoot elongation, such as aspartate aminotransferase, elongation factor, calreticulin-3, and cell division cycle protein 48. Ex vitro rooting was obtained in all treatments of IBA, and the use of auxin was not necessary. The BA used for shoot multiplication significantly affected rooting, reducing the induction and number of roots but increasing the length of roots. This study showed the relevance of cytokinin, PAs and proteomic profiles on *in vitro* shoot development, as well as the auxin and cytokinin balance on *ex vitro* rooting in *D. nigra.*

Key Message

Micropropagation of *Dalbergia nigra* (Fabaceae)

Introduction

Several native wood species from the Atlantic rainforest are endangered, including *Dalbergia nigra* (Vell.) Allemão ex Benth, commonly known as Jacarandá-da-Bahia. Due to the intense exploitation of woody plants and the lack of reforestation programs, this species has been included as vulnerable on the Red List of the International Union for Conservation of Nature (IUCN 2021). Appropriate biotechnological and sustainable conservation strategies for many woody species need further research and development, and *in vitro* propagation technologies can be applied for conservation, with a great going to a global economic and ecological impact on sustaining tropical forest woody biodiversity (Pijut et al. 2012).

Biotechnological tools, such as micropropagation, have been applied in studies aiming at the propagation of woody species (Fermino-Junior and Scherwinski-Pereira 2012; Perveen et al. 2013). Micropropagation is the commercially efficient propagation of species in a short period of time (Gupta et al. 2014), enabling clonal production and conservation of germplasm (Giri et al. 2004; Shukla et al. 2008; Kodym and Leeb 2019). Knowledge of the biochemical and molecular aspects of *in vitro* morphogenesis, such as the cellular mechanisms involved in the growth and development of these species, is necessary for the propagation and preservation of threatened species with difficulties in propagation by conventional methods (Pijut et al. 2007; Dias et al. 2012; Stuepp et al. 2018).

The steps of *in vitro* propagation involve manipulation of the type of explants as well as the components of the culture medium, such as plant growth regulators (PGRs), to achieve optimal conditions for shoot multiplication and root induction (Bunn et al. 2011). Among the PGRs, cytokinins and auxins are the most commonly used in plant tissue cultures for shoot and root development (Phillips and Garda 2019). In the propagation of woody species, benzyladenine (BA) is the cytokinin most commonly used to promote the development of axillary buds, breaking apical dominance and stimulating shoot proliferation (Giri et al. 2004; Pijut et al. 2012). Studies have shown the relationship of PGRs with other compounds, such as polyamines (PAs), in shoot development (Aragão et al. 2016; Aragão et al. 2017b).

PAs are low molecular weight, aliphatic, polycationic compounds with positively charged nitrogen atoms naturally occurring in plants (Baron and Stasolla 2008). These compounds can interact with negatively charged macromolecules, such as DNA, RNA, phospholipids, cell wall components, and proteins (Baron and Stasolla 2008). Thus, PAs are essential for various physiological and developmental processes in plants (Santa-Catarina et al. 2007; Dutra et al. 2013), including shoot development (Aragão et al. 2017b; Lerin et al. 2019; Oliveira et al. 2020). Changes in endogenous PA contents induced by exogenous addition, especially putrescine (Put), demonstrated its relevance for shoot development in *C. fissilis* (Aragão et al. 2017b).

In addition to PAs, specific proteins have been candidate markers associated with morphogenic competence during *in vitro* plant morphogenesis (Reis et al. 2016; Heringer et al. 2018). Proteomic studies revealed the involvement of multiple proteins with specific functions in competence for the *in vitro* development of shoots (Mitrović et al. 2012; Ghosh and Pal 2013). Changes in the accumulation of some proteins involved mainly in metabolic and cellular processes, such as cell division, during *in vitro* shoot development induced or not induced by exogenous putrescine (Put) demonstrated an important relationship of specific proteins with shoot development in *C. fissilis* (Aragão et al. 2016; Aragão et al. 2017b).

In addition to shoot development, the formation of adventitious roots is an essential step of *in vitro* plant propagation. Auxin plays an essential role in rooting, and indole-3-butyric acid (IBA) is the most commonly used due to its higher root-inducing capacity and greater stability to light (Pacurar et al. 2014). IBA use is well documented during *in vitro* and *ex vitro* rooting in several woody species (Pijut et al. 2012). *Ex vitro* rooting has the advantage of lower cost and saves time, reducing the cost of a micropropagation protocol up to 70% compared to *in vitro* rooting(Yan et al. 2009; Ranaweera et al. 2013; Patel et al. 2014). In addition, another advantage of *ex vitro* rooting is that plantlets do not need additional acclimatization, exhibiting good root development and improved plantlet survival compared to *in vitro* rooting (Yan et al. 2009; Gupta et al. 2014).

Studies on the *in vitro* propagation of *D. nigra* have not yet been developed. In this sense, the establishment of *in vitro* propagation for this species can contribute to conservation programs and the repositioning of impacted areas. In addition, biochemical and molecular approaches can improve the knowledge of *in vitro* morphogenesis competence. Thus, the aim of this work was to establish the *in vitro*

propagation and *ex vitro* rooting of *D. nigra* and evaluate the alterations in PA contents and protein profiles during shoot development.

Materials And Methods

Plant material

Mature seeds obtained from Caiçara Comércio de Sementes LTDA located in Brejo Alegre, SP, Brazil (21°10'S and 50°10'W) were used for in vitro germination. Forty-five-day-old seedlings in vitro germinated were used as the source of apical and cotyledonary nodal explants for the shoot development experiments. Forty-five-day-old micropropagated shoots were used for the ex vitro rooting experiments.

Effect of plant culture medium on in vitro seed germination

For in vitro germination, seeds were surface disinfected according to Santa-Catarina et al. (2001), with modifications. First, seeds were washed with 250 mL distilled water, followed by immersion in 70% ethanol for 1 min and incubation in 2.5% sodium hypochlorite solution supplemented with the fungicide Derosal® 500 SC (Bayer; São Paulo, Brazil; active ingredient carbendazim 500 g L^{-1}; 200 µL of commercial solution per liter of water) for 30 min. Seeds were washed five times for 10 min each in sterile distilled water in a flow chamber. After disinfection, seeds were transferred to Murashige and Skoog (MS; Phytotechnology Lab, Overland Park, USA) (Murashige and Skoog 1962) and Woody Plant Medium (WPM; Phytotechnology) (Lloyd and McCown 1981) culture media, both supplemented with 20 g L^{-1} sucrose (Synth; São Paulo, Brazil) and 2 g L^{-1} Phytagel® (Sigma–Aldrich, St. Louis, USA). The pH of the culture medium was adjusted to 5.7 before the use of Phytagel and autoclaved at 121 °C for 15 min. Then, seeds were incubated in a 16-h photoperiod at a light intensity of 55 µmol m^{-2} s^{-1} and a temperature of 25 ± 2 °C. The germination (%) and morphology of seedlings were analyzed after 30 days of incubation. Each treatment consisted of five repetitions, with 20 seeds of each repetition.

Effect of explant, plant culture medium and BA concentration on shoot development

Forty-five-day-old seedlings germinated *in vitro* were used as the source of explants. Apical and cotyledonary nodal segments (± 2 cm) were isolated from seedlings and cultured on MS and/or WPM culture medium supplemented with 20 g L^{-1} sucrose, 2 g L^{-1} Phytagel® and different concentrations (0, 2.5 and 5 µM) of BA (Sigma–Aldrich). The pH of the culture medium was adjusted to 5.7 and autoclaved at 121 °C for 15 min. The explants were transferred to the culture medium with different treatments and maintained at a 16-h photoperiod under a light intensity of 55 µmol m^{-2} s^{-1} and a temperature of 25 ± 2 °C. Eight repetitions per treatment were used, with four explants of each repetition. The induction (%), number of shoots per explant and length of the first and second shoots were analyzed after 45 days of incubation. Samples were collected for PA and proteomic analyses and maintained at -80 °C until analysis.

Effect of IBA on ex vitro rooting of shoots and plant acclimatization

Shoots from apical and cotyledonary nodal segments (± 2 cm) cultured in WPM culture medium supplemented without and with 2.5 µM BA were used for shoot rooting. Shoots containing the apical meristem and leaves were treated for 30 s with different concentrations (0, 100 and 500 µM) of IBA (Sigma–Aldrich). Then, shoots were transferred to 50 mL plastic pots containing a mixture of PlantMax substrate (DDL Agroindustria, Paulínia, Brazil) with vermiculite (2:1; v/v). The shoots were maintained in plastic trays covered with a plastic film to maintain the high humidity needed for root development during *ex vitro* rooting and acclimatization. These plastic trays were kept in the growth room under a 16-h photoperiod, a light intensity of 55 µmol m^{-2} s^{-1}, and a temperature of 25 ± 2 °C. After 30 days, the humidity was gradually reduced until 40 days, when rooted shoots were considered acclimatized. The induction of rooting (%), number of roots initiated per shoot and root length were recorded after 45 days. Each treatment consisted of eight repetitions, with four shoots in each repetition.

Free PA determination

Determination of free PAs was performed according to Santa-Catarina et al. (2006) using samples of shoots from apical and cotyledonary nodal segments with 45 days of incubation without (control) and with 2.5 µM BA in WPM medium. Samples (200 mg fresh matter – FM – each, in triplicate) were ground in 1.2 mL of 5% perchloric acid (Merck Millipore, Darmstadt, Germany). After 1 h of incubation at 4 °C, the samples were centrifuged for 20 min at 20,000×g at 4 °C. The supernatant was collected, and free PAs were determined directly from the supernatant by derivatization with dansyl chloride (Merck Millipore) and identified by high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) using a 5-µm C18 reverse-phase column (Shimadzu Shin-pack CLC ODS). The HPLC column gradient was created by adding increasing volumes of absolute acetonitrile (Merck Millipore) to a 10% aqueous acetonitrile solution with the pH adjusted to 3.5 with hydrochloric acid (Merck Millipore). The absolute acetonitrile concentration was maintained at 65% for the first 10 min, increased from 65 to 100% between 10 and 13 min, and maintained at 100% between 13 and 21 min. The mobile phase was added at a flow rate of 1 mL min^{-1} and 40 °C. The PA concentration was determined using a fluorescence detector at 340 nm (excitation) and 510 nm (emission). The peak areas and retention times of the samples were measured through comparisons with the standard Pas putrescine (Put), spermidine (Spd), and spermine (Spm) (Sigma–Aldrich).

Protein extraction and digestion

Proteins were extracted from samples (three biological triplicates, 300 mg FM per sample) of shoots from apical and cotyledonary nodal explants grown without (0 µM - control) and with 2.5 µM BA at 45 days of incubation in WPM medium. Proteins were extracted using the trichloroacetic acid (TCA)/acetone method with modifications (Damerval et al. 1986). Initially, the samples were pulverized in liquid nitrogen using a ceramic mortar and pestle. The resulting powder was resuspended in 1 mL of chilled extraction buffer containing 10% (w/v) TCA (Sigma) in acetone with 20 mM dithiothreitol (DTT; GE Healthcare, Piscataway, USA) and vortexed for 5 min at 8 °C. Next, the samples were kept at −20 °C for 1 h before centrifugation at 16,000 x *g* for 30 min at 4 °C. The resulting pellets were washed three times with cold acetone plus 20

mM DTT and centrifuged for 5 min each time. The pellets were air dried and resuspended in buffer containing 7 M urea (GE Healthcare), 2 M thiourea (GE Healthcare), 2% Triton X-100 (GE Healthcare), 1% DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma–Aldrich), and complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), vortexed for 30 min at 8 °C, and centrifuged for 20 min at 16,000 x *g* at 4 °C. The supernatants were collected, and the protein concentrations were determined using a 2-D Quant Kit (GE Healthcare).

Before the trypsin digestion step, protein samples were precipitated using the methanol/chloroform methodology (Nanjo et al. 2012). After protein precipitation, the samples were resuspended in 7 M urea/2 M thiourea solution. Aliquots of 100 µg of protein were subjected to tryptic digestion using the filter-aided sample preparation (FASP) methodology (Reis et al. 2021). Next, the peptides were resuspended in 100 µL solution containing 95% 50 mM ammonium bicarbonate, 5% acetonitrile and 0.1% formic acid and quantified by A205 nm protein and peptide methodology using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific). The samples were transferred to Total Recovery Vials (Waters) for mass spectrometry analysis.

Mass spectrometry analysis

Mass spectrometry was performed using a nanoAcquity UPLC connected to a Q-TOF SYNAPT G2-Si instrument (Waters, Manchester, UK) according to Passamani et al. (2018). Runs consisted of three biological replicates of 1 µg of peptide samples. During separation, samples were loaded onto the nanoAcquity UPLC M-Class Symmetry C18 5 µm trap column (180 µm × 20 mm) at 5 $\mu L.min^{-1}$ for 3 min and then onto the nanoAcquity M-Class HSS T3 1.8 µm analytical reversed-phase column (75 µm × 150 mm) at 400 $nL.min^{-1}$, with a column temperature of 45 °C. For peptide elution, a binary gradient was used, with mobile phase A consisting of water (Tedia, Fairfield, Ohio, USA) and 0.1% formic acid (Sigma–Aldrich) and mobile phase B consisting of acetonitrile (Sigma Aldrich) and 0.1% formic acid. The gradient elution started at 7% B, then ramped from 7% B to 40% B until 91.12 min, then ramped again from 40% B to 99.9% B until 92.72 min, then remained at 99.9% until 106.00 min, then decreased to 7% B until 106.1 min, and finally remained at 7% B until the end of experiment at 120 min. Mass spectrometry was performed in positive and resolution mode (V mode), 35,000 full widths at half maximum, with ion mobility separation (IMS), and in data-independent acquisition mode ($HDMS^E$). The ion mobility wave was set to a velocity of 600 m $s^{-1,}$ and helium and IMS gas flows were 180 and 90 mL min^{-1}, respectively. The transfer collision energy ramped from 19 to 55 V in high-energy mode; the cone and capillary voltages were 30 and 2750 V, respectively; and the source temperature was 70 °C. Regarding the time of flight (TOF) parameters, the scan time was set to 0.5 s in continuum mode with a mass range of 50 to 2000 Da. Human [Glu1]-fibrinopeptide B at 100 $fmol.\mu L^{-1}$ was used as an external calibrant, and lock mass acquisition was performed every 30 s. Mass spectra were acquired by MassLynx v4.0 software.

Proteomics data analysis

Spectra processing and database search conditions were performed using ProteinLynx Global Server (PLGS) software v.3.0.2 (Waters). The PLGS was processed by the following parameters: Apex3D of 150 counts for low-energy threshold, 50 counts for elevated-energy threshold, and 750 counts for intensity threshold; two missed cleavages; minimum fragment ions per peptide equal to three; minimum fragment ions per protein equal to seven; minimum peptides per protein equal to two; fixed modifications of carbamidomethyl (C) and variable modifications of oxidation (M) and phosphoryl (STY); default false discovery rate (FDR) was set to a maximum of 1%. We used the *Arachis hypogaea* protein databank from UniProtKB (http://www.unipr ot.org) for protein identification, as it is the largest databank with proximity to *D. nigra*. Label-free quantification analysis was performed using ISOQuant workflow software v.1.7 (Distler et al. 2014). Briefly, the following parameters were used to identify proteins: FDR 1%, a peptide score greater than six, a minimum peptide length of six amino acids, and at least two peptides per protein were considered for label-free quantitation using the TOP3 approach, followed by the multidimensional normalized process within ISOQuant. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al. 2022) partner repository with the dataset identifier PXD031999. To ensure the quality of the results after data processing, only the proteins that were either present or absent (for unique proteins) in all three biological replicates were considered for differential accumulation analysis. Data were analyzed using Student's t test (two-tailed). Proteins with ANOVA ($P < 0.05$) were considered up-accumulated if the $\log_2$ value of the fold change (FC) was greater than 0.60 and down-accumulated if the $\log_2$ value of the FC was less than -0.60. Functional annotations were performed using OmicsBox software 1.0.34 and UniProtKB (http://www.uniprot.org).

Data analyses

The experimental design was completely randomized. Data were analyzed using analysis of variance ($P < 0.05$) followed by the Student-Newman–Keuls (SNK) test using the R Environment (R Core Team 2014).

Results

Effects of plant culture medium on in vitro germination and seedling development

Both culture media tested showed no significant difference on germination, resulting in similar percentages for MS (80%) and WPM (81%) (Fig. 1a). However, the seedlings from WPM culture medium showed well-developed leaves (Fig. 1c), while those obtained in the MS culture medium showed more senescence of leaves (Fig. 1b).

Effects of explants, plant culture media and BA on shoot development

Due to the difference in length between the first (Figs. 2a and 2b) and second shoots (Figs. 2c and 2d) developed, they were measured separately. BA addition promoted the elongation of the first shoot developed from both types of explants, the apical and cotyledonary nodal segments, in both culture media, MS (Fig. 2a) and WPM (Fig. 2b), compared to shoots obtained without BA (control). Moreover, no

significant difference was observed between the two types of explants in the same BA concentration and same culture medium or between the two culture media used (Figs. 2a and 2b). As BA is essential for shoot growth and there were no significant differences in the length of shoots between 2.5 and 5 µM BA in either culture medium, the two types of explants can be used for *in vitro* propagation of *D. nigra*, considering the elongation of the first shoot developed. The second shoot showed lower elongation (Figs. 2d and 2e) than the first shoot (Figs. 2a and 2b). In MS culture medium, the BA concentration increased the length of the second shoot from cotyledonary nodal segments compared to the control, while no significant effects were observed in shoots from apical nodal segments (Fig. 2c). In WPM culture medium, the 2.5 µM BA treatment showed lower elongation of second shoots from cotyledonary nodal segments compared to the control and 5 µM BA (Fig. 2d).

There were no significant differences in the number of shoots between the two types of explants at each BA concentration (0 or 2.5 µM) or in either culture medium, MS (Fig. 2e) or WPM (Fig. 2f), except for apical explants in the control treatment (without BA) in MS culture medium, which showed a significantly lower number of shoots (Fig. 2e). On the other hand, no significant differences were observed for the number of shoots from apical and cotyledonary nodal segments incubated with WPM culture medium (Fig. 2f). For shoot induction, no significant differences were observed in explant type, culture medium or BA concentration, showing 100% shoot induction in all treatments (data not shown).

Effect of BA and explant type on endogenous PA contents during shoot development

Free PAs were quantified in 45-day-old shoots from apical and cotyledonary nodal segments incubated without and with 2.5 µM BA and WPM culture medium (Fig. 3). Free Put content was significantly higher when shoots were grown in 2.5 µM BA compared to the control in both types of explants, being significantly higher in shoots from apical nodal segments compared to cotyledonary nodal segments (Fig. 3a). Higher free Spd content was observed in shoots from apical explants grown in 2.5 µM BA, differing statistically from shoots in the control treatment (Fig. 3b). Moreover, a higher free-Spm content was observed in shoots from cotyledonary nodal segments in the 2.5 µM BA treatment than in the control (Fig. 3c). The content of total free PAs was significantly higher in shoots from both types of explants incubated in 2.5 µM BA than in shoots from the control treatment (Fig. 3d).

Effect of BA and the type of explant on the proteomic profile during shoot development

Proteomic analysis was performed comparing the effects of BA (by the comparisons BA2.5_Apical/BA0_Apical and BA2.5_Cotyledonary/BA0_Cotyledoenary) and the type of explant (by the comparisons BA2.5_Cotyledonary/BA2.5_Apical and BA0_Cotyledonary/BA0_Apical) on shoot development. A total of 1232 proteins were identified (Supplementary Table 1). Among the DAPs, some proteins were highlighted according to their relevance to cell division and shoot growth.

Comparing the BA concentrations (0 and 2.5 µM) in shoots obtained from apical nodal segments (BA2.5_Apical/BA0_Apical), a total of 292 proteins were differentially accumulated (DAPs), and 923 were unchanged. Among the DAPs, 93 proteins were up- and 158 down-accumulated, with 13 unique

in shoots grown under BA2.5_Apical and 28 unique in shoots under BA0_Apical (Fig. 4a; Supplementary table 1). In the comparison of BA concentration treatment in shoots from cotyledonary nodal segments (BA2.5_Cotyledonary/BA0_Cotyledonary), a total of 374 proteins were DAPs, and 846 were unchanged. Among the DAPs, 179 were up- and 158 were down-accumulated. In addition, 18 proteins were unique in shoots from cotyledonary nodal segments incubated with 2.5 µM BA (BA2.5_Cotyledonary), and 19 proteins were unique in shoots from cotyledonary nodal segments without BA (BA0_Cotyledonary) (Fig. 4b; Supplementary table 1).

Among these proteins, some were up-accumulated in shoots from both types of explants (apical and cotyledonary nodal segments) incubated with 2.5 BA compared to shoots without BA (comparisons BA2.5_Cotyledonary/BA0_Cotyledonary and BA2.5_Apical/BA0_Apical), as the pre-mRNA-splicing factor ATP-dependent RNA helicase DEAH7-like (A0A444YGB9), calreticulin-3 (A0A445A993), aspartate aminotransferase 1 (A0A445B4C1), protein elongation factor 1-alpha (A0A444Y7Y0) and cell division control protein 48 homolog D (A0A444Z3W1) (Supplementary Table 1). In addition, the proteins phosphoenolpyruvate carboxylase 2 (A0A445BXQ0), phosphoribosylamine-glycine ligase isoform X1 (A0A445E7J0), FT-interacting protein 3 (A0A444ZYV6), dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48 kDa subunit (A0A445A8R7), and 60S ribosomal protein L35a-1 (A0A444ZT56) were up-accumulated in shoots from cotyledonary nodal segments incubated in 2.5 µM BA compared to shoots without BA (comparison BA2.5_Cotyledonary/BA0_Cotyledonary) (Supplementary Table 1).

The effect of explant type at the same BA concentration on the protein profile was evaluated. Comparing cotyledonary shoots from apical nodal segments incubated at 2.5 µM BA (comparison BA2.5_Cotyledonary/BA2.5_Apical), 242 proteins were DAPs, and 973 were unchanged. Among the DAPs, 154 were up- and 46 were down-accumulated, with 28 proteins unique to shoots under the BA2.5_Cotyledonary treatment and 14 proteins unique to shoots under the BA2.5_Apical treatment (Fig. 4c). Comparing the shoots from cotyledonary to apical nodal segments incubated without BA (comparison BA0_Cotyledonary/BA0_Apical), a total of 231 proteins were DAP, and 997 were unchanged (Fig. 4d). Among the DAPs, 89 were up- and 90 down-accumulated, presenting 26 unique proteins in shoots from cotyledonary nodal segments without BA (BA0_Cotyledonary treatment) and 26 unique proteins in shoots from apical nodal segments without BA (BA0_Apical treatment) (Supplementary table 1). Among the DAPs, the malate dehydrogenase 2, peroxisomal (A0A445DRP9), ATP synthase CF1 beta subunit (A0A445AH22) proteins were up-accumulated in shoots from cotyledonary nodal segments compared to those from apical segments at both BA concentrations. The bifunctional dTDP-4-dehydrorhamnose 3,5-epimerase/dTDP-4-dehydrorhamnose reductase (A0A444Z945) protein was unique in *D. nigra* shoots from BA-treated cotyledonary nodal segments compared to shoots without BA (BA2.5_Cotyledonary/BA0_Cotyledonary) and shoots from BA-treated apical nodal segments (BA2.5_Cotyledonary/BA2.5_Apical).

Effect of IBA on *ex vitro* rooting of shoots and acclimatization

The ex vitro root induction of shoots was not significantly affected by IBA concentrations or types of explants (Fig. 5). However, the induction of roots was significantly affected by BA supplementation on the culture medium of shoot development, with the induction of roots being significantly higher in shoots grown in culture medium without BA (Fig. 5a) compared to 2.5 µM BA (Fig. 5b). The number of roots was also significantly affected by the BA concentration used on shoot multiplication, being significantly higher in shoots multiplied without BA (Fig. 5c) compared to BA (Fig. 5d). The length of shoots was significantly affected by BA treatment during shoot multiplication and was significantly higher in shoots from cotyledonary nodal explants grown with BA (Figs. 5e and 5f). On the other hand, the length of roots was not significantly affected by IBA concentrations used in shoots from either type of explant used (Figs. 5e and 5f).

Discussion

The establishment of the best culture medium for *in vitro* seed germination is relevant to obtaining explants for micropropagation. In addition to no significant differences in seed germination (Fig. 1a), the WPM culture medium resulted in seedlings with better growth (Fig. 1c). In *Cariniana legalis*, the WPM culture medium improved the percentage of *in vitro* seed germination, which was significantly higher than that of MS culture medium (Aragão et al. 2017a). The WPM culture medium has only 25% of the concentrations of nitrate and ammonium ions present in MS culture medium, in addition to more potassium and a high level of sulfate ions, which are widely used for micropropagation of woody species (Hazubska-Przybył 2019). The lower concentrations of total nitrogen and ammonium in WPM culture medium reduce the possibility of toxicity to ammonium, which can contribute to the development of seedlings in some woody species (Phillips and Garda 2019), as observed in *D. nigra* in the present work.

For shoot development, BA is the most common cytokinin used for the proliferation of axillary buds in many plant species (Sahai and Shahzad 2013), including several trees, such as *Santalum album* (Mujib 2005), *Cedrela fissilis* (Aragão et al. 2016; Aragão et al,. 2017b), *Azadirachta excelsa* (Foan and Othman 2006), *Sapium sebiferum* and *Calophyllum brasiliensis* (Stein et al. 2017). The structural stability of BA and the ability of plant cells to easily assimilate make this cytokinin an efficient promoter of plant development (Ahmad et al. 2013). Our results showed that BA addition was essential to increase the length of shoots from both types of explants (apical and cotyledonary nodal segments) and culture media (MS and WPM) (Fig. 2) in *D. nigra*. The use of BA also promoted longer shoots in *Juglans nigra* (Stevens and Pijut 2018) and *Rauvolfia tetraphylla* (Hussain et al. 2018). This promotion in the length of BA-induced shoots may be associated with the effects of cytokinins in the control of cell division, providing greater growth and development (Wybouw and De Rybel 2019). In addition to the positive effects of BA on shoot length, this cytokinin showed no effects on shoot induction (%) or the number of shoots per explant (Fig. 2) in *D. nigra*. Similarly, no significant effects of BA on the number of shoots were observed for *C. legalis* (Aragão et al. 2017a). On the other hand, in *Dalbergia sisso*, the use of 4.4 µM BA provided a greater number of shoots compared to the control (Sahu et al. 2014). These results

show that the *in vitro* morphogenic response induced by BA is intrinsic to the species, and this response may be different even within species of the same genus as that observed between *D. nigra* and *D. sissoo*.

In addition to cytokinins, PAs are involved in plant growth and development, as they can act in various physiological processes, such as the promotion of cell division, differentiation, and elongation, which are essential to embryo development, seed germination, rhizogenesis and shoot development in woody plants (Santa-Catarina et al. 2006; Kusano et al. 2008; Pieruzzi et al. 2011; Aragão et al. 2016; Lerin et al. 2019). The higher content of free Put (Fig. 3a) in shoots from cotyledonary and apical nodal segments incubated with BA was correlated with the higher length of shoots in *D. nigra* (Figs. 2c and 2d). Similar results were observed during *in vitro* shoot development in other species, such as *Bixa orellana* (Parimalan et al. 2011) and *C. fissilis* (Aragão et al. 2016; Oliveira et al. 2020). A high content of Put was shown to be directly related to cell cycle progression at the G1/S transition, stimulating the synthesis of proteins such as tubulins, which contribute to cell growth (Tiburcio et al. 2014). Cross-talk among PAs and other plant hormones, such as cytokinin, has been proposed, as BA can affect PA metabolism and thereby their homeostasis by changing the expression of the genes responsible for PA biosynthesis, catabolism, or both (Ahanger et al. 2020). Therefore, the modulation of endogenous PA contents is relevant for shoot elongation in *D. nigra.*

Comparative proteomics is an important tool for the comprehension of physiological and molecular processes during *in vitro* morphogenesis, as it is possible to compare DAPs under different treatments (Heringer et al. 2018). This approach was applied in the present work to comprehend the effects of BA and the type of explant (apical and cotyledonary nodal segments) (Fig. 4) on protein accumulation during shoot development in *D. nigra*. The accumulation of some proteins was significantly affected by BA addition in the shoots of *D. nigra* from both types of explants (Supplementary Table 1), such as the up-accumulation of the factor ATP-dependent RNA helicase DEAH7 (A0A444YGB9). This protein is involved in the expression of genes related to auxin-mediated development, such as the apical-basal standardization of embryonic development and vascular development in *Arabidopsis* (Tsugeki and Terada 2015). Thus, the accumulation of this protein under BA treatment can improve shoot elongation in *D. nigra,* probably interacting with auxin metabolism, as this protein is related to auxin polar transport. Auxin polar transport is essential for cell elongation of the embryo scutellum owing to auxin-induced cell acidification and elongation to the plasma membrane, enabling growth (Chen et al. 2010).

Calreticulin is a molecular calcium-binding chaperone that promotes folding, oligomeric assembly and quality control in the endoplasmic reticulum through the calreticulin/calnexin cycle. Calcium is an important stabilizing agent in the control of plant cell metabolism, playing a role in the structure and permeability of cell membranes, cell division and elongation, translocation of carbohydrates and nitrogen metabolism, presenting a direct effect on plant growth (Ahmad et al. 2016). In this sense, the increase in the accumulation of calreticulin-3 (A0A445A993) protein BA induced in shoots from both types of explants may be related to calcium and other compounds important for shoot elongation observed in *D. nigra.* In addition, calreticulin proteins are able to transiently interact with almost all monoglucosylated glycoproteins necessary for the accumulation of elongation factor receptors (Ahmad et al. 2016).

In the present work, the up-accumulation of elongation factor 1-alpha (A0A444Y7Y0) protein in shoots from both types of explants under BA treatment compared to shoots grown without BA (Supplementary Table 1) could be relevant for the best shoot development in *D. nigra*. This protein works as a promoter of GTP-dependent binding of aminoacyl-tRNA to ribosome sites during protein biosynthesis, an important process in growth and development (White et al. 2019). In addition, the up-accumulation of protein cell division cycle protein 48 homolog (A0A444Z3W1) in shoots from both types of explants could be relevant for the higher shoot elongation BA-induced in *D. nigra*. This protein is directly related to cell division, cytokinesis and growth processes in plants (Rancour et al. 2002).

Nitrogen (N) is essential to carbon skeletons for the biosynthesis of the primary amino acids glutamine and glutamate, which serve as N donors for the biosynthesis of major N compounds in plants, including other amino acids, nucleic acid bases, PAs, and chlorophyll (de la Torre et al. 2014). The aspartate aminotransferase protein is important for aspartate biosynthesis and plays a key role in the metabolic regulation of carbon and nitrogen metabolism in all organisms (Cánovas et al. 2007). The induced gene silencing of aspartate aminotransferase in *Nicotiana benthamiana* causes a reduction in growth and chlorosis symptoms and decreases the levels of chlorophyll and lignin (de la Torre et al. 2014). Moreover, aspartate aminotransferase activity was involved in biomass increments in *Brassica napus* (McAllister et al. 2016). The up-accumulation of aspartate aminotransferase 1 (A0A445B4C1) protein in shoots from both types of explants grown under BA treatment (Supplementary Table 1) may be important for the increase in biomass due to the higher length of shoots in *D. nigra* induced by BA.

The protein phosphoribosylamine-glycine ligase is associated with N assimilation in bacterial nitrogen fixation (Resendis-Antonio et al. 2011), and the accumulation of this protein (A0A445E7J0) in shoots from BA-treated cotyledonary nodal segments can promote the elongation of *D. nigra* shoots, altering nitrogen metabolism. In addition to nitrogen, carbohydrate metabolism is essential for energy supply in plants. The phosphoenolpyruvate carboxylase protein is an important cytosolic enzyme situated at a crucial branch point of plant carbohydrate metabolism (Scholl et al. 2020). Phosphoenolpyruvate carboxylase 2 also fulfils essential nonphotosynthetic functions, particularly the replenishment of tricarboxylic acid (TCA) cycle intermediates consumed during biosynthesis and N assimilation (Scholl et al. 2020). Some developmental or metabolic processes that require these organic acids will benefit from increased carbon flux through the phosphoenolpyruvate carboxylase 2 reaction (Willick et al. 2019). Thus, the up-accumulation of phosphoenolpyruvate carboxylase 2 (A0A445BXQ0) could be relevant for the elongation of shoots from BA-treated cotyledonary nodal segments compared to those without BA in *D. nigra* (Supplementary Table 1). Another up-accumulated protein in shoots from cotyledonary nodal explants incubated with BA compared to those without BA was the FT-interacting protein (A0A444ZYV6) (Supplementary Table 1). This protein plays an essential role in mediating the proliferation and differentiation of shoot stem cells in *Arabidopsis* (Liu et al. 2018). FT-interacting protein prevents intracellular trafficking of a key regulator, SHOOTMERISTEMLESS, to the plasma membrane in cells in the peripheral shoot meristem region. This facilitates SHOOTMERISTEMLESS recycling to the nucleus to maintain stem cells and accelerates stem cell differentiation (Liu et al. 2018).

In this sense, these proteins may be interesting to shed light on BA signaling for the promotion of higher shoot elongation in *D. nigra*.

In addition, the dolichyl-diphosphooligosaccharide glycosyltransferase protein is known to be involved in protein glycosylation and protein modification and participates in biological processes relevant for plant growth and development, such as mechanisms controlling the assembly of cell wall polymers, protein N-linked glycosylation through asparagine and cell growth (Lerouxel et al. 2005). An increase in the accumulation of dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48 kDa subunit (A0A445A8R7) protein in shoots from cotyledonary nodal segments BA-treated can be related to cytokinin promotion of the higher elongation of shoots in *D. nigra*.

Citrate synthase mitochondrial (mtCS) (A0A445EVE9 and A0A445CF35) was another protein up-accumulated only in shoots from BA-treated cotyledonary nodal segments. The citrate synthase mitochondrial (mtCS) protein has an enhanced ability to excrete citric acid, and the overexpression of mtCS in carrot cells results in better cell growth than that in wild-type cells (Koyama et al. 1999). It appears that the overexpression of citrate synthase in *Arabidopsis* improves growth in phosphorus-limited soils due to the increased excretion of citrate from the roots (Koyama et al. 2000). This evidence may suggest that the increase in the accumulation of citrate synthase mitochondrial (mtCS) (A0A445EVE9 and A0A445CF35) proteins in shoots from cotyledonary explants treated with BA can regulate oxidative metabolism, promoting the elongation of shoots in *D. nigra*.

Another up-accumulated protein, the phosphoribosylamine-glycine ligase chloroplastic (A0A445E7J0), is involved in enzymes in the *de novo* purine biosynthesis pathway (Zhang et al. 2018). Plants can degrade purines, and the final products glyoxylate and ammonia are recovered to synthesize organic molecules for new growth (Amarante et al. 2006). The increase in accumulation of this protein may be involved in the biosynthesis of organic molecules necessary for the higher growth of *D. nigra* shoots from cotyledonary nodal segments incubated with BA. The 60S ribosomal protein L35a-3 (A0A444ZT56) observed in *D. nigra* shoots from cotyledonary nodal segments treated with BA (Supplementary Table 1) is a structural constituent of ribosomes and has cytoplasmic translation and ribosomal large subunit biogenesis as a biological function (Xiao et al. 2019). The eukaryotic ribosome is a complex structure composed of several ribosomal RNAs and ribosomal proteins (r-proteins) (Taylor et al. 2009), which are responsible for protein synthesis necessary for cell growth, division, and development (Barakat et al. 2001). It has been shown that genetic defects in ribosomal components, such as a reduction in the levels of individual r-proteins, can induce deleterious effects on the development of plants (Barakat et al. 2001). Thus, a higher accumulation of 60S ribosomal protein L35a-3 (A0A444ZT56) could be relevant to maintaining higher levels of r-proteins and, consequently, higher elongation of *D. nigra* shoots from cotyledonary nodal segments incubated with BA.

The bifunctional dTDP-4-desidrorhamnose 3,5-epimerase/dTDP-4-desidrorhamnose reductase (A0A444Z945) protein was unique in *D. nigra* shoots from BA-treated cotyledonary nodal segments compared to shoots without BA (BA2.5_Cotyledonary/BA0_Cotyledonary) and to shoots from BA-

treated apical nodal segments (BA2.5_Cotyledonary/BA2.5_Apical). This protein is involved in dTDP-L-rhamnose biosynthesis, which is part of carbohydrate metabolism (Watt et al. 2004). The analysis of sugar composition and the study of gene expression at different stages of growth indicate that the synthesis of rhamnose-containing glycans is under specific tissue regulation (Martinez et al. 2012). In addition, this protein is also present in the cell wall organization process, which can be an interesting factor associated with the differential elongation of *D. nigra* shoots incubated with BA.

Some proteins identified were associated with the type of explant, being more accumulated in shoots from cotyledonary nodal segments compared to apical segments, both with (BA2.5_Cotyledonary/BA2.5_apical comparison) or without BA (BA0_cotyledonary/BA0_apical comparison) (Supplementary Table 1). The malate dehydrogenase 2 protein catalyzes a reversible NAD-dependent dehydrogenase reaction involved in central metabolism and redox homeostasis between organelle compartments (Tomaz et al. 2010) and is also required for the maintenance of photosynthetic rates under photorespiratory conditions (Cousins et al. 2008). ATP synthase subunit beta, chloroplastic (A0A445AH22), can be found in the plasma membrane of eubacteria, in thylakoids of chloroplasts and in the inner mitochondrial membrane of eukaryotic cells (Mulkidjanian et al. 2009). Loss of ATP synthase assembly defective in the β subunit results in mitochondria deprived of cristae structures, and when ATP2 is silenced, cells show a peculiar organization of thylakoid stacks in the chloroplast with a reduced number of lamellae compared to wild-type, harming plant development (Lapaille et al. 2010). The up-accumulation of dehydrogenase 2 (peroxisome) (A0A445DRP9) and ATP synthase subunit beta and chloroplastic (A0A445AH22) in shoots of *D. nigra* from cotyledonary nodal segments treated or not with BA shows that this type of explant better regulates redox homeostasis between organelle compartments and ATP biosynthesis, an essential process for growth.

Rooting is a critical phase of *in vitro* propagation, and overcoming this phase can ensure the success of the process (Zeng et al. 2019). Usually, exogenous auxins are necessary to promote root induction in some species, as observed for *Malus domestica* rootstocks (Meng et al. 2019) and *Populus alba* (Zeng et al. 2019). *Dalbergia nigra* has the possibility of propagation using the cutting method, however, reaching rates below 50%. In our work, *ex vitro* rooting was efficient for plantlet production using shoots from both types of explants, with no necessity of IBA use (Figs. 5a and 5b). IBA was also not necessary for root induction or the number of roots in *Prunus persica* and *Prunus davidiana* (Zhou et al. 2010). Thus, it was possible to reach a rate higher than 80% rooting, showing better results when compared to the cutting technique used in the species.

Moreover, the balance between auxins and cytokinins is important for root induction (Růžička et al. 2009; Jing and Strader 2019), and differences in rooting may occur due to the accumulation of cytokinins in plant tissues (Da Costa et al. 2013). In the present work, a comparison between shoots multiplied in culture medium without (control) and with 2.5 µM BA was performed to analyze whether shoot multiplication in BA concentrations affects the induction of roots. The use of 2.5 µM BA is essential for shoot elongation; however, the treatments with cytokinin significantly affected the root induction and number of roots in *D. nigra* compared to the treatment without BA (Figs. 5c and 5d). In this way, we can

infer that the balance between auxins and cytokinins influences *D. nigra* shoot rooting. This balance adjustment was also considered important in *Ceropegia bulbosa*, where different concentrations of cytokinin (BA) and auxins, such as naphthalene acetic acid (NAA) and IBA, were tested, demonstrating the importance of crosstalk among hormones (Phulwaria et al. 2013). Unlike in *Albizia lebbeck,* the use of 250 µM IBA promoted the largest number and longer length of roots from shoots grown under concentrations of the cytokinin thidiazuron (Perveen et al. 2013), showing that endogenous hormones present in the explant have an important role in plant organogenesis (Pal et al. 2012; Zeng et al. 2019). Thus, we can infer that the results obtained for rooting depend on the species and concentrations of PGRs for root induction, as well as those used in the shoot multiplication step.

Conclusions

The WPM culture medium promoted the best seedling growth. The addition of BA is necessary for longer shoot lengths for both types of explants. BA addition promoted an increase in endogenous Put content, which induced the higher growth of shoots in both explants. Some proteins involved in central metabolism, redox homeostasis, maintenance of photosynthetic rates and carbon flow during photorespiration conditions were differentially accumulated in shoots from cotyledonary nodal explants with and without BA and are important for the growth of these shoots. *Ex vitro* rooting of shoots can be performed without IBA in both types of explants. This work enabled the production of seedlings that were directed to an ecological reserve. Furthermore, the first results demonstrated the involvement of PAs and proteomic profiles in the development of *D. nigra* shoots.

Abbreviations

BA	benzyladenine
CDC48	Cell division cycle protein 48 homolog
DAPs	Differentially accumulated proteins
DTT	Dithiothreitol
FDR	False discovery rate
IBA	Indole-3-butyric acid
MS	Murashige and Skoog
mtCS	Citrate synthase mitochondrial
PA	Polyamines
PGRs	Plant growth regulators

Put	Putrescine
TCA	Trichloroacetic acid
Spd	Spermidine
Spm	Spermine
WPM	Woody Plant Medium

Declarations

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Author contributions LPS and CSC conceived the study, designed the experiments and wrote the manuscript. LSP was responsible for the in vitro culture of shoots and ex vitro rooting experiments and performed the statistical analyses. LSP and VPMA were responsible for PA analyses. LSP, TRO and VS were responsible for the proteomic analyses. All the authors read and approved the final manuscript.

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Data availability The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD031999. All identified proteins are available in the supplementary material.

Code availability PXD031999.

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Figures

Figure 1

Germination (%) of *Dalbergia nigra* seeds after 30 days of incubation in MS and WPM culture media (a) and morphological aspects of 30-day-old seedlings germinated in MS (b) and WPM (c) culture media. (n = 5; coefficient of variation = 13.18%). Bars in figures b and c = 1 cm.

Figure 2

Number of shoots (a and b) and length (cm) of first (c and d) and second (e and f) shoots obtained from apical and cotyledonary nodal segments of *Dalbergia nigra* after 45 days of incubation in MS (a, c, and e) and WPM (b, d and f) culture media. Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test. Capital letters denote significant differences comparing the same type of explant (apical and cotyledonary) at different BA concentrations (0, 2.5 and 5 µM). Lowercase letters denote significant differences between the two types of explants (apical and cotyledonary) at the same BA concentration (0, 2.5 or 5 µM). Asterisks (*) denote significant differences comparing the MS and WPM culture media for apical nodal segment explants at the same BA concentration (0, 2.5 or 5 µM). CV = Coefficient of Variation. (n = 8; CV of shoot number = 21.13%; CV of length of first shoot = 29.82%; CV of length of second shoot = 66.4%).

Figure 3

Free Put (a), Spd (b) and Spm (c) contents ($\mu g.g^{-1}$ FM) in shoots obtained from apical and cotyledonary nodal segments of *Dalbergia nigra* at 45 days of incubation on WPM culture medium without and with 2.5 µM BA. Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test. Capital letters denote significant differences between the same type of explant (apical or cotyledonary) at different BA concentrations (0 and 2.5 µM). Lowercase letters denote significant differences between different types of explants (apical and cotyledonary) at the same BA concentration (0 or 2.5 µM). CV = Coefficient of Variation. (n = 3; CV of Put = 8.33%, CV of Spd = 10.99%, CV of Spm = 15.16%, CV of total free PAs = 7.57%).

Figure 4

Differentially accumulated proteins (DAPs) in 45-day-old shoots of *Dalbergia nigra* obtained from apical and cotyledonary nodal segments used as explants in the following comparisons: BA2.5_Apical/BA0_Apical (a), BA2.5_Cotyledonary/BA0_Cotyledonary (b), BA2.5_Cotyledonary/BA2.5_Apical (c) and BA0_Cotyledonary/BA0_Apical (d).

Figure 5

Root induction (a, b), root number (c, d) and root length (cm) (e, f) in shoots from two types of explants (apical and cotyledonary nodal segments) of *Dalbergia nigra* obtained in WPM culture medium without (0 µM) and with 2.5 µM BA at 45 days in acclimatization. Capital letters denote significant differences comparing the same type of explant (shoots from apical or cotyledonary nodal segments) at different IBA concentrations. Lowercase letters denote significant differences comparing shoots from the two types of explants (apical and cotyledonary nodal segments) at the same IBA concentration. Asterisks (*) denote significant differences for shoots from apical and cotyledonary nodal segments comparing the BA treatment (0 and 2.5 µM) at each IBA concentration. (n = 8; CV of root induction = 27.4%; CV of root number = 24.92%; CV of root length = 24.4%)

Supplementary Files

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- TableS1.xlsx