The inhibition of putrescine synthesis affects the in vitro shoot development of Cedrela fissilis Vell. (Meliaceae) by altering endogenous polyamine metabolism and the proteomic profile

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Research Article

Keywords: D-arginine , Enzymatic activities , Polyamine biosynthesis inhibitor , Morphogenic competence

Posted Date: September 16th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2049319/v1

Abstract

Polyamines (PAs) and proteins have been demonstrated to be fundamental for *in vitro* shoot development of *Cedrela fissilis.* We evaluated the influence of 6-benzyladenine (BA) and putrescine (Put) on the growth of shoots, PA metabolism and proteomic profiles of *C. fissilis*.The longest shoots were obtained under 2.5 µM BA + 2.5 mM Put treatment. The inhibition of Put synthesis by D-arginine (D-arg) reduced the activities of the Put biosynthesis enzymes arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) and the endogenous contents of free Put, resulting the reduced shoot growth. The ODC activity was higher than that ofADC, which is the main enzyme in the synthesis of Put in *C. fissilis*. Inhibition of Put synthesis affected the proteomic profile, reducing the accumulation of the ubiquitin receptor RAD23c, peroxidase 15, ADP-ribosylation factor 1, ADP-ribosylation factor-like protein 8a, profilin-4, profilin-2, glucan endo-1,3-beta-glucosidase, and expansin-like B1 and increasing the accumulation of V-type proton ATPase catalytic subunit A and methionine gamma-lyase, highlighting the relevance of these proteins in promoting the length of shoots. Moreover, the transport protein SEC13 homolog B and the basic isoform glucan endo-1,3-beta-glucosidase, unique proteins in shoots treated with BA+Put, were related to the promotion of shoot growth. Our results show that the modulation of endogenous PAs and proteomic profiles is necessary to regulate *in vitro* morphogenesis in *C. fissilis*. Moreover, the ODC enzyme is highly involved in the synthesis of Put during *in vitro* shoot development and is described for the first time in this species.

Introduction

Polyamines (PAs) are aliphatic, low-molecular-weight molecules containing positively charged nitrogen atoms that can interact with macromolecules, such as DNA, RNA, phospholipids, cell wall components, and proteins, regulating the growth of plants (Baron and Stasolla 2008). Studies have shown that PAs are associated with several essential processes in plant growth and development, including cell division and differentiation, growth, regulation of gene expression and cell signaling, stability of nucleic acids, membrane structure and plant survival (Kusano et al. 2008; Chen et al. 2019). In higher plants, the three main PAs are putrescine (Put), spermidine (Spd), and spermine (Spm), which are involved in plant morphogenesis. In PA biosynthesis, Put is synthesized from arginine (Arg) by the action of arginine decarboxylase (ADC), which constitutes the main Put synthesis pathway in plants, and from ornithine (Orn) by the action of ornithine decarboxylase (ODC) (Chen et al. 2019).

The use of PAs as plant growth regulators has been linked to increased rates of *in vitro* development for some species, such as *Bixa orellana* and *Glycine max* (Parimalan et al. 2011; Arun et al. 2014). For *Cedrela fissilis* Vell. (Meliaceae), an endangered woody species from the Brazilian Atlantic rainforest (IUCN 2020), *in vitro* propagation studies and *ex vitro* rooting have been developed (Nunes et al., 2002; Aragão et al. 2016; Aragão et al. 2017; Oliveira et al. 2020; Oliveira et al. 2022; Ribeiro et al. 2022). Studies have shown that the addition of 6-benzyladenine (BA) to culture media increased the endogenous contents of free Put, which is related to shoot growth (Aragão et al. 2016). In addition, the addition of exogenous Put contributes significantly to the elongation of shoots and modulation of both endogenous

PAs and proteomic profiles in this species (Aragão et al. 2017). However, the effects of BA and Put, as well as the inhibition of Put synthesis, on shoot development from axillary buds have not yet been studied in *C. fissilis* but are relevant to understanding the interaction between cytokinins and PAs on *in vitro* morphogenesis in this species.

The use of inhibitors of PA synthesis can be an important approach to understanding the functions of these substances in developing plants, as PAs are considered biochemical markers of morphogenic competence and plant regeneration ability (Rey et al. 1994; Shoeb et al. 2001). Among the inhibitors of PA synthesis, D-arginine (D-arg) is a competitive inhibitor of the activity of the ADC enzyme, which is responsible for Put synthesis from Arg and has been used in *in vitro* morphogenesis studies in plants (Königshofer and Lechner 2002; Liu et al. 2009; Cheng et al. 2015). The use of D-arg inhibited the growth of *Malus sylvestris* shoots (Hao et al. 2005; Liu et al. 2009). Moreover, the inhibition of Put biosynthesis by D-arg alters protein synthesis during the *in vitro* germination of pollen (Falasca et al. 2010). Taken together, these studies show that D-arg can be used as a tool for *in vitro* morphogenesis responses associated with biochemical investigations, such as proteomic profiling.

Proteomics approaches have been applied to understand the qualitative and quantitative changes in proteins during *in vitro* morphogenesis (Ghosh and Pal 2013; Reis et al. 2016; Aragão et al. 2017), allowing the identification of proteins that are associated with specific *in vitro* development processes (Heringer et al. 2015; Reis et al. 2016; Passamani et al. 2017, 2018). The acquisition of morphogenic competence for *in vitro* shoot development involves specific responsive explants, such as cotyledons obtained from mature seeds or cotyledonary nodal segments obtained from young seedlings (Ghosh and Pal 2013; Aragão et al. 2017). In *C. fissilis*, the addition of Put affects shoot development from cotyledonary nodal segments, altering the accumulation of proteins, including those related to cell division (Aragão et al. 2017). The identification of possible candidate protein markers associated with morphogenic competence via comparative proteomics is important for understanding *in vitro* shoot development. In this sense, the inhibition of Put biosynthesis can provide important information about how modulation of PA metabolism and protein accumulation can promote *in vitro C. fissilis* shoot growth. Thus, the aim of this work was to evaluate the influence of BA and Put on the shoot growth, endogenous PA metabolism and proteomic profiles of *C. fissilis*.

Materials And Methods

Plant material

C. fissilis seeds were obtained in September 2016 from the Caiçara Comércio de Sementes LTDA located in Brejo Alegre, SP, Brazil (21°10'S and 50°10'W). The mature seeds were germinated *in vitro* according to the methods of Aragão et al. (2016) to obtain aseptic seedlings, which were used as explant sources for the experiments.

Effects of exogenous BA and Put on *in vitro* shoot development

To evaluate the effects of BA and Put on shoot development, cotyledonary and apical nodal segments (explants approximately 2 cm in length) were isolated from 60-day-old seedlings and cultured in Murashige and Skoog (MS) (Murashige and Skoog 1962) media (M519; Phytotechnology Lab, Lenexa, USA) supplemented with 20 g L^{-1} sucrose (Vetec, Rio de Janeiro, Brazil), 2 g L^{-1} Phytagel (Sigma–Aldrich, St. Louis, USA), and Put (0 or 2.5 mM) (Sigma–Aldrich) together with different concentrations (0, 0.5, 1.0, 2.5 or 5.0 μM) of BA (Sigma–Aldrich). Together, there were six treatments: a control (absence of BA and Put); 0 μM BA + 2.5 mM Put; 0.5 μM BA+2.5 mM Put; 1.0 μM BA+2.5 mM Put; 2.5 μM BA+2.5 mM Put and 5.0 μM BA+2.5 mM Put. The pH of the culture media was adjusted to 5.7 before autoclaving at 121 °C and 1.5 atm for 15 min. Put was prepared in water, with the pH adjusted to 5.7, and incorporated into the MS culture media by filter sterilization using a 0.22-μm filter (Biofil, Guangzhou, China). Each culture medium (30 mL) was aliquoted into 150-mL glass culture vessels (8 cm in height × 5 cm in diameter; Aapace, São Paulo, Brazil). The explants were then transplanted into the culture media composing the different treatments and incubated in a growth chamber with a temperature of 25±2 °C and a photoperiod of 16 h of light (55 μmol m^{-2} s^{-1}) for 30 days according to a protocol by Aragão et al. (2017). Each treatment comprised eight replicates, and each replicate consisted of one glass culture vessel with five explants. The length (cm) of the shoots, induction (%) and number of shoots per explant were evaluated after 30 days of incubation.

Effects of the inhibition of Put synthesis on *in vitro* shoot development

To evaluate the effects of the inhibition of Put synthesis on shoot development, the inhibitor D-arg was used according to the methods of Liu et al. (2009), with modifications. Cotyledonary and apical nodal segments were isolated and transferred to MS culture media supplemented with 20 g L^{-1} sucrose, 2 g L^{-1} Phytagel, BA (0 or 2.5 μM) and Put (0 or 2.5 mM) together with different concentrations (0, 1.0 or 5.0 mM) of D-arg (Sigma–Aldrich). Together, there were six treatments: a control (absence of BA, Put and D-arg); 1.0 mM D-arg, 5.0 mM D-arg; 2.5 μM BA+2.5 mM Put; 2.5 μM BA+2.5 mM Put+1.0 mM D-arg and 2.5 μM BA+2.5 mM Put+5.0 mM D-arg. Put and D-arg were prepared in water, with the pH adjusted to 5.7, and were incorporated into the MS culture media after filter sterilization using a 0.22-μm filter. Each treatment was aliquoted into 150-mL culture vessels (30 mL per culture vessel). After they were transplanted into the culture media, the explants were maintained in a growth chamber under the same conditions as those described previously. Each treatment comprised eight replicates, and each replicate consisted of one glass culture vessel with five explants. For all the treatments, the length (cm) of the shoots, induction (%) and number of shoots per explant were evaluated after 30 days of incubation.

Three biological samples of whole shoots (including the stem + leaves and apical meristem) after 30 days of incubation were collected to analyze the endogenous PA content and ADC and ODC enzyme activities. With respect to proteomic analysis, three biological samples of whole shoots from cotyledonary nodal segments were collected from the treatment with the highest shoot length and from the treatment in which D-arg significantly inhibited shoot growth. All biological samples were stored at -80 °C until analysis.

Free PA determination

The free PA contents were determined according to the methods of Aragão et al. (2016). Samples (three biological replicates of 200 mg fresh matter [FM] per sample) from each treatment were ground in 1.3 mL of 5% perchloric acid (Merck, Darmstadt, Germany). After incubation for 1 h at 4 °C, the samples were centrifuged at 20,000×*g* for 20 min at 4 °C, and free PAs were collected directly from the supernatant. Free PAs were derivatized with dansyl chloride (Merck) and identified by high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan); the HPLC instrument was equipped with a 5-µm C18 reverse-phase column (Shimadzu Shin-pack CLC ODS). The HPLC column gradient was generated by adding increasing volumes of absolute acetonitrile (Merck) to a 10% acetonitrile solution, and the pH was adjusted to 3.5 with hydrochloric acid (Merck). 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 performed at a flow rate of 1 mL min^{-1} at 40 °C. A fluorescence detector at 340 nm (excitation) and 510 nm (emission) was used to determine the PA content. The peak areas and retention times of the samples were measured by comparisons with PA Put, Spd, and Spm standards (Sigma–Aldrich).

ADC and ODC enzyme assays

The activities of the ADC and ODC enzymes were determined according to the methods of de Oliveira et al. (2017), with modifications. Three biological replicates from each treatment were used, consisting of 500 mg FM per sample. Samples were homogenized in liquid nitrogen using an ice-cold mortar and then transferred to 500 µL of extraction buffer composed of 50 mM Tris-HCl (Invitrogen, Carlsbad, USA) (pH 8.5), 0.5 mM pyridoxal-5-phosphate (Sigma–Aldrich), 0.1 mM ethylenediaminetetraacetic acid (EDTA) (Sigma–Aldrich) and 5 mM dithiothreitol (DTT) (Bio-Rad, Hercules, USA). The samples were vortexed and centrifuged at 13,000×*g* for 20 min at 4 °C, and then the supernatant was used for ADC and ODC enzyme assays. For the assays, a reaction mixture containing 100 µL of protein extract, 8.3 µL of extraction buffer, 12 mM unlabeled L-Arg (Sigma–Aldrich) or L-Orn (Sigma–Aldrich) and 25 nCi of either L-[^{14}C(U)]-Arg (specific activity of 274.0 mCi $mmol^{-1}$) (PerkinElmer, Houten, The Netherlands) or L-[1-^{14}C]-Orn (specific activity of 57.1 mCi $mmol^{-1}$) (PerkinElmer) was used. Samples containing only 100 µL of extraction buffer were used as blanks. The reaction mixtures were incubated in glass tubes fitted with rubber stoppers and filter paper discs (GE Healthcare, Piscataway, USA). The mixtures were subsequently soaked in 2 N KOH (Vetec). The mixtures were kept at 37 °C with rotation at 120 rpm via an orbital shaker for 90 min. Each reaction was stopped by the addition of 200 µL described above. Filter paper containing $^{14}CO_2$ was immersed in 1 mL of scintillation fluid (PerkinElmer), and the radioactivity was measured using a scintillation counter (Tri-Carb 2910 TR, PerkinElmer). The protein content was measured using the Bradford method (Bradford 1976), with bovine serum albumin (Sigma–Aldrich) as the standard. The activities were expressed as nanomoles of $^{14}CO_2$ per milligram of protein per hour.

Proteomic analysis

Protein extraction was performed using the trichloroacetic acid (TCA)/acetone method according to a protocol by Damerval et al. (1986), with modifications. Three biological samples of shoots containing leaves and apical meristems (300 mg FM per sample) were frozen in liquid nitrogen and then ground into a fine powder. Then, 300 milligrams of fine powder per sample was resuspended in 1 mL of chilled solution containing 10% (w/v) TCA (Sigma–Aldrich) in acetone (Sigma–Aldrich) together with 20 mM DTT (GE Healthcare) and vortexed for 5 min at 8 °C. The mixture was maintained at - 20 °C for 1 h before centrifugation at 16,000×*g* for 30 min at 4 °C. Then, the supernatant was discarded, and the resulting pellets were washed three times with cold acetone plus 20 mM DTT and centrifuged for 5 min for each wash. The pellets were air dried and resuspended in buffer consisting of 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). Next, the samples were vortexed for 30 min at 8 °C and then centrifuged at 16,000 × *g* for 20 min. The supernatant from each sample was collected, and the protein concentration was measured by a 2-D Quant Kit (GE Healthcare).

Before the trypsin digestion step, the protein samples were precipitated with methanol/chloroform (Nanjo et al. 2012) and resuspended in a solution of 7 M urea/2 M thiourea. Protein digestion was performed using 100 µg of protein from each sample 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. The resulting peptides were quantified according to the A205 nm protein and peptide methodology using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, USA). The samples were transferred to Total Recovery Vials (Waters) for mass spectrometry analysis.

A nanoACQUITY ultraperformance liquid chromatograph (UPLC) connected to a Q-TOF SYNAPT G2-Si instrument (Waters, Manchester, UK) was used for electrospray-liquid chromatography-tandem mass spectrometry analysis according to (Passamani et al. 2018). For each treatment, three biological replicates of 1 µg of digested peptide per sample were used. For separation, the samples were loaded onto a nanoACQUITY UPLC M-Class Symmetry C18 5 µm trap column (180 µm × 20 mm) at 5 µL min^{-1} for 3 min and then onto a nanoACQUITY M-Class HSS T3 1.8-µm analytical reversed-phase column (75 µm × 150 mm) at 400 nL min^{-1} and 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 and mobile phase B consisting of acetonitrile and 0.1% formic acid. The gradient elution started with 7% B, increased from 7 to 40% B until 91.12 min, increased again from 40 to 99.9% B until 92.72 min, and then remained at 99.9% until 106.00 min, followed by a decrease to 7% B until 106.1 min, and then remained at 7% B until 120 min, the end of the run. Mass spectrometry was performed in positive and resolution mode (V mode), 35,000 full width at half maximum (FWHM), with ion mobility separation (IMS), and in data-independent acquisition mode ($HDMS^E$). The ion mobility wave was set to 600 m s^{-1}, with helium and IMS gas flow rates of 180 and 90 mL min^{-1}, respectively. The external calibrant used was human [Glu1]-fibrinopeptide B

(100 fmol μL^{-1}). Lock mass acquisition was performed every 30 s, and mass spectra were acquired via MassLynx version 4.1 software.

Spectral processing and comparative analysis were performed according to Passamani et al. (2020). Spectra processing and database searching were performed using ProteinLynx Global Server (PLGS) software version 3.0.2 (Waters), and comparative label-free quantification was performed using ISOQuant software version 1.7 (Distler et al. 2014). For ISOQuant, the following parameters were used to identify proteins: a 1% FDR, a peptide score greater than six, a minimum peptide length of six amino acids, and at least two peptides per protein were required for label-free quantitation using the TOP3 approach, followed by the multidimensional normalized process within ISOQuant. For protein identification, the obtained data were processed against the content in the nonredundant *Cedrela fissilis* database (Oliveira et al. 2020). 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 PXD036504.

To ensure the quality of the results after data processing, only the proteins present or absent (for unique proteins) in all three runs were considered for differential accumulation analysis via Student's t test (two-tailed; $P < 0.05$). Proteins with a significant t test result 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. Finally, the sequences of proteins were used in BLAST searches against the Nonredundant (nr) Green Plants/Viridiplantae Protein Sequences database via OmicsBox software (https://www.biobam.com/omicsbox) for the description of high-throughput functional annotation (Gotz et al. 2008) and UniProtKB (http://www.uniprot.org).

Statistical analysis

The data concerning shoot development, free PA content, and the enzymatic activity of ADC and ODC were analyzed by analysis of variance (ANOVA) ($P < 0.05$) followed by the Tukey test using the R statistical environment (R Core Time 2018).

Results

Effects of exogenous BA and Put on *in vitro* shoot development

The combination of BA and Put significantly affected the development of shoots from both cotyledonary and apical nodal segments (Fig. 1). For cotyledonary nodal segments, a higher shoot length (1.51 cm) was observed under the 2.5 µM BA+2.5 mM Put treatment, while a lower length (0.47 cm) was observed under the control treatment, without BA and Put supplementation (Figs. 1a and 2). In addition, no significant differences among the treatments were observed in terms of shoot induction, with percentages ranging from 90 to 100% (Fig. 1b), or the number of shoots per explant, with values ranging from 1.62 to 1.85 (Fig. 1c).

With respect to apical nodal segments, the highest shoot lengths were observed for the 1 μM BA+2.5 mM Put (0.60 cm) and 2.5 μM BA+2.5 mM Put (0.72 cm) treatments (Fig. 1d). The combination of BA and Put did not significantly affect the induction of shoots from this type of explant, with percentages ranging from 90 to 100% (Fig. 1e). The highest number of shoots was observed for the 2.5 mM Put combined with 1, 2.5 and 5 μM BA treatments (with 1.85, 1.85 and 1.80 shoots per explant, respectively) (Fig. 1e), while the lowest number of shoots was observed under the 2.5 mM Put treatment (1.09 shoots per explant) (Fig. 1e).

Effects of the inhibition of Put synthesis on *in vitro* shoot development

The effects of the Put synthesis inhibitor D-arg on the growth of shoots from cotyledonary and apical nodal segments of *C. fissilis* after 30 days of incubation were evaluated (Fig. 3). With respect to the cotyledonary nodal segments, the use of 5 mM D-arg promoted stronger inhibition of shoot length (0.16 cm), while the highest shoot length was obtained under the 2.5 μM BA+2.5 mM Put treatment (1.51 cm) (Fig. 3a). In addition, the use of 1 or 5 mM D-arg combined with 2.5 μM BA+2.5 mM Put significantly decreased the shoot length (1.10 and 1 cm, respectively) compared to that under the 2.5 μM BA+2.5 mM Put treatment (1.51 cm) (Fig. 3a). On the other hand, there were no significant differences in shoot induction, with values ranging from 97 to 100% (Fig. 3b), or the number of shoots per explant, with values ranging from 1.93 to 2.00 (Fig. 3c), among all the treatments involving cotyledonary nodal segments.

With respect to the apical nodal segments, the use of 5 mM D-arg strongly inhibited shoot length (0.15 cm), while the use of 1 or 5 mM D-arg combined with 2.5 μM BA+2.5 mM Put did not significantly affect the shoot length; the shoot length was greatest under these treatments (Fig. 3d). No significant differences among the treatments tested were observed for shoot induction, with percentages ranging from 95 to 100% (Fig. 3e). However, there was a significant inhibition in the number of shoots per explant under 1 or 5 mM D-arg (1.11 and 1.08 shoots, respectively) (Fig. 3f), while these D-arg concentrations combined with 2.5 μM BA+2.5 mM Put resulted in the highest number of shoots per explant (Fig. 3f).

Figure 4 shows the morphology of shoots obtained from cotyledonary nodal segments cultured in the presence of 2.5 μM BA+2.5 mM Put, which yielded the greatest shoot length (Fig. 4a), and in the presence of BA and Put combined with 5 mM D-arg, which significantly inhibited shoot growth (Fig. 4b).

Effects of a Put synthesis inhibitor on endogenous free PA contents during shoot development

Due to the greater length of shoots from cotyledonary nodal segments than from the other segments, the free PA contents were analyzed in shoots from cotyledonary nodal segments after 30 days of incubation with 2.5 μM BA+2.5 mM Put and 2.5 μM BA+2.5 mM Put+5 mM D-arg (Figs. 5 and 6). The use of D-arg combined with Put and BA significantly reduced the endogenous contents of free Put (Fig. 5a) and free Spd (Fig. 5b) in the shoots. Compared with the other treatments, the treatments with D-arg (0, 1 and 5 mM) but without BA and Put yielded lower contents of free Put and free Spd (Figs. 5a and 5b). On the other hand, compared with the other treatments, the treatments with 1 or 5 mM D-arg but without BA and Put induced the highest contents of endogenous free Spm (Fig. 5c). Moreover, compared with 2.5 μM

BA+2.5 mM Put, 1 or 5 mM D-arg combined with 2.5 μM BA+2.5 mM Put significantly decreased the endogenous contents of total free PAs (Fig. 6a) and significantly decreased the PA ratio [Put/(Spd+Spm)] (Fig. 6b).

Effects of the inhibition of Put synthesis on ADC and ODC enzyme activities in the shoots

The activities of ADC and ODC were evaluated in the shoots from cotyledonary nodal segments under the 2.5 μM BA+2.5 mM Put and 2.5 μM BA+2.5 mM Put+5 mM D-arg treatments (Fig. 7). Supplementation with a Put synthesis inhibitor (5 mM D-arg) together with BA and Put induced a significant reduction in ADC activity in the shoots from cotyledonary nodal segments compared to shoots under the control and BA + Put treatments (Fig. 7a). On the other hand, the ODC activity increased in shoots incubated under BA + Put treatments compared to the control and BA + Put + D-arg treatments (Fig. 7b). The ODC activity was higher than the ADC activity, indicating that ODC is preferential for Put synthesis during *in vitro* shoot development in this species (Fig. 7).

Effects of a Put synthesis inhibitor on the proteomic profile of the shoots

Proteomic analysis was performed on shoots from cotyledonary nodal segments incubated in the 2.5 μM BA+2.5 mM Put treatment (BA+Put treatment), which promoted increased shoot length, and in the 5 mM D-arg together with 2.5 μM BA+2.5 mM Put treatment (BA+Put+D-arg treatment), which reduced the shoot length.

The inhibition of Put synthesis by D-arg affects the accumulation of proteins. A total of 926 proteins were identified (Supplementary Table S1). Among them, 701 were unchanged, and 218 differentially accumulated, with 91 proteins down- and 127 up-accumulated in the shoots treated with D-arg (BA+Put+D-arg) compared with the shoots grown in the absence of D-arg (BA+Put). In addition, four proteins were unique in shoots grown under the treatment containing D-arg (BA+Put+D-arg), and three proteins were unique in the treatment without D-arg (BA+Put) (Supplementary Table S1). Among the differentially accumulated proteins (up- or down-accumulated and unique), some are highlighted due to their relationship with plant growth and development processes (Supplementary Table S1).

Some proteins were down-accumulated in the shoots incubated in the treatment containing the Put synthesis inhibitor compared to shoots grown without the inhibitor (comparison of BA+Put+D-arg with BA+Put). Among these proteins, two RAD23c ubiquitin receptors (Ce_fissilis.004398.1 and Ce_fissilis.004398.2), two peroxidase 15 proteins (Ce_fissilis.015096.1 and Ce_fissilis.013923.1), ADP-ribosylation factor 1 (Ce_fissilis.009793.1), ADP-ribosylation factor-like protein 8a (Ce_fissilis.012754.1), profilin-4 (Ce_fissilis.007056.1), profilin-2 (Ce_fissilis.011671.1), expansin-like B1 (Ce_fissilis.010254.1) and glucan endo-1,3-beta-glucosidase 4 (Ce_fissilis.001287.1) were down-accumulated in shoots grown in the presence of the Put synthesis inhibitor (BA+Put+D-arg) (Supplementary Table S1).

On the other hand, the use of D-arg (BA+Put+D-arg) induced an up-accumulation of proteins compared with those in shoots grown in the absence of D-arg (BA+Put); these proteins

included V-type proton ATPase catalytic subunit A (Ce_fissilis.011699.1) and methionine gamma-lyase (Ce_fissilis.014973.1) (Supplementary Table S1).

Some proteins, such as the transport protein SEC13 homolog B (Ce_fissilis.003056.1) and glucan endo-1,3-beta-glucosidase, basic isoform (Ce_fissilis.005316.1), were unique in shoots grown under the BA+Put treatment, which promoted increased shoot length (Supplementary Table S1).

Discussion

The synergistic effect between BA and Put can increase the cellular divisions necessary for efficient shoot growth in *C. fissilis*, resulting in longer shoots under treatment with 2.5 µM BA + 2.5 mM Put from both types of explants: those from cotyledonary and apical nodal segments. The associations between cytokinins, BA and Put could be relevant to increase the cellular divisions necessary for the growth of shoots, as Put stimulates cell division, promoting the transition through the G1/S and G2/M cell cycle phases (Kuznetsov et al. 2002; Weiger and Hermann 2014). Moreover, BA also promotes cell division, which is necessary for shoot organogenesis and shoot proliferation during *in vitro* development (Hart et al. 2016). Among the cytokinins, BA has most frequently been used to promote the development of axillary buds, which involves breaking apical dominance and stimulating shoot proliferation, of several tree species (Pijut et al. 2012). Additionally, it has been shown that Put is involved in the growth of *M. sylvestris* and *C. fissilis* shoots (Liu et al. 2009; Aragão et al. 2016, 2017). Moreover, it has been shown that 2.5 µM BA can induce an increase in endogenous free Put contents, which is related to increased shoot development in this species (Aragão et al. 2016). Thus, BA can interact with Put, improving the development of *C. fissilis* shoots.

The use of the Put inhibitor D-arg reduced both the endogenous free Put contents and the activity of ADC and ODC enzymes, resulting in a reduction in the length of *C. fissilis* shoots. Thus, the inhibition of Put synthesis could be an important tool for understanding PA metabolism during *in vitro* morphogenesis in this species. Similarly, the use of D-arg (1–10 mM) has been shown to affect the endogenous contents of PAs, especially Put, in several species, such as *Oryza sativa* (Sung et al. 1994), M. *sylvestris* (Hao et al. 2005; Liu et al. 2006), *Prunus persica* (Liu and Moriguchi 2007) and *Pringlea antiscorbutica* (Hummel et al. 2002). In *Cucumis sativus*, the addition of D-arg (1 or 5 mM) significantly decreased adventitious shoot formation from cotyledons of seedlings, reducing endogenous Put contents (Zhu and Chen 2005). In addition, the growth of *M. sylvestris* shoots was inhibited with the addition of 1 or 5 mM D-arg, which induced a decrease in endogenous Put and Spd contents (Liu et al. 2009). Put is produced by two enzymes in plants: ADC and/or ODC (Chen et al. 2019). Our results show that the addition of Put and BA significantly increased the ODC activity and, consequently, the shoot length, while the use of D-arg resulted in a significant reduction in the activity of ADC and ODC enzymes and consequently reduced the synthesis of free Put, compromising shoot development in this species. Under the D-arg treatment (BA + Put + D-arg), the activity of ODC may have been reduced by the low conversion of Arg into Orn. It has been reported that a lower conversion of Arg into Orn through arginase action can occur, or Arg can be

redirected to other pathways (de Oliveira et al. 2018). Thus, we suggest that both ADC and ODC are required for the development of *C. fissilis* shoots.

In our work, we observed that the ODC enzyme presented a higher activity than the ADC enzyme in the shoots of *C. fissilis*, which is the first time Put synthesis-related enzymes have been analyzed in this species. Similarly, in the woody species *Araucaria angustifolia*, the coexistence of both ADC and ODC has been reported in the Put biosynthesis pathway during zygotic and somatic embryogenesis (de Oliveira et al. 2017, 2018), with ODC being the main pathway for Put biosynthesis during somatic embryogenesis in this species (de Oliveira et al. 2018). According to Kevers et al. (2000) and Vuosku et al. (2006), ODCs are particularly active in cell proliferation, whereas ADCs are involved in embryo and organ differentiation. Smith (1985) suggested that ODC is important for Put synthesis in actively dividing cells, while ADC is generally linked to cell elongation. The results described in our work show that the addition of BA + Put significantly increased ODC activity, increasing the endogenous contents of free Put and consequently promoting increased shoot elongation. On the other hand, the use of D-arg reduced the endogenous contents of free Put by reducing ADC and ODC activities, inhibiting the elongation of *C. fissilis* shoots. Similarly, the use of 5 mM Put increased the shoot growth and ODC activity in *M. sylvestris*, and the addition of 5 mM D-arg resulted in a decrease in shoot length and reduced the activity of both the ADC enzyme and Put synthesis (Liu et al. 2009). Our results demonstrated that Put is an important PA involved in the growth of *C. fissilis* shoots and that ODC is preferred over ADC for Put synthesis during *in vitro* shoot development.

In addition, the total free PA content and the PA ratio [Put/(Spd + Spm)] decreased in the presence of the Put synthesis inhibitor. It was previously shown that BA increased the endogenous total free PA content and free Put content in *C. fissilis* (Aragão et al. 2016), while exogenous Put also increased the total free PA content (Aragão et al. 2017), which was related to the greatest shoot growth in *C. fissilis*. The PA ratio could be used as a biochemical marker for morphological processes (Shoeb et al. 2001). In our work, we show that a decrease in the PA ratio caused by a decrease in endogenous Put content in the presence of a Put synthesis inhibitor negatively affected the development of *C. fissilis* shoots. In this sense, an increase in the PA ratio due to the increase in Put content is relevant for proper shoot development in this species.

In addition to PAs, the involvement of differentially accumulated proteins in *in vitro* shoot development was analyzed. Several proteins were down-accumulated when the shoots were incubated with the Put synthesis inhibitor, suggesting that they are important for the growth of *C. fissilis* shoots. Among the proteins that were down-accumulated are the ubiquitin receptor RAD23c (Ce_fissilis.004398.1 and Ce_fissilis.004398.2). Rad23 proteins are characterized as DNA repair factors required for nucleotide excision repair and perform a key role in recognizing damage to DNA (Shuck et al. 2008) and in transcription and proteolysis (Dantuma et al. 2009). Rad23 proteins were found to play essential roles in the cell cycle and morphology in *Arabidopsis thaliana*; thus, Rad23 is essential for normal growth (Farmer et al. 2010). Compared with wild-type *Pinus sylvestris* seedlings, *P. sylvestris* var. *mongolica*, a natural dwarf-type mutant, showed decreased accumulation of Rad23 proteins, indicating that these proteins are

among the main proteins responsible for the reduction in growth in dwarf-type mutants (Ning et al. 2013). These authors suggested that Rad23 proteins may play an important role in signaling cascades involved in the regulation of plant growth, particularly apical dominance. Indeed, these proteins may be involved in the auxin-mediated regulation of apical dominance regulating plant growth (Ning et al. 2013). Thus, this protein may be important for the proper development of *C. fissilis* shoots, and the decreased accumulation induced by the D-arg treatment (BA + Put + D-arg) could be related to the lower shoot length observed compared to that under the Put + BA treatment.

In addition, peroxidases are important for cell wall extension and cell division necessary for shoot organogenesis (Liszkay et al. 2003; Rajeswari and Paliwal 2008). It has been shown that these enzymes are involved in *in vitro* growth and differentiation, including direct organogenesis (Viu et al. 2009). In *Curcuma longa*, the PAs Put, Spd and Spm can induce an increase in peroxidase activity and promote differentiation (Viu et al. 2009). In *Centaurium erythraea*, peroxidase activity was strongly induced during *in vitro* morphogenesis, resulting in increased shoot development from the leaves (Filipović et al. 2015). In addition, the inhibition of peroxidase activity can decrease the cell division necessary for differentiation processes in *Nicotiana tabacum* (Marco and Roubelakis-Angelakis 1996). In this sense, the reduction in the accumulation of peroxidase enzymes (Ce_fissilis.013923.1 and Ce_fissilis.015096.1) in the shoots in the presence of a Put synthesis inhibitor (BA + Put + D-arg treatment) suggests that these enzymes can be regulated by BA + Put treatment to promote the development of *C. fissilis* shoots.

ADP-ribosylation factors have a fundamental role in plant growth and development (Naramoto et al. 2010). Studies in plants have revealed that the crucial roles of these factors mainly involve vesicular transport, such as protein trafficking out of the endoplasmic reticulum and other organelles, such as the Golgi apparatus (Pimpl et al. 2003; Stefano et al. 2006; Myung et al. 2013). In addition, these proteins are involved in several plant growth and development processes, including auxin transport, apical dominance, determination of cellular polarity, cell proliferation and elongation, and cell cycle regulation (Gebbie et al. 2005; Xu and Scheres 2005; Naramoto et al. 2010). It is possible that ADP-ribosylation factors are required for normal shoot growth and development in *C. fissilis*, since the decreased accumulation of these proteins (Ce_fissilis.009793.1 and Ce_fissilis.012754.1) negatively affects the endogenous free Put content and the cell divisions necessary for shoot growth, reducing shoot elongation under D-arg treatment.

The use of a Put synthesis inhibitor decreased the accumulation of two profilin (Ce_fissilis.007056.1 and Ce_fissilis.011671.1) proteins, indicating that profilin proteins are important and required for the *in vitro* growth of *C. fissilis* shoots. Profilin is an actin monomer-binding protein that causes either polymerization or depolymerization of actin filaments (Kovar et al. 2000). In higher plants, profilin proteins are involved in cell elongation (Vidali et al. 2007). In shoots of *Camellia sinensis* multiplied vegetatively by single-node cuttings, a significant increase in the accumulation of profilin occurred to promote growth (Liu et al. 2017).

The most documented function of expansins in plants is their regulation of cell wall extensibility, which promotes the growth and differentiation of plant cells (Cho and Cosgrove 2000). In *A. thaliana*, expansins play a role in cell enlargement and organ morphogenesis. This fact supports the hypothesis that the cell wall-loosening activity of expansins is an important control point for the regulation of plant cell growth (Cho and Cosgrove 2000). Expansins are also needed for cell wall softening during the growth of the apical meristem, root hairs, shoots, and fruits; for the initiation of leaf primordia; and for the regulation of embryogenesis and germination (Sharova 2007). In our work, the reduction in the accumulation of the expansin protein (Ce_fissilis.010254.1) in shoots treated with D-arg, which ultimately reduced shoot growth, suggests that expansins may play a key role in the control of *C. fissilis* shoot growth.

Some identified proteins accumulated more in shoots treated with the inhibitor of Put synthesis (BA + Put + D-arg) than in shoots treated with BA + Put, and these proteins, such as the V-type proton ATPase catalytic subunit A (Ce_fissilis.011699.1) protein, could be negatively related to *C. fissilis* shoot growth. The V-type ATPase plays important roles in the cell growth and development of plants, participating in the uptake and release of solutes across the vacuolar membrane and in the regulation of many cellular processes, including osmoregulation, signal transduction, metabolic regulation and pH (Sze et al. 1992; Kluge et al. 2003). Vacuoles contain V-type ATPases that catalyze the transport of H^+ into the organelle, maintaining cytosolic pH homeostasis, which is important for several enzymes that exhibit their highest activity at a pH of 7 in this compartment (Martinoia et al. 2007). In sugarcane (*Saccharum* spp.), V-ATPase activity was higher in nonembryogenic callus than in embryogenic callus, suggesting that somatic embryogenesis in this species is negatively affected by greater V-ATPase activity (Passamani et al. 2018). In contrast, in embryogenic suspension cultures of *A. angustifolia*, exogenous Put treatment stimulated V-ATPase activity and cellular growth, while Spd and Spm addition inhibited the activity of these proton pumps, decreasing cellular growth (Dutra et al. 2013). According to Dutra et al. (2013), the relationship between PAs and proton pump activities in plants and the mechanism of PA action in the regulation of these pumps need to be investigated. Our results suggest that the inhibition of Put synthesis by D-arg modulates endogenous PA, reducing the endogenous content of free Put and consequently increasing the accumulation of V-ATPases in *C. fissilis*, which may be related to a reduction in shoot length.

In plants, methionine gamma-lyase is a protein that breaks down methionine into methanethiol and 2-ketobutyrate, with 2-ketobutyrate being a precursor for isoleucine biosynthesis (Joshi et al. 2010; Huang et al. 2014). Spd and Spm are produced from Put by the addition of aminopropyl groups, which are provided by methionine (Chen et al. 2019). In our work, the increased accumulation of methionine gamma-lyase (Ce_fissilis.014973.1) in the shoots grown under the Put synthesis inhibitor treatment (BA + Put + D-arg) suggests a greater degradation of methionine, which may have affected PA metabolism, reflected mainly by the decrease in free Spd content compared to that in the shoots under BA + Put treatment. Our results are in agreement with those reported by Aragão et al. (2015), who found an increase in methionine and Spd contents to promote growth during early seedling growth in *C. fissilis*.

In addition, some proteins, such as the transport protein SEC13 homolog B (Ce_fissilis.003056.1), were unique in shoots grown under the 2.5 µM BA + 2.5 mM Put treatment, which promoted an increase in the length of *C. fissilis* shoots. Intracellular protein transport between organelles is mediated by several vesicles (Hino et al. 2014). Among the components of these vesicles is the transport protein SEC13 homolog B, which allows proteins to exit the endoplasmic reticulum toward the Golgi apparatus through a vesicular complex called COPII (Cevher-Keskin 2013). In *A. thaliana*, this protein contributes to the development of young leaves, the root apical region, and lateral root primordia (Hino et al. 2014). According Brandizzi (2018), the COPII machinery, including the transport protein SEC13 homolog B, may partially account for differences in the metabolic activity of the cells during growth; however, the connection between the endoplasmic reticulum and Golgi apparatus ensures that the protein synthesis necessary for plant growth can occur. Another unique protein in the shoots treated with BA + Put, which increased in length, was the glucan endo-1,3-beta-glucosidase, basic isoform (Ce_fissilis.005316.1). In addition, a glucan endo-1,3-beta-glucosidase 4 (Ce_fissilis.001287.1) protein was down-accumulated in the shoots under D-arg treatment, which ultimately reduced growth, suggesting that these proteins are relevant to the promotion of *C. fissilis* shoot elongation. The glucan endo-1,3-beta-glucosidase enzyme is regulated in part by auxin, cytokinins, and ethylene (Sperisen et al. 1991). In addition, this protein participates in several physiological processes, such as cell division and seed germination (Simmons 1994), and it is involved in cell wall assembly and reorganization (Minic 2008). Thus, our results suggest that these proteins may be important for *C. fissilis* shoot growth.

In summary, our results indicate that a Put synthesis inhibitor modulates the endogenous metabolism of PAs through the activity of ADC and ODC enzymes and alters the proteomic profile, which affects the development of *C. fissilis* shoots (Fig. 8).

Conclusions

This manuscript provides new insights into morphogenic competence in *C. fissilis* regulated by the inhibition of Put synthesis, as well as by BA and Put addition. Our results showed that 2.5 µM BA combined with 2.5 Mm Put increased the shoot length and ODC activity. The use of 1 or 5 mM D-arg combined with BA and Put inhibited shoot elongation by decreasing the endogenous free Put content, the total free PA content, the PA ratio and ADC and ODC activities. The ODC activity was higher than the ADC activity in this species. Moreover, compared with ADC, ODC is preferred for the synthesis of Put during *in vitro* shoot development and is described for the first time in this species. The inhibition of Put synthesis reduced the abundance of proteins related to plant growth and development, such as the ubiquitin receptor RAD23c, peroxidase 15, ADP-ribosylation factor 1, ADP-ribosylation factor-like protein 8a, Profilin-4, Profilin-2, expansin-like B1, V-type proton ATPase catalytic subunit A and methionine gamma-lyase, all of which are related to decreased shoot length in this species. In addition, glucan endo-1,3-beta-glucosidase, which was unique to the BA + Put treatment and down-accumulated when BA + Put was combined with D-arg, was associated with an increase in shoot length. These data suggest that Put is essential for morphogenic competence in *C. fissilis* by modulating the endogenous Put content and

proteomic profiles and that effective cell homeostasis at the PA and protein levels is necessary to regulate the growth and development of this species.

Abbreviations

ADC Arginine decarboxylase

ODC Ornithine decarboxylase

BA 6-Benzyladenine

D-arg D-arginine

FM Fresh matter

IAA Indole-3-acetic acid

MS Murashige and Skoog

PA Polyamine

Put Putrescine

Spd Spermidine

Spm Spermine

Declarations

Acknowledgments The authors thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) for funding. This study was also financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) - Finance Code 001. VPMA, KRS and TRO acknowledge the scholarship funded by FAPERJ.

Author contributions CSC and VPMA conceived the study, designed the experiments and wrote the manuscript. VPMA and TRO were responsible for the *in vitro* culture. VPMA and CSC were responsible for the PA experiments and analyses. EISF, LFO, VPMA and TRO were responsible for the ADC and ODC enzyme assays. VPMA, KRS and VS were responsible for the proteomic analyses. All authors read and approved the final manuscript.

Funding This research was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (444453/2014-8; 309303/2019-2) and the Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) (E26/202.969/2016; E26/202.533/2019). This study

was also financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brazil (CAPES)—Finance Code 001.

Data availability The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD036504. All identified proteins are available in the supplementary material.

Code availability PXD036504.

Financial interests The authors declare they have no financial interests.

Conflicts of interest/Competing interests The authors have no conflicts of interest to declare that are relevant to the content of this article.

References

1. Aragão VPM, Navarro BV, Passamani LZ, Macedo AF, Floh EIS, Silveira V, Santa-Catarina C (2015) Free amino acids, polyamines, soluble sugars and proteins during seed germination and early seedling growth of *Cedrela fissilis* Vellozo (Meliaceae), an endangered hardwood species from the Atlantic Forest in Brazil. Theor Exp Plant Physiol 27:157-169
2. Aragão VPM, Ribeiro YRS, Reis RS, Macedo AF, Floh EIS, Silveira V, Santa-Catarina C (2016) *In vitro* organogenesis of *Cedrela fissilis* Vell. (Meliaceae): the involvement of endogenous polyamines and carbohydrates on shoot development. Plant Cell Tissue Organ Cult 124(3):611-620
3. Aragão VPM, Reis RS, Silveira V, Santa-Catarina C (2017) Putrescine promotes changes in the endogenous polyamine levels and proteomic profiles to regulate organogenesis in *Cedrela fissilis* Vellozo (Meliaceae). Plant Cell Tissue Organ Cult 130:495-505
4. Arun M, Subramanyam K, Theboral J, Ganapathi A, Manickavasagam M (2014) Optimized shoot regeneration for Indian soybean: the influence of exogenous polyamines. Plant Cell Tissue Organ Cult 117:305-309
5. Baron K, Stasolla C (2008) The role of polyamines during in vivo and *in vitro* development. *In vitro* Cell Dev Biol Plant 44:384-395
6. Bradford M (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254
7. Brandizzi F (2018) Transport from the endoplasmic reticulum to the Golgi in plants: Where are we now?. Semin Cell Dev Biol 80:94-105
8. Burrieza HP, Rizzo AJ, Vale EM, Silveira V, Maldonado S (2019) Shotgun proteomic analysis of quinoa seeds reveals novel lysine-rich seed storage globulins. Food Chem 293:299-306
9. Cevher-Keskin B (2013) ARF1 and SAR1 GTPases in endomembrane trafficking in plants. Int J Mol Sci 14(9):18181-18199

10. Chen D, Shao Q, Yin L, Younis A, Zheng B (2019) Polyamine function in plants: metabolism, regulation on development, and roles in abiotic stress responses. Front Plant Sci 9:1945
11. Cheng WH, Wang FL, Cheng XQ, Zhu QH, Sun YQ, Zhu HG, Sun J (2015) Polyamine and its metabolite H_2O_2 play a key role in the conversion of embryogenic callus into somatic embryos in upland cotton (*Gossypium hirsutum* L.). Front Plant Sci 6:1063
12. Cho HT, Cosgrove DJ (2000) Altered expression of expansin modulates leaf growth and pedicel abscission in Arabidopsis thaliana. Proc Natl Acad Sci 97(17):9783-9788
13. Damerval C, De Vienne D, Zivy M, Thiellement H (1986) Technical improvements in two-dimensional electrophoresis increase the level of genetic variation detected in wheat-seedling proteins. Electrophoresis 7(1):52-54
14. Dantuma NP, Heinen C, Hoogstraten D (2009) The ubiquitin receptor Rad23: at the crossroads of nucleotide excision repair and proteasomal degradation. DNA Repair 8:449-60
15. de Oliveira LF, Elbl P, Navarro BV, Macedo AF, dos Santos ALW, Floh EIS (2017) Elucidation of the polyamine biosynthesis pathway during Brazilian pine (*Araucaria angustifolia*) seed development. Tree Physiol 37:116-130
16. de Oliveira LF, Navarro BV, Cerruti GV, Elbl P, Minocha R, Minocha SC, dos Santos ALW, Floh EIS (2018) Polyamine-and amino acid-related metabolism: the roles of arginine and ornithine are associated with the embryogenic potential. Plant Cell Physiol 59(5):1084-1098
17. Distler U, Kuharev J, Navarro P, Levin Y, Schild H, Tenzer S (2014) Drift time-specific collision energies enable deep-coverage data-independent acquisition proteomics. Nat methods 11:167-170
18. Dutra NT, Silveira V, de Azevedo IG, Gomes-Neto LR, Facanha AR, Steiner N, Guerra MP, Floh EIS, Santa- Catarina C (2013) Polyamines affect the cellular growth and structure of pro-embryogenic masses in *Araucaria angustifolia* embryogenic cultures through the modulation of proton pump activities and endogenous levels of polyamines. Physiol Plant 148 (1):121-132
19. Falasca G, Franceschetti M, Bagni N, Altamura MM, Biasi R (2010) Polyamine biosynthesis and control of the development of functional pollen in kiwifruit. Plant Physiol Biochem 48:565-573
20. Farmer LM, Book AJ, Lee KH, Lin YL, Fu HY, Vierstra RD (2010) The RAD23 family provides an essential connection between the 26S proteasome and ubiquitylated proteins in arabidopsis. Plant Cell 22(1):124-142
21. Ferry JG (2010) The gamma class of carbonic anhydrases. Biochim Biophys Acta 1804:374-381
22. Filipović BK, Simonović AD, Trifunović MM, Dmitrović SS, Savić JM, Jevremović SB, Subotić AR (2015) Plant regeneration in leaf culture of *Centaurium erythraea* Rafn. Part 1: The role of antioxidant enzymes. Plant Cell Tissue Organ Cult 121(3):703-719
23. Gebbie LK, Burn JE, Hocart CH, Williamson RE (2005) Genes encoding ADP-ribosylation factors in *Arabidopsis thaliana* L. Heyn.; genome analysis and antisense suppression. J Exp Bot 56:1079-1091
24. Ghosh S, Pal A (2013) Proteomic analysis of cotyledonary explants during shoot organogenesis in *Vigna radiata*. Plant Cell Tissue Organ Cult 115:55-68

25. Gotz S, Garcia-Gomez JM, Terol J, Williams TD, Nagaraj SH, Nueda MJ, Robles M, Talon M, Dopazo J, Conesa A (2008) High-throughput functional annotation and data mining with the Blast2GO suite. Nucleic Acids Res 36:3420-3435
26. Hao YJ, Kitashiba H, Honda C, Nada K, Moriguchi T (2005) Expression of arginine decarboxylase and ornithine decarboxylase genes in apple cells and stressed shoots. J Exp Bot 56(414):1105-1115
27. Hart DS, Keightley A, Sappington D, Nguyen PT, Chritton C, Seckinger GR, Torres KC (2016) Stability of adenine-based cytokinins in aqueous solution. *In vitro* Cell Dev Biol Plant 52(1):1-9
28. Heringer AS, Barroso T, Macedo AF, Santa-Catarina C, Souza GHMF, Floh EIS, Souza-Filho GA, Silveira V (2015) Label-free quantitative proteomics of embryogenic and non-embryogenic callus during sugarcane somatic embryogenesis. PLoS ONE 10:e0127803
29. Hernandez-Valladares M, Aasebø E, Mjaavatten O, Vaudel M, Bruserud Ø, Berven F, Selheim F (2016) Reliable FASP-based procedures for optimal quantitative proteomic and phosphoproteomic analysis on samples from acute myeloid leukemia patients. Biol Proced Online 18:13
30. Hino T, Tanaka Y, Kawamukai M, Nishimura K, Mano S, Nakagawa T (2011) Two Sec13p homologs, AtSec13A and AtSec13B, redundantly contribute to the formation of COPII transport vesicles in *Arabidopsis thaliana*. Biosci Biotechnol Biochem 75(9):1848-1852
31. Huang T, Joshi V, Jander G (2014) The catabolic enzyme methionine gamma-lyase limits methionine accumulation in potato tubers. Plant Biotechnol J 12(7):883-893
32. Hummel I, Couée I, El Amrani A, Martin-Tanguy J, Hennion F (2002) Involvement of polyamines in root development at low temperature in the subantarctic cruciferous species *Pringlea antiscorbutica*. J Exp Bot 53:1463-1473
33. IUCN (2020) The IUCN Red List of threatened species. International Union for Conservation of Nature. http://www.iucnredlist.org. Accessed 10 January 2020
34. Joshi V, Joung JG, Fei Z, Jander G (2010) Interdependence of threonine, methionine and isoleucine metabolism in plants: accumulation and transcriptional regulation under abiotic stress. Amino Acids 39(4):933-947
35. Kevers C, Le Gal N, Monteiro M, Dommes J, Gaspar T (2000) Somatic embryogenesis of *Panax ginseng* in liquid cultures: a role for polyamines and their metabolic pathways. Plant Growth Regul 31:209-214
36. Kluge C, Lahr J, Hanitzsch M, Bolte S, Golldack G, Dietz K-J (2003) New insight into the structure and regulation of the plant vacuolar H+-ATPase. J Bioenerg Biomembr 35:377-388
37. Königshofer H, Lechner S (2002) Are polyamines involved in the synthesis of heat-shock proteins in cell suspension cultures of tobacco and alfalfa in response to high-temperature stress? Plant Physiol Biochem 40(1):51-59
38. Kovar DR, Drøbak BK, Staiger CJ (2000) Maize profilin isoforms are functionally distinct. Plant Cell 12(4):583-598
39. Kusano T, Berberich T, Tateda C, Takahashi Y (2008) Polyamines: essential factors for growth and survival. Planta 228:367-381

40. Kuznetsov VV, Rakitin VY, Sadomov N, Dam D, Stetsenko L, Shevyakova N (2002) Do polyamines participate in the long-distance translocation of stress signals in plants? Russ J Plant Phys 49:120-130
41. Lipecka J, Chhuon C, Bourderioux M, Bessard MA, Endert P, Edelman A, Guerrera IC (2016) Sensitivity of mass spectrometry analysis depends on the shape of the filtration unit used for filter aided sample preparation (FASP). Proteomics 16:1852-1857
42. Liszkay A, Kenk B, Schopfer P (2003) Evidence for the involvement of cell wall peroxidase in the generation of hydroxyl radicals mediating extension growth. Planta 217:658-667
43. Liu S, Gao J, Chen Z, Qiao X, Huang H, Cui B, Zhu Q, Dai Z, Wu H, Pan Y, Yang C, Liu J (2017) Comparative proteomics reveals the physiological differences between winter tender shoots and spring tender shoots of a novel tea (*Camellia sinensis* L.) cultivar evergrowing in winter. BMC Plant Biol 17(1):206
44. Liu JH, Nada K, Honda C, Kitashiba H, Wen XP, Pang XM, Moriguchi T (2006) Polyamine biosynthesis of apple callus under salt stress: importance of the arginine decarboxylase pathway in stress response. J Exp Bot 57(11):2589-2599
45. Liu JH, Moriguchi T (2007) Changes in free polyamine titers and expression of polyamine biosynthetic genes during growth of peach *in vitro* callus. Plant Cell Rep 26(2):125-131
46. Liu JH, Nada K, Kurosawa T, Ban Y, Moriguchi T (2009) Potential regulation of apple *in vitro* shoot growth via modulation of cellular polyamine contents. Sci Hortic 119:423-429
47. Marco AD, Roubelakis-Angelakis KA (1996) Hydrogen peroxide plays a bivalent role in the regeneration of protoplasts. J Plant Physiol 149:109-114
48. Martinoia E, Maeshima M, Neuhaus HE (2007) Vacuolar transporters and their essential role in plant metabolism. J Exp Bot 58(1):83-102
49. Minic Z (2008). Physiological roles of plant glycoside hydrolases. Planta 227:723-740
50. Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473-497
51. Myung KM, Mihue J, Myounghui L, Junho L, Kyungyoung S, Yongjik L, KwanYong C, David GR, Inhwan H (2013) Recruitment of Arf1- GDP to golgi by Glo3p-Type ArfGAPs is crucial for Golgi maintenance and plant growth. Plant Physiol 161:676-691
52. Nanjo Y, Skultety L, Uváčková L, Klubicová, K, Hajduch M, Komatsu S (2012) Mass spectrometry-based analysis of proteomic changes in the root tips of flooded soybean seedlings. J Proteome Res 11:372-385
53. Naramoto S, Kleine-Vehn J, Robert S, Fujimoto M, Dainobu T, Paciorek T, Ueda T, Nakano A, Marc CE, Montagu V, Fukuda H, Friml, J (2010) ADP-ribosylation factor machinery mediates endocytosis in plant cells. Proc Natl Acad Sci 107(50):21890-21895
54. Ning DL, Lu TC, Liu GF, Yang CP, Wang BC (2013) Proteomic analysis points to a role for RAD23 in apical dominance in *Pinus sylvestris* var. mongolica. Plant Mol Biol Rep 31(6):1283-1292

55. Oliveira TR, Aragão VPM, Moharana KC, Fedosejevs E, Amaral FP, Sousa KR, Thelen JJ, Venâncio TM, Silveira V, Santa-Catarina C (2020) Light spectra affect the *in vitro* shoot development of *Cedrela fissilis* Vell. (Meliaceae) by changing the protein profile and polyamine contents. BBA-Proteins Proteom 1868:140529
56. Oliveira TR, Balfagón D, Sousa KR, Aragão VPM, de Oliveira LF, Floh EIS, Silveira V, Gómez-Cadenas A, Santa-Catarina C (2022) Long-term subculture affects rooting competence via changes in the hormones and protein profiles in *Cedrela fissilis* Vell.(Meliaceae) shoots. Plant Cell Tissue Organ Cult 148(1):137-153
57. Parimalan R, Giridhar P, Ravishankar G (2011) Enhanced shoot organogenesis in *Bixa orellana* L. in the presence of putrescine and silver nitrate. Plant Cell Tissue Organ Cult 105:285-290
58. Passamani LZ, Barbosa RR, Reis RS, Heringer AS, Rangel PL, Santa-Catarina C, Grativol C, Veiga CFM, Souza-Filho GA, Silveira V (2017) Salt stress induces changes in the proteomic profile of micropropagated sugarcane shoots. PloS one, 12(4):e0176076
59. Passamani LZ, Bertolazi AA, Ramos AC, Santa-Catarina C, Thelen JJ, Silveira V (2018) Embryogenic competence acquisition in sugar cane callus is associated with differential H+-pump abundance and activity. J Proteome Res 17:2767-2779
60. Passamani LZ, Reis RS, Vale EM, Sousa KR, Aragão VPM, Santa-Catarina C, Silveira V (2020) Long-term culture with 2,4-dichlorophenoxyacetic acid affects embryogenic competence in sugarcane callus via changes in starch, polyamine and protein profiles. Plant Cell Tissue Organ Cult. 140(2):415-429
61. Perez-Riverol Y, Bai J, Bandla C, García-Seisdedos D, Hewapathirana S, Kamatchinathan S, Kundu Deepti J, Prakash A, Frericks-Zipper A, Eisenacher M, Walzer M, Wang S, Brazma A, Vizcaíno Juan A (2022) The PRIDE database resources in 2022: a hub for mass spectrometry-based proteomics evidences. Nucleic Acids Res. 50(D1):D543-D552
62. Pijut PM, Beasley RR, Lawson SS, Palla KJ, Stevens ME, Wang Y (2012) *In vitro* propagation of tropical hardwood tree species-a review (2001-2011). Prop Ornam Plants 12:25-51
63. Pimpl P, Hanton SL, Taylor JP, Pinto-DaSilva LL, Denecke J (2003) The GTPase ARF1p controls the sequence-specific vacuolar sorting route to the lytic vacuole. Plant Cell 15:1242-1256
64. R Core Team (2018) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna
65. Rajeswari V, Paliwal K (2008) Peroxidase and catalase changes during *in vitro* adventitious shoot organogenesis from hypocotyls of *Albizia odoratissima* L.f. (Benth). Acta Physiol Plant 30:825-832
66. Reis RS, Vale EM, Heringer AS, Santa-Catarina C, Silveira V (2016) Putrescine induces somatic embryo development and proteomic changes in embryogenic callus of sugarcane. J Proteom 130:170-179
67. Reis RS, Vale EM, Sousa KR, Santa-Catarina C, Silveira V (2021) Pretreatment free of 2,4-dichlorophenoxyacetic acid improves the differentiation of sugarcane somatic embryos by affecting the hormonal balance and the accumulation of reserves. Plant Cell Tissue Organ Cult. 145:101–115

68. Rey M, Díza-Sala C, Rodríguez R (1994) Comparison of endogenous polyamine content in hazel leaves and buds between the dormancy and flowering annual phases of growth. Physiol Plant 91:45-50
69. Ribeiro YRS, Aragão VPM, Sousa KR, Macedo AF, Floh EIS, Silveira V, Santa-Catarina C (2022) Involvement of differentially accumulated proteins and endogenous auxin in adventitious root formation in micropropagated shoot cuttings of *Cedrela fissilis* Vellozo (Meliaceae). Plant Cell Tissue Organ Cult 148(1):119-135
70. Sharova EI (2007) Expansins: proteins involved in cell wall softening during plant growth and morphogenesis. Russ J Plant Physiol 54(6):713-727
71. Shoeb F, Yadav JS, Bajaj S, RajamMV (2001) Polyamines as biomarkers for plant regeneration capacity: improvement of regeneration by modulation of polyamine metabolism in different genotypes of indica rice. Plant Sci 160:1229-1235
72. Shuck SC, Short EA, Turchi JJ (2008) Eukaryotic nucleotide excision repair: from understanding mechanisms to influencing biology. Cell Res 18:64-72
73. Simmons CR (1994) The physiology and molecular biology of plant 1,3-β-glucanases and 1,3;1,4- β-glucanases. Crit Rev Plant Sci 13:325-387
74. Smith TA (1985) Polyamines. Ann Rev Plant Physiol 36:117-143
75. Sperisen C, Ryals J, Meins F (1991) Comparison of cloned genes provides evidence for intergenomic exchange of DNA in the evolution of a tobacco glucan endo-1, 3-beta-glucosidase gene family. Proc Natl Acad Sci 88(5):1820-1824
76. Stefano G, Renna L, Chatre L, Hanton SL, Moreau P, Hawes C, Brandizzi F (2006) In tobacco leaf epidermal cells, the integrity of protein export from the endoplasmic reticulum and of ER export sites depends on active COPI machinery. Plant J 46:95-110
77. Sung HI, Liu LF, Kao CH (1994) The induction of α-amylase activity by sucrose starvation in suspension-cultured rice cells is regulated by polyamines. Physiol Plant 91(2):137-140
78. Sze H, Ward JM, Lai S (1992) Vacuolar H+-translocating ATPases from plants: structure, function, and isoforms. J Bioenerg Biomembr 24(4):371-381
79. Vidali L, Augustine RC, Kleinman KP, Bezanilla M (2007) Profilin is essential for tip growth in the moss *Physcomitrella* patens. Plant Cell 19(11):3705-3722
80. Viu AF, Viu MA, Tavares AR, Vianello F, Lima GP (2009). Endogenous and exogenous polyamines in the organogenesis in *Curcuma longa* L. Sci Hortic 121(4):501-504
81. Vuosku J, Jokela A, Laara E, Saaskilahti M, Muilu R, Sutela S, Altabella T, Sarjala T, Häggman H (2006) Consistency of polyamine profiles and expression of arginine decarboxylase in mitosis during zygotic embryogenesis of Scots pine. Plant Physiol 142:1027-1038
82. Weiger TM, Hermann A (2014) Cell proliferation, potassium channels, polyamines and their interactions: a mini review. Amino Acids 46:681-688

83. Wiśniewski JR, Zougman A, Nagaraj N, Mann M (2009) Universal sample preparation method for proteome analysis. Nat Methods 6:359-362
84. Xu J, Scheres B (2005) Dissection of Arabidopsis ADP-RIBOSYLATION FACTOR 1 function in epidermal cell polarity. Plant Cell 17:525-536
85. Zhu C, Chen Z (2005) Role of polyamines in adventitious shoot morphogenesis from cotyledons of cucumber *in vitro*. Plant Cell Tissue Organ Cult 81(1):45-53

Figures

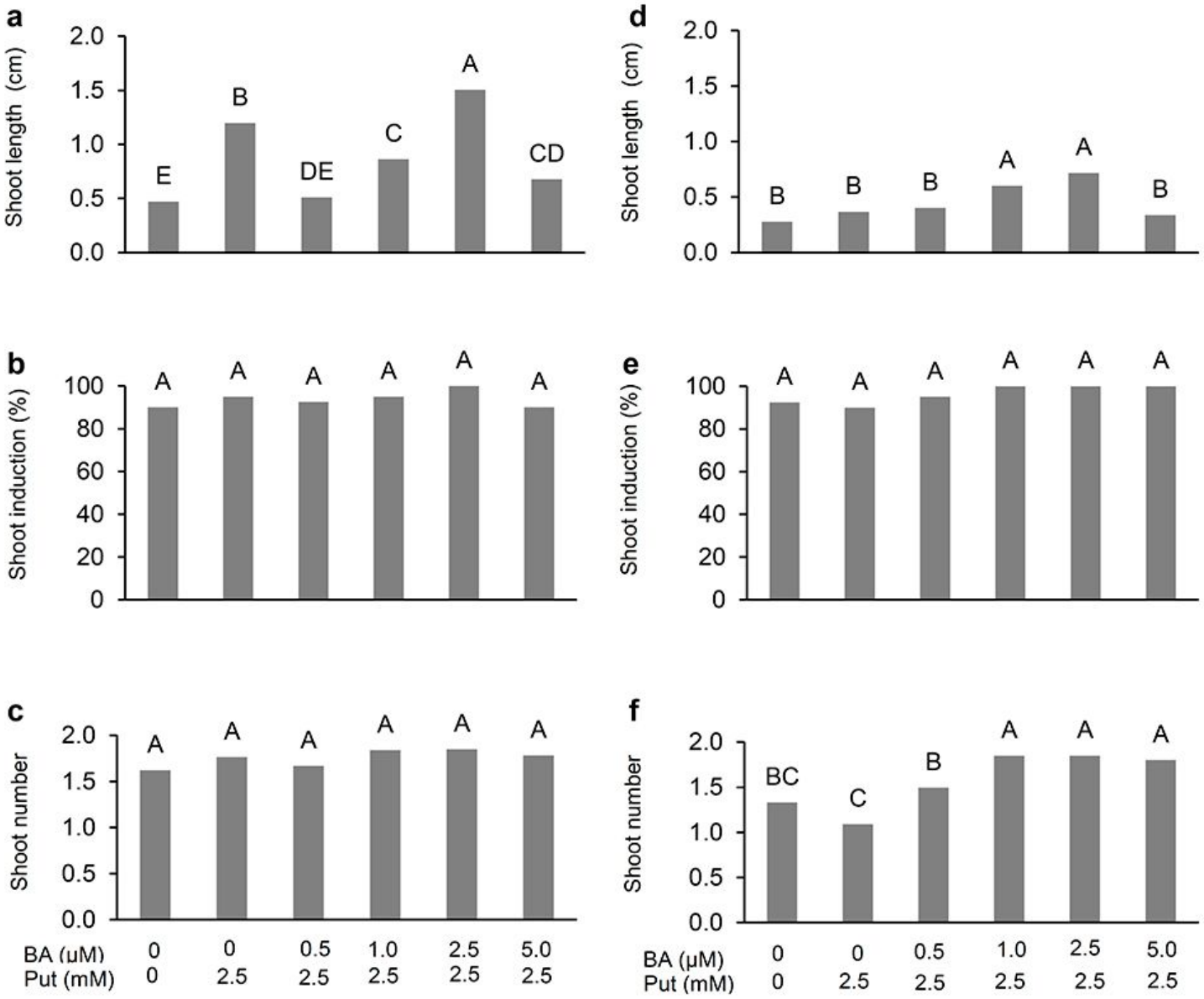


Figure 1

Length (a, d), induction (%) (b, e) and number of shoots per explant (c, f) from cotyledonary (a, b, c) and apical (d, e, f) nodal segments of *Cedrela fissilis* after 30 days of incubation in different concentrations of BA and Put. The means followed by different letters are significantly different ($P < 0.05$) according to the Tukey test. CV = coefficient of variation. (n = 8; CV a = 15.3%; CV b = 10.8%; CV c = 10.4%; CV d = 26.7%; CV e = 8.7%; CV f = 10.4%).

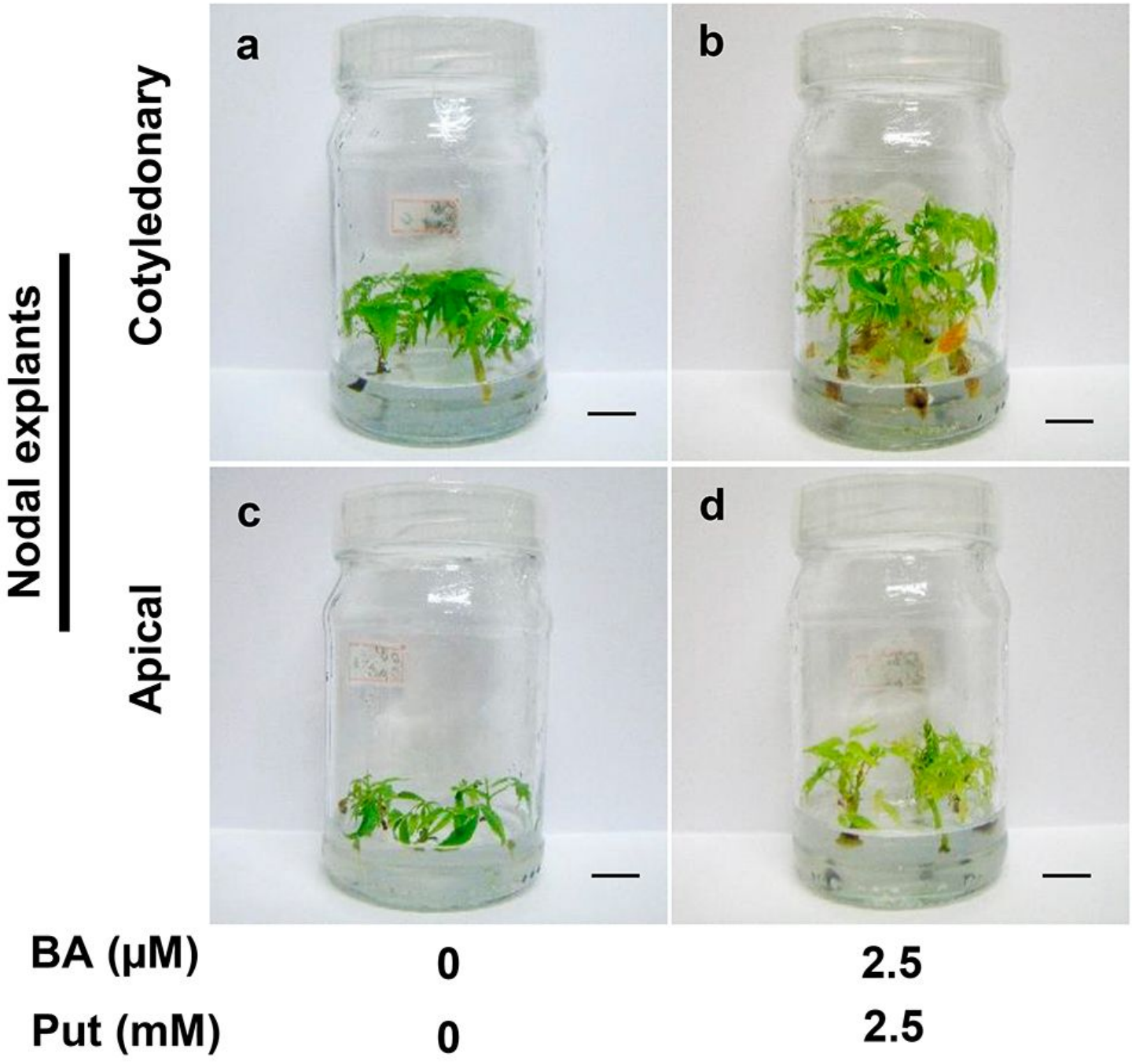


Figure 2

Morphology of shoots obtained from cotyledonary (a, b) and apical nodal segments (c, d) of *Cedrela fissilis* after 30 days of incubation in the absence (control) and presence of BA (2.5 µM) and Put (2.5

mM). Bars = 1 cm.

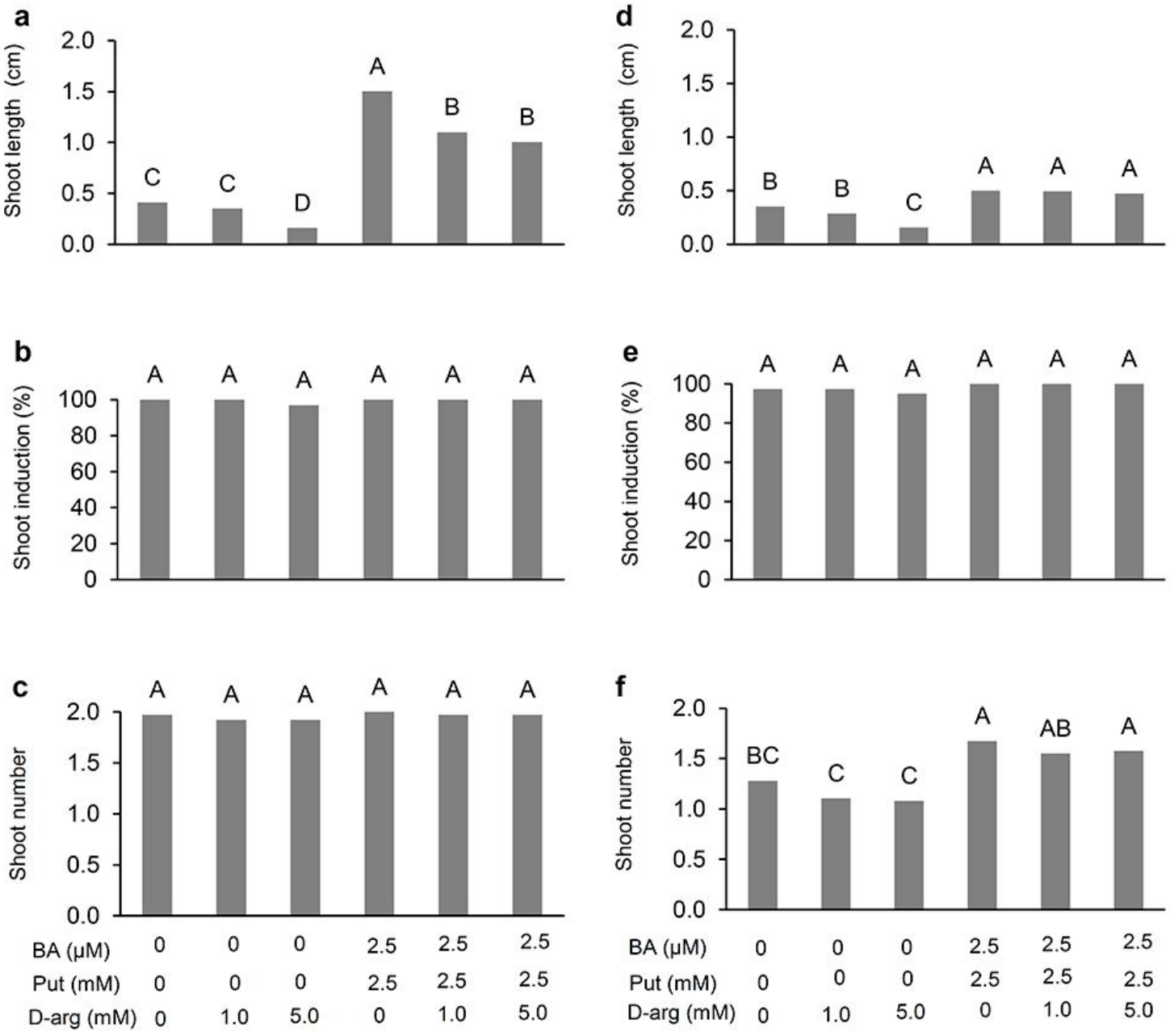
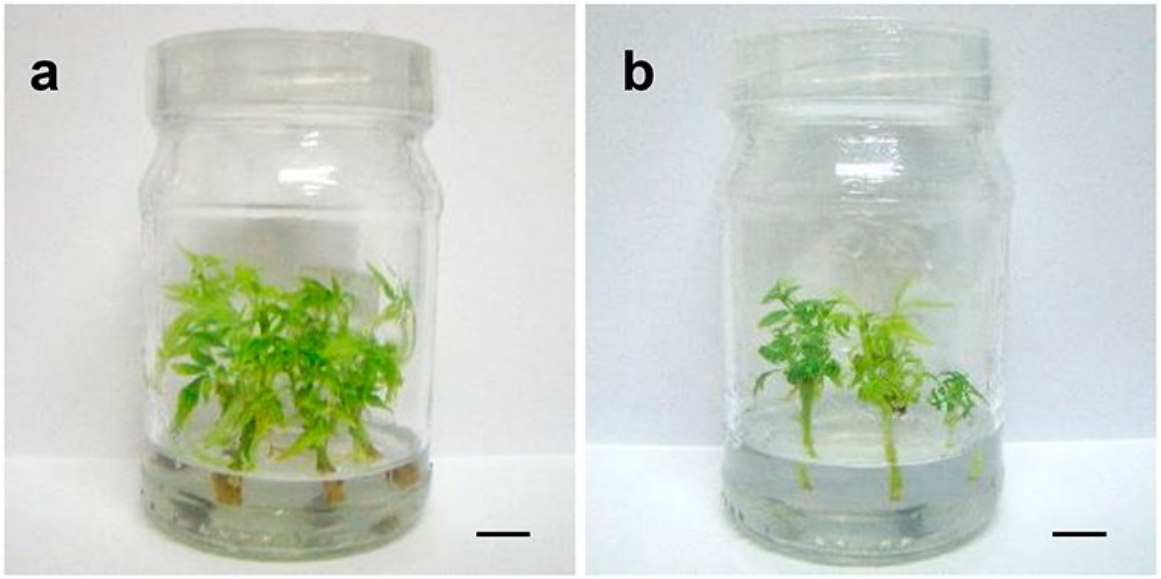


Figure 3

Length (a, d), induction (%) (b, e) and number of shoots per explant (c, f) in shoots from cotyledonary (a, b, c) and apical (d, e, f) nodal segments of *Cedrela fissilis* after 30 days of incubation under different concentrations of BA, Put and D-arg. The means followed by different letters are significantly different ($P < 0.05$) according to the Tukey test. CV = coefficient of variation. (n = 8; CV a = 13.5%; CV b = 1.1%; CV c = 3.9%; CV d = 14.9%; CV e = 5.6%; CV f = 13.1%).



BA (μM)	2.5	2.5
Put (mM)	2.5	2.5
D-arg (mM)	0	5.0

Figure 4

Morphology of shoots obtained from cotyledonary nodal segments of *Cedrela fissilis* after 30 days of incubation under treatment with 2.5 μM BA+2.5 mM Put (a) and 2.5 μM BA+2.5 mM Put+5 mM D-arg (b). Bars = 1 cm.

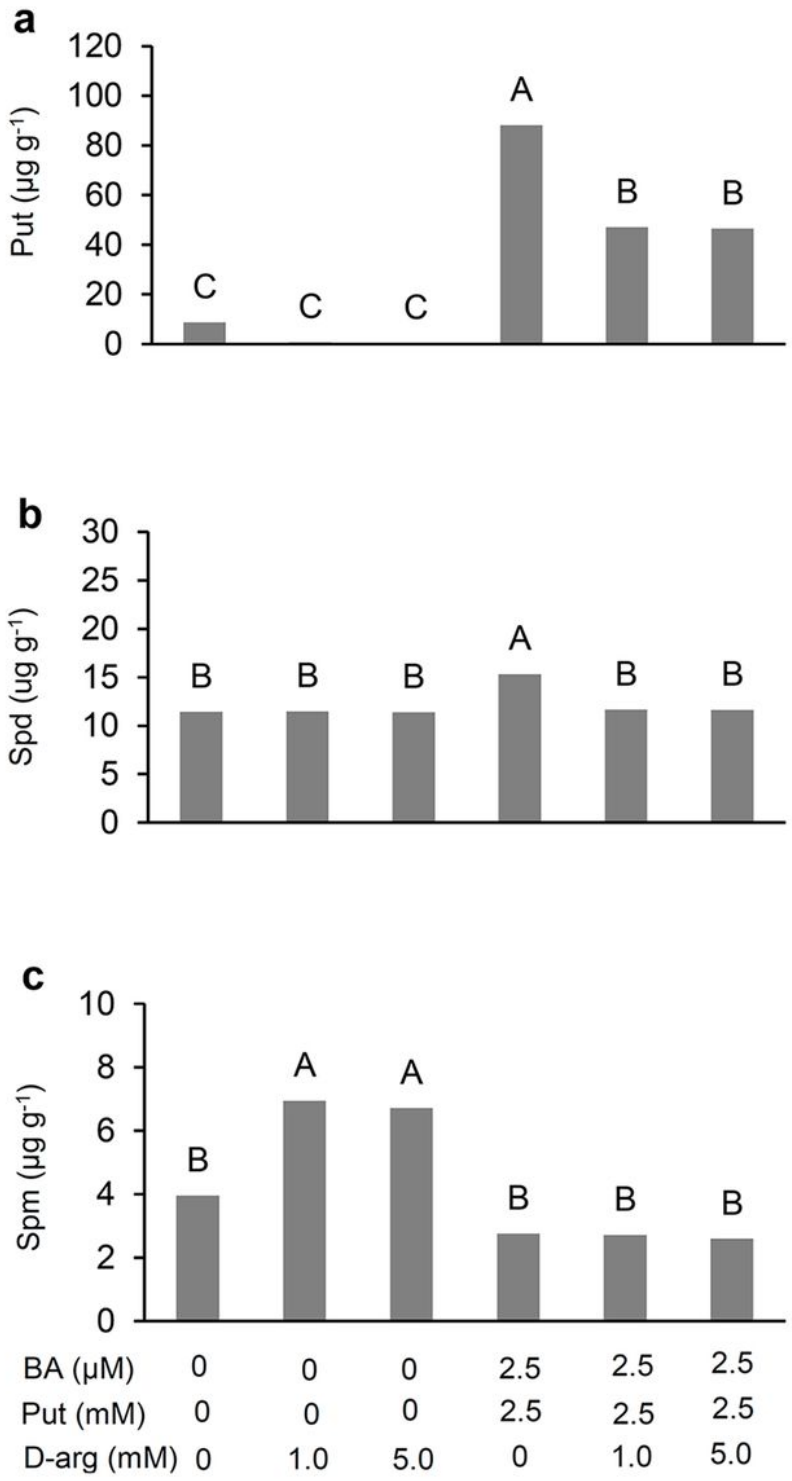


Figure 5

Endogenous contents (µg g-1 FM) of free Put (a), Spd (b), and Spm (c) in the shoots of *Cedrela fissilis* obtained from cotyledonary nodal explants after 30 days of incubation under different concentrations of BA, Put and D-arg. The means followed by different letters are significantly different ($P < 0.05$). CV = coefficient of variation. (n = 3; CV for Put = 12.5%; CV for Spd = 8.2%; CV for Spm = 13.5%).

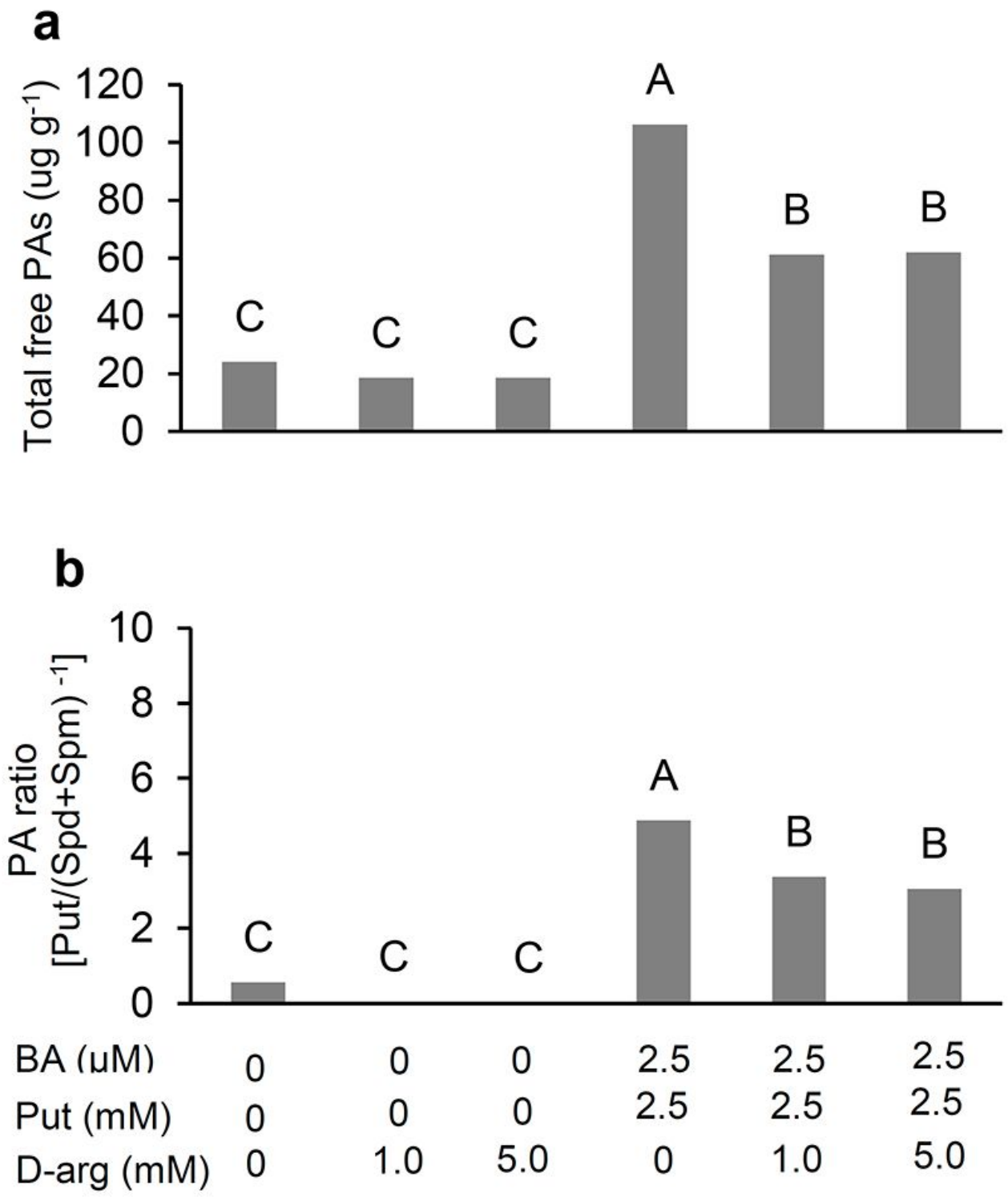


Figure 6

Endogenous contents (µg g-1 FM) of total free PAs (a) and the PA ratio (b) of shoots of *Cedrela fissilis* obtained from cotyledonary nodal explants after 30 days of incubation under different concentrations of BA, Put and D-arg. The means followed by different letters are significantly different ($P < 0.05$) according to the Tukey test. CV = coefficient of variation. (n = 3; CV for total free PAs = 10.3%; CV for the PA ratio= 14.2%)

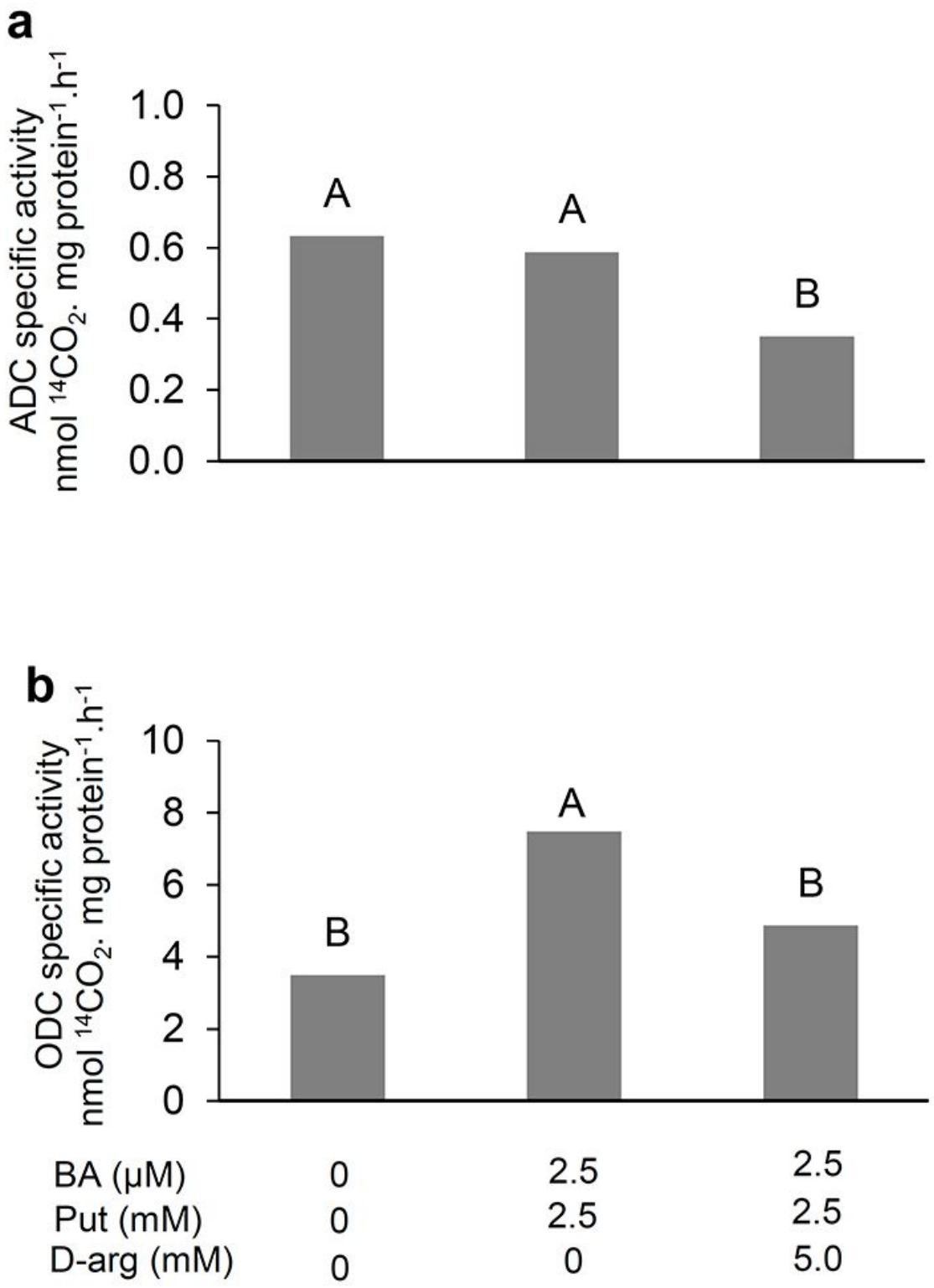


Figure 7

Specific enzymatic activity of ADC (a) and ODC (b) in the shoots of *Cedrela fissilis* obtained from cotyledonary nodal explants after 30 days of incubation under different concentrations of BA, Put and D-arg. The enzymatic activities were analyzed by measuring [14]CO2released from decarboxylated radiolabeled L-Arg and L-Orn. The means followed by different letters are significantly different ($P < 0.05$) according to the Tukey test. CV = coefficient of variation. (n = 3; CV of ADC = 7.0%; CV of ODC= 13.8%).

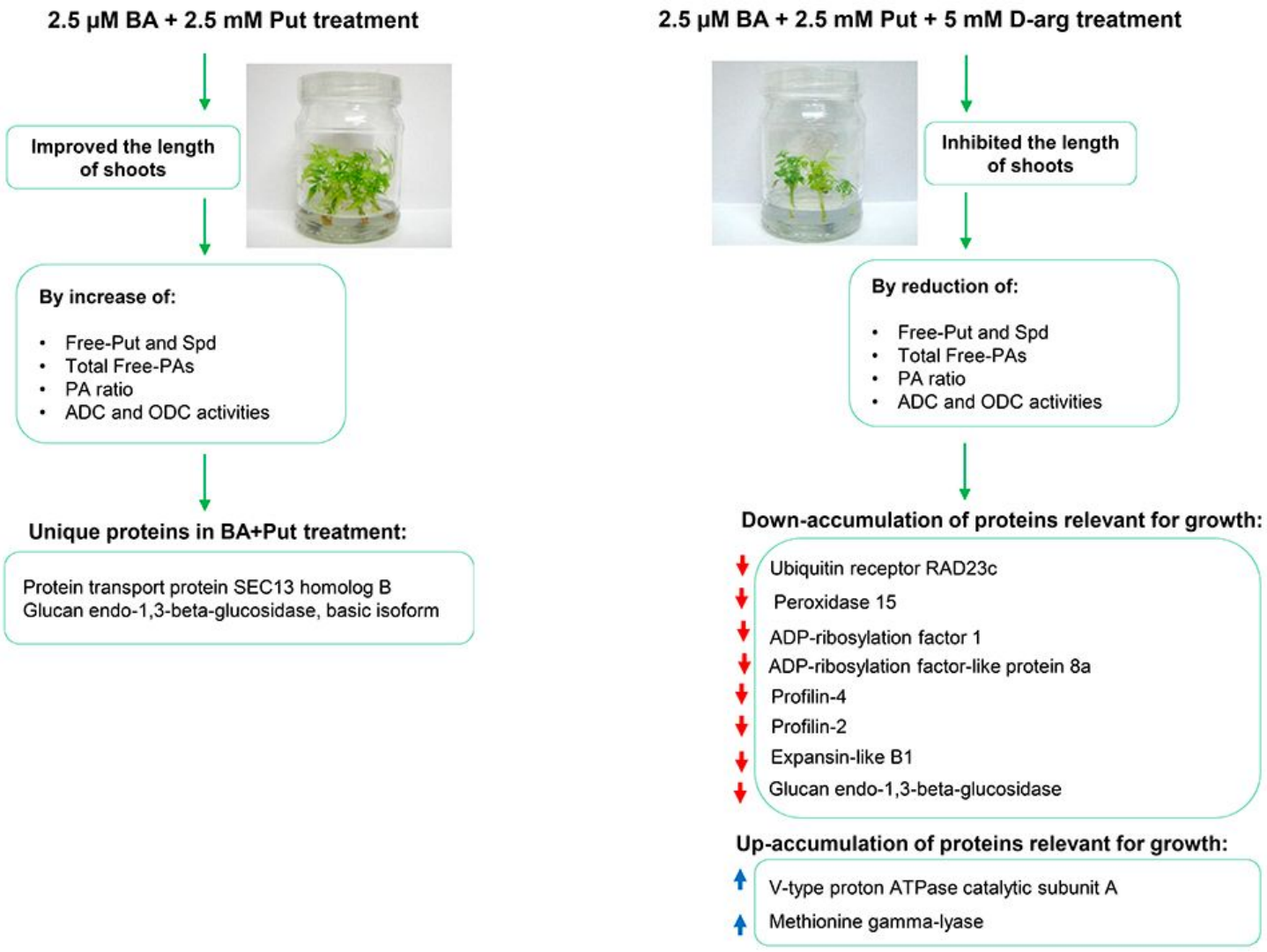


Figure 8

Proposed scheme of the metabolic modulation of PAs, ADC and ODC enzymes, and proteins by the use of a Put synthesis inhibitor, which affected the development of *Cedrela fissilis* shoots. The blue and red arrows represent proteins whose abundance increased and decreased, respectively.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarytable1.xlsx