2,3,5-Triiodobenzoic acid affects endogenous polyamine and indol-3-acetic acid levels and changes the proteomic profile, modulating the in vitro growth response of axillary buds and affecting the adventitious rooting of Cedrela fissilis Vell. (Meliaceae)

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Abstract

Endogenous levels of some molecules, such as polyamines (PAs) and auxin, can be associated with the control of shoot and root development. The effects of 6-benzyladenine (BA), putrescine (Put) and the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) on the development of *Cedrela fissilis* shoots, as well as the effects on endogenous alteration of PAs and variations in protein abundance, were investigated. Furthermore, the effect of Put and TIBA on ex vitro root development and the endogenous content of PAs was investigated. The BA + Put combined with TIBA treatment promoted an inhibition of shoot growth from cotyledonary nodal segments, decreasing endogenous total free-PA levels and increasing IAA-free content compared to shoots treated with BA + Put without TIBA. A comparative proteomic analysis of BA + Put + TIBA-treated shoots showed reduced accumulation of proteins mainly related to cytoskeleton organization, carbon metabolism, and photosynthesis and increased accumulation of proteins related to the degradation of valine, leucine and isoleucine compared to those without TIBA. The percentage and number of rooted shoots were significantly reduced by Put + TIBA treatment, showing that auxin inhibitors and not Put are responsible for the decrease in rooting. These results show that the combination of BA + Put with the auxin transport inhibitor TIBA interferes with endogenous PA metabolism and alters protein abundance, thereby altering shoot development. Moreover, Put is not related to rooting induction in *C. fissilis*.

Key Message

Effects 2,3,5-triiodobenzoic acid in modulate in vitro growth response of axillary buds and in adventitious rooting of *Cedrela fissilis*

1 Introduction

Cedrela fissilis Vellozo (Meliaceae), popularly known as “cedro rosa”, is a native woody species from the Brazilian Atlantic Forest and has been explored for decades. Due to intense exploitation over the years, this species is currently included on the list of threatened species and is placed in the vulnerable category (Barstow 2018). *C. fissilis* has economic and ecological importance, and alternative propagations by in vitro culture can improve the conservation of this species. Studies on in vitro propagation have been performed, showing the effects of several factors on in vitro shoot development (Nunes et al. 2002, Aragão et al. 2016, Aragão et al. 2017, dos Reis de Oliveira et al. 2022) and ex vitro rooting (Ribeiro et al. 2022).

The propagation of forest trees in vitro can provide a large number of plantlets in a short time throughout the year (Shahzad et al. 2017). During in vitro propagation, shoot induction and root development are two important steps. Shoot growth is an important characteristic that determines the morphology, thus highlighting the astonishing regenerative potential of plant somatic cells (Ikeuchi et al. 2016). Improvements to in vitro plant development are essential for optimizing shoot development and understanding basic physiological processes (Aragão et al. 2017). However, plant regeneration can follow

various pathways, with shoot regeneration being a prerequisite for plant biotechnologies (Zeng et al. 2019). In this sense, plant growth regulators (PGRs) play critical roles in regulating shoot development, with exogenous and/or endogenous signaling important to promote in vitro morphogenesis.

Auxin is a PGR that regulates the lateral branching of plant shoots and is transported basipetally from the primary shoot apex, suppressing axillary bud outgrowth (Hayward et al. 2009). This PGR is involved in diverse aspects of plant growth and development, including elongation, growth, vascular differentiation, root induction, and shoot branching inhibition (Hobbie and Estelle 1994). In addition to great advances in the characterization of auxin transport and the roles of these PGRs in tissue pattern formation (Verna et al. 2019, Koike et al. 2020), some aspects of cross-talk between auxin and polyamine (PA) pathways in vitro shoot development and root induction remain unclear. Thus, knowledge about the physiological and molecular mechanisms of auxin and PA interactions will increase the possibilities for controlling plant growth and development.

Studies with auxin inhibitors are important for understanding the functions of these substances in plant growth and development (Ma et al. 2018). 2,3,5-Triiodobenzoic acid (TIBA), an auxin transport inhibitor, acts specifically in the inhibition of the polar transport of auxins, regulating the binding to the plasma membrane of PIN-FORMED (PIN) transport proteins of IAA efflux (Geldner et al. 2001). Thus, the use of TIBA in vitro studies can be an important tool to investigate the influence of polar auxin transport on shoot development in *C. fissilis* once the effect of exogenous TIBA on the shoot growth of this species remains unknown to date. On the other hand, previous studies have shown the effects of TIBA inhibiting the ex vitro rooting of micropropagated shoot cuttings in *C. fissilis* (Ribeiro et al. 2022). Furthermore, the involvement of auxin and PA metabolism in in vitro shoot development and rooting is not well understood.

PAs are ubiquitous molecules present in all organisms and have been considered a new type of plant signaling involved in various physiological processes in plants, including organogenesis, embryogenesis, rooting, stress response and in vitro morphogenesis (Santa-Catarina et al. 2006, Martínez Pastur et al. 2007, Chen et al. 2018, Pessanha et al. 2022). Putrescine (Put), spermidine (Spd) and spermine (Spm) are the most common PAs in plants (Todorova et al. 2014). Due to the cationic nature of PAs, they can interact with negatively charged macromolecules (Kuznetsov and Shevyakova 2007), helping to stabilize proteins, nucleic acids, and the plasma membrane (Spormann et al. 2021), including transcription factors, protein kinases and phospholipases (Miller-Fleming et al. 2015), as well as ion transport proteins (Pottosin and Shabala 2014). Specifically, in *C. fissilis*, investigations reported the increased content of endogenous PAs, especially Put, by the addition of 6-benzyladenine (BA) to the culture medium, promoting shoot development (Aragão et al. 2016). It has also been shown that exogenous Put promotes in vitro shoot elongation in *C. fissilis* (Aragão et al. 2017), whereas Put synthesis is inhibited by D-arg, resulting in reduced shoot growth (Aragão et al. 2023).

Moreover, synergistic or antagonistic interactions between plant hormones, such as auxin, and PAs have been described. Studies have shown that an increase in Put contents may regulate some genes related to

IAA biosynthesis, auxin-related transcription factors and other genes related to signal transduction during abiotic stress in transgenic plants of *Arabidopsis thaliana* (Marco et al. 2011). In addition, it was shown that Put modulated the expression of PIN1 and PIN2 auxin transporters in *Arabidopsis* (Hashem et al. 2021). On the other hand, Spm is related to the regulation of auxin and cytokinin signaling genes in *Arabidopsis* (Anwar et al. 2015). Thus, the use of an auxin inhibitor, such as TIBA, can be an interesting tool for investigating the relationship between auxin and PA metabolism. Although studies show the involvement of PAs in shoot development and rooting (Tang and Newton 2005, Aragão et al. 2017), little is known about how PAs modulate these morphological processes and whether they interact with auxin metabolism in *C. fissilis*.

Proteomic approaches are interesting for characterizing a complete set of proteins present in a cell, organ or organism at a given time (Eldakak et al. 2013). It is an important tool used in studies during in vitro morphogenesis responses, such as shoot development (Aragão et al. 2017, Lerin et al. 2019) and rooting of shoots (Lerin et al. 2021, Ribeiro et al. 2022) in tree species. These studies have allowed us to better understand the physiological events during in vitro morphogenesis. More specifically, studies in *C. fissilis* showed that exogenous Put induces proteomic changes in the abundance of proteins related to the response to stimuli associated with oxidative and abiotic stress, energy and cell division and increases shoot length (Aragão et al. 2017). In this context, comparative proteomics plays a crucial role in identifying potential markers of candidate proteins associated with morphogenetic competence, thereby enhancing our understanding of the in vitro development of shoots.

Therefore, this study aimed to evaluate the influence of BA, Put and TIBA on shoot development in *C. fissilis* and on the endogenous contents of PAs and IAA and the proteomic profile. In addition, we investigated the effect of Put and TIBA on ex vitro root development and on endogenous contents of PAs in this species.

2 Materials and methods

Plant material

Mature *C. fissilis* seeds obtained in the Sementes Caiçara nursery located in Brejo Alegre, SP, Brazil (21°10'S and 50°10'W), were germinated in vitro as described by Aragão et al. (2016), and 60-day-old seedlings were used as the explant source of cotyledonary and apical nodal segments for the experiments.

Effect of auxin transport inhibitor on in vitro shoot development from cotyledonary and apical nodal segments and on endogenous PA contents and proteomic profiles

Cotyledonary and apical nodal segments (± 2 cm) were isolated from 60-day-old seedlings and inoculated in MS (Murashige and Skoog 1962) culture medium (M519; Phytotechnology Lab, Lenexa, KS, USA) supplemented with 20 g L^{-1} sucrose (Vetec, Rio de Janeiro, Brazil), 2 g L^{-1} Phytagel® (Sigma–Aldrich, St. Louis, MO, USA) and different concentrations of BA (0 or 2.5 µM; Sigma–Aldrich), Put (0 or 2.5

mM; Sigma–Aldrich), combined or not with TIBA (0, 10 or 20 µM; Sigma–Aldrich). The experiment consisted of six treatments: control (absence BA, Put and TIBA), 10 µM TIBA, 20 µM TIBA, 2.5 µM BA + 2.5 mM Put, 2.5 µM BA + 2.5 mM Put + 10 µM TIBA and 2.5 µM BA + 2.5 mM Put + 20 µM TIBA. Put and TIBA were incorporated into the MS culture medium after filter sterilization using a 0.22-µm filter (Biofil, Guangzhou, China). The pH of the culture medium was adjusted to 5.7. The MS culture medium (30 mL) was aliquoted into 150 mL culture vessels (8 cm in height × 5 cm in diameter; Aapace, São Paulo, Brazil). The explants were incubated in a growth room at 25 ± 2°C with a 16-h photoperiod (55 µmol m^2 s^{-1}) every 30 days. Each treatment was composed of eight replicates, with each replicate being one glass culture vessel with five explants. For all treatments, the shoot length (cm), induction (%) and number of shoots per explant were evaluated after 30 days of culture. Three biological samples of shoots derived from cotyledonary nodal segments were collected after a 30-day incubation for endogenous PA, IAA and proteomic analysis. All biological samples were stored at -80°C until analysis.

Effect of Put on ex vitro rooting of micropropagated shoots

Forty-five-day-old in vitro shoot cuttings (1.5 to 2.0 cm) containing the apical meristem and four main leaves were used to evaluate the effect of exogenous Put on ex vitro rooting. The bases of the shoot cuttings were immersed in different concentrations (1 and 5 mM) Put for 90 min. After the treatment, the shoot cuttings were transferred to plastic cups (50 ml) containing the substrate Basaplant and vermiculite (1:1; v/v), maintained in plastic trays (39.4 x 31.9 x 15.4 cm) (Pleion, São Paulo, Brazil) covered with PVC-type plastic film (Lumipam, São Paulo, Brazil) to maintain high relative humidity, and kept in a growth room at 25 ± 2°C under a photoperiod of 16 h, with a light intensity of 55 µmol m^{-2} s^{-1} provided by LED lamps (Koninklijke Philips Electronics NV). After seven days, to simultaneously achieve the rooting of shoots and acclimatization, the PVC parafilm plastic was perforated until complete removal at 10 days of incubation, according to Ribeiro et al. (2022). Each treatment was composed of eight replicates, with five shoot cuttings per replicate, for a total of forty shoot cuttings per treatment. After 13 days, the percentage of rooted shoot cuttings and the number and length (cm) of roots per shoot cutting were evaluated.

Effect of the auxin transport inhibitor combined with exogenous Put on ex vitro rooting and endogenous PA contents in micropropagated shoots

To evaluate the effect of auxin transport inhibition on PA metabolism, the contents of endogenous PAs in shoots treated without and with 200 µM TIBA, which inhibited rooting in *C. fissilis* according to results previously shown by Ribeiro et al. (2022), were evaluated. In vitro shoot cuttings (1.5 to 2.0 cm) with the apical meristem of the shoots and four main leaves from forty-five-day-old plants were used to evaluate the interaction of auxin and Put on ex vitro rooting. The bases of the shoot cuttings were immersed in 200 µM TIBA, 5 mM Put, and 5 mM Put + 200 µM TIBA for 90 min. Then, the shoot cuttings were transferred to substrate and incubated under the same conditions previously described by Ribeiro et al. (2022). Three biological samples containing the bases (0.5 cm) of shoots incubated in the treatments

described before (day 0) and after 3, 6, and 10 days during rooting were collected and stored at − 80°C until free-PA analysis.

Free PA determination

For PA analysis, three biological samples (200 mg fresh matter - FM- from each sample) of whole shoots after 30 days of culture from cotyledonary nodal segments of all treatments and the base of the shoot cuttings during rooting in all treatments were collected and stored at -20°C until free-PA assessment. The free PAs were determined according to Aragão et al. (2016). The samples were ground in 1.3 mL of 5% perchloric acid (Merck, Darmstadt, Germany), incubated at 4°C for 1 h, and then centrifuged for 20 min at 20,000×*g* at 4°C. Free PAs were obtained from the supernatant, derivatized with dansyl chloride (Merck) 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) to a 10% aqueous acetonitrile solution with the pH 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, and this 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 Put, Spd, and Spm (Sigma—Aldrich).

IAA determination

For endogenous IAA analysis, three biological samples (500 g FM each sample) were obtained of whole shoots after 30 days of culture from cotyledonary nodal segments collected from control (absence PGRs), treatment with highest shoot length and from treatment with TIBA that induced significant inhibition of shoot growth, being maintained at -80°C until performing the analyses.

The quantification of endogenous IAA levels was performed according to Álvarez-Flórez et al. (2017) and Silveira et al. (2004) with modifications. Samples were lyophilized, and IAA was extracted with chilled extraction solution containing 2.5 mL of methanol (Merck): isopropanol (Merck) (20:80, v/v) with 1% glacial acetic acid. IAA[3H] was added as an internal standard. Then, the samples were vortexed for 5 min and centrifuged at 11,000 x g for 20 min at 4°C. The supernatants containing IAA were collected, and the pellets were re-extracted with 2.5 mL of extraction solution and centrifuged again. Then, the supernatants were combined and dried completely in a speed vac at 45°C. Each sample was then resuspended in 150 µL of solution containing 10% methanol (Merck) plus 0.5% glacial acetic acid (Merck). Next, samples were filtered through a 0.2 µm Minisart filter (Sartorius Stedim Biotech; Goettingen, Germany). Finally, 10 µL of each sample was used for analysis by high-performance liquid chromatography (HPLC) using a 5-µm reversed-phase column (Shim-pack CLC ODS; Shimadzu, Kyoto, Japan). The gradient was developed by a mixture of increasing proportions of absolute methanol with a water solution containing 10% methanol plus 0.5% glacial acetic acid. The absolute methanol gradient was set to 10% in the first 5 min,

from 10 to 20% between 5 and 7 min, from 20 to 30% between 7 and 15 min, from 30 to 45% between 15 and 21 min and 100% from 21 to 35 min with a flow rate of 1 mL.min^{-1} at 40°C. The IAA concentration was determined using a fluorescence detector at 280 nm (excitation) and 350 nm (emission). The fraction containing the IAA peak was collected and analyzed by a Tri-Carb Liquid Scintillation counter (Packard Instrument Co., Meriden, USA) to estimate the losses. The IAA retention areas and times were evaluated by comparison with known concentrations of this hormone.

Proteomic analysis

To determine the proteomic profile, three biological samples (300 mg FM each sample) of whole shoots from cotyledonary nodal segments were collected from the treatment with the highest shoot length and from the treatment with TIBA that induced significant inhibition of shoot growth. Protein extracts were prepared in biological triplicate. Proteins were extracted using the trichloroacetic acid (TCA)/acetone method according to Damerval et al. (1986), with modifications. Shoots were frozen in liquid nitrogen and ground to a fine powder using a ceramic mortar and pestle. The resulting powder was resuspended in 1 mL of chilled solution containing 10% (w/v) TCA (Sigma–Aldrich) in acetone (Sigma–Aldrich) with 20 mM dithiothreitol (DTT; GE Healthcare) and vortexed for 5 min at 8°C; the mixture was kept at − 20°C for 1 h before centrifugation at 16,000×*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 wash. The pellets were air dried, 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 × *g*. 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 to remove any interferents from the samples (Nanjo et al. 2012). After protein precipitation, samples were resuspended in a solution of 7 M urea/2 M thiourea for proper resuspension. 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 of 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.

A nanoAcquity ultraperformance liquid chromatograph (UPLC) connected to a Synapt G2-Si HDMS mass spectrometer (Waters) was used for electrospray-liquid chromatography-tandem mass spectrometry analysis. 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 µL min^{-1} for 3 min and then onto the nanoAcquity 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 and mobile phase B consisting of acetonitrile and 0.1% formic acid. The gradient elution started at 7% B, then ramped from 7 to 40% B until 91.12 min, then ramped again from 40 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 width 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 of 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 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 μL^{-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.

Spectra processing and database search conditions were performed using ProteinLynx Global Server (PLGS; software version 3.0.2) (Waters). The HDMSE analysis utilized 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) of 1%, automatic peptide and fragment tolerance. For protein identification, the obtained data were processed against the nonredundant *Cedrela fissilis* databank (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 PXD043790. Label-free quantification analyses were performed using ISOQuant software v.1.7 (Distler et al. 2014). Briefly, we used the following parameters: peptide and protein FDR 1%, sequence length of at least 6 amino acid residues, and minimum peptide score equal to six. Samples were normalized by a multidimensional normalization process, which corrects peak intensities based on the intensity and retention time domains. The software performed relative protein quantification based on the TOP3 method. Based on the relative abundances of uniquely assigned peptides, the abundances of shared peptides were redistributed to the respective source proteins, followed by TOP3-based quantification (Distler et al. 2014). To ensure the quality of the results after data processing, only proteins present or absent (for unique proteins) in all three runs were considered for the differential accumulation analysis using Student's t test (two-tailed; $P < 0.05$). Differentially abundant proteins were considered up-accumulated if the log2 value of their fold change (FC) was greater than 0.6 and if the log2 value of their FC was lower than − 0.6 according to Student's t test. Functional annotations were performed using OmicsBox (Götz et al. 2008) and UniProtKB (http://www.uniprot.org). The predicted interaction networks of DAPs were constructed using *C. fissilis* identified through a STRING search, with confidence > 0.7. The network of KEGG pathway enrichment by STRING for differentially accumulated proteins (DAPs) resulted in the enrichment of biological processes.

Statistical analysis

The data were presented by analysis of variance (ANOVA) ($P < 0.05$) followed by Tukey's test using the program R (R Foundation for Statistical Computing, version 3.4.4, 2018, Vienna, Austria) (R Core 2018).

3 Results

Effect of auxin transport inhibitor on in vitro shoot development from cotyledonary and apical nodal segments

The highest inhibition of shoot growth was observed in cotyledonary nodal segments with 20 μM TIBA (0.26 cm), while the highest shoot length (1.4 cm) was obtained in the 2.5 μM BA + 2.5 mM Put treatment (Fig. 1a). The use of 10 or 20 μM TIBA combined with BA + Put significantly decreased the length of shoots (0.58 and 0.48 cm, respectively) compared to treatment with 2.5 μM BA + 2.5 mM Put (1.4 cm) (Fig. 1a). In addition, for shoot induction (Fig. 1b) and the number of shoots per explant (Fig. 1c), a significant reduction was observed in the 20 μM TIBA treatment. Using apical nodal segments, the highest shoot length (0.45 cm) was observed in the treatment with 2.5 μM BA + 2.5 mM Put (Fig. 1d), while for shoot induction, higher inhibition was observed in treatments containing only TIBA at 10 and 20 μM (Fig. 1e). For the number of shoots per explant, there were no significant differences among all treatments tested (Fig. 1f).

Figure 2 shows the morphological aspects of shoots obtained from cotyledonary and apical nodal segments cultured with 2.5 μM BA + 2.5 mM Put and 2.5 μM BA + 2.5 mM Put + 20 μM TIBA.

Effect of auxin transport inhibitor on endogenous free PA levels in shoots from cotyledonary nodal segments

The highest endogenous Put content was observed in the treatment with 2.5 μM BA + 2.5 mM Put, while a significant reduction in Put was observed in treatments with 10 or 20 μM TIBA combined with BA + Put compared to the 2.5 μM BA + 2.5 mM Put treatment (Fig. 3a). In addition, a lower content of this PA was observed in the control and in the 10 and 20 μM TIBA treatments (Fig. 3a). The endogenous Spd levels were significantly highest in the treatments with 2.5 μM BA + 2.5 mM Put, 2.5 μM BA + 2.5 mM Put + 10 μM TIBA, 2.5 μM BA + 2.5 mM Put + 20 μM and with 10 μM TIBA alone (Fig. 3b), while the other treatments exhibited significantly lower levels (Fig. 3b). For Spm, significant differences were observed in the 2.5 μM BA + 2.5 mM Put combined with 10 or 20 μM TIBA treatments compared to the control and 10 μM TIBA treatments (Fig. 3c). Additionally, the highest endogenous total free PA levels were observed in the treatment containing 2.5 μM BA + 2.5 mM Put (Fig. 4a). The combination of 10 or 20 μM TIBA with 2.5 μM BA + 2.5 mM Put significantly decreased endogenous total free PA levels (Fig. 4a). Shoots from the control, 10 or 20 μM TIBA treatments showed lower endogenous total free PAs (Fig. 4a) and PA ratios [Put/(Spd + Spm)] (Fig. 4b).

Effect of auxin transport inhibitor on IAA levels in shoots from cotyledonary nodal segments

In the present study, the effects of TIBA on endogenous IAA levels were evaluated in shoots from cotyledonary nodal segments of *C. fissilis* after 30 days of culture (Fig. 5). The endogenous IAA levels were significantly higher in shoots grown under 2.5 µM BA + 2.5 mM Put + 20 µM TIBA, while between the control and 2.5 µM BA + 2.5 mM Put, there were no significant differences, with the lowest IAA levels observed (Fig. 5).

Effect of auxin transport inhibitor on the proteomic profile in shoots from cotyledonary nodal segments

A total of 607 proteins were identified in shoots from cotyledonary nodal segments grown under 2.5 µM BA + 2.5 mM Put + 20 µM TIBA (BA + Put + TIBA) compared to shoots from 2.5 µM BA + 2.5 mM Put (BA + Put) (Supplementary Table S1). Among them, 121 were differentially accumulated proteins (DAPs), while 486 were unchanged. Of the DAPs, 44 were down-accumulated and 75 were up-accumulated in the shoots treated with TIBA-treated compared to those without TIBA treatment. In addition, one unique protein was identified only in shoots from the TIBA treatment, and one unique protein was only identified in shoots grown without TIBA (BA + Put) (Fig. 6).

From the DAPs, we highlight some down- and up-accumulated in the comparison of BA + Put + TIBA with BA + Put due to their direct relationship with shoot growth and development in *C. fissilis*. Among the down-accumulated proteins, two were identified, tubulin beta-1 chain (Ce_fissilis.006840.1) and actin (Ce_fissilis.012078.1), and one unique protein, peroxidase 15 (Ce_fissilis.013923.2), in shoots under the use of BA + Put + TIBA (Supplementary Table S1). On the other hand, some proteins were up-accumulated, such as membrane steroid-binding protein 1 (Ce_fissilis.005206.1) and stem-specific protein TSJT1 (Ce_fissilis.006747.1), in shoots treated with TIBA compared to shoots without TIBA, while the mitochondrial carnitine/acylcarnitine carrier-like protein (Ce_fissilis.012378.1) was unique in shoots treated without TIBA (BA + Put) (Supplementary Table 1).

The network of KEGG pathway enrichment by STRING for DAPs resulted in the enrichment of biological processes (Fig. 7). In the predicted protein—protein interaction network, the identified protein was 14-3-3 protein 4 (Ce_fissilis.010535.1), which was down-accumulated in shoots under the use of BA + Put + TIBA. This protein showed an interaction with several down-accumulated proteins related to carbon metabolism biological processes, such as 3-phosphoglycerate dehydrogenase (Ce_fissilis.013598.1), glycerate dehydrogenase HPR, peroxisomal (Ce_fissilis.001892.1), malate dehydrogenase, glyoxysomal (Ce_fissilis.014972.1) and with proteins related to photosynthesis, such as chlorophyll a-b binding protein 13 chloroplastic (Ce_fissilis.000974.1) and chlorophyll a-b binding protein CP29.2, chloroplastic (Ce_fissilis.015248.1) (Fig. 7). On the other hand, in the biological process of valine, leucine and isoleucine degradation, proteins were up-accumulated such as 2-oxoisovalerate dehydrogenase subunit beta 1, mitochondrial (Ce_fissilis.014783.1), probable acetyl-CoA acetyltransferase, cytosolic 2 (Ce_fissilis.014293.1, Ce_fissilis.015933.1) and probable 3-hydroxyisobutyrate dehydrogenase, mitochondrial (Ce_fissilis.018195.1), in shoots treated with TIBA (Fig. 7).

Effect of exogenous Put on ex vitro rooting

Treatment with Put did not significantly affect the induction (Fig. 8a), number (Fig. 8b) or length (Fig. 8c) of roots, except for the shoot cuttings treated with 5 mM Put, which showed a significant increase in the length of the roots (Fig. 8c).

Effect of the auxin transport inhibitor combined with exogenous Put on ex vitro rooting

As the cuttings treated with 5 mM Put showed a significant increase in the length of the roots (Fig. 8c), the effects of Put and its combination auxin transport inhibitor TIBA on rooting were evaluated using shoot cuttings from cotyledonary nodal segments. The percentage of rooted shoot cuttings (Fig. 9a) and the number of roots per shoot cutting (Fig. 9b) were significantly reduced by Put treatment combined with TIBA, while the length of roots was significantly increased by Put treatment (Fig. 9c).

Effect of Put and auxin transport inhibitor on endogenous PA contents

As treatment with 200 µM TIBA induced a reduction in rooting in *C. fissilis* cuttings (Ribeiro et al. 2022), the endogenous contents of free PAs Put, Spd and Spm were analyzed in shoot cuttings from cotyledonary nodal segments treated without and with 5 mM Put, combined or not with TIBA (Fig. 10). Among the analyzed PAs, the endogenous free Put contents increased on the 3rd day of rooting in shoot cuttings under 200 µM TIBA and 5 mM Put. On the 6th rooting, the shoot cuttings treated with Put + TIBA showed higher Put content, while on the 10th rooting, this result was observed in the TIBA treatment (Fig. 10a).

A higher content of free Spd was observed in shoot cuttings treated with Put + TIBA on the 3rd and 6th days of rooting, while on the 10th day, the highest Spd content was observed in the TIBA treatment (Fig. 10b). On the other hand, the Put treatment increased the endogenous contents of free-Spm in the 3rd and 10th cuttings, while in the 6th cuttings, the Put and control treatments increased the endogenous contents of free-Spm compared to shoot cuttings before root induction (Fig. 10c).

4 Discussion

Our results showed a reduction in shoot growth from cotyledonary and apical nodal segments using TIBA, an inhibitor of auxin transport. This inhibitor acts by altering the cytokinin/auxin ratio, i.e., can cause an increase in IAA contents, which in turn inhibits the proliferation of axillary shoots (Rubery 1987). It is likely that TIBA can interfere with organized cell division by competing with endogenous auxin (Dhaliwal et al. 2004). Our study showed that the auxin transport inhibitor affected the endogenous content of PAs in shoots from apical and cotyledonary nodal segments, with lower endogenous free Put contents. Put stimulates cell division, promoting the transition through the G1/S and G2/M cell cycle phases (Weiger and Hermann 2014), and the synergistic effect between BA and Put can increase the cellular divisions necessary for higher shoot growth in *C. fissilis* (Aragão et al. 2016, Aragão et al. 2017). In this sense, the auxin inhibitor negatively affects the endogenous contents of Put, which can be related to the crosstalk between the cytokinin/auxin ratio and PA contents in the shoots of *C. fissilis*.

The addition of the auxin transport inhibitor TIBA (BA + Put + TIBA treatment) also resulted in significant changes in the proteomic profile in shoots from cotyledonary nodal segments compared to that without TIBA (BA + Put). The down-accumulation of tubulin beta-1 chain (Ce_fissilis.006840.1) and actin (Ce_fissilis.012078.1) proteins in shoots treated with auxin inhibitor (BA + Put + TIBA) compared without TIBA could be relevant for shoot growth. Tubulin is the main constituent of microtubules (Weisenberg et al. 1968). Mechulam et al. (2009) demonstrated that PAs are potent agents for inducing tubulin self-attraction, which is relevant to the regulation of the microtubule network. Additionally, actin is involved in several crucial cellular processes in plants, including determination of the division plane, cell elongation and differentiation (Staiger et al. 2013). In our analysis, the reduced levels of endogenous free Put and total free PAs, along with the decreased abundance of tubulin and actin proteins in shoots treated with TIBA, may be associated with impaired shoot elongation. This could be a consequence of a diminished G1/S transition within the cell cycle, resulting in reduced cell divisions and hindered shoot growth. The relationship between increased Put contents and elevated tubulin abundance was found to be associated with enhanced in vitro shoot elongation in *Cariniana legalis* under various light spectra provided by LED lamps (Lerin et al. 2019). Consistent with this study, our results demonstrate that the application of an auxin transport inhibitor combined with BA and Put (BA + Put + TIBA) led to a decrease in Put content and tubulin abundance, ultimately hindering shoot growth in *C. fissilis*.

The use of 20 µM of the auxin transport inhibitor TIBA resulted in a reduction in the abundance of 14-3-3 protein 4 (Ce_fissilis.010535.1) in shoots. This protein is known to interact with target proteins, thereby regulating enzymatic activity, protein stability, and protein–protein interactions (Camoni et al. 2018). It plays a vital role as a regulatory protein during plant growth and development (Zhang et al. 2010) (Guo et al. 2022). Moreover, it has been observed to modulate carbon and nitrogen metabolism enzymes (Huber et al. 2002), which aligns with the protein–protein interaction network revealed in our study. Our findings demonstrate that the 14-3-3 protein interacts with several other proteins associated with metabolic pathways, carbon metabolism, and photosynthesis (Fig. 7). Consequently, a decrease in the accumulation of this protein can potentially disrupt critical interactions involved in the growth of *C. fissilis* shoots.

Carbon and nitrogen are crucial elements that act as limiting factors for plant growth. The 3-phosphoglycerate dehydrogenase protein (Ce_fissilis.013598.1) is one of the key enzymes involved in carbon metabolism, which catalyzes the oxidation of 3-phosphoglycerate derived from glycolysis and the Calvin cycle in autotrophic, heterotrophic, and photosynthetic cells (Ros et al. 2013). Another important enzyme identified, the glycerate dehydrogenase HPR, peroxisomal (Ce_fissilis.001892.1), is responsible for the reduction of hydroxypyruvate into glycerate within the photorespiratory core cycle (Timm et al. 2008). Additionally, the malate dehydrogenase glyoxysomal (Ce_fissilis.014972.1) protein plays a vital role in maintaining optimal photorespiration rates by reducing NAD + to NADH through the oxidation of malate, which is supplied from the cytoplasm to oxaloacetate (Cousins et al. 2008). Our results revealed an accumulation of these proteins, indicating that shoots treated with TIBA (BA + Put + TIBA) may exhibit smaller elongation due to alterations in the photorespiratory cycle caused by the reduced accumulation of these proteins. Furthermore, alterations in physiological processes related to photosynthesis can

contribute to an imbalance between light energy absorption, the Calvin cycle, and carbohydrate metabolism.

The down-accumulation of chlorophyll a-b binding protein 13 chloroplastic (Ce_fissilis.000974.1) and chlorophyll a-b binding protein CP29.2, chloroplastic (Ce_fissilis.015248.1) in shoots treated with TIBA suggests disturbances in these processes. These proteins are part of the antenna complex and play a crucial role in absorbing light and transferring excitation energy to the central photosystem II (PSII) complexes, which drive photosynthetic electron transport (Jansson 1999). Moreover, in our work, a unique protein in shoots under the use of BA + Put + TIBA, peroxidase 15 (Ce_fissilis.013923.1), was identified. The activity of peroxidases can be related to changes in photosynthesis (MacFarlane and Burchett 2001), which has consequences on plant growth. Consequently, the observed decrease in the shoot length of *C. fissilis* may be associated with changes in processes related to photosynthesis.

In our analyses, we observed a higher accumulation of membrane steroid-binding protein 1 (Ce_fissilis.005206.1) and stem-specific protein TSJT1 (Ce_fissilis.006747.1) in shoots treated with TIBA compared to untreated shoots. Hu et al. (2016) have previously reported that the stem-specific protein TSJT1 can function as a negative regulator of castor internode development. Similarly, in *Arabidopsis thaliana*, a membrane steroid-binding protein was identified as a negative regulator of cell elongation (Yang et al. 2005). Thus, the higher accumulation of these proteins may be related to the shorter length of shoots under TIBA treatment compared to those without TIBA treatment in *C. fissilis*.

Furthermore, by the analyses from the network of KEGG pathway enrichment, we observed some up-accumulated proteins related to the degradation of valine, leucine, and isoleucine pathways in shoots treated with TIBA, such as 2-oxoisovalerate dehydrogenase subunit beta 1, mitochondrial (Ce_fissilis.014783.1), probable acetyl-CoA acetyltransferase, cytosolic 2 (Ce_fissilis.014293.1, Ce_fissilis.015933.1) and probable 3-hydroxyisobutyrate dehydrogenase, mitochondrial (Ce_fissilis.018195.1). Previous studies have highlighted the significance of branched-chain amino acid degradation, including the valine and leucine pathways, in maintaining amino acid homeostasis (Araújo et al. 2010, Peng et al. 2015, Gipson et al. 2017). Although Zhao et al. (2021) have shown the critical role of this pathway in coleoptile elongation in *Zea mays*, in *C. fissilis*, a shorter shoot length was observed. Thus, the protein-related degradation of valine, leucine, and isoleucine may affect shoot development in *C. fissilis*.

One unique protein identified in shoots without TIBA is the mitochondrial carnitine/acylcarnitine carrier-like protein (Ce_fissilis.012378.1). This protein plays a crucial role in facilitating the uptake of the acetyl group generated during the β-oxidation of fatty acids in the mitochondria (Picault et al. 2004). Within the β-oxidation pathway, each turn of the cycle generates one molecule of acetyl-CoA, which is subsequently converted by the glyoxylate cycle into succinate, which can enter the tricarboxylic acid (TCA) cycle, glyoxylate cycle, and other biosynthetic pathways (Harwood and Moore 1989). The presence of this protein suggests that the proper functioning of these pathways supports the energy demands and growth requirements necessary for optimal shoot development in *C. fissilis* in the absence of auxin transport.

The rooting of shoot cuttings is a crucial step in the micropropagation process, as it is fundamental to produce plantlets. In *C. fissilis*, the rooting of shoots can be achieved even without the application of exogenous auxin, such as indole-3-butyric acid (IBA), as endogenous levels of auxin are sufficient to promote this morphological process (Ribeiro et al. 2022). Additionally, PAs also play a significant role in rooting, and studies suggest a possible interaction between these molecules and auxin (Tonon et al. 2001). Therefore, we analyzed the effect of Put, combined or not with an auxin inhibitor, on the rooting of shoot cuttings and its effects on the endogenous content of PAs in these treatments. The percentage of rooted shoots and number of roots per shoot cuttings were significantly reduced in shoots under Put combined with auxin inhibitor (Put + TIBA) treatments, while only the length of roots was significantly increased by Put treatment without TIBA. This increased length of roots by exogenous Put treatment can be justified by the involvement of this PA with greater cell division and expansion (Tang and Newton 2005), which is consequently associated with longer roots in the study species but not with root induction and number.

The endogenous free Put contents increased in the shoot cuttings treated with TIBA or 5 mM Put on the 3rd day of rooting, while on the 10th day of rooting, this result was observed in the TIBA. According to our endogenous contents of PAs, we suggest that the increase in endogenous Put content may be the result of the auxin inhibitor effect and not the cause of root inhibition, as Put is not related to the inhibition of rooting in *C. fissilis*. Alterations in PA metabolism can impact amino acid levels in living cells (Majumdar et al. 2016). Homeostasis of PAs, which adjusts the levels of Put, Spd and Spm, is regulated by reprogramming the anabolic and catabolic pathways of this molecule's metabolism. A substantial part of the effects of PA can be attributed to its catabolites, such as γ-aminobutyric acid (GABA), which in plants is produced via decarboxylation of glutamate or by a two-step catabolism of Put. According to Shelp et al. (2012), an increase in diamine oxidase expression can lead to an increase in GABA production. GABA inhibits malate efflux from roots by directly binding to the anion transporter aluminum-activated malate transporters (ALMT1) Ramesh et al. (2015), causing hyperpolarization in *Hordeum vulgare* roots (Shabala et al. 2014). In addition, data show that GABA negatively regulates the development of adventitious roots in *Populus* (Xie et al. 2019). In *Brassica napus*, endogenous GABA content contributed to the decrease in primary root growth (Deleu et al. 2013). In this sense, a higher content of Put can inhibit rooting by increasing GABA from its catabolism, and further studies can be developed during AR induction in *C. fissilis* to show this interaction. Interestingly, in maize mesocotyl epidermal tissues, it has been shown that the auxin inhibitors TIBA and naphthylphthalamic acid (NPA) increased the expression of polyamine oxidase (PAO), an enzyme of PA catabolism, after treatment with light and reverted by exogenously supplied auxin, showing that both light and auxin adjust PAO expression in cell wall differentiation (Cona et al. 2003). Moreover, Put depletion affects the root phenotype through the antagonistic actions of hormonal signaling, with auxin and reactive oxygen species accumulation (Hashem et al. 2021). Based on our data, which demonstrate Put content in the presence of auxin inhibitor treatment during shoot rooting in *C. fissilis*, it is plausible to suggest that it can affect the metabolism of certain amino acids, such as GABA, due to alterations in PA content, particularly Put. Consequently, the perturbation of PAs may lead to a rebalancing of their biosynthesis, thereby affecting

the outcomes of phenotypic characteristics through multiple direct and indirect pathways. Future research could focus on understanding whether the intracellular formation of amino acids, such as GABA, can be triggered by changes in PA content during rooting in *C. fissilis* and investigating their potential involvement in the rooting process. By exploring these aspects, we can enhance our understanding of the intricate relationship between PAs, amino acids, and rooting mechanisms in this species.

5 Conclusion

The findings of our work revealed that inhibition of shoot growth by auxin transport inhibition was related to a reduction in endogenous total free PA levels and an increase in IAA-free content. Additionally, TIBA induced changes in the abundance of proteins related to the inhibition of shoot growth. The reduction in the accumulation of proteins related to cytoskeleton organization, carbon metabolism, and photosynthesis and the increase in the accumulation of proteins related to the degradation of valine, leucine and isoleucine in shoots treated with auxin inhibitors were related to the inhibition of shoot growth. The percentage and number of roots were significantly reduced by Put + TIBA treatment, whereas endogenous free Put contents increased in the shoot cuttings treated with TIBA or 5 mM Put during rooting. From these findings, we can infer that Put is not directly related to rooting induction but rather to the length of roots in *C. fissilis*.

Declarations

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Author contributions YRSR and VPMA CSC conceived the study, designed the experiments and wrote the manuscript. YRSR and VPMA were responsible for the in vitro culture of shoots and ex vitro rooting experiments and performed the statistical analyses. YRSR and RCS performed the polyamine analyses. YRSR, AFM and EISF were responsible for IAA analyses. KRS 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 identifer PXD043790. All identifed proteins are available in the supplementary material.

Code availability PXD043790

Conflict of interest The authors of the manuscript have no competing interests to declare that are relevant to the content of this article.

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Figures

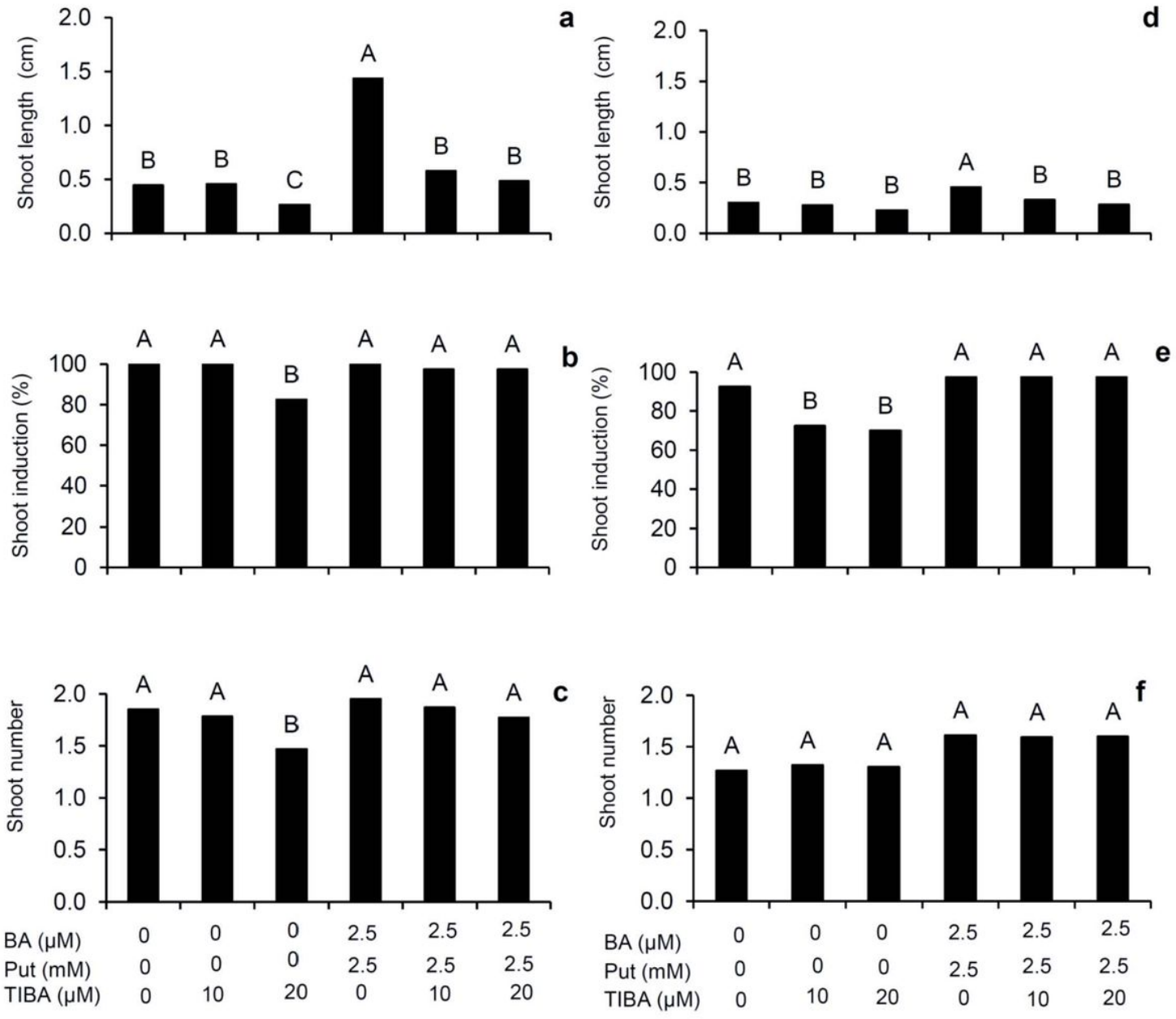

Figure 1

Length (a) induction (b) and number (c) of shoot cuttings from apical and cotyledonary nodal segments of *Cedrela fissilis* after 30 days of in vitro incubation. Means followed by the same letter do not differ statistically according to Tukey's test ($P < 0.05$). Different capital letters show significant differences. CV = coefficient of variation (n = 8; CV of a = 16.62%, CV of b = 8.25%, CV of c = 9.92%, d = 22.99%, CV of e = 12.35%, CV of f = 17.98%)

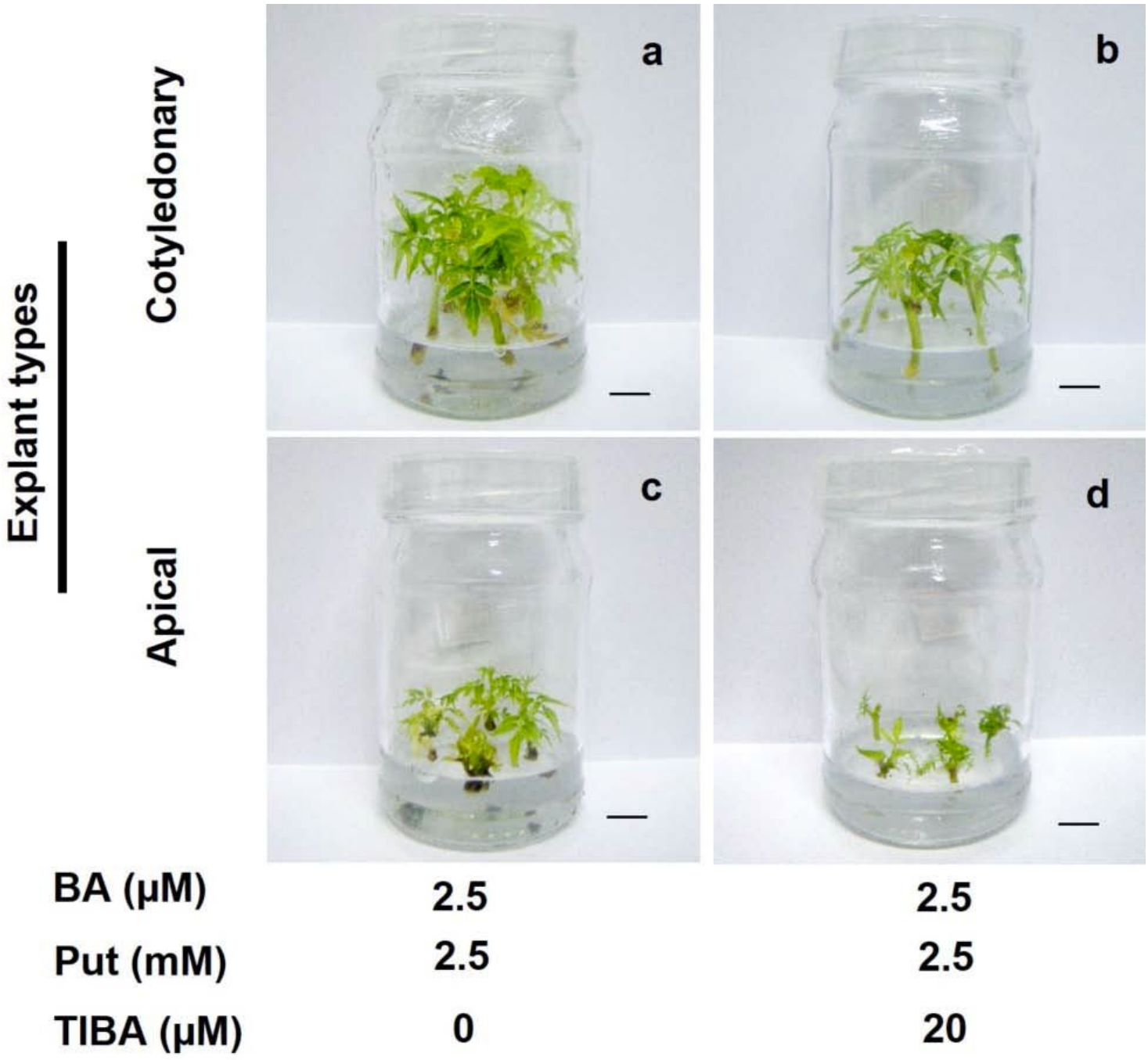

Figure 2

Morphological aspects of shoots obtained from apical (c, d) and cotyledonary nodal (a, b) segments of *Cedrela fissilis* after 30 days of in vitro culture with 2.5 µM BA+2.5 mM Put (a, c) and 2.5 µM BA+2.5 mM Put+20 µM TIBA. Bars 1 cm

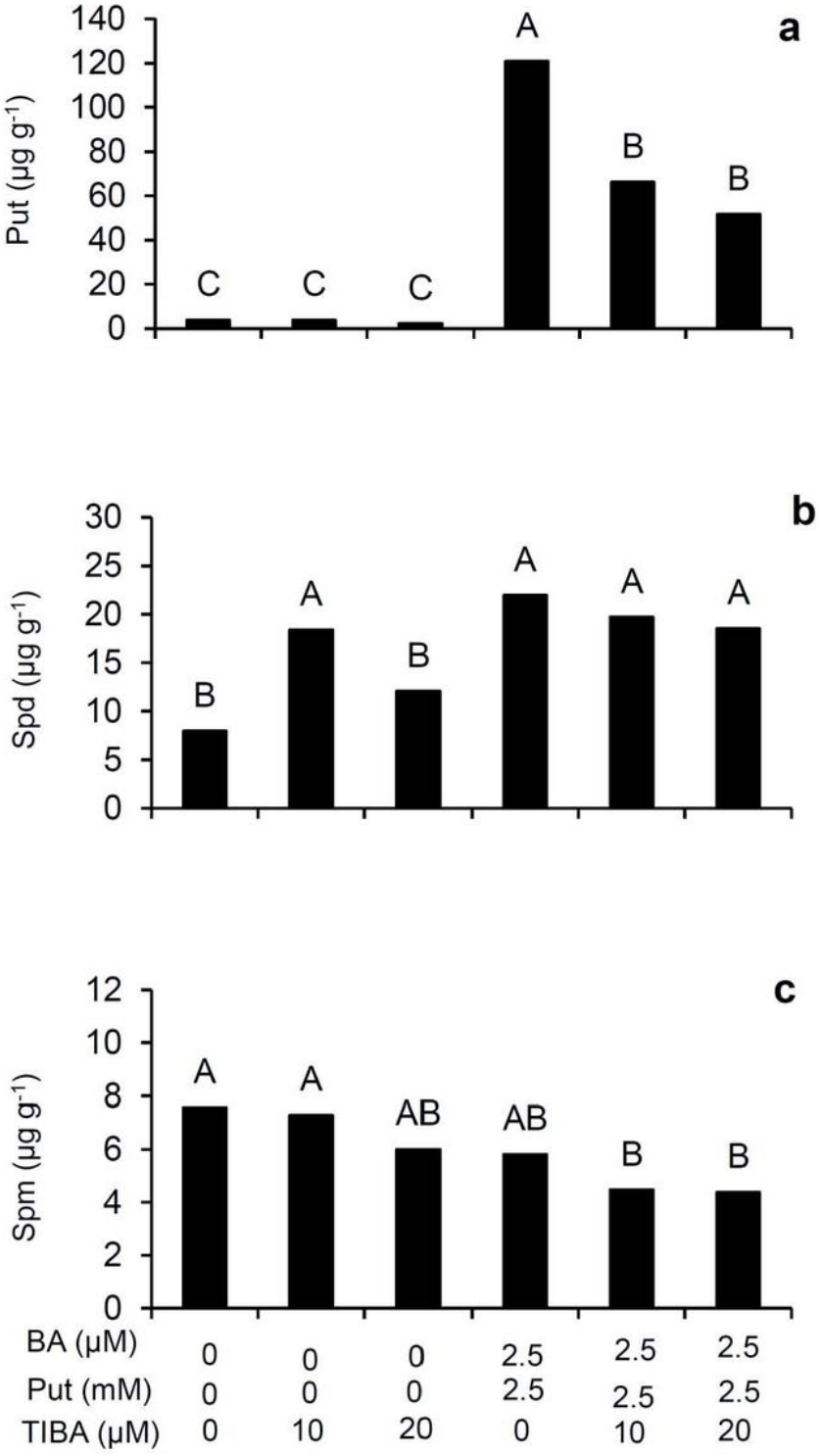

Figure 3

Endogenous levels (μg g^{-1} FM) of free Put (a), Spd (b), and Spm (c) in the shoots from cotyledonary nodal segments of *Cedrela fissilis* cultured in the absence (0 mM) or presence (2.5 mM) of Put in the absence (0 mM) or presence (2.5 mM) of BA combined or not with TIBA (10 or 20 μM) after 30 days of in vitro culture. Means followed by different letters are significantly different ($P < 0.05$) according to Tukey's test. CV coefficient of variation (n = 3; CV for Put = 15.15%; CV for Spd = 11.38%; CV for Spm = 13.90%)

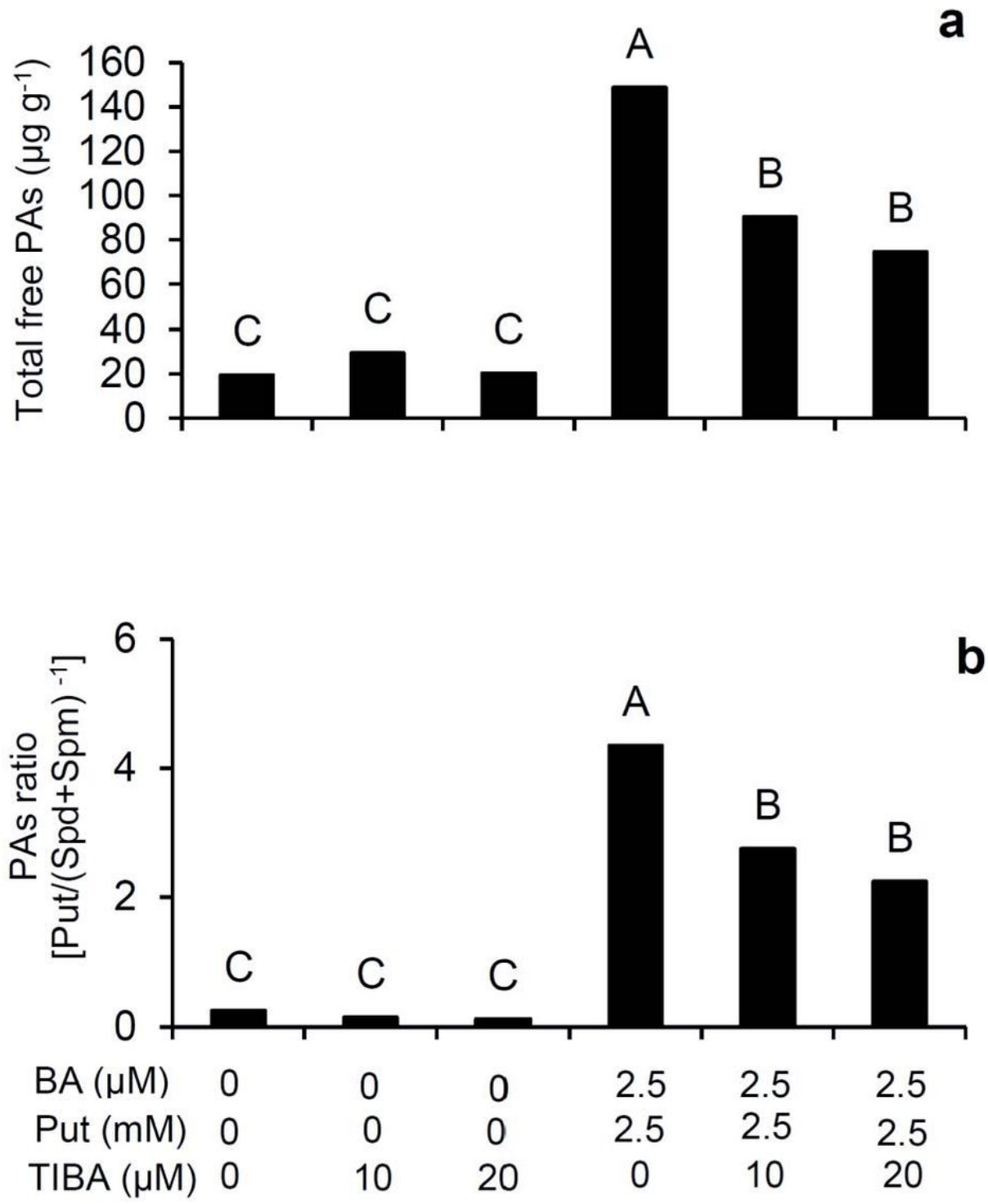

Figure 4

Endogenous levels (μg g^{-1} FM) of total free PAs (a) and PA ratio (b) in shoots from cotyledonary nodal segments of *Cedrela fissilis* incubated in the absence (0 mM) or presence (2.5 mM) of Put in the absence (0 mM) or presence (2.5 mM) of BA combined or not with TIBA (10 or 20 μM) at 30 days of in vitro culture. Means followed by different letters are significantly different (P < 0.05) according to Tukey's test. CV coefficient of variation (n = 3; CV for total free PAs = 10.99%; CV PA ratio = 13.62%)

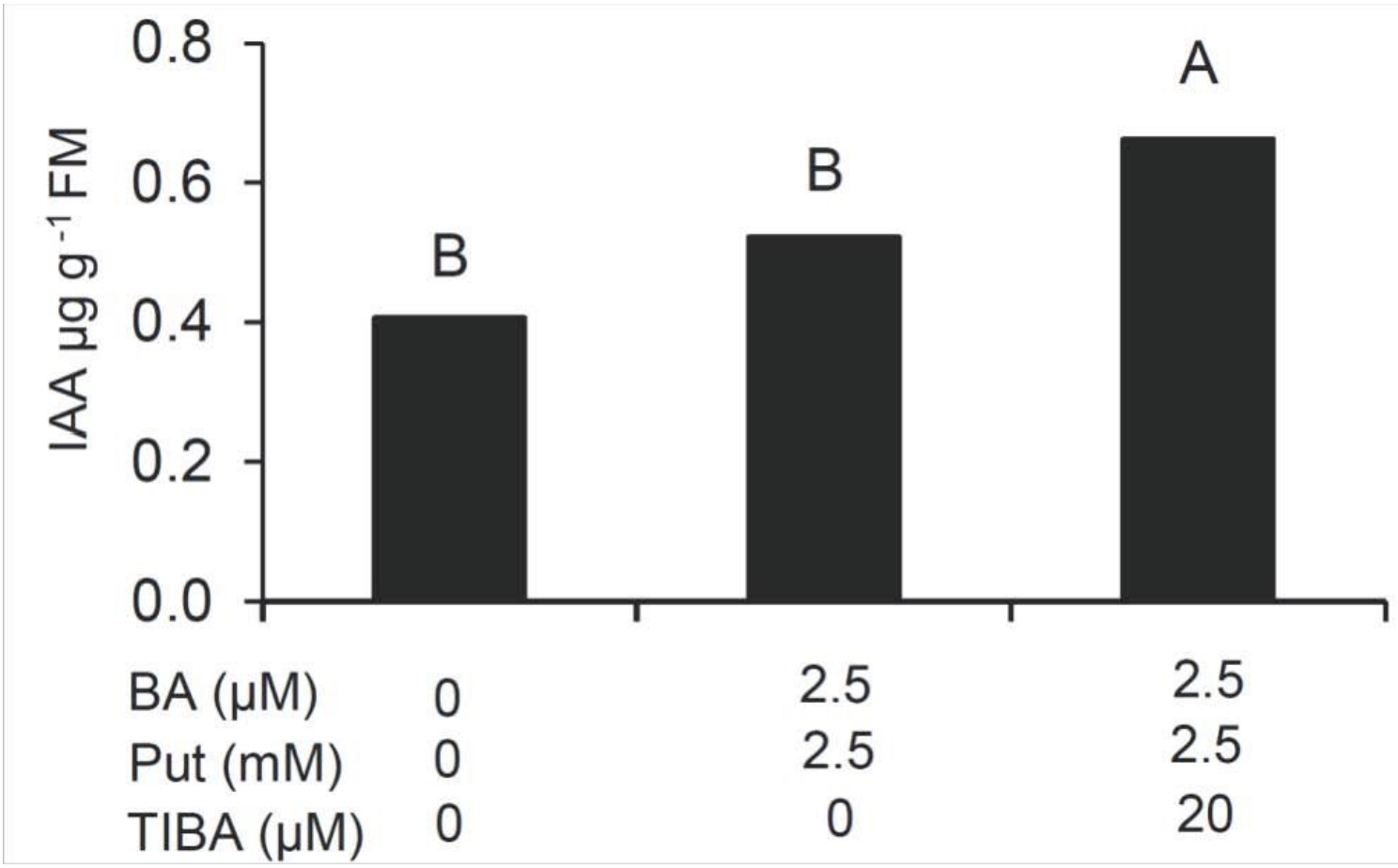

Figure 5

IAA contents (μg g^{-1} FM) of shoots from cotyledonary nodal segments of *Cedrela fissilis* in the absence or presence of 2.5 μM BA+2.5 mM Put and 2.5 μM BA+2.5 mM Put+20 μM TIBA at 30 days of in vitro culture. Means followed by the same letter do not differ statistically according to Tukey's test (P < 0.05). CV = coefficient of variation. (n = 3, CV = 6.53%)

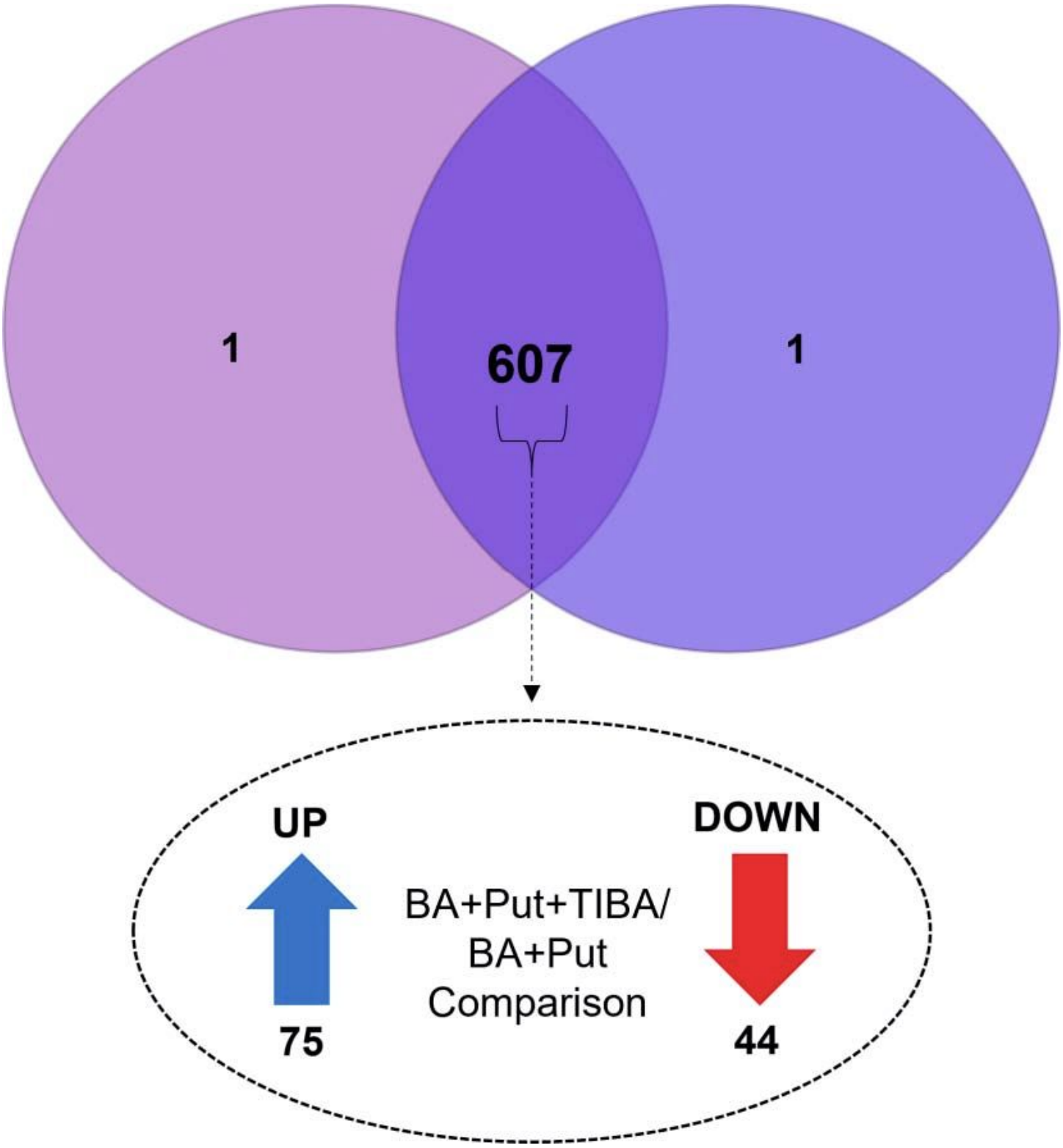

Figure 6

Venn diagram of proteins identified in *Cedrela fissilis* shoots at 30 days of treatment with 2.5 µM BA+2.5 mM Put+20 µM TIBA compared to 2.5 µM BA+2.5 mM Put.

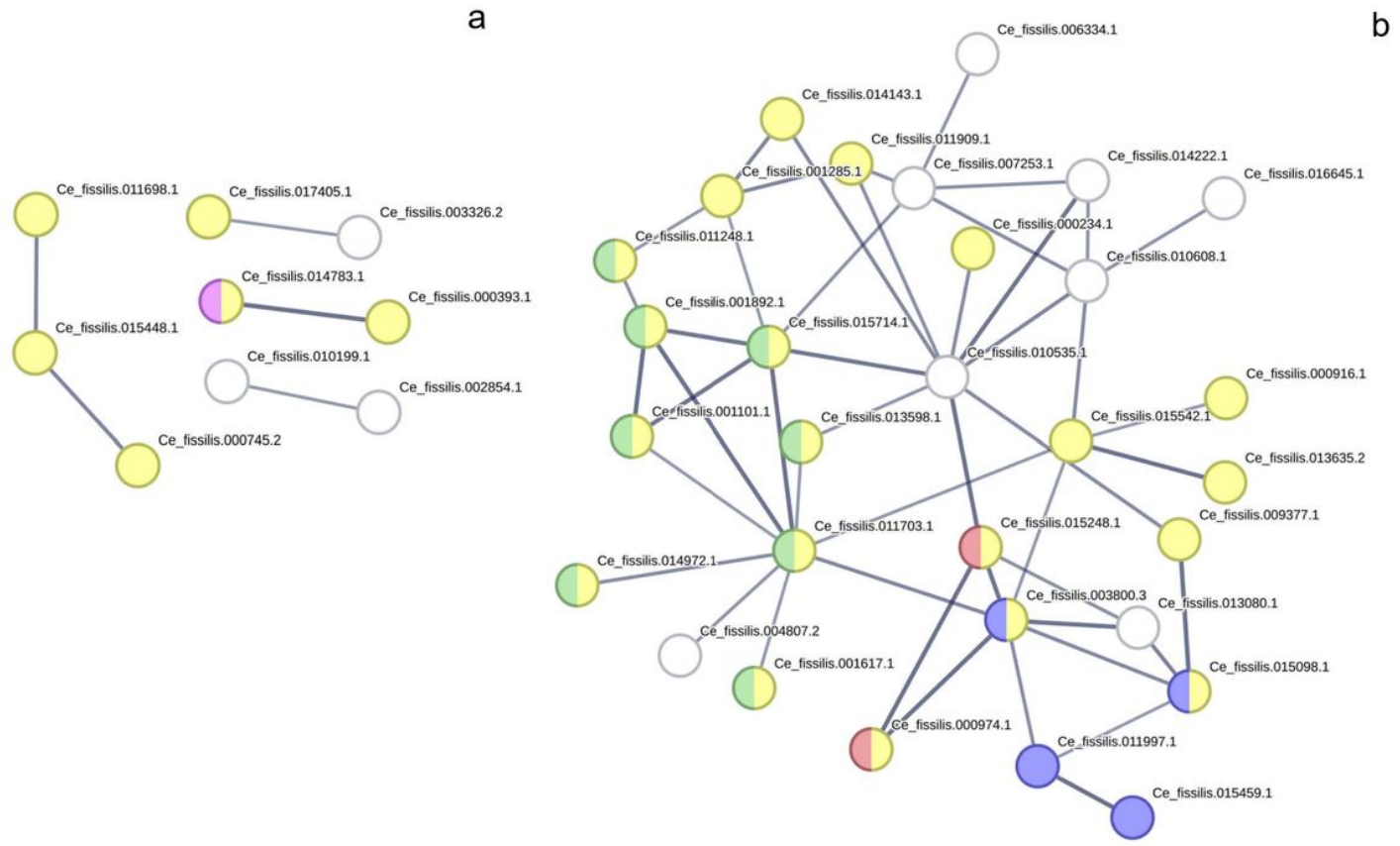

Figure 7

Protein–protein interaction network between differentially accumulated proteins up-accumulated (a) and down-accumulated (b) in *Cedrela fissilis* at 30 days of treatment with 2.5 µM BA+2.5 mM Put+20 µM TIBA compared to 2.5 µM BA+2.5 mM Put. Nodes colored in yellow indicate metabolic pathways, nodes colored in green indicate carbon metabolism, nodes colored in blue indicate photosynthesis, nodes colored in red indicate proteins related to photosynthesis – antenna proteins, and nodes colored in pink indicate proteins related to valine, leucine and isoleucine degradation.

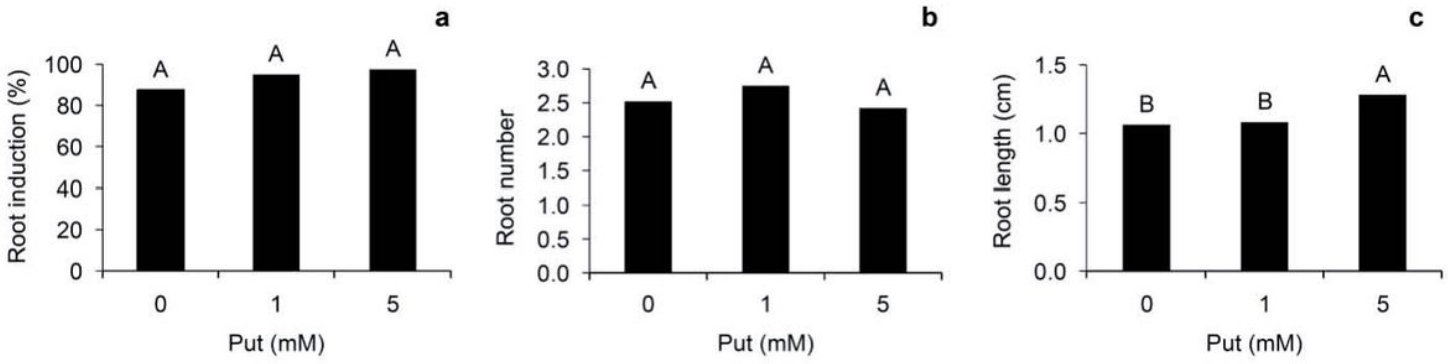

Figure 8

Percentage of rooted shoot cuttings (a), number (b) and length (c) of roots per shoot cuttings in the absence, 1 mM, and 5 mM Put treatments of *Cedrela fissilis* at 13 days of rooting. Means followed by the same letter do not differ statistically according to Tukey's test (P<0.05). CV = coefficient of variation (n = 8; CV a = 9.64%, CV b = 16.11%, CV c = 12.93%).

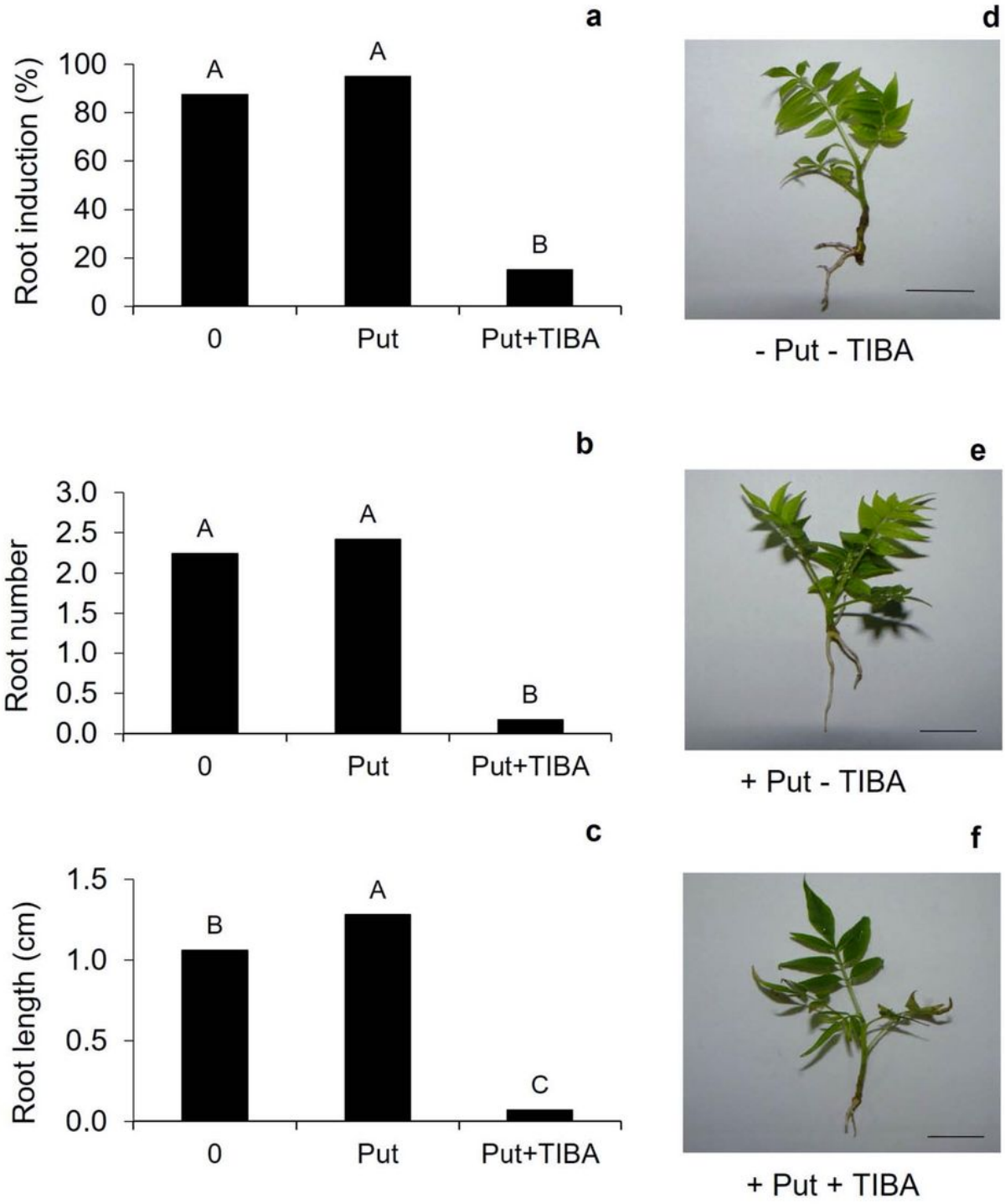

Figure 9

Percentage of rooted shoot cuttings (a), number (b) length (c) of roots per shoot cuttings and (d) rooted shoot cutting in the absence of Put and TIBA, 5 mM Put (e), and 5 mM Put + TIBA (f) treatments of *Cedrela fissilis* at 13 days of rooting. Means followed by the same letter do not differ statistically according to the SNK test ($P < 0.05$). CV = coefficient of variation (n = 8; CV a = 12.6%, CV b = 22.5%, CV c = 16.5%). Bars = 1 cm.

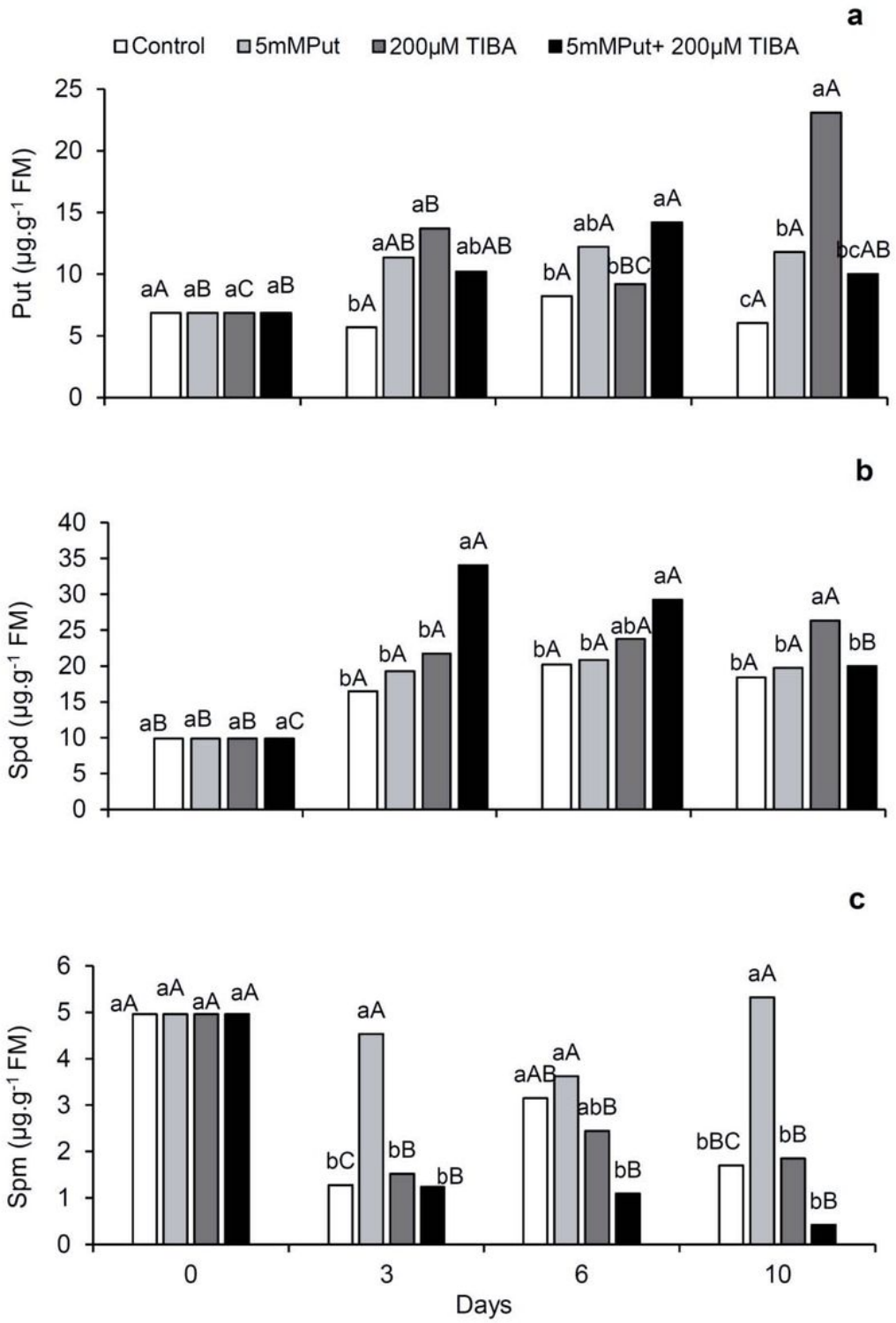

Figure 10

Endogenous content (µg.g^{-1} MF) of putrescine (a), spermidine (b) and spermine (c) in *Cedrela fissilis* shoot cuttings before (time 0) and after 3, 6 and 10 days of rooting in the control, 200 µM TIBA, 5 mM Put and 5 mM Put +200 µM TIBA treatments. Means followed by the same letter do not differ statistically from each other according to Tukey's test (P<0.05). Different capital letters show significant differences comparing the days of rooting in each treatment. Different lowercase letters show significant differences comparing treatments on each day of rooting. CV = coefficient of variation. FM = fresh matter; Put = putrescine; Spd = spermidine; Spm = spermine. (n = 3, CV a = 21%, CV b = 14.9%, CV c = 27.3%).

Supplementary Files

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

- SupplementaryTable1.xlsx