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ORIGINAL ARTICLE

Involvement of differentially accumulated proteins and endogenous auxin in adventitious root formation in micropropagated shoot cuttings of *Cedrela fissilis* Vellozo (Meliaceae)

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Abstract

Investigation of the biochemical and molecular changes during rooting in woody species can reveal how specific molecules are involved in adventitious root (AR) induction. We investigated the effects of indole butyric acid (IBA) and inhibitors of polar auxin transport and auxin signaling on ex vitro rooting of micropropagated shoots of *Cedrela fissilis* and evaluated the proteomic profile and endogenous contents of indole acetic acid (IAA) during AR initiation. We observed that exogenous IBA was not necessary for ex vitro rooting, and root induction was significantly reduced by auxin signaling (p-chlorophenoxyisobutyric acid—PCIB) and polar auxin transport (triiodobenzoic acid—TIBA) inhibitors. The presence of meristematic cells and the formation of meristematic centers in the base of shoot cuttings at 3 days of rooting are necessary for AR formation. The accumulation of cell division- and auxin-related proteins and the presence of unique proteins related to cell wall modification in shoot cuttings at 3 days of rooting were associated with higher IAA contents and AR initiation. In addition, an accumulation of proteins related to dephosphorylation, glycolytic and tricarboxylic acid cycle pathways and a reduction in the accumulation of proteins from isoflavonoid metabolism were associated with the promotion of AR initiation. These results show the relevance of these proteins for AR initiation in this species. This is the first study showing the involvement of protein accumulation and endogenous IAA contents in adventitious rooting initiation in *C. fissilis*, an easily rooted endangered woody species from the Brazilian rainforest.

Key message

Auxin contents and proteomic analyses during adventitious rooting of in vitro propagated *Cedrela fissilis* shoots.

Keywords Adventitious rooting · Auxin signaling inhibitor · Comparative proteomics · Indole-acid acetic · In vitro shoot propagation · Polar auxin transport inhibitor

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Abbreviations

ACR4	ARABIDOPSIS CRINKLY 4
AIR12	Auxin-induced in root cultures protein 12
AR	Adventitious root
Aux/IAA	Auxin/IAA repressor protein
BA	6-Benzyladenine
BSA	Bovine serum albumin
CDC48	Cell division cycle protein 48 homolog
DAPs	Differentially accumulated proteins
DTT	Dithiothreitol
EXPB1	Expansin-like B1
FAA-50	Formalin-acetic acid-alcohol
FM	Fresh matter
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid

PBS	Phosphate-buffered saline
PCIB	2-Chlorophenoxy-2-methyl propionic acid
PMSF	Phenylmethylsulfonyl fluoride
TCA	Trichloroacetic acid

Introduction

Native woody species from the Brazilian Atlantic Forest, including *Cedrela fissilis* Vellozo (Meliaceae), popularly known as "cedro rosa", have 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). Due to the economic and ecological importance of *C. fissilis*, initial studies on in vitro propagation have been performed for this species (Aragão et al. 2017, 2016; Nunes et al. 2002). However, no studies have been performed using ex vitro rooting of in vitro propagated shoots in this species.

The establishment of rooting is essential for plantlet production in a micropropagation system (Kasthurirengan et al. 2013). The process of adventitious root (AR) development can be divided into three successive phases: (a) induction, the period prior to any histological event; (b) initiation, with the occurrence of cell divisions leading to the formation of root meristems, characterized by the presence of small cells with large nuclei and dense cytoplasm; and (c) expression, characterized by root primordium formation from the internal cells formed at the initiation phase and culminating in root emergence (Li et al. 2009; Pacurar et al. 2014). According to Pijut et al. (2011), an additional step before the induction phase, called dedifferentiation, is necessary for adventitious rooting. In this phase, cells such as parenchymal cells close to the vascular bundles of the stem shoots are dedifferentiated, acquiring competence to respond to an AR-inducing signal that evokes proliferation and differentiation into meristematic type cells followed by differentiation into the complete root body through the stages described (Druege et al. 2019).

Ex vitro rooting provides several advantages in relation to in vitro rooting, with a cost reduction of up to 70% (Ranaweera et al. 2013). Thus, the ex vitro rooting method can be highly efficient, and the rooted plantlets showed a better quality of developed roots than those established in vitro (Yan et al. 2010), conferring greater tolerance of plantlets to the stress caused by the external environment (Phulwaria et al. 2013). Ex vitro rooting has been applied for some woody species, and it is often necessary to use exogenous auxin to promote rooting (Husain and Anis 2009; Phulwaria et al. 2013), as observed for *Tecomella undulata* (Varshney and Anis 2012) and *Albizia lebbeck* (Perveen et al. 2013), both of which were treated with 200 µM IBA for 30 min. The use of exogenous auxin is necessary if the endogenous IAA content is insufficient to promote rooting. However, in *Tectonia grandis*, ex vitro rooting also occurred in the absence of IBA (Fermino Júnior et al. 2011). Thus, in some species, the content of endogenous auxin, which occurs naturally in the shoots, is sufficient to promote rooting (Da Costa et al. 2013). If endogenous auxin contents are optimal to promote rooting, the exogenous application of this regulator is unnecessary and may be inhibitory (Yin et al. 2011).

The use of chemical inhibitors has been an effective tool for studying auxin responses (Ma et al. 2018), both in signaling (Oono et al. 2003) and polar auxin transport (Ahkami et al. 2013) during rooting. *p*-Chlorophenoxyisobutyric acid (PCIB) has been widely used to impair the auxin signaling pathway, regulating the stability of the auxin/IAA repressor protein (Aux/IAA) and inhibiting root formation in Arabidopsis (Oono et al. 2003). Triiodobenzoic acid (TIBA) is an inhibitor of auxin efflux transport involved in the inhibition of auxin trafficking across membranes (Kleine-Vehn et al. 2006) as well as in blocking the recycling of the PIN-FORMED (PIN) protein between endosomes and plasmatic membranes (Geldner et al. 2001). Thus, it is suggested that auxin is needed to stimulate the founder cells involved in root formation during the induction and initiation phases of AR (Druege et al. 2016, 2019; Guan et al. 2019).

Proteins and enzymes are synthesized and regulated by a cascade of events triggered by auxin and may also be related to the initiation of AR, which allows root primordia formation. Proteins related to carbohydrate and energy metabolism, photosynthesis, cell structure, and degradation were found to play a key role in the formation of new roots (Liu et al. 2013). Similarly, proteins involved in glucose metabolism, flavonoid biosynthesis, cell wall modification, hormone regulation, cellular cytoskeleton formation, and protein degradation/folding and import showed potential contributions to adventitious rooting (Tang et al. 2016; Zhang et al. 2015). Thus, these studies illustrate that proteomic analyses are an effective tool for identifying relevant proteins involved in the regulation of biological processes associated with rooting.

In this sense, we investigated the effects of IBA and inhibitors of polar auxin transport and auxin signaling on ex vitro rooting of micropropagated shoots of *C. fissilis* and evaluated the proteomic profile and endogenous contents of IAA during AR initiation.

Materials and methods

Plant material

Mature seeds of *C. fissilis* obtained from the Caiçara Comércio de Sementes LTDA located in Brejo Alegre, SP, Brazil (21° 10′ S and 50° 10′ W) were germinated in vitro, and 60-day-old seedlings were used as a source of explants

(apical and cotyledonary nodal segments) for shoot development. Micropropagated shoots at 45 days of in vitro culture were used for ex vitro rooting experiments.

In vitro seed germination and shoot propagation

For germination, seeds were submitted to a disinfection process according to Aragão et al. (2016), with modifications. Disinfection of 250 seeds was performed with 250 mL of distilled water containing 60 µL of neutral commercial detergent (Limpol; São Paulo, Brazil) and rinsed in 100 mL of water 5 times. Then, seeds were immersed in 250 mL of 70% ethanol (Tupi; São Paulo, Brazil) for 1 min. Subsequently, seeds were immersed in 100% bleach (QBoa®; São Paulo, Brazil) containing 1.8 to 2.5% active chlorine, supplemented with 7 g L^{-1} fungicide (Cercobin® 700 wp; São Paulo, Brazil) for 90 min. Subsequently, seeds were submitted to five washes with autoclaved distilled water (200 mL each) in a laminar flow chamber. The seeds were sown in MS culture medium (Murashige and Skoog 1962) (PhytoTechnology Laboratories®; Overland Park, USA) supplemented with 20 g L^{-1} sucrose (Synth; São Paulo, Brazil) and 2.0 g L^{-1} Phytagel® (Sigma-Aldrich; St. Louis, USA). The pH of the culture medium was adjusted to 5.8, and then the medium was autoclaved at 121 °C at 1.5 atm for 15 min. In vitro sowed seeds were maintained in a growth room at 25 ± 2 °C under a 16 h photoperiod, with a light intensity of 55 µmol m^{-2} s^{-1} provided by light-emitting diodes (LED) lamps (Koninklijke Philips Electronics NV; Amsterdam, Netherlands).

Sixty-day-old seedlings were used as a source of explants (apical and cotyledonary nodal segments) for the induction of shoots. The explants were isolated in a flow chamber and transferred to MS culture medium supplemented with 20 g L^{-1} sucrose, 2 g L^{-1} Phytagel and 2.5 µM BA (Sigma-Aldrich) according to Nunes et al. (2002). The pH of the culture medium was adjusted to 5.8 and autoclaved at 121 °C at 1.5 atm for 15 min. After being transferred into the culture medium, the explants were maintained in a growth room at 25 ± 2 °C under a 16 h photoperiod, with a light intensity of 55 µmol m^{-2} s^{-1} provided by LED lamps (Koninklijke Philips Electronics NV). After 45 days, shoots from apical and cotyledonary nodal segments were used for ex vitro rooting.

Effect of IBA on ex vitro rooting of micropropagated shoots

Forty-five-day-old shoots obtained by in vitro culture from apical and cotyledonary nodal segments were separated into cuttings (1.5 to 2.0 cm), retaining the apical meristem of the shoots and four main leaves. The bases of the shoot cuttings were immersed in solutions containing different concentrations (0 [distilled water], 50, 100, 250 and 500 µM) of IBA (Sigma-Aldrich) for 30 s. The shoot cuttings were then transferred to plastic cups (50 mL; TotalPlast, Santa Catarina, Brazil) containing the substrate Basaplant® (São Paulo, Brazil) and vermiculite (Basil Minérios; Goiás, Brazil) (1:1; v/v) and maintained in plastic trays (39.4 × 31.9 × 15.4 cm) (Pleion; São Paulo, Brazil) covered with PVC-type plastic film (Lumipam; São Paulo, Brazil) to maintain high relative humidity. The trays were maintained 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 fifteen days, aiming to reduce the moisture inside trays and stimulate the start of an acclimatization process simultaneously with rooting induction, the PVC parafilm plastic was perforated. This procedure was carried out until the complete removal of the PVC at 24 days after the start of perforation.

The experiment followed a completely randomized design in a 2 × 5 factorial arrangement, with shoot cuttings originating from two types of explants (cotyledonary and apical nodal segments) under five concentrations of IBA. Each treatment consisted of eight replicates, with each replicate containing four shoot cuttings, with a total of thirty-two shoot cuttings per treatment. After 30 days, the percentage of rooted shoot cuttings and the number and length (cm) of roots per shoot cuttings were evaluated.

Effect of the auxin inhibitors PCIB and TIBA on ex vitro rooting of micropropagated shoots

The effects of the auxin signaling inhibitor PCIB (Sigma-Aldrich) and polar auxin transport inhibitor TIBA (Sigma-Aldrich) on the rooting of shoot cuttings from cotyledonary nodal segments were evaluated. Forty-five-day-old in vitro shoot cuttings (1.5 to 2.0 cm) with the apical meristem of the shoots and four main leaves were used. The bases of the shoot cuttings were immersed in different concentrations of PCIB (0, 400 and 800 µM) and TIBA (0, 100 and 200 µM) for 90 min. For the control, i.e., without PCIB or TIBA, the bases of the shoot cuttings were immersed in distilled water for 90 min. PCIB and TIBA inhibitors were dissolved in 1 N NaOH (Sigma-Aldrich) and diluted in distilled water, and the pH was adjusted to 7.0.

After the treatment, the shoot cuttings were transferred to plastic cups (50 mL) containing substrate Basaplant® and vermiculite (1:1; v/v), maintained in plastic trays (39.4 × 31.9 × 15.4 cm) (Pleion) covered with PVC-type plastic film (Lumipam) to maintain high relative humidity, and kept in a growth room at 25 ± 2 °C under a 16 h photoperiod, 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, as described before.

The experiment followed a completely randomized design, with three concentrations of each inhibitor (TIBA or PCIB). 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 cuttings were evaluated. In addition, samples containing the base (0.5 cm) of shoot cuttings were fixed for histomorphological analysis to establish the phases of rooting, as well as for IAA immunolocalization analysis. Moreover, samples containing the bases (0.5 cm) of shoot cuttings were collected for proteomic profile and endogenous IAA quantification analysis and maintained at − 80 °C until the analyses were performed.

Determination of adventitious root development phases by histomorphological analysis

To determine the phases of rooting, the bases (0.5 cm) of shoot cuttings originating from cotyledonary nodal segments incubated in the treatments without inhibitors (control treatment) and with 800 µM PCIB and 200 µM TIBA were collected each day during 10 days of incubation. These samples were fixed in fixative solution containing 2.5% glutaraldehyde (Sigma-Aldrich) and 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M phosphate buffer (Sigma-Aldrich), pH 7.2, at room temperature for 24 h. The samples were then dehydrated with an increasing ethanol series (30, 50, 70, 90 and 100%) twice for 1 h each. Subsequently, the samples were infiltrated in 1:1 (v/v) Historesin (Leica, Heidelberger, Germany) and 100% ethanol (Merck; Darmstadt, Germany) for 12 h, followed by 100% Historesin for 24 h and hardened in 100% Historesin. Sections (5 µm) were obtained on a microtome (Leica), mounted on slides (Sail Brand; Zhejiang, China) and then stained with 1% aqueous toluidine blue (Synth) solution (Silveira et al. 2013). The sections were observed under an AxioImager M2 microscope (Carl Zeiss; Oberkochen, Germany) with the AxioVision 4.8 program (Carl Zeiss) and photographed with the aid of an AxioCam MR3 camera (Carl Zeiss) coupled to the equipment.

Proteomics analysis

For proteomic analysis, samples in biological triplicates (300 mg fresh matter—FM—each, in triplicate) containing the bases (0.5 cm) of shoot cuttings originating from cotyledonary nodal segments before (0 day) and after 3 days of treatment without and with 800 µM PCIB and 200 µM TIBA were used. Proteins were extracted using the trichloroacetic acid (TCA)/acetone method according to Damerval et al. (1986), with modifications. Initially, the samples were pulverized in liquid nitrogen using a ceramic mortar and pestle. The resulting powder was resuspended in 1 mL of chilled solution containing 10% (w/v) TCA (Sigma) in acetone with 20 mM dithiothreitol (DTT; GE Healthcare; Piscataway, USA) and vortexed for 5 min at 8 °C. Next, the samples were kept at − 20 °C for 1 h before centrifugation at 16,000×*g* for 30 min at 4 °C. The resulting pellets were washed three times with cold acetone plus 20 mM DTT and centrifuged for 5 min each time. The pellets were air dried and resuspended in buffer containing 7 M urea (GE Healthcare), 2 M thiourea (GE Healthcare), 2% Triton X-100 (GE Healthcare), 1% DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich), and a complete protease inhibitor cocktail (Roche Diagnostics; Mannheim, Germany), vortexed for 30 min at 8 °C, and centrifuged for 20 min at 16,000×*g* at 4 °C. The supernatants were collected, and the protein concentrations were determined using a 2-D Quant Kit (GE Healthcare).

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

Mass spectrometry was performed using a nanoAcquity UPLC connected to a Q-TOF SYNAPT G2-Si instrument (Waters, Manchester, UK) according to Passamani et al. (2018). Runs consisted of three biological replicates of 1 µg of digested peptides. 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 M-Class HSS T3 1.8 µm analytical reversed-phase column (75 µm × 150 mm) at 400 nL min^{-1}, with a column temperature of 45 °C. For peptide elution, a binary gradient was used, with mobile phase A consisting of water (Tedia; Fairfield, Ohio, USA) and 0.1% formic acid (Sigma-Aldrich) and mobile phase B consisting of acetonitrile (Sigma-Aldrich) and 0.1% formic acid. The gradient elution started at 7% B, then ramped from 7 B to 40% B until 91.12 min, then ramped again from 40 B to 99.9% B until 92.72 min, then remained at 99.9% until 106.00 min, then decreased to 7% B until 106.1 min, and finally remained at 7% B until the end of run at 120 min. Mass spectrometry was performed in positive and resolution mode (V mode), at 35,000 FWHM, with ion mobility, and in data-independent acquisition mode ($HDMS^E$). 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 v 4.0 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 v. 3.0.2 (Waters), and comparative label-free quantification was performed using ISOQuant software v. 1.7 (Distler et al. 2014, 2016). Briefly, 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 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. 2019) partner repository with the dataset identifier PXD021070.

To ensure the quality of the results after data processing, only the proteins present or absent (for unique proteins) in all three of the biological replicates were considered for differential accumulation analysis using 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, proteins were used in BLAST searches against the Nonredundant (nr) Green Plants/Viridiplantae Protein Sequences database using OmicsBox software (https://www.biobam.com/omicsbox) for the description of high-throughput functional annotation (Götz et al. 2008).

Quantification of IAA

The quantification of endogenous IAA contents in the bases of shoot cuttings from cotyledonary nodal segments was performed according to Álvarez-Flórez et al. (2017) and Silveira et al. (2004), with modifications. For this analysis, the bases (0.5 cm) of shoot cuttings originating from cotyledonary nodal segments before (0 days) and after 3 days of treatment without and with 800 µM PCIB and 200 µM TIBA were used. The samples (500 mg FM each, in triplicate) 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 (Merck). IAA[3H] was added as an internal standard. Then, the samples were vortexed for 5 min and centrifuged at 11,000×*g* for 20 min at 4 °C. The supernatants containing the 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 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.

Immunolocalization of IAA

IAA immunolocalization analysis was performed according to Pence and Caruso (1987) and De Diego et al. (2013), with modifications. For this analysis, samples containing the bases (0.5 cm) of shoot cuttings originating from cotyledonary nodal segments before (0 days) and after 3 days of treatment without and with 800 µM PCIB and 200 µM TIBA were used, with the times selected from the histomorphological analysis. The samples were prefixed for 2 h in 4% 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma-Aldrich) at 4 °C in a vacuum. The samples were then fixed in formalin-acetic acid-alcohol (FAA-50) solution containing 4% formaldehyde (Merck), 5% acetic acid (Merck) and 50% ethanol (Merck) in aqueous solution at 4 °C in a vacuum. Afterwards, samples were dehydrated by an increasing ethanol series (50, 70, 90 and 100%) twice for 12 h each. Subsequently, the samples were infiltrated in LR White resin (Sigma-Aldrich) and polymerized at 57 °C for 24 h. Seven-micrometer-thick sections were cut using a rotary microtome (Leica). The sections were also hydrated for 5 min in 10 mM phosphate-buffered saline (PBS) containing 138 mM NaCl, 2 mM KH_2PO_4, and 10 mM $Na_2HPO_4 7.H_2O$. Next, the sections were incubated in blocking solution containing 10 mM PBS, 0.1% Triton-X 100, 1.5% glycine and 5% bovine serum albumin (BSA) for 1 h. Sections were further incubated in 10 mM PBS for 1 h. To determine the localization of endogenous IAA, sections were incubated for 6 h in anti-IAA rabbit polyclonal antibody (Agrisera AB; Vännäs, Sweden) and diluted 1:100 in a 10 mM PBS solution containing 10% BSA and 1% sodium azide. The sections were

washed once for 5 min in PBS I solution containing 10 mM PBS, 0.1% Triton-X 100, and 0.8% BSA and twice for 5 min each in PBS II solution containing 10 mM PBS and 0.8% BSA. Next, for IAA signal verification, sections were incubated for 1 h in Alexa Fluor 488-labeled anti–rabbit immunoglobulin G antibody (Agrisera AB) diluted 1:300 in 10 mM PBS solution containing 10% BSA and 1% sodium azide. Sections were washed in Milli-Q-type water for 15 min once and assembled on slides with gold antifade reagent (Invitrogen—Molecular probes; Eugene, USA). The slides were covered and sealed with nail varnish. Sections were viewed with a confocal laser scanning microscope (Carl Zeiss).

Statistical analysis

Rooting and IAA content data were submitted to analysis of variance ($P < 0.05$), and the means were compared using a Student–Newman–Keuls (SNK) test (Sokal and Rohlf 1995) in the R program (R Core 2018) (R Foundation for Statistical Computing, version 3.4.4, 2018, Vienna, Austria).

Results

Effect of IBA on ex vitro rooting of micropropagated shoots

No significant effects of the different IBA concentrations tested were observed on the percentage of rooted shoot cuttings from both types of explants, cotyledonary and apical nodal segments (Fig. 1a). However, the different IBA concentrations significantly affected the number (Fig. 1b) and length (Fig. 1c) of roots per shoot cuttings from apical and cotyledonary nodal segments. Shoots from cotyledonary nodal segments showed a higher number of roots with 50 µM IBA, while in shoots from apical nodal segments, the number of roots was significantly reduced with 500 µM IBA when compared to that under other treatments (Fig. 1b). When the two types of explants (i.e., shoots from cotyledonary and apical nodal segments) were compared at the same concentration, no significant differences were observed for the number of roots (Fig. 1b). The highest length of roots was observed at concentrations of 50 and 250 µM IBA in shoots from apical and cotyledonary nodal segments, respectively, whereas the shortest roots were formed at the highest IBA concentration of 500 µM in the shoot cuttings from both segments (Fig. 1c).

Effect of PCIB and TIBA inhibitors on ex vitro rooting of micropropagated shoots

Shoot cuttings of *C. fissilis* can be rooted without IBA treatment, without significant differences among the types of explants used, i.e., cotyledonary and apical nodal segments (Fig. 1a). In this sense, the effects of the auxin signaling inhibitor PCIB and the auxin transport inhibitor TIBA on rooting were evaluated using shoot cuttings from cotyledonary nodal segments. The percentage of rooted shoot cuttings was significantly reduced by 800 µM PCIB (Fig. 2a). The number (Fig. 2b) and length (Fig. 2c) of roots were significantly reduced by 400 and 800 µM PCIB. In addition, the percentage of rooted shoot cuttings (Fig. 2d), number (Fig. 2e) and length (Fig. 2f) of roots per shoot cuttings were significantly reduced by 100 and 200 µM TIBA.

Establishment of adventitious root development phases by histomorphological analysis

Histomorphological analysis was performed on shoot cuttings from cotyledonary nodal segments without (control) and with 800 µM PCIB and 200 µM TIBA during 10 days of incubation. In this analysis, samples at the beginning (i.e., day 0) and after 3, 6 and 10 days of incubation (Fig. 3) were used to describe the phases of AR development in *C. fissilis*. Several cells dividing at the bases of shoot cuttings not treated with the inhibitors PCIB and TIBA were observed at 3 days of rooting. These cells were induced from parenchymal cells close to vascular bundles, forming regions called meristematic centers (Fig. 3b, arrow), allowing the development of the root primordium observed at 6 days of rooting (Fig. 3c) and the elongation of the root observed at 10 days of rooting (Fig. 3d). In contrast, the shoots treated with PCIB and TIBA (Fig. 3f, g, h, j, k, l) did not present the histological events observed in the shoots that were not treated with auxin inhibitors.

Proteomic analysis

Proteomic analysis was performed comparing the bases of shoot cuttings from cotyledonary nodal segments at the beginning (0 days, initial time) and shoots at 3 days of rooting (3 days) (3-day/0-day comparison) to analyze the alterations in protein accumulation during the rooting initiation phase. In addition, we can analyze the alterations in protein accumulation induced by the PCIB and TIBA treatments in shoot cuttings at 3 days of rooting, with the goal of verifying proteins related to the inhibition of the root development process in this species.

From the proteomic analysis, a total of 1096 proteins were identified in at least one treatment (Table S1). Among these proteins, 528 were differentially accumulated proteins (DAPs) in the 3-day/0-day comparison, with 374 up- and 112 down-accumulated, 36 unique at 3 days and 6 unique at 0 days (Fig. 4). Comparing shoot cuttings at 3 days PCIB/3 days, 82 were DAPs, with 51 up- and 26 down-accumulated, 3 unique at 3 days of PCIB treatment and 2 unique

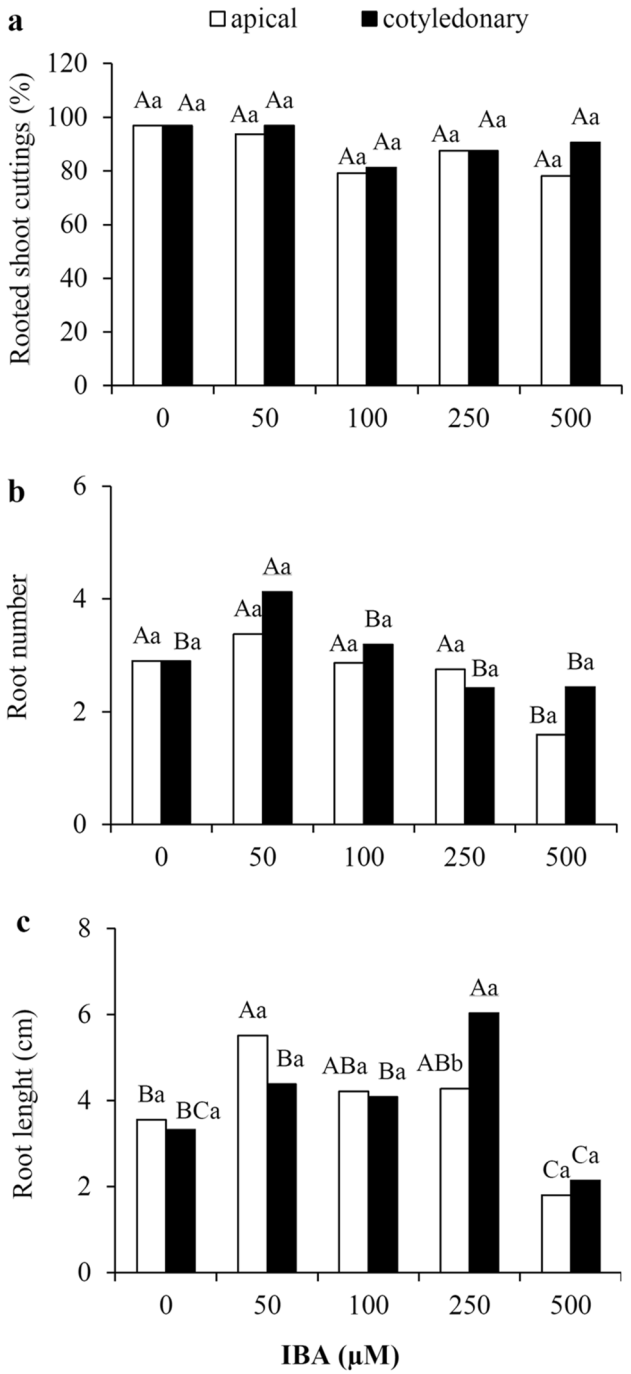

Fig. 1 Percentage of rooted shoot cuttings (**a**), number (**b**) and length (**c**) of roots per shoot cuttings from apical and cotyledonary nodal segments of *Cedrela fissilis* at 30 days of rooting. Means followed by the same letter do not differ statistically according to the SNK test ($P < 0.05$). Different capital letters show significant differences comparing the different IBA concentrations in each type of shoot (apical or cotyledonary). Different lowercase letters show significant differences comparing the two types of shoots (apical and cotyledonary) under each IBA concentration. CV = coefficient of variation (n = 8; CV of a = 17.5%, CV of b = 31.8%, CV of c = 33.5%)

at 3 days of shoot cutting rooting (Fig. 4). In addition, 159 were DAPs, with 80 up- and 72 down-accumulated, 5 unique at 3 days of TIBA treatment and 2 unique in shoots at 3 days of rooting compared with 3-days shoot cuttings treated with TIBA (3-days TIBA/3-days) (Fig. 4). Among the accumulated proteins, some were highlighted and discussed in this work according to their relevance in shoot rooting, mainly due to their relationship with auxin, cell division and cell wall organization (Table 1).

Considering the DAPs during the root initiation phase, in the 3-days/0-days comparison, some proteins were up-accumulated, such as Chorismate synthase, chloroplastic (Ce_fissilis.015065.1), Peroxidase 12 (Ce_fissilis.017405.2), Peroxidase 4 (Ce_fissilis.019038.1), Auxin-induced in root culture protein 12 (AIR12) (Ce_fissilis.015339.1), Patellin-3-like (Ce_fissilis.016593.1), Cell division cycle protein 48 homolog (CDC48) (Ce_fissilis.012572.1), Xylose isomerase (Ce_fissilis.014702.1), and Serine/threonine-protein phosphatase PP2A-4 catalytic subunit (Ce_fissilis.014280.1). On the other hand, the Vestitone reductase (Ce_fissilis.002557.2) protein was down-accumulated in this comparison. In addition, expansin-like B1 (EXPB1) (Ce_fissilis.010254.1) and probable xyloglucan endotransglucosylase/hydrolase protein 5 (XTH5) (Ce_fissilis.012480.1) were unique in shoot cuttings at 3 days of rooting (Table 1).

Considering the DAPs during root inhibition under the use of PCIB and TIBA inhibitors, some were DAPs in the 3-day PCIB/3-day comparison and 3-day TIBA/3-day comparison. Among the proteins, Vestitone reductase (Ce_fissilis.002557.2) was up-accumulated in both comparisons of shoots treated with PCIB and with TIBA, showing that both inhibitors affect the accumulation of this protein in addition to reducing rooting. Similarly, the protein serine/threonine-protein phosphatase PP2A-4 catalytic subunit (Ce_fissilis.014280.1) was down-accumulated in shoot cuttings treated with inhibitors (3-day PCIB/3-day and 3-day TIBA/3-day comparisons), showing that PCIB and TIBA decreased the accumulation of this protein and decreased rooting (Table 1). However, as this protein was up-accumulated in shoot cuttings during root initiation, i.e., in the 3-day/0-day comparison, the relevance of this protein for rooting promotion in this species seems likely. In addition, the phosphoglycerate kinase cytosolic (Ce_fissilis.016667.1) and pyruvate kinase 1 cytosolic (Ce_fissilis.011754.1) proteins were down-accumulated in shoot cuttings treated with both inhibitors (3-day PCIB/3-day and 3-day TIBA/3-day comparisons) (Table 1; Fig. S1).

In shoot cuttings at 3 days treated with TIBA (TIBA/3-day comparison), the xylose isomerase (Ce_fissilis.014702.1), glyceraldehyde-3-phosphate dehydrogenase, cytosolic (Ce_fissilis.011648.1), and succinate dehydrogenase subunit 6 mitochondrial (Ce_fissilis.015355.1) proteins were down-accumulated (Fig. S1). In addition, the cell division cycle protein 48 homolog (CDC48) (Ce_fissilis.012572.1) and chorismate

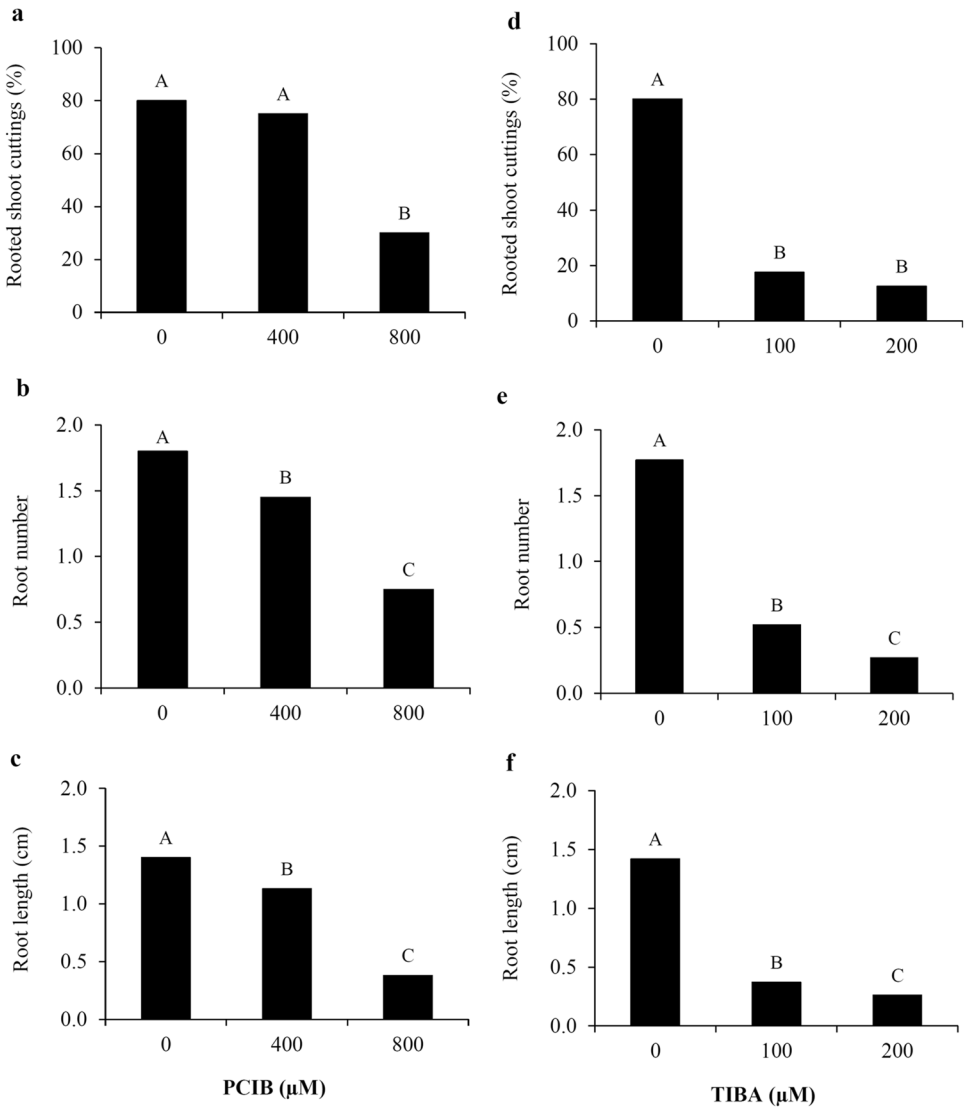

Fig. 2 Percentage of rooted shoot cuttings (%), number and length (cm) of roots in shoot cuttings from cotyledonary nodal segments of *Cedrela fissilis* as affected by exposure to different concentrations (0, 400 and 800 µM) of PCIB (**a**, **b**, **c**) and different concentrations (0, 100 and 200 µM) of TIBA (**d**, **e**, **f**) after 13 days of ex vitro 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 of a = 13.2%, CV of b = 18.0%; CV of c = 16.3%, CV of d = 19.7%; CV of e = 16.6%, CV of f = 13.8%)

synthase and chloroplastic (Ce_fissilis.015065.1) proteins were down-accumulated in the shoot cuttings at 3-day TIBA/3-day comparison, showing that IAA efflux by the TIBA inhibitor reduced the accumulation of this protein and root initiation. However, these proteins were up-accumulated in shoot cuttings at 3 days of rooting compared with shoot cuttings before the start of the experiment (3-day/0-day comparison), suggesting their relevance to the initiation of roots in *C. fissilis*.

Endogenous IAA quantification and immunolocalization

As many proteins identified were related to auxin and rooting in plants (Table 1), the endogenous contents and immunolocalization of IAA were evaluated in the base of shoot cuttings from cotyledonary nodal segments before (0 days) and after 3 days of rooting without and with PCIB and TIBA treatments (Fig. 5).

The endogenous contents of IAA accumulated in the base of shoot cuttings at 3 days of rooting compared with shoot cuttings at day 0, whereas this increase was prevented in shoot cuttings at 3 days of rooting when auxin transport TIBA or auxin signaling PCIB were used (Figs. 5 and 6). The base of shoot cuttings at 3 days of rooting showed a more intense signal fluorescence around the cell (Fig. 6d), and some cells accumulated IAA on the intracellular cortex parenchyma (inset in Fig. 6d), suggesting IAA transport between cells. Some small cells from the meristematic center of shoot cuttings at 3 days of rooting showed an especially intense IAA fluorescence signal (Fig. 7, arrow), showing

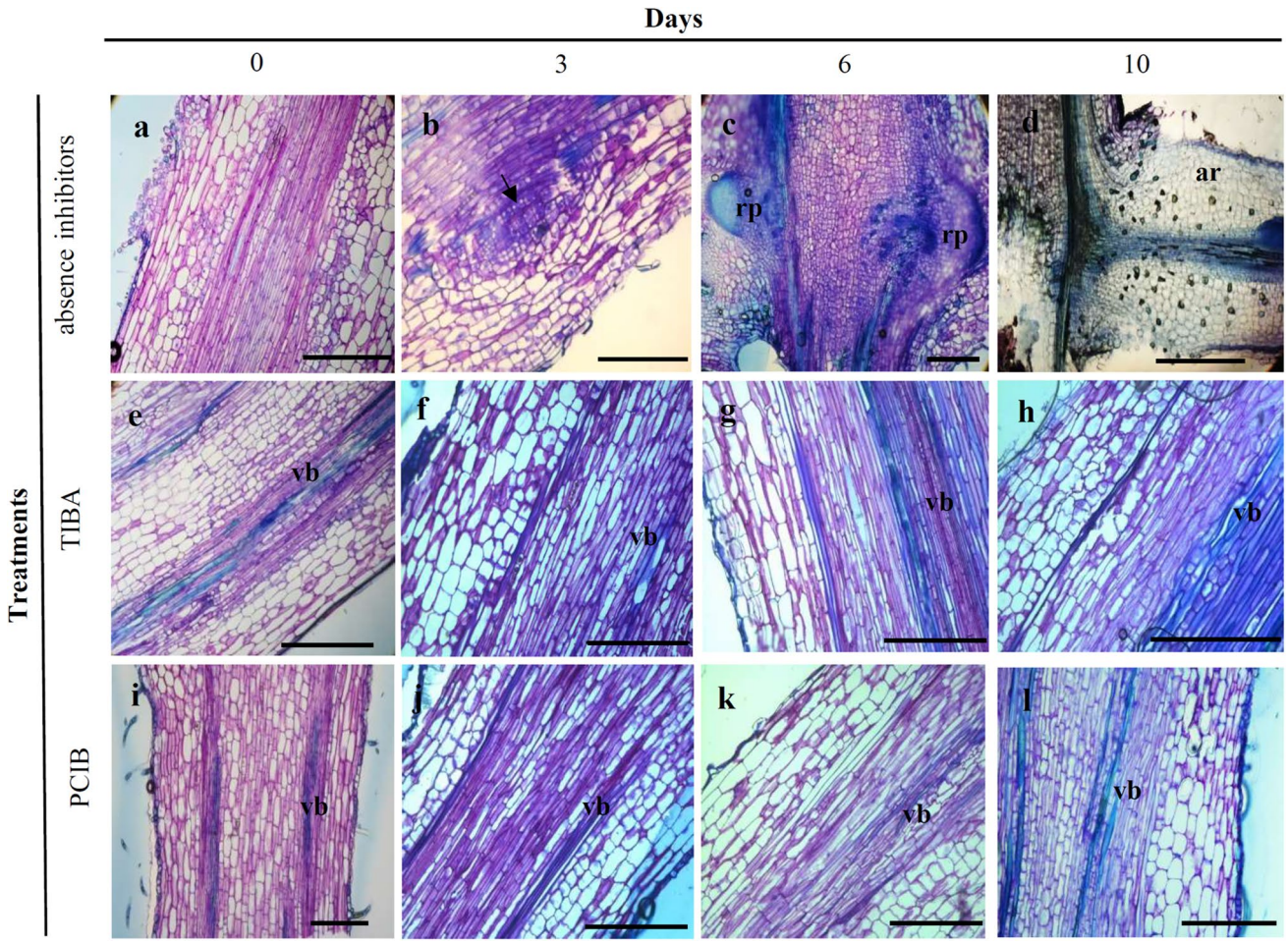

Fig. 3 Histomorphological aspects during ex vitro rooting of *Cedrela fissilis* shoot cuttings from cotyledonary nodal segments before (0 days) and after 3, 6 and 10 days of root induction in the absence (control) and presence of TIBA (200 µM) and PCIB (800 µM). Arrows indicate cell divisions. rp = root primordium, vb = vascular bundle, ar = adventitious root. Bars = 200 µm

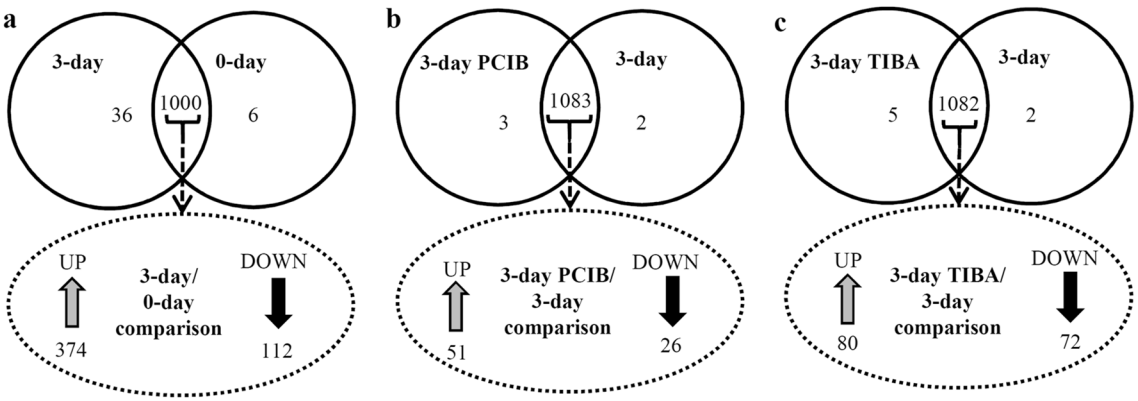

Fig. 4 Venn diagram of proteins identified in *Cedrela fissilis* shoots during root initiation comparing cotyledonary nodal segments before (0-day) and after 3 days of rooting (3-day/0-day comparison) (**a**) and in shoot 3 at days of rooting without and with PCIB (3-day PCIB/3-day comparison) (**b**) and TIBA (3-day TIBA/3-day comparison) (**c**)

Table 1 Highlighted differentially accumulated proteins related to rooting of *Cedrela fissilis* shoot cuttings

Accession	Reported peptide	Description	Biological process	Accumulation 3-day/0-day	Accumulation 3-day PCIB/3-day	Accumulation 3-dayTIBA/3-day
Ce_fissilis.019038.1	18	Peroxidase 4	Hydrogen peroxide catabolic process, oxidation–reduction process	UP	UNCHANGED	UNCHANGED
Ce_fissilis.017405.2	15	Peroxidase 12	Hydrogen peroxide catabolic process, oxidation–reduction process	UP	UNCHANGED	UNCHANGED
Ce_fissilis.015065.1	7	Chorismate synthase, chloroplastic	Aromatic amino acid family biosynthetic process	UP	UNCHANGED	DOWN
Ce_fissilis.010254.1	3	Expansin-like B1	Cell wall organization	Unique 3-days	UNCHANGED	UNCHANGED
Ce_fissilis.016593.1	16	Patellin-3-like	Cell cycle, cell division	UP	UNCHANGED	UNCHANGED
Ce_fissilis.012572.1	8	Cell division cycle protein 48 homolog	Mitotic spindle disassembly, cell cycle	UP	UNCHANGED	DOWN
Ce_fissilis.014280.1	12	Serine/threonine-protein phosphatase PP2A-4 catalytic subunit	Protein dephosphorylation, cellular protein modification process	UP	DOWN	DOWN
Ce_fissilis.011648.1	14	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	Glycolytic process, carbohydrate metabolic process	UP	UNCHANGED	DOWN
Ce_fissilis.016667.1	18	Phosphoglycerate kinase, cytosolic	Glycolytic process, carbohydrate metabolic process	UP	DOWN	DOWN
Ce_fissilis.011754.1	11	Pyruvate kinase 1, cytosolic	Carbohydrate metabolic process	UP	DOWN	DOWN
Ce_fissilis.015355.1	3	Succinate dehydrogenase subunit 6, mitochondrial	Tricarboxylic acid cycle	UP	UNCHANGED	DOWN
Ce_fissilis.014702.1	8	Xylose isomerase	Carbohydrate metabolic process	UP	UNCHANGED	DOWN
Ce_fissilis.015339.1	4	Auxin-induced in root cultures protein 12	Auxin-activated signaling pathway, metabolic process	UP	UNCHANGED	UNCHANGED
Ce_fissilis.012480.1	5	Probable xyloglucan endotransglucosylase/hydrolase protein 5	Cell wall biogenesis	Unique 3-days	UNCHANGED	UNCHANGED
Ce_fissilis.002557.2	7	Vestitone reductase	Flavonoid biosynthesis, biosynthetic process	DOWN	UP	UP

3-day/0-day comparison = shoot cuttings at 3 days of rooting compared to shoot cuttings at the beginning of rooting (0-day). 3-day PCIB/3-day comparison = shoot cuttings at 3 days of rooting treated with PCIB compared to shoot cuttings at 3 days of rooting without PCIB. 3-day TIBA/3-day comparison = shoot cuttings at 3 days of rooting treated with TIBA compared to shoot cuttings at 3 days of rooting without TIBA

that the endogenous levels of IAA on the 3rd day of root initiation are important for AR initiation in this species.

Discussion

Tree species often require the use of plant growth regulators, especially auxins, such as IBA, to promote adventitious rooting (Husain and Anis 2009; Phulwaria et al. 2013). However, our results showed that exogenous auxin is not necessary for adventitious rooting in micropropagated shoots of *C. fissilis*, since the IBA concentrations used did not present a significant effect on root induction (Fig. 1a). This ability to promote rooting without exogenous auxin can be explained by the existence of endogenous IAA metabolism and signaling, which can trigger root induction (Wilson 1994), and this hypothesis was confirmed in AR development of *C. fissilis* shoots by the IAA analyses performed (Fig. 5). Auxin has

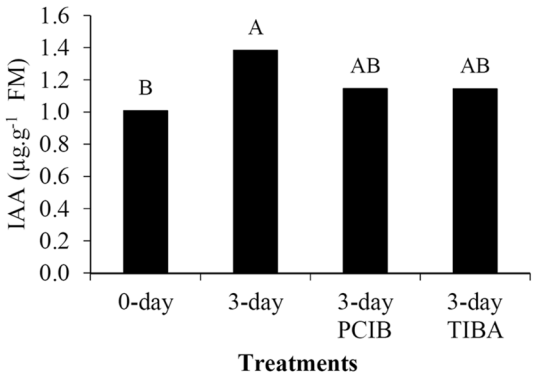

Fig. 5 Quantification of IAA at the bases of *Cedrela fissilis* shoot cuttings from cotyledonary nodal segments before (0-day) and after 3 days of rooting without and with 800 µM PCIB and 200 µM TIBA. Means followed by the same letter do not differ statistically according to the SNK test ($P < 0.05$). CV = coefficient of variation. (n = 3, CV = 11.7%)

long been considered a key factor in root development (Da Costa et al. 2013; Pacurar et al. 2014), and auxin activity may be related to the cell cycle during AR formation, as auxin-dependent transcription factors regulate the control of G1/S and G2/M progression (Magyar et al. 2005). Any interference with endogenous auxin movement may alter cell cycle progression and influence AR formation (Wang and Ruan 2013). In our study, shoot cuttings of *C. fissilis* at 3 days of rooting were found to well represent the initiation phase, presenting regions with cell divisions characterized as meristematic centers, followed by the formation of root primordia at 6 days and root elongation at 10 days of rooting (Fig. 3), and the finding that those events were inhibited by the PCIB and TIBA treatments highlights the functional role of auxin in this process. There are increasing experimental data supporting the stimulating function of auxin during AR induction and early processes of AR initiation in root competent cells (Druege et al. 2019; Hu and Xu 2016; Sheng et al. 2017). During the phase of root initiation in shoots of *C. fissilis*, at 3 days, a higher content of IAA (Figs. 5 and 6) was critical to trigger adventitious rooting, as also demonstrated by Li et al. (Li et al. 2009). The finding that IAA accumulation in shoot cuttings at 3 days of rooting was prevented by the application of TIBA and by PCIB demonstrates the important role of auxin transport and auxin signaling in the auxin balance (Druege et al. 2019). The inhibition of polar auxin transport eliminated the IAA peak and root formation in *Petunia hybrida* shoots, showing the contribution of auxin in the stem base as dependent on polar auxin transport essential for subsequent AR formation (Ahkami et al. 2013). Thus, the endogenous auxin produced at the apex is conveyed basipetally to the cut surface of the stem shoot, acting as a stimulus for rooting (Da Costa et al. 2013). The use of chemical tools, such as the inhibitors TIBA and PCIB, can improve the identification of biochemical alterations modulating polar auxin transport and signaling involved in the rooting of micropropagated shoots of *C. fissilis*.

Proteomic analysis showed that the treatments significantly affected the accumulation of some DAPs during rooting of shoot cuttings in *C. fissilis*. Chorismate synthase is an important protein in the final step of the chiquimate pathway that gives rise to the main precursor of IAA, tryptophan (Tzin and Galili 2010). The increase in accumulation of chorismate synthase and chloroplastic (Ce_fissilis.015065.1) protein in the *C. fissilis* shoot cuttings at 3 days of rooting compared to 0-day shoot cuttings can be related to the increase in endogenous IAA content, which was observed at this time (Figs. 5 and 6), thus promoting root initiation. In addition, the higher accumulation of AIR12 (Ce_fissilis.015339.1) in shoot cuttings at 3 days of rooting compared to 0 days could be related to AR initiation in *C. fissilis*. This protein is a b-type cytochrome that can facilitate a redox link between the cytoplasm and apoplast, interacting with other redox partners within the plasmatic membrane (Preger et al. 2009). Redox regulation affects hormonal signaling pathways controlling meristem maintenance and organogenesis, such as renewal and differentiation of stem cells (Beveridge et al. 2007).

Endogenous auxin accumulation at the bases of stem shoots can also lead to cell division and expansion (Da Costa et al. 2013). An auxin-induced acidic pH is required to activate expansins, which are responsible for the disintegration and loosening of the connections between cellulose microfibrils (Cosgrove 2005; Majda and Robert 2018), while XTH cleaves and reconnects xyloglucan polymers, promoting cell wall loosening and extensibility (Cosgrove 2005). This process may be important for cell division since these cell wall proteins are necessary for normal cell expansion during the growth of various plant organs. In our work, the higher accumulation of the EXPB1 (Ce_fissilis.010254.1) and XTH5 (Ce_fissilis.012480.1) proteins in shoot cuttings of *C. fissilis* at 3 days of rooting (Table 1) and the simultaneous increase in endogenous IAA (Fig. 5) could be related to the loosening and extensibility of cell walls necessary for cell division and elongation, which are essential for AR development in *C. fissilis*.

The higher accumulation of the Patellin-3-like (Ce_fissilis.016593.1) and CDC48 (Ce_fissilis.012572.1) proteins in shoot cuttings at 3 days rooting, which also occurred with higher endogenous IAA contents (Fig. 5), can be related to cell divisions during the formation of meristematic centers (Figs. 3b and 7), promoting the formation of ARs in *C. fissilis* shoots. Patellin is a plasma membrane-localized protein required for auxin-induced PIN1 localization and multiple developmental processes, and patellin has been verified in different tissues going through mitosis or initiating differentiation (Tejos et al. 2018). The CDC48 protein may regulate

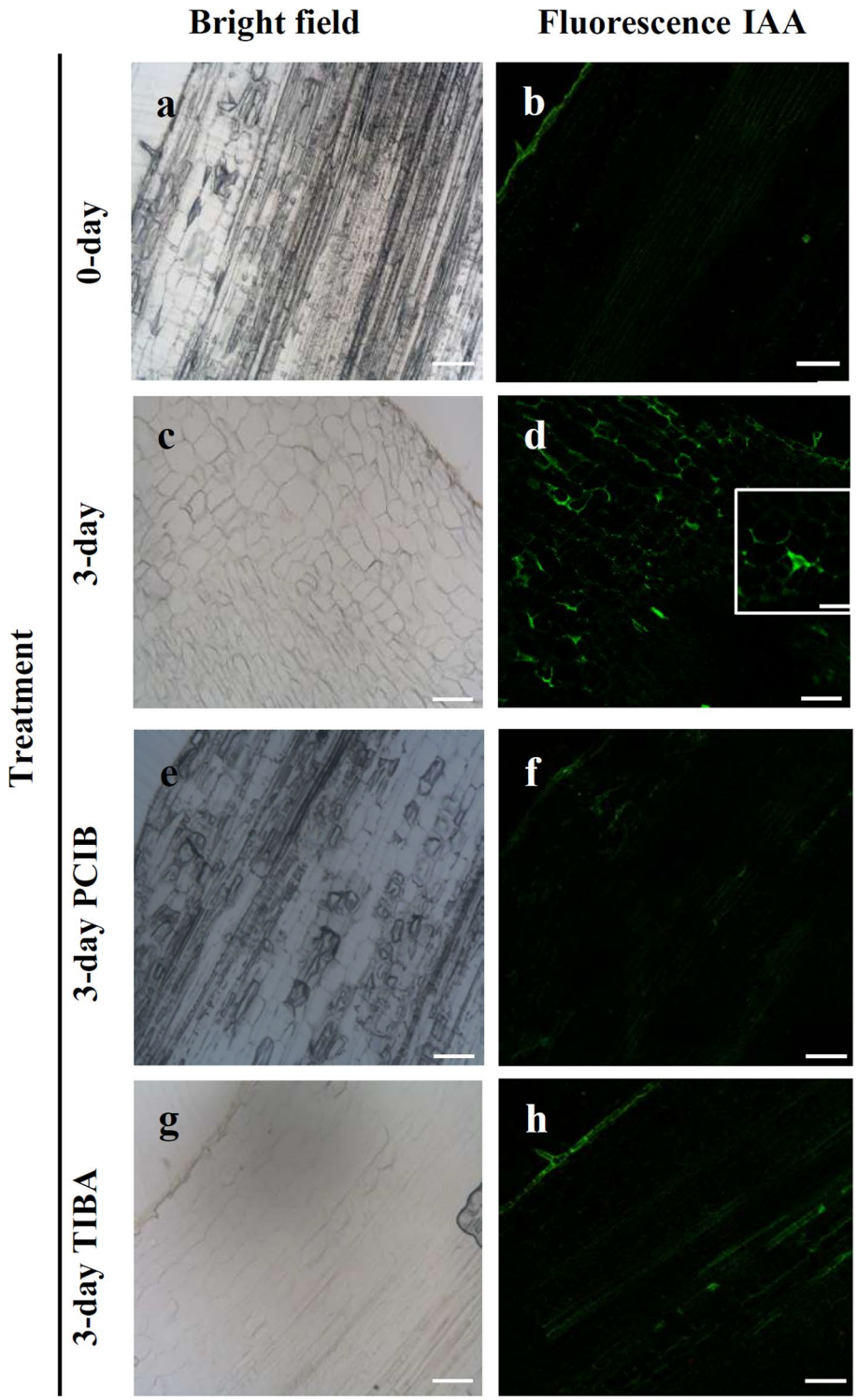

Fig. 6 Immunolocalization of IAA in the bases of *Cedrela fissilis* stem shoots obtained from cotyledonary nodal segments before root initiation (0-day) (**a**, **b**), after 3 days of rooting (**c**, **d**), and at 3 days of rooting with 800 µM PCIB (**e**, **f**) and 200 µM TIBA (**g**, **h**). Inset in **d**: details of intracellular IAA presence. Bars **a**–**h** = 100 µm; Bar inset **d** = 50 µm

endoplasmic reticulum assembly during cytokinesis and may also be involved in cell expansion and differentiation (Park et al. 2008). Thus, we propose that the accumulation of these proteins is related to the accumulation of endogenous IAA and induces the promotion of root initiation in *C. fissilis*.

Moreover, some peroxidases (Ce_fissilis.017405.2 and Ce_fissilis.019038.1) were highly accumulated in shoot cuttings at 3 days of rooting compared to 0 days. It is thought that the oxidative decarboxylation of IAA by plant peroxidases may be involved in controlling the content of endogenous IAA (Savitsky et al. 1999) and thus could be important

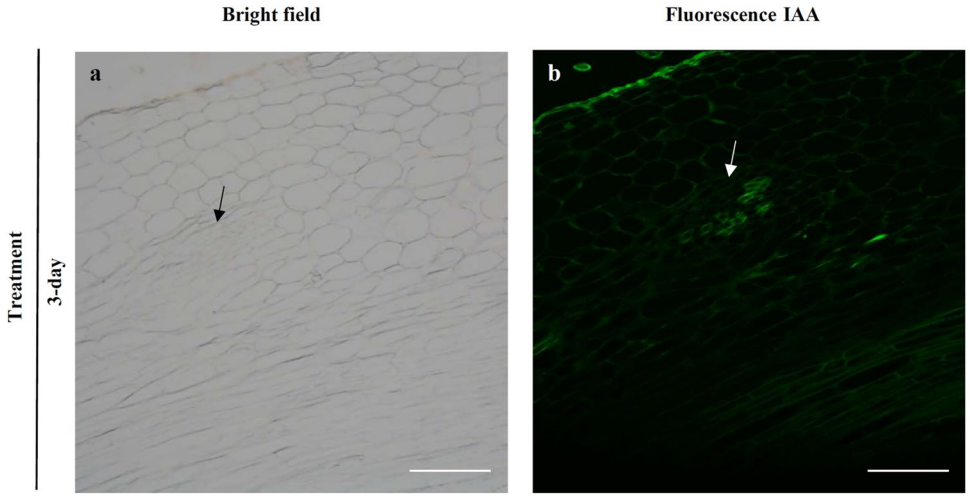

Fig. 7 Immunolocalization of IAA in the bases of *Cedrela fissilis* stem shoots obtained from cotyledonary nodal segments at 3 days of root initiation showing the cells of the meristematic center in bright field (**a**) and IAA fluorescence microscopy (**b**). Arrow indicate meristematic center. Bars = 100 µm

for AR initiation in *C. fissilis* via the regulation of endogenous auxin contents. In this sense, the higher accumulation of auxin-related proteins (such as chorismate synthase chloroplastic, AIR12, peroxidase 4 and 12), cell division proteins (CDC48 and patellin-3-like) and cell wall modification proteins (EXPB1 and XTH5) can be markers for the initiation of AR in *C. fissilis.*

In addition, inhibition of rooting promoted by inhibitors of polar auxin transport (TIBA) and auxin signaling (PCIB) modulated the accumulation of some proteins that could be related to shoot rooting in *C. fissilis*. Among them, the reduction in the accumulation of the protein serine/threonine-protein phosphatase PP2A-4 catalytic subunit (Ce_fissilis.014280.1) in shoot cuttings at 3 days of treatment with PCIB and TIBA compared to untreated shoot cuttings suggests that this protein is relevant for rooting promotion in this species. PP2A may interact with plasma membrane components and can also interact with PIN proteins (Blakeslee et al. 2008). PINs are transporters acting in the efflux of auxin from cells at the plasma membrane, and their polarity determines the directionality of polarized intercellular auxin flow (Křeček et al. 2009). The polarization of PINs can be regulated by PP2A proteins by dephosphorylation (Li et al. 2011) and hence alter auxin flow and plant patterning (Ballesteros et al. 2013). In addition, the activity of PP2A is reduced in the presence of the N-1-naphthylphthalamic acid auxin transport inhibitor in Arabidopsis (Deruere et al. 1999), suggesting that PP2A may play a role in the regulation of auxin transport (Ludwig-Müller et al. 2005). In addition, it was shown that PP2A also acted by dephosphorylating other components of the plasma membrane by regulating their location and possibly their activity, such as that of Arabidopsis Crinkly 4 (ACR4). The ACR4 protein is part of a mechanism that controls cell division during the formation of roots in *Arabidopsis* (Yue et al. 2016). In this sense, our results suggest that the inhibition of rooting by the TIBA and PCIB treatments may be related to changes in the dynamics of membrane-associated protein dephosphorylation, which is essential for signal transduction during root development and can affect rooting in *C. fissilis*, dependent on auxin flow. Moreover, the TIBA and PCIB inhibitors affected the accumulation of Vestitone reductase (Ce_fissilis.002557.2) protein related to isoflavonoid metabolism (Table 1). This protein is involved in the biosynthesis of medicarpin, a type of natural isoflavonoid (Guo and Paiva 1995). Some authors have suggested that flavonoids may act as nonessential auxin transport inhibitors (Brown et al. 2001; Peer and Murphy 2007), affecting the localization of PIN proteins (Santelia et al. 2008). In this sense, this protein (Fig. 8) could be related to rooting inhibition in *C. fissilis* via the inhibition of auxin transport and efflux.

In addition to proteins related to auxin, proteins related to carbohydrates are important to provide the energy during cell divisions necessary for AR formation. The reduction in the accumulation of some proteins from the glycolysis and tricarboxylic acid cycle pathways, such as phosphoglycerate kinase cytosolic (Ce_fissilis.016667.1), pyruvate kinase 1 cytosolic (Ce_fissilis.011754.1), xylose isomerase (Ce_fissilis.014702.1) and glyceraldehyde-3-phosphate dehydrogenase cytosolic (Ce_fissilis.011648.1) (Fig. S1), promoted by chemical inhibitors, was related to the inhibition of AR formation and is relevant for energy supplies during AR initiation. These proteins act on the glycolysis pathway and are important for energy production in plant cells (Muñoz-Bertomeu et al. 2009), and during the phase of AR initiation, high levels of energy and carbon skeletons are required to promote cell division and growth (Ahkami et al. 2009; Kromer and Gamian 2000). In addition, the down-accumulation of succinate dehydrogenase subunit 6 mitochondria (Ce_fissilis.015355.1) was observed in shoot cuttings treated with TIBA at 3 days of rooting compared to shoot cuttings at 3 days without TIBA, while the up-accumulation in shoot cuttings at 3 days of rooting compared to shoot cuttings at 0 days of rooting was observed

(Table 1). Succinate dehydrogenase, a protein of the tricarboxylic acid cycle pathway (Fig. S1), catalyzes the oxidation of succinate to fumarate (Hagerhall 1997) in the tricarboxylic acid cycle pathway, which, like glycolysis, is involved in the production of energy. In this sense, the reduction in the accumulation of these proteins induced by polar auxin transport and auxin signaling inhibitors, which leads to the inhibition of rooting, suggests the relevance of these proteins to the energy production needed for cell division and differentiation during AR initiation in *C. fissilis* (Fig. 8).

Conclusion

The use of IBA is not necessary for ex vitro rooting of in vitro propagated shoot cuttings. The proliferation of meristematic-type cells was observed on the 3rd day of rooting, leading to AR initiation. At this time, the up-accumulation of auxin-related proteins, cell division and unique proteins of cell wall modification was observed and can be markers of AR initiations. The higher accumulation of auxin-related proteins could increase the endogenous IAA content and AR

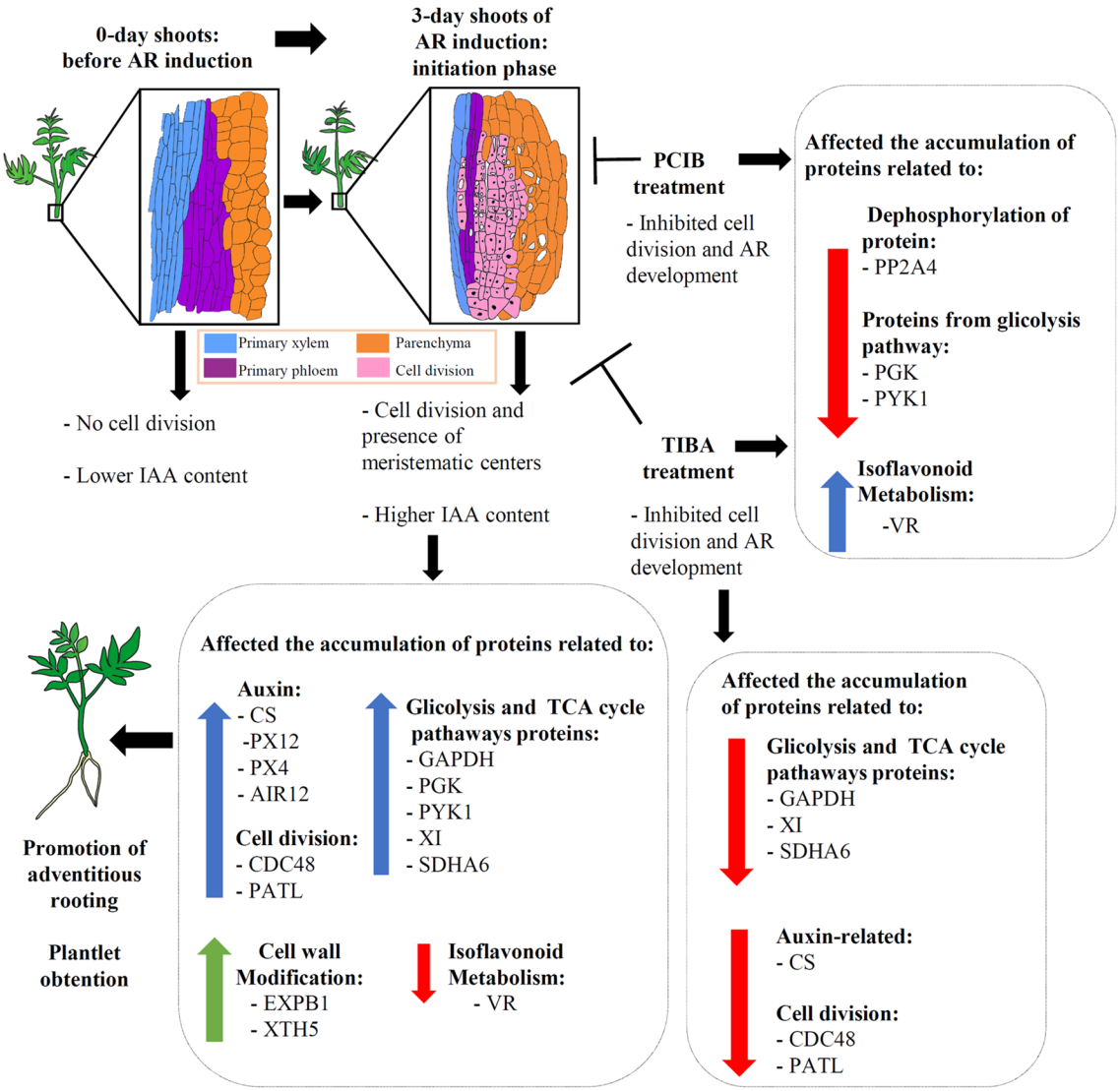

Fig. 8 Overview of biochemical and proteomic alterations during adventitious root initiation in shoots of *Cedrela fissilis*. The arrow in red indicates down-accumulated proteins. The arrow in blue indicates the up-accumulated proteins. The arrow in green indicates the unique proteins. AIR12, Auxin-induced root culture protein 12 PP2A-4; Protein serine/threonine-protein phosphatase catalytic subunit; CDC48, Cell division cycle protein 48 homolog; EXPB1, Expansin-like B1; CS, Chorismate synthase, chloroplastic; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase, cytosolic; PATL, Patellin-3-like; PGK, Phosphoglycerate kinase; PX12, Peroxidase 12; PX4, Peroxidase 4; PYK1, Pyruvate kinase 1, cytosolic; SDHA6, Succinate dehydrogenase subunit 6, mitochondrial; VR, Vestitone reductase; XI, Xylose isomerase; XTH5, Probable xyloglucan endotransglucosylase/hydrolase protein 5

initiation on shoot cuttings on the 3rd day of rooting than on day 0. Proteins related to dephosphorylation and the glycolytic and tricarboxylic acid cycle pathways were up-accumulated and down-accumulated, respectively, in shoot cuttings at 3 days of rooting and were relevant for AR development in this species. These findings reveal that there was a dynamic relationship between auxin-related proteins and IAA contents controlling AR initiation. Our results are the first to show the involvement of highlighted auxin-responsive proteins and endogenous auxin that contribute to the activation of meristematic-type cells, leading to meristematic center formation and AR initiation in micropropagated shoots of *C. fissilis*, an easy-to-root woody species.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11240-021-02171-7.

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Author contributions YRSR and CSC conceived the study, designed the experiments and wrote the manuscript. YRSR was responsible for the in vitro culture of shoots and ex vitro rooting experiments and performed the statistical analyses. YRSR and CSC performed the histomorphological analyses. YRSR, VPMA, AFM and EISF were responsible for IAA analyses. YRSR, 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 identifier PXD021070. All identified proteins are available in the supplementary material.

Code availability PXD021070.

Declarations

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

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