Long-Term Subculture Affects Rooting Competence via Changes in the Hormones and Protein Profiles in *Cedrela Fissilis* Vell. (Meliaceae) Shoots

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Research Article

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Abstract

Long-term subculture plays an essential role in the large-scale multiplication and production of somatic plantlets. We investigated the effects of long-term subculture on *in vitro* shoot development and *ex vitro* rooting associated with changes in the hormones and protein profiles in *C. fissilis*. The number of subcultures of shoots induced a decrease in the *ex vitro* rooting response. The reduction in adventitious root (AR) formation was associated with decreases in the contents of indole-3-acetic acid (IAA), abscisic acid (ABA), 12-oxo phytodienoic acid (OPDA), putrescine (Put), and spermine and increases in jasmonic acid (JA), jasmonoyl-isoleucine, trans-cinnamic acid, and salicylic acid contents in shoots at the fourth subculture compared to the first. The ornithine decarboxylase enzyme preferentially functions in the Put biosynthesis pathway and was related to the highest AR formation in shoots at the first subculture. Down-accumulation of the auxin-binding protein ABP19a in shoots from the fourth subculture compared to the first subculture was related to a decrease in both IAA contents and AR formation. In addition, down-accumulation of glucose-6-phosphate isomerase, glutamine synthetase leaf isozyme chloroplastic, 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase, L-ascorbate peroxidase, cytosolic, monodehydroascorbate reductase, and 2-Cys peroxiredoxin BAS1-like, chloroplastic and up-accumulation of caffeoyl-CoA O-methyltransferase 1 and isoforms of peroxidase 4 proteins in shoots from the fourth relative to the first subculture were associated with a reduction in AR formation. These results showed that the understanding of hormonal and molecular mechanisms related to the potential of AR formation in shoots under successive subcultures is relevant to improving large-scale plantlet production in *C. fissilis*.

Key Message

Long-term subculture affects the competence to *ex vitro* rooting of *Cedrela fissilis* shoots by changes in endogenous hormones and protein profiles.

Introduction

Cedrela fissilis Vellozo (Meliaceae) species is a native woody tree from the Brazilian Atlantic Forest. Due to its high economic value for commercial wood production, this species has been included in the Red List of Endangered Species by the International Union for Conservation of Nature (IUCN), in which it is classified as vulnerable (Barstow 2018). *In vitro* propagation can be used to establish preservation strategies and can be used for the large-scale production of clonal plants. Micropropagation systems using nodal segments as explants for shoot development have been established for *C. fissilis* (Nunes et al. 2002). In addition, the relationship of cytokines and polyamines (PAs) in improving shoot development has been evaluated for this species (Aragão et al. 2017). In addition, no studies have been developed relating the long-term subculture on shoot development and adventitious rooting in *C. fissilis*.

Several factors are associated with *in vitro* morphogenic competence and significantly affect plant regeneration, including the explant type, culture medium composition, plant growth regulators (PGRs),

plant genotype and the number of subcultures (Duclercq et al. 2011; Shemer et al. 2015). Long-term subculture is an important factor for the optimization of micropropagation systems and plays an essential role in multiplication and large-scale plantlet production (Pastelín Solano et al. 2019). However, species present differences in their competence to produce shoots with an increasing number of subcultures (Rocha et al. 2007; Hamad and Taha 2008). In addition, during *in vitro* initiation cultures and the subculture steps for multiplication, the isolation of explants induces wounding of tissues and can cause oxidative stress, which may explain reductions in *in vitro* shoot development, affecting the propagation potential. Wounding induces several cellular responses, including the production of plant hormones, loss of cell-to-cell communication and disruption of long-distance signaling, and can be considered a trigger of both cell division and damaging oxidative bursts (Cassells and Curry 2001; Ikeuchi et al. 2016).

In addition to shoot development, long-term exposure can also affect the rooting of shoots. For rooting, auxins play a central role in root induction and are often applied exogenously to promote the development of adventitious root (AR) formation on stem cuttings of difficult-to-root species (De Almeida et al. 2020). Auxins can interact with other hormones, such as abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) (Zhang et al. 2007; Albacete et al. 2008; Gutierrez et al. 2012). There is considerable evidence that the crosstalk between indole-3-acetic acid (IAA) and ABA plays an important role in improving root growth under abiotic stress conditions (Albacete et al. 2008). Beyond the evidence showing that ABA activates the expression of genes encoding antioxidant enzymes (Gomez-Cadenas et al. 2015), little is known about the involvement and effects of ABA on rooting. In addition, the crosstalk between IAA and JA plays an important role in the regulation of AR formation in etiolated hypocotyls, in which IAA controls AR initiation by negatively regulating JA signaling (Gutierrez et al. 2012). Furthermore, JA has been related to injuries induced by cuts in isolated plant tissues, in which it influences stress-induced reprogramming and is one of the factors associated with hormonal variation (Da Costa et al. 2013). An intermediate in JA biosynthesis, 12-oxo-phytodienoic acid (OPDA), is also involved in signaling and induces defense and wounding responses independently (Stenzel et al. 2003), although the precise functions of OPDA in physiological events, including rooting, are still largely unknown. SA is another plant hormone involved in root development and shows a potential role in AR formation in some species (Gutierrez et al. 2012; Yang et al. 2013).

In addition, PAs modulate the morphogenetic response and can be involved in rooting competence (Kusano et al. 2008; Ahkami et al. 2009; Wang et al. 2020). PAs are low-molecular-weight aliphatic compounds (Lenis et al. 2017) that are essential for several processes involved in plant growth (Kevers et al. 2000; Vuosku et al. 2006; Kusano et al. 2008). Among PAs, the diamine putrescine (Put) is synthesized directly from ornithine decarboxylase (ODC) or from arginine decarboxylase (ADC) via two additional synthesis steps (Bais and Ravishankar 2002). The coexistence of ADC and ODC in the Put biosynthetic pathway in some plant species may be related to their different contributions to development and tissue-specific processes (Vuosku et al. 2006). In plants, some studies have indicated that specific PAs are associated with different processes; e.g., spermidine (Spd) and spermine (Spm) have been associated with cell differentiation, whereas Put is more closely related to cell division (Santa-

Catarina et al. 2006; Aragão et al. 2017). Although significant progress has been made in understanding the regulation of PA metabolism and signal transduction (Tun et al. 2006; Lasanajak et al. 2014; Majumdar et al. 2016), little is known about the roles of these compounds in shoots and AR development in woody species (Acosta et al. 2005).

In addition to plant hormone analysis, a proteomic approach can provide information related to the protein profile during morphogenetic responses (Hochholdinger et al. 2006; Takáč et al. 2011), such as *in vitro* shoot development (Aragão et al. 2017) and AR formation (De Almeida et al. 2020). The application of 2.5 mM Put improved *in vitro* shoot development in *C. fissilis* and induced an alteration in the abundance of proteins related to metabolic and cellular proteins associated with cell division (Aragão et al. 2017). During AR development, proteomic studies have revealed the roles of several proteins involved in different biological pathways, mainly oxidative stress, energy metabolism and photosynthesis (De Almeida et al. 2020). In addition, some proteins that can act as positive regulators of ARs, such as hydrogen peroxide or starch metabolism-related proteins, have been shown to exhibit higher accumulation in *Eucalyptus grandis* than in *Eucalyptus globulus*, which are species with different rooting potentials (De Almeida et al. 2020).

The hormonal and molecular mechanisms controlling the competence for shoot development and rooting during successive *in vitro* subcultures are still poorly understood in woody plants, such as *C. fissilis*. The assessment of changes in the endogenous contents of plant hormones and proteomic profiles during increasing numbers of subcultures can provide important information about the AR competence of this woody plant. Thus, the aim of this work was to investigate the effects of long-term subculture on *in vitro* shoot development and *ex vitro* rooting associated with changes in the hormones and protein profiles of *C. fissilis*.

Materials And Methods

Plant material

Mature seeds of *C. fissilis* obtained from Caiçara Comércio de Sementes LTDA located in Brejo Alegre (21°10’S and 50°10’W), São Paulo State, Brazil, were germinated *in vitro* according to Oliveira et al. (2020). Sixty-day-old seedlings were used as the source of apical and cotyledonary nodal segment explants to obtain the shoots that were used for rooting in each subculture.

In vitro germination

In vitro germination was performed according to Oliveira et al. (2020). First, the seeds were surface disinfected and then transferred to glass test tubes (15 x 2.5 cm; Laborglas; São Paulo, Brazil) containing 10 mL of MS (Murashige and Skoog 1962) culture medium (M519; Phytotechnology Lab; Lenexa, USA) supplemented with 20 g L^{-1} sucrose (Vetec; Rio de Janeiro, Brazil) and 2 g L^{-1} Phytagel (Sigma-Aldrich; St. Louis, USA). The pH of the culture medium was adjusted to 5.7 before being autoclaved at 121 °C for 15

min. The seeds were incubated in a growth room at 25 ± 2 °C under a 16-h photoperiod (55 µmol m^{-2} s^{-1}) for a culture period of 60 days. Sixty-day-old seedlings were used as a source of explants (cotyledonary and apical nodal segments) for the experiment.

In vitro multiplication of shoots during successive subcultures

The explants (apical and cotyledonary nodal segments) obtained from 60-day-old seedlings were inoculated into glass test tubes (15 x 2.5 cm; Laborglas) containing 10 mL of MS culture medium supplemented with 20 g L^{-1} sucrose, 2 g L^{-1} Phytagel, and 2.5 µM 6-benzyladenine (BA; Sigma-Aldrich), according to Oliveira et al. (2020). The pH of the culture medium was adjusted to 5.7 before autoclaving at 121 °C for 15 min. The explants were incubated in a growth room at 25 ± 2 °C under a 16-h photoperiod (55 µmol m^{-2} s^{-1}) for a culture period of 45 days. This step corresponded to the shoots obtained in the first subculture using nodal segments obtained from 60-day-old seedlings as explants. For the second, third, and fourth subcultures, the shoots grown from apical and cotyledonary nodal segments after 45 days of incubation were excised (approximately 2 cm), cutting the leaves and apical meristem to obtain the explants and were then used for the respective subculture. The explants were inoculated in the same culture medium again under the same conditions used for the first subculture. Intervals of 45 days were maintained between each subculture under the same conditions of incubation for the growth of shoots. After 45 days of incubation, at the end of each subculture, shoots were collected and used for *ex vitro* rooting.

Effect of subculture on the *ex vitro* adventitious rooting of micropropagated shoots

To study the effect of the number of subcultures on *ex vitro* rooting, shoots (approximately 2 cm) obtained from apical and cotyledonary nodal segments after 1, 2, 3 and 4 subcultures were used. The shoots were removed from the culture medium and transferred to disposable polypropylene plastic cups (50 mL; Orleans, Santa Catarina, Brazil) containing Basaplant® (Artur Nogueira; São Paulo, Brazil) substrate and vermiculite (Mineração Pedra Lavrada LTDA; Atibaia, Brazil) in a 2:1 (v/v) proportion. The plastic cups were maintained in plastic trays (50 x 60 x 10 cm) covered with plastic film to maintain high humidity for 15 days. Thereafter, the humidity level was gradually reduced through perforation of the plastic film, and after 25 days, the plastic film was removed. The explants were maintained in a growth room at 25 ± 2 °C under a 16 h photoperiod at 55 µmol m^{-2} s^{-1} for 45 days.

After rooting, the plantlets were transplanted to disposable polypropylene plastic cups (250 mL) containing Basaplant® substrate and vermiculite in a 2:1 (v/v) proportion and were maintained under a 16 h photoperiod at 55 µmol m^{-2} s^{-1} at 25 ± 2 °C for 15 days. Then, the rooted plantlets were transferred to a greenhouse with relative air humidity higher than 85% and temperatures between 20 and

30 °C (measured using an Extech RHT10 USB Datalogger, Extech; Waltham, USA). After 90 days, plantlet survival (%) was analyzed.

From the first to the fourth subcultures, root induction (%), the number of roots per explant, and the length (cm) of roots were evaluated after 45 days of rooting. Each treatment (subculture) was composed of eight replicates, with five shoots per replicate. For PA analyses, shoots from cotyledonary nodal segments with leaves were collected after 45 days of culture across four subcultures. For ADC, ODC, hormones and comparative proteomic analyses, shoots from cotyledonary nodal segments with leaves were collected after 45 days of culture in the first and fourth subcultures. Samples of the shoots used in the proteomic and PA analyses were macerated with liquid nitrogen until a fine powder was obtained, which was then stored at −80 °C until assessment. Samples for hormonal analysis were macerated and lyophilized for storage until assessment.

Free PA determination

Free PA contents were determined according to Santa-Catarina et al. (2006) using three biological replicate samples (200 mg fresh matter – FM - each) of shoots in each subculture (1, 2, 3 and 4). The samples were ground in 1.2 mL of 5% perchloric acid (Merck; Darmstadt, Germany), incubated at 4 °C for 1 h, and centrifuged for 20 min at 20,000 × *g* at 4 °C. The supernatant containing free PAs was obtained, followed by derivatization with dansyl chloride (Merck) and identification by high-performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan) using a 5-µm C_{18} 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 at pH 3.5, adjusted with hydrochloric acid (Merck). The absolute acetonitrile concentration was maintained at 65% for the first 10 min, increased from 65 to 100% between 10 and 13 min, and maintained at 100% between 13 and 21 min; the mobile phase was added at a flow rate of 1 mL min^{-1} at 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).

Determination of ADC and ODC activities

ODC and ADC activities were determined according to De Oliveira et al. (2017) with some modifications using three biological replicate samples (500 mg FM per sample) of shoots at the first and fourth subcultures. Each sample was homogenized in an ice-cold mortar with liquid nitrogen and transferred to 500 µL of extraction buffer containing 50 mM Tris–HCl (Invitrogen; Carlsbad, USA) (pH 8.5), 0.5 mM pyridoxal-5-phosphate (Sigma-Aldrich), 0.1 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich) and 5 mM dithiothreitol (DTT; Bio-Rad, Hercules, USA). The sample mixtures were vortexed and centrifuged for 20 min at 13,000 × *g* at 4 °C, and the supernatant was used for the ADC and ODC

enzymatic assays. A reaction mixture containing 100 µL of protein extract, 8.3 µL of extraction buffer, 12 mM unlabeled L-Arg or L-Orn, and 25 mCi of either L-[^{14}C(U)]-Arg (specific activity 274.0 mCi mmol^{-1}; PerkinElmer; Waltham, USA) or L-[1-^{14}C]-Orn (specific activity 57.1 mCimmol^{-1}; PerkinElmer) was used. For blank samples, 100 µL of extraction buffer was used. The reaction mixtures were incubated in glass tubes fitted with rubber stoppers and filter paper discs (GE Healthcare; Piscataway, USA) soaked in 2 N KOH (Vetec). The material was incubated at 37 °C at 120 rpm in an orbital shaker for 90 min, and the reaction was stopped by adding 200 µL of 5% (v/v) perchloric acid, followed by further incubation for 15 min under the same conditions. Filter paper containing $^{14}CO_2$ was immersed in 1 mL of scintillation fluid (PerkinElmer). Radioactivity was then measured using a scintillation counter (Tri-Carb2910TR; PerkinElmer). Protein content was measured using the Bradford method (Bradford 1976) with bovine serum albumin as the standard. The specific enzymatic activities of ADC and ODC were expressed as nmol $^{14}CO_2$ mg protein^{-1} h^{-1}.

Plant hormone analysis

Plant hormone analysis was performed according to Durgbanshi et al. (2005) with slight modification using three biological replicate samples (30 mg dry matter (DM) for each sample) of shoots in the first and fourth subcultures. Before extraction, a mixture containing 50 ng of [$^2H_6$]-ABA, [C_{13}]-SA, dihydrojasmonic acid and 5 ng of [$^2H_2$]-IAA (all from Sigma-Aldrich) was added to each sample as an internal standard. [$^2H_2$]-IAA was used to determine IAA contents, [$^2H_6$]-ABA was used to determine ABA contents, [^{13}C]-SA was used to determine SA and trans-cinnamic acid (t-CA) contents, and dihydrojasmonic acid was used to determine JA, OPDA and jasmonoyl-isoleucine (JA-Ile) contents. The previously macerated and lyophilized samples were immediately homogenized in 2 mL of ultrapure water in a ball mill (MillMix20; Domel; Železniki, Slovenija) at a frequency of 14 Hz for 10 min. After centrifugation at 4,000 × *g* and 4 °C for 10 min, the supernatants were recovered, and the pH was adjusted to 3.0 with 30% (v/v) acetic acid (Labkem; Barcelona, Spain). The water extract was partitioned twice against 2 mL of diethyl ether (Labkem), and the organic layer was recovered and evaporated under vacuum in a centrifuge concentrator (Speed Vac, Jouan SA; Saint-Herblain, France). Once dried, the samples were resuspended in a 10% (v/v) methanol (Fisher Scientific; Loughborough, UK) solution via gentle sonication. The resulting solution was filtered through 0.22-µm polytetrafluoroethylene membrane syringe filters (PTFE 13-mm diameter; Kinesis Ltda; Cambridgeshire, UK) and directly transferred to vials for mass spectrometry analysis.

LC-electrospray ionization (ESI)-MS/MS analysis was performed using an Acquity Ultra-High Performance Liquid Chromatography (UPLC) system (Waters; Milford, USA) coupled to a tandem Xevo TQ-XS triple quadrupole mass spectrometer (Waters) using an orthogonal Z-Spray ESI interface operated in negative-ion mode. Chromatographic separations were carried out in a reversed-phase C_{18} column (50 × 2.1 mm, 1.6-µm particle size; Phenomenex Luna Omega; Madrid, Spain) at a flow rate of 300 µL min^{-1} with a column temperature of 40 °C. A binary gradient was used for elution: mobile phase A consisted of

ultrapure water and 0.1% acetic acid, and mobile phase B consisted of 99.9% (v/v) methanol (Fisher Scientific) and 0.1% (v/v) acetic acid. Gradient elution was performed sequentially as follows: maintenance of 10% B for 2 min, followed by ramping from 10 to 90% B at 6 min and a decrease to 10% B at 7 min, after which 10% B was maintained until the end of the run at 8 min. The drying gas and the nebulizing gas were both nitrogen (Praxair; Valencia, Spain). The cone gas flow was set to 250 L h^{-1}, and the desolvation gas flow was set to 1200 L h^{-1}. For operation in tandem MS (MS/MS) mode, the collision gas was 99.995% pure argon (Praxair). The cone voltage and collision energies were adjusted depending on the compound under investigation, as described by Durgbanshi et al. (2005) with few modifications. The desolvation gas temperature was 650 °C, the source temperature was 150 °C, and the capillary voltage was 2 kV. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. Masslynx v4.1 software was used for mass spectral acquisition, and growth regulators were measured through comparisons with the internal standard for each deuterium-labeled growth regulator.

Protein extraction and digestion

Comparative proteomic analyses were performed with three biological replicate samples (300 mg FM per sample) of shoots from the first and fourth subcultures. Total protein extraction was performed according to Damerval et al. (1986) with some modifications. The samples, previously macerated to a fine powder with liquid nitrogen, were resuspended in 1 mL of chilled solution containing 10% (w/v) trichloroacetic acid (TCA; Sigma-Aldrich) in acetone (Merck) and 20 mM DTT (GE Healthcare, Piscataway, USA), and the mixture was vortexed for 5 min at 4 °C and kept at - 20 °C for 1 h, followed by centrifugation at 16,000 x *g* for 20 min at 4 °C. The resulting pellets were washed three times with cold acetone containing 20 mM DTT and centrifuged for 5 min in each wash step. 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 (GE Healthcare), and 1 mM phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich), vortexed, and incubated on ice for 30 min. Following incubation, the samples were centrifuged at 16,000 × *g* for 20 min at 4 °C. The supernatants containing total proteins were collected, and the protein concentration was measured using a 2-D Quant Kit (GE Healthcare).

Before the digestion step, the extracted proteins (100 µg from each biological replicate) were first precipitated using methanol/chloroform (Nanjo et al. 2012). Then, the samples were resuspended in a solution of 7 M urea (GE Healthcare) and 2 M thiourea (GE Healthcare), and protein digestion was performed with trypsin (50 ng μL^{-1}; V5111, Promega, Madison, USA) using the filter-aided sample preparation (FASP) method (Reis et al. 2021). The digested samples were transferred to Total Recovery Vials (Waters) for mass spectrometry analysis.

Nano-LC-electrospray ionization (ESI)-MS/MS analysis was performed using a nanoAcquity UPLC system (Waters) coupled to a Synapt G2-Si mass spectrometer (Waters). First, a chromatography step was performed by loading 1 µg of the digested samples according to Oliveira et al. (2020) to normalize the relative protein quantification results. To ensure standardized molar values for all conditions, the

normalization among samples was based on stoichiometric measurements of the total ion counts (TICs) from MSE scouting runs prior to the analyses using ProteinLynx Global SERVER v. 3.0 (PLGS; Waters). After sample normalization, the peptide mixtures were separated via liquid chromatography using a nanoAcquity UPLC 5 µm C18 trap column (180 µm × 20 mm; Waters) at 5 µL min^{-1} for 3 min, followed by a nanoAcquity HSS T3 1.8 µm analytical reverse-phase column (75 µm × 150 mm; Waters) at 400 nL min^{-1} at 45 °C. A binary gradient of mobile phase A containing water (Tedia; Fairfield, USA) and 0.1% formic acid (Sigma-Aldrich) and mobile phase B containing absolute acetonitrile (Sigma-Aldrich) and 0.1% formic acid was used for peptide elution. Gradient elution started with 7% B, followed by 7 to 40% B until 91.12 min, 40 to 99.9% B until 92.72 min, holding at 99.9% B until 106 min, then a decrease to 7% B until 106.1 min and holding at 7% B until the end of the run at 120 min. Mass spectrometry was performed with the following settings: positive and resolution mode (V mode), 35,000 full width at half maximum ion mobility separation (IMS), and data-independent acquisition (DIA) mode ($HDMS^E$). IMS was performed using a wave velocity of 600 m s^{-1}. The helium and IMS gas flow rates were 180 and 90 mL/min, respectively. The transfer collision energy was ramped from 19 to 55 V in high-energy mode, with cone and capillary voltages of 30 and 2750 V, respectively, and a source temperature of 70 °C. For the time of flight (TOF) parameters, the scan time was set to 0.5 s in continuum mode, with a mass range of 50 to 2000 Da. Human [Glu^1]-fibrinopeptide B (Sigma-Aldrich; 100 fmol $µL^{-1}$) was used as an external standard, and lock mass acquisition was performed every 30 s. Mass spectral acquisition was performed for 90 min using MassLynx v4.0 software.

Proteomic data analysis

Spectral processing and database searching were performed using the ProteinLynx Global Server (PLGS; version 3.0.2) (Waters). The Apex3D parameters were set to a low-energy threshold of 150 counts, an elevated-energy threshold of 50 counts, and an intensity threshold of 750 counts. In addition, the analysis settings included the following: one missed cleavage, minimum fragment ion per peptide equal to 3, minimum fragment ions per protein equal to 7, minimum peptide per protein equal to 2, automatic peptide and fragment tolerance, a fixed modification of carbamidomethyl and variable modifications of oxidation and phosphoryl. The false discovery rate (FDR) was set to a maximum of 1%. Protein identification was performed against a nonredundant protein databank for *C. fissilis* generated by transcriptome sequencing and de novo assembly (Oliveira et al. 2020). Comparative label-free quantification analysis was performed using ISOQuant software v.1.7 (Distler et al. 2014). 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. The mass spectrometry proteomic data have been deposited with ProteomeXchange (Deutsch et al. 2019). Consortium via the PRIDE (Perez-Riverol et al. 2019) partner repository with the dataset identifier PXD023173.

To ensure the quality of the results after data processing, only the proteins that were present or absent (for unique proteins) in all three runs of biological replicates were considered in the differential accumulation analysis using Student's *t*-test (two-tailed; $P < 0.05$). Differentially accumulated proteins were considered to be up-accumulated if the $\log_2$ value of their fold change (FC) was greater than 0.6 and down-accumulated if the $\log_2$ value of their FC was lower than -0.6 according to Student's *t*-test. Finally, the proteins were subjected to BLASTp searches against the Nonredundant (nr) Green Plants/Viridiplantae Protein Sequences database using OmicsBox software (https://www.biobam.com/omicsbox) for high-throughput functional annotation (Götz et al. 2008).

Statistical analysis

The rooting of shoots was performed using a completely randomized design. The data on root induction, the numbers and lengths of roots, plantlet survival, free PAs, ADC/ODC enzyme activities and plant hormones were analyzed by analysis of variance (ANOVA) ($P < 0.05$) followed by the Student-Newman-Keuls (SNK) test (Sokal and Rohlf 1995) in the R statistical environment (R Core Team 2017).

Results

Effect of the number of subcultures on the *ex vitro* adventitious rooting of micropropagated shoots

The number of subcultures significantly affected the rooting of the shoots, resulting in a significant reduction in the development of ARs on shoots from apical and cotyledonary nodal segments (Figs. 1 and 2).

Shoots obtained from cotyledonary nodal segments showed a significant reduction in the induction of *ex vitro* rooting (Fig. 1a) and the number of roots formed (Fig. 1b) in the third and fourth subcultures, whereas those obtained from apical nodal segments showed a significant reduction in both parameters beginning in the second subculture (Fig. 1). In addition, a significant decrease in the length of roots on shoots from the two types of explants was observed with an increase in the number of subcultures (Fig. 1c).

A comparison of shoots from the apical and cotyledonary nodal segments used for *ex vitro* rooting revealed a significant difference in the percentage of root induction (Fig. 1a) and number of roots per shoot (Fig. 1b) beginning in the second subculture. In addition, shoots from cotyledonary nodal segments presented a significantly higher percentage of root induction and number of roots per shoot than shoots from apical nodal segments (Fig. 1a). Moreover, shoots obtained from cotyledonary nodal segments showed longer roots than those initiated from apical nodal segments in the first, second and third subcultures (Fig. 1c).

The highest survival rates of the plantlets were obtained with shoots from the first subculture, with no significant difference between the two types of explants used (Fig. 1d). In the second subculture, the survival rate was significantly higher for shoots initiated from cotyledonary nodal segments than for those initiated from apical nodal segments, while in the other subcultures (third and fourth), a significant decline in plantlet survival occurred among the shoots from both types of explants used (Fig. 1d). Therefore, shoots generated from cotyledonary nodal segments collected in the first and fourth subcultures were used for PA enzyme activity, proteomic and hormonal analyses.

Effect of the number of subcultures on the endogenous contents of free PAs

The number of subcultures significantly affected the endogenous contents of PAs in shoots from cotyledonary nodal segments (Fig. 3). The contents of free Put (Fig. 3a), Spd (Fig. 3b) and Spm (Fig. 3c) decreased significantly in shoots from the first to the second subcultures. The endogenous contents of total free PAs were significantly higher in the first subculture, when greater rooting was observed, and they then significantly decreased in shoots with increasing number of subcultures, when the rooting potential also decreased (Fig. 3d).

Effect of the number of subcultures on ADC and ODC enzyme activities

Because of the results regarding endogenous PA contents, especially free Put, enzyme activity analysis was performed on shoots generated from cotyledonary nodal segments from the first and fourth subcultures. The activity of ODC was predominant in this species. A significant reduction in ODC enzyme activity was observed in the fourth subculture relative to the first (Fig. 4), resulting in a significant reduction in the free Put content (Fig. 3a). The activity of the ADC enzyme did not show significant differences between the first and fourth subcultures (Fig. 4).

Effect of the number of subcultures on the endogenous contents of IAA, ABA, OPDA, JA, JA-Ile, t-CA and SA

Decreases in the endogenous contents of IAA, ABA and OPDA (Figs. 5a-c) were observed in shoots from cotyledonary nodal segments from the fourth subculture relative to those from the first, showing that successive subculture reduces the contents of these hormones, which are necessary for AR formation. In contrast, the endogenous contents of JA, JA-Ile, t-CA and SA (Figs. 6a-d) were significantly higher in shoots from cotyledonary nodal segments from the fourth subculture than in those from the first subculture.

Effect of the number of subcultures on the proteomic profile

A total of 858 proteins were identified (Supplementary Table 1) using shoots obtained from cotyledonary nodal segments from the first and fourth subcultures. The comparison of shoots from the fourth subculture with those from the first subculture (S4/S1) revealed 321 differentially accumulated proteins (DAPs), including 86 up- and 218 down-accumulated proteins, 2 proteins unique to the shoots from the fourth subculture (S4) and 15 proteins unique to the shoots from the first subculture (S1) (Supplementary Table 1).

The DAPs were classified into five groups according to biological processes: photosynthesis, energy metabolism, nitrogen compound metabolic process, response to stimulus, and response to stress (Fig. 7). Among these proteins, some were highlighted due to their relationship to AR formation in *C. fissilis*.

Some proteins related to photosynthesis were down-accumulated in shoots from the fourth subculture relative to those from the first subculture (Fig. 7). Among these proteins, five photosystem I (PSI) reaction center subunit (Ce_fissilis.001790.1, Ce_fissilis.003800.3, Ce_fissilis.004692.1, Ce_fissilis.018999.1 and Ce_fissilis.015428.1) proteins were down-accumulated in the shoots from the fourth subculture relative to those from the first subculture (Supplementary Table 1). Moreover, in PSII, the photosystem II 22 kDa protein chloroplastic (CP22; Ce_fissilis.000704.1), photosystem II CP43 reaction center protein (CP-43; Ce_fissilis.006118.1) and photosystem II CP47 reaction center protein (CP-47; Ce_fissilis.001288.2) were down-accumulated in the shoots from the fourth subculture relative to those from the first subculture (Supplementary Table 1).

Proteins associated with energy metabolism in plants (Fig. 7) were down-accumulated in the shoots from the fourth subculture relative to those from the first (Supplementary Table 1); these proteins included two ribulose bisphosphate carboxylase/oxygenase activase 1, chloroplastic (RuBisCO; Ce_fissilis.018513.1 and Ce_fissilis.018684.1), as well as ribulose bisphosphate carboxylase small chain, chloroplastic (Ce_fissilis.000071.1), and ribulose bisphosphate carboxylase small chain, chloroplastic (Ce_fissilis.000411.1). Furthermore, in this same group, we found proteins such as phosphoglycerate kinase 2, chloroplastic (Ce_fissilis.007229.1), phosphoglycerate kinase, cytosolic (Ce_fissilis.016667.1), phosphoglucomutase, cytoplasmic (Ce_fissilis.009200.1; Ce_fissilis.011316.1), putative glucose-6-phosphate 1-epimerase (Ce_fissilis.000392.1), transketolase-2, chloroplastic (Ce_fissilis.005337.1), glucose-6-phosphate isomerase (PGI), cytosolic (Ce_fissilis.016775.1) and sedoheptulose-1,7-bisphosphatase, chloroplastic (Ce_fissilis.007537.1) (Supplementary Table 1).

In the nitrogen compound metabolic process group (Fig. 7), proteins related to ammonium fixation, such as glutamine synthetase cytosolic isozyme 2 (Ce_fissilis.008404.1), glutamine synthetase leaf isozyme, chloroplastic (Ce_fissilis.008156.1), and glutamate dehydrogenase 1 (GDH; Ce_fissilis.009844.1), were down-accumulated in shoots from the fourth subculture relative to those from the first subculture (Supplementary Table 1). Furthermore, methionine synthetase and 5-

methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (MET; Ce_fissilis.001903.1 and Ce_fissilis.012853.1), involved in the production of methionine, a precursor of Spd and Spm, were down-accumulated in the shoots from the fourth subculture relative to those from the first subculture (Supplementary Table 1).

We found three proteins that were up-accumulated in the shoots from the fourth subculture relative to those from the first and were related to the response to the stimulus process group (Fig. 7); these proteins included caffeoyl-CoA O-methyltransferase 1 (CCoAOMT; Ce_fissilis.006261.1) and two peroxidase 4 proteins (POX; Ce_fissilis.008801.1 and Ce_fissilis.015659.1) (Supplementary Table 1).

Among the proteins related to stress responses (Fig. 7), some important antioxidant enzymes were identified, such as L-ascorbate peroxidase, cytosolic (APX; Ce_fissilis.017763.1), monodehydroascorbate reductase (MDHAR; Ce_fissilis.000462.1) and 2-Cys peroxiredoxin BAS1-like, chloroplastic (Prxs 2-Cys; Ce_fissilis.010608.1), which were down-accumulated in the shoots from the fourth subculture (when AR formation was reduced) relative to those from the first subculture (Supplementary Table 1).

Additionally, proteins related to plant hormones were identified and could be related to the reduction in AR formation observed in the fourth subculture relative to the first. Additionally, two isoforms of auxin-binding protein ABP19a proteins related to the auxin-activated signaling pathway, the auxin-binding protein ABP19a (Ce_fissilis.009318.1 and Ce_fissilis.010810.1), were down-accumulated in shoots from the fourth subculture relative to those from the first subculture and were associated with a reduction in the potential for AR formation in this species (Supplementary Table 1). Allene oxide cyclase, chloroplastic (AOC; Ce_fissilis.001385.1) protein, related to JA biosynthesis, was identified as a protein unique to shoots from the fourth subculture (Supplementary Table 1) and could be involved in the reduction in AR formation observed in this species.

Discussion

The number of subcultures affects the AR formation

Studies on biochemical and molecular factors controlling AR formation potential in shoots under successive subcultures in woody species are rare (Moura et al. 2012). The number of subcultures was shown to affect *ex vitro* rooting in *C. fissilis*, resulting in significant decreases in root induction, root numbers and lengths and resulting in lower plantlet survival rates (Figs. 1 and 2). Similar results were observed in other woody species, such as *Cabralea canjerana* (Rocha et al. 2007) and *Tectona grandis* (Raposo et al. 2010). It has been shown that wounding due to the excision of explants during subcultures can trigger stress in the plant and increase tissue differentiation, which induces the physiological aging of tissues (Leakey and Storeton-West 1992; Iwase et al. 2015; Ikeuchi et al. 2016). Moreover, the injuries caused by cutting can lead to stress-induced reprogramming of shoot cell fate and metabolic readjustment, which is one of the factors related to hormonal variation (Da Costa et al. 2013). In this

regard, wounding due to the excision of explants during subcultures in *C. fissilis* may be associated with changes in the hormonal balance in shoots and result in different potentials for AR development.

Hormonal balance affects AR formation along subcultures

The plant hormone auxin is important for the development of ARs on stem cuttings, and an appropriate hormonal balance of the auxin/cytokinin ratio is necessary to reprogram tissues to stimulate the cell division necessary for AR development (Gutierrez et al. 2012; Legué et al. 2014). The reduction in rooting with an increase in the number of subcultures was associated with a reduction in endogenous IAA contents in the shoots of *C. fissilis* (Fig. 5a). This reduction in the IAA content was consistent with the down-accumulation of two isoforms of the auxin-binding protein ABP19a (Ce_fissilis.009318.1 and Ce_fissilis.010810.1) in shoots from the fourth subculture relative to those from the first subculture (Supplementary Table 1).

It has been shown that ABP1 is required for the auxin response, acting as a key regulator of auxin action on the cell cycle and elongation and controlling auxin-mediated gene expression on root growth in *Arabidopsis thaliana* (Tromas et al. 2009). ABP19a protein was also associated with the auxin-induced signaling pathway for AR development in *E. grandis*, an easy-to-root species (De Almeida et al. 2020). According to these authors, the ABP19a protein probably acts as an auxin-binding molecule similar to ABP1 and can modulate responses to auxin. These results indicated that the down-accumulation of ABP19a protein in shoots from the fourth subculture compared to the first subculture could be associated with a decrease in the IAA content and with a reduction in the potential for AR formation in *C. fissilis*. In addition, IAA has been indicated to engage in crosstalk with other plant hormones, such as ABA (Albacete et al. 2008), JA (Gutierrez et al. 2012) and SA (Zhang et al. 2007). Similar to IAA, the content of endogenous ABA was higher in the shoots of *C. fissilis* from the first subculture than in those from the fourth subculture (Fig. 5b). While ABA is known to play a role in the maturation and dormancy of seeds, little is known about its involvement in rooting. It has been shown that crosstalk between IAA and ABA during abiotic stress conditions plays an important role in improving the growth of roots in *Solanum lycopersicum* plants cultivated under high salinity (Albacete et al. 2008). Moreover, the endogenous contents of JA (Fig. 6a) and JA-Ile, the active conjugated form of JA (Fig. 6b), were higher in shoots from the fourth subculture relative to those from the first subculture, which could be related to the lower AR development observed. In *A. thaliana*, crosstalk between IAA and JA has been observed to play an important role in the regulation of AR formation in etiolated hypocotyls, in which IAA controls AR initiation by negatively regulating JA signaling (Gutierrez et al. 2012). In *Petunia hybrida*, the excision of cuttings leads to a fast, transient increase in endogenous JA, showing the involvement of this hormone in the wound response during AR formation (Ahkami et al. 2009). Through proteomic analysis, we identified one of the main enzymes related to JA biosynthesis, AOC, chloroplastic (Ce_fissilis.001385.1), which was unique to the shoots from the fourth subculture (Supplementary Table 1). The AOC enzyme gives rise to OPDA, the first biologically active compound in the JA pathway (Schaller et al. 2008). The decreased OPDA content in shoots from the fourth subculture (Fig. 5c) could be related to the increases in JA and JA-Ile contents (Figs. 6a and 6b). Studies have shown that

OPDA plays a role in wounding response-related signaling that is independent of JA (Stintzi et al. 2001; Taki et al. 2005), indicating a positive feedback response between OPDA and JA (Stenzel et al. 2003). Our results clearly showed an increase in the endogenous content of JA in shoots
at the fourth subculture relative to those from the first subculture, negatively affecting rooting, and the presence of the AOC protein could play an essential role in the production of JA induced by the increase in the number of subcultures via a reduction in the OPDA content.

In addition, studies have shown that SA could play a role in AR development. Previously, SA was shown to inhibit IAA-induced AR formation in the shoots of *Malus* 'Jork 9' during the first few days posttreatment as a result of IAA decarboxylation promoted by exogenous SA (De Klerk et al. 2011). In the present study, we observed lower IAA and higher SA contents (Figs. 5a and 6d) in the shoots of *C. fissilis* at the fourth subculture. The increase in SA content in shoots from the fourth subculture is also associated with a higher content of t-CA, one of the first compounds in the phenylpropanoid pathway and a precursor of SA (Sendon et al. 2011). In our work, the lowest t-CA and SA contents in shoots were identified in the first subculture (Figs. 6c and 6d), when greater rooting was observed, while the higher contents of these compounds in the fourth subculture were related to a reduction in the rooting potential of *C. fissilis* shoots (Fig. 1). On the other hand, AR formation in SA-deficient *A. thaliana* mutants was found to be lower than that in the wild type (Gutierrez et al. 2012), and exogenous SA treatments improved AR formation in *Phaseolus radiates* hypocotyl cuttings (Yang et al. 2013).

Changes in the endogenous contents of free PAs have been shown to be determinants of AR induction *in in vitro*-propagated shoots from *Juglans regia* (Heloir et al. 1996). Recently, cuttings of *Taxus chinensis* 'Mairei' with an increased potential for AR formation were found to show a higher free Put content in the basal stem portion of rooted cuttings as well as a higher IAA content relative to cuttings with a low rate of AR formation (Fei and Tang 2018). In our work, the number of subcultures decreased AR induction, root length and the number of roots per shoot as well as plantlet survival (Fig. 1), which was associated with decreases in the endogenous contents of free Put (Fig. 3a), Spd (Fig. 3c) and total free PAs (Fig. 3d). Studies have shown that Put stimulates the cell division and elongation of shoots (Kuznetsov et al. 2002; Aragão et al. 2017) since Put can induce the G1/S to G2/M cell phase transition (Weiger and Hermann 2014). Thus, the reduction in endogenous free Put contents could be directly associated with the reduction in *ex vitro* rooting in the shoots of *C. fissilis*, suggesting that Put plays an important role in this process. In addition, the significantly higher activity of ODC relative to the ADC enzymes (Fig. 4) showed that ODC is the main enzyme affecting Put biosynthesis during AR induction in *C. fissilis* shoots, an observation made for the first time to our knowledge for this species.

Differential accumulation of proteins associated with AR formation during subcultures

The increase in the number of subcultures induced a differential accumulation of proteins in the shoots of *C. fissilis* (Supplementary Table 1). Eight proteins related to PSI and PSII complexes were down-accumulated in shoots from the fourth subculture relative to those from the first subculture. These proteins are associated with photosynthesis, which plays a fundamental role in energy transduction by

trapping light energy and converting it into biochemical energy (Amunts and Nelson 2008) and is a determinant of higher AR formation (Druege et al. 2019).The down-accumulation of these proteins could be related to the reduced growth of shoots at the fourth subculture compared to the first, thus affecting the current net photosynthesis and the initial carbohydrate reserves in the leaves of shoots in *C. fissilis*.

During rooting, high inputs of energy and carbon skeletons are necessary to support cell division, new root meristem establishment and AR formation (Ahkami et al. 2009; Da Costa et al. 2013). Among the identified proteins, glucose-6-phosphate isomerase, cytosolic (PGI) was down-accumulated in shoots from the fourth subculture relative to those from the first subculture, suggesting that this protein may play a positive role in the development of roots in *C. fissilis*. A higher accumulation of the PGI protein was relevant for higher rooting in *E. grandis*, an easy-to-root species, than in *E. globulus,* a difficult-to-root species, with this protein being a positive regulator of AR formation (De Almeida et al. 2020). Moreover, five proteins related to carbohydrate metabolism involved in the Calvin cycle and carbohydrate metabolic processes, such as the synthesis of starch and sucrose in plants, were down-accumulated in shoots from the fourth subculture relative to those from the first subculture. Studies assessing the effects on carbohydrate dynamics on rooting in two *Eucalyptus* species with differences in recalcitrance pointed to a key role of a higher proportion of carbohydrate allocation at the base of shoot cuttings, increasing energy and carbon availability for new AR formation and therefore contributing to overcoming rooting recalcitrance (Ruedell et al. 2013).

Nitrogen is a key element in plant growth, nutrition and reproduction and an essential building block of nucleic acids and proteins (Stitt and Schulze 1994; Bernard and Habash 2009), and glutamine synthetase (GS) plays a major role in fixing ammonium to produce the amino acid glutamine (Bernard and Habash 2009). Two GS proteins were down-accumulated in the shoots of *C. fissilis* from the fourth subculture relative to those from the first subculture, which was associated with the lower *ex vitro* rooting potential observed. *E. globulus,* a difficult-to-root species, showed a down-accumulation of GS*,* which was related to lower AR formation compared to *E. grandis*, an easily rooting species (Corrêa et al. 2005). In addition, the down-accumulation of 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (MET) proteins in shoots from the fourth subculture compared to the first subculture was associated with reduced rooting in *C. fissilis*. Our results revealed the highest endogenous contents of total free PAs and free Spm in the first subculture, in agreement with MET differential accumulation. MET is an enzyme that catalyzes the transfer of a methyl group leading to the production of methionine, which may be the substrate for PA biosynthesis (Garras et al. 1991).

Wounding during the process of shoot multiplication can be a trigger for cell division (Sangwan et al. 1992). Our results showed the up-accumulation of proteins related to the response to the stimulus process group in the shoots from the fourth subculture relative to those from the first subculture in *C. fissilis*, such as CCoAOMT (Supplementary Table 1; Fig. 7). CCoAOMT plays a role in the synthesis of feruloylated polysaccharide-producing compounds such as monolignols, the building units of lignin, and other compounds implicated in plant development and interactions with the environment (Pinçon et

al. 2001). An increase in the accumulation of lignin inhibits the growth of *A. thaliana* (Zhou et al. 2009). The up-accumulation of CCoAOMT could be linked to defense mechanisms and adaptive responses to wounding, reducing AR formation in *C. fissilis* shoots at the fourth subculture. Plant cells can detect mechanical disturbances and initiate a defense reaction in response (Muday and Brown-Harding 2018). POX, a wound-induced protein, has been shown to exhibit increases in activity or mRNA levels upon mechanical wounding in several plants, such as *Oryza sativa* (Hiraga et al. 2000b; Ito et al. 2000), *Nicotiana tabacum* (Hiraga et al. 2000a) and *Ipomoea batatas* (Kim et al. 1999). Two POXs were found up-accumulated in the *C. fissilis* shoots from the fourth relative to those from the first subculture and are associated with a reduced rooting observed during subcultures. Adventitious rooting is marked by lower POX activities during AR induction and initiation in microcuttings of *Bacopa monnieri* (Goel et al. 2018), while no apparent correlation between the activity of POX and the ability of the cuttings to form roots has been found in *Populus nigra*, *P. alba* or *P. tremula* (Güneș 2000). The down-accumulation of POX proteins was associated with a reduced rooting potential in *C. fissilis*.

Other proteins related to the stress response (Fig. 7), some antioxidant enzymes, such as APX and MDHAR, showed down-accumulation in the shoots from the fourth subculture relative to those from the first subculture (Supplementary Table 1). These enzymes play a crucial role during organ regeneration in plants (Ghosh and Pal 2013), in addition to being important enzymes related to stress responses, such as wounding responses (Hiraga et al. 2001). In addition, the Prxs 2-Cys protein is part of the 2-Cys peroxyredoxin family of enzymes, which are widely distributed among all organisms (Rouhier and Jacquot 2005). Transgenic antisense *A. thaliana* plants with reduced levels of Prx 2-Cys show developmental retardation and transient damage to the photosynthetic membrane, suggesting that Prxs 2-Cys is important for seedling development (Baier and Dietz 1999). In our study, the down-accumulation of Prxs 2-Cys in the shoots from the fourth subculture relative to those from the first subculture was associated with the higher AR formation potential of shoots from the first subculture.

Taken together, the data support an initial working model of the roles of key protein differential accumulation and hormonal alterations in the regulation of rooting competence (Fig. 8).

Conclusion

Long-term subculture affected the potential for AR development. The reduction in AR formation was associated with decreases in the endogenous contents of IAA, ABA, 12-oxo phytodienoic acid, and free Put and Spm. In contrast, increases in JA, JA-Ile, t-CA, and SA contents could negatively affect AR development in shoots from the fourth subculture. The AOC protein, unique in shoots from the fourth subculture, could increase JA accumulation, resulting in a reduction in AR formation during successive subcultures. The ODC enzyme (rather than ADC) plays the predominant role in Put synthesis during adventitious rooting in *C. fissilis*. Additionally, a down-accumulation of the ABP19a protein in shoots from the fourth subculture compared to the first subculture was associated with a decreased IAA content and reduced AR formation potential in shoots from the fourth subculture. Among the down-accumulated proteins, photosystem I reaction center subunits and PGI were related to maintenance and energy

transduction in PSI and PSII and Calvin–Benson, respectively. The GS and MET proteins were down-accumulated, and correlated with the nitrogen compound metabolic process, and PA signaling pathways. Furthermore, antioxidant proteins, such as APX, MDHAR, and Prxs 2-Cys, were correlated with reduced AR potential in shoots from the fourth subculture relative to those from the first subculture. For up-accumulated proteins in shoots from the fourth subculture relative to those from the first subculture, the CCoAOMT and POX proteins are related to adaptive responses to wounding caused by the long-term subculture, showing the role of proteins in the AR formation process. This work is the first to report the effect of long-term subculture on AR development potential in the shoots of *C. fissilis* and its crosstalk with endogenous plant hormone contents and the protein profile.

Abbreviations

ABA	Abscisic acid
ADC	Arginine decarboxylase
ARs	Adventitious roots
BA	6-benzyladenine
DAPs	Differentially accumulated proteins
DM	Dry matter
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FM	Fresh matter
HPLC	High-performance liquid chromatography
IAA	Indole-3-acetic acid
JA	Jasmonic acid
JA-Ile	Jasmonoyl-isoleucine
MS	Murashige and Skoog culture medium
ODC	Ornithine decarboxylase
PAs	Polyamines
Put	Putrescine

SA	Salicylic acid
Spd	Spermidine
Spm	Spermine
TCA	Trichloroacetic acid

Declarations

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Code availability: PXD023173.

Authors' contributions: CSC and TRO conceived the study, designed the experiments, and wrote the manuscript. TRO was responsible for the *in vitro* culture experiments, PA analyses and statistical analyses. TRO, KRS and VS were responsible for the proteomic analyses. TRO, DB and AGC were responsible for the plant hormone analyses at SCIC from UJI - Spain. TRO, VPMA, LFO and EISF were

responsible for the ADC and ODC enzyme analyses. All the authors read and approved the final manuscript.

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Figures

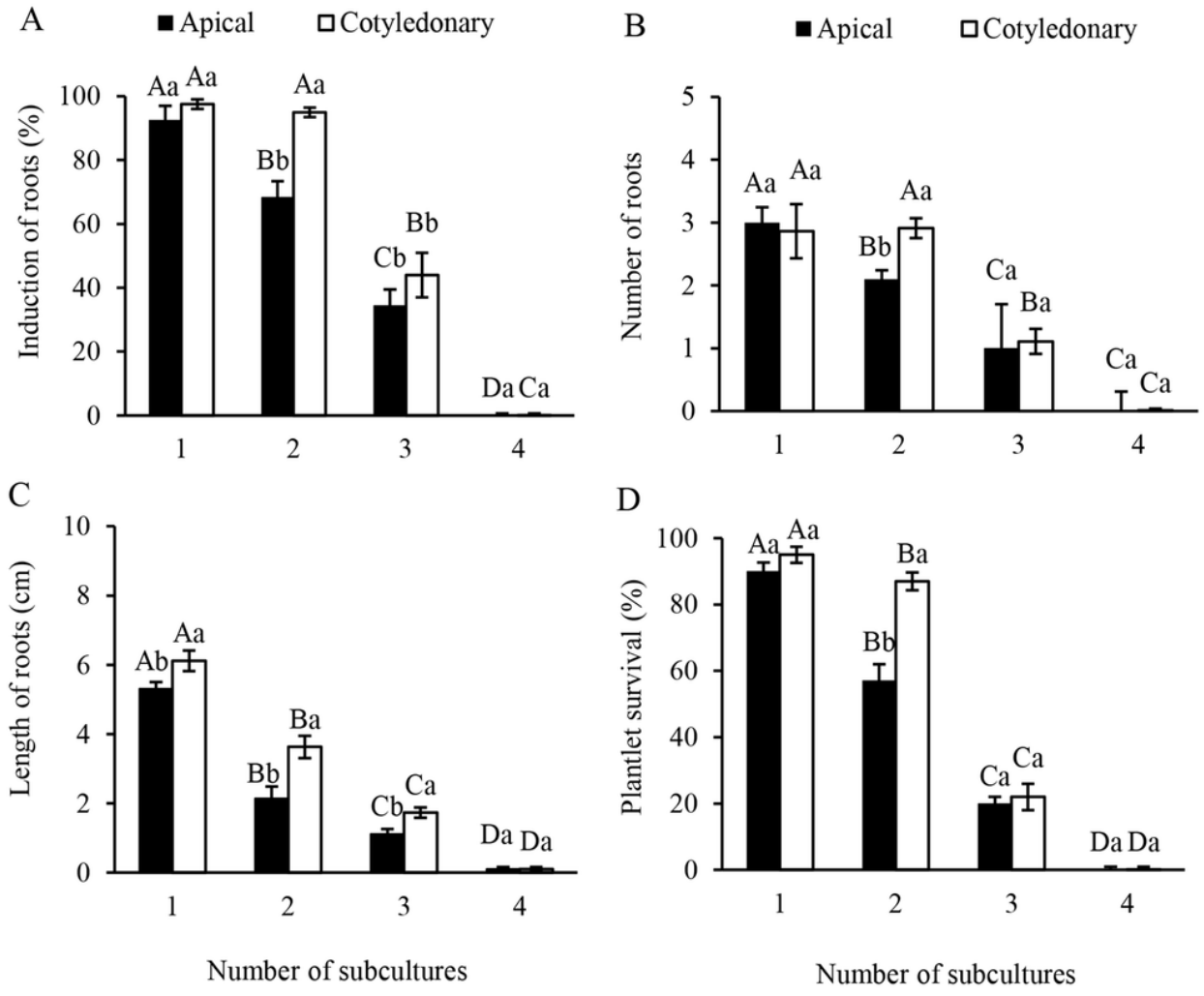

Figure 1

Effects of the number of subcultures and the type of initial explant (apical or cotyledonary nodal segments) on the ex vitro rooting of micropropagated Cedrela fissilis shoots. The induction of roots (a), number of roots per shoot (b), length of roots (c), and plantlet survival throughout the subculture (d) were analyzed in the shoots of C. fissilis during four subcultures with 45-day intervals in each subculture. Means followed by different letters show significant differences ($P < 0.05$) according to the SNK test. Capital letters show significant differences between subcultures according to the initial explant type (apical or cotyledonary nodal segments). Lowercase letters show significant differences between initial explant types (apical or cotyledonary nodal segments) in each subculture. CV = coefficient of variation. (n = 8; CV for the induction of roots = 10.4%; CV for the number of roots = 16.3%; CV for the length of roots = 14.5%; CV for plantlet survival = 14.3%). Error bars indicate standard deviation.

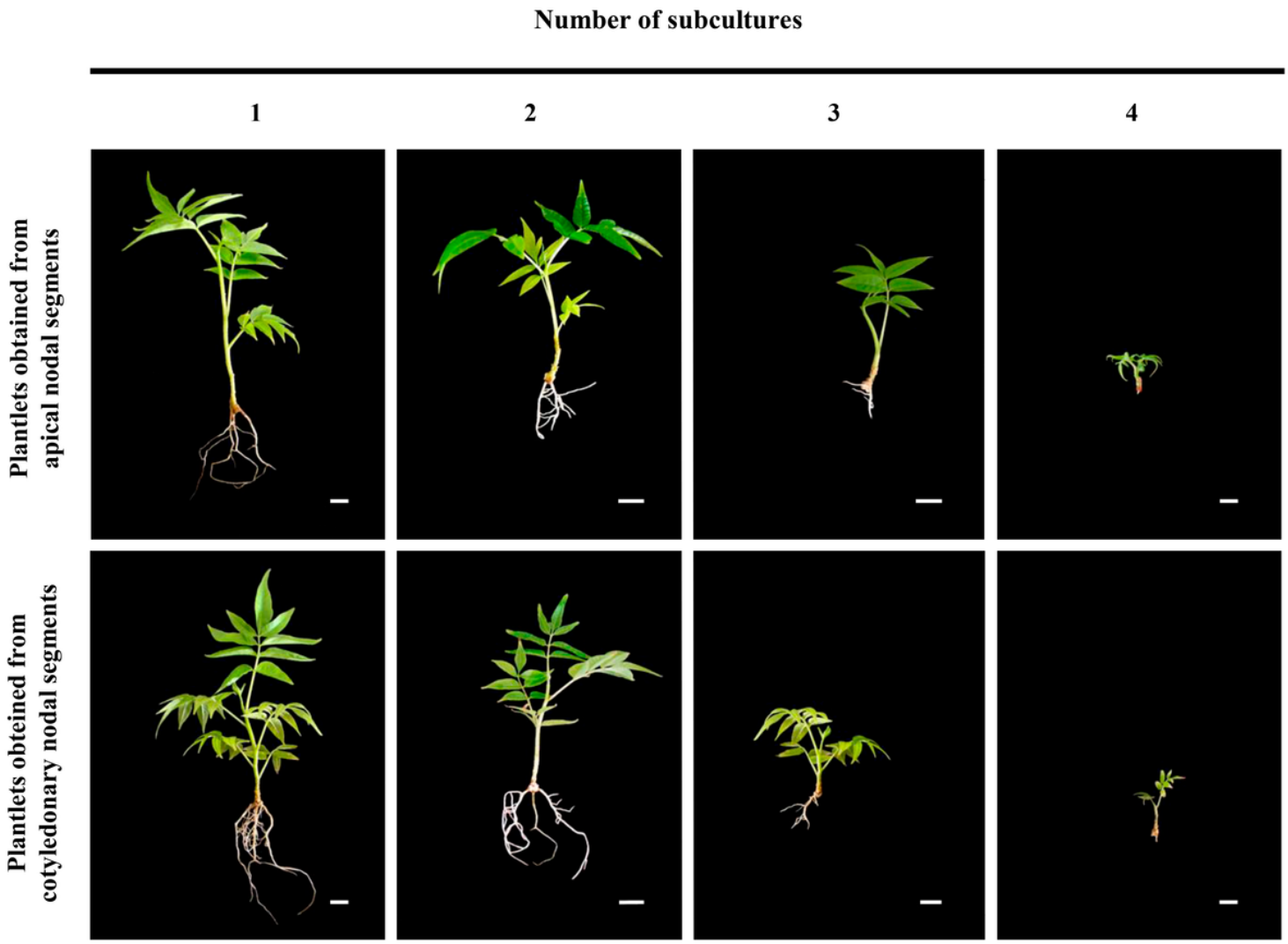

Figure 2

Morphological aspects of the plantlets and adventitious root development in the shoots of Cedrela fissilis initiated from apical and cotyledonary nodal segments during four subcultures, with 45-day intervals in each subculture. Bars = 1 cm.

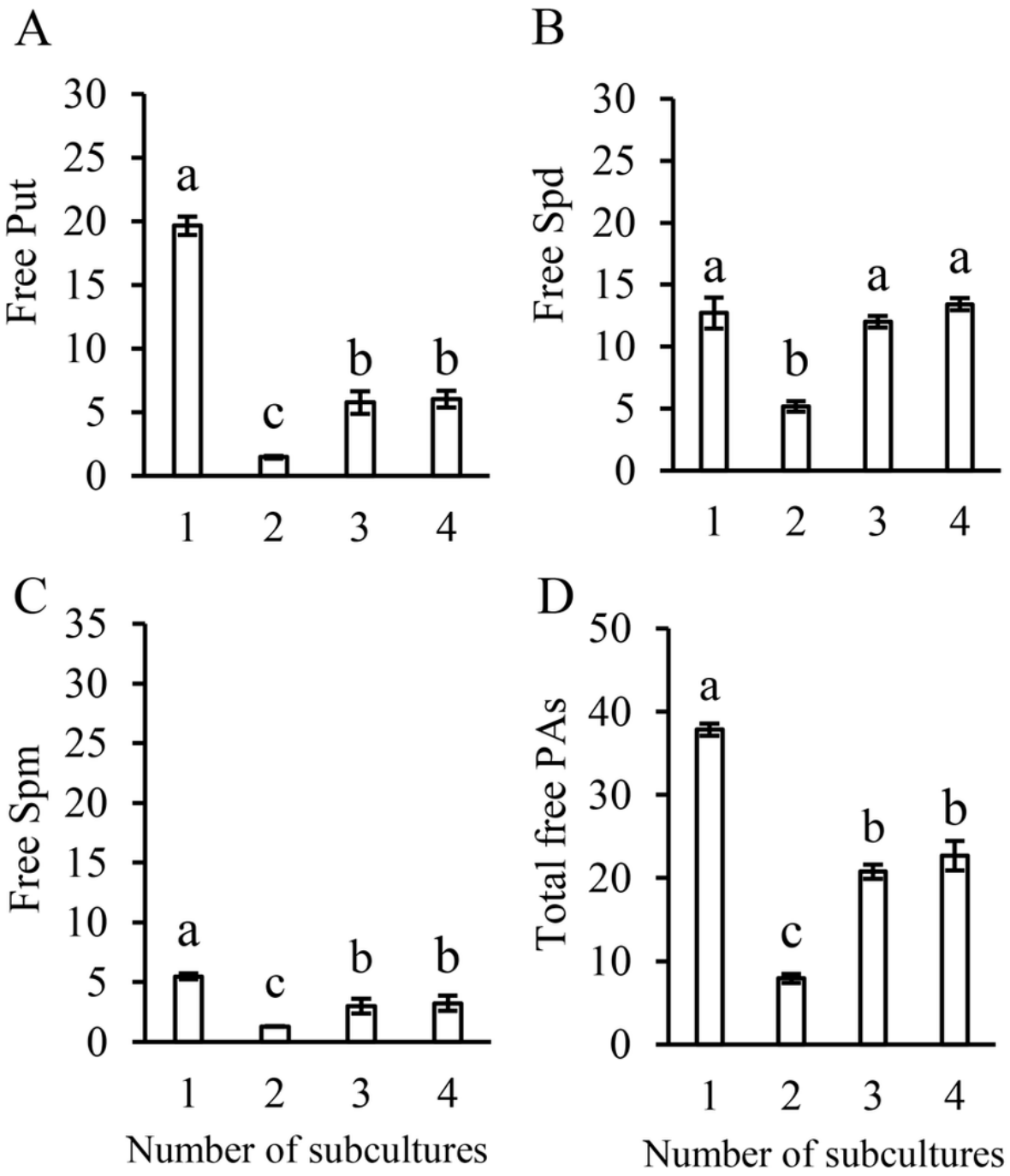

Figure 3

Endogenous contents (μg g-1 FM) of free Put (a), Spd (b), Spm (c) and total free PAs (d) in shoots from cotyledonary nodal segments of Cedrela fissilis during four subcultures. Means followed by different letters show significant differences ($P < 0.05$) according to the SNK test. CV = coefficient of variation. (n = 3; CV for Put = 7.56%; CV for Spd = 5.41%; CV for Spm = 11.45; CV for total free PAs = 4.63%). Error bars indicate standard deviations.

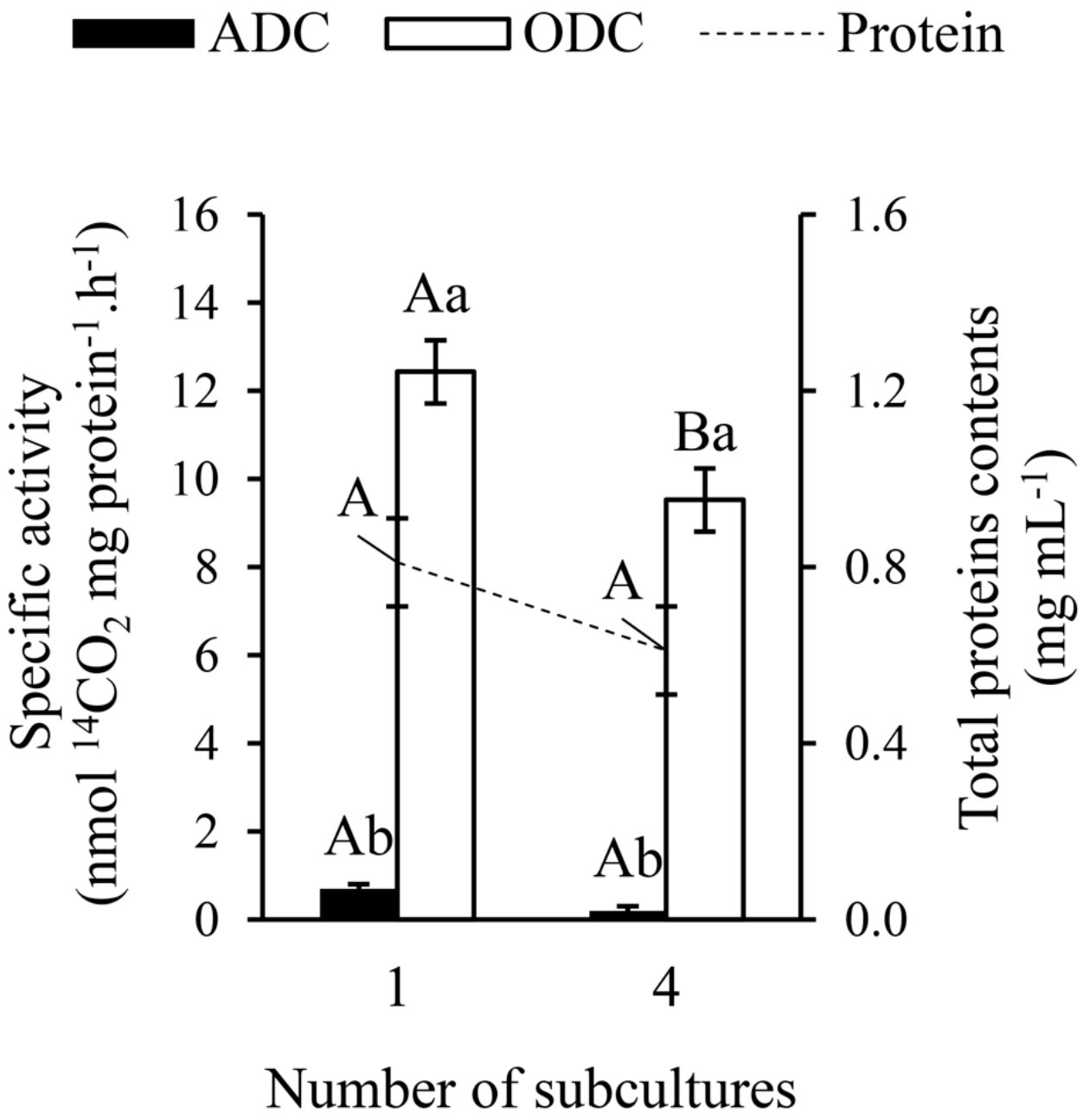

Figure 4

Enzymatic activities of arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) in the shoots of Cedrela fissilis obtained from cotyledonary nodal segments in the first and fourth subcultures. Means followed by different letters show significant differences ($P < 0.05$) according to the SNK test. Capital letters show significant differences between subcultures (first and fourth) for each enzyme (ADC or ODC). Lowercase letters show significant differences comparing the activities of enzymes (ADC and ODC) in

each subculture (first or fourth). CV = coefficient of variation. (n =3; CV = 23.04%). Error bars indicate standard deviations.

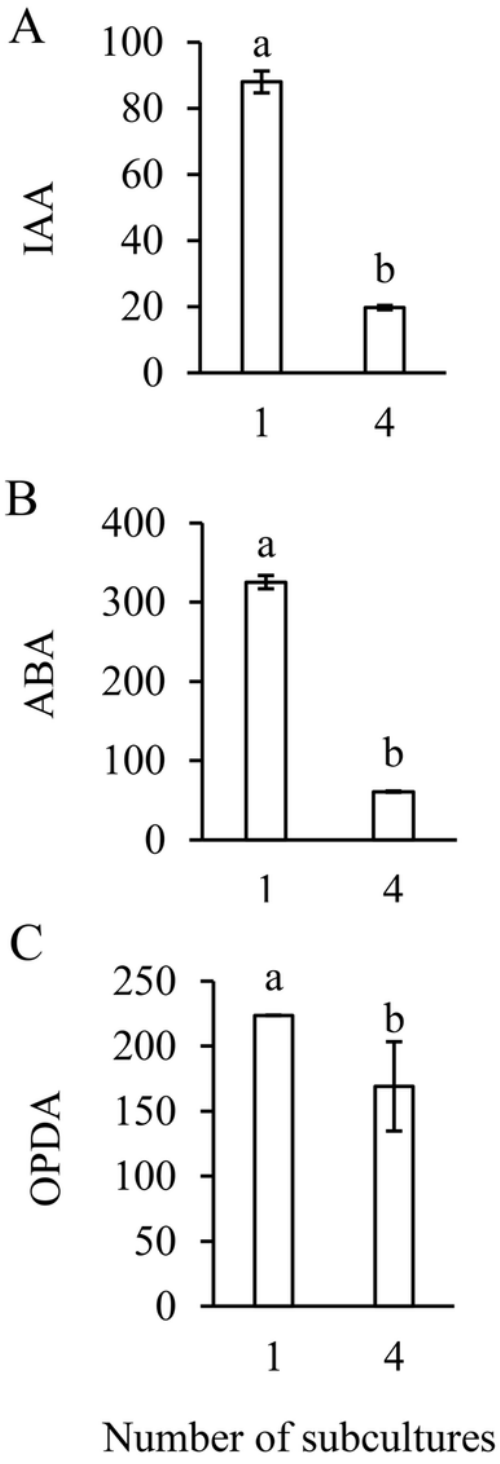

Figure 5

Endogenous contents (ng g-1) of IAA (a), ABA (b) and OPDA (c) in the shoots of Cedrela fissilis obtained from cotyledonary nodal segments at the first and fourth subcultures. Means followed by different letters

show significant differences (P < 0.05) according to the SNK. CV = coefficient of variation. (n =3; CV for IAA = 19.56%; CV for ABA = 4.33%; CV for OPDA = 18.78%). Error bars indicate standard deviations.

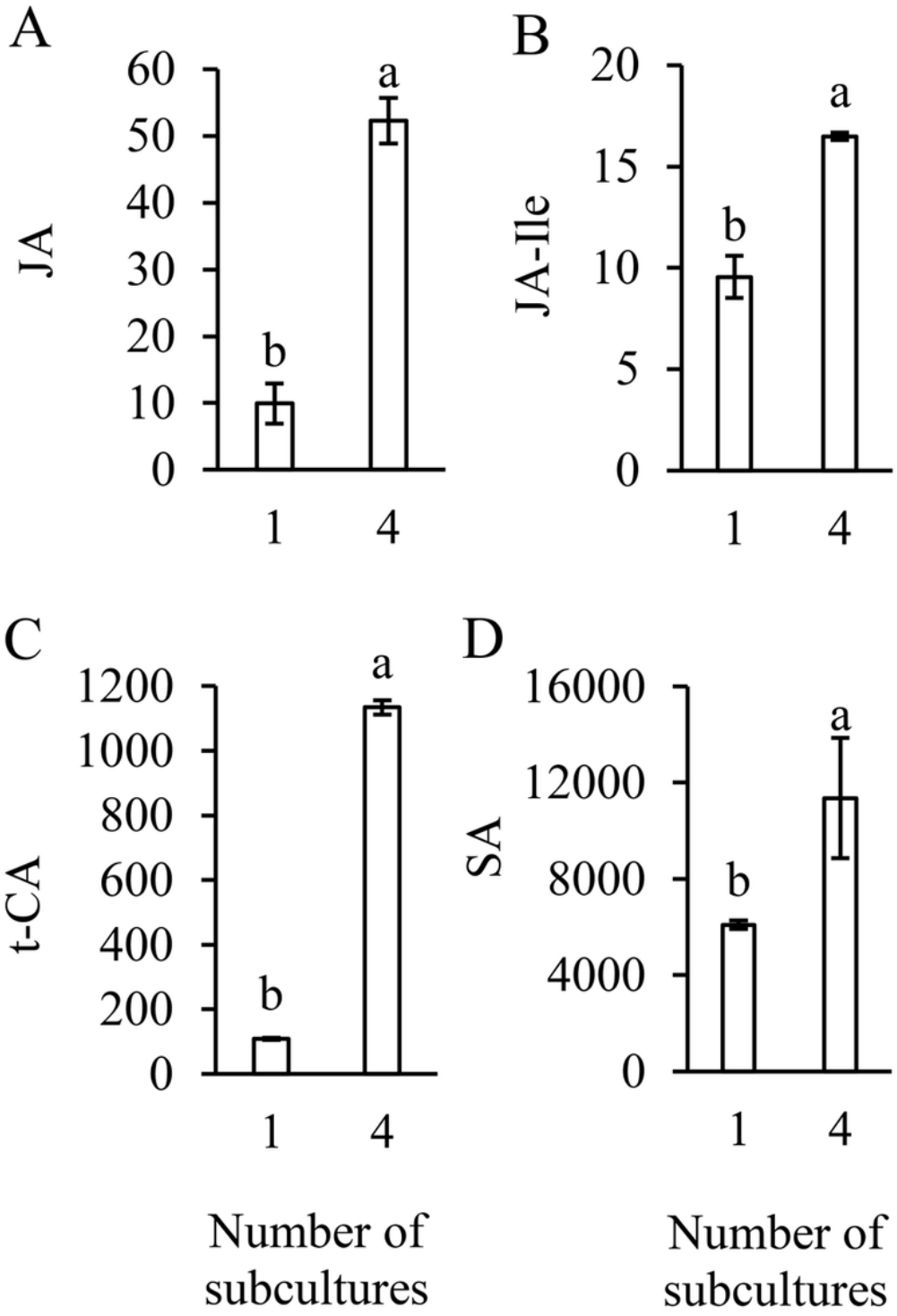

Figure 6

Endogenous contents (ng g-1) of JA (a), JA-Ile (b), t-CA (c) and SA (d) in the shoots of Cedrela fissilis obtained from cotyledonary nodal segments in the first and fourth subcultures. Means followed by different letters show significant differences (P < 0.05) according to the SNK. CV = coefficient of variation.

Error bars indicate standard deviations. (n =3; CV for JA = 17.46%; CV for JA-Ile = 6.69%; CV for t-CA = 5.54%; CV for SA = 13.27%).

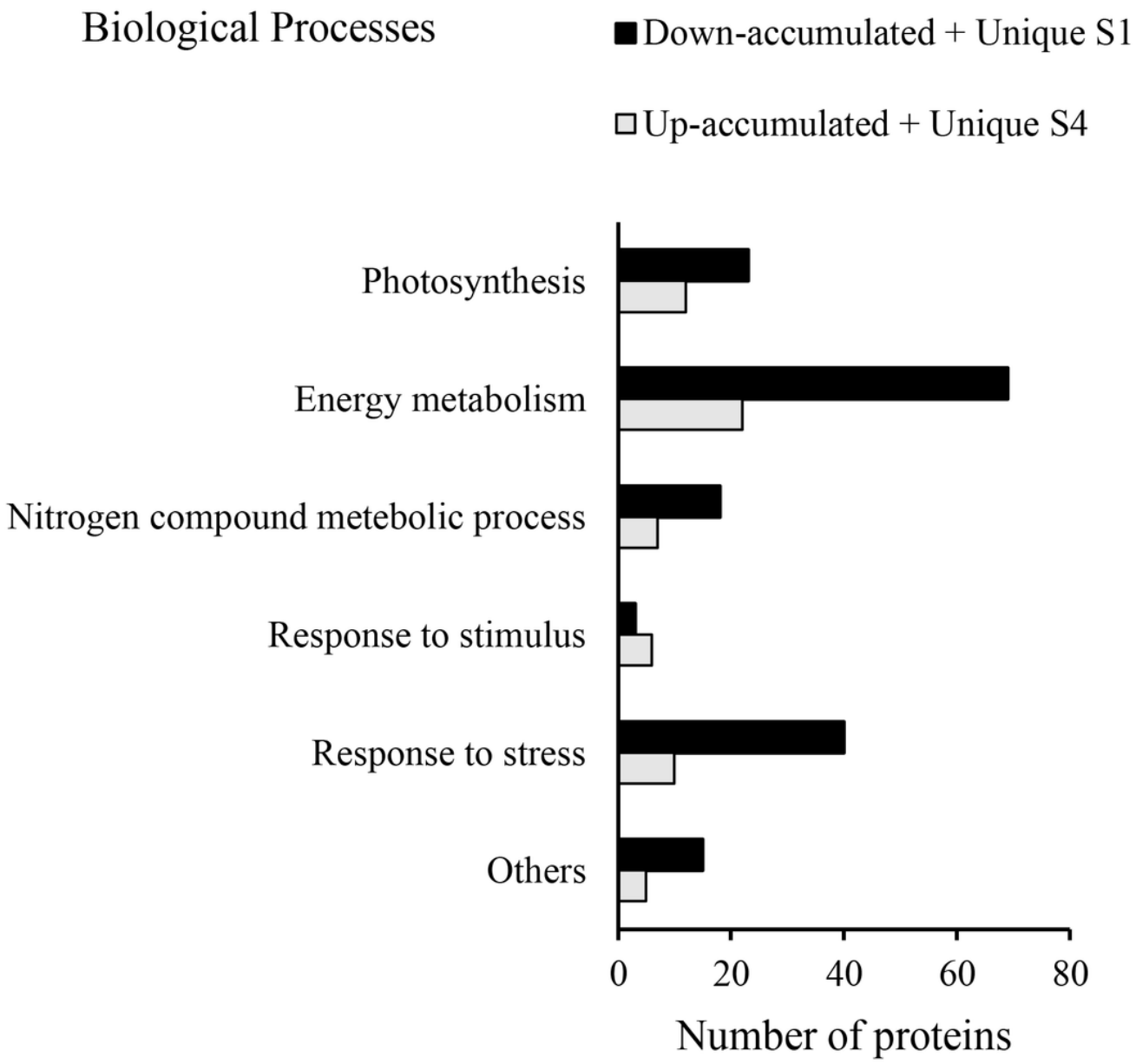

Figure 7

Main biological processes of differentially accumulated proteins identified in the shoots of Cedrela fissilis obtained from cotyledonary nodal segments in the comparison of shoots from the fourth subculture with those from the first subculture (S4/S1).

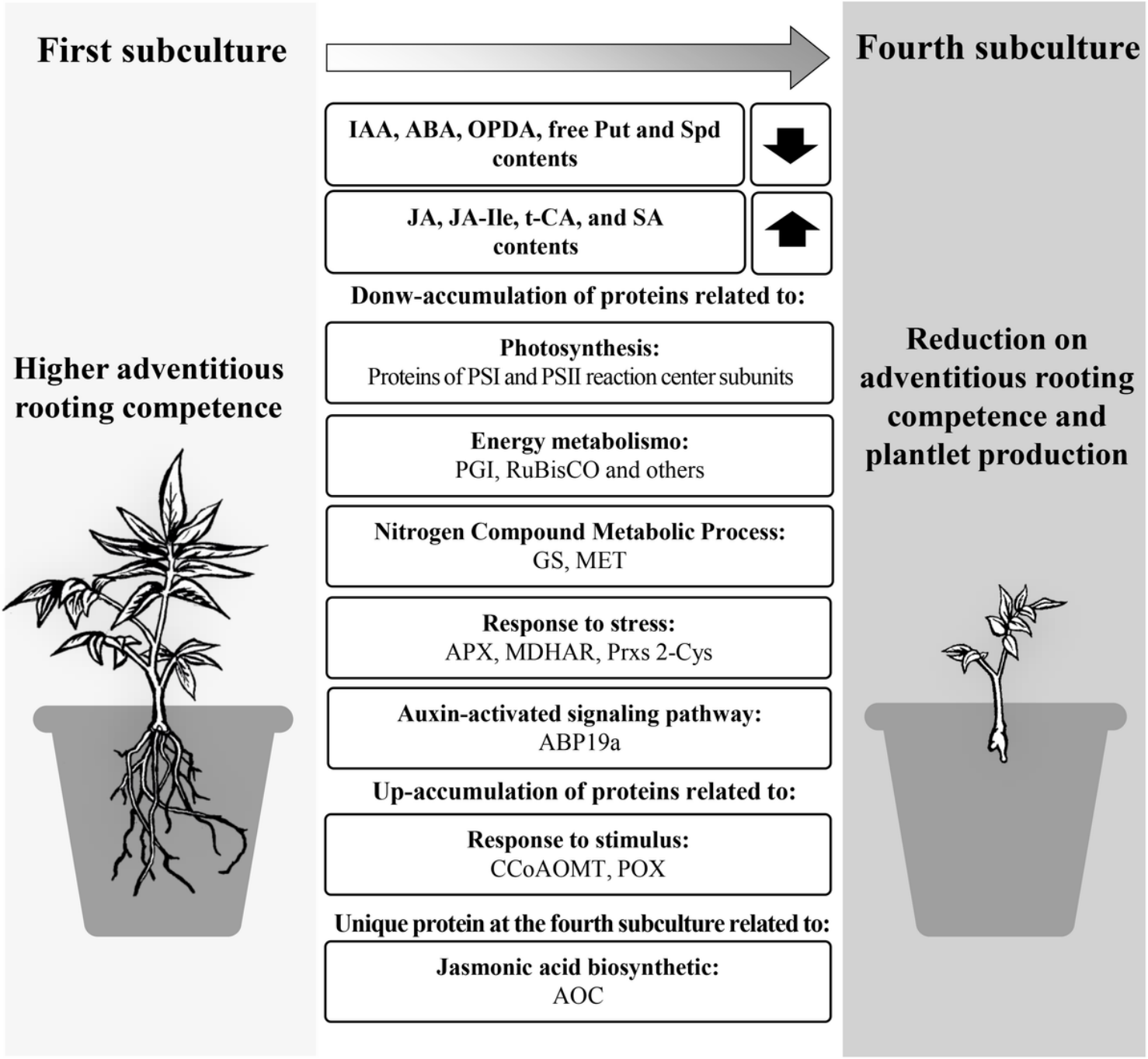

Figure 8

Positive and negative regulators of AR formation based on comparison of differentially accumulated proteins and plant hormones in shoots of Cedrela fissilis from the first and fourth subcultures. Endogenous IAA, ABA, and OPDA contents decreased during subcultures, showing their positive regulation of AR formation in C. fissilis, as higher contents of these hormones were observed in shoots at the first subculture, with higher AR formation, compared to shoots from the fourth subculture. However, JA, JA-Ile, t-CA, and SA can act as negative regulators of AR formation in this species, as an increase in its contents was observed in shoots from the fourth subculture relative to those from the first subculture. The auxin-binding protein ABP19a is an important protein related to IAA modulation and AR formation in C. fissilis, since the down-accumulation of ABP19a accumulation in shoots at the fourth subculture was

related to reduced rooting. This IAA modulation may in turn influence the AOC, which are proteins associated with the auxin signaling pathway and responses to JA, suggesting that these proteins play a role in AR competence in C. fissilis. The donw-accumulation of some proteins (such as PSI and PSII reaction center subunit proteins and PGI, RuBisCo, GS and MET proteins) during subcultures shows that these proteins can act as positive regulators of AR formation in this species, and their reduction during long-term subcultures affects AR formation. In addition, antioxidant enzymes for H2O2 detoxification, such as APX, MDHAR, and Prxs 2-Cys proteins, were down-accumulated in shoots at the fourth subculture compared to those from the first subculture, showing that these proteins can act as positive regulators for adequate AR formation in this species. On the other hand, an increasing number of subcultures resulted in up-accumulation of CCoAOMT and some peroxidase isoform proteins involved in adaptive responses to wounding in shoots from the fourth subculture compared to the first, showing that the greater accumulation of these proteins can act as negative regulators of AR development in this species.

Supplementary Files

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

- Supplementarytable1.xlsx