



Stem-cutting anatomy and biochemical responses associated with competence for adventitious root differentiation in *Acca sellowiana* (Myrtaceae)

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Abstract

Key Message Anatomical evidences suggest that differences in rooting ability among *Acca sellowiana* materials are explained by earlier phase change in difficult to root genotypes.

Abstract Successful development of adventitious roots (AR) in cuttings imposes an important limitation to the propagation of woody plants and in some species, the ability to form AR is strongly affected by genotype. However, we lack an understanding of the differences among genotypes underlying such different responses in various species. We examined the anatomical and biochemical effects of exogenous indol-3-butyric acid and type of cutting in rooting experiments of two *Acca sellowiana* genotypes with contrasting rooting ability. New meristems developed outside the cambial ring, without callus formation by day fourteen and new adventitious roots grew through the cortex emerging by day 28. Both anatomically in vivo and biochemically in vitro, cuttings from the different genotypes behaved differently. We found anatomical differences between the genotypes that might explain the differences in rooting ability. An earlier development of a periderm was present in the difficult-to-root genotype. This secondary dermal tissue could be used as a reliable phase-change marker to distinguish juvenile from mature plant parts which have lost rooting capacity.

Keywords Indol-3-butyric acid (IBA) · Phase change · Rooting · Stem-cutting anatomy · Vegetative propagation

Introduction

Propagation by stem cuttings, which relies on adventitious roots (AR) formed in response to wounding is the most widely used method of vegetative propagation around the world and plays a central role in asexual propagation of forest and fruit crops (Steffens and Rasmussen 2016). The success of breeding programmes of woody plants depends on the availability of a cost effective method for vegetative propagation. However, the ability to form AR is variable

among genotypes and in some cases it remains the most important limitation for the commercial propagation of elite genotypes. For this reason, a better understanding of the AR differentiation process is needed to explain the great variability found amongst genotypes and to design a strategy to overcome it. This is the case of *Acca sellowiana*, a fruit species native to Uruguay and Brazil with outstanding organoleptic traits (Fischer 2003; Thorp and Bielecki 2005) but little background information available.

In wild plants of *A. sellowiana*, the ability to induce adventitious roots is strongly affected by genotype (Fischer 2003; Cabrera et al. 2010; Ross et al. 2017). Adventitious roots emerge from groups of cells named root initials (Evert 2006). These cells can be found in different tissues of the stem depending on the species (Geiss et al. 2009) including phloem, parenchyma, or other cell types (Tarrago et al. 2005; Riov et al. 2013). The process is triggered by numerous factors both endogenous and exogenous, and different genotypes vary in their requisites and needs along the process of re-establishing a root system (Di Battista et al. 2019).

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Three successive phases are usually recognized in the physiological pathway leading to AR, each with specific requirements: induction, initiation and expression (Pacurar et al. 2014). The induction phase comprises a period of time with no visible histological events during which biochemical changes occur prior to the first cell divisions. During this phase, several events take place at the base of the cutting including a local increase in auxin levels, the establishment of a sink for carbohydrates and transient changes in the activity of several enzymes (de Klerk et al. 1999; Arena et al. 2003; Porfirio et al. 2016a). Consequently, although no visible histological events can be observed, biochemical markers can be used to characterize this period in a particular species or genotype and establish its onset and duration.

In difficult to root genotypes, AR initiation may be triggered by the application of exogenous auxins. Biochemical studies indicate that exogenous IBA induces changes in the metabolism of enzymes, carbohydrates and proteins (Elmongy et al. 2018; de Almeida et al. 2020). Increased levels of total soluble carbohydrates in the rooting zone of stem cuttings after exogenous application of auxin have been reported (Agulló-Antón et al. 2014; Elmongy et al. 2018; Goel et al. 2018). Particularly in *A. sellowiana*, IBA has been found to promote in vitro rooting (Guerra et al. 2012; Ross et al. 2017). During the initiation phase, cell divisions leading to the formation of a new root meristem can be observed. A higher number of proteins has been identified in *Eucalyptus* during this phase that reflect these important cellular changes (de Almeida et al. 2020). Protein content increases significantly along the rooting process in response to exogenous IBA, and the maximum levels have generally been related to increased synthesis of enzymatic proteins during the initiation of the root regeneration process (Husen and Pal 2007; Elmongy et al. 2018). The expression phase involves the growth of AR through the cortex and out of the epidermis, and the establishment of vascular connections of the new root with the stem cutting (da Costa et al. 2013). According to Zhang (2009), poor rooting ability of *A. sellowiana* cuttings is associated with the presence of phloem fibres, which prevent growth of the newly formed meristem. Poor rooting has been related to the presence of sclerenchyma or periphloematic fibres in a variety of species including *Quercus macrocarpa* (Amisshah et al. 2008), *Juglans nigra* (Stevens and Pijut 2017) and several species of *Eucalyptus* (Goulart et al. 2014; Bryant and Trueman 2015). On the other hand, the development of tissues that may potentially represent physical barriers, may be associated to phase change, and therefore loss of cell competence to form a new AR meristem altogether. In many plant species, both Angiosperms and Gymnosperms the ability to form AR is strongly affected by juvenile to mature phase change; stem anatomy differs markedly in woody plants at different maturational stages (Husen and Pal 2006; Wendling

et al. 2014). Phase changes may be observed anatomically by the presence of tissues such as phellem to the inner side of the phloem fibres (Beakbane 1961).

To understand the causes of poor rooting in wild *A. sellowiana* genotypes, we studied temporal changes in carbohydrate and protein levels in response to IBA during AR formation in cuttings and microcuttings of two genotypes with contrasting rooting ability. We examined the stem anatomy at the base of the cuttings in both easy-to-root (R) and difficult-to-root (NR) genotypes using microscopic methods, throughout the AR process and in cuttings with different numbers of nodes to relate rooting ability with maturational stage.

Materials and methods

Plant material

Trials were performed using selected wild genotypes of *A. sellowiana* with contrasting rooting ability, previously identified as R (easy-to-root) and NR (difficult-to-root) (Ross et al. 2017). Four-year-old mother plants were cultured in the greenhouse, under natural light conditions, without heating. Average temperature and humidity during the growing season were 28 °C and 60%, respectively, obtained from data registered with RHT10 datalogger (EXTECH Instruments). Fungicide (Benlate®, 0.2%) and Phostrogen® [NPK(MgO3-SO3): 14-10-27 (2.5–7.5)] were applied periodically to promote pathogen-free vigorous sprout growth. Because all observations were destructive, separate experiments were established to analyze the response to IBA, anatomical evolution in multinodal cuttings and biochemical characterizations.

In vitro culture

An experiment was established to evaluate biochemical differences between R and NR genotypes and their response to exogenous IBA. To facilitate the analysis and obtain the amount of plant material required, this experiment was carried out in vitro. Apical microshoots without leaves were established in vitro on semi-solid Woody Plant basal medium (WPM) (Lloyd and McCown 1980), supplemented with MS vitamins (Murashige and Skoog 1962), 0.44 µM BAP (6-benzylaminopurine; Sigma B3408, Sigma-Aldrich, St. Louis MO, USA) and 0.054 µM NAA (1-naphtalenacetic acid; Sigma N0640, Sigma-Aldrich, St. Louis MO, USA), as previously reported (Ross and Grasso 2010). Multiplication medium was supplemented with 9.8 µM 2iP (6-γ,γ-Dimethylallylamino purine; Sigma D7674, Sigma-Aldrich, St. Louis MO, USA). Two rooting conditions

were compared: IBA 9.8 μM (Sigma I5386, Sigma-Aldrich, St. Louis MO, USA) added to the rooting induction media and media without IBA; explants from both conditions were subcultured on auxin-free media after 7 days. Conditions in the growth chamber were 25 ± 2 °C, 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and 16-h photoperiod. The in vitro rooting experiment was a 2×2 factorial design with five replicates, where the factors were two genotypes (R and NR) and two IBA levels (0 and 9.8 μM).

Biochemical tests

Soluble carbohydrates were determined colorimetrically using the anthrone method (Yemm and Willis 1954). Fresh samples (0.10 g) were collected weekly from the basal portion of microcuttings after transfer to auxin-free medium, ground with mortar and pestle and extracted with 1 mL ethanol (80% v/v). After centrifugation for 10 min at 5000g, 500 μL of the ethanolic supernatant was diluted in 2.5 mL anthrone (Sigma319899, Sigma-Aldrich, St. Louis MO, USA) and incubated at 100 °C for 10 min. After cooling, 50 μL of sample was diluted in 950 μL anthrone and absorbance was read at 625 nm in a Shimadzu spectrophotometer using as blank ethanol diluted in anthrone at the same concentration. The calibration curve was obtained from dilutions prepared from a glucose solution (0.24 mM). Analyses were carried out in triplicate, with five cuttings per biological replicate, and values expressed as mean \pm standard error.

Protein determination was carried out in samples collected weekly after transfer to auxin-free medium from the basal portion (1 cm) of microshoots, and ground with mortar and pestle in liquid nitrogen. Extraction was done with buffer containing 50 mM sodium acetate (Sigma302406, Sigma-Aldrich), 2.0 mM ethylenediamine-tetra-acetic acid (EDTA) (SigmaEDS, Sigma-Aldrich), 1.0 mM magnesium chloride (SigmaM8266, Sigma-Aldrich) and 1.0 mM phenylmethylsulfonyl fluoride (PMSF) (SigmaP7626, Sigma-Aldrich) at pH 5.5. After centrifugation (20 min at 10,000g and 4 °C), the supernatant was used as crude extract for quantification of total protein. Analyses were carried out in triplicate, with five cuttings per biological replicate, and values expressed as mean \pm standard error.

Protein concentration was determined according to the method by Bradford (1976), using bovine serum albumin (BSA) (SigmaA2153, Sigma-Aldrich) as a standard. Homogenate (20 μL) was thoroughly mixed with 1 mL Coomassie Brilliant Blue stain reactant and absorbance was read at 595 nm using a Shimadzu spectrophotometer. To obtain the standard curve, absorbance at 595 nm was plotted versus known protein concentration of BSA solution.

Anatomical analysis and response to exogenous IBA

Two different experiments were carried out using semi-hardwood cuttings of the two genotypes under evaluation R and NR. In experiment 1, we emphasized the evaluation of rooting response and the basic anatomical differences between the R and NR genotypes and in response to exogenous IBA application. In experiment 2, we emphasized the evaluation of the anatomical differences between the genotypes along more mature nodes and its response to exogenous IBA. Cuttings for both experiments were collected from the basal branches of greenhouse-grown mother plants once the current season's growth had stopped (February–March, southern hemisphere) following Thorp and Bielecki (2005). Average temperature in the greenhouse at the time of harvest was 28.05 °C and relative humidity 58.97%. To reduce water loss through transpiration, all but the top two leaves half-trimmed were removed (Fig. 1). Cuttings were placed on a propagation bed with bottom heating and overhead mist (Aphos SRL manufacturers), using perlite as rooting substrate, under natural light conditions. Substrate temperature was maintained at 27 ± 1 °C and air relative humidity at $90 \pm 5\%$ using an intermittent mist system throughout the rooting experiment.

In experiment 1, only apical uninodal cuttings were used (Fig. 1a) in a 2×2 factorial design with three replicates, where the factors were two genotypes (R and NR) and two IBA levels (0 and 12.3 mM). IBA was applied by dipping the base of the each cutting for 5 s in 20 ml of a non-sterile solution of 12.3 mM IBA (Sigma I5386, Sigma-Aldrich) to induce rooting. Ten cuttings were used for each biological replicate. Rooting (%), number of roots per explant and length (cm) of roots were recorded weekly for 5 weeks. Samples for stem anatomy observations were

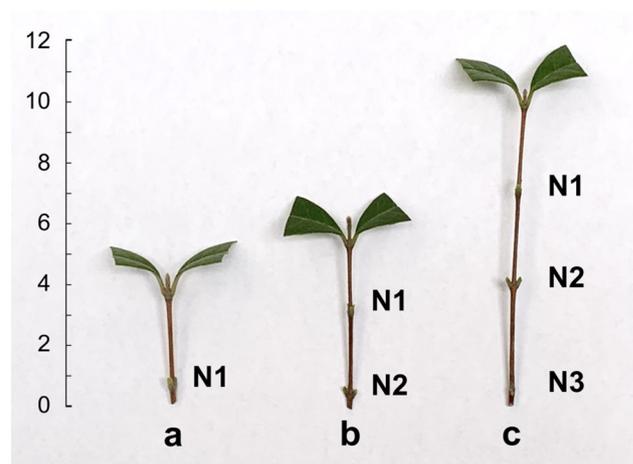


Fig. 1 Type of apical cuttings of *Acca sellowiana* used for in vivo rooting experiments. **a** Uninodal cutting; **b** two-nodes cutting; **c** three-nodes cutting. Nodes are indicated as N1, N2 and N3. Scale bar in centimetres

collected weekly for 4 weeks, trimming the basal portion (0.5 cm length) of three cuttings per factor combination (genotype \times IBA level). Samples were fixed in a formalin-acetic acid–ethanol 70% (FAA, 5:5:90) solution, dehydrated through a series of graded ethanol baths and then infiltrated and embedded in paraffin blocks (D'Ambrogio de Argüeso 1986). Serial sections 10–12 μm thick were cut using a rotary microtome (Slee Medical, Cut 4062), dried, stained with safranin–fast green (Johansen 1940) and finally mounted on microscope slides with Canada balsam mounting medium (Sigma C1795, Sigma-Aldrich). To establish the boundary between the cortex and the stele, we used stem hand sections (12 μm -thick) and employed two different approaches: localization of the starch sheath using Lugol reagent (D'Ambrogio de Argüeso 1986) and detection of callose to identify the phloem, mounting the hand sections in a drop of high-pH solution of aniline blue (Sigma95290, Sigma-Aldrich) (Zarlavsky 2014).

In experiment 2, samples were harvested from cuttings with different sizes: the apical meristem plus one, two or three nodes (Fig. 1a–c) arranged in a $2 \times 2 \times 3$ factorial design with three replicates, where the factors were two genotypes (R and NR), two IBA levels (0 and 12.3 mM) and three sizes of cuttings named N1, N2 and N3 (one, two or three nodes below the apex, respectively). Three cuttings were used for each biological replicate. IBA was applied as in experiment 1.

Samples were collected weekly for stem anatomy observations at each node level and processed in the same way as described above. An exploratory analysis was done in branches of both genotypes, R and NR up to node six. For detection of lignified tissues, histochemical staining of lignin with phloroglucinol/HCl (SigmaP3502, Sigma-Aldrich) was performed on hand sections, 12 μm -thick (D'Ambrogio de Argüeso 1986). Number, width and continuity of periphloematic lignified rings were recorded in photographs of transversal section (Electronic supplementary material 1). The gap distance between fibre strands was used to calculate the percentage gap, later correlated with the percentage of rooting (Amisshah et al. 2008), according to the equation:

$$\% \text{Gap} = \frac{\text{Length of fibre free gaps}}{\text{Length of the circumferential arc}} \times 100$$

A Nikon E100 light microscope was used for observation of the samples. Selected cross-sections were photographed with Dino Eye 2.0 digital camera and Dino Capture 1.5.27.A (Electronic Corp.) software. Samples mounted in aniline blue were observed in an epifluorescence microscope (Labotec, exciting 330–380, dichroism 400 and cut-off 420) and photographs were taken using ScopeImage 9.0 software.

Statistical analysis

Data were analysed statistically by analysis of variance (ANOVA) and means were compared by Tukey's test, with a confidence level of $p \leq 0.05$, using Infostat® statistical software. Arcsine transformation was applied to response data before analysis.

Results

Response to IBA

The results of ANOVA from experiment 1 showed a significant effect of exogenous IBA, genotype and interaction genotype \times treatment ($p < 0.0001$) (Electronic supplementary material 1). Exogenous IBA (12.3 mM) improved rooting of uninodal cuttings from experiment 1 in both genotypes (R and NR) but the effect was stronger in the NR genotype (Tukey, $p < 0.05$) (Fig. 2). Rooting of R genotype increased from 58.8% (without IBA) to 68.3% (with IBA). When IBA was added to the NR genotype, rooting increased from 1.67 to 56.2%. This value is not different from that of the R genotype without IBA (Tukey, $p < 0.05$) (Fig. 2a). The NR genotype had fewer roots per rooted explant (Fig. 2b), but neither root number nor root length was significantly affected by IBA within each genotype (Fig. 2b, c). Root emergence was not observed earlier than 28 days regardless of genotype, and neither of them formed callus at the base of the cuttings.

Biochemical markers of AR

For biochemical analysis, materials were multiplied in vitro. Rooting of R and NR genotypes in response to IBA in vitro was not different from uninodal cuttings (Tukey, $p < 0.05$). Rooting of NR genotype increased from less than 5–63% when IBA was added to the rooting medium while R genotype rooting was 71% and 63%, with and without IBA, respectively (Table 1).

We found a significant effect of exogenous IBA on both carbohydrate and protein levels ($p < 0.0001$) but there was no significant effect of genotype and the interaction genotype \times treatment was significant only for protein levels (Electronic supplementary material 2). Carbohydrate (CHO) levels increased from day 0 to day 7 in both genotypes and treatments, and decreased gradually from day 7 onwards. Auxin treatment significantly increased carbohydrate levels in both genotypes but no significant differences were found between R and NR genotypes (Tukey, $p < 0.05$) (Fig. 3a). The maximum level of protein was detected at day 14 in both genotypes and treatments. From day 14 onwards protein content at the base of the cuttings decreased gradually. The NR genotype without IBA had the maximum content of

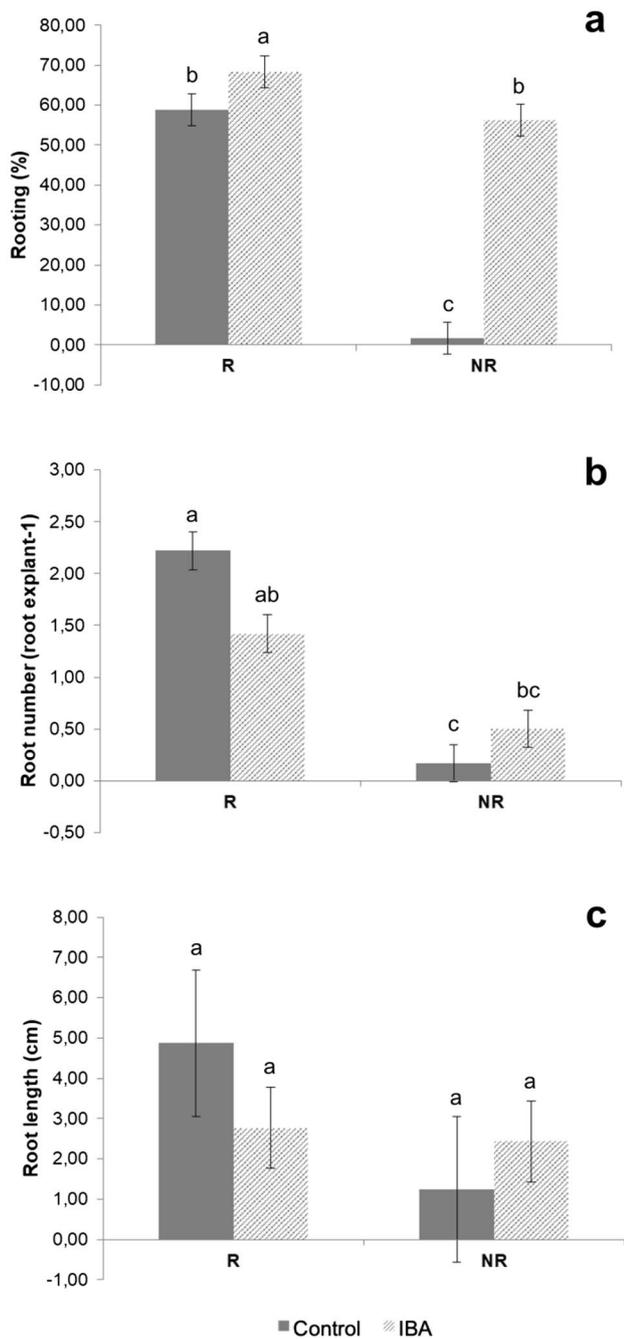


Fig. 2 Effect of IBA (12.3 mM) on: **a** rooting percentage; **b** root number per rooted explant; **c** root length; of two genotypes of *Acca sellowiana* uninodal cuttings with contrasting rooting ability, experiment 1. R (easy-to-root); NR (difficult-to-root). Data are presented as mean ± SE. Different letters indicate significant differences between genotype/treatment combinations (Tukey, $p < 0.05$)

protein and was the only condition (combination genotype × treatment) that differed significantly (Tukey, $p < 0.05$). The addition of exogenous IBA induced a significant decrease in protein content, which reached levels similar to the R genotype. On the other hand, protein content in the R genotype

Table 1 In vitro rooting of two genotypes of *Acca sellowiana* with contrasting rooting ability in response to 9.8 μM IBA

Genotype	IBA (μM)	Rooting (%)
R	0	63.05 ± 3.10
	9.8	71.00 ± 3.50
NR	0	3.65 ± 2.85
	9.8	62.66 ± 3.86

Data are presented as mean ± sd

R easy-to-root, NR difficult-to-root

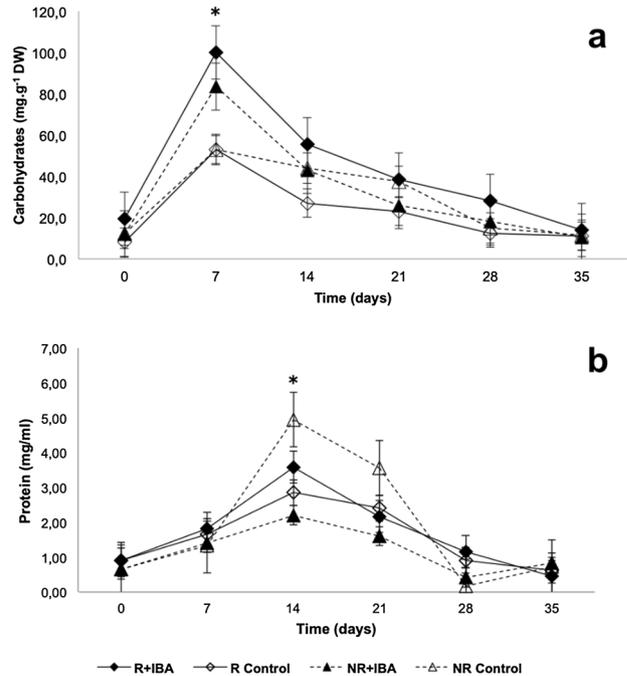


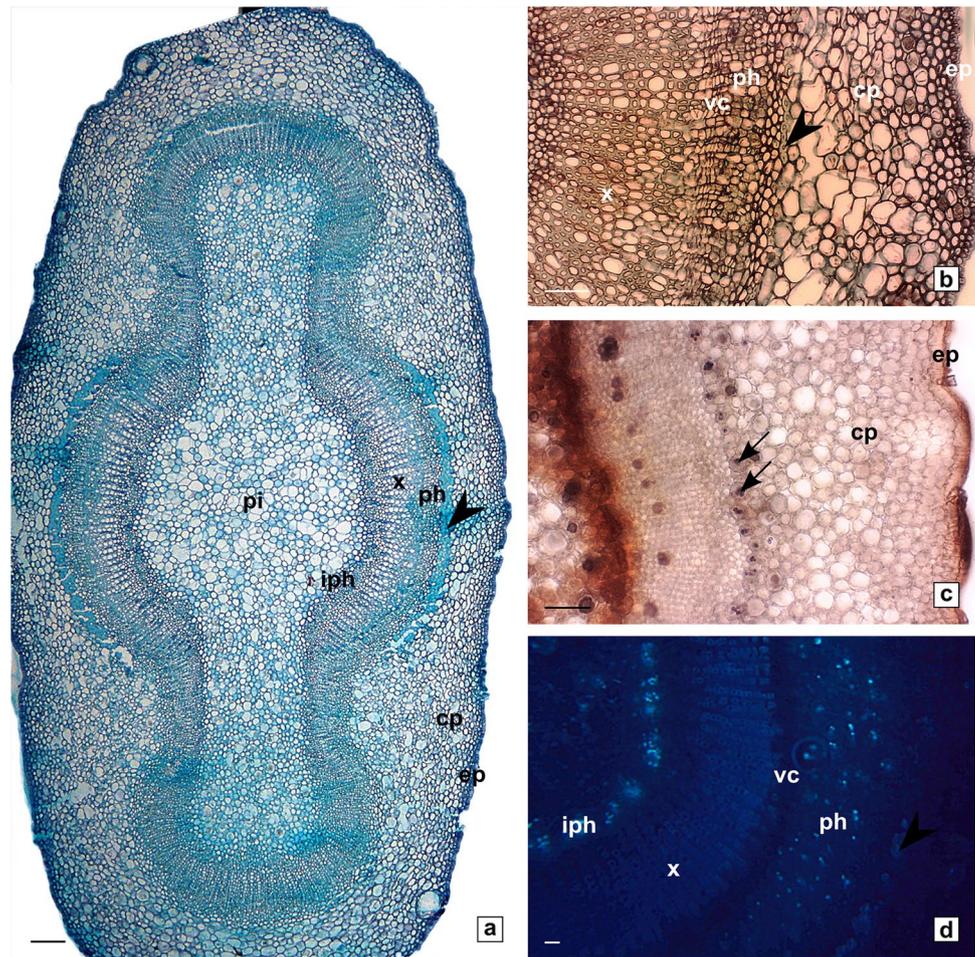
Fig. 3 **a** Soluble carbohydrate content; **b** protein content; throughout adventitious root development in uninodal cuttings of *Acca sellowiana* genotypes (R: easy-to-root and NR: difficult-to-root) with or without IBA (9.8 mM). Asterisks indicate where significant differences between genotypes and treatments were found (Tukey, $p < 0.05$). Data are presented as mean ± SE

was not affected by the presence of exogenous IBA in relation to the control (Fig. 3b).

Anatomical studies of stem cuttings

In the uninodal cuttings, a uniseriate epidermis covers the stem surface of both genotypes and a cortical parenchyma is found below it (Fig. 4a, b). An internal cortical starch sheath, detected by the presence of amyloplasts inside the cells with Lugol staining, surrounds the stele ring (Fig. 4c). Vascular secondary growth is already present at this level of the cuttings. Xylem is continuous with intraxylary phloem and surrounds the central parenchymous pith (Fig. 4a).

Fig. 4 Micrographs showing stem anatomy at the base of *Acca sellowiana* uninodal stem cuttings (cross sections) from experiment 1 of the R (easy-to-root) genotype on day 0. **a** Panoramic view with safranin-fast green staining; **b** detail of tissue location showing phloem fibres with safranin-fast green staining (black arrowhead); **c** detail showing starch-sheath (black arrows) with lugol staining; **d** micrograph showing epifluorescence of callose staining with aniline blue, black arrowhead points at phloem fibres. *cp* cortical parenchyma, *ep* epidermis, *iph* internal phloem, *ph* phloem, *pi* pith, *vc* vascular cambium, *x* xylem. Scale bars in panel **a** 100 μ m; in panels **b–d** 50 μ m



The internal and external phloem was evidenced by callose detection using aniline blue fluorescence of phloem cellular elements (Fig. 4d). An external discontinuous ring of phloem fibres is present in both materials adjacent to the cortical starch sheath (Fig. 4a, b). No evidence of preformed root primordium was detected and uninodal cuttings of both genotypes had a similar anatomical structure at their bases.

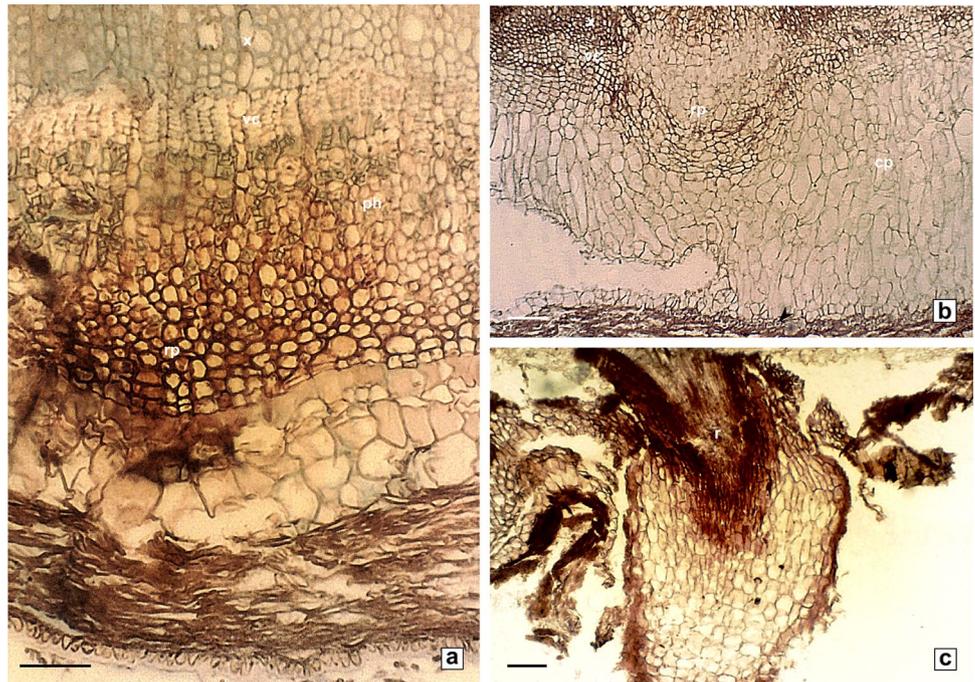
For the first 2 weeks of rooting experiment 1, we found no evidence of anatomical modifications. Although safranin-fast green staining did not show a clear contrast, mitotic figures were first observed in groups of cells within the outer secondary phloem by day 14 (Fig. 5a). Root primordia grew through the cortex (Fig. 5b) and emerged through the epidermis 28 days after cutting establishment (Fig. 5c). AR formation was direct, without callus formation in both genotypes.

Anatomical differences between genotypes were observed in multinodal cuttings below the second node. All cross-sections showed the same basic anatomical structure and rooting behaviour described above, and both genotypes had a discontinuous ring of phloem fibres (Fig. 6a, b). AR initials were also observed as in the previous experiment only in nodes 1 and 2. In the NR genotype, we observed the

differentiation of a multistratified tissue with initially thin-walled cells arising by the second node below the apical meristem of the cutting (Fig. 6b, d, white arrowheads). This tissue differentiated from a cell layer located immediately below the phloem fibres. The cells of this layer presented slightly thicker walls than the other phloem cells and were bigger in the NR genotype than in the R genotype (Fig. 6d). The multistratified tissue formed varied from one to six continuous layers of tangentially flattened cells without intercellular spaces throughout the cross-section. The number of layers increased towards the third node (Fig. 6d, f) and the outermost layers showed thickened cell walls. To explore the progress of this ring towards more basal nodes, we analysed cross-sections at nodes 4–6 (Fig. 6g, h), and found evidence that this multiseriate tissue forms a periderm (internal ring of lignified tissue) which was always present in the NR genotype but seldom appeared in the R genotype.

To compare the position and development of these tissues between R and NR genotypes, we used stem cross sections stained with phloroglucinol/HCl for a better visualization of lignified tissues (Electronic supplementary material 3). Statistical analysis of the data obtained after image processing

Fig. 5 Light micrographs showing stem anatomy at the base of *Acca sellowiana* uninodal stem cuttings (cross sections) of the R genotype (easy-to-root), at different stages during adventitious root differentiation from experiment 1. **a** Day 14, neof ormation of a root meristem outside the cambial ring; **b** day 21, root primordium elongating through the cortex (black arrowheads point at the phloem fibres); **c** day 28, AR emerging. *cp* cortical parenchyma, *ph* phloem, *r* root, *rp* root primordium, *vc* vascular cambium, *x* xylem. Scale bars in panel **a** 50 μ m; in panels **b** and **c** 100 μ m



showed that the principal effect of genotype was significant on the number of rings of lignified tissues and width of the internal ring ($p < 0.0001$); exogenous IBA affected the width of the internal ring and the proportion of fibre-free gaps of the external ring; width of internal ring was also affected by node. The interaction genotype \times node was significant for the three variables. (Electronic supplementary material 4). Depending on the node, the different genotypes (R and NR) had significantly different number of rings with lignified cells (Fig. 7a) and width of the internal ring (Fig. 7b), but no differences were detected on the proportion of fibre-free gaps of the external ring (Fig. 7c). Addition of exogenous IBA affected the internal ring width (Fig. 7b) and the proportion of fibre-free gaps of the external ring in the first two nodes of the R genotype (Fig. 7c) (Tukey, $p < 0.05$). These differences were first detected 2 weeks after IBA treatment.

Discussion

Our results show that the R and NR genotypes of *A. sellowiana* show anatomical differences *in vivo* and different biochemical responses to IBA *in vitro*, possibly related to their different rooting ability. In spite of these differences, exogenous IBA was effective to improve the performance of difficult-to-root genotypes which reached levels similar to those of the R genotype without exogenous IBA. Although separate experiments were established to record different characteristics, the differential response to exogenous IBA of R and NR genotypes was maintained both *in vitro* and

in vivo. We had previously obtained a very similar rooting response to IBA for the same R and NR genotypes in a similar *in vitro* experiment (Ross et al. 2017). The duration of the whole differentiation process did not differ between R and NR genotypes, and no roots were visible in either genotype earlier than 28 days *in vivo* similar to our previous results *in vitro*. Zhang et al. (2009) observed new meristem formation by day 20 and reported that it took 30 days for *A. sellowiana* roots to emerge from cuttings. Similar durations of the whole process of AR formation has been reported in several species of *Eucalyptus* (Baltierra et al. 2004; Bryant and Trueman 2015). In *E. grandis*, Abu-Abied (2012) achieved maximum rooting 35 days after IBA treatment.

In response to wounding, a new sink for CHO is established, providing the structural elements as well as the energy resources needed for development (Kozłowski 1992; Druege et al. 2016). However, the demands for energy and carbon skeletons to support rooting vary between species and depend on the type of cutting (Haissig 1986) and it has been suggested that rooting can be related to individual CHO pools rather than total content in cuttings (Druege 2009). In our experiments, the content of CHO of both genotypes *in vitro* evolved and responded similarly to exogenous IBA and reached its maximum levels 7 days after cutting establishment. This increase in CHO levels in *A. sellowiana* cuttings was the first evidence of metabolic changes following wounding that we detected. Exogenous auxin treatments have been found to increase total soluble sugar levels in several species (Steffens and Rasmussen 2016) which in turn modulate gene expression (Rolland et al. 2002; Gibson

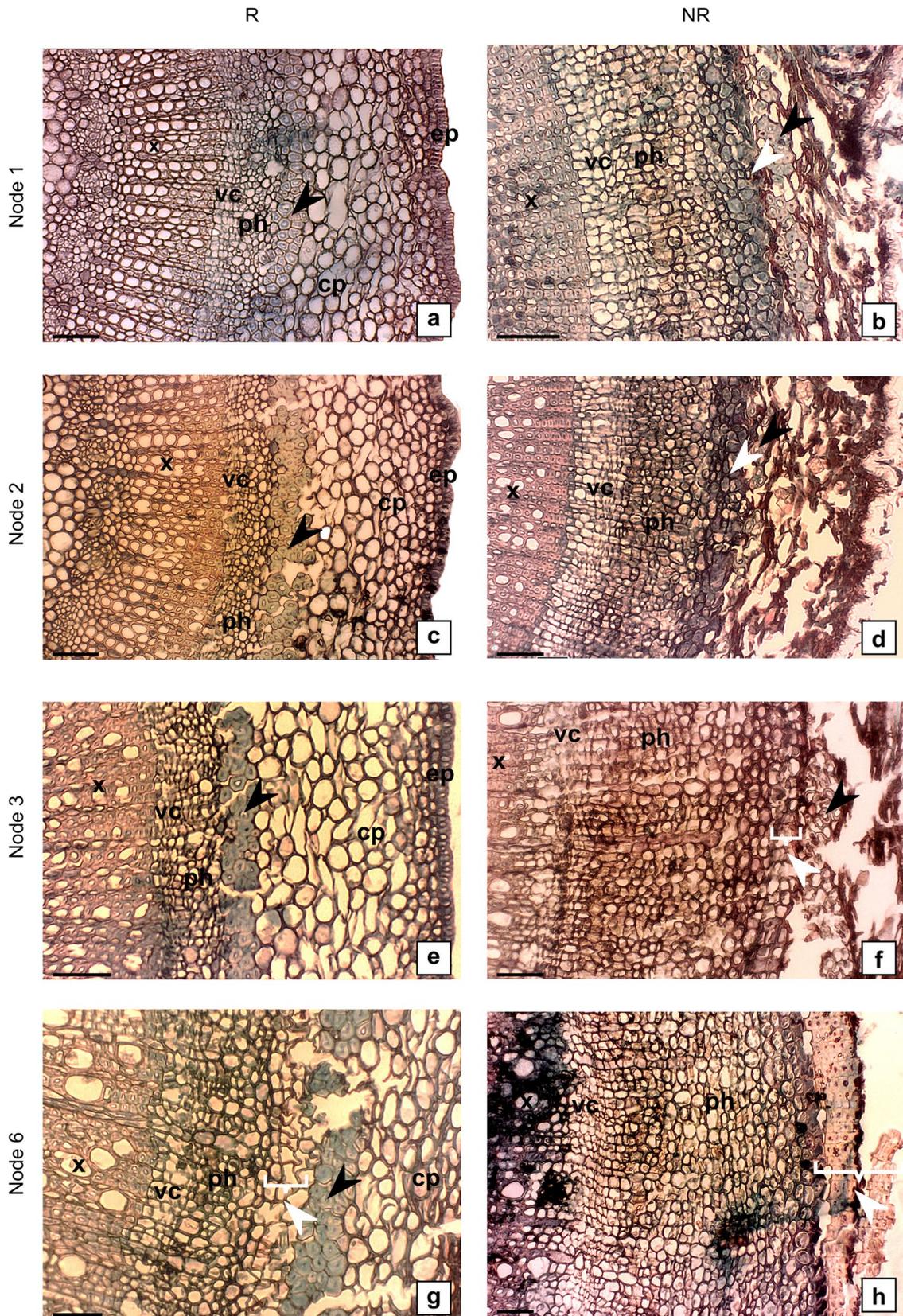


Fig. 6 Representative light micrographs of stem-cutting cross-sections on day 0 and at different nodes below the apical bud, of two genotypes of *Acca sellowiana* with contrasting rooting ability from experiment 2: R (easy-to-root) and NR (difficult-to-root). **a** R genotype, Node 1; **b** NR genotype, Node 1; **c** R genotype, Node 2; **d** NR genotype, Node 2; **e** R genotype, Node 3; **f** NR genotype, Node 3; **g** R genotype, Node 6; **h** NR genotype, Node 6. Black arrowheads point at the ring of phloem fibres. White arrowheads point at the initial development of the periderm. *cp* cortical parenchyma, *ep* epidermis, *pe* periderm, *ph* phloem, *vc* vascular cambium, *x* xylem. Scale bars 50 μ m

2005; Geiss et al. 2009; Ruedell et al. 2013; De Almeida et al. 2017; Wojtania et al. 2019). In our experiments, cuttings exposed to exogenous IBA showed the highest levels of CHOs, irrespective of genotype. However, the specific role of CHOs in AR formation is controversial and their specific role in the process is not completely clear (Druege 2009). Indeed, even CHO depletion induced by darkness may cause an increase in soluble nitrogen metabolites that can accelerate AR formation (Zerche et al. 2019). Therefore, the content of CHOs per se, cannot explain the different ability to form adventitious roots of the R and NR genotypes. In *E. globulus* cuttings, excised from donor plants before and after they had lost rooting capacity, the concentration of soluble CHOs did not show significant differences (Aumond et al. 2017). Therefore, it is more likely that the increase in CHOs that we observed is a consequence of wounding and physical isolation from the mother plant after excision of the cuttings which leads to the accumulation of substances otherwise transported downwards.

Protein contents showed a very different response suggesting that the nature of the changes induced by IBA were different on both genotypes. The increase in the levels of proteins by day 14 most likely reflects metabolic changes that occur in response to wounding which are generally related to the induction phase of AR formation (da Costa et al. 2013). This increase was similar in all the material that was expected to form AR, i.e., cuttings of the R genotype and the NR genotype with IBA. After cutting excision, proteins belonging to several different biological pathways are up or down regulated. In *Eucalyptus*, most of the proteins identified were related to oxidation–reduction processes followed by proteins involved in energy metabolism (de Almeida et al. 2020). Protein content in the NR genotype, on the other hand, was higher to that of the R genotype during the second and third weeks; however, it decreased to levels similar to those in the R genotype with IBA treatment. We did not investigate the identity of the proteins accumulated in each type of cutting in vitro and we cannot confirm whether the same or different genes were responsible for protein accumulation in the R and NR cuttings. However, our results show that internal factors in the untreated cuttings of the NR genotype are responsible for their different

biochemical response, and that this further increase in proteins synthesized in response to wounding is then not related to processes associated to rooting at least in vitro.

Cuttings of *A. sellowiana* of the two genotypes with contrasting rooting ability and taken from the same position from mother plants with the same chronological age, differed anatomically. A multistratified tissue that developed between the phloem and the discontinuous ring of phloem fibres was observed in uninodal cuttings of the NR genotype but not in the R genotype. This tissue presented one to several inner continuous layers of thin-walled prismatic cells and several outer thick-walled prismatic cells compactly arranged, lacking intercellular spaces. This pattern corresponds with periderm development during secondary stem growth of several woody species, which may form near the epidermis or deeper into the cortex up to the outer secondary phloem (Metcalfe and Chalk 1950; Evert 2006). A pericycle generally including a sub-continuous ring of fibres is a diagnostic feature of the Myrtaceae stem (Metcalfe and Chalk 1950) and it is coincident with the origin of the periderm found in our anatomical study. Recent investigations in other species of the Myrtaceae family have shown similar results relating reduced rooting capacity and vigour of cuttings with stem anatomy (Abu-Abied et al. 2012; Goulart et al. 2014; Bryant and Trueman 2015; Wendling et al. 2015).

For *A. sellowiana*, poor AR formation has been attributed to the presence of fibre cells in the phloem of stem cuttings that affect the metabolic activity of the new root meristem and mechanically prevent growth of the root primordia (Zhang et al. 2009). Coincidentally, we could not find any indication of root meristem formation in any of the cuttings where this periderm was present (data not shown). However, the proportion of fibre-free gaps of the external ring in uninodal cuttings of both genotypes was not significantly different. The main difference was the earlier development of a periderm in the NR genotype and how its width increased from the apex towards the lower nodes and in response to exogenous IBA. Except for this earlier presence of a periderm, both types of cuttings were anatomically similar. According to these results, it is unlikely that this tissue represents a physical barrier to AR development. The lack of AR in this genotype seems to be the absence of meristem formation itself, as observed for several recalcitrant fruit species (Altamura 1996). The development of this tissue by the second node, accompanied by loss of rooting capacity may indicate that the NR genotype actually undergoes an earlier phase change, from juvenile to mature.

The developmental programme of AR differentiation has been inversely linked to the xylogenesis programme in several forest species such as pine (Abarca et al. 2014), peach palm tree (de Almeida et al. 2012) and chestnut (Vielba et al. 2016). It is a consequence of an alteration in auxin homeostasis associated with phase change that negatively affects

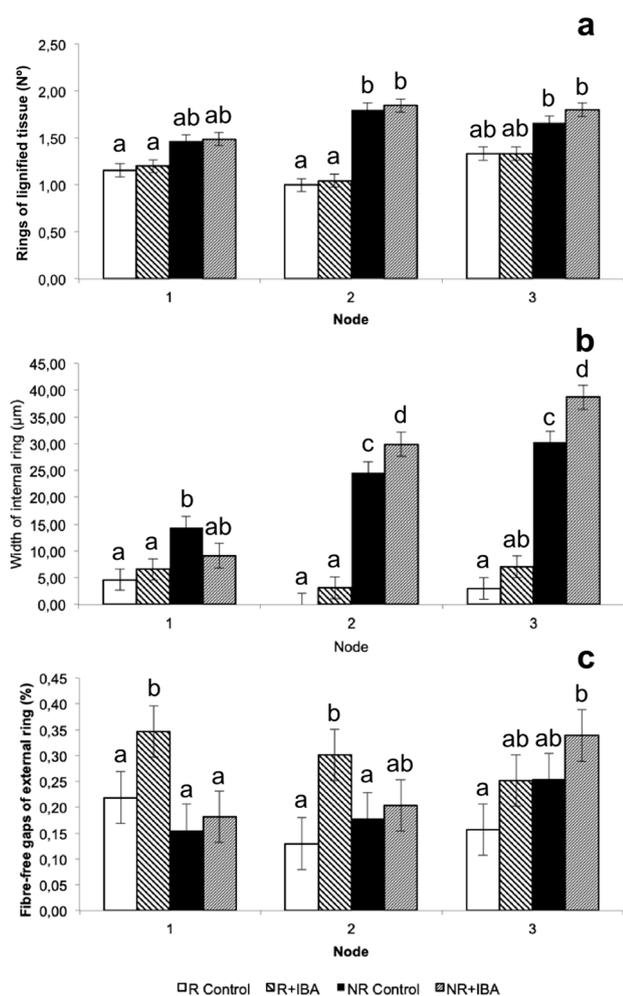


Fig. 7 Characteristics of lignified tissue after 28 days of incubation, at different nodes (1, 2 and 3 from the apex), in two genotypes of *Acca sellowiana* cuttings with contrasting rooting ability: R (easy-to-root) and NR (difficult-to-root), with or without exogenous IBA (12.3 mM) from experiment 2. **a** Mean number of rings; **b** width of internal ring; **c** fibre-free gaps of external ring. Thirty-six stem cross-sections, corresponding to 12 cuttings, were examined for each combination of genotype-treatment-node. Variables are presented as mean \pm SE

AR (Rasmussen et al. 2014). Consequently, high doses of IBA may inhibit rooting of cuttings by promoting lignification and secondary growth (Wendling et al. 2015). In *A. sellowiana*, cuttings of the NR genotype with only one node responded to exogenous IBA improving AR differentiation from less than 10% to more than 50%. However, from the second node downwards AR meristems failed to form, and instead the appearance and width of a periderm tissue was promoted by IBA. In *Eucalyptus*, the difference in rooting capacity among species was related to differences in the metabolism of auxins (Fett-Neto et al. 2001). This apparently contradictory effect of IBA can be understood as a change in sensitivity and response to IBA associated with

phase change. Also associated with phase change is the pattern of protein synthesis. Major changes in protein expression accompany phase-change in *E. grandis*; the expression of about 600 up and down regulated genes was significantly different in juvenile and mature cuttings and multiple transcripts related to different regulatory processes, had different expression between juvenile and mature cuttings in response to exogenous auxin (Abu-Abied et al. 2012, 2014). The difference in the pattern of protein expression observed in vitro and lack of AR formation of the untreated NR genotype both in vivo and in vitro is then probably due to its earlier phase change which can be partially reverted by exogenous IBA in the first node when tissues are still responsive.

Conclusions

In our study, we found no evidence of preformed root meristems in *A. sellowiana* cuttings of the wild genotypes used. Instead, AR meristems developed de novo outside the cambial ring. Exogenous application of IBA was effective in promoting rooting rates compatible with commercial propagation in uninodal cuttings. Anatomy of stem cuttings differed between genotypes. However, we found these differences to be more likely a consequence of phase change, from juvenile to mature, and loss of rooting ability of the NR genotype, associated with an earlier development of the periderm.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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