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RESEARCH REPORT

Comparative analysis of anthraquinone biosynthesis and auxin homeostasis in one-year-old and 25-year-old calli of *Rubia cordifolia* L

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Abstract

The present work is devoted to the study of dynamic changes in the biosynthesis of anthraquinones (AQs) during multiple years of cultivation in the calli of the medicinal plant *Rubia cordifolia* L. (Rubiaceae). Eight AQ derivatives were identified only in the 1-year-old calli. Only 4 components were detected in the 25-year-old calli and adventitious root extracts. No AQ was detected in the leaf or stem extracts. We have shown that 1-year-old calli contain the major compound, AQ, presumably of the "emodin type". "Emodin"-type anthraquinones contain a radical on the A-ring, and their presence in the genus *Rubia* has not been previously demonstrated. However, our mass spectrometry data suggest subsequent confirmation by NMR spectroscopy. The expression patterns of the enzymes involved in AQ biosynthesis were correlated with the AQ content. Untreated 1-year-old calli produce up to 0.5% DW, whereas 25-year-old calli produce up to 0.03% DW. In addition, derivatives of ferulic acid, coumaroylquinic acid, caffeoylquinic acid, and rutin were detected in 1-year-old calli. In the leaf extracts, all the listed compounds were found, with the exception of the ferulic acid derivatives, which were specific to the 1-year-old calli. The extract of 25-yr-old calli did not contain any phenylpropanoids. During long-term cultivation, the level of endogenous indole-3-acetic acid increased significantly, almost to the level of the native plant, whereas the level of IAA as well as the expression of IAA marker genes in the 1-year-old culture was significantly lower. Further research into auxin homeostasis will allow the optimization of conditions for the productive long-term cultivation of plant cell cultures.

Keywords "alizarin type" anthraquinone · Callus culture · "emodin type" anthraquinone · Indole-3-acetic acid · Rubiaceae

Introduction

Plant cell culture technology can address some of these issues by providing efficient and sustainable sources of phytochemicals with reduced energy and carbon footprints, allowing the conservation of valuable endangered species (Krasteva *et al*. 2021). The main success of this method depends on the careful selection and optimization of nutrient media, mainly plant growth regulators (Vasil 2008; Sidik et al. 2024). The development of organs and tissues *in vitro* culture depends on the concentration and balance of exogenously supplied phytohormones, and the impact of an external supply of different concentrations of auxins depends on the concentrations of the auxins of the plant tissue itself (Li, *et al*. 2017). Research on secondary metabolism based on cell culture is particularly popular (Pantchev *et al*. 2018). The production of secondary metabolites (including pharmacologically active compounds) in native plants is low (usually less than 1% of dry weight) and depends mainly on the physiological state and stage of development of the plant (Dixon 2001; Georgiev *et al*. 2008; Pantchev *et al*. 2018). The dynamics of the biosynthesis of secondary metabolites during long-term multiyear cultivation have been poorly studied. There are few publications on the phytochemical content of calli that are more than 10 years old; however, a dynamic comparison of "young" and "old" calli is lacking (Federici *et al*. 2003; Malarz *et al*. 2013). In our work,

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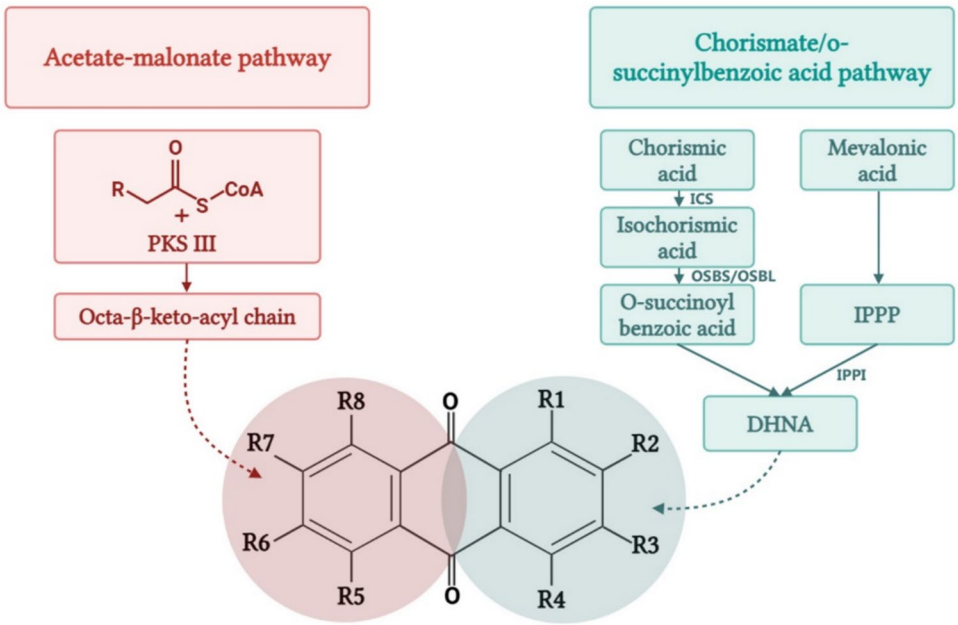


Figure 1. Simplified scheme of AQ biosynthesis and the skeleton structure of anthraquinoid derivatives (according to Caro *et al* 2012). The pink left part of the scheme is the acetate-malonate pathway of biosynthesis of the "emodin-type AQ": PKS III and III-type polyketidesynthases are among the most important enzymes in this pathway. The turquoise right part of the scheme is the chorismate/*o*-succinylbenzoate pathway of biosynthesis of the "alizarin-type AQ": ICS, isochorismate synthase; OSBS, *O*-succinylbenzoate synthase; OSBL, *O*-succinylbenzoate ligase; DHNA, 1,4-dihydroxy-2-naphthoic acid; IPPP, isopentenyl diphosphate; IPPI, isopentenyl diphosphate isomerase.

we have shown that with age, the accumulation of different classes of phytochemicals significantly decreases in multi-year-maintained calli of various plants (Veremeichik *et al*. 2024; 25a, b). In addition, the homeostasis of endogenous auxins during long-term cultivation of plant callus cultures has been poorly studied. Such studies are carried out mainly on newly obtained calli or during the first year (Ikeuchi *et al*. 2017; Cheng *et al*. 2025). The present work is devoted to the study of dynamic changes in the level of biosynthesis of secondary metabolites during long-term multiyear cultivation in comparison with changes in the homeostasis of endogenous auxins in the cell culture of the valuable medicinal plant *Rubia cordifolia* L. (Rubiaceae).

R. cordifolia is a perennial medicinal plant whose rhizome has been used in traditional Asian medicine for many years. In recent decades, many scientific groups around the world have actively studied the phytochemical composition, pharmacological properties, and molecular mechanisms of the biosynthesis of pharmacologically active compounds. An analysis of publications by the keyword *R. cordifolia* up to 2022 in the Google Scholar, Web of Science and PubMed databases revealed that studies are aimed mainly at studying pharmacological activity (Wen *et al*. 2022). A rapid surge in publications devoted to *R. cordifolia* indicates the high relevance of *R. cordifolia* as an object of research (Wen *et al*. 2022). This interest in *R. cordifolia* is primarily due to the pronounced pharmacological properties of the main secondary metabolites of madder, anthraquinones (AQs). The most well-known pharmacological properties of *R cordifolia* root extracts are neuroprotective effects (Joy and Nair 2008; Lodi *et al*. 2011;); cosmetic effects, such as hyperpigmentation, psoriasis, and inflammatory processes; and osteoblastic activity (Shivakumar *et al*. 2012; Di Pompo *et al*. 2014; Baek *et al*. 2015). Alizarin and emodin are used as antioxidants in foods (Essaidi *et al*. 2017). Alizarin exhibits chemoprotective activity against nitrosodiethylamine (NDE)-induced lung tumors by eliminating excess free radicals that promote lipid peroxidation and by enhancing the antioxidant defense of molecules such as GST and GSH (Bhardwaj *et al*. 2001). Moreover, mollugin inhibits cell proliferation and promotes apoptosis in breast and ovarian cancer cells by suppressing FAS expression through the modulation of the HER2/Akt/SREBP-1c signalling pathway. Furthermore, mollugin can inhibit HER2 expression by suppressing NF-κB activation (Do *et al*. 2013).

Anthraquinones are a class of compounds of the quinone family that consists of several hundred compounds whose nature and positions differ. Anthraquinoid derivatives are derivatives of the basic structure 9,10-anthracenedione or 9,10-dioxoanthracene, i.e., tricyclic aromatic organic compounds with the formula C14H8O2 and whose ketone groups are on the central ring at positions C-9 and C-10. The skeleton structure of anthraquinoid derivatives and a simplified scheme of AQ biosynthesis (according to Caro *et al*. 2012) are shown in Fig. 1. The pink left part of the scheme is the acetate−malonate pathway of biosynthesis of the "emodin-type AQ": PKS III, III-type polyketide synthetases, are among the most important enzymes in this pathway. The turquoise right part of the scheme is the chorismate/*o*-succinylbenzoate pathway for the biosynthesis of "alizarin-type AQ": ICS, isochorismate synthase; OSBS, O-succinylbenzoate synthase; OSBL, O-succinylbenzoate ligase; DHNA, 1,4-dihydroxy-2-naphthoic acid; IPPP, isopentenyl

diphosphate; IPPI, isopentenyl diphosphate isomerase. The shikimate and terpenoid pathways (MVA and MEP, the mevalonate and 2-c-methyl-d-erythritol-4-phosphate pathways, respectively) are sources of the immediate precursors of anthraquinones, 1,4-dihydroxy-2-naphnoic acid and dimethyl aliphatic phosphate, respectively. α-Ketoglutarate, a product of the tricarboxylic acid cycle, is also involved in the formation of 1,4-dihydroxy-2-naphnoic acid (Han *et al*. 2001, 2002). The natural HAQN pigments formed *via* the chorismate/o-succinylbenzoic acid pathway have substitutions on only one aromatic ring, such as alizarin and purpurin. In contrast, the HAQN pigments that are synthesized *via* the polyketide pathway have substituents on both aromatic rings and at least two hydroxyl groups at both the R1 and R8 positions, similar to emodin (Caro *et al*. 2012). In extracts of native plants and cell cultures, anthraquinones of the "alizarin-type" type are usually detected, and research has focused mainly on the chorismate/o-succinylbenzoate pathway of biosynthesis of the "alizarin-type AQ" (Murthy *et al*. 2022).

Thus, the general goal of the present work was to investigate the biosynthesis of the main phytochemicals in newly obtained and long-term continuously cultivated R. cordifolia calli in comparison with the original plant. For the first time, we investigated the changes in the AQ composition and content in 1- and 30-year-old calli. Moreover, in addition to AQ, we investigated the phenylpropanoid composition in plant and callus tissues. To confirm the obtained data, we also analysed the expression of key enzymes involved in AQ biosynthesis. Given that the main precursor of AQ, chorismate, is also a precursor of the important PGR IAA, we also performed a comparative analysis of the endogenous IAA content and performed an expression analysis of key genes of the IAA signalling system.

Materials and methods

Plant and callus tissue Materials of the wild-grown plants were collected in July, 2024 and 2025 in the southern area of Primorsky Krai, Russia (43° 06′ 54″ N 131° 53′ 07″ E). Both cell cultures were obtained from leaf explants of the wild-grown parent plants collected under the same natural conditions. A callus culture of *R. cordifolia* was previously obtained more than 25 years ago (1998) and was denoted by R (Mischenko *et al*. 1999). In 2024, a new callus culture was obtained and designated as RN (Rubia New). For the present analysis, an RN callus culture was used after 1 year of continuous cultivation. Both types of callus culture were induced in the dark from leaf explants *via* agar media supplemented with 0.5 mg L^{-1} 6-benzylaminopurine and 2.0 mg L^{-1} α-naphthaleneacetic acid. For the cultivation of both types of callus cultures, the following conditions were used: the calli were subcultured every 30 days in the dark on the same agar media in the dark at 70% humidity. For analyses, plant materials (leaves, stems and adventitious roots) were harvested from three different plants in July 2014 and 2025. Calli cultures were harvested on the 30th day of passage, weighed, and photographed. For chemical analyses, the tissues were dried (45°C for 12–16 h); for molecular analyses, they were used immediately. The study employed specimens (strains) deposited in the Bioresource Collection of the Federal Scientific Centre of East Asia Terrestrial Biodiversity of the Far East Branch of the Russian Academy of Sciences (reg. number 2797657).

Secondary metabolite determination

chemicals. HPLC-grade acetonitrile was obtained from "Neo-Froxx" (Einhausen, Germany). Ultragradient HPLC grade methanol was obtained from "J.T. Baker" (Gliwice, Poland). Formic acid was obtained from Sigma–Aldrich (Taufkirchen, Germany). Deionized water was purified using a Milli-Q Simplicity water purification system (Millipore, Molsheim, France). Analytical standards of chlorogenic acid (Sigma–Aldrich, St. Louis, MO) and rutin trihydrate (Fluka, Shanghai, China) were used in our study. A standard sample of alizarin was previously isolated from *R. cordifolia* cell cultures and characterized at the laboratory of natural quinonoid chemistry of the Elyakov Pacific Institute of Bioorganic Chemistry (Far Eastern Branch, Russian Academy of Sciences) (Mischenko *et al*., 1999), where it was used for anthraquinone quantification.

HPLC–PDA–MS assay. Sample preparation and HPLC−PDA−MS analysis of *R. cordifolia* plant and callus tissues were carried out exactly according to a previously described protocol (Veremeichik *et al*. 2024). Briefly, all samples (dry and crushed plant tissue) were extracted in 80% methyl alcohol using ultrasonic treatment, centrifuged, and filtered (0.45 μm membrane, Millipore, Bedford, MA, USA) prior to analysis. An Agilent 1260 Infinity instrument (Agilent Technologies, Santa Clara, CA) equipped with an analytical Zorbax C18 column (150 mm, 2.1 mm i.d., 3.5 μm; Agilent Technologies) and a PDA detector was used for metabolite profiling. A binary gradient from A (0.1% aqueous formic acid) to B (acetonitrile with 0.1% formic acid) with a flow rate of 0.2 ml min^{-1} was applied. The chromatograms for quantification were recorded at wavelengths of 430 nm and 320 nm. High-resolution MS data were collected using a Shimadzu LCMS-IT-TOF hybrid instrument (Shimadzu, Kyoto, Japan) operating in electrospray ionization mode with negative ion detection. An external standard method was used to quantify the detected compounds. All the discovered anthraquinone derivatives were quantified as alizarin equivalents. An analytical standard of chlorogenic acid was used to evaluate the hydroxycinnamic acid derivatives.

RNA isolation, cDNA synthesis, sequencing and real-time PCR *RNA extraction.* To analyse AQ biosynthesis and IAA marker gene expression, samples of R and RN cultures were harvested from 30-day-old cell cultures; wild-grown plant material (leaves, stems, and adventitious roots) was harvested in July. For all RT analyses, we used three independent RNA extractions. Total RNA was isolated, and first-strand cDNA synthesis was carried out as described previously (Shkryl *et al*. 2008). All RNA samples were treated with DNAse (Biolabmix, Novosibirsk, Russia) according to the manufacturer's protocol.

Cloning of the IAA biosynthesis-related genes. To analyse IAA marker gene expression, the sequences of the closest homologues of the IAA marker genes of *A. thaliana* (Mano and Nemoto, 2012) were resequenced *via* next-generation sequencing (NGS) (NCBI accession number PRJNA1079016SRR28040871). Using PCR, cDNA fragments of *RcTAA* (tryptophan aminotransferase-related protein), *RcYUC* (indole-3-pyruvate monooxygenase YUCCA-like), and *RcARF* (auxin response factor) of predicted lengths were amplified. The PCRs were carried out in an iCycler amplifier (Bio-Rad Laboratories, Hercules, CA) programmed for the following reaction conditions: preliminary denaturation at 95 °C for 3 min; 35 cycles of 95 °C for 30 s, 55 °C for 20 s, and 72 °C for 60 s; and a final elongation at 72 °C for 3 min. For cloning, the PCR products were purified by isolation with a low-melting agarose gel (Sigma, St. Louis, MO) using phenol extraction (Sambrook *et al*. 1989) and cloned and inserted into a pJET vector using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific, Waltham, MA) and then sequenced as described earlier (Shkryl *et al*. 2008) at the Instrumental Centre of Biotechnology and Gene Engineering of FSCEATB FEB RAS using an ABI 3130 Genetic Analyser (Applied Biosystems, Foster City, CA). The search for homologues of sequenced DNA fragments was carried out in the GenBank sequence database using the BLAST program (Altschul *et al*. 1990). Amino acid sequences corresponding to the sequenced cDNA fragments were determined using the GeneRunner v3.0 program. The GenBank accession numbers of the obtained sequences and specific primer pairs used for qPCR are listed in Supplementary Table S1.

Real-time PCR. The expression of genes involved in anthraquinone biosynthesis (*RcICS*, isochorismate synthase; *RcOSBL*, *o*-succinylbenzoate-CoA ligase; *RcIPPi*, isopentenyl diphosphate isomerase) was measured *via* gene-specific primer pairs described previously (Veremeichik *et al*. 2019). Gene-specific primer pairs for housekeeping reference genes such as actin and 18S have been previously described (Shkryl *et al*. 2011). A CFX96 instrument (Bio-Rad Laboratories, Inc., Hercules, CA) was used with 2.5×SYBR Green PCR Master Mix (Biolabmix) for quantitative real-time PCR (qPCR) examination. Three biological and three technical replicates from three distinct RNA extractions were used for analysis. A 10-µl volume containing 250 nM of each primer, 1 µL of the diluted cDNA sample, and 2 mM $MgCl_2$ was used for the reactions. To confirm that there was no contamination, qPCR analysis was performed with both the RNA-RT and no-template controls. CFX Manager Software (Version 1.5; Bio-Rad Laboratories Inc., Hercules, CA) was used to analyse the data. With the use of the formula $2^{-\Delta\Delta Ct}$, the lower-expressing sample was given a value of 1 in the relative mRNA calculation. The reaction conditions were as follows: preliminary denaturation at 95 °C for 3 min; 35 cycles at 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 30 s; final elongation at 72 °C for 3 min; denaturation at 95 °C for 10 min; and melting curves in the range of 75–90°C with a temperature increase of 0.2°C, with 100 repetitions. To verify that there were no nonspecific products or primer−dimer artifacts in the samples, melting curve analysis and product visualization *via* electrophoresis on a 1% agarose gel stained with ethidium bromide were employed.

IAA ELISA To compare the IAA contents of 1-year- and 25-year-old cultured calli *via* ELISA, sample preparation was performed according to previously described methods (Veselov *et al*. 1992). The plants and callus cultures were ground into powder with liquid nitrogen and immediately weighed to 300 mg. Extraction was performed in 1 ml of 80% ethanol for 16 h at 4°C. After incubation, the extracts were centrifuged, and the supernatant was dried (Concentrator Plus, Eppendorf). The dried pellet was dissolved in 1 mL of water acidified with HCl (pH 2–3). IAA was subsequently concentrated twice with diethyl ether. The ether phase was dried; the pellet was dissolved in 50 µI of 80% ethanol and used immediately for the ELISA. ELISA was performed *via* an ELISA kit for indole-3-acetic acid (IAA) (Cloud-Clone Corp., Hubei China) according to the manufacturer's instructions. The samples were analysed in a 96-well plate using a spectrophotometer (Benchmark Plus Microplate Reader, Bio-Rad) at a wavelength of 450 nm. The results were obtained from a calibration curve constructed according to the optical density of a standard diluted to concentrations of 320, 1600, 8000, and 40000 ng mL^{-1}. The analysis of IAA content was repeated three times.

Statistical analysis All values are presented using Statistica 10.0 (StatSoft Inc., Tulsa, OK) as the mean ± SE. A significant difference was defined as $P < 0.05$. Student's t test was used to compare two independent categories, and ANOVA and multiple comparisons were used to evaluate data across multiple groups. For the intergroup comparisons, Fisher's protected least significant difference (PLSD) *post hoc* test was used.

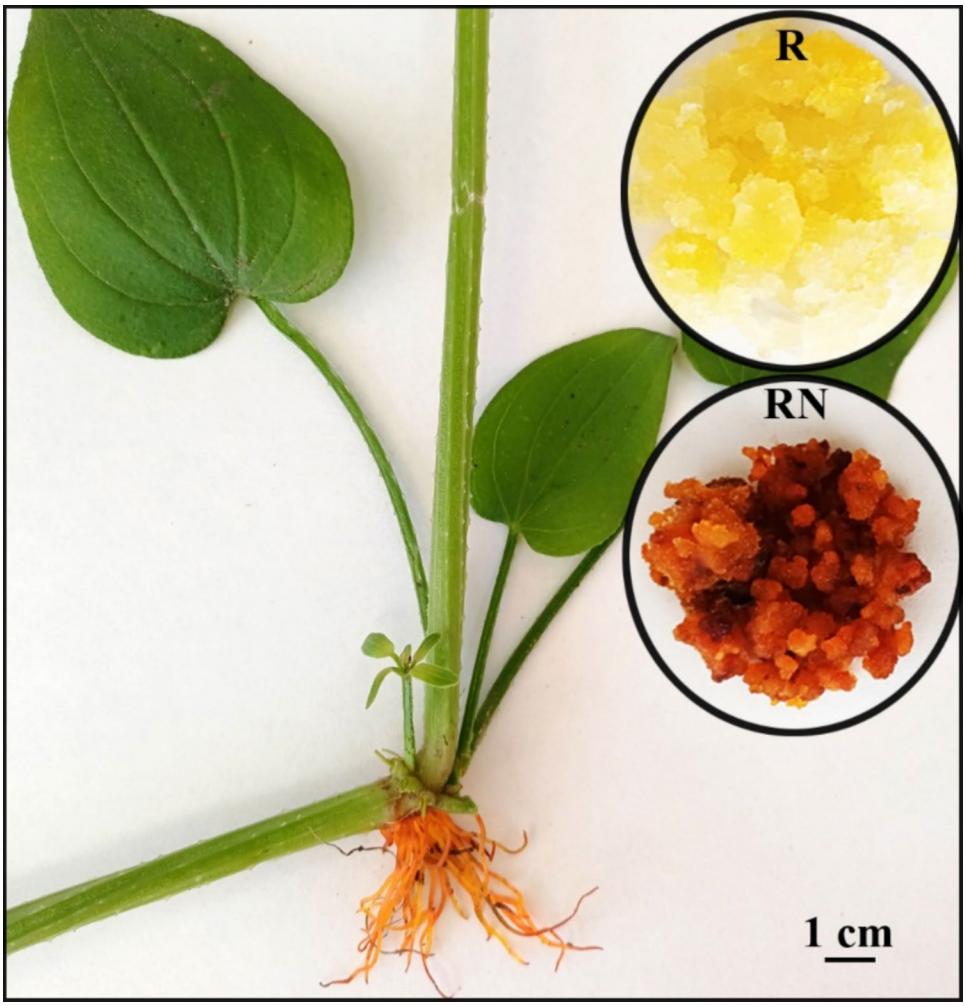


Figure 2. Phenotypes of the *R. cordifolia* plants and callus cultures used in this work. Underground part of the wild-grown *R. cordifolia* plant with adventive roots. The 25-yr-old calli (R) and newly obtained 1-year-old calli (RN) of *R. cordifolia* (*upper* and *lower circles*, respectively). The color of the callus cultures (yellowish of R and reddish of RN) depends on the content of the anthraquinones.

Results

AQ compositions of *R. Cordifolia* callus lines and plant tissues As stated above, the aim of the present study was to analyse AQ biosynthesis in newly obtained and long-term-maintained callus cultures of *R. cordifolia* in comparison with the original wild-grown plant tissues. For these purposes, we used 25-yr-old long-term maintained calli designated "R" and newly obtained 1-year-old calli designated "RN". For analyses, plant materials (leaves, stems and adventitious roots) were harvested from three different plants. The morphology of the plant materials used is shown in Fig. 2. As shown in Fig. 2, the R culture is characterized by loose, finely dispersed tissue with a light yellow color, whereas the RN culture is characterized by heterogeneous, large aggregate tissue rich in red−orange color. The saturation of the color depends on the AQ content, which is evident from the color of the adventitious roots, which are also orange−red, in contrast to the leaves and stems of the plant (Fig. 2).

The anthraquinone derivatives of the samples were studied by high-performance liquid chromatography coupled with PDA and MS detection. High-resolution mass measurements were performed *via* a time-of-flight mass spectrometer, and molecular formulas were developed for the detected compounds with a mass error of less than 1.1 mDa. The chromatographic profiles of the anthraquinoid pigments are shown in Fig. 3. A total of eight major peaks were assigned to the anthraquinone class. Therefore, six of these compounds were identified as we described earlier (Veremeichik *et al*. 2024): pseudopurpurin primveroside (A1), pseudopurpurin glucoside (A2), munjistin glucoside (A4), lucidin primveroside (A5), ruberitrinic acid (A6), munjistin and pseudopurpurin (A8). In addition, two new compounds were found to be anthraquinone derivatives on the basis of their characteristic UV−Vis profiles. Therefore, compound A7, with a retention time of 33.2 min and a molecular formula of $C_{15}H_{10}O_6$ ($[M\text{-}H]^-$ at *m/z* 285.0402), was assigned as methyl-tetrahydroxy-anthraquinone, trihydroxy-methoxy-anthraquinone, or an isomer (Caro *et al*. 2012). Compound A3, with a retention time of 26.3 min and a molecular

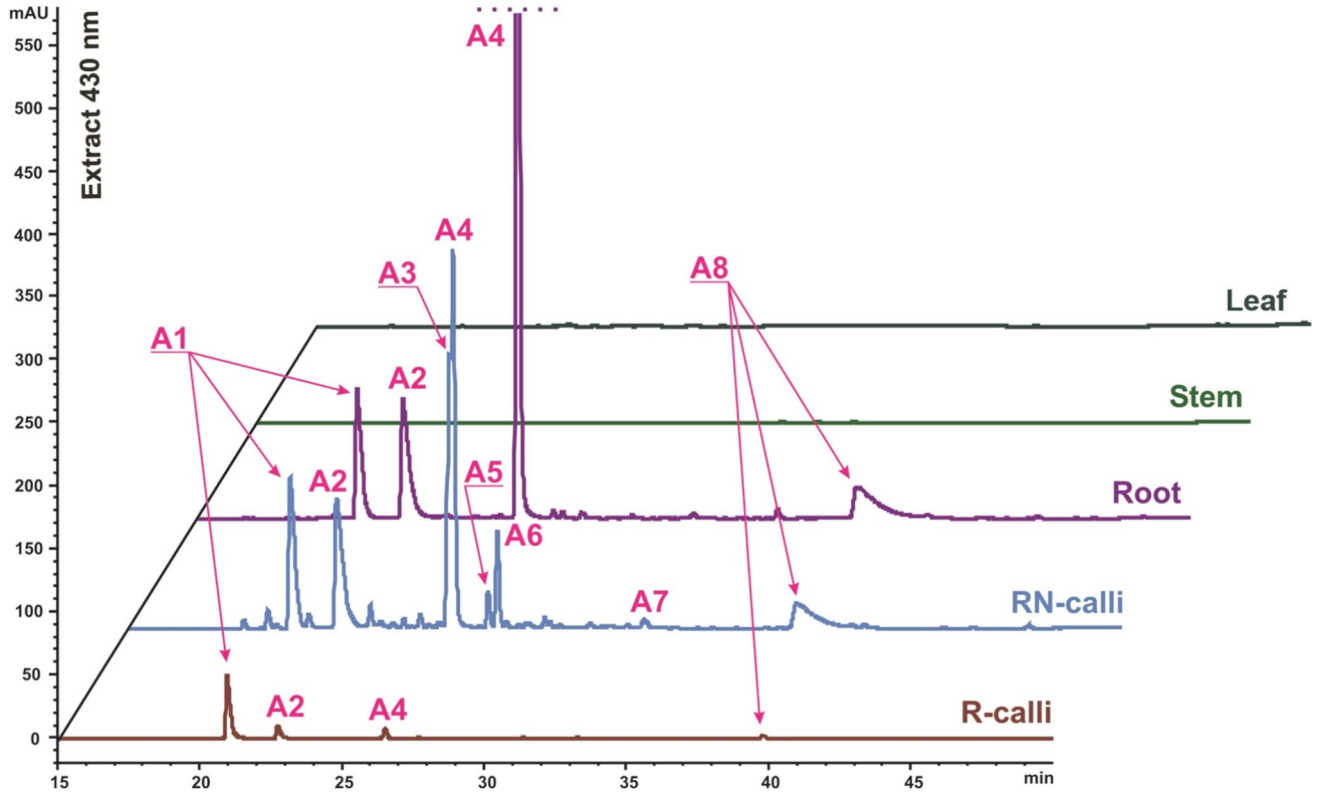


Figure 3. HPLC (430 nm) profiling of anthraquinone derivatives determined in the crude extract of *R. cordifolia* callus lines and plant tissues. The peak numbers correspond to those listed in Supplementary Table S2. Analysis was performed to compare callus lines (R, long-term-cultured 25-yr-old callus culture; RN, newly obtained callus culture of *R. cordifolia*) and plant tissues (leaf, stem, and root). Peaks corresponding to AQ derivatives are designated in *pink letters*.

formula of $C_{21}H_{20}O_{11}$ ([M-H]$^-$ at *m/z* 447.0927), was characterized as a monosaccharide derivative of compound A7. All chromatographic and mass spectrometric data required for identification are listed in Supplementary Table S2 and Supplementary Figures S1–4.

As shown in Fig. 3, all 8 AQ derivatives were identified only in the RN line extract. Only 4 components were detected in the R line and adventitious root extracts. No AQ was detected in the leaf or stem extracts. Interestingly, two of the identified AQ derivatives in RN tissues were "emodin-type": methyl-tetrahydroxy-anthraquinone and its glucoside, compounds A7 and A3, respectively. Previously, this AQ derivative was identified as catenarin (Caro et al. 2012). Catenarin is known to be an "emodin-type" AQ, whereas the other AQs identified in this study are "alizarin-type" AQs (Murthy *et al*. 2022).

Phenylpropanoid composition of *R. cordifolia* callus Lines and plant tissues A study of the chromatographic profiles of the leaf and RN callus extracts revealed a number of phenylpropanoid compounds (Fig. 4). Among them, two flavonoids and nine hydroxycinnamic acid derivatives were detected. Thus, compounds P5 and P11 were undoubtedly identified as chlorogenic acid and rutin, respectively, according to their exact matches with available analytical standards. Compound P10 was assigned as an isomer of rutin on the basis of its UV and MS characteristics, which are consistent with those of P11. The identification of other phenylpropanoids was based on MS/MS^2 experiments (Supplementary Table S3, Supplementary Figures S5-10). Thus, two caffeoylquinic acids (P1 and P8) and three coumaroylquinic acids (P3, P7 and P9) were recognized. Several ferulic acid derivatives were also detected: one monosaccharide (P4) and two disaccharide derivatives (P2 and P6).

As shown in Fig. 4, phenylpropanoid compounds were detected in the extracts of the leaves, stems, roots and the RN callus line, whereas the extracts of the R callus line contained only AQ. These 10 phenylpropanoid derivatives can be divided into four groups according to their order in the common phenylpropanoid biosynthesis pathway: ferulic acid derivatives, coumaroylquinic acid derivatives, caffeoylquinic acid derivatives, and rutin derivatives. Ferulic acid derivatives are synthesized from *p*-coumaric acid at the early third stage of the common phenylpropanoid biosynthesis pathway. Caffeoylquinic derivatives are synthesized from *p*-coumaroyl-CoA at the fourth stage of the

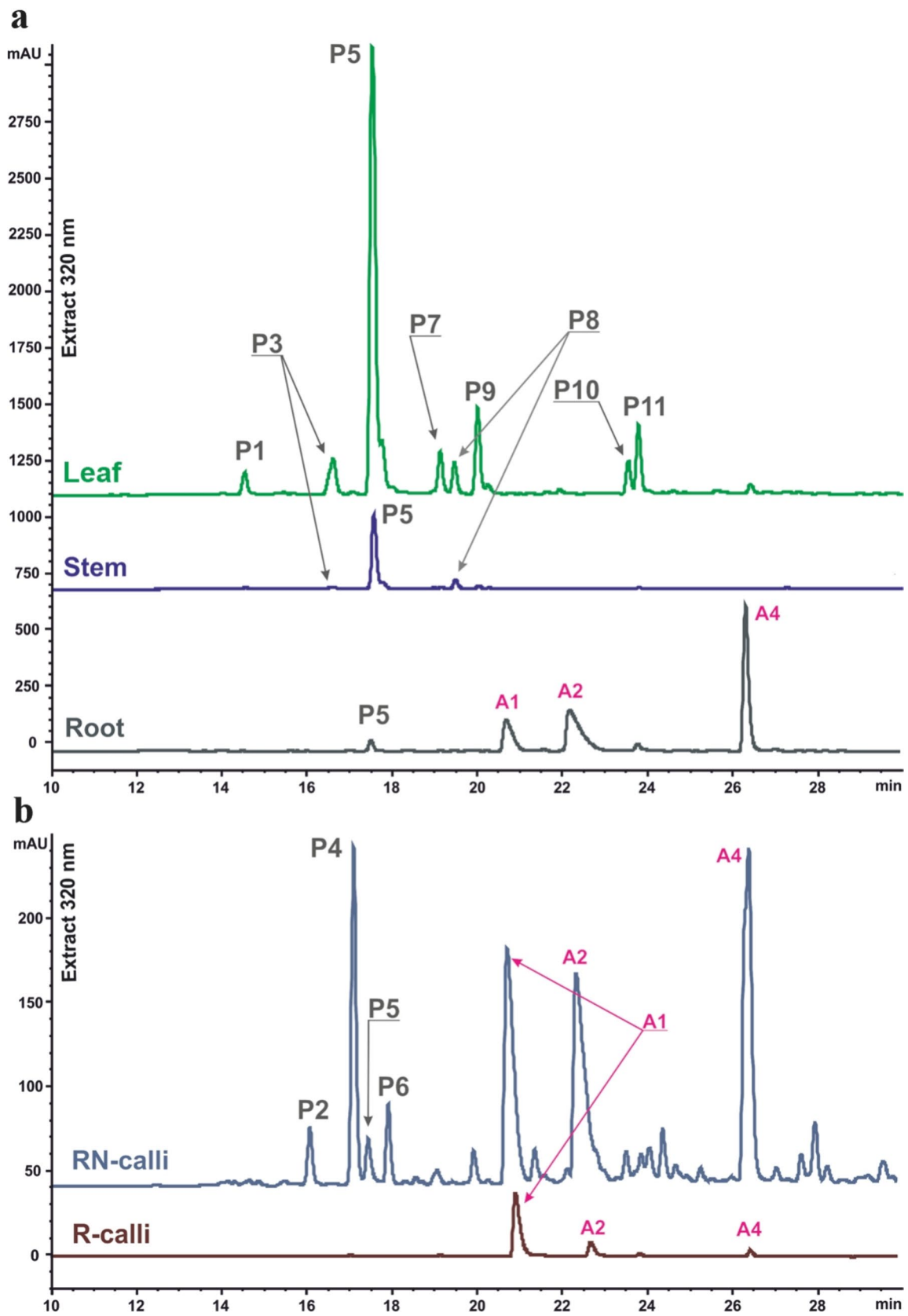
common phenylpropanoid biosynthesis pathway, where coumaroylquinic acid derivatives are intermediate compounds. Rutin is the final product of the flavonol branch of the common phenylpropanoid biosynthesis pathway. Therefore, as shown in Fig. 4, ferulic acid derivatives (compounds P2, P4, and P6), products of the earlier biosynthetic stage, were found only in the RN callus extract. In addition to these compounds, chlorogenic acid (compounds P5) was present in both the RN callus extract and the extracts of all the analysed plant tissues. Moreover, in the root extracts, no other phenylpropanoid compounds were detected. The steam extract included three coumaroylquinic and caffeoylquinic acid derivatives (compounds P3, P4, and P9). In the leaf extracts, all the listed compounds were found, with the exception of the ferulic acid derivatives (compounds P2, P4, and P6), which were specific to the 1-year-old calli RN. The extract of 25-yr-old calli did not contain any phenylpropanoid compounds.

AQ biosynthesis and productivity of *R. cordifolia* callus lines and plant tissues Quantitative analysis of the individual AQ derivative contents was performed *via* high-performance liquid chromatography (HPLC), and the results were quantified as the alizarin equivalent. As shown in Table 1, the major AQ compound in the root tissues was munjistin glucoside (A4), whose content was approximately 7000 μg g^{-1} DW. In R, with long-term culture in 25-yr-old callus culture, and in RN, a newly obtained callus culture of *R. cordifolia*, its content decreased 150- and tenfold, respectively. The contents of the other AQ derivatives (the three derivatives of pseudopurpurin, A1, A2, and A8) detected in the roots were more than three times lower. The same compounds were detected in the extract of R, a long-term-cultured 25-yr-old callus culture. However, the major AQ was pseudopurpurin primveroside, whose concentration reached 250 μg g^{-1}, whereas the concentrations of the other three AQs did not exceed 50 μg g^{-1}. In 1-year-old RNs callus culture, the major compound was the glucoside methyl-tetrahydroxy-anthraquinone or catenarin. Its content was approximately 1500 μg g^{-1} DW, whereas the content of munjistin glucoside and pseudopurpurin primveroside and pseudopurpurin glucoside was no more than 700 μg g^{-1} DW. In addition, ruberitrinic acid was detected in the 1-year-old callus culture (RN); its concentration was approximately 200 μg g^{-1} DW. The minor AQ compounds found in the RN extracts were lucidin primveroside and catenarin (no more than 100 μg g^{-1} DW).

To evaluate the productivity of the 1-year-old calli RN and 25-year-old calli R, the total AQ content and growth parameters were analysed (Fig. 5). Both the fresh and dry weights of R calli were greater than those of RN calli by 4 and 2 times, respectively (Fig. 5*a*). Compared with that of R calli, the total AQ content of RN calli was more than 10 times greater—up to 4.5 mg g^{-1} DW (Fig. 5*b*). Therefore, the total AQ productivity of the RN calli was 5 times greater than that of the R calli, with up to 15 mg of total AQ per 1 L of culture medium when 1 g of calli was inoculated on 50 mL of culture medium (Fig. 5*c*). The mRNAs of AQ biosynthesis genes (*RcICS*, isochorismate synthase; *RcOSBL*, *o*-succinylbenzoate-CoA ligase; *RcIPPi*, isopentenyl diphosphate isomerase) in R and RN calli of *R. cordifolia* were measured by real-time PCR (Fig. 4*d*). Compared with those in the leaves and stems, the expression of the AQ biosynthesis genes *RcICS*, *RcOSBL*, and *RcIPPi* in the roots was 7, 8 and 6 times greater, respectively. In R calli, the expression of these genes was slightly lower than that in roots. In the RN calli, the expression pattern of the AQ biosynthesis genes was similar to that in the roots (Fig. 5).

Phenylpropanoid contents of *R. cordifolia* callus lines and plant tissues As noted above, phenylpropanoid derivatives were found in all the plant tissues and RN calli of *R.Cordifolia*. A relatively high abundance of phenylpropanoid derivatives was detected in the leaves of *R.Cordifolia*, and the concentration of total phenylpropanoids was greater than 50 μM g^{-1} DW (Table 2). Among the total amount, 67% is represented by the major compound, chlorogenic acid. Its concentration reached 35 μM g^{-1} DW. Moreover, chlorogenic acid was detected in other plant tissues and in the RN calli of *R. Cordifolia*. This was the only compound found in the roots at concentrations up to 0.5 μM g^{-1} DW. In the stems, chlorogenic acid was also the major compound; however, its concentration was ten times lower than that in the leaves, reaching 4 μM g^{-1} DW. The concentration of phenylpropanoids in the RN line was comparable to that in the stem and was 3 μM g^{-1} DW, which was 17 times lower than that in the leaves. However, in the RN line, phenylpropanoids are represented mainly by derivatives of ferulic acid, which are synthesized from *p*-coumaric acid at the earlier third stage of the common phenylpropanoid biosynthesis pathway.

IAA homeostasis in *R. cordifolia* callus lines and plant tissues Given that the main precursor of AQ, chorismate, is also a precursor of the important PGR IAA, we also performed a comparative analysis of the endogenous IAA content and an expression analysis of key genes of the IAA signalling system. To analyse IAA marker gene expression, the sequences of the closest homologues of the IAA marker genes of *A. thaliana* (Mano and Nemoto, 2012) for *R. cordifolia* were resequenced *via* next-generation sequencing (NGS). Using PCR, cDNA fragments of *RcTAA* (tryptophan aminotransferase-related protein), *RcYUC* (indole-3-pyruvate monooxygenase YUCCA-like), and *RcARF* (auxin response factor) of predicted lengths were amplified and sequenced, and the amino acid sequences of RcTAA, RcYUC, and RcARF to related sequences of *A. thaliana*


a
mAU
Extract 320 nm
Leaf
Stem
Root
P1
P3
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P7
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b
mAU
Extract 320 nm
RN-calli
R-calli
P2
P4
P5
P6
A1
A2
A4
min

◂**Figure 4.** HPLC (320 nm) profiling of phenylpropanoids determined in the crude extract of *R. cordifolia* callus lines (*a*) and plant tissues (*b*). The peak numbers correspond to those listed in Supplementary Table S3. Analysis was performed to compare callus lines (R, long-term-cultured 25-yr-old callus culture; RN, newly obtained callus culture of *R. cordifolia*) and plant tissues (leaf, stem, and root). Peaks corresponding to phenylpropanoid derivatives are designated in *gray letters*, and those corresponding to AQ are designated in *pink letters*.

were 70% (TAA1, GenBank № NP_173746.1), 63% (YUC6, GenBank № OAO91776.1), and 86% (ARF6, GenBank № NP_001031115.1), respectively.

Analysis was performed to compare the callus lines R, long-term-cultured 25-year-old callus cultures and newly obtained RN callus cultures and plant tissues (leaves and roots) of *R. cordifolia*. The mRNA levels of IAA marker genes (*RcTAA*, tryptophan aminotransferase-related protein; *RcYUC*, indole-3-pyruvate monooxygenase YUCCA-like; *RcARF*, auxin response factor) were measured by real-time PCR and are reported as relative expression. The expression levels of the IAA biosynthesis genes *RcTAA* and *RcYUC* were the same in the leaves of the plants and R calli, whereas in the RN calli, they were dramatically lower, by more than 10 and 20 times, respectively (Fig. 6*a*, *b*). However, the expression of *RcARF* decreased in both the R and RN callus lines by 2 and 4 times, respectively (Fig. 6*c*). The IAA content was measured using ELISA. Compared with that in the plants, the content of exogenous IAA slightly decreased in the R calli and significantly decreased in the RN calli (Fig. 6*d*).

Discussion

An applied branch of biotechnology, such as plant cell culture, has a 100-year history. Given the need to preserve biodiversity, one cannot fail to appreciate the advantages of an alternative source of phytochemicals on the basis of long-term maintenance of plant cell cultures. The first industrial process for producing the bioactive secondary metabolite shikonin from a red-rooted ginseng plant (*Lithospermum erythrorhizon* Siebold & Zucc.) was developed in 1983 (Curtin 1983). Large-scale production of ginseng biomass was successfully launched within five years (Pantchev *et al*. 2018). On the basis of the promising results obtained at that time, a series of successful large-scale cultivations of various plant cell suspensions in bioreactors with a volume of up to 50,000 L were performed as follows: (*Duboisia* sp.), podophyllotoxin (*Podophyllum* sp.), protoberberin (*Coptis japonica* (Thunb.) Makino.), rosmarinic acid (*Coleus blumei* Benth.), geraniol (*Gramineae* sp.), arbutin (*Catharanthus roseus* L.), and vanilin (*Vanilla planifolia* Andrews.) (Tulecke and Nickell, 1960). Japan is the leader in the development of industrial production based on *in vitro* plant biotechnology; production is represented by corporations such as Japan Tobacco Inc., Meiji Seika, Nitto Dko Co., Ajinomoto, and Nippon Shinyaku, which have developed their technologies in close collaboration with academic research groups (Yesil-Celiktas *et al*. 2010).

However, despite the popularity of research in the field of plant cell culture, there is no information on the dynamics of molecular and biochemical processes during long-term multiyear cultivation. Some studies have focused on regenerative potential during long-term cultivation, which is important for the breeding of cultivated plants (Häsler *et al*., 2003; Peng *et al*., 2022). Thus, we managed to find several publications on the dynamics of the productivity of secondary metabolites during the long-term cultivation of cell cultures of some plants. For example, with 5 years of cultivation of a suspension culture of *Buddleja cordata* Kunth, the productivity of verbascoside remains unchanged (Arano-Varela *et al*., 2020). However, a duration of 5 years cannot be considered a long period of cultivation. Rather, it is more of a case of stabilization of the culture. Therefore, after more than 10 yrs of continuous long-term maintenance, the biosynthesis of bioactive compounds is significantly reduced (Veremeichik *et al*. 2024; 2025a, b). In the present work, we elucidate not only the dynamics of biosynthetic parameters but also endogenic auxin alterations in *R. cordifolia* calli during 25 yrs of cultivation.

The most common traditional source of red dyestuffs is *R. cordifolia*, a well-known plant used in oriental medicine. Given the present reduction in the natural resources of *R. cordifolia*, the cultivation of *R. cordifolia* is essential (Lian *et al*., 2025). In addition to *R. cordifolia*, other members of the genus Rubia are of research interest, with more than 70 species from the Americas, Europe, Africa, and Asia and approximately 15 species from India. Some of the most common variants include *R. akane* Nakai., *R. tinctorum* L., and *R. peregrine* L. (Eskandarzadeh *et al*., 2022). In the present work, for the first time, we have shown that newly obtained 1-year-old *R. cordifolia* calli contain the major compound AQ of the "emodin type". The anthraquinone derivatives of the samples were studied using high-performance liquid chromatography coupled with PDA and MS detection. High-resolution mass measurements were performed *via* a time-of-flight mass spectrometer, and molecular formulas were developed for the detected compounds with a mass error of less than 1.1 mDa. In *R. cordifolia* tissues, "alizarin-type" AQs are usually found (Caro *et al*. 2012). AQs of the "emodin type" are synthesized *via* the acetate–malonate pathway with the participation of PKS III- and III-type polyketide synthetases. This type of AQ is detected in plants of families such as Polygonaceae, Rhamnaceae, and Fabaceae (Caro *et al*. 2012). In the Rubiacea family, the "emodin type" AQ robustaquinone B was detected by

Table 1. Anthraquinone derivatives determined in the crude extract of *R. cordifolia* callus lines and plant tissues (μg g^{-1} DW)

Peak №	Compound assignment	R	RN	Root	Leaf	Steam
1	Pseudopurpurin primveroside-433	223.5 ± 28.6^{c}	784.1 ± 35.1^{b}	1324.7 ± 80.2^{a}	ND	ND
2	Pseudopurpurin glucoside-433	41.3 ± 16.1^{c}	712.1 ± 49.9^{b}	2225.2 ± 424.9^{a}	ND	ND
3	Dihydroxy Rubiadin glucoside	ND	1372.5 ± 94.2	ND	ND	ND
4	Munjistin glucoside-404	30.7 ± 6.1^{c}	709.3 ± 57.5^{b}	6978.1 ± 503.5^{a}	ND	ND
5	Lucidin primveroside-404	ND	76.1 ± 3.8	ND	ND	ND
6	Ruberitrinic acid-416	ND	187.3 ± 15.3	ND	ND	ND
7	Dihydroxy Rubiadin	ND	71.2 ± 5.9	ND	ND	ND
8	Munjistin + Pseudopurpurin 420	49.1 ± 5.1^{c}	618.8 ± 56.6^{b}	1199.5 ± 48.1^{a}	ND	ND
	Total AQ	344.6 ± 50.1	4531.6 ± 484.1	$11{,}727.5 \pm 957.1$	ND	ND

*Catenarin or Trihydroxy-methoxy-anthraquinone glucoside

**Catenarin or Trihydroxy-methoxy-anthraquinone

ND not detected

The peak numbers correspond to those listed in Supplementary Table S2. Analysis was performed to compare callus lines (R, long-term-cultured 25-year-old callus culture; RN, newly obtained callus culture of *R. cordifolia*) and plant tissues (leaf, stem, and root). Different *letters* indicate statistically significant differences ($P < 0.05$, Fisher's LSD)

one- and two-dimensional NMR spectroscopy in *Cinchona succirubra* Pav. ex Klotzsch; however, it was confirmed by labelling that rings A and B of robustaquinone B are formed from chorismate and aketoglutarate *via* o-succinylbenzoate (Han *et al*. 2002). In another case, anthraquinone rubiquinone and its derivatives were described in the roots of several *Rubia* species as containing a hydroxyl group on the A-ring (Hu *et al*. 2020). However, we were unable to find any NMR spectroscopy data (Qiao *et al*. 1990). Therefore, the biosynthesis of "emodin-type" AQs has not been studied in the Rubiaceae family. The structure of the compound described in the present work also needs to be determined by NMR spectroscopy and pathway analysis by labelling. Using this young culture, it is possible to study the biosynthetic pathway and the main enzymes involved in biosynthesis. Furthermore, the pharmacological properties of the discovered compounds are no less interesting.

The AQ composition of the 25-year-old R calli was the same as that of the adventitious roots of the wild-type plants. However, while the total AQ content in 1-year-old calli RN was approximately twofold lower than that in roots, 25-year-old calli R contained 36 times less AQ than did roots did. Both types of cell cultures were obtained from parent plant leaf explants collected under the same natural conditions (43° 06′ 54″ N 131° 53′ 07″ E), but the differences were 25 years. Interestingly, the just-obtained callus line R contained approximately 0.8% DW of the total AQ (Mischenko *et al*. 1999). After 25 years, the total AQ content in the R callus line decreased more than 10 times to 0.05% DW. Unfortunately, at that time (1999), no qualitative analysis of the composition of AQ had been conducted, and we cannot compare how it has changed over time. In the aboveground part of *R. cordifolia* plants, no AQ was detected. However, traces of purpurin were detected in the aboveground parts of *R. cordifolia* plants growing in an experimental field located in the Henan Medicinal Botanical Garden of Henan University of Chinese Medicine (113°63′15'' E, 34°74′99'' N, 107 m). At this location, in root materials, only purpurin and mollugin were detected (Lian *et al*., 2025). However, despite the differences in qualitative composition, the total content of AQ in the roots was similar to our results, approximately 1% of DW. Differences in the qualitative composition of AQ in plant roots can be explained by different growing conditions and different growth stages. The presence of new compounds in young cell cultures suggests that the qualitative composition of AQ in roots also varies significantly depending on the growth stage. Research in this area is interesting and relevant, as it may lead to the discovery of new compounds with interesting properties.

The expression patterns of the enzymes involved in AQ biosynthesis were correlated with the AQ content. We investigated the expression of the key enzymes of the chorismate/*o*-succinylbenzoate pathway involved in the biosynthesis of the "alizarin-type AQ": ICS, isochorismate synthase; OSBL, O-succinylbenzoate ligase; and IPPI, isopentenyl diphosphate isomerase (Veremeichik et al. 2019). Thus, we can discuss the potential of *R. cordifolia* cell cultures as alternative sources of AQs. First, the quality of the AQ composition is important since AQ can have toxic activities and mutagenic and carcinogenic properties (Caro *et al*. 2012). As we have shown, monitoring the qualitative composition over time during long-term cultivation is important. On the other hand, temporary stabilization of the cell culture was accompanied by a decrease in the AQ content and a significant improvement in growth indicators. Thus, to judge the productivity of

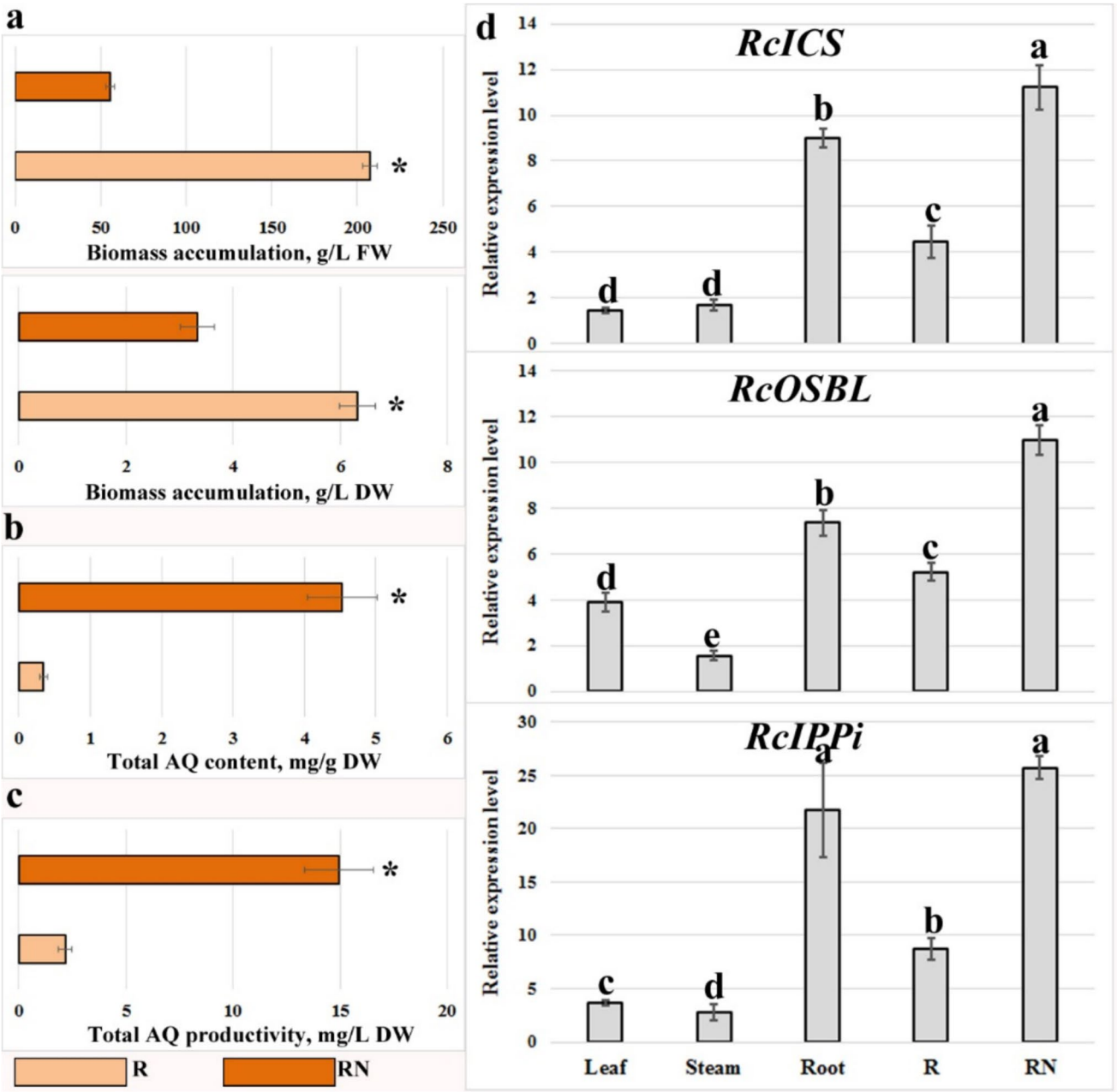

Figure 5. Growth, AQ productivity and AQ biosynthesis gene expression in *R. cordifolia* callus lines and plant tissues. Analysis was performed to compare callus lines (R, long-term-cultured 25-year-old callus culture; RN, newly obtained callus culture of *R. cordifolia*) and plant tissues (leaf, stem, and root). Biomass accumulation (*a*) for fresh (FW) and dry (DW) tissues was calculated as g per litter of agarized medium when 1 g of initial calli was inoculated on 50 mL. The total AQ content (*b*) in the R and RN calli of *R. cordifolia* was calculated as the equivalent of alizarin obtained from each chromatogram (recorded at a wavelength of 430 nm). The total AQ productivity (*c*, mg L^{-1}) was calculated as described in the Materials and Methods section. The mRNA levels of AQ biosynthesis genes (*RcICS*, isochorismate synthase; *RcOSBL*, *o*-succinylbenzoate-CoA ligase; *RcIPPi*, isopentenyl diphosphate isomerase) in R and RN calli of *R. cordifolia* were measured by real-time PCR and are reported as relative expression. The values were computed from the qPCR data using the $2^{-\Delta\Delta.Ct}$ method. The analysis was repeated three times. The data are presented as the means ± standard errors. Different *letters* above the *bars* indicate significantly different means ($P < 0.05$; Fisher's LSD). The *asterisks* above the *bars* denote statistically significant differences (*t*-test, $P < 0.05$).

a callus culture and compare various published data, it is necessary to consider both parameters. However, most often, % DW is indicated, which does not allow for an objective assessment of productivity while taking into account the increase. Thus, untreated *R. cordifolia* cultures can yield up to 0.5% DW. Genetic transformation

Table 2. Phenylpropanoid derivatives determined in the crude extract of *R. cordifolia* callus lines and plant tissues (μM g^{-1} DW)

Peak №	Compound assignment	R	RN	Root	Leaf	Steam
1	Caffeoylquinic acid	ND	ND	ND	1.45 ± 0.07	ND
2	Ferulic acid sucroside	ND	0.38 ± 0.02	ND	ND	ND
3	Coumaroylquinic acid	ND	ND	ND	2.48 ± 0.21[a]	0.16 ± 0.01[b]
4	Ferulic acid O-glucoside	ND	1.65 ± 0.29	ND	ND	ND
5	Chlorogenic acid	ND	0.44 ± 0.02[c]	0.47 ± 0.09[c]	35.19 ± 1.01[a]	3.83 ± 0.19[b]
6	Ferulic acid derivative	ND	0.57 ± 0.06	ND	ND	ND
7	Coumaroylquinic acid isomer	ND	ND	ND	2.31 ± 0.13	ND
8	Caffeoylquinic acid isomer	ND	ND	ND	1.85 ± 0.07[a]	0.42 ± 0.02[b]
9	Coumaroylquinic acid isomer	ND	ND	ND	4.19 ± 0.28[a]	0.16 ± 0.01[b]
10	Rutine isomer (260 nm)	ND	ND	ND	1.51 ± 0.04	ND
	Rutine (260 nm)	ND	ND	ND	3.39 ± 0.14	ND
	Total	±	3.06 ± 0.26[c]	0.47 ± 0.09[d]	52.39 ± 0.91[a]	4.58 ± 0.24[b]

ND not detected

The peak numbers correspond to those listed in Supplementary Table S3. Analysis was performed to compare callus lines (R, long-term-cultured 25-year-old callus culture; RN, newly obtained callus culture of *R. cordifolia*) and plant tissues (Leafleaf, stem, and root). Different *letters* indicate statistically significant differences ($P < 0.05$, Fisher's LSD or *t* test)

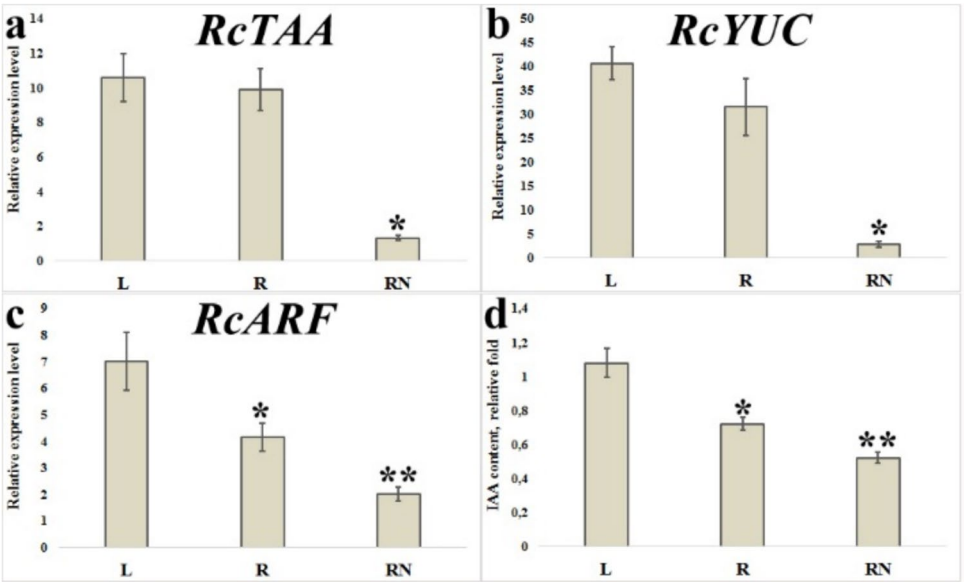

Figure 6. IAA biosynthesis gene expression and IAA content in *R. cordifolia* callus lines and plant tissues. Analysis was performed to compare callus lines (R, long-term-cultured 25-year-old callus culture; RN, newly obtained callus culture of *R. cordifolia*) and plant tissues (leaf and root). The mRNA levels of IAA marker genes (*a*) *RcTAA*, tryptophan aminotransferase-related protein; (*b*) *RcYUC* , indole-3-pyruvate monooxygenase YUCCA-like;(*c*) *RcARF*, auxin response factor) in R and RN calli of *R. cordifolia* were measured by real-time PCR and are reported as relative expression. The values were computed from the qPCR data using the $2^{-\Delta\Delta.Ct}$ method. The IAA content was measured using ELISA (*d*). The analysis was repeated three times. The data are presented as the means ± standard errors. The asterisks above the bars denote statistically significant differences ($P < 0.05$; Fisher's LSD).

and gamma irradiation can increase the AQ content to 5% DW (Murthy *et al.*, 2022). In our work, we have shown that untreated 1-year-old calli produce up to 0.5% DW, whereas 25-year-old calli produce up to 0.03% DW. Taking into account growth indicators, 2.5 and 15 mg of AQs were added per L of culture medium.

A study of the chromatographic profiles of the leaf and RN callus extracts revealed a number of phenylpropanoid compounds (Fig. 4). Among them, two flavonoids and nine hydroxycinnamic acid derivatives were detected. These 10 phenylpropanoid derivatives can be divided into four groups according to their order in the common

phenylpropanoid biosynthesis pathway: ferulic acid derivatives, coumaroylquinic acid derivatives, caffeoylquinic acid derivatives, and rutin derivatives. Ferulic acid derivatives are synthesized from *p*-coumaric acid at the early third stage of the common phenylpropanoid biosynthesis pathway and are found only in RN callus extracts. Caffeoylquinic derivatives are synthesized from *p*-coumaroyl-CoA at the fourth stage of the common phenylpropanoid biosynthesis pathway, where coumaroylquinic acid derivatives are intermediate compounds. Rutin is the final product of the flavonol branch of the common phenylpropanoid biosynthesis pathway (Bulgakov *et al*., 2018). In addition to these compounds, chlorogenic acid was present in both the RN callus extract and the extracts of all the analysed plant tissues. Moreover, in the root extracts, no other phenylpropanoid compounds were detected. In the leaf extracts, all the listed compounds were found, with the exception of the ferulic acid derivatives, which were specific to the 1-year-old calli RN. The extract of 25-year-old calli did not contain any phenylpropanoid compounds. Many plants contain high quantities of chlorogenic acid that respond to a range of pathological issues and have a variety of therapeutic benefits, especially those associated with age-related disorders and chronic metabolic diseases. It has a variety of multifaceted properties, such as anti-inflammatory, antioxidant, antipathogen, antitumour, and heart disease effects; skin conditions; diabetes mellitus; liver and kidney damage; and neuroprotective effects against neurodegenerative diseases and diabetic peripheral neuropathy (Nguyen *et al*., 2024). As a flavonoid found in many fruits and vegetables, rutin is commonly ingested by people. Numerous studies of the multisystem chemopreventive qualities of rutin in the biomedical literature have sparked significant consumer interest in this substance (Calabrese *et al*., 2024). In preclinical pharmacological methods, ferulic acid derivatives have shown encouraging antitumour properties (Ahmmed *et al*., 2025). Thus, in the absence of AQs, the above-ground parts of *R. cordifolia* plants contain large amounts of bioactive phenylpropanoids. Moreover, 1-year-old calli of *R. cordifolia* contain both AQs and phenylpropanoids. However, over time, the phytochemical composition of leaf-derived cell cultures mimics that of roots.

Since chorismate is a major precursor in both AQ biosynthesis and the biosynthesis of the major growth regulator IAA (Mano and Nemoto, 2012), we investigated IAA homeostasis in 1- and 25-year-old calli and plant tissues of *R. cordifolia*. With long-term cultivation, the level of endogenous IAA increases significantly, almost to the level of the native plant. Moreover, the level of IAA and the expression of IAA marker genes in the 1-year-old culture were significantly lower. This is a consequence of the fact that, during prolonged, multiyear cell culture, endogenous IAA increases almost to the level of the native plant. In a one-year-old culture, the IAA level was almost two times lower. The expression of IAA homeostasis marker genes in the 25-yr-old culture was just as widespread as that in the same plants, whereas in the one-yr-old culture, it was significantly lower. It can be assumed that cellular resources are redistributed over time in favour of IAA biosynthesis, which ensures stable and better growth. Furthermore, it can be hypothesized that modulating the concentration of exogenous IAA may influence AQ biosynthesis in 25-yr-old cultures. The culture requirements for exogenous IAA may have changed significantly. In any case, research in this area may shed light on the possibility of redistributing cellular resources to modulate the productivity of long-term cultured calli.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11627-026-10685-y.

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Author Contribution GNV Conceptualization, Data curation, Project administration, Supervision, Validation, Visualization, Writing—original draft.

VPB, Resources.

GNV, TOG, SAS, OAT, AAK, VPG, EVB, Investigation, Writing—original draft, Methodology, Formal Analysis.

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Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

Code Availability Not applicable.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication All the authors whose names appeared on the submission approved the version to be published and agreed to be accountable for all aspects of the work in ensuring that the questions related to the accuracy of integrity of any part of the work were appropriately investigated and resolved.

Conflict of interest The authors declare that they have no competing interests.

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