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EFFECTS OF CINNAMYL ALCOHOL ON THE PHENYLPROPENOID CONTENT IN *RHODIOLA ROSEA* PLANTS CULTIVATED *IN VITRO* AND *EX VITRO*

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The effect of cinnamyl alcohol (CA) on the biosynthesis of rosavins was examined in *Rhodiola rosea* L. plants during *in vitro* or *ex vitro* culture. An additional factor–a source of carbohydrates–was introduced into the *in vitro* culture experiments. The study revealed a high concentration of rosin in the *in vitro* culture during treatment with CA (67–98% of total rosavins). It was shown that glucose does not have a stimulatory effect on the production of rosavins in the *in vitro* culture but changes its ratio. In the *ex vitro* culture, an increase in the amount of total rosavins after the introduction of CA was caused by enhanced biosynthesis of rosavin and rosarin, whereas the level of rosin did not differ from a control. Additionally, a stimulatory effect of CA on the growth of root biomass and of the above-ground part of the plants was noted under *ex vitro* conditions. Thus, we demonstrated that the levels and ratio of rosavins differ significantly between the CA group and control group during *in vitro* and *ex vitro* culture. *In vitro* culture of regenerated *R. rosea* plants after treatment with CA can serve as a source of rosin (0.56%).

Keywords: roseroot, cinnamyl alcohol glycosides, biotransformation, glucose, HPLC.

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Introduction

Cinnamyl alcohol (CA) glycosides (rosin, rosarin, and rosavin: collectively known as rosavins) and salidroside are characteristic compounds of all formulations based on rhizomes or roots of *Rhodiola rosea* L. [1]. Rapid growth of the market of products based on *R. rosea* and the increasing demand for its raw materials pose a serious threat to populations of wild *R. rosea* plants around the world, thereby prompting extensive research to find alternative sources for the production of these biologically active compounds. Plant cell culture represents a promising alternative platform for the production of rosavins and salidroside. There are several strategies aimed at increasing the production of specialized metabolites in cultivation systems of plant cells, tissues, and organs: selection and optimization of nutrient media, the use of metabolic precursors (biotransformation), exposure to chemical and physical factors (elicitation), polyploidization, genetic engineering, and immobilization of plant cells [1–3]. In recent years, microbial synthesis emerged as a new strategy to produce bioactive phytochemicals [4, 5]. *De novo* biosynthesis of rosavin at 7.5 g/L has been achieved in *Escherichia coli* [6].

In *in vitro* cultures, a significant enhancement of the production of rosin and its derivatives is observed when the cultures are fed with a metabolic precursor: CA, cinnamaldehyde, or cinnamic acid. Furmanova et al. [7] have shown that CA can be transformed into its glycosides by roseroot compact callus aggregate cultures. Krajewska-Patan et al. [8] have proved that the transformation of CA can be performed on solid media as well. Mirmazloum et al. [9] have reported that in addition to CA, *trans*-cinnamic acid and cinnamaldehyde affect the production of CA

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glycosides in *R. rosea* callus culture. Javid et al. [10] by means of *in vitro*–cultured *R. rosea* plants have demonstrated that CA and cinnamaldehyde are beneficial because they improve the production rate of rosin and rosavin by 13.8- and 6.9-fold or by 92.7- and 8.0-fold, respectively, whereas *trans*-cinnamic acid does not affect the biosynthesis significantly. Hairy-root cultures of *R. kirilowii* (Regel) Maxim. supplemented with CA exhibit greater potential for the production of rosin and its derivatives in comparison to field-cultivated plants [11]. György and Hohtola [12] have shown that it is possible to increase, even to double the biotransformation rate of CA by the addition of glucose to the medium. Those authors have noticed that rosavin is not produced at all when only sucrose is used.

To date, the highest rosavin concentration in *in vitro* cultures of *R. rosea* (cell suspension cultures) has been achieved in a study by Furmanova et al. [7] and amounts to 10.01 mg/g under the influence of 2.5 mM *trans*-CA, whereas in natural specimens, this parameter is up to 37 mg/g [11]. At the same time, in *R. rosea* compact callus aggregate cultures, concentrations of other rosavins—rosin and rosarin—are even higher than those in field-cultivated plants [11]. In our study, a comparative analysis of effects of CA on the biosynthesis and ratio of rosavins in regenerated plants during *in vitro* and *ex vitro* culture is carried out for the first time.

The aim of the work was to investigate the effects of CA on the levels and ratio of rosavins in *R. rosea* plants *in vitro* and *ex vitro* culture and to find optimal processing conditions.

Materials and Methods

Plant Material. Rhizomes and seeds of *R. rosea* served as the plant material for the experiments. The sampling site was located in Barun-Khemchiksky District (Tuva Republic, Russia), near the Kara-Sug River, 51°41'57.0"N 89°53'35.0"E, in a subalpine meadow, 1248 m above sea level [13].

In Vitro Propagation. Seeds were surface-sterilized with a 1% sodium hypochlorite solution (Macklin, Shanghai, China) for 20 min with continuous mixing on an Elmi orbital shaker (100 rpm; Elmi, Latvia), followed by three rinses with sterile distilled water. The MS medium (HopeBio, Qingdao, China) with a halved content of macro- and microelements (½ MS) and supplemented with 6 g/L agar (Coolaber Science & Technology Co., LTD., Beijing, China) and 30 g/L sucrose was used for seed germination. Thirty days after the germination, the plants were transplanted onto the MS medium supplemented with 0.5 μM 6-benzylaminopurine (BAP) (Coolaber Science & Technology Co., LTD., Beijing, China). pH was adjusted to 5.8 before sterilization of the medium by autoclaving at 121 °C for 20 min. On this medium, the plants developed a good root system, and a separate rooting procedure was not needed.

The Precursor Feeding Experiment. Regenerated plants were transferred to the 1/2 MS liquid nutrient medium supplemented either with 2 mM CA (Aladdin, Shanghai, China) and 30 g/L sucrose or with 2 mM CA, 20 g/L sucrose, and 10 g/L glucose. The 1/2 MS liquid medium with 30 g/L sucrose served as a control. Plants were cultivated on the Elmi shaker (Elmi, Latvia) at 100 rpm for 24, 36, 72, or 96 h. For the cultivation, 100 mL flasks with 50 mL of the medium were employed. Five plants were placed in each flask. CA was dissolved in water with constant stirring and heating. The precursor solutions were filter-sterilized using a syringe and a 0.22 μm pore size filter (Jet Biofil, China). After sterilization, the precursor solutions were mixed with the already autoclaved and slightly cooled 1/2 MS media.

Cultivation conditions were as follows: illumination 3000 lux, the 16 h/8 h light/dark cycle, and temperature 22±2 °C.

Adaptation to Ex Vitro Conditions. Regenerated plants were cleansed from agar with running water and were planted in plastic containers at 10 specimens per container filled with vermiculite (0.5–2.0 mm fraction, Crewix, Uzbekistan). During the first 10 days, the containers were covered with plastic lids to ensure high humidity. Distilled water was utilized for watering and spraying. A month after the adaptation, the substrate was watered with distilled water containing 2 mM CA. Plants that were watered only with distilled water served as a control. The watering was performed at 10 mL per plant. After the addition of CA, the plants were grown for 24, 36, 72, or 96 h. CA was dissolved in water with constant stirring and heating.

Cultivation conditions were as follows: illumination 3000 lux, the 16 h/8 h light/dark cycle, temperature 22±2 °C.

Drying of Plant Material. Drying of plant material was carried out according to Peschel et al. [14]. The plant material after treatment and the control specimens were washed once in distilled water and then dried in a LOIP LF 240/300-VS1 thermostat at 45 °C (LIOP, Russia) until stable weight (for 3 days).

The natural material (rhizome) was cleaned of soil, then cut into 3-mm-thick slices and dried in the LOIP LF 240/300-VS1 thermostat at 45 °C (LIOP, Russia) until stable weight (for 5–6 days).

Extraction and HPLC Analysis of Rosavins. Double extraction was performed to extract phenolic compounds. An exactly weighed sample (0.2 g) of the crushed material was extracted via maceration with 10 mL of aqueous 50% ethanol for 5 days, and then with 20 mL of 70% ethanol for 60 min in a water bath at 60–70 °C [15]. The combined extract was concentrated (via evaporation) to 20 mL and passed through a membrane filter with a pore diameter of 0.45 μm. HPLC analysis of the aqueous–ethanol extracts was carried out using an Agilent 1200 system with a diode array detector and the ChemStation software for data processing (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was performed at 25 °C on a Zorbax SB-C18 column (4.6 × 150 mm, 5 μm internal diameter) (Agilent Technologies). The mobile phase consisted of MeOH (solvent A) and 0.1% orthophosphoric acid in water (solvent B). The gradient was started with an A–B mixture at 22 : 78 (v/v) followed by a linear gradient to 70 : 30 (v/v) for the first 30 min, and then to 100 : 0 (v/v) from minute 30 to minute 32. A return of the mobile phase to 22 : 78 (v/v) was implemented from minute 32 to minute 36. The flow rate was set to 1 mL/min. The sample injection volume was 10 μL. Tracking of chromatograms was conducted by means of absorbance at 220, 255, 270, 290, 325, 340, 350, 360, and 370 nm. Concentrations of substances were calculated by detection at 255 nm. Quantification of rosavins was carried out according to the calibration curve for rosavin (Aobious, Gloucester, MA, USA) in the concentration range of 10–300 μg/mL.

Statistical Analysis. All the data were processed in the STATISTICA 6.0 software (Statsoft Inc., Tulsa, OK, USA), are reported as mean ± standard error (SE) of three biological replicates, and were compared using ANOVA followed by Duncan's multiple-range test. Differences between the means were considered statistically significant at $p \leq 0.05$.

Results and Discussion

Growth Parameters. During the *in vitro* culture on the MS medium supplemented with 0.5 μM BAP, on average 51.75-mm-tall plants were obtained with 5–7 leaves and a well-developed root system (root length of 43.23 mm and dry biomass of 38.63 mg on average; Figure 1a). After treatment with CA and glucose, the biomass of the roots and aboveground parts of the plants in the *in vitro* culture did not statistically differ from the control (Table 1).

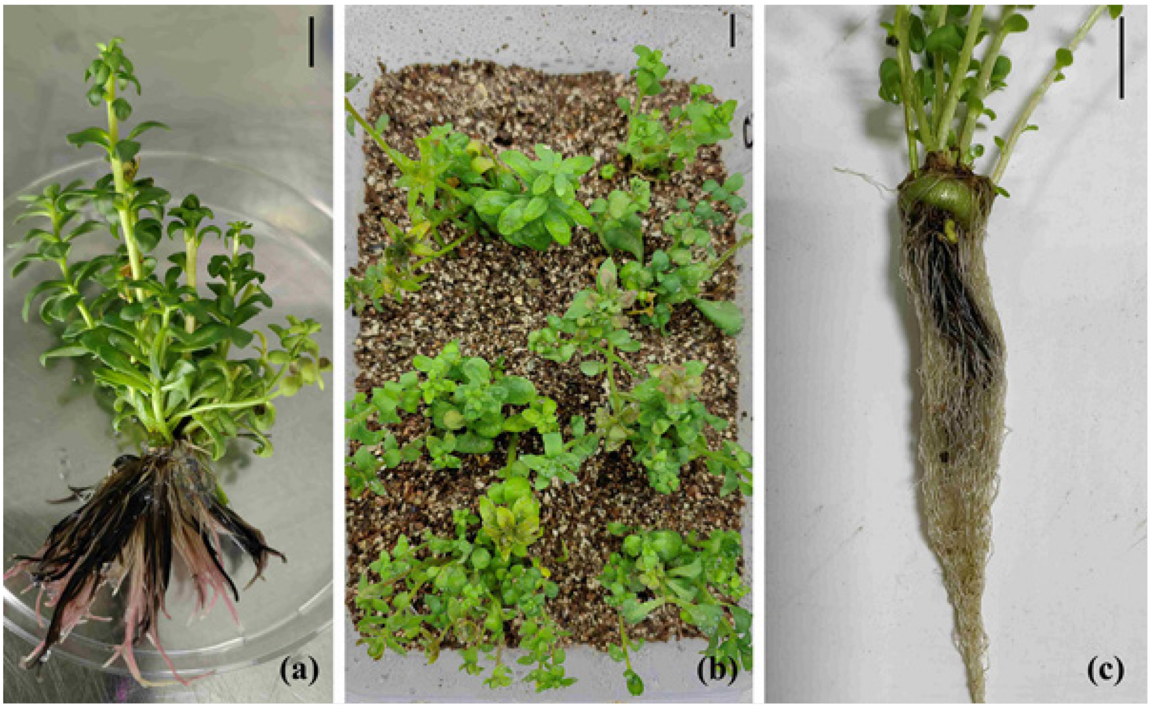

Fig. 1. *Rhodiola rosea* plants in *in vitro* culture on the MS medium supplemented with 0.5 μM BAP (a) and in *ex vitro* culture in vermiculite after 30 days of cultivation: general view (b) and the formed rhizome with roots (c). Scale bar: 1 cm

After a month of cultivation under the *ex vitro* conditions in vermiculite, rhizome formation was noted, the height of the plants was 72.45 mm on average, and the number of leaves 6–7 (Fig. 1b). Under the *ex vitro* conditions during treatment with CA, an increase in the biomass of the aboveground part by 1.4-fold and in the biomass of roots by 1.9-fold was noted after 96 h of the cultivation; the weight of the rhizome did not differ from the control (Table 2).

Our data on growth parameters are not consistent with results of other researchers, who have observed a negative effect of CA on *in vitro* culture of *Rhodiola* species. For instance, for the culture of native roots and hairy roots of *R. kirilowii*, a decrease in root growth of ~48% and ~55%, respectively, has been registered after the addition of CA [11], and for compact callus aggregate culture of *R. rosea*, a decrease in biomass by 20–50% has been observed depending on the concentration of CA [16].

Levels of Rosavins. The Natural Specimens. Relative abundance of rosavins in the natural specimens of *R. rosea* was 2.36%; in particular, relative abundance of rosavin was 1.73%, of rosarin 0.47%, and of rosin 0.16%. The rosavin : rosarin : rosin ratio was 73 : 20 : 7. In a study by Kotsupiy et al. [15], it was found that variation of the total level of rosavins in natural specimens of *R. rosea* from the Altai Mountains is 21–30%, and the greatest variation was registered for rosin: up to 40%. Previously, we have reported that under the conditions of introduction into the forest-steppe zone of Western Siberia, the predominant phenylpropanoid in *R. rosea* is rosavin, except for two specimens in which rosin was the main phenylpropanoid, and the variation of the total concentration of rosavins was ~50% [17].

Table 1. Biomass of *R. rosea* plants in *in vitro* culture during treatment with 2 mM CA and 1% glucose (GL) for 24, 48, 72, or 96 h

Treatment	Aboveground part, mg	Underground part, mg
Control 96 h	47.14±1.25a	38.18±2.37a
CA 24 h	44.21±2.32a	45.26±0.95a
CA 48 h	53.41±0.98a	46.08±1.14a
CA 72 h	43.09±1.15a	42.21±1.75a
CA 96 h	46.30±2.06a	44.19±2.04a
CA+GL1% 24 h	44.15±2.13a	43.16±0.99a
CA+GL1% 48 h	43.14±1.47a	44.21±1.52a
CA+GL1% 72 h	45.22±2.27a	46.23±2.01a
CA+GL1% 96 h	48.34±1.35a	42.15±1.14a

Values followed by the same letter(s) within a column are not significantly different at $p \leq 0.05$ according to Duncan's multiple-range test.

Table 2. Biomass of *R. rosea* plants under *ex vitro* conditions during treatment with 2 mM CA for 24, 48, 72, or 96 h

Treatment	Aboveground part, mg	Roots, mg	Rhizome, mg
Control 96 h	45.14±1.17b	15.09±1.12b	51.23±2.13a
CA 24 h	56.21±2.06b	17.11±0.98b	46.19±1.23a
CA 48 h	53.18±1.91b	22.13±1.25ab	50.14±1.45a
CA 72 h	61.23±1.21a	26.18±1.17a	41.25±1.63a
CA 96 h	63.15±1.35a	28.15±0.98a	49.21±1.47a

Values followed by the same letter(s) within a column are not significantly different at $p \leq 0.05$ according to Duncan's multiple-range test.

In Vitro Culture. Relative abundance of rosavins in the underground part of our plants during *in vitro* culture ranged from 0.016% in the control to 0.623% during treatment with CA for 72 h (Fig. 2a). It was noted that treatment with CA without the addition of glucose leads to greater accumulation of rosavins. The highest relative abundance of rosavins in both supplementation groups was achieved after 72 h.

In the control specimens, after treatment with CA or with CA together with glucose, the main rosavin was rosin: relative abundance of 67% to 98% of total rosavins (Fig. 2b), in contrast to the natural specimens, where the main CA glycoside was rosavin, and rosin constituted only 7% of the total rosavins. Relative abundance of rosin in the natural specimens was 0.160%, whereas during the *in vitro* culture in the control, it was 0.012%, and during treatment with CA for 72 h: 0.562% (46.83 times more than in the control and 3.51 times more than in the natural specimens). Earlier, György and Hohtola [12] have shown that it is possible to increase and even to double the biotransformation rate of CA by the addition of glucose to the medium. We noticed that the introduction of glucose into the nutrient medium shifts the ratio of rosavins toward a higher proportion of rosavin but does not significantly increase relative abundance of this compounds.

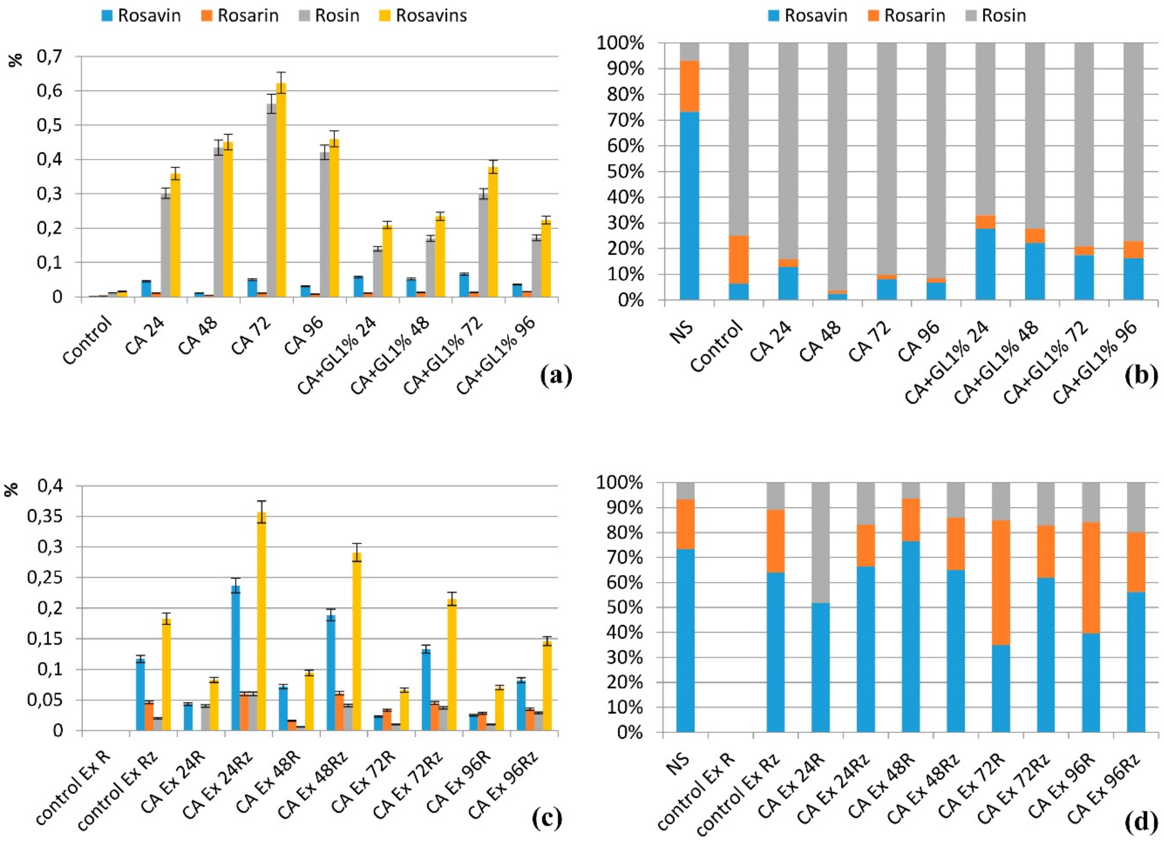

Fig. 2. Relative abundance (a, c) and the ratio (b, d) of rosavins during *in vitro* (a, b) and *ex vitro* (c, d) culture of *R. rosea* after treatment either with 2 mM CA or with 2 mM CA and 1% glucose (GL) for 24, 48, 72, or 96 h

Ex Vitro Culture. Under *ex vitro* conditions, the ratio of rosavins in the rhizome of the regenerated plants was similar to that in the natural specimens: rosavin 68%, rosarin 21%, and rosin 11% of total rosavins (Fig. 2d). When CA was added, an increase in relative abundance of rosavins was noted in the rhizome (maximum: 1.95 times after 24 h of treatment) and roots (maximum: 5.88 times after 48 h of treatment). In this context, the upregulation of rosavins was due to higher relative abundance of rosavin in the rhizome and of rosavin and rosarin in the roots. The highest relative abundance of rosavin was 0.237% in the rhizome after 24 h of treatment, whereas in the roots: 0.072% after 48 h of treatment. By contrast, at 72 h after treatment with CA, relative abundance of rosavins dropped to the control level (Fig. 2c).

It should be pointed out that without the addition of the biosynthesis precursor, rosavins were synthesized in the *in vitro* culture of *R. rosea* at very low relative abundance (0.016%), consistently with previously obtained data [3, 17]. Nonetheless, at the stage of adaptation to *ex vitro* conditions, in the control, relative abundance of rosavins was already 0.180% and was linked with the formation of the rhizome during this growth period.

We noticed that relative abundance of rosavins significantly decreased at 96 h of treatment with CA during *in vitro* culture and at 72 h of CA treatment during *ex vitro* culture. In a study by Grech-Baran et al. [11], it was shown that approximately 80–95% of CA glycosides are released into the medium during culturing of *R. kirilowii*. Furthermore, the highest yield of rosavin (505±106 mg/L) was observed in the culture of hairy roots with the addition of CA on the day of inoculation of the culture and the addition of sucrose on the 14th day of cultivation. We can hypothesize that the secretion of rosavins from cells (roots) is explained by little or no secondary growth of the underground part and a small number of specialized storage cells within plants *in vitro* and within young plants *ex vitro*. It is well known that the concentration of rosavins increases with the age of plants (explained by lignification processes among other things) and under the influence of severe soil/climatic conditions [14, 18]. In our opinion, the finding of the secretion of rosavins into the culture medium deserves further thorough research. Besides, it is known that not only *R. rosea* contains rosavins; this class of substances is also present in *R. quadrifida* (Pall.) Fisch. & C.A.Mey., *R. sachalinensis* Boriss., and *R. kirilowii*, and *in vitro* culture of some of them exceeds the productivity of *in vitro R. rosea* culture in terms of rosavins. Creation of the most optimal biotechnological system for the production of rosavins probably depends not so much on the choice of an initial specimens containing the highest level of rosavins but on the selection of *in vitro* cultures of *Rhodiola* representatives that are capable of launching their own enzymatic systems for effective conversion of metabolic precursors into cinnamyl alcohol glycosides.

Conclusions

In our work, a stimulatory effect of CA on the production of rosavins was observed in both *in vitro* and *ex vitro* cultures. Additionally, it was found that the supplementation with CA increases the biomass of the roots and of the aboveground part in *R. rosea* under *ex vitro* conditions. A significant increase in relative abundance of rosin in the roots of *R. rosea* was detected during *in vitro* culture after treatment with CA for 72 h (46.83-fold as compared to the control and 3.51-fold in comparison with the natural specimens). At the same time, in *ex vitro* culture, an increase in relative abundance of rosavins during CA treatment was due to higher relative abundance of rosavin in the rhizome and of rosavin and rosarin in the roots, whereas relative abundance of rosin did not differ from the control. In *in vitro* culture, the main rosavin was rosin (up to 98% of total rosavins), and in *ex vitro* culture, the ratio of rosavins in the rhizome of the regenerated plants was similar to that in the natural specimens: rosavin 68%, rosarin 21%, and rosin 11%. *In vitro* culture of regenerated *R. rosea* plants after treatment with CA for 72 h can serve as a source of rosin (relative abundance: 0.56%).

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Conflict of Interest

The authors of this work declare that they have no conflicts of interest.

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