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Enhancing the Accumulation of Rosavins in *Rhodiola rosea* L. Plants Grown In Vitro by Precursor Feeding

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Abstract: *Rhodiola rosea* produces nearly 150 bioactive compounds. Cinnamyl alcohol glycosides (CAGs) are among the most important secondary metabolites which are specific to this plant species, exhibiting adaptogenic properties along with salidroside. However, raw material supplies for the pharmaceutical industry are hindered by limited access to the plant material. The species is endangered and protected in many areas: cultivation is long and ineffective. Precursor feeding has been found to be an effective strategy for improving the production of secondary metabolites in various plant tissues cultures, including in *Rhodiola* species. In this study, whole *R. rosea* plants grown in vitro were subjected to three different precursor treatments, including with *trans*-cinnamic acid, cinnamaldehyde and cinnamyl alcohol at 2 mM concentrations. The different treatments affected the secondary metabolite production differently. *Trans*-cinnamic acid did not affect the synthesis significantly, which contradicts earlier studies with cell suspensions. On the other hand, cinnamyl alcohol and cinnamaldehyde were beneficial, improving the production rate of rosin and rosavin by 13.8- and 6.9-fold, and 92.7- and 8.0-fold, respectively. The significant improvement in CAG accumulation due to cinnamaldehyde treatment was unexpected based on previous studies. In addition, cinnamaldehyde triggered the production of rosarin, which the other two treatments failed to do. The study presents the beneficial application of precursors to whole plants grown in vitro.

Keywords: biotransformation; cinnamyl alcohol glycosides; HPLC; precursor feeding; roseroot

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1. Introduction

Cinnamyl alcohol glycosides (CAGs) are a group of compounds specific to *Rhodiola rosea*. Collectively named rosavins, they are used to treat broad range of medical complications like stress, anxiety, fatigue, and depression [1]. In addition to their proven neuroprotective [2], hepatoprotective [3], anti-aging, anti-cancer and immuno-stimulant properties [4], it has been used in traditional Asian and Scandinavian medicine for centuries [5]. *Rhodiola rosea* belongs to the family Crassulaceae, which is a perennial, dioecious herb that grows in high altitudes and Arctic regions of Asia and Europe. It is also known as kings crown, rose root or golden root. *R. rosea* contains a wide diversity of secondary metabolites mainly produced in the root and rhizome tissues, among which salidroside (glycoside of tyrosol) and CAGs are important for attributing the pharmaceutical properties to this plant species. Due to its various pharmaceutical properties and slow growth (usually 4–6 years), it has been overharvested, and has been declared endangered in many regions.

The slow growth and depletion from the natural environment have necessitated protection of the species and the search for alternatives to produce the pharmaceutically important compounds. Plant cell culture represents an effective tool for producing these compounds against extraction from whole plants (natural habitat) or by chemical synthesis [6,7]. In vitro cultures provide a possibility to enhance the synthesis, but the production of glycosides is far lower than that produced in the natural habitat [8]; or can even be

completely absent [9]. Low productivity, biosynthetic instability, and problems in the scaling up of compounds are some of the reasons [10]; among which, low product yield is a universal phenomenon [11,12]. Efforts have been made to increase productivity following different strategies, including precursor feeding and elicitation, in situ product removal, immobilization, the screening and selection of high-production cell lines, hairy root cultures, etc. [13–15]. In addition to applying elicitors, precursor feeding is an effective method to boost bioactive compound biosynthesis either alone or in combination with elicitors. This strategy has been successfully used with cell suspension cultures of *Rhodiola* species. Xu et al. [16] found that both tyrosine and tyrosol improved the salidroside content of *R. sachalinensis* CCA cultures, which was also confirmed by Wu et al. [17]. Furmanova et al. [18,19] and György [20] studied the same biotransformation reaction with *R. rosea* cell cultures and found that tyrosol is very effectively transformed into salidrosid. Krajewska-Patan et al. [21] proved that tyrosol can be taken up and transformed from solid media by *R. rosea* callus. The production of the cinnamyl alcohol glycosides by biotransformation is less well studied, which can be explained by the fact that these compounds are only produced in *R. rosea*. Furmanova et al. [8,22] showed that cinnamyl alcohol can be transformed to its glycosides (CAGs) by roseroot CCA cultures. Krajewska-Patan et al. [23] proved that the transformation of cinnamyl alcohol can be performed on solid media as well. Recently, Mirmazloum et al. [24] showed that in addition to cinnamyl alcohol, *trans*-cinnamic acid and cinnamaldehyde affected the CAG production in *R. rosea* callus culture.

In this study, the effects of different precursors involved in CAG biosynthesis pathways of *R. rosea* were explored, to enhance the production of rosavins in root and rhizome tissues of plants in vitro.

2. Materials and Methods

2.1. Plant Material, Germination and Development

The mature seeds of *Rhodiola rosea* were collected from a wild population (Hochkar, Austria 47°43′21.5″ N, 14° 55′26.8″ E), surface-sterilized by soaking in 40% (*v/v*) NaOCl for 7 min and then immersed in 70% (*v/v*) ethanol for 30 s, followed by washing three times with sterile distilled water. For germination, half-strength MS (Murashige and Skoog, 1962) medium containing 25 g/L sucrose was prepared and solidified with 6.5 g/L plant agar (Duchefa, Haarlem The Netherland). The pH was adjusted to 5.8 before sterilizing the medium by autoclaving at 121 °C for 20 min. After sterilization, 15 mL of medium was distributed into plastic Petri dishes in a laminar hood. Seeds were germinated at 24 °C under a photoperiod of 16 h light and 8 h dark [24]. *R. rosea* seeds germinated on hormone-free half-strength MS medium within one week. The seedlings exhibited a germination rate 80% and are a promising explant type for in vitro mass propagation of the species. In vitro plantlets were sub-cultured for 5–6 cycles after every three to four weeks, and well-rooted plants, approximately 6 months old, were used for the experiment. Plants selected for the experiment were 6–7 cm tall with well-developed root systems. The applied precursors were incorporated in the MS media in a final concentration of 2 mM for biotransformation. Plants possessing a sufficient root system were used for precursor feeding experiments.

2.2. Precursor Feeding Experiment

The final concentration of each precursor compounds in liquid culture was 2 mM, based on our previous results [20]. *Trans*-cinnamic acid was dissolved in water, whereas cinnamaldehyde and cinnamyl alcohol were dissolved first in a few drops of ethanol and then diluted with water. The precursor solutions were filter-sterilized using a syringe and a 0.22 µm pore size filter. After sterilization, the precursor solutions were mixed with the already autoclaved and slightly cooled MS media.

The explants were put into glass jars containing 35 mL of biotransformation MS media with or without (control) 2 mM of each precursor in six replicates (3 × 6 jars plus 6 controls). Plants were carefully taken out from the culture jars, and roots were gently cleaned with

forceps to remove the traces of media. The control plants were cultured on precursor-free MS media. After 1, 2, 4 or 7 days of treatment, root samples were harvested and washed three times in distilled water to remove the residual precursor and medium. Then, roots were separated from shoots and dried in an oven at 40 °C for 24 h. The roots (100 mg) were ground with a pestle and mortar and the dry weight was recorded.

2.3. Extraction for HPLC Analysis

Oven-dried grounded roots of *R. rosea* were extracted three times with 2 mL methanol at room temperature with an ultrasonic bath for 30 min followed by centrifugation for 5 min at 14,000 rpm. HPLC analysis was carried as per the earlier report of Javid et al. [25] The supernatant was filtered through a 0.22 µm PTFE filter (Nantong FilterBio Membrane Co., Ltd., Nantong, Jiangsu, China), then used for HPLC analysis. The samples were analyzed on a Waters 2690 HPLC system with a Waters 996 diode array detector (Waters Corporation, Milford, MA, USA) equipped with a Luna C18 column (150 × 4.6 mm, 5 µm) by Phenomenex (Torrance, CA, USA). Eluents consisted of 0.1% *v/v* formic acid (A) and acetonitrile (B). The gradient program of Avula et al. [26] was applied: 0.0 min, 5% B; 30.0 min, 19% B; 35 min, 19% B. The solvent flow rate was 1.0 mL/min, and the column temperature was set to 25 °C; 10 µL extracts were subjected to analyses on the column.

Individual stock solutions of the standards (rosarin, rosavin and rosin) (Goldenroot, *Rhodiola rosea* standards kit, LGC Standards, UK) were prepared to a final concentration of 1 mg/mL by dissolving the compounds in methanol. Equal parts of the standard solutions were mixed to gain the stock solution. For the preparation of calibration standards, stock solution was diluted with methanol. The calibration curves were constructed over the wide concentration range of 0.1–100 µg/mL. All peaks were integrated on their absorption maxima (251 nm for rosarin, rosavin and rosin, 275 nm for tyrosol and salidroside). The triplicate analysis of each working solution at 10 concentration levels was performed for calibration, i.e., 100 µg/mL, 60 µg/mL, 30 µg/mL, 10 µg/mL, 6 µg/mL, 3 µg/mL, 1 µg/mL, 0.6 µg/mL, 0.3 µg/mL and 0.1 µg/mL. The calibration curves were plotted using a 1/x-weighted linear model for the regression of peak area vs. analyte concentration. The determined linearity ranges can be seen in Table 1, along with the regression equations and the coefficients of determination. The limit of detection (LOD) and limit of quantitation (LOQ) values were determined at 3 and 10 times the signal-to-noise ratio, respectively (Table 1).

Table 1. The validation parameters of the standard compounds.

Compound	Rt (min)	Equation	LOQ (µg/mL)	LOD (µg/mL)
Rosarin	25.09	y = 23,313x + 24,628	1.49	0.447
Rosavin	25.91	y = 27,503x + 11,329	0.56	0.168
Rosin	26.76	y = 34,359x + 16,492	0.84	0.252

Rt—retention time, LOQ—limit of quantification. LOD—Limit of detection. R^2—The determination coefficients for rosarin, rosavin and rosin were 0.9985, 0.9976 and 0.9979, respectively.

2.4. Statistical Analysis

Statistical processing of the results and analysis of the obtained data was carried out using MS Excel. All experiments were repeated with six independent replicates. Outlier data were excluded after application of the Nalimov test. For comparison of the metabolites at each time point, Students' *t*-tests were used. The significance level was set at $p \leq 0.05$. The data are presented as the mean values and standard deviation.

3. Results

3.1. Precursor Treatment and Analysis

Three precursors (*trans*-cinnamic acid, cinnamaldehyde and cinnamyl alcohol) were tested for transformation into rosavins (rosin, rosarin and rosavin). Root samples were harvested 1, 2, 4 and 7 days after the precursor feeding. All three rosavins could be detected

both in the control and in the treated samples, although contents in the control samples were in smaller magnitudes than in the treated samples. The individual differences were high, showing high standard deviations (Table 2).

Table 2. The contents of studied rosavins in the control (Ctrl) and the *trans*-cinnamic acid (T1)-, cinnamaldehyde (T2)- and cinnamyl alcohol (T3)-treated plants 1/2/4/7 days after the precursor feeding (in % *m/m*). Mean values ± standard deviation were calculated based on five treated individuals.

	Day	Rosarin Mean	Rosavin Mean	Rosin Mean
Ctrl	1	0.0014 ± 0.0019	0.0078 ± 0.0056	0.0010 ± 0.0017
	2	0.0013 ± 0.0018	0.0090 ± 0.0105	0.0004 ± 0.0009
	4	0.0046 ± 0.0035	0.0129 ± 0.0070	0.0006 ± 0.0009
	7	0.0022 ± 0.0034	0.0119 ± 0.0082	0.0000 ± 0.0000
T1	1	0.0049 ± 0.0058	0.0146 ± 0.0121	0.0026 ± 0.0000
	2	0.0094 ± 0.0086	0.0174 ± 0.0158	0.0034 ± 0.0033
	4	0.0021 ± 0.0034	0.0072 ± 0.0097	0.0031 ± 0.0069
	7	0.0070 ± 0.0048	0.0107 ± 0.0180	0.0000 ± 0.0036
T2	1	0.0010 ± 0.0023	0.0092 ± 0.0107	0.1648 ± 0.0968
	2	0.0065 ± 0.0052	0.0211 ± 0.0056	0.2711 ± 0.1025
	4	0.0063 ± 0.0064	0.0361 ± 0.0129	0.3230 ± 0.1307
	7	0.0199 ± 0.0192	0.0616 ± 0.0624	0.1873 ± 0.1544
T3	1	0.0121 ± 0.0079	0.0230 ± 0.0099	0.1009 ± 0.0505
	2	0.0066 ± 0.0049	0.0132 ± 0.0047	0.1048 ± 0.0396
	4	0.0066 ± 0.0056	0.0371 ± 0.0174	0.1136 ± 0.0903
	7	0.0128 ± 0.0063	0.0467 ± 0.0275	0.0996 ± 0.0893

3.2. Effect of Trans-Cinnamic Acid Feeding on the Production of Rosavins

The addition of *trans*-cinnamic acid enhanced the content of none of the studied cinnamyl alcohol glycosides (Figure 1). All compounds were detected in very small amounts (0.001–0.018%). The contents of rosavin and rosarin seemed to be elevated in the treated samples. However, the statistical analysis did not prove it to be significant, because of the big standard deviation, i.e., the large individual differences among the plantlets.

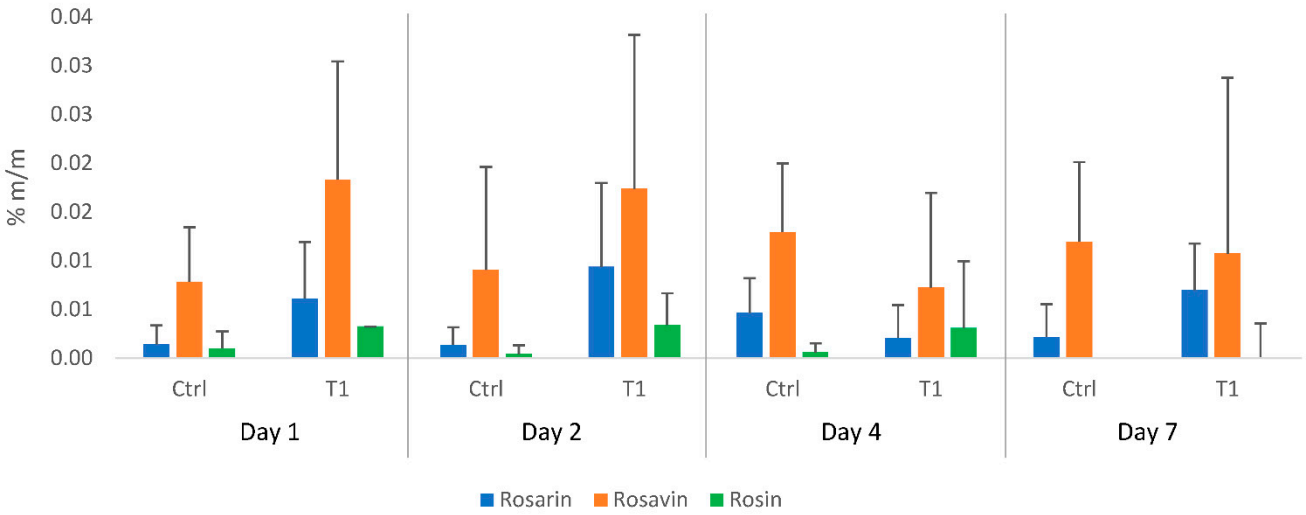

Figure 1. Effect of *trans*-cinnamic acid feeding on the production of rosavins. The abbreviations of letters indicate: Ctrl—control, T1—*trans*-cinnamic acid treatment.

3.3. Effect of Cinnamaldehyde Feeding on the Production of Rosavins

In the experiment when 2 mM cinnamaldehyde was supplemented to the media, the rosin content increased significantly even by the first day (0.16%) after the treatment (Figure 2). The highest rosin content was recorded four days after the treatment (0.32%), and then by the seventh day, it decreased to 0.18% (*m/m*), because of its conversion to rosavin and rosarin by some enzymatic modification with the addition of arabinose and/or arabinofuranose. Additionally, the content of rosavin and rosarin increased continuously during the seven-day observation. Due to the high standard deviations, the increase in rosarin was not significant, and for rosavin only the content on day 4 was significantly higher (0.03%) compared to the control (0.01%). The contents of rosavin in control samples were negligible. The accumulations of rosin, rosavin and rosarin in the treated samples after seven days were 374-, 5- and 9-fold higher, respectively, as compared with the control samples.

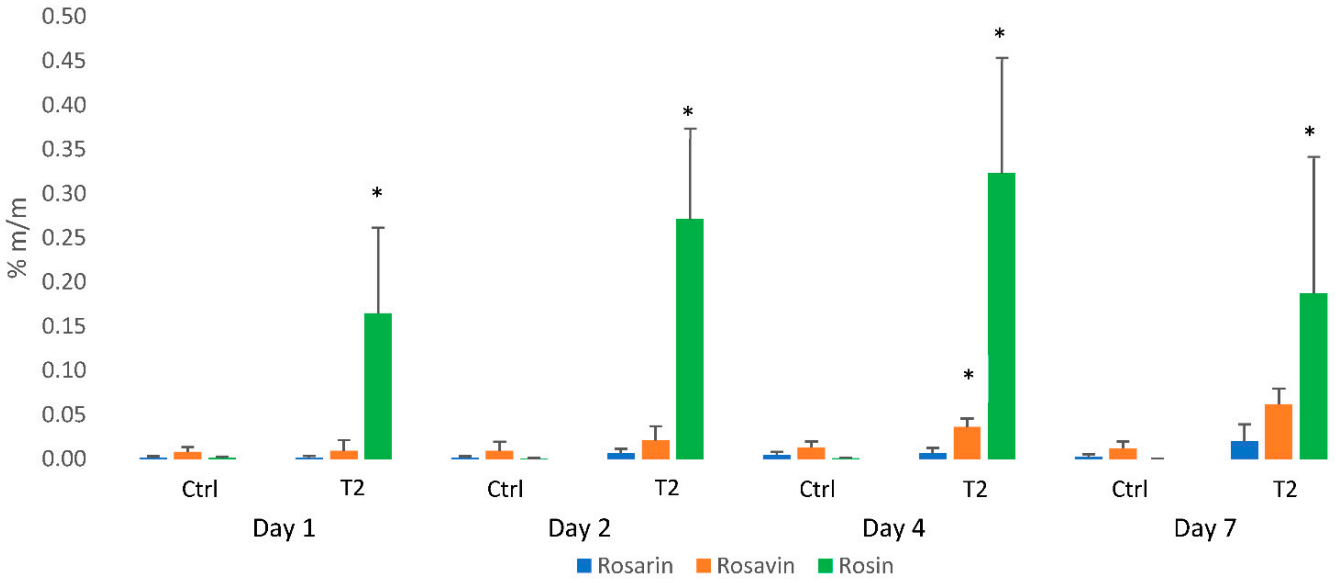

Figure 2. Effect of cinnamaldehyde feeding on the production of rosavins. The abbreviations of letters indicate: Ctrl—control, T2—cinnamaldehyde treatment. * significant ($p < 0.05$) deviation from the control based on Student's *t*-tests.

3.4. Effect of Cinnamyl Alcohol Feeding on the Production of Rosavins

In the experiment when 2 mM cinnamyl alcohol was supplemented to the media, the content of all three rosavins increased significantly and continuously over the course of seven days. The highest accumulation of rosin (0.13%) occurred again four days after the treatment, as in case with cinnamaldehyde feeding (Figure 3); then, it slightly decreased to 0.09% (*m/m*) due to it being further transformed into rosarin and rosavin, with the highest contents of rosavin (0.04%) and rosarin (0.012%) on day seven. The accumulations of rosin, rosavin and rosarin in the treated samples 7 days after the treatment were 200-, 4- and 6-fold higher, respectively, as compared with the control samples.

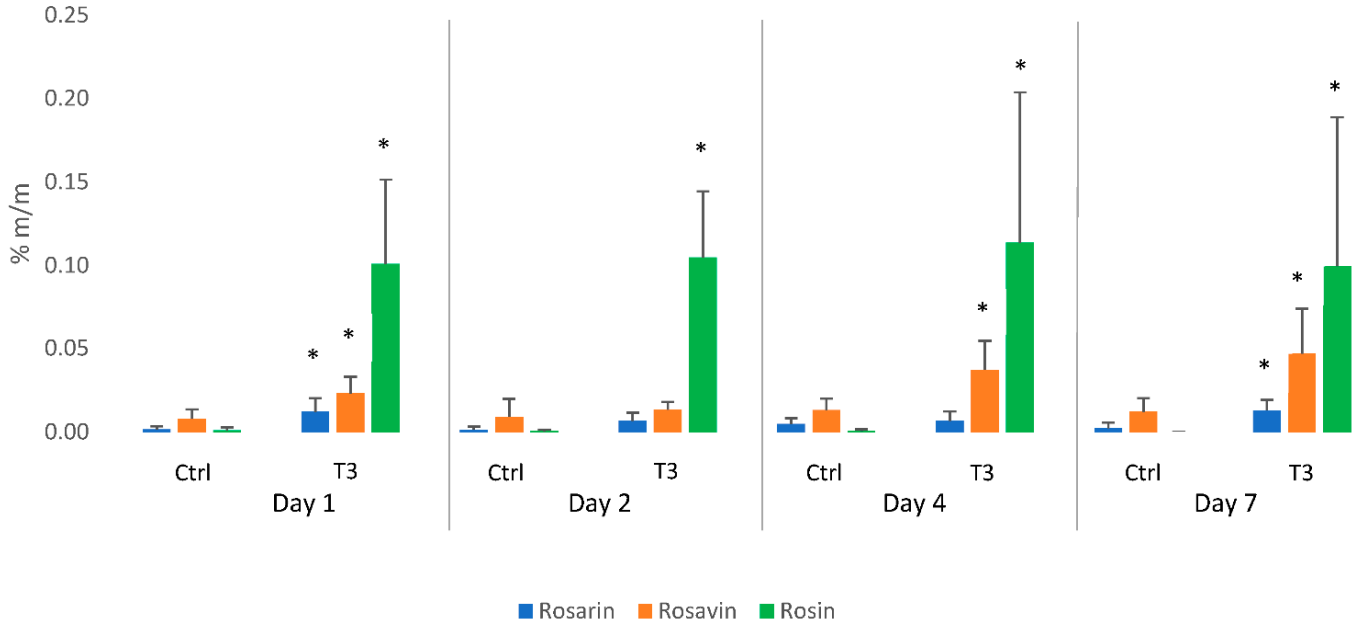

Figure 3. Effect of cinnamyl alcohol feeding on the production of rosavins. The abbreviations of letters indicate: Ctrl—control, T3—cinnamyl alcohol treatment. * significant ($p < 0.05$) deviation from the control based on Student's *t*-test.

4. Discussion

The biosynthesis of CAGs starting from phenylalanine involves five key substrate-specific enzymes responsible for the formation of rosin and its derivatives (rosarin and rosavin). The conversion of phenylalanine to *trans*-cinnamic acid is catalyzed by phenyl alanine ammonium-lyase (Ec no.: 4.3.1.24) based on the KEGG database. *Trans*-cinnamic acid is further catalyzed by 4-coumarate CoA ligase to cinnamyl CoA, which is further reduced to cinnamaldehyde by cinnamoyl CoA reductase, an oxidoreductive enzyme utilizing NADPH and a proton and releases $NADP^+$ CoA in the process. Cinnamyl alcohol dehydrogenase further catalyzes the reaction by reducing cinnamaldehyde to cinnamyl alcohol which, by the further addition of glucose, results in the formation of rosin. Rosavin and rosarin are the compounds derived from rosin by the addition of arabinose and arabinofuranose, respectively [20] (Figure 4). The effects of three of these precursors (*trans*-cinnamic acid, cinnamaldehyde and cinnamyl alcohol) have been examined in the present study. The results are in accordance with previous studies on roseroot callus or CCA cultures both in liquid and on solid media, which confirmed that the biotransformation of exogenously added cinnamyl alcohol could be transformed into CAGs [8,22,23]. Mirmazloum et al. [24] found that as well as the addition of cinnamyl alcohol, cinnamaldehyde and *trans*-cinnamic acid positively affected the accumulation of CAGs in callus cultures of *R. rosea*. In addition to callus cultures, hairy root cultures of *R. kirilowii* supplemented with cinnamyl alcohol also exhibited potential for the production of rosin and its derivatives at higher amounts [27].

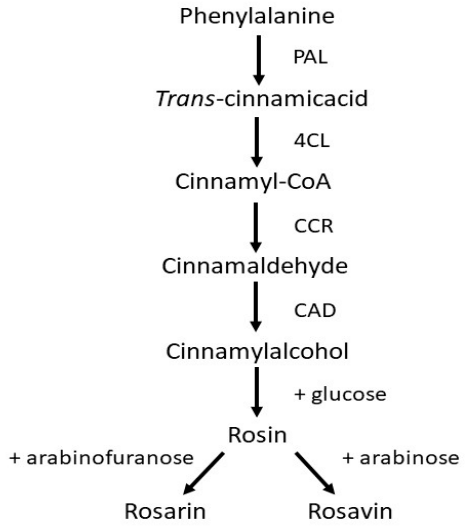

Figure 4. The proposed biosynthetic pathway for CAGs in *R. rosea* (based on György [20]).

For the first time, in the present study, *R. rosea* plants grown in vitro have been studied for their biotransformation capacity. Plants were grown on MS media and fed with different precursors at a concentration of 2 mM for 7 days with four sampling intervals. The mean value of five individuals was used in the analysis, showing high standard deviation resulting from the high genetic diversity of the species, which was reported previously [28]. Even though Mirmazloum et al. [28] detected a 75-fold increase in rosin content after *trans*-cinnamic acid feeding to cell suspensions, in the course of our study, the production of CAGs in plants grown in vitro was not affected by this precursor. However, plants were capable of efficiently utilizing cinnamyl alcohol, which is the aglycone of CAGs, and performed biotransformation to rosavins, with the highest amount of rosin, as was reported previously from callus cultures and compact callus aggregates of *R. rosea* [8,22–24,28,29]. Rosavin contents also increased, whereas no significant effect of cinnamyl alcohol was observed on rosarin content. The addition of 2 mM cinnamaldehyde, which preceded cinnamyl alcohol in the CAG biosynthesis pathway, was also beneficial, as previously shown with cell cultures [24,28]. Cinnamyl aldehyde increased the content of rosin and rosavin by 374- and 5-fold compared to the control. It was observed that cinnamaldehyde was more effective than cinnamyl alcohol, which increased the accumulation of rosin and rosavin 1.87 and 1.25-fold in comparison to the cinnamyl alcohol. In addition, plants fed with cinnamaldehyde showed an increase in rosarin content, whereas it was not observed in cases of cinnamyl alcohol treatment, which did not affect the rosarin accumulation. The content of rosin started to decrease after the fourth day, whereas the contents of rosavin and rosarin increased in both the cinnamyl alcohol and cinnamaldehyde treatments, which can be explained by the conversion of rosin to rosavin and rosarin along with other glycosides, as was previously reported by Tolonen et al. [29]. The highest amount of rosin content was detected as 0.32% (*m/m*) on the fourth day after cinnamaldehyde feeding. In wild-growing plants (age not known) the rosavin content is the highest, followed by rosarin, and the content of rosin is usually lowest [30,31]. Alperth et al. [30] compared the secondary metabolite profiles of *R. rosea* rhizomes collected from Austrian habitats and concluded that the chemical composition of *R. rosea* rhizomes varies much, depending on the growth site and phenological phase. In their study, the contents of rosin were found to be between 0.028% and 0.042% (*m/m*), the contents of rosavin varied between 0.7% and 1.58% (*m/m*), and the contents of rosarin varied between 0.25% and 0.47% (*m/m*) using 85% methanol for the extraction. Marchev et al. [31] also studied the metabolic differences of *R. rosea* rhizomes in Bulgarian natural habitats. The contents of rosarin, rosavin and rosin were 0.37%, 1.97% and 0.04%, respectively. Precursor feeding to in vitro roseroot plants turned out to be a successful approach for the production of CAGs. Further studies are needed to

optimize the biotransformation reaction. Higher amounts of the precursor might further increase the production of CAGs. Increasing the duration of the biotransformation reaction might favor the transformation of rosin to rosavin and rosarin; therefore, the ratio of the CAGs will possibly be similar to natural plants. Our future study will focus on how to make the production more efficient, possibly omitting in vitro cultures.

5. Future Perspective

The primary source of important glycosides in *R. rosea* are the roots and rhizome. However, this plant species is slow-growing; therefore, attempts are needed for a more efficient production system. One approach is to directly increase the production of important secondary metabolites by precursor feeding. In addition, further strategies should be developed to increase the root and rhizome biomass. Hydroponic systems could be an option to increase the biomass of roots and rhizome, which may be directly associated with the increasing content of SMs. Cinnamyl alcohol dehydrogenase (CAD) involved in the biosynthetic pathway is responsible for the production of cinnamyl alcohol; therefore, utilizing cinnamaldehyde in the reaction might be an interesting candidate for further study in this pathway.

Author Contributions: A.J. and F.G. performed the experiments and wrote the manuscript; N.G. performed the chemical analyses; Z.G. designed the experiment, analyzed and discussed the results, and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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