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ANALYSIS OF THE GLYCOSIDE BIOSYNTHESIS IN *RHODIOLA ROSEA* L.

DOCTORAL (Ph.D.) DISSERTATION

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The applicant met the requirement of the Ph.D regulations of the SZENT ISTVÁN UNIVERSITY and the thesis is accepted for the defense process.

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INTRODUCTION AND OBJECTIVES

Adaptogens, referred to as rejuvenating herbs and natural compounds, have attracted the attention of many herbalists and the pharmaceutical industries for their ability to decrease stress levels and relieve depression. The concept of ‘‘adaptogen’’ was phrased as a substance that nonspecifically increases resistance by Brekhman and Dardymov (1969). *Rhodiola rosea* (roseroot) is one of the most famous adaptogen plants and recently getting more and more popular.

The pharmaceutical usage of *R. rosea* and more notably its bioactive secondary metabolites, namely rosine, rosavin, rosarin and salidroside, are extensively investigated in many different aspects by scientists worldwide (Panossian *et al.*, 2010; Hung *et al.*, 2011).

As more recent promising experimental results emerge from the clinical trials, the consumption demand for roseroot-originated drugs grows faster than that of natural production of plant raw materials can compensate for. The very slow development of roseroot in its natural sites of alpine climate in one hand, and the growing demand for plant raw material on the other hand has resulted in a rapid and severe depletion of its natural sources and has necessitated a legal protection for this species (Weglarz *et al.*, 2008).

The optimal cultivation system of roseroot needs quite a long time (at least 5 years) between planting and harvesting the underground parts of the plants, resulting in remarkable financial requirements during this long period (Galambosi, 2006). Another restriction factor for cultivation is the high level of heterozygosis that results in high morphological, developmental and chemical variability even intraspecifically (Weglarz *et al.*, 2008). These cultivation difficulties encourage the investigation *in vitro* cultures of *R. rosea* to obtain a faster and more efficient way to its phytopharmaceuticals. Despite the numerous advantages of *in vitro* techniques in favor of enhancing the plant’s natural

compounds and secondary metabolites production, "potential" is still the word most frequently used to describe this technology as it has met very limited commercial success. Not surprisingly in many cases, the rigorously controlled *in vitro* plant cultures cannot generate the same valuable natural products because they are not necessary for the maintenance of the fundamental life processes in the plants. Secondary metabolites are usually synthesized in plants to cope with environmental conditions and stresses. Roseroot callus also does not produce the pharmaceutically active metabolites similar to the wild plants under regular *in vitro* culture condition, as was proved in several studies (György, 2006).

The overall objectives of this research were as follows:

- Analysis of the intra-population variation of the phytochemicals in different roseroot individuals during the vegetation period *in situ* and *ex situ* (in phytotron environment).
- Roseroot *in vitro* culture (solid and liquid) establishment, development and optimization.
- Following the biotransformation capacity after precursor feeding in roseroot cell culture.
- Transgenic callus culture establishment.
- Knowledge update and validation of salidroside and cinnamyl-alcohol glycosides biosynthetic pathways.
- Molecular cloning and characterization of two hypothesized genes in salidroside biosynthetic pathway.
- Relative transcriptional expression analysis of the key genes in salidroside biosynthetic pathway.

LITERATURE REVIEW

Rhodiola rosea L. botany

Rhodiola rosea L. (roseroot or golden root) is a perennial plant of the Crassulaceae plant family (Figure 1.). *R. rosea* L. (Sedum roseum (L.) Scop., S. rhodiola DC.), is a herbaceous and dioecious species, distinguished among the other almost 200 species of *Rhodiola* genus and honored for its outstanding pharmacological importance and use (Tasheva and Kosturkova, 2012).

Conservation

In Europe, *R. rosea* is considered as endangered in Great Britan, Czech Republic, Bosnia-Herzegovina, vulnerable in Slovakia and Bulgaria (Galambosi and Galambosi, 2015; Tasheva and Kosturkova, 2013). The increasing demands and susceptible status of the endangered *R. rosea* led to more intensive research in domestication, cultivation, and even biotechnological techniques such as *in vitro* culture and biotransformation (precursor feeding) to find a more promising and feasible way to obtain the main compounds of the plant (Galambosi, 2014).

R. rosea, as a traditional medicine and as a modern adaptogen

Roseroot has a long history as a valuable medicinal plant having appeared in the Materia Medica of a number of European countries. According to the literature, *R. rosea* was known and was in use as far back as the Vikings' time for its medicinal properties and for its strengthening action on hard work (Panossian *et al*., 2010). Today, preparations of *R. rosea* are officially accepted as medicine in several countries (Mashkovskij, 1977; Muravijeva, 1978; Turova and Sapozhnikova, 1984; National Pharmacopoeia of the USSR, 1990; National Pharmacopoeia Committee, 1996; Estonian Ministry of Health Affairs, 1998). *Rhodiola rosea* is one of the most popular plant adaptogens utilized in Russia today, and has gained much publicity (Panossian *et al*., 2010). As a dietary supplement, numerous preparations of *R. rosea* extracts are used world-wide (Khanum *et al*., 2005).

Active constituents and secondary metabolites

The initial investigation of roseroot phytochemical profile began in the 1960s. Most of the research was carried out by Russian and Scandinavian researchers and publications are mostly not in English (Ahmed *et al.*, 2014). The application of more advanced analytical methods such as HPLC and GC-MS revealed more about roseroot phytochemical profile and so far about 140 compounds from different natural product classes have been isolated and identified (Panossian *et al*., 2010). Different phytochemical compounds of roseroot are presented in Table 1.

Table 1. Phytochemical classes of *Rhodiola rosea* L.

Chemical group	Phytochemicals	Reference
Phenylpropanoid glycosides	Rosarin, Rosavin, Rosin	Zapesochnaya and Kurkin 1982, Brown *et al.*, 2002, Tolonen *et al.*, 2003
Monoterpenes/Glycosides	Rosiridol, Rosiridin, Sachalinol A, Rhodioloside A&B	Kurkin *et al.*, 1985, Avula *et al.*, 2009,
Phenylmethanoids	Benzyl-O-β- D-glucopyranoside	Avula *et al.*, 2009, Mudge *et al.*, 2013
Phenylethanoids	Salidroside, Tyrosol, Mongrhoside, Viridoside	Troshchenko and Kutikova 1967, Avula *et al.*, 2009
Flavonoids	Rhodionin, Rhodiolinin, Tricin, Kaempferol, Herbacetin, Rhodiologidin, Rhodiolin, Rhodalidin, Quercetin-3'/4'-rhamnose	Zapesochnaya and Kurkin 1983, Kurkin *et al.*, 1984, Brown *et al.*, 2002, Petsalo *et al.*, 2006, Avula *et al.*, 2009
Triterpenes	Daucosterol, β-Sitosterol	Kurkin *et al.*, 1985. Dubichev *et al.*, 1991
Phenolic acids	Caffeic acid, Chlorogenic acid, Gallic acid esters	Kurkin *et al.*, 1991, Brown *et al.*, 2002
Cyanogenic glycosides	Lotaustralin, Rhodiocyanoside A	Akgul *et al.*, 2004, Diermen *et al.*, 2009
Oligomeric/polymeric proanthocyanidins	Prodelphinidin gallates/esters	Yousef *et al.*, 2006

Modified from György, 2006.

Biosynthethic pathways for *Rhodiola* phytopharmaceuticals

Salidroside and cinnamyl alcohol glycosides (rosavins) are the most precious bioactive compounds of roseroot that have arisen from the basic phenylpropanoid metabolism via the biosynthesis of aromatic L-amino acids (phenylalanine and tyrosine) in shikimate pathway (György, 2006). The plausible biosynthetic pathways of salidroside and cinnamyl alcohol glycosides (CAGs) are presented in Figure 1. There are different hypothetical pathways for salidroside biosynthesis in the literature but none of them have been completely supported by molecular experimental results.

In the proposed CAGs biosynthesis pathway (György, 2006), trans-cinnamic acid is converted to cinnamoyl-CoA (Figure 1, A type arrows) by the activity of an enzyme named 4-coumarate: CoA ligase (4CL) via a two-step reaction mechanism that involves the hydrolysis of ATP (Gross and Zenk, 1974). 4CL catalyzes the activation of 4-cinnamate and various other cinnamic acid derivatives by forming their corresponding CoA thioesters (Kumar and Ellis, 2003). In higher plants, 4CL typically occurs as gene family consisting of two to three members. Furthermore, the reduction of cinnamoyl-CoA to cinnamaldehyde is catalyzed by the enzyme cinnamoyl-CoA oxidoreductase (CCR).

Subsequently, cinnamyl alcohol dehydrogenase (CAD) reduces the cinnamaldehyde to cinnamyl alcohol. The enzyme(s) that take part in the formation of the glycosides of cinnamyl alcohol are not yet described. Rosin is the simplest glycoside of roseroot which is formed when one molecule of glucose attaches to the cinnamyl alcohol. From rosin by the connection of an arabinose molecule, rosavin and by the connection of an arabinofuranose molecule, rosarin is formed. Depending on the sugar type and the site it is attached to, further glycosides may be formed (György, 2006).

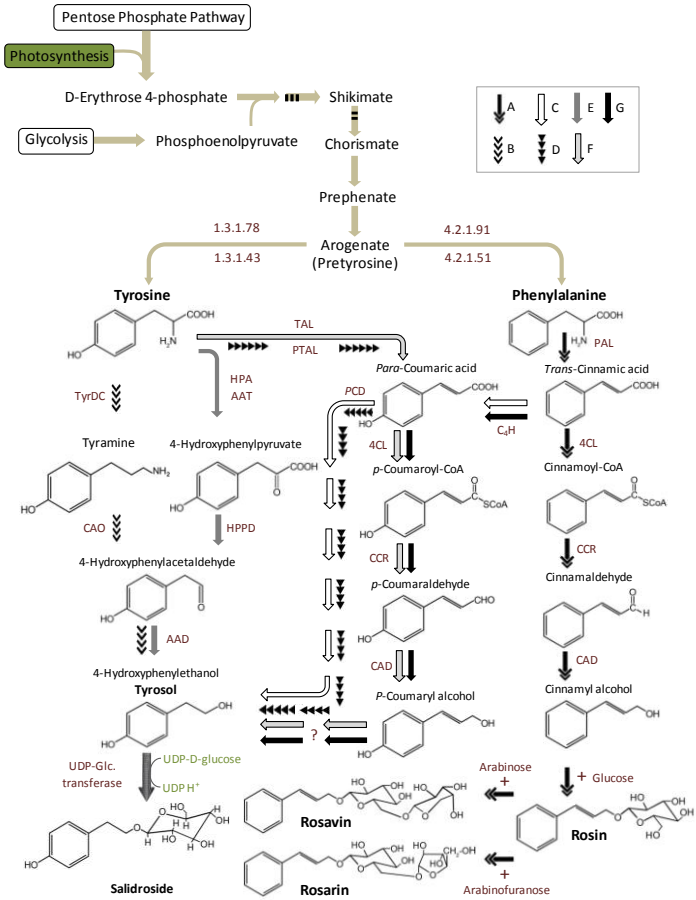

Figure 1. Attainable biosynthetic pathway of salidroside and cinnamyl alcohol glycosides in *Rhodiola* spp. (Modified from Ling-ling *et al.*, 2007; Zhang *et al.*, 2011; KEGG Database, 2011). **Enzyme abbreviations**: PAL: Phenylalanine ammonia-lyase (4.3.1.24); TAL: Tyrosine ammonia-lyase (4.3.1.23); PTAL: Phenylalanine/tyrosine ammonia-lyase (4.3.1.25); TyrDC: Tyrosine decarboxylase (4.1.1.25); HPA: Histidinol-phosphate transaminase (2.6.1.9); AAT: Aromatic-amino-acid transaminase (2.6.1.57); HPPD: 4-hydroxyphenylpyruvate decarboxylase (4.1.1.80); CAO: Primary-amine oxidase (1.4.3.21); AAD: Aryl-alcohol dehydrogenase (1.1.1.90); 4CL: 4-Coumarate: CoA ligase (6.2.1.12); C_4H: Trans-cinnamate 4-monooxygenase (1.14.13.11); CCR: Cinnamoyl-CoA reductase (1.2.1.44); CAD: Cinnamyl-alcohol dehydrogenase (1.1.1.195); PCD: *P*-coumaric acid decarboxylase (4.1.1.-)

The enhancement of roseroot secondary metabolites in *in vitro* cultures

The utilization of callus cultures for the production of the bioactive agents is considered as an alternative way, fast and independent from environmental conditions. Callogenesis has been obtained from roseroot on MS medium supplemented with different plant hormones. Furmanowa *et al*. (1995) concluded that the most effective callogenesis is from leaf explants on BAP along with NAA or IBA or IAA containing medium. The best combination for induction and growth of callus was BAP and IBA. In their work, two types of calli were described: a deep green and a light cream colored (Furmanowa *et al.* 1995)

Different biotechnological approaches have been also utilized to increase the roseroot active agents including roseroot cell, tissue and organ culture on solid and liquid medium with different compositions (Grech-Baran et al., 2015). Many authors applied different biotechnological approaches, including precursor feeding in *in vitro* cultures to enhance the content of the active phytochemicals which are mostly missing from such cultures.

MATERIALS AND METHODS

Plant materials

Different roseroot plant materials including seeds, leaves, rhizome and whole plants have been obtained and used for different experiments in this research. Seeds of *R. rosea* L. were kindly provided by Botanical Garden of University of Oulu (Finland). Samples from leaves and rhizomes of different plants were collected from a natural population on the Hochkar, Göstling Alps; Austria.

***In vitro* experiments of roseroot**

To establish *in vitro* culture, roseroot seeds were surface sterilized before germination on half-strength MS medium supplemented with 30 g/l sucrose, solidified with 4.5 g/l agar. Seeds were germinated and grown aseptically at 22 ± 2°C under a 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 60 µmol.m-2 m^{-2}.at culture level, provided by cool-white fluorescent lamps.

Transgenic callus culture establishment

Twelve pieces of callus masses were placed on solid MS medium in Petri dish for the transformation. The callus was transformed using an *A. tumefaciens* EHA101 strain containing pTd33 vector, (Szegedi *et al.,* 2001). The T-DNA of pTd33 binary vector plasmid harbors a neomycin phosphotransferase II (*npt*II) gene conferring resistance to antibiotics, and a *uidA* (GUS) reporter gene (Tinland *et al.,* 1995) under the control of cauliflower mosaic virus (CaMV) 35S promoter. For the co-cultivation, small volumes of bacterial suspension were placed onto the surface of the prepared callus. After the co-cultivation of the callus with bacteria, calli were transferred onto solid MS medium containing 20 mg/l kanamycin, 200 mg/l carbenicillin and 300 mg/l claforan. Calli were transferred to fresh selection medium of the same composition in each 20 days for selecting the 100% transgenic cells in response to direct contact with kanamycin.

Histochemical GUS assay

For verification of the transformation, callus samples were transferred to assay solution (Jefferson *et al.*, 1987). The reaction mix was kept in a shaker-incubator for 1h at 37°C. Transient GUS expression has been tested for 20 pieces of transformed calluses from 10 co-cultivation Petri dishes.

Precursor feeding and biotransformation

Different hypothesized precursor compounds of salidroside and cinnamyl alcohol glycosides biosynthesis pathway (tyramine, 4-hydroxyphenylpyruvate, tyrosol, phenylalanine, trans-cinnamic acid, cinnamaldehyde and cinnamyl alcohol) were purchased from Sigma-Aldrich. The precursors were dissolved in different solvents and prepared to be of 2 mM concentration in the culture medium. Callus samples were harvested (in triplicates) 1, 6, 12, 24, 48 and 96 h after culture initiation along with controls including samples without any precursor and media samples supplemented with each precursor without any calli.

Phytochemical analysis (HPLC)

Samples (~5 g) were dried at 45°C overnight. From each sample 0.5 g of dried plant tissues was grounded and 70% methanolic extraction was used for HPLC. Using an ultrasonic bath for 1 hour a clear extract was obtained by centrifugation (8000 rpm) and used for HPLC. The HPLC was performed by Waters 1525 binary pump, 717 autosampler with 2998 PDA detector on a reversed phase Thermo Hypersil ODS 250×4.6 5 µm column at 40°C.

Statistical analysis

The deviation in the phytochemical content of roseroot plants ($n = 7$ grown in phytotron) was very high and so the regular repeated measures ANOVA method was insufficient to detect significant differences. Therefore, we focused not on the nominal values of the contents but on the characters of the accumulation, i.e. whether any of the compounds have increased, decreased at a fixed rate (say, 10%) or are stable between time points.

Molecular biology experiments

All of the PCRs have been carried out in a Swift MaxPro thermocycler. All of the PCR products to be sequenced were ligated to pJET1.2/blunt vector. The ligation mix was transferred into 50 µl of chemically competent DH5α *E. coli*.

Twenty of the positive colonies were analyzed and three colonies which showed the expected size were selected from the master plates and subjected for bacterial plasmid extraction and sequencing.

RNA extraction

Total RNA was extracted according to a CTAB-based protocol from 0.5-1 g fresh plant material and their quality was determined. RNA samples were treated with DNase I enzyme and the RNA concentration was quantitatively normalized to 5 µg/30 µl of reaction mix for all of further molecular studies.

Reverse transcription (cDNA synthesis)

DNase treated RNAs were reverse-transcribed to cDNA by M-MuLV RT enzyme using Maxima Reverse Transcriptase kit. Random hexamer or oligo $(dT)_{17}$ primers were used relevant for different purpose of molecular methods in this work.

Rapid amplification of cDNAs end (RACE)

The 3′ and 5′ RACE method was used to amplify the flanking regions of each transcript.

Isolation of full length *UDPG* and *AAD* open reading frame

The complete *UDPG* and *AAD* ORF was PCR amplified with *pfu* DNA polymerase using the corresponding transcript primer pairs. The following PCR program was performed: 3 min 95°C, 30 cycles: 30 s 95°C, 45 s 60°C, 3 min 72°C and a final extension for 7 min at 72°C.

Heterologous expression of *UDPG*

An engineered *E. coli* expression vector of the pONE series, made by Dr. Laszlo Beinrohr, based on pET-24d backbone was used for producing recombinant proteins. The *Bam*HI, *Nhe*I and *Not*I were chosen as non-cutter enzymes for roseroot *UDP-glycosyltransferase* open reading frame. PCRs were performed with designed primers. The amplified PCR products have been inserted in the vector to make the construct.

Expression induction and SDS-PAGE

The *UDPG*s insert + engineered pET-24 vector construct was used for transformation of BL21 cells. After 2-3 h of bacterial culture when the OD_{600} was about 0.8-l, 1 mM IPTG was added to the exponentially growing cells to induce the gene expression. The harvested culture samples were centrifuged and the pellets were frozen in liquid nitrogen. The cells were re-suspended in extraction buffer and disrupted by sonication on ice cold water. The lysate was further centrifuged and each sample supernatant was loaded on 10 % SDS-PAGE gel.

Affinity chromatography

Gravity flow affinity chromatography was applied to purify the recombinant protein from transgenic BL21 strains. *E. coli* lysate was thoroughly centrifuged and the supernatant was collected for chromatography. The lysate was applied onto the column and the eluted recombinant polypeptide of each collected fraction used for SDS PAGE analysis.

Western Blotting

Lysate samples (Non-transformed bacteria, *UDPG*-His transformed bacteria, transformed bacteria with empty vector, *UDPG*-His transformed bacteria with different IPTG induction time purified with affinity chromatography) were loaded in duplicates in 10% polyacrylamide gels by SDS-polyacrylamide gel electrophoresis (Bio-Rad) and run under reducing condition. The fractionated proteins of the gel were transferred to a nitrocellulose membrane were visualized by incubating with BCIP-T and NBT substrate solution (Thermo Sientific).

***UDPG* and *AAD* gene expression analysis**

The expression of UDPG and AAD genes in proposed salidroside formation pathway were studied in the callus samples from the biotransformation experiment. For qPCR analysis the normalized cDNAs from oligo dT primed reactions as outlined earlier were used. The expression of the gene of interest is then normalized with the reference gene (*actin*).

Gene expression analysis in rosavins biosynthesis pathway

The collected plant materials from the Austrian *R. rosea* papulation were used for gene expression analysis. The expression of four genes - *phenylalanine ammonia-lyase* (*PAL*), *4-coumarate:CoA ligase* (*4CL*), *cinnamoyl-CoA reductase* (*CCR*) and *cinnamyl alcohol dehydrogenase* (*CAD*) - was studied by real-time PCR in relevance to *actin* gene.

Analysis of the gene expression data

The ratio of target genes expression to *actin* expression signal was defined as relative expression, where the expression of *PAL* in the rhizomes from the first sampling date was considered as 1. Expression data was processed with REST software pairwisely. In order to express the similarity and dissimilarity of the character of gene expression process in time, we introduced a three-dimensional code for each one of the 4 genes (*PAL, 4CL, CCR* and *CAD*) in leaf and rhizome of all the 4 plants, that is to say we calculated 4*2*4 4*2*4 three-dimensional codes: $C_k^l(i) = \left(c_k^l(i)_1, c_k^l(i)_2\ c_k^l(i)_3\right), k = 4\text{CL, CAD, CCR or PAL},$

$l = \text{rhizome or leaf}, i = \text{U, M, L, G}.$

The value of a code is equal to +1 or -1 if the gene expression is increasing or decreasing and the absolute increase/decrease is above the 10% of the mean of the gene expression measured in three phenological stages, respectively.

RESULTS

***In vitro* experiments of roseroot**

Roseroot callus was obtained on MS medium supplemented with different combination of plant growth regulators from leaf and stem explants. The highest callus induction rate (80%) was on medium supplemented with 1.0 mg/l NAA and 0.5 mg/l BAP, 3 mg/l 2iP + 0.3 mg/l IAA and 0.6 mg/l NAA + 3 mg/l 2iP. The leaf explants gave the best growth rate and high quality callus which was used in further sub-cultures and experiments.

Transgenic roseroot callus culture establishment

To test the capacity of roseroot callus for genetic alteration, the selected calli were co-cultivated with *Agrobacterium*. More than 50% of the calli survived the selection medium. In the second and further selection sub-cultures, 100% stable antibiotic resistant callus has been obtained.

Molecular analysis showed the amplified fragment of the inserted neomycin phosphotransferase II (*npt*II) gene in all of the samples from transformed callus pieces. As a more quantitative expression indicator, GUS test was performed. The test clearly showed positive results by expressing blue color indicating the inserted reporter gene which was visible in all parts of the callus.

Precursor feeding and biotransformation of intermediate compounds

Three proposed precursors of salidroside (tyramine, 4HPP, and tyrosol) and cinnamyl alcohol glycosides (phenylalanine, trans-cinnamic acid, cinnamaldehyde and cinnamyl alcohol) were studied for their properties and biotransformation possibilities. Based on our findings, none of the precursors in salidroside pathway was released into the medium from the plant cells. Tyrosol significantly increased the salidroside content for about 17 fold comparing to the control in a measurement after 24 hours (Figure 2).

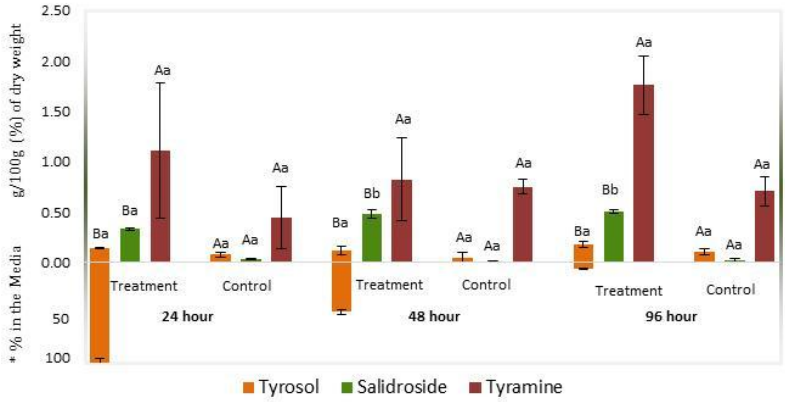

Figure 2. Effect of 2 mM tyrosol treatment on tyrosol, tyramine and salidroside content in roseroot callus culture. Upper case letters: different letters are for significantly different treatments assumed a fixed interval of elapsed time ($p<0.05$). Lower case letters: different letters are for significantly different intervals of elapsed time assumed a fixed treatment, according to Games-Howell's post hoc test ($p<0.05$). Error bars represent standard deviation of the mean among 3 biological replicates. * The detected content from 2 mM tyrosol after 24h considered as 100%.

The only compound in cinnamyl alcohol glycosides pathway which was released into the media from plant cells was rosin and only after cinnamyl alcohol treatment. 2 mM phenylalanine (Phe); showed no effect on CAGs biosynthesis even after 96 h. The treatment with Phe significantly increased its content in the treated samples during the experiment when compared with the controls (Figure 3). Only after 24 h, the tyrosol content significantly increased in comparison with the control. Surprisingly, Phe induced a significant change also in tyrosine content after 96 h compared to the control.

No cinnamldehyde was found nor in the media neither in the cells after 24 h of treatment (Figure 4). It was not present in the controls either. But cinnamyl alcohol was synthesized and detected in the treated samples. Around 130 fold increase in rosin content was recorded when 2 mM cinnamaldehyde was given to the culture after 96 h compared to the controls.

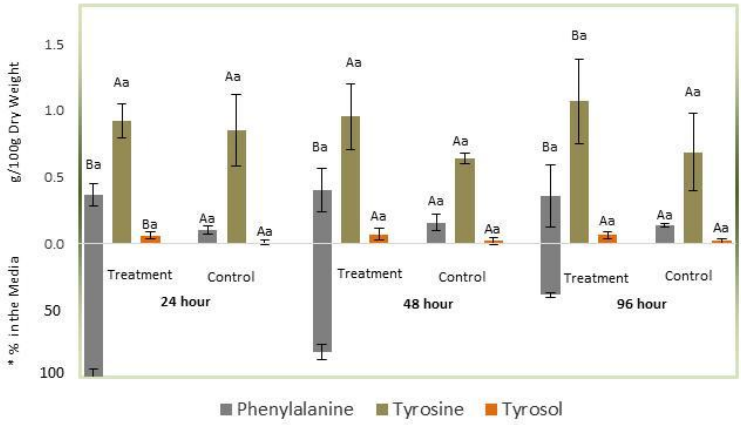

Figure 3. Effect of 2 mM phenylalanine treatment on phenylalanine, tyrosine and tyrosol content in roseroot callus culture. Upper case letters: different letters are for significantly different treatments assumed a fixed interval of elapsed time ($p<0.05$). Lower case letters: different letters are for significantly different intervals of elapsed time assumed a fixed treatment, according to Games-Howell's post hoc test ($p<0.05$). Error bars represent standard deviation of the mean among 3 biological replicates. * The detected content from 2 mM phenylalanine after 24h considered as 100%.

The results of 2 mM cinnamyl alcohol treatment on the content of rosin, rosarian and rosavin are presented in Figure 5. Cinnamyl alcohol is the last compound to be converted to rosin in the biosynthestic pathway of rosavins. After 48 h of biotransformation approximately 200 fold increases was recorded in rosin biosynthesis comparing to the control. These results revealed an interesting pattern where precursor's content declined from the media and desired compounds were formed in the plant cells during the experiment.

As it can be seen in the Figure 8, there was a significant increase in rosavin content with the control and also a significant rosavin increase among the treated samples in correlation with a decrease of rosin content after 48 hours.

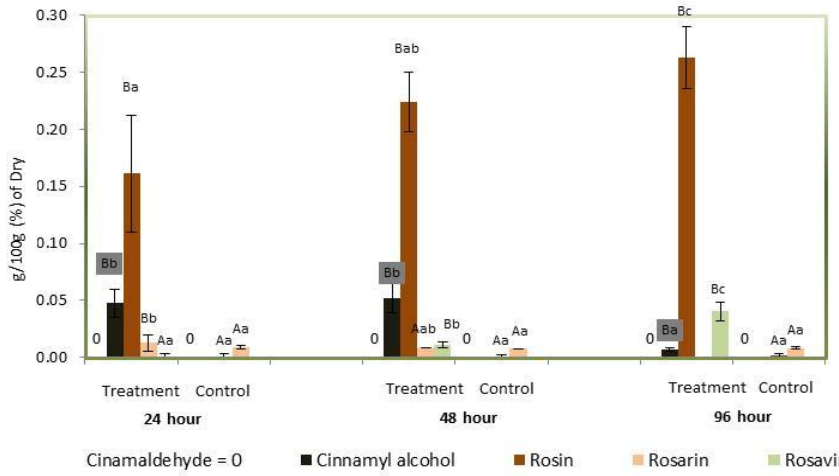

Figure 4. Effect of 2 mM cinnamaldehyde treatment on cinnamyl alcohol, rosin, rosarin and rosavin content in roseroot callus culture. Upper case: different letters are for significantly different treatments assumed a fixed interval of elapsed time ($p<0.05$). Lower case: different letters are for significantly different intervals of elapsed time assumed a fixed treatment, according to Games-Howell's post hoc test ($p<0.05$). Error bars represent standard deviation of the mean among 3 biological replicates.

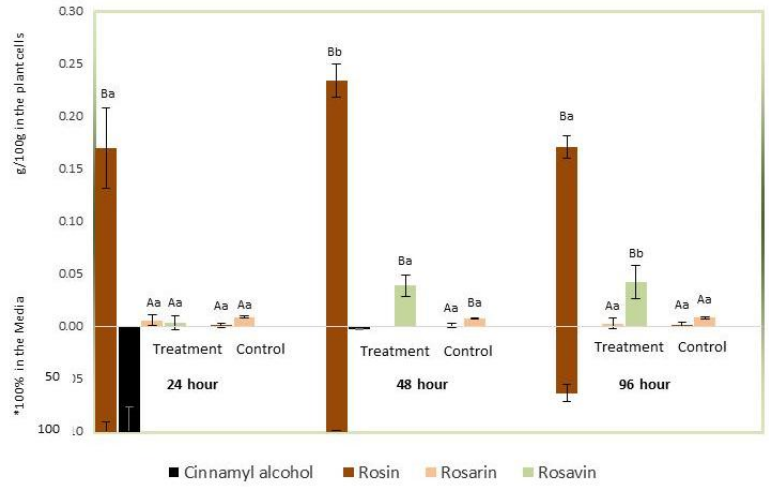

Figure 5. Effect of 2 mM cinnamyl alcohol treatment on rosin, rosarian and rosavin content in roseroot callus culture. Upper case: different letters are for significantly different treatments assumed a fixed interval of elapsed time ($p<0.05$). Lower case: different letters are for significantly different intervals of elapsed time assumed a fixed treatment, according to Games-Howell's post hoc test ($p<0.05$). Error bars represent standard deviation of the mean among 3 biological replicates. * The detected content from 2 mM cinnamyl alcohol after 24h considered as 100%.

Roseroot phytochemical analysis during the vegetation period

HPLC analysis of roseroot leaf and rhizome samples was conducted during the vegetation period to detect the important roseroot phytochemicals. All compounds of our interest have been detected in the roots and rhizomes with salidroside being the highest in both organs. The content of all compounds were 2-3 times more in the rhizomes than in the root samples. Significant differences were hard to detect, because of the big variation in the amount of the metabolites.

To achieve a better understanding of the metabolites formation pattern, we examined the direction of the changes of the content of each compound. Figure 6, is showing the results of studied compounds in in regard with their accumulation pattern during the sampling times.

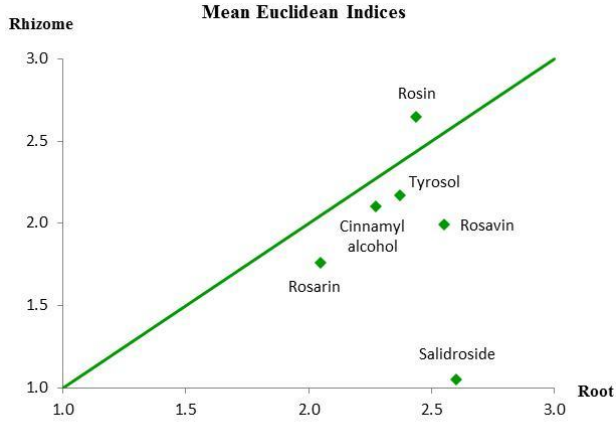

Figure 6. Pairs of dissimilarity indices of studied compounds. $P_k = \left(\overline{I_k^{root}}; \overline{I_k^{rhizome}}\right)$ (k = rosin, rosavin, rosarin, cinnamic alcohol, salidroside and tyrosol). The indices are calculated using the distances between the characteristic codes of the accumulation processes introduced in section *Statistical analysis*. The closer a point is to the origin, the more similar characters of accumulation patterns are detectable among the plants. The points under the diagonal line represent compounds the accumulation processes of which are more similar in rhizome while if a point is above the diagonal line, the similarity is more expressed in root.

Molecular biology experiments

Isolation of *R. rosea UDP- glycosyltransferase* gene

We isolated a full length *UDP- glycosyltransferase* gene for the first time from *R. rosea*. The full-length cDNA contains a 1425 bp long coding sequence of 474 deduced amino-acid polypeptide residues with a calculated molecular mass of 53.05 kDa and a predicted isoelectric point (pI) of 5.90. The full length *RrUDPG* transcript contains 53 nucleotides in its 5′ (5′UTR) and 119 nucleotides in its 3′ (3′UTR) untranslated regions. Following the recommended nomenclature, UGT73B16 was given as an official name to *RrUDPG* gene. The full-length cDNA of UGT73B16 was submitted to NCBI (http://www.ncbi.nlm.nih.gov) database (GenBank accession number: KM396888).

Isolation of *R. rosea Aryl Alcohol Dehydrogenase* (*AAD*) genec

AAD is an enzyme known to catalyze the conversion of 4-hydroxyphenylacetaldehyde to tyrosol. Here we present a full length transcript nucleotide sequence and genomic organization of *AAD* from *R. rosea* for the first time. The *AAD* full-length cDNA contains 1155 bp long coding sequence to encode 384 deduced amino-acid residues with a calculated polypeptide molecular mass of 42.95 kDa and a predicted isoelectric point (pI) of 8.17.

The full length *RrAAD* transcript contains 34 nucleotides in its 5′ (5′UTR) and 259 nucleotides in its 3′ (3′UTR) untranslated regions. The full-length cDNA of *RrAAD* was submitted to NCBI database (GenBank accession number: KP686072). When genomic DNA was used as template with *AAD* ORF primers in PCR, a new and larger PCR product (~3100 bp) was amplified. Using the online Splign computing tool, the cDNA and genomic DNA sequences were aligned from which the presence of 8 introns in this gene have been revealed. All of the intron/exons splicing sites contained the common splicing signals (AG<exon>GT).

Heterologous expression of *R. rosea UDPG* gene

The *UDPG* ORF was digested from pJET-UGT73B16 construct with *Nhe*I and *Xho*I restriction enzymes and inserted into the expression vector (engineered pET-24). The expression construct was checked and confirmed for in-frame fusion by DNA sequencing. The gene constructs (UGT73B16- engineered pET-24) were transformed into competent BL21 (DE3) cells and their expressions was induced by the addition of 1 mM IPTG at OD_{600} = 0.8-1. This resulted in the appearance of a new fusion polypeptide with an expected molecular mass of 96.55 kDa when fractioned on SDS-PAGE (Figure 7).

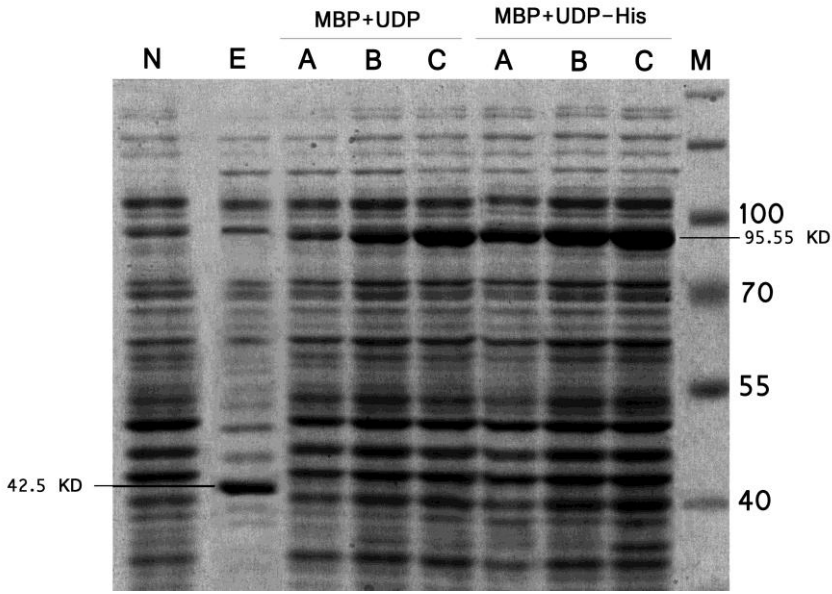

Figure 7. Heterologous expression of UGT73B16 in *E. coli*. Total protein extracts of *E. coli* cells were used for SDS-PAGE analysis (stained with coomassie blue). Lane N: non-transformed BL 21; Lane E: Transformed with empty vector (control); A, B and C: expression of recombinant protein (MBP+*UDPG* without histidine tag and MBP+*UDPG* with histidine tag) after 1 h, 2 h and 4 h of induction with 1 mM IPTG at 37°C. Lane M: Protein molecular weight marker.

The recombinant proteins were further purified by affinity chromatography. The bacterial cell lysate from non-transformed BL21 (DE3), transformed with the empty vector and transformed with *UDPG* expression construct were subjected for gravity column chromatography. An amylose resin bead were used to purify the expressed maltose binding protein (MBP~42.5 kDa) and MBP+HDPG-His (~95.55 kDa) fusion protein after expression induction with 1 mM IPTG for 4 h (Figure 8).

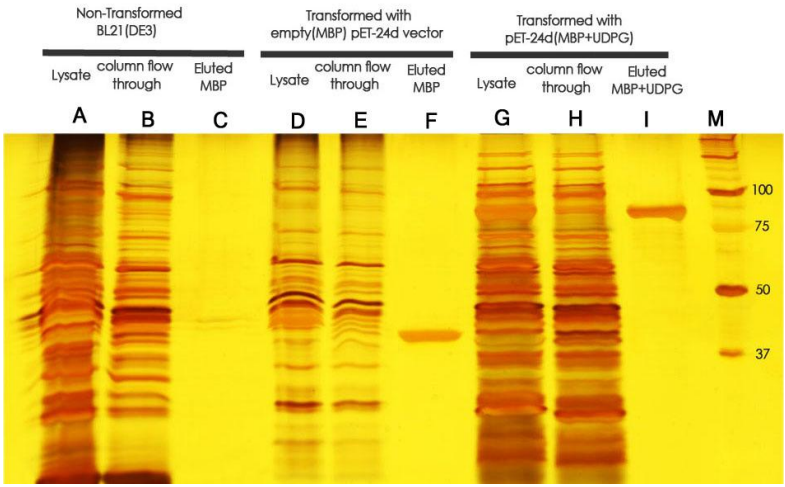

Figure 8. Purification of recombinant proteins with affinity chromatography (Expression induced by IPTG and for 4 hours and the SDS gel was stained with silver salt). A: total protein extract from non-transformed BL 21 before chromatography, B: Amylose resin column flow trough of A, C: Purified MBP after eluting with maltose containing elution buffer; D: total protein extract from transformed BL 21 with engineered pET-24d vector (containing MBP) before chromatography, E: Amylose resin column flow trough of D, F: Purified recombinant MBP after eluting with maltose containing elution buffer; G: total protein extract from transformed BL 21 with engineered pET-24d vector construct (MBP+*UDPG*-His) before chromatography, H: Amylose resin column flow trough of G, I: Purified recombinant MBP+*UDPG* fusion protein after eluting with maltose containing elution buffer; M: Protein molecular weight marker.

The western blot analysis confirmed the authenticity of expressed *UDPG* protein from *R. rosea* in *E. coli*. Two parallel gels were run with the same samples for western blotting. The figure 12 shows the SDS-PAGE analysis and western blot analysis of a non-transformed BL21 (DE3), a transformed BL21 (DE3) with empty vector (containing MBP) and transformed BL21 (DE3) with our expression construct (MBP+*UDPG*-His) before and after affinity chromatography. As can be seen in Figure 9, the histidine tagged proteins showed the exact expected molecular weight and no signal on the blotting paper appeared from the non-transformed bacterial cell lysate.

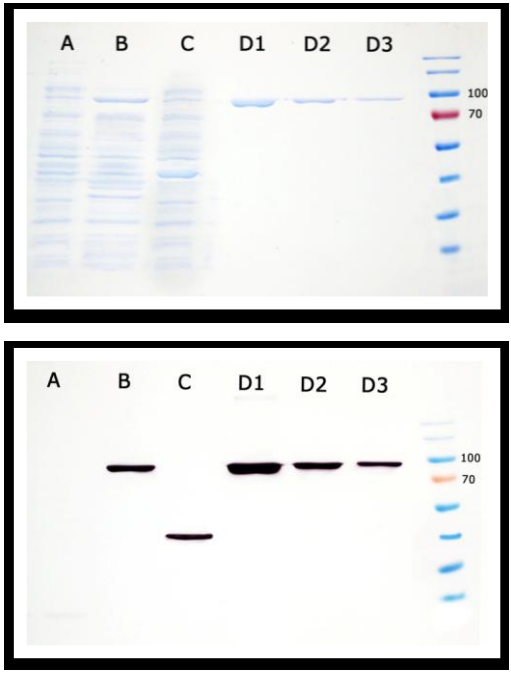

Figure 9. SDS-PAGE analysis (upper) and western blot analysis (lower) of MBP and MBP+*UDPG* proteins. **A**: Non-Transformed BL21 *E.coli* cells lysate, **B**: MBP+*UDPG*-His transformed cell lysate, **C**: Empty vector (with MBP~42.5 kDa) transformed cell lysate, **D1-D3**: Purified MBP+*UDPG*-His recombinant protein with affinity chromatography after 4, 2 and 1h IPTG induction time.

UDPG and *AAD* gene expression analysis *in vitro*

The expression pattern of roseroot specific *UDPG* and *AAD* genes were studied in response to precursor feeding in callus cultures. The ANOVA test revealed highly significant effect both for treatment and for the elapsed time (UDPG: $F_{treatm}(1;24)>28$; $p<0.001$; $F_{time}(5;24)>26$; $p<0.001$; AAD: $F_{treatm}(1;24)>233$; $p<0.001$; $F_{time}(5;24)>24$; $p<0.001$).

Since the interaction was also significant, we tested the treatment effect for each interval of elapsed time and also the elapsed time effect for controls and treatments, separately.

According to our results, the gene expression was instantly effected when the fresh medium was given to the calli. The addition of 2 mM tyrosol to the media increased the expression of *UDPG* significantly after one hour when compared to the control (Figure 12.).

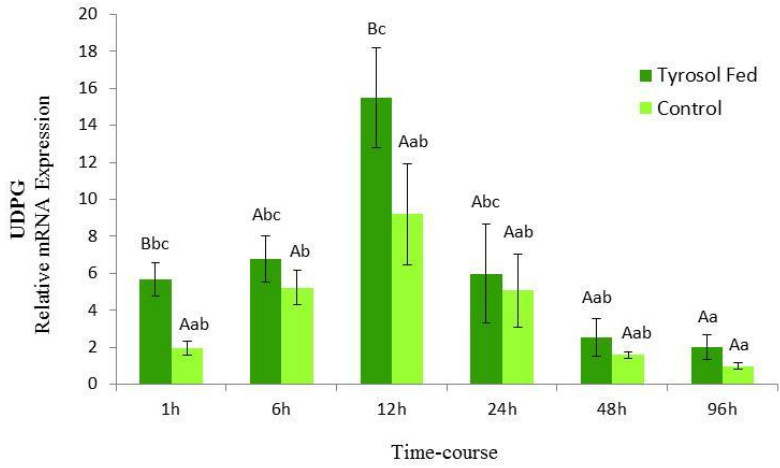

Figure 10. Upper case: different letters are for significantly different treatments assumed a fixed interval of elapsed time ($p<0.05$). Lower case: different letters are for significantly different intervals of elapsed time assumed a fixed treatment, according to Games-Howell's post hoc test ($p<0.05$). Error bars represent standard deviation of the mean among 3 biological replicates.

Tyramine and 4-hydroxyphenylpyruvate (4HPP), the possible precursors for tyrosol biosynthesis also significantly altered the *UDPG* expression level. Significantly higher *UDPG* expression was recorded after 1 and 12 hour in tyramine fed samples comparing to the controls (Figure 11)

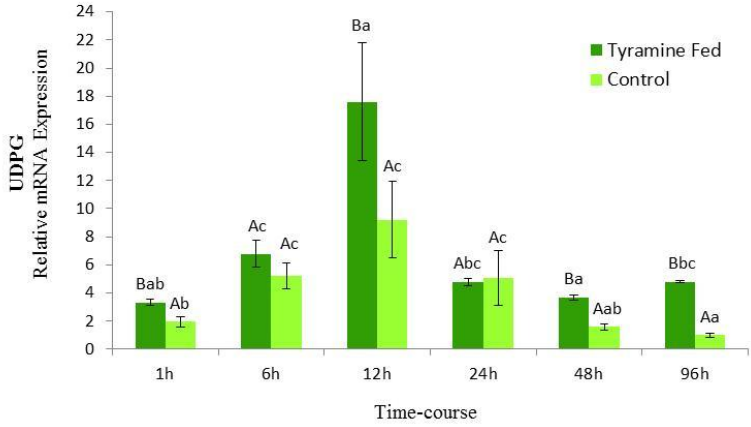

Figure 11. Upper case: different letters are for significantly different treatments assumed a fixed interval of elapsed time ($p<0.05$).Lower case: different letters are for significantly different intervals of elapsed time assumed a fixed treatment, according to Games-Howell's post hoc test ($p<0.05$). Error bars represent standard deviation of the mean among 3 biological replicates.

Aryl alcohol dehydrogenase (*AAD*) is the penultimate enzyme thought to be involved in salidroside biosynthesis pathway of roseroot plants. The expression pattern of *AAD* was also studied in response to presence of tyrosol, tyramine and 4HPP. A significant downregulation of *AAD* gene was revealed when 2 mM tyrosol, tyramine or 4HPP was given to the culture media comparing to the controls. A constant and very low expression level was measured during the experiment when tyrosol or 4HPP was in the culture media. Tyramine also suppressed the expression of *AAD* in a similar manner to tyrosol and 4HPP but in a lower degree.

Gene expression analysis *in vivo*

With qPCR, 104, 127, 108 and 102 bp fragments from *PAL*, *4CL*, *CCR* and *CAD* genes, respectively were amplified and their relative expression were studied. Squared Euclidean distances of all pairs of curves for leaf and rhizome

studied genes were calculated and represented by points as curve characteristic codes (Figure 12, left panel). To show the expression pattern of the studied genes, the graphs were then summarized to mean values for all plants (Figure 12, right panel)

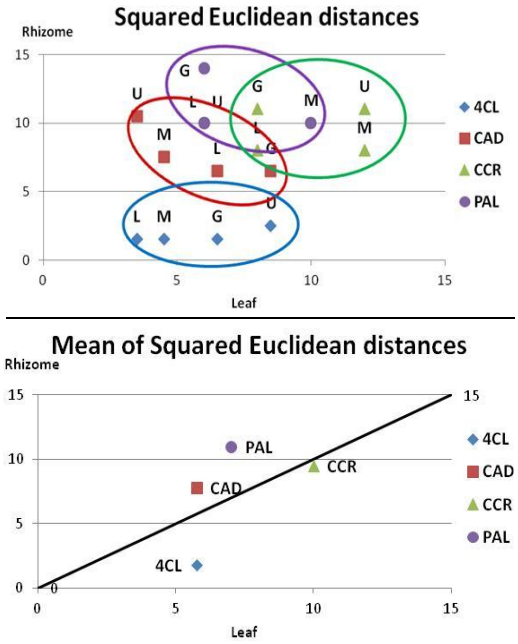

Figure 12. Squared Euclidean distances of plants i ($i = \mathrm{U, M, L, G}$) of curve characteristic codes represented as points $P_k(i) = \left(I_k^{leaf}(i); I_k^{rhizome}(i)\right)$, $k = 4\mathrm{CL, CAD, CCR}$ and PAL (left panel) and their means $P_k = \left(\overline{I_k^{leaf}}; \overline{I_k^{rhizome}}\right)$, $k = 4\mathrm{CL, CAD, CCR}$ and PAL calculated for all plants (right panel).

The expression of the studied genes was not following meaningful tendency in regard with the expected products of their activity. This can be explained with the possibility that there may be different organ specific versions of the studied genes or more isozymes that can be involved which we were not aware of. This is also an indicator of how dynamic the CAGs pathway is in wild growing roseroot plant surrounded with enormous number of environmental stresses.

SUMMARY

Rhodiola rosea (roseroot) is a plant species extensively applied as an adaptogenic, anti-fatigue, antidepressant, antioxidant, anti-inflammatory, antinociceptive, and anticancer agent, and modulator of immune functions. Application of a safe and effective alternative therapy utilizing natural products could be of public health relevance for many individuals unable, or unwilling, to use conventional therapies. Numerous clinical trials provide important information for the applicability, safety and clinical relevance of *R. rosea* active constituents (salidroside, rosin, rosavin, rosarin). Intensive research on its molecular mechanisms is going on for understanding the signaling and molecular network affected by adaptogens at the cellular level in order to rationalize their beneficial effect on emotional behavior, psychological, neurological and metabolite disorders, as well as, mental and physical performance under stress.

Regarding the protected status of *R. rosea*, *in vitro* cultures of this species are relevant and sustainable alternative for production of the pharmaceutically important salidroside and cinnamyl alcohol glycosides (CAGs). Current optimization procedures of the growth medium components and the process of cell cultivation show promise to increase the biosynthesis of these secondary metabolites. Nevertheless, exploring the mechanisms that regulate the biosynthesis of these metabolites is key factor in understanding and controlling their biosynthetic pathways in the plant as well as in cultures.

These approaches, along with the emerging "omics" platforms (metabolomics in particular), could be successfully implemented in the manufacturing and chemical profiling of *R. rosea* preparations based on *in vitro* cultures.

In the current research project we tried to address the limiting factors for obtaining roseroot medicinal constituents (salidroside, tyrosol, rosin, rosarin and rosavin). The investigation of phytochemical profile of roseroot individuals in

their natural habitats and also the samples from a controlled environment showed significant and remarkable variability among the individuals during the vegetation period. Our results in accordance with the existing reports with similar objectives led us to study the formation of roseroot metabolites *in vitro* not only to achieve a more stable production strategy but also to come up with a more environmental friendly approach. The difficulties of roseroot *in vitro* cultures were also discussed in details. Different methods and biotechnological tools (culture medium optimization, precursor feeding and biotransformation, *Agrobacterium* mediated genetic transformation) were applied and studied. Parallel with the callus culture experiments, we also studied the biosynthetic pathways of the roseroot phytopharmaceuticals and identified the genes that are most likely involved in their formation.

Variety of different molecular techniques such as PCR, qPCR, RACE, DNA cloning, reverse transcription, DNA restriction site digestion, agarose and SDS-PAGE electrophoresis, recombinant DNA construction and heterologous gene expression, HPLC and protein purification were applied during this research to gain more information about the molecular mechanisms of the phytopharmacuticals' biosynthesis of roseroot. The phytochemical analysis of wide growing roseroot individuals showed a remarkable variation in their active constituents. The plants that were grown in a controlled environment (phytotron) as well did not follow a similar pattern for their phytochemicals biosynthesis. These results emphasized the phytochemical diversity of wild growing individuals and more practically on the weak principles of wild collection strategies. To summarize the phytochemical analysis we strongly recommend the cultivation of roseroot and to develop more alternative production systems like *in vitro* cultures.

During this research we optimized the callus culture condition for *R. rosea* in which we tried the precursors feeding experiments.

We also conducted an *Agrobacterium* mediated genetic transformation and established a transgenic callus culture. The proposed compounds in salidroside and CAGs biosynthesis pathways were given to the callus cells in 2 mM concentration and for 96 hours. Significant increase in biosynthesis rate of salidroside, tyrosol, and rosavins were recorded as result of this experiment. This showed a remarkable possibility to boost the active metabolites of roseroot in a very short period of time and based on desired content of final plant materials.

Our molecular experiments resulted in full identification of 2 new genes (UDP-glucosyltransferase (*UDPG*) and aryl-alcohol dehydrogenase (*AAD*)) in salidroside biosynthesis pathway and partial identification of 4 new genes (phenylalanine ammonia-lyase, 4-coumarate: CoA ligase, cinnamoyl-CoA oxidoreductase and cinnamyl alcohol dehydrogenase) in CAGs biosynthesis pathway from *R. rosea* for the first time. The UDP-glucosyltransferase expression was studied during the biotransformation of tyrosol and the results of phytochemical analysis showed a meaningful correlation with the expression of this gene. The validity of the identified *UDPG* was confirmed by its heterologous expression in *E. coli*. The complete genomic and transcript sequence of *AAD* and *UDPG* was deposited in the GenBank database.

Our research showed a promising potential for *in vitro* cultures of roseroot. The intensive growth in the scientific reports on roseroot medicinal properties leave no other option to meet the demand for this valuable phytochemicals if one considers the endangered situation of wild populations.

NEW SCIENTIFIC ACHIEVEMENTS

1. **Optimization and conduction of genetic transformation of *Rhodiola rosea* L. callus and transgenic callus culture establishment.**

   This research introduced a reliable and promising method of genetic transformation which gives the possibility for bioengineering the glycosides' biosynthetic pathway of *Rhodiola rosea*.

2. **Enhancing the accumulation of the phytochemicals of roseroot in callus cultures by precursor feeding and biotransformation of intermediate compounds.**

   2 mM Cinnamic acid, cinnamaldehyde and cinnamyl alcohol significantly increased the rosin content by more than 75, 130 and 200 fold, respectively. Addition of 2 mM tyrosol to the culture increased the salidroside production rate by 26 fold compared to the control.

3. **A full length UDP-glucosyltransferase (*UDPG*) gene was isolated and identified from *R. rosea* for the first time.**

   The UDPG heterologous expression and molecular characterization showed that this is a valid transcript and in combination with HPLC results, its activity in glycosylation of tyrosol and salidroside biosynthesis was strongly supported.

4. **A full length putative aryl-alcohol dehydrogenase (*AAD*) gene was isolated and identified from *R. rosea* for the first time**.

   The *AAD* transcript and its genomic organization was characterized and reported for the first time from a plant species.

5. **Four other genes** (*PAL*, *4CL*, *CCR* and *CAD*) of the hypothetical biosynthetic pathway of cinnamyl alcohol glycosides from *R. rosea* were partially identified for the first time and their sequence analysis showed a significant similarity with their counterparts in different plant species.

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