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

DOCTORAL (Ph.D.) DISSERTATION

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CONTENT

LIST OF ABBREVIATIONS

2iP	N^6-(2-Isopentenyl) adenosine
4CL	4-Coumarate: CoA Ligase
4HPP	4-HydroxyPhenylPyruvate
AAD	Aryl-Alcohol Dehydrogenase
AAT	Aromatic-Amino-Acid Transaminase
BAP	6-BenzylAminoPurine
C4H	Trans-Cinnamate 4-Monooxygenase
CAD	Cinnamyl-Alcohol Dehydrogenase
CAO	Primary-Amine Oxidase
CCR	Cinnamoyl-CoA Reductase
cDNA	Complementary DNA
dNTP	DeoxyriboNucleotide TriPhosphate
GAPDH	GlycerAldehyde-3-Phosphate Dehydrogenase
GBIF	Global Biodiversity Information Facility
GUS	β-Glucuronidase
HPA	Histidinol-Phosphate Transaminase
HPLC	High-Performance Liquid Chromatography
HPPD	4-HydroxyPhenylPyruvate Decarboxylase
IAA	Indole-3-Acetic Acid
IBA	Indole-3-Butyric Acid
MS	Murashige and Skoog
NAA	α-NaphthaleneAcetic Acid
ORF	Open Reading Frame
PAL	Phenylalanine Ammonia-Lyase
PCD	*P*-Coumaric Acid Decarboxylase
PCR	Polymerase Chain Reaction
PTAL	Phenylalanine/Tyrosine Ammonia-Lyase
qPCR	Quantitative Polymerase Chain Reaction
RACE	Rapid Amplification of cDNA End
REST	Relative Expression Software Tool
TAE	Tris base, Acetic acid and EDTA Buffer
TAL	Tyrosine Ammonia-Lyase
TBE	Tris base, Boric acid and EDTA Buffer
T_m	Primer Melting Temperature (defined as the temperature at which 50% of the helices are dissociated)
TyrDC	Tyrosine DeCarboxylase
UDPG	Uridine Diphosphate Glycosyltransferase

1. INTRODUCTION AND OBJECTIVES

The 21st century with all of its tremendous advantages spontaneously provided several mental discomforts and excessive fatigue for many people throughout the world. Nowadays almost every country has a remarkable proportion of non-native inhabitants who are suffering from various types of mental to physical disorders in their new environment that were previously unrecognized in their families. Besides that, living in the overpopulated and polluted metropolises with all of their complicated and stressful work places raises concern for adverse implications and consequences associated with such environmental stressors (Mirmazloum and György, 2012).

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. Nikolai Lazarev, a Russian scientist is known for his painstaking study in 1947, in which he and his team attempted for the first time to develop a group of medicinal compounds that increase nonspecific resistance to adverse influences in an organism helping to adopt to physical, chemical or biological stressors. The concept of ‘‘adaptogen’’ therefore was phrased by Dr. Lazarev as a substance that nonspecifically increases resistance (Brekhman and Dardymov, 1969). Several research experiments and clinical trials have been carried out with different plant species to identify the biologically active constituents and their effects concerning this phenomenon. According to the most common definition for adaptogens (Kelly, 2001) not many medicinal plant species possess adaptogenic properties. Some of the most well-known adaptogen plants are *Schisandra chinensis*, *Withania somnifera*, *Panax ginseng*, *Astragalus membranaceus*, *Lycium chinense*, *Eleutherococcus senticosus*, *Rhaponticum carthamoides*, *Aralia mandshurica*, *Sterculia platanifolia* and last but not least *Rhodiola rosea* (roseroot), which is currently 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). There are many published results available from a tremendous number of research experiments and human clinical studies proving the multipurpose medicinal character of roseroot (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 of *R. rosea in vitro* cultures 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). Hence, different strategies should be considered to enhance the formation of roseroot bioactive metabolites in *in vitro*.

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.

2. LITERATURE REVIEW

2.1. *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, 2012a). The root is spindle-shaped becoming thicker at the base, developing into short rhizomes with brown or blackish cortex in which the bioactive glycosides and other secondary metabolites are usually stored (Kylin, 2010). Roots and rhizomes have rosaceous odor when wounded or cut due to the terpenoid compounds that are also stored here. The leaves are orbicular-ovate to linear-oblong, dentate, without hair or more or less waxy with a green-bluish color. Leaf-shape is variable (long and narrow to short and broad) depending on geographical location (Tutin, 1964).

Inflorescences are corymbiform or capitates, unisexual and usually being 4-merous (Tutin, 1964). Several shoots grow from the same thick rhizomes. The round, 2-6 mm thick flowering stems are erect and 10-40 cm in height. The male flowers are yellow with sometimes reddish nuance and are larger and more prominent during the flowering (Kylin, 2010). Male plants are larger and heavier then female plants (Galambosi *et al.*, 2009). Each female flower has four red follicles, 6-12 mm long. Brown seeds are 1-1.5 mm wide, 2 mm long and lanceolate in shape. From the end of summer, leaves and stems wither, leaving buds on rhizomes ready to burst in early spring (Tasheva and Kosturkova, 2012a). Chromosome number is 2n=22 (Lid 1985).

Figure 1. *Rhodiola rosea* L. (Roseroot)
Left: Slovakia, High Tatras, 2013; Right: www.adaptoherbs.com

The species was first described by Carl von Linné (Linnaeus, 1753); however, his description was not linked to a type specimen and was typified later by Ohba (1993). It is broadly acknowledged that the *Rhodiola* species are highly polymorphic, and confident differentiation of *R. rosea* from related taxa is problematic due to a variety of phenotypic features in members of this section (Ohba 1989). Numerous ecological and/or geographical races of *R. rosea* are recognized as distinct species (e.g., *R. sachalinensis* Boriss., *R. arctica* Boriss., *R. iremelica* Boriss. and some others) by some authors, and others regard them as intraspecific taxa or even synonyms (Ohba, 1981; 2005).

According to GBIF (2015) the genus of *Rhodiola* comprises of 233 accepted species followed by 32 synonyms, while The Plant List (http://www.theplantlist.org) includes 192 hits for the *Rhodiola* genus of which 72 are accepted species names and 89 are considered as synonyms and the rest are unresolved or unclarified. Even though *R. rosea* is an economically and pharmaceutically important species, still not much is known about the genetic structure of its naturally occurring populations and their diversity. Recently, genotyping and chemotaxonomic markers have been used to identify *Rhodiola* species within the genus (Liu *et al.*, 2013; Elameen *et al.*, 2008; György *et al.*, 2013; 2014) which may help to identify the more reliable and highly characterized representative species from the phytopharmaceutical point of view.

2.2. Conservation

As a result of recent industrialization, urbanization, and modern agricultural systems, natural plant populations are considered to be highly threatened throughout the world. In some countries, more than two-thirds of the existing habitat types are considered to be endangered. Human activity is the primary cause of risk for almost 80% of all endangered plant species. Habitat destruction and loss are also a problem because they lead to the fragmentation of the remaining plant population and their further isolation (Tasheva and Kosturkova, 2013).

On the other hand, during the last 15 years or so, an intense interest has emerged for "nutraceuticals" or "functional foods" in which phytochemical constituents can have long-term health promoting or medicinal properties. This, along with the smoother flow of information (internet) and easier accessibility to foreign traditional medicine literature resulted that the demand for medicinal herbs which had mostly been consumed locally remarkably increased and became international. Consequently, an attractive market is getting formed in which the consumption demands for herbal products grow faster than natural resources can supply for.

Roseroot which is mostly known for its adaptogenic properties is also getting to lights of international markets and demands for its plant raw materials and extracts increased dramatically in recent years. Up to now, most of the raw material for production of medical preparations is taken from the natural populations. The largest population of *R. rosea* in the world is found in Altai, south Siberia (Galambosi 2006). As the genus is also known as an old Chinese traditional medicine, the accelerated and uncontrolled use of *Rhodiola* species during the 1980s in southwestern China was so severe that they are now listed as endangered in whole China (Lei *et al.*, 2006). Other factors reported to threaten *Rhodiola sp*. is deforestation of natural habitats and excessive grazing (Xia *et al.*, 2007). Galambosi (2006) confirms the same pattern in several countries, among them Russia, wherein roseroot is nowadays on the Russian Red List and collection is restricted. Despite being among the red listed species, estimated quantity of *Rhodiola* roots exported from Russia was around 20-30 tons/year (Galambosi *et al.*, 2006) and according to personal communications all are coming from natural sites.

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).

2.3. *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, *Rhodiola 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). In an old book of useful plants from Iceland (Halldórsson, 1783; *cited in* Panossian *et al.*, 2010) the following statement is written about *Rhodiola*; ''Infusion of stone crop taken dries and astringes, heals pain in the mouth, heals kidneys from sand which forms stones, stops diarrhea and cures headache and also strengthens head and also hair growth if the head is washed with it''.

The root may also be suitable for severe skin conditions. Grinded, pressed and mixed with butter it is considered to relieve swellings and decreases back pain and pain in joins and in other painful conditions. The dried root has been used to cure swellings, remove freckles and strengthen the head (Panossian *et al.*, 2010). Roseroot was also used to ''enhance the intellect'', to be ''tonic

against infirmity'' and to ''restore weak nerves'' (Halldórsson, 1783 cited in Panossian *et al.*, 2010). Alm (2004) mentioned the use of *R. rosea* in folk medicine against scurvy, being also medically used as a stimulant and an astringent in France. It is also known to have been used against headaches and for washing hair. In the textbook of pharmacology for dispenser training in Sweden, *R. rosea* is mentioned as a plant with stimulant effect. Also in the Swedish Pharmaceutical Book (Lakemedelsboken) 97/98 *R. rosea* is mentioned as one of the most common herbal medicines and its effect is specified as a ''general strengthener'' and ''psychostimulantium'' (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).

It was first recommended in 1969 by the Pharmacological Committee of the Ministry of Health of the USSR to be used as a stimulant against fatigue by patients who suffered asthenic states and by healthy people who showed asthenia during periods of high mental exertion or after intensive physical work. The drug can also be applied in cases of borderline nervous-mental diseases, neuroses, neurotic disorders and psychopathies. In psychiatric practice, extracts of *R. rosea* are indicated for the correction of neurological side-effects associated with psychopharmacological therapy, and for the intensification and stabilization of remissions of asthenic and apathistical-aboulictype schizophrenia patients (Saratikov *et al.*, 1965; Mikhailova, 1983; Brichenko *et al.*, 1986; Saratikov and Krasnow, 1987). As a dietary supplement, numerous preparations of *R. rosea* extracts are used world-wide (Khanum *et al.*, 2005).

The clinical trials with animals and humans using the alcohol-aqueous extract of roots and rhizome of roseroot revealed various range of medicinal effects which are summarized and listed in Table 1.

Table 1. Pharmacological effects of alcohol-aqueous extract of *Rhodiola rosea* L.

Medicinal properties	**Type of Experiment**	**Reference**
Stimulates the central nervous system	*in vivo*	Sokolov *et al.*, 1985
Improves learning and long-term memory	*in vivo*	Petkov *et al.*, 1986
Antitumor and antimetastatic effect	*in vivo*	Dementieva and Iaremenko 1987
Antitumor and antimetastatic effect	*in vivo*	Bocharova *et al.*, 1995
Inhibits the growth of tumors in liver by 39%	*in vivo*	Udintsev and Shakhov 1991
Anti-arrhythmia effect	*in vivo*	Lishmanov *et al.*, 1993
Prevents stress-induced cardiac damage	*in vivo*	Maslova *et al.*, 1994
Improves coronary flow	*in vitro*	Lishmanov *et al.*, 1997
Improves physical fitness and general wellbeing	*in vivo*	Spasov *et al.*, 2000
Antihyperglycaemic and insulin stimulating activity	*in vivo*	Molokovskij *et al.*, 2002
Hepatoprotective effect	*in vivo*	Laremii and Grigoreva 2002
Antifatigue effect	*in vivo*	Darbinyan *et al.*, 2000; Shevtsov *et al.*, 2003
Stimulates bone marrow erythropoiesis during paradoxical sleep deprivation	*in vivo*	Provalova *et al.*, 2002
Prevents the ischemic brain damage development	*in vivo*	Pogorelyi and Makarova 2002
Anti-inflammatory effect, protects muscle tissue	*in vivo*	Abidov *et al.*, 2004
Protects against hypochlorous acid induced oxidative damage	*in vitro*	De Sanctis *et al.*, 2004
Expedites the recovery after acute non-specific pneumonia	*in vivo*	Narimanian *et al.*, 2005
Improves 5-HT level in hippocampus in depressive rats, repairing the injured neurons athippocampus.	*in vivo*	Chen *et al.*, 2009
Has potent anti-depressant activity by inhibiting MAO A and may also help in the control of senile dementia	*in vitro*	Diermen *et al.*, 2009
Possesses antiviral activities against CVB3 and it may represent a potential therapeutic agent for Viral Myocarditis	*in vitro and in vivo*	Wang *et al.*, 2009
Decreasing the growth of bladder cancer cell lines	*in vivo*	Liu *et al.*, 2011
For mental and physical fatigue	*in vivo*	Punja *et al.*, 2014
Anti-Inflammatory and neuroprotective effects	*in vitro*	Lee *et al.*, 2013
Antagonized both induction and reinstatement of nicotine place preference in mice	*in vivo*	Titomanlio *et al.*, 2014
Free radical-scavenging activities	*in vivo*	Zhou *et al.*, 2014
Melanogenesis inhibition	*in vivo*	Chiang *et al.*, 2014
Treatment of mild to moderate mepression	*in vivo*	Ross, 2014

2.4. 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). The rhizome and root of *R. rosea* L. accumulate the most of the pharmaceutically active secondary metabolites which belong to different chemical groups (Table 2.). Kurkin and co-workers (1984) investigated the flavonoid compounds of *Rhodiola rosea* and from its rhizomes they isolated rhodionin, rhodiosin, rhodiolin, 8-methylherbacetin, acetylrhodalgin, kaempferol, 7-O-α-L-rhamnopyranoside and methyl gallate. They also identified 7 flavonoid compounds (rhodionin, rhodionidin, rhodiolgin, rhodiolgidin, rhodalin, rhodalidin and caffeic acid) from the aerial parts of *R. rosea* (Kurkin *et al.*, 1985). The chemical structures of those compounds were also identified (Zapesochnaya *et al.,* 1985).

Table 2. 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, Ma *et al.,* 2006
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, Jeong *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.

The essential oil content of *R. rosea* dried rhizome is around 0.05% including 86 different compounds, mainly from the chemical classes of monoterpene hydrocarbons (25.40%), oxygenated monoterpenes (23.61%) and straight chain aliphatic alcohols (37.54%), (Rohloff, 2002). In the studied samples, *n*-decanol (30.38%), geraniol (12.49%) giving the characteristic rose-like fragrance, and 1,4-*p*-menthadien-7-ol (5.10%) were the most abundant volatile compounds. An essential oil analysis of Finnish *R. rosea* samples showed that myrtenol (36.9%), trans-pinocarveol (16.1%), geraniol (12.7%) and dihydrocumin alcohol (12.1%) were the most abundant volatiles in the oil (Héthelyi *et al.*, 2005). The variation in the metabolite composition which is commonly referred to as due to the effect of plant origin, the chosen plant extraction and analytical methods and even the harvests time, is a well-known phenomenon and is also pronounced in case of roseroot essential oil constituents.

Salidroside, the most studied phytochemical compound of roseroot was first isolated in 1967 by Troshchenko and Kutikova along with its aglycone, tyrosol and was named rhodioloside. Rhodioloside was later reidentified as salidroside (Thieme, 1969), which was first found in *Salix triandra* and even the name salidroside is derived from the *Salix* name (Brigel and Beguin, 1926; György, 2006). Zapesochnaya and Kurkin (1982) isolated phenylpropanoid glycosides from *R. rosea* rhizome. They were identified as the glycosides of cinnamyl alcohol and named rosin, rosarian and rosavin which are collectively called ‘‘rosavins’’. A phytochemical profile comparison of 21 *Rhodiola* species revealed the specificity of cinnamyl alcohol glycosides to *Rhodiola rosea*, which can distinguish it from all other *Rhodiola* species (Kurkin *et al.*, 1986).

Besides the above mentioned compounds, also coumarins, lactones (Furmanowa *et al.*, 1995) and phenolic acids: chlorogenic-, hydroxycinnamic- and gallic acid (Brown *et al.*, 2002) were found in *R. rosea*. Recently lotaustralin was isolated from *R. rosea* roots (Akgul *et al*, 2004). Today rosin, rosavin, rosarin and salidroside are considered as diagnostic marker compounds for *R. rosea*, all demonstrating adaptogenic activity (Furmanowa *et al.*, 1995; Germano *et al.*, 1999; Panossian and Wagner, 2005). Extracts used in most clinical trials are standardized to minimum 3% (rosavins) and 0.8-1% salidroside as the naturally occurring ratio of these compounds in the plant rhizomes is approximately 3:1 (Brown *et al.*, 2002). Enzymatic synthesis of salidroside was reported by Tong and co-workers (2004) using apple seed meal as a source of β–glucosides obtaining 15.8% salidroside yield and by Zhang and co-workers (2005), who isolated the enzyme from *Aspergillus niger* and obtained 10% salidroside yield. Kishida and Akita (2005) constructed the rosavin framework based on the Mizoroki-Heck type reaction and reached 82% yield.

2.5. Mechanism of action

From several identified compounds in roseroot, salidroside, tyrosol, and rosavins are the most studied for their pharmacological properties. Many biological effects of salidroside like neuroprotective (Chen *et al.*, 2008), hepatoprotective (Wu *et al.*, 2009), antioxidant (Chen *et al.*, 2009), antiviral (Wang *et al.*, 2009), anticancer (Hu *et al.*, 2010) and anti-inflammatory (Guan *et al.*, 2011) effects and many more have been reported which are recently reviewed by Chiang *et al.*, (2015) and Marchev *et al.*, (2016).

The roseroot adaptogenic properties and stress reducing effect are suggested to be in correlation with its antioxidant potentials. The protective effect of salidroside against oxidative stress (commonly induced by hydrogen peroxide) have been reported in many cellular and animal models (Zhang *et al.*, 2007; Cai *et al.*, 2008; Chen *et al.*, 2009; Yu *et al.*, 2010; Zhu *et al.*, 2011; Shi *et al.*, 2012; Zhang *et al.*, 2013; Xiao *et al.*, 2014; Shi *et al.*, 2015). Some of the salidroside action mechanisms are referred to its role of inhibiting the reactive oxygen species (ROS) accumulation, reducing lipid peroxidation and DNA damage, intracellular Ca^{2+} ion level stabilization, and inhibiting the activity of certain caspases (Ahmed *et al.*, 2015).

Like salidroside, tyrosol has also various biological properties, including antioxidative, cancer preventive and anti-inflammatory properties (Tuck and Hayball, 2002). It was reported to be protective against adrenaline and $CaCl_2$-induced arrhythmia (Maimeskulova and Maslov, 1998). Tyrosol exhibits antioxidant activity, scavenges DPPH free radicals, and has an IC50 of 4.7 mg/mL (Ko *et al.*, 2011). It is likely that tyrosol remains stable during the digestion process with no dramatic changes in its structure and properties (Dinnella *et al.*, 2007).

Rosavins (rosin, rosarin and rosavin) are the essential constituents of *R. rosea* extracts along with salidroside, but are less studied for their pharmacological effects and action mechanisms. Most of the reports on rosavins are studies in the context of *R. rosea* extracts in which obviously salidroside was also present. Cybulska *et al.*, (2011) reported that *R. rosea* extracts containing salidroside and rosavin demonstrated some inhibitory activity (*in vitro*) against *Neisseria ghonorrhea* isolates with different antimicrobial resistance phenotypes. In the case of human urinary bladder cancer, *R. rosea* extract and salidroside component exhibited a selective ability to inhibit the growth of p53 knockout primary mouse embryo fibroblasts (p53-/- MEFs) compared to their wild-type counterparts (Liu *et al.*, 2011).

The antidepressant activity of rosavins *in vivo* was demonstrated in rats in a forced swimming test by significantly increasing the freezing time and the swimming duration compared to the untreated controls (Kurkin at al., 2006). The action mechanism of roseroot extract and salidroside in human is hypothesized by Asea *et al.*, (2013) as a neuroendocrine mechanism for stress protection (Figure 2.). The idea was further developed and is currently a subject of some research experiments (Panossian and Wikman, 2014; Panossian *et al.*, 2014).

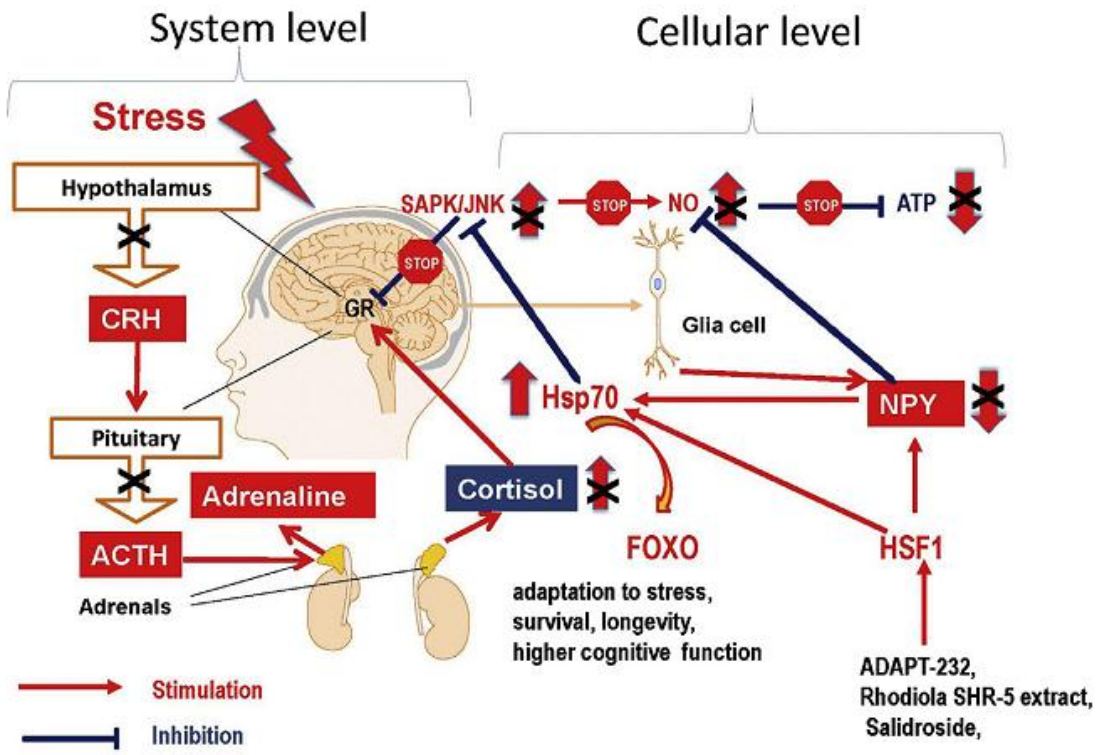

Figure 2. Hypothetical neuroendocrine mechanism of stress protection by *Rhodiola rosea* extract and salidroside (Asea *et al.*, 2013).

As a result of stress induction, corticotropin-releasing hormone (CRH) is secreted from hypothalamus followed by adrenocorticotropic hormone (ACTH) release from the pituitary, which simulates the release of adrenal hormones and neuropeptide Y (NPY) to mobilize energy resources and cope with stress. Feedback regulation of overreaction is initiated by cortisol release from the adrenal cortex followed by binding to glucocorticoid receptors (GR) in the brain. This signal stops the release of hormones and brings the cortisol down to the normal level. Stress is normally associated with generation of oxygen-free radicals like nitric oxide (NO); an inhibitor of ATP formation. Stress activated protein kinases (SAPK/JNK/MAPK) inhibit GR consequently the down regulation feedback is blocked and the blood cortisol level remain high during stressful conditions. Adaptogens normalize the stress-induced elevated level of cortisol, NO and SAPK as a result of up-regulation in NPY expression. Consequently NO formation is reduced and ATP generation is no longer suppressed. These pathways contribute to adaptogenic effects like anti-fatigue and improved functions.

2.6. 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 3. There are different hypothetical pathways for salidroside biosynthesis in the literature but none of them have been completely supported by molecular experimental results.

The shikimate pathway is initiated by condensation of phosphoenolpyruvate and erythrose 4-phosphate, derived from glycolysis and the pentose phosphate pathway, respectively. The pathway continues by several reactions to yield chorismate which is considered as a central branch point molecule in the synthesis of primary aromatic amino acids and secondary metabolites. Chorismate is then converted to prephenate and finally to arogenate (pretyrosine). The conversion of chorismate to prephenate can be catalyzed by chorismate mutase, while prephenate to arogenate is catalyzed by prephenate aminotransferase (Vermerris and Nicholson, 2006). Although the prephenate aminotransferase (PAT) enzymatic activity for converting the prephenate into arogenate was first reported in plants almost 20 years ago (Siehl *et al.*, 1986; De-Eknamkul and Ellis, 1988), the first supportive molecular research result for a plant gene encoding such an activity has been published just recently (Graindorge *et al.*, 2010).

Arogenate can be converted to either phenylalanine (Phe) by the activity of enzymes arogenate dehydratase (EC: 4.2.1.91) and prephenate dehydratase (EC: 4.2.1.51), or tyrosine (Tyr) by the activity of arogenate dehydrogenase (EC: 1.3.1.78) enzyme, where the pathway bifurcates (Vermerris and Nicholson, 2006). Phenylalanine formation from the prephenate precursor by using phenylpyruvate as substrate is also suggested to be possessed by an endogenous Aromatic-Amino-Acid Transaminase (AAT) activity in *Arabidopsis* plant (Tzin *et al.*, 2009). Yet, no gene encoding for such an enzyme (AAT) that can specifically convert phenylpyruvate into Phe has been identified so far in plants and the final enzymatic steps for this conversion are still not entirely elucidated (Tzin and Galili, 2010).

Phenylalanine and tyrosine are important basic precursors for a large and diverse group of phenylpropanoids. The key regulatory functions of the enzymes responsible for the conversion of these primary aromatic amino acids to secondary metabolites are remarkable for all downstream reactions involved in the natural products biosynthesis (Facchini *et al.*, 2000).

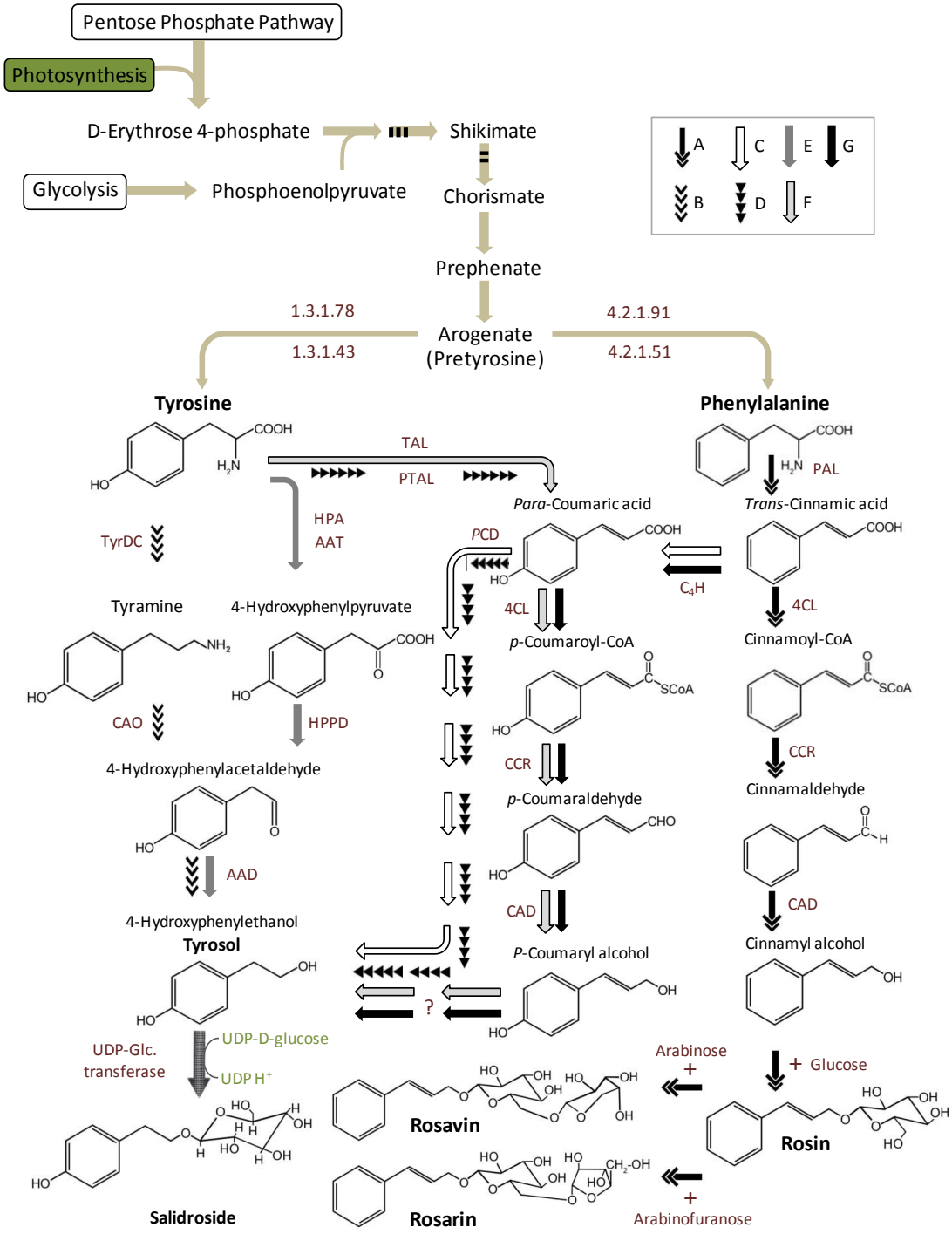

Figure 3. Attainable biosynthetic pathway of salidroside and cinnamyl alcohol glycosides in *Rhodiola* spp. (Modified from Ling-ling *et al.*, 2007; Zhang *et al.*, 2011; KEGG Database, 2011). **Enzyme abbreviations**: PAL: Phenylalanine ammonia-lyase (4.3.1.24); TAL: Tyrosine ammonia-lyase (4.3.1.23); 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.-)

Phenylalanine ammonia-lyase (PAL) is the first enzyme that can catalyze the phenylalanine deamination and trans-cinnamic acid production (Hahlbrock and Scheel, 1989) which is the common precursor for the synthesis of both CAGs and salidroside. PAL activity has been found in all higher plants and several research (Lois *et al.*, 1989; Subramaniam *et al.*, 1993; Pellegrini *et al.*, 1994; Butland *et al.*, 1998; Kumar and Ellis 2001; Cochrane *et al.*, 2004) have been carried out to study the enzyme characteristics and its corresponding gene(s). In poplar, an organ-specific expression of three of the PAL-genes has been suggested to be involved in different functions (Kao *et al.*, 2002). Two genes are expressed in lignifying tissues but only one is involved in lignin formation. The second PAL-gene is specifically involved in tannin formation. The third gene is associated with flowering, although its functional role remains to be understood (Hamberger *et al.*, 2007).

Ma and co-workers (2008) reported the cloning and expression pattern of a cDNA encoding a PAL (PALrs1) from *Rhodiola sachalinensis*. It turned out that PALrs1 protein, in amino acid level shows about 80% identity with the prior isolated cDNA clones from different investigated dicotyledons (Ma *et al.*, 2008). No molecular work has been reported in regard with PAL gene(s) in the case of *R. rosea*. The further steps depending on the downstream enzymatic activities involved in the pathways lead to the formation of either cinnamoyl-CoA in CAGs biosynthesis or *p*-coumaric acid (trans-p-hydroxycinnamic acid) in salidroside (4-hydroxyphenethyl O-β-D-glucopyranoside) biosynthesis.

2.6.1. Cinnamyl alcohol glycosides (CAGs) biosynthesis pathway

In the proposed CAGs biosynthesis pathway (György, 2006), trans-cinnamic acid is converted to cinnamoyl-CoA (Figure 3, 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. The pivotal activity of 4CL is widely studied in lignin biosynthesis but no gene encoding 4CL activity has been reported from any *Rhodiola* species concerning the CAGs synthesis.

Furthermore, the reduction of cinnamoyl-CoA to cinnamaldehyde is catalyzed by the enzyme cinnamoyl-CoA oxidoreductase (CCR). CCR was first purified from soybean (Wegenmayer *et al.*, 1976), and later on efficiently isolated from lignifying cambium of eucalypts (*Eucalyptus gunnii*) (Goffner *et al.*, 1994). A CCR cDNA from *E. gunnii* was identified in a cDNA library that was

screened with oligonucleotide derived from the peptide sequence of the CCR protein. CCR is considered to be the first enzyme committed towards the biosynthesis of monolignols (hydroxycinnamyl alcohols) and shows homology to the flavonoid biosynthetic gene flavonol 4-reductase (Lacombe *et al.*, 1997).

Subsequently, cinnamyl alcohol dehydrogenase (CAD) reduces the cinnamaldehyde to cinnamyl alcohol. CAD is also encoded by a multigene family in *Arabidopsis* (Raes *et al.*, 2003; Goujon *et al.*, 2003), rice (*Oryza sativa)* (Tobias and Chow, 2005), and probably in many other plant species. Conversion of hydroxy-cinnamaldehydes to their corresponding alcohols appears to be catalyzed by a combination of CAD isoforms, some of which have a preference towards one of the available substrates. The combination of isoforms varies depending on the developmental stage and the tissue (Sibout *et al.*, 2003).

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).

There are a few studies dealing with the production of the cinnamyl alcohol glycosides in callus cultures by biotransformation. Furmanowa and co-workers (1999) added 2.5 mM cinnamyl alcohol to cell suspensions of roseroot, of which more than 90% was transformed into several products, but only rosavin was identified. After 72 h, 0.03 to 1% rosin accumulated in the cells, and was not excreted into the medium. The results obtained by György and co-workers (2004, 2005) are more detailed. Beside rosin, also rosavin and four new products were identified after precursor feeding to *in vitro* roseroot callus cultures. The optimal cinnamyl alcohol concentration was found to be 2 mM, since the resulting rosin content was the highest at that concentration. This amount of cinnamyl alcohol did not have any adverse effect on the biomass growth, as seen with higher concentrations, and all cinnamyl alcohol was converted at this level, whereas using higher concentrations, residual amounts were detected in the medium. The rosin content was the highest, three days after the precursor addition and it decreased when the cells were further cultivated. The maximum rosin contents achieved were between 0.4 and 1.25%. The repeated addition of 2 mM cinnamyl alcohol at 3 days intervals did not improve the rosin production like it was demonstrated with salidroside by Xu and co-workers (1998a); but it even inhibited the production (György *et al.*, 2004).

The four new compounds identified (Tolonen *et al.*, 2004) are all closely related to rosin and rosavin (opening the double bond in the middle of the propyl chain of the aglycone, extra hydroxyl group at the C-8 position, extra hydroxyl group on the 3rd carbon of the second glucose compared to rosavin). The presence of the many closely related products after the biotransformation indicates that either several enzymes take part in the glycosylation of cinnamyl alcohol or at least some of the products form spontaneously. For increasing the glycosylation of cinnamyl alcohol and tyrosol, a simple trick was applied (György *et al.*, 2005). Since the MS medium contains only sucrose as a sugar source, glucose was added into the medium to be directly used in the glycosylation reaction. This approach was very effective and beneficial for the production of the cinnamyl alcohol glycosides; yields were doubled compared to the control. In their experiment rosavin was only produced in the glucose containing media.

2.6.2. Salidroside biosynthesis pathway

Salidroside is a product of dehydration between the hemiacetal hydroxyl of glucose and the ethanol hydroxyl of tyrosol (4-Hydroxyphenylethanol) catalyzed by the activity of a glycosyltransferase. Therefore, the biosynthesis of salidroside can be divided into two steps: biosynthesis of tyrosol and the combination of glucose and tyrosol to form salidroside. There are six different possible routes for tyrosol formation in plants based on the literature and the KEGG database utilizing both phenylalanine and tyrosine as precursor, indicated by different arrow types (B-G) in Figure 3.

P-cumaric acid is one of the precursors for tyrosol biosynthesis that can arise either from Phe or from Tyr by different enzymatic activities. Trans-cinnamate 4-monooxygenase (C4H); a cytochrome P450 (Anterola and Lewis, 2002), catalyzes the conversion of Phe-derived trans-cinnamic acid to *p*-cumaric acid in plants. C4H has been found to exist in two distinct classes, which are functionally identical but show only around 60% sequence similarity (Millar *et al.*, 2007). Some species such as *Arabidopsis* have only one class of them (Costa *et al.*, 2003) whereas, *Solanaceous* species, have both (Ehlting *et al.*, 2006). The presence and activity of this enzyme have been reported in many plant species eg.: *Ruta graveolens* (Gravot *et al.*, 2004); *Popolus sp.* (Lu *et al.*, 2006); *Catharanthus roseus* (Mahroug *et al.*, 2006); *Lycopersicum aesculentum* (Millar *et al.*, 2007); *Fragaria* × *ananassa* (Cao *et al.*, 2010) and the co-localization of C4H and PAL has been precisely described by Achnine and co-workers (2004).

P-cumaric acid, as mentioned earlier can be arisen also from tyrosine by the activity of several enzymes: tyrosine ammonia-lyase (TAL), phenylalanine/tyrosine ammonia-lyase (PTAL) and also

PAL enzyme with TAL activity (Rosler *et al.*, 1997). Most of the members in this aromatic amino acid lyase family have been proved to be active in monocots and bacteria (Dewick, 2009). Even though the presence of TAL enzyme was reported in *Glycine max* (Khan *et al.*, 2003) and strawberry (Cao *et al.*, 2010) but a tyrosine-specific TAL activity in dicot plants is still a matter of debate (Mirmazloum and György, 2012). Watts *et al.* (2006) discovered an interesting substrate selectivity switch (a single point mutation) that can change the TAL activity to PAL activity. They also showed that the identity of the amino acid at the switch position can serve as a guide to predict substrate specificities of the annotated aromatic amino acid lyases (Watts *et al.*, 2006).

A possible activity of *p*-coumaric acid decarboxylase (PCD), for a direct conversion of *p*-coumaric acid to tyrosol in salidroside biosynthesis has been proposed by Ling-Ling and co-workers (2007), but no experimental results are published to prove their statement (Figure 3, C type arrows). However, the PCD activity has only been described in microorganisms (Cavin *et al.*, 1997; Rodríguez *et al.*, 2010); but no molecular characterization and genetic information have been reported so far in plant species (Ma *et al.*, 2008). *P*-coumaric acid can be further converted to its corresponding alcohol (*p*-coumaryl alcohol) by the activity of the same enzymes; 4CL, CCR and CAD (Figure 3, F and G type arrows) which have been described in CAGs proposed biosynthetic pathway. The enzyme catalyzing the conversion of p-coumaryl alcohol to tyrosol which has also been proposed by Ling-Ling and co-workers (2007), (indicated by a question mark (?) in their paper), is completely unknown.

Two other pathways for the tyrosine-derived tyrosol formation (Figure 3, B and E type arrows) for salidroside biosynthesis have been also proposed which are more plausible as both are supported by some molecular studies in roseroot species (Ma *et al.*, 2008; György *et al.*, 2009; Zhang *et al.*, 2011).

The first one (Figure 3, B type arrows) is initiated by the activity of tyrosine decarboxylase (TyrDC) enzyme which can catalyze the formation of tyramine. The enzyme activity and its characteristics have been studied in roseroot species. A deduced amino acid sequence of *R. sachalinensis TyrDC* showed around 60% identity with those of other *TyrDCs* of plant origin (Zhang *et al.*, 2011) and 79% identity with studied *R. rosea TyrDC* by György and co-workers (2009). Tyramine, then can be converted to 4-hydroxyphenylacetaldehyde as a result of primary-amine oxidase (CAO) enzyme activity. The presence of CAO in plant species (Tipping and McPherson, 1995; Padiglia *et al.*, 2004; Longu *et al.*, 2005; An *et al.*, 2008) catalyzing the oxidation of primary amines (tyramine) to aldehydes, have been cited in many enzyme databases, but no report with its new EC number

(1.4.3.21) is published in the literatures. The activity of another enzyme named, tyramine oxydase (EC: 1.4.3.4) was also proposed by Landtag and co-workers (2002) for the conversion of tyramine to 4-hydroxyphenylacetaldehyde, which is not known to exist in plant species by this EC number.

In the second one (Figure 3, E type arrows), two enzymes; histidinol-phosphate transaminase (HPA) and aromatic-amino-acid transaminase (AAT) is thought to be responsible for catalyzing the formation of 4-hydroxyphenylpyruvate. The isolation and characterization of the first plant cDNA encoding HPA protein has been reported from *Nicotiana tabacum* plantlets (El Malki *et al.*, 1998). AAT is also purified and its characterization has been reported from *Vigna radiate* (Simpson *et al.*, 1997). 4-Hydroxyphenylpyruvate can then be converted to 4-hydroxyphenylacetaldehyde by the activity of another enzyme named, 4-hydroxyphenylpyruvate decarboxylase (HPPD). The HPPD activity was reported only once in *Berberis* plant species by Rueffer and Zenk (1987).

Finally, aryl-alcohol dehydrogenase (*AAD*) catalyzes the conversion of 4-hydroxyphenylacetaldehyde to tyrosol according to KEGG database, although no precise scientific evidence exists, supporting such an activity in plant species. Among all these mentioned enzymes only the role of TyrDC has been supported in roseroot plants by molecular research results. György and co-workers (2009) have isolated a cDNA encoding for TyrDC and found that in plants with higher salidroside content the expression of this gene was considerably higher than in the low salidroside producer line. The expression of the gene was higher in the roots (where salidroside accumulates) than in the leaves. Zhang and co-workers (2011) reported a cloning and expression pattern of a cDNA encoding TyrDC from *R. sachalinensis*. The biochemical assays of recombinant *RsTyrDC* and the effects of sense and antisense overexpression of endogenous *RsTyrDC* in *R. sachalinensis*, demonstrated that the *RsTyrDC* is most likely to have an important function in the initial steps of the salidroside biosynthesis pathway.

The functioning role of TyrDC in tyrosol formation and salidroside biosynthesis is also supported by the results of Ma and co-workers (2007) in which they proved that tyrosol biosynthesis does not have any direct linkage with *p*-coumaric acid accumulation in *R. sachalinensis* and these results demonstrate that it is highly unlikely that *p*-coumaric acid is a precursor for tyrosol biosynthesis (Figure 3, C, D, F and G type arrows). Another supportive result for this hypothesis is in the work of Landtag and co-workers (2002), where potato was transformed to express parsley *TyrDC* to study what role tyramine plays in response to *Phytophthora infestans* infection and whether the expression leads to higher tyramine-derived compounds. However, the reaction did not

turn out as expected. Instead, it led to the accumulation of another compound, which has not been reported from potato previously, and was identified as tyrosol glycoside, i.e. salidroside.

The final step in salidroside formation is the glycosilation of tyrosol. It is likely that *UDP-glycosyltransferase* is the enzyme catalyzing the final reaction by means of deploying UDP-Glc (a typical activated sugar in plants) as a glucose donor and tyrosol as its aglycon (Ma *et al.*, 2007). Currently the Carbohydrate-Active Enzyme database (CAZy, http://www.cazy.org) has collected over 12,000 sequences classified in 90 families, encoding glycosyltransferases (GTs) in different organisms. Among the GTs contributing to the many different glycosyl transfer reactions, those glycosylating natural products and small lipophylic molecules belong to the GT1 family. Sugars can be transferred to the OH, SH, NH and carboxyl groups of the acceptors (Vaistij *et al.*, 2009). Glycosides may also act as subject for further glycosylation. The glycosylation profile of an acceptor can vary between different species within a plant genus (Frydman *et al.*, 2004). Natural products such as shikimate derivatives (phenylpropanoids, benzoates, and flavonoids), alkaloids and many amino acid derivatives exist as glycosides in plants. Glycosylation can alter the solubility and transport of the compounds within the cell; stabilizing the product; and modulate bioavailability and storage (Vogt and Jones, 2000).

In connection with salidroside formation, Ma and co-workers (2007) reported an expression pattern of a cDNA encoding a *UDP-glycosyltransferase* that regulates the conversion of tyrosol to salidroside in *R. sachalinensis*. Overexpression of the endogenous *UDP-glycosyltransferase* (*UGT73B6*) increased the salidroside content up to two folds compared with that of untransformed controls in transgenic *R. sachalinensis* although, the lack of tyrosol supply may be a limiting factor for further accumulation of salidroside (Ma *et al.*, 2007).

Yu and co-workers (2011) studied the over-expression of two endogenous *UDP-glycosyltransferases*; *UGT72B14* and *UGT74R1* in hairy root lines of *R. sachalinensis*. *UGT72B14* transcripts were mainly detected in roots and exhibited the highest level of activity for salidroside production *in vitro* and *in vivo*. Accordingly, *UGT72B14* has been suggested to have an important function in this pathway. Compared to *UGT74R1*, *UGT73B6* (Ma *et al.*, 2007) showed a higher level of activity for salidroside production, and this isozyme was highly expressed in the roots, so it is very likely that *UGT73B6* contributes to salidoroside synthesis.

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

Biotechnological achievements provide the possibility to produce standardized phytochemicals independent from environmental conditions. Plant cell culture technologies were introduced at the end of the 1960s as a possible tool for both studying and producing plant secondary metabolites (Tatli, 2012). Organ cultures are also appropriate for germplasm conservation and propagation. *In vitro* systems are mostly following common strategies:

1. Search for cell lines with high production capacity of desired metabolites.
2. Optimizing the culture media and physico-chemical parameters by means of cell permeability, organelle targeting, eliciting systems, nutritional adjustments, cell immobilization, cellular biotransformation and even genetic alteration.
3. Commercialization (scaling up the *in vitro* cultures to bioreactors considering sterility and adequate oxygen supply for the cells).

All these procedures are developed as a viable option to increase the synthesis of secondary metabolites. Another important aspect regarding the commercial exploitation of *in vitro* systems is the maintenance of the productivity over time, which can be changed depending on the inherent genetic and epigenetic instability of plant cell cultures (Phillips *et al.*, 1994; Vázquez, 2001).

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).

2.7.1. *In vitro* culture of *R. rosea*

In vitro culture of *Rhodiola rosea* has a history of about 30 years. First studies have been made in the former Soviet Union which are mostly about the effect of different culture media and different plant hormones on the various types of explants; sterilization of different explant types, callus induction, organogenesis and regeneration. In 1981, Aleksandrova *et al.* (cited by Furmanowa *et al.*, 1995) patented the method for root regeneration from the callus, but did not provide any data on the callus induction and its maintenance. The only information was that MS medium (Murashige and Skoog, 1962) was used for root induction from callus supplemented with sucrose (2.5-3.5%) thiamine HCl, mesoinositol, NAA (0.8-1.1 mg/l), adenine (0.08-0.013 mg/l) and kinetin (0.01-0.1 mg/l) and 25-28 days were necessary for the root formation. First scientific publications about callus induction appeared in the mid 80's which are cited by Bykov *et al.*(1999).

2.7.2. Explants for *in vitro* culture

Contamination of *R. rosea* explants is a major problem. The most often and most successfully used explants are leaves of *in vitro* grown seedlings developed from *in vitro* sown seeds (Tasheva and Kosturkova, 2010 and 2012b). Furmanowa *et al.* (1995) germinated immature seeds after being washed in running water for 1 h, dipped into 70% ethanol for 1 min, soaked in 5% solution of calciumhypochlorite for 10 min and finally rinsed three times with sterile water. Ishmuratova (1998) used both seeds and buds successfully for *in vitro* culture establishment. The sterilization protocol for both, included 1 min in 70% ethanol and 5 min in either 3% hydrogen peroxide or 7 min in aqueous 0.1% mercuric chloride.

Tasheva and Kosturkova (2010) conducted a broad study with 6 schemes for sterilizing not only seeds but also buds and shoot or rhizome segments. For seed decontamination, immersion for 3 min in 70% ethanol and 15 min in 20% (v/v) bleach is recommended by them. Also apical buds immersed for 1 min in 70% ethanol, 20 min in 15% bleach and 15 min in 0.2% mercuric chloride and rhizome buds that immersed for 1 min in 70% ethanol, 17 min in 20% bleach were successfully sterilized and used. Ghiorghita *et al.* (2011) reported about difficulties during the decontamination of roseroot explants. Mature rhizome buds, apices and shoot segments of young plants were treated with short immersion in 5% chloramine-T solution. Only those explants remained viable after the sterilization process that was first submitted to low temperature. Based on these reports a general protocol cannot be determined. The different working groups have contradictory results; what worked for one, not necessarily worked for the other. Probably the genotype and the original environment of the plant have a major effect on the success of the decontamination.

2.7.3. Callus induction and tissue culture

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 on MS medium supplemented with different plant hormones. Furmanowa *et al.* (1995; 1998) 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.

György *et al.* (2004; 2005) also used leaf explants and the combination of 1.5 mg/l BAP and 0.5 mg/l NAA. Krajewska-Patan *et al.* (2007 a,b) induced callus from hypocotyls of *in vitro* grown seedlings on MS medium supplemented with also BAP, NAA and adenine chloride.

Martin *et al.* (2010) used epicotyls for callus induction and liquid medium instead of solid. The most effective combination was 1 mg/l 2,4-D with 1 mg/l IBA or 0.1 or 1 mg/l 2,4-D alone. The main aim of Tasheva and Kosturkova (2010) was the micropropagation of roseroot, but during their thorough experiments an optimized callus inducing medium composition was also achieved. MS medium containing 0.2 mg/l BAP and 0.1 mg/l IAA resulted in compact, green callus from apical bud explants. Leaf explants on zeatin containing medium (2 or 0.2 mg/l zeatin and 0.2 mg/l IAA) produced poor growing and soft callus. On 2-iP containing medium (3 mg/l 2-iP and 0.3 mg/l IAA or NAA) 78% of leaf explants formed compact green callus and 22% pale and friable callus. The already mentioned two types of roseroot calli were also observed by Ghiorghita *et al.* (2011). A compact, green callus was formed on MS medium supplemented with 1 mg/l BAP and 0.5 mg/l 2,4-D from internode fragments, while a semi-compact cream colored callus developed from leaf fragments on MS medium with 1 mg/l BAP and 0.5 mg/l 2,4-D.

György *et al.* (2004) reported the initiation and cultivation of compact callus aggregates (CCA). In order to establish suspension culture, calli grown on solid media were gently broken using forceps and were transferred into liquid MS medium containing 0.5 mg/l BAP and 1 mg/l NAA, shaken on an orbital shaker at 135 rpm. Subcultures were carried out in every 8-10 days by decanting all medium from the flask and adding fresh medium to the cultures. The culture was composed of green or light green, spherical, smooth surfaced callus aggregates as described similarly by Xu *et al.* (1999) in case of *Rhodiola sachalinensis*.

The secondary metabolite production of tissue cultures of *Rhodiola rosea* has been investigated since the late 1970s. In the earliest reports, 13 compounds and their structures were reported from the callus of *R. rosea* by Kurkin *et al.* (1991). In the suspension culture, the main constituent was triandrine with 0.19% (dry weight) and neither salidroside and tyrosol nor cinnamyl alcohol glycosides have been found.

Furmanowa *et al.* (1995; 1998) reported only traces of these glycosides in addition to triandrine and caffeic acid. The results presented in the study of György *et al.* (2004) confirm that there is no production of the pharmaceutically important glycosides in the callus cultures of *R. rosea* and not even in compact callus aggregates.

3. MATERIALS AND METHODS

3.1. Plant materials

Different roseroot plant materials including seeds, leaves, rhizome cuttings, whole plants, calli and rhizomes, have been obtained and used for different experiments in this research.

Seeds of *Rhodiola rosea* L. were kindly provided by Botanical Garden of University of Oulu (Finland) originated from an Austrian population. Samples from leaves and rhizomes of different individual plants were collected from a natural population on the Hochkar, Göstling Alps; Austria (47°48′N, 14°56′E) in 2012-2013. The collected plant materials for molecular studies (leaves, roots and rhizomes) were fast frozen in dry ice at the collection site and kept at -70°C upon arrival in the lab until further analysis.

The collected rhizome cuttings of equal size (right after snow melting, 7.06.2013) were transferred quickly to the lab and subjected for phytotron experiment. The cuttings were planted in 1:1:1 mixture of perlite, black peat and lime soil in 2 liter containers. The plants were kept in plant growth chamber (Weiss Technik SGC-120.UK) where the temperature and relative humidity was kept at 17/12°C and 60/80% day/night, respectively and with 14 h of day length. Light was provided by fluorescent light tubes (12×36W Philips 840 TL-D 1G; 4000K) with a light intensity of 10 klx at the level of plants. Root and rhizome samples were taken every 3 weeks at 12 a.m. individually from each plant during the experiment period. All of the samples were immediately frozen in liquid nitrogen and kept at -70°C for further studies.

3.2. *In vitro* experiments of roseroot

3.2.1. *In vitro* cultures

To establish an *in vitro* culture, roseroot seeds were surface sterilized by being washed in running tap water, immersed in 70% ethanol for 3 min followed by submerging in 50% sodium hypochlorite for 4 min and then rinsed four times in sterile distilled water. For germination, half-strength MS medium (Murashige and Skoog, 1962) supplemented with 30 g/l sucrose, solidified with 4.5 g/l agar was used. The pH was adjusted to 5.8 before sterilizing the medium by autoclaving at 121°C for 18 min. 25 ml of medium was distributed into glass baby food jars. 40 sterilized seeds were sown in each jar. 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}.s^{-1} at culture level, provided by cool-white fluorescent lamps. After seed germination, the seedlings were sub-cultured on the same medium every 6 weeks (appearance of the 5th leaf).

For callus induction, three individual seedlings were selected based on good vigor 16 weeks after germination. Leaves of 4-6 mm in diameter were cut from the seedlings and scratched at the edges by using a sharp sterile scalpel blade. The leaves were put on the surface of fresh MS medium enriched with 30 g/l sucrose and gelled with 4.5 g/l agar at the same pH, supplemented with NAA, BAP, IAA, IBA and 2iP phytohormones with different concentration and combinations in glass Petri dishes. Callus formation occurred under the same light and temperature conditions during the following 6 weeks. The maintenance of the callus lines was insured by subcultures on the same media during the whole experiment and was utilized in further experiments.

3.2.2. Transgenic callus culture establishment

3.2.2.1. Transformation of the callus

Twelve pieces of the most vigorous callus masses (6-8 mm in diameter) were placed on solid MS medium without any growth regulators in Petri dish, 2 days before the transformation. The callus was transformed using an *Agrobacterium 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 aminoglycoside antibiotics such as kanamycin, and a *uidA* (GUS) reporter gene, encoding ß-glucuronidase enzyme (Tinland *et al.,* 1995). Both the reporter and selection marker genes are under the control of cauliflower mosaic virus (CaMV) 35S promoter. The bacterium containing the gene construct was placed on solid AB medium (Lichtenstein and Draper 1986) 48 h before the transformation to obtain fresh growing bacterial clone. For the co-cultivation, small volumes (20-30 µl) of bacterial suspension (10^8 cells in 1 ml) were placed onto the surface of the prepared callus.

The co-cultivation of the callus with bacteria was conducted in dark for 24, 36 or 48 h at 26-28^{o}C, then the calli were transferred onto solid MS medium containing 20 mg/l kanamycin, 200 mg/l carbenicillin, 300 mg/l claforan, 4 g/l insoluble polyvinylpyrrolidone (Perl *et al.,* 1996; Mozsár *et al.,* 1998) and 0.1 g/l dithioerythritol (Bornhoff and Harst 2000). Calli were transferred to fresh selection medium of the same composition in each 20 days for selecting the transgenic cells. Final selection was conducted by culturing the calli in shaking liquid medium to obtain 100% transgenic cells in response to direct contact with kanamycin.

3.2.2.2. DNA extraction and polymerase chain reaction (PCR)

To determine the foreign gene insertion in the callus, genomic DNA was individually extracted from 20 different transgenic callus pieces and one of the non-transformed callus (Figure 4.) from the 5th sub-cultures (transformed calluses) by using SP Plant mini kit (Omega Bio-tek, USA). PCR was performed in 25 µl reaction volume containing 20-80 ng DNA, 10X PCR reaction buffer, 2.5 mM $MgCl_2$, 0.02 mM dNTP mix, 1 µM of each forward (5'-GAGGCGAGGCGGCTATGACTG-3') and reverse (5'-ATCGGGAGCGGCGATACCGTA-3') primers for *npt*II gene (Hoffmann *et al.*, 1997), 1 unit of *Taq* DNA polymerase (Fermentas) and sterile distilled water. The reactions were carried out in a Swift MaxPro thermocycler (Esco Healthcare, Pte, Singapore). For amplification of the transgene fragment the following program was used: initial denaturation at 94°C for 4 min; followed by 30 cycles of 94°C for 60 s, 54°C for 60 s, 72°C for 90 s; and a final synthesis at 72°C for 3 min. For positive control, pTd33 plasmid DNA was used as a template in the reaction mix to control the PCR performance, negative control without any DNA template was also included. The PCR products were applied on a 1% (w/v) ethidium bromide-stained agarose gel in 1xTBE buffer with xylencyanol loading buffer to verify the occurrence of the amplification. 10 µl of the PCR products stained with 2 µl of loading dye were run for 1 h at 80 V. Amplicons were scored visually for presence (1-20 and P, in Figure 10.) or absence (N-C, in Figure 10.) of bands of 700 bps in length. DNA samples were also tested with VC Forward (5′-ATCATTTGTAGCGACT-3′) and VC Reverse (5′-AGCTCAAACCTGCTTC-3′) primers, designed to amplify fragments of the Ti and Ri plasmid encoding *virC1* and *virC2* regions of *Agrobacterium tumefaciens* (Sawada *et al.*, 1995) to verify the absence of any *Agrobacterium* in the medium or in the calli.

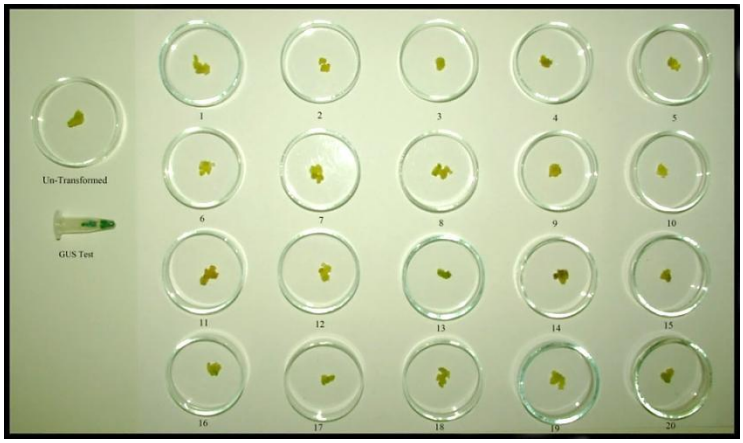

Figure 4. Callus samples subjected for DNA extraction and genetic transformation verification

3.2.2.3. Histochemical GUS assay

For verification of the transformation, callus samples were transferred to 1 ml of assay solution (Jefferson *et al.*, 1987; Oláh, 2005) in 1.5 ml Eppendorf tubes. The solution contained 150 µl of 100 mM Na-Phosphate buffer (pH 7.0, 50 mM Na_2HPO_4 and 50 mM KH_2PO_4), 100 µl of 50 mM Na-EDTA, 25 µl of 5 mM K-ferricyanide, 25 µl of 5 mM K-ferrocyanide, 100 µl of 0.005% Triton X-100, 25 µl of 0.3% X-Gluc and 575 µl of distilled water. 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 (2 out of 12 pieces from each Petri dish) 5 and 15 days after transformation time.

3.2.3. Hormonal effects of antibiotics on callus culture

After optimizing the liquid culture medium (MS supplemented with 30 g/l sucrose, 1 mg/l NAA and 0.5 mg/l BAP), different concentrations of carbenicillin and claforan (300, 400, 450, 500 and 550 mg/l) were added separately and also in equal combinations to the culture medium. Twenty ml of different liquid media and 1 g of roseroot callus were inoculated in 100 ml Erlenmeyer flasks and placed on an orbital shaker at 120 rpm in 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 60 $\mu mol.m^{-2}.s^{-1}$ at 25°C in a light room. The experiment lasted for 30 days and the media were refreshed at 10 days intervals. After the laps of experiment, calluses were removed, washed with distilled water, rinsed on paper towel and their fresh weight was measured.

3.2.4. 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. Stock solutions of 4 mM were prepared according to the recommended protocols for dissolving and molarity adjustment. Around 500 g of green homogenous roseroot callus was produced for biotransformation experiments in the optimized medium (MS + 1 mg/l NAA + 0.5 mg/l BAP + 30 g/l sucrose). 4 g of callus tissues were measured aseptically and put into 8 ml of biotransformation media in 50 ml Erlenmeyer flasks.

The precursors were dissolved in different solvents and prepared to be of 2 mM final concentration in the culture medium. The pH was adjusted to 5.8 using HCl and NaOH. The final medium was filter sterilized using 0.22 µm pore size, Millex GP filter unit from Millipore.

Due to the large scale of this experiment, the precursor feedings were conducted in 2 separate (but similar) experimental conditions; using the compounds from salidroside (tyramine, 4HPP, tyrosol) and cinnamylcohol glycosides biosynthesis pathway (phenylalanine, *para*-cumaric acid, *trans*-cinnamic acid, cinnamaldehyde, cinnamyl alcohol), respectively. The flasks were kept on an orbital shaker at 120 rpm in 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 60 $\mu mol.m^{-2}.s^{-1}$ at 25°C in a light room. 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. Five ml of each culture medium was also taken at the start of the experiment and stored at -20°C for HPLC analysis and as original reference. The harvested samples were divided to two parts; half of them were washed with distilled water (3 times) and surface dried on paper towel and finally dried in an oven overnight at 45°C, while the other half were just washed with nuclease free water (2 times), cut to very thin flakes (to ease the freeze grinding of samples with mortar and pestle), fast frozen in liquid nitrogen and stored at -70°C. The dried calluses were used for HPLC analysis and the frozen samples were subjected for RNA extraction and gene expression analysis.

3. 3. Phytochemical analysis

3.3.1. Extraction and HPLC

For all of our phytochemical analysis, samples (~5 g) were dried at 45°C overnight. From each sample 0.5 g of dried plant tissues was grounded for extraction. Different extraction solvents were tested to obtain the optimal result. The 70% methanolic extraction was selected as best for chromatographic analysis. To disrupt the plant cell physical barriers, extraction was carried out in an ultrasonic bath for 1 hour. A clear extract (supernatant) was obtained by centrifugation (8000 rpm) and used for HPLC analysis. The chromatography 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 with a neutral mobile phase (purified water and acetonitrile) gradient system at a flow rate of 1.0 ml/min and UV detection at 205, 222, 254 and 275 nm simultaneously. Peaks were identified by comparison of retention time and spectral data with adequate parameters of standards. The *R. rosea* Standards Kit (Chroma Dex, USA) contained rosin, rosavin, rosarin, salidroside and, tyrosol. Tyrosine, phenylalanine, tyramine, 4-hydroxyphenylpyruvate, *p*-cumaric acid, *trans*-cinnamic acid, cinnamaldehyde and cinnamyl alcohol were purchased from Sigma. Quantification was performed based on the peak area. The content of the determined compounds was calculated as mg/100g dry matter.

3.3.2. Statistical analysis

The results of HPLC analysis were subjected for different statistical methods and the obtained data were processed and validated using different approaches in each experiment. 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.

3.3.3. Definition and comparison of the values of dissimilarity index

In order to learn the similarity and express the dissimilarity of the character of compound accumulation process in time, we introduced a ten-dimensional characteristic code of accumulation process for each of the 6 compounds in root and rhizome of all the 7 plants, that is to say we calculated 6*2*7 ten-dimensional codes $C_k^l(i) = \left(c_k^l(i)_{12}, c_k^l(i)_{13}, \ldots, c_k^l(i)_{ts}, \ldots, c_k^l(i)_{45}\right)$, where *k* is for rosin, rosavin, rosarin, cinnamic alcohol, salidroside and tyrosol, *l* is for rhizome or root and $i = 1, 2, \ldots, 7$ is for the plants. The way of calculation was as follows: dimension *ts* was for the change from sampling time *t* to *s* ($t = 1, 2, \ldots, 4$, $s = 1, 2, \ldots, 5$, that is to say we have $ts = 12, 13, 14, 15, 23, 24, 25, 34, 35, 45$). The value of a code is equal to +1 or -1 if the compound content is increasing/decreasing and the rate of increase/decrease is above the 10% of the mean of the glycoside content measured in five sampling times, respectively. The value of a code is equal to 0 if the absolute change of glycoside content is below the 10% of the mean (Figure 26.).

3.3.4. The characteristic code of accumulation process

$C_k^l(i)$ is suitable to describe the process for a fixed type of compounds, plant and place of accumulation; moreover, we can compare the plants, the compounds and the rhizome-root pairs, according to their dissimilarity expressed by the distance of the characteristic codes.

Therefore, we calculated the Euclidean distances of all pairs of curves as dimensionless quantities:

$$D_k^l(j,i) = D_k^l(i,j) = \sqrt{\sum_{t=1}^{4}\sum_{s=2}^{5}\left(c_k^l(i)_{ts} - c_k^l(j)_{ts}\right)^2}$$

(again, *k* is for the compounds, *l* is for rhizome or root, *i* is for the individuals) and took the sum of dissimilarity measures $D_k^l(j,i)$ for all the individuals $i = 1, 2, \ldots, 7$:

$$I_k^l(i) = \sum_{j \neq i} D_k^l(i, j).$$

$I_k^l(i)$ is called dissimilarity index of plant i, calculated for compound k. Let us denote by $\overline{I_k^l}$ the mean of values $I_k^l(i)$ taken over all plants and call it *mean dissimilarity index* of compound k:

$$\overline{I_k^l} = \frac{1}{7} \sum_{i=1}^{7} I_k^l(i).$$

If we represent 6 points $P_k = \left(\overline{I_k^{root}}; \overline{I_k^{rhizome}}\right)$ for *k* as rosin, rosavin, rosarin, cinnamyl alcohol, salidroside and tyrosol, we can state that the closer a point is to the origin, it represents the more similar set of curves (Figure 26.). Moreover, if a point is under the identity line, the curves for rhizome are more similar while if it is above the identity line, the similarity is more expressed in root.

For the data analysis and representation, MS Excel 2013 was used.

3.4. Molecular biology experiments

All of the PCRs have been carried out using a Swift MaxPro thermocycler (Esco Healthcare, Pte, Singapore).

All of the PCR products were applied on a 1% (w/v) ethidium bromide-stained agarose gel in 1×TBE/TAE buffer (depending on our need) with xylencyanol loading buffer to verify the occurrence of the amplification.

All of the PCR products to be sequenced were ligated to pJET1.2/blunt vector (CloneJET PCR Cloning Kit, Thermo Scientific) according to the kit protocol for positive selection. Five µl of the ligation mix was transferred into 50 µl of chemically competent DH5α *E. coli* (Zymo Research, Irvine, CA, USA) and held on ice for 15 minutes. Fifty seconds of heat shock at 42°C was used for the transformation, and then the bacteria were put back on the ice for another 15 minutes. The transformation mix was diluted with 300 µl LB-ampicillin liquid media and kept at 37°C shaker for half an hour.

As this vector contains a lethal gene, which is disrupted by ligation of the DNA insert into the cloning site, only cells with recombinant plasmids are able to propagate, eliminating the need for blue/white screening. One hundred µl of the competent cells were spread on pre-warmed (37°C) LB culture plate containing 100µg/ml (0.01%) ampicillin. The plate was kept overnight at 37°C for the colonies to grow. Twenty of the positive colonies were selected and re-plated to fresh LB-ampicillin

medium. The same pipetting tips that were used for re-plating were used to give each of the templates DNA for colony PCR.

The colony PCR analysis was carried out according to the CloneJET PCR Cloning Kit protocol and with pJET1.2 forward and reverse primers to confirm the expected and correct amplicon size. Three colonies which showed the expected size were selected from the master plates and subjected for another overnight culture in 2 ml liquid LB medium with 1% ampicillin.

The bacterial plasmids were isolated using a Plasmid Mini Kit I (Omega Bio-tek, USA) according to the manufacturer's protocol. The plasmid concentration was assessed using NanoDrop 1000 spectrophotometer (NanoDrop Technologies, USA) at 260 nm. Finally, ABI PRISM® 3100 Genetic Analyzer (Applied Biosystem) automatic DNA sequencer was utilized for plasmid sequencing at the BayGen Institute in Szeged.

3.4.1. Genomic DNA extraction

Roseroot genomic DNA was extracted from frozen leaves, roots, rhizomes and callus using E.Z.N.A. SP Plant Mini Kit (Omega, VWR, Budapest, Hungary) according to the manufacturer's instructions. DNA quality and quantity was measured respectively by ethidiumbromide stained 1% TBE agarose gel electrophoresis and NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies, USA).

3.4.2. RNA extraction

Deep frozen leaves, roots, rhizomes and callus of *Rhodiola rosea* (kept at -70°C), were ground in liquid N_2 using a sterile mortar and pestle. Total RNA was extracted according to a CTAB-based protocol (Jaakola *et al.*, 2001) from 0.5-1 g fresh plant material and their quality was determined on an ethidium bromide stained 1% agarose gel. RNA concentration was assessed using NanoDrop 1000 spectrophotometer (NanoDrop Technologies, USA) at 260 nm.

To eliminate any possible genomic DNA contamination, all samples were treated with DNase I enzyme (Fermentas) and the RNA concentration was quantitatively normalized to 5 μg/30 μl of reaction mix for all of further molecular studies. DNase treated RNA concentration was measured again using NanoDrop spectrophotometer for reverse transcription (cDNA synthesis) and stored at -70°C for further studies.

3.4.3. Reverse transcription (cDNA synthesis)

DNase treated RNAs were reverse-transcribed to cDNA by M-MuLV RT enzyme using Maxima Reverse Transcriptase kit (Thermo Scientific). Half and 1 μg of total RNA was used in a final reaction volume of 30 μl using gene specific, random hexamer or oligo $(dT)_{17}$ primers relevant for different purpose of molecular methods in this work.

3.4.4. Rapid amplification of cDNAs end (RACE)

3.4.4.1. 3′ cDNA end amplification

To amplify the 3′ cDNA end, the following strategy was applied. Specific primers from the known area of the targeted gene plus an adaptor-oligo (dT) primer were synthesized and used in the following procedure:

1. First strand cDNA synthesis was initiated at the poly(A) tail of mRNAs (Figure 5, 1.) using the adapter primer AP (5′-GGCCACGCGTCGACTAGTAC$T_{(17)}$-3′).
2. After first strand cDNA synthesis, the original mRNA template was destroyed with RNase H, which is specific for RNA:DNA heteroduplex molecules (Figure 5, 2.).
3. Amplification was performed, using two primers: one**,** our gene specific primer (GSP); eg, UDP3′a (5′-CCAGACTCCGTCGTGTACGTTTG-3′) that anneals to a site located within the identified cDNA molecule; and the other was a universal amplification primer UAP (5′-CUACUACUACUAGGCCACGCGTCGACTAGTAC-3′) that targets the 3′ end of the newly synthesized complementary DNA (Figure 5, 3.).
4. As the AP primer would lead to first strand cDNA synthesis from all polyadenylated mRNAs, the sequence specificity in the amplification reaction is therefore derived solely from the GSP. Therefore, a second "nested" gene specific primer; eg; UDP3′b (5′-GGAGAAGTTCCGAGTCAGGATC-3′) was utilized in conjunction with the AUAP (5′GGCCACGCGTCGACTAGTAC-3′) in a second amplification reaction to give more specificity to 3′ RACE procedure (Figure 5, 4.).

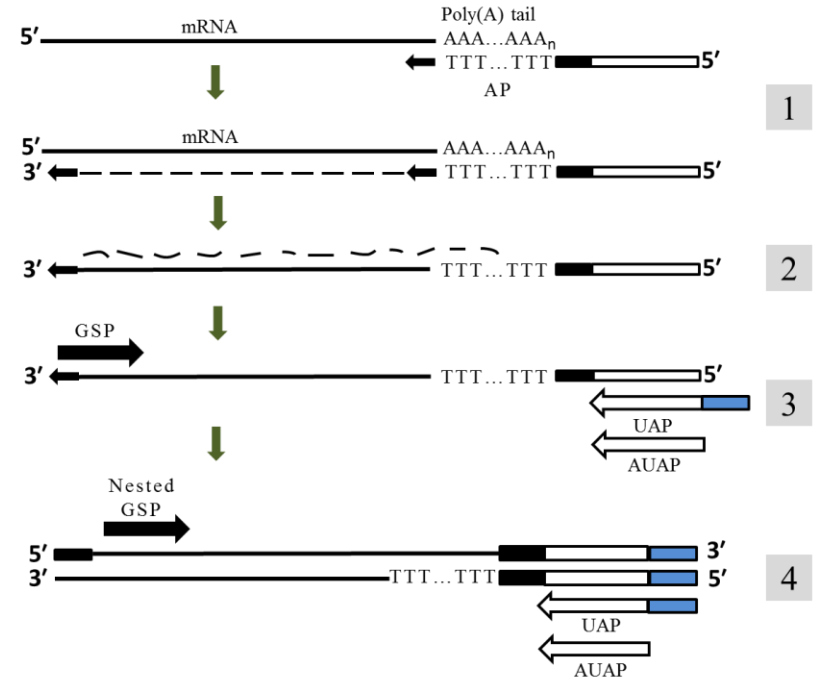

Figure 5. Overview of the 3' RACE procedure.

3.4.4.2. 5′ cDNA end amplification

To amplify the 5′ cDNA end, the following strategy was applied:

1. Complementary DNA (cDNA) was synthesized (Figure 6, 1.) using a gene specific primer (GSP1) as described at (3.4.3).
2. After first strand cDNA synthesis, the original mRNA template was removed by treatment with the RNase H and RNase T1 which cleaves the 3′ of single-stranded G residues (Figure 6, 2.). Unincorporated dNTPs, GSP1, and proteins were separated from cDNA using S.N.A.P. Column (Thermo Scientific).

A homopolymeric tail was then added to the 3' end of the purified cDNA using dCTP and terminal transferase enzyme (Thermo Scientific) (Figure 6, 3.).

3. By using GSP2 primer from the known region of our target gene; and an abridged anchor primer; AAP (5′-GGCCACGCGTCGACTAGTAC$G_{(16)}$-3′) the complete 5′ cDNA end was PCR amplified (Figure 6, 4.).
4. By using a nested GSP2 primer the PCR efficiency was improved due to an increased specificity and prevention of “primer-dimer” amplification of residual GSP1 that may have been carried over through the cDNA purification procedure (Figure 6, 5.).

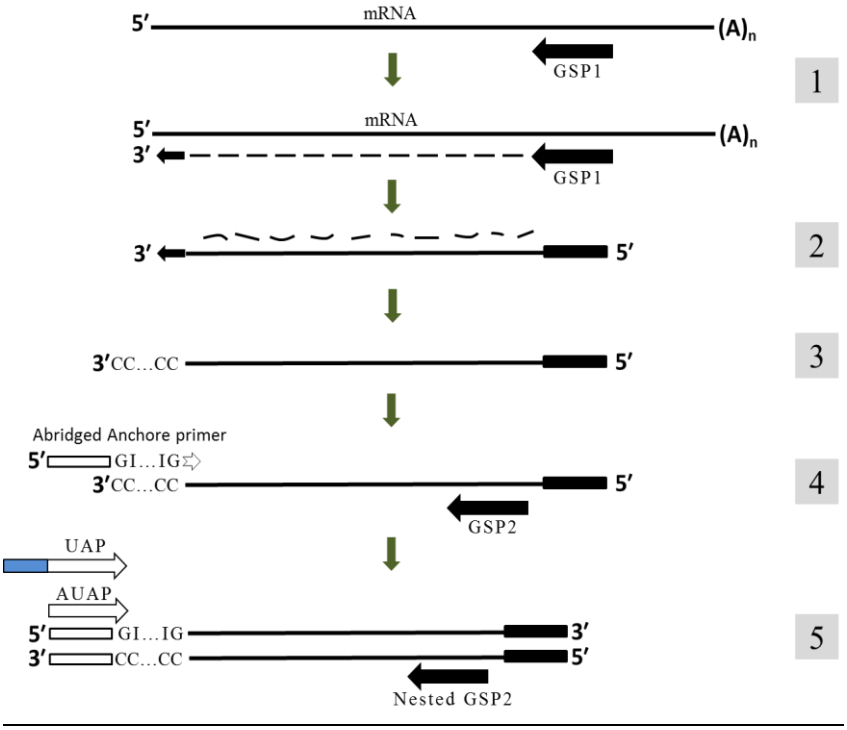

Figure 6. Overview of the 5' RACE procedure.

3.4.5. Data mining and degenerate primer design

To identify the two hypothesized genes (*UDPG* and *AAD*) involved in salidroside biosynthesis, the work started with datamining in the NCBI database using the gene names as keywords (*UDP-glycosyltransferase* and *aryl alcohol dehydrogenase*). The upcoming results were further filtered to mRNA sequences and for "green plants" and "eudicots". The search results with complete coding DNA sequence (cds) tags were selected and aligned in Mega 6 software (Tamura *et al.*, 2013) for similarity analysis.

Degenerate primers were designed based on the most conserved regions of the aligned sequences and synthesized. Using *R. rosea* genomic DNA and cDNA as templates, PCR was performed (3 min, 95°C; 30 cycles of 30 s, 95°C, 60 s, 58-65°C, 1-2 min, 72°C and a final extension for 7 min at 72°C) for different primer pairs with slight program modifications. PCR products were run on agarose gel and the expected size amplicons were excised from the gel, purified with EZ10 Spin Column DNA Gel Extraction Kit (BIO BACIC INC., Canada), cloned and sequenced as described earlier (3.4). The obtained sequence was analyzed by NCBI BLAST similarity search.

3.4.6. *R. rosea* specific *UDPG* and *ADD* primer design

R. rosea specific *UDPG* and *AAD* primer pairs were designed after that significant similarity of sequenced nucleotides (amplified by using degenerate primers) were confirmed by blasting the sequences in NCBI database and comparison with other plant *UDPG*s and *AAD*s. The *UDPG* specific primers (0SP-F: 5′-GAATGTGTTGAACATGAAAGCG-3′ and 0SP-R: 5′-GATCCTGACTCGGAACTTCTCCTG-3′) were designed to amplify a 273 bp long fragment. Whereas, the *AAD* specific primers (0SP-F: 5′-GGAGAAGGTAAAATCAAGACTAT-3′ and 0SP-R: 5′ -CACCCATTACTGTTCCATA-3′) were designed to amplify a 196 bp long fragment. To confirm the primary results, the same PCR program was applied and the products were subjected for similar electrophoresis, gel excision, cloning and sequencing (3.4).

The 3′ cDNA end nucleotide sequence was PCR amplified as explained in 3.4.4.1. Amplicons of approximately 750 bp long for *UDPG* and 800 bp long for *AAD* were amplified, transferred to *E.coli*, cloned and sequenced as described before (3.4). The 5′ cDNA end sequence was also found (3.4.4.2) using *UDPG* and *AAD* gene specific reverse primers (UDP5′a-R- UDP5′c-R; *AAD5′*aR, *AAD5′*bR) and an abridged anchor forward primer. Approximately 850 bp (*UDPG*) and 800 bp (*AAD*) long PCR product was amplified, cloned and sequenced as described earlier (3.4).

Having the 3′ and 5′ sequences, the full length cDNA was ultimately PCR amplified using UDORF-F (5′-ATGGGTTCTGATTCACGG-3′) and UDORF-R (5′-CTAGGACAAAGTCTCTCTTCTC3-′) primers for *UDPG* and AAORF-F (5′-ATGTCGCTCCACGTCTAC-3′) and AAORF-R (5′-TTATGCGCGTCTGTACTCA-3′) primers for *AAD* (Table 3. and 4.), synthesized from the beginning and the end of the open reading frame.

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

The complete *UDPG* and *AAD* ORF was PCR amplified with *pfu* DNA polymerase (Thermo Scientific). The 50 µl reaction volume contained 1µl cDNA (corresponding to 50 ng of DNase treated RNA), 10× *Pfu* Buffer with $MgSO_4$, 0.2 mM dNTP mix, 1 µM of forward (ORF-F) and reverse (ORF-R) primers (Table 3. and 4.). 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. Negative control, without cDNA template was also included in the set. 10 µl of PCR products stained with 2 µl of loading dye were applied on agarose gel in 1×TBE buffer for 45 min at 80 V. A single and sharp band of 1425 bp for *UDPG* and 1155 bp for *AAD* were scored visually as expected. 1µl of each PCR product was ligated to pJET1.2/blunt vector, transferred into *E. coli* and cloned as described earlier (3.4).

From the isolated and quantified bacterial plasmid, 0.5 µg was digested with 1 µl of Fast Digest *Bgl*II restriction enzyme (Thermo Scientific) for 20 min at 37°C and run on 1% agarose gel in 1×TBE buffer to finally confirm the presence of the expected amplicons before sequencing. As the amplicon size was more than 1 kb the sequencing was carried out from both 3′ and 5′ end using the pJET1.2 forward and reverse primers. The *UDPG* nomenclature (Mackenzie *et al.*, 1997) was used for naming the *R. rosea* UDP glycosyltransferase and finally both *UDPG* and *AAD* sequences were uploaded to the NCBI Genebank.

3.4.8. Genomic sequence of *UDPG* and *AAD*

To identify the genomic organization of *R. rosea*'s *UDPG* and *AAD* and to detect the possible presence of intron/s or any post transcriptional modification, roseroot genomic DNA was used in a separate set of PCR using the *UDPG* and *AAD* ORF primer pairs (Table 3. and 4.). The PCR products were subjected for electrophoresis, bacterial transformation, cloning, plasmid isolation, restriction site digestion and finally sequencing similar to what described for full length cDNA amplification (3.4).

Table 3. Nucleotide primer sequences used for *UDP-glucosyltransferase* study

Transcript	Primer Name	Primer Sequence	Fragment Size (bp)	Reference
UDPG	0DG-F	5′-TTATWGWRAACAGCWTCWWSGARYTSG-3′	510	This study
	0DG-R	5′-TCCAAWRTYGAGTTCCAYCCRCAATG-3′		
UDPG	0SP-F	5′-GAATGTGTTGAACATGAAAGCG-3′	273	This study
	0SP-R	5′-GATCCTGACTCGGAACTTCTCCTG-3′		
UDPG	UDORF-F	5′-ATGGGTTCTGATTCACGG-3′	1425	This study
	UDORF-R	5′-CTAGGACAAAGTCTCTCTTCTC-3′		
UDPG	1RT-F	5′-GGCTGAGGAGAGGCGTGG-3′	221	This study
	1RT-R	5′-TGCATCCGGAGAACAATGGA-3′		
Actin	ACT-F	5′-CTCCGTGTTGCTCCAGAAG-3′	112	GenBank: JX431891
	ACT-R	5′-CAGGCACGTTGAAGGTTTC-3′		
UDPG	UDP3′a-F	5′-CCAGACTCCGTCGTGTACGTTTG-3′	728	This study
UDPG	UDP3′b-F	5′-GGAGAAGTTCCGAGTCAGGATC-3′	613	This study
UDPG	UDP5′a-R	5′-TTTCTGGTTGAAAAAC3′	*	This study
UDPG	UDP5′b-R	5′-GATCCTGACTCGGAACTTCTCC-3′	1006	This study
UDPG	UDP5′c-R	5′-GCTTTCATGTTCAACACATTC-3′	754	This study
Universal	3′RA-UAP	5′-GGCCACGCGTCGACTAGTACT$_{(17)}$-3′	**	Invitrogen
Universal	AAP	5′-GGCCACGCGTCGACTAGTACG$_{(16)}$-3′	***	Invitrogen
UDPG	UDHE-F	5′-CGGGATCCGCTAGCATGGGTTCTGATTCACgg-3′	1453	This study
UDPG	UDHE-R	5′-cgGGATcctgcggccgcCTAGGACAAAGTCTCTCTTCTC-3′		This study
UDPG	UDHE-R$_{His}$	5′-cgGGATCCtgcggccgcGGACAAAGTCTCTCTTCTCaac-3′		This study

* *UDPG* specific primer used for reverse transcription of *R. rosea* mRNAs in 5′ RACE; ** Oligo-dT-Adaptor primer used for *UDPG* 3′ RACE; *** Abridged Anchor Primer used for 5′ RACE .

Table 4. Nucleotide primer sequences used for *aryl alcohol dehydrogenase* (*AAD*) study

Transcript	Primer Name	Primer Sequence	Fragment Size (bp)	Reference
AAD	0DG-F	5′-GRAGAAGGTAAARTCAAGACTRT3′	195	This study
	0DG-R	5′-CACSCATTACTDTWCCATA3′		
AAD	0SP-F	5′-GGAGAAGGTAAAATCAAGACTAT3′	196	This study
	0SP-R	5′-CACCCATTACTGTTCCATA-3′		
AAD	AAORF-F	5′-ATGTCGCTCCACGTCTAC-3′	1155	This study
	AAORF-R	5′-TTATGCGCGTCTGTACTCA-3′		
AAD	0RT-F	5′-TCCCATTGTCAGCAACCAG-3′	119	This study
	0RT-R	5′-CACCCATTACTGTTCCATA-3′		
AAD	1RT-F	5′-CCTCTGATCATCGATAGGCAAC-3′	132	This study
	1RT-R	5′AAAAGGCTCCATCGATCAATTC-3′		
Actin	ACT-F	5′-CTCCGTGTTGCTCCAGAAG-3′	112	GenBank: JX431891
	ACT-R	5′-CAGGCACGTTGAAGGTTTC-3′		
AAD	*AAD3′*a-F	5′-TCCCATTGTCAGCAACCAG-3′	776	This study
AAD	*AAD5′*aR	5′-TCCTTTTGTACTTTTG-3′	*	This study
AAD	*AAD5′*bR	5′-TATGGAACAGTAATGGGTG-3′	791	This study
Universal	3′RA-UAP	5′-GGCCACGCGTCGACTAGTACT$_{(17)}$-3′	**	Invitrogen
Universal	AAP	5′-GGCCACGCGTCGACTAGTACG$_{(16)}$-3′	***	Invitrogen

AAD* Gene specific primer used for reverse transcription of *R. rosea* mRNAs in 5′ RACE; ** Oligo-dT-Adaptor primer used for *AAD* 3′ RACE; * Abridged anchor primer used for 5′ RACE.

3.4.9. Phylogenetic trees

BLASTP was applied for the obtained deduced amino acid sequence of *UDP-glucosyltransferase* (*UGT73B16*) and *aryl alcohol dehydrogenase* (*AAD*) using the Align/Do Blast search in MEGA6.06. Twenty-two hits from different plant specific secondary metabolite glucosyltransferases (*e*-value 0.0, no filter query sequence) and 14 hits from *AAD* sequences were selected for ClustalW alignment. Befor the alignments the gaps were removed. The maximum-likelihood approach was applied to construct a phylogenetic neighbor-joining tree with 500 bootstrap replicates (Tamura *et al.*, 2011; 2013). Bootstrap values higher than 60% were indicated above the nods.

3.4.10. Heterologous expression of *UDPG*

3.4.10.1. Plasmid vector and restriction sites

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 ORF sequence of *UGT73B16* was searched for the absence of engineered pET-24d restriction sites sequences. The *Bam*HI, *Nhe*I and *Not*I were chosen as non-cutter enzymes for roseroot *UDP-glycosyltransferase* open reading frame.

3.4.10.2. Primer design and PCR amplification

Two restriction sites (*Bam*HI, *Nhe*I) were added to the forward primer; upstream of the start codon of genes ORF. For the reverse primer, two restriction sites (*Bam*HI, *Not*I) were added downstream of the ORF stop codon. To facilitate the Histidin tagging strategy, another reverse primer was designed in which the two restriction sites were added before the stop codon of our gene ORF. In the oligonocleotides, the *Bam*HI, *Nhe*I and *Not*I sites are underlined, bolded and italicized, respectively when appear.

Two PCRs were performed in 50 µl reaction volume containing 50 ng of template DNA (already sequenced and confirmed full length ORF of *UGT73B16* plasmid), 2× fusion master mix, 0.5 µM of forward: UDHE-F (5′-CGGGATCC**GCTAGC**ATGGGTTCTGATTCACGG-3′) and each of the two reverse primers: UDHE-R (5′-GGGATCCT*GCGGCCGC*CTAGGACAAAGTCTCTCTTCTC-3′) and UDHE-RHis (5′-CGGGATCCT*GCGGCCGC*GGACAAAGTCTCTCTTCTCAAC-3′), 1 unit of Phusion High-Fidelity DNA Polymerase (Thermo Scientific) and sterile distilled water. The reactions were carried out in a Swift MaxPro thermocycler (Esco Healthcare, Pte, Singapore).

For amplification of the desired fragments the following program was used: initial denaturation at 98°C for 1 min; followed by 30 cycles of 98°C for 30 s, 60°C for 30 s, 72°C for 90 s; and a final synthesis at 72°C for 5 min. Two reaction mixes without any DNA template were also included as no-template PCR control. 10 µl of the PCR products stained with 2 µl of xylencyanol loading dye was applied on a 1% (w/v) ethidium bromide-stained agarose gel in 1xTBE buffer and run for 1 h at 70 V to verify the occurrence of the amplification. Amplicons were scored visually for presence of nucleotide fragments of ~1450 base pair length. One µl of each two PCR product was ligated into pJET1.2/blunt vector (CloneJET PCR Cloning Kit, Thermo scientific) according to the protocol of the kit for cloning. Five µl of the ligation reaction mix was transformed into 50 µl of chemically competent DH5α *E. coli* (Zymo Research, Irvine, CA, USA).

The transformation and cloning were performed as described before (3.4). The colony PCR analyses were carried out according to the CloneJET PCR Cloning Kit protocol using pJET1.2 forward and reverse primers. Three colonies from each PCR products that showed the expected amplicon size were selected and subjected for another overnight culture in 2 ml liquid LB medium supplemented with 0.1% ampicillin. The bacterial plasmid was isolated and quantified as described earlier (3.4). The ABI PRISM® 3100 Genetic Analyzer (Applied Biosystem) automatic DNA sequencer was utilized for plasmid sequencing. As the amplicon size was more than 1 kb the sequencing was carried out from the both 3′ and 5′ side using the pJET1.2 forward and reverse

primers. The error free plasmids containing our desired restriction sites in frame were marked and kept in -70°C for expression experiment.

3.4.10.3. Preparation of the expression vector for ligation

One µl of the engineered pET-24d expression vector was kindly assured by Dr. Laszlo Beinrohr (TTK/MTA) and transformed into 50 µl of chemically competent DH5α *E. coli* (Zymo Research, Irvine, CA, USA) on ice. The transformation reaction mix was diluted in 400 µl LB liquid media and kept at 37°C shaker (150 rpm) for half an hour. 100 µl of the diluted competent cells was spread on pre-warmed (37°C) LB culture plate containing 50 µg/ml kanamycin. The plate was kept overnight at the same temperature (37°C). From the positive colonies, one was selected and propagated in overnight liquid culture in fresh LB-kanamycin (50 µg/ml) medium at 37°C. The next day, plasmids were purified from 4 ml liquid culture using a E.Z.N.A.® Plasmid Mini Kit I (Omega Bio-tek, USA) according to the kit protocol and eluted in 30 µl buffer. The isolated plasmid concentration was measured using NanoDrop 1000 spectrophotometer at 260 nm.

Fifteen µl (~1.5 µg) of plasmid was sequentially digested first with fast digest *Not*1 enzyme (Thermo Scientific) for 30 min at 37°C and in 20 µl of reaction volume. Two µl of the digestion mix was run on 1% agarose gel in 1×TBE buffer to confirm the first digest before 2nd digestion. The 2nd digestion with fast digest *Nhe*I enzyme (Thermo Scientific) was done in the same reaction tube according to the company's protocol. The linearized double digested empty vector was further cleaned and purified using the spin column from a E.Z.N.A.® Plasmid Mini Kit I (Omega Bio-tek, USA) and stored in -70°C.

3.4.10.4. Ligation cloning of *UDPG* into pET-24 vector

For the insert ligation, three ligation reactions were prepared. For the first two ligation reactions, ~200 ng (7-8 µl) of each *UDPG*s (His-tagging and not His-tagging), PCR amplified ORF plasmid DNA was separately mixed with ~50 ng (3 µl) engineered pET-24 vector and 1 µl of ligation buffer in 15 µl reaction volume. The third ligation reaction was set up without the insert DNA and just with empty vector. The reaction tubes were heated up to 42°C for a few minutes (to denature the annealed plasmids and annealed inserts with sticky ends) and cooled down to 4°C. Five units (1 µl) of T4 DNA ligase (Thermo Scientific) was added to the solutions, mixed properly and incubated at 4°C overnight. Ten µl from each ligation reactions were transferred and cloned in chemically induced competent DH5α *E. coli* cells (Zymo Research, Irvine, CA, USA) as described earlier (3.4).

Two hundred µl from each transformation solution were spread on two plates (100 µl each) of fresh LB medium supplemented with kanamycin (50 mg/l) at 37°C. Three positive clones were selected from each plate; and were propagated in 2 ml liquid culture. Plasmid DNA was isolated, quantified, and subjected for sequential restriction digestion with *Nhe*I and *Xho*I enzymes (Thermo Scientific) according to the supplier's manuals. From each digestion reaction, 1 µl was loaded on 1% agarose gel to see if the inserts were cut out. Having confirmed the presence of both inserts (*UDPG*s), the constructed plasmid was marked and kept at -20°C for further use.

3.4.10.5. Cloning of *UDPG* in BL21 (DE3) pLysS *E. coli*

An *E.coli* expression strain [BL21 (DE3) pLysS] was kindly assured by Dr. Laszlo Beinrohr (TTK/MTA). The bacteria were applied on an LB plate supplemented with chloramphenicol (34 mg/l) and the plate was incubated overnight at 37°C. Next day a 5 ml liquid culture was started using fresh grown bacteria and let to grow overnight at 37°C. The preparation of competent cells was done according to Sambrook and Russell (2001).

Two ml from the fresh liquid cultured bacteria was added into 200 ml fresh LB-liquid media supplemented with chloramphenicol (34 mg/l) in 500 ml Erlenmeyer flask and kept shaking for almost 3 hours until the OD_{600} reached 0.4. The BL21 competent cells were obtained using $CaCl_2$ treatment. The competent cells (in Eppendorf tubes) were stored at -70°C in 0.1 M $CaCl_2$ containing 15% glycerol. In another round of transformation, 10 µl from each *UDPG*s insert + engineered pET-24 vector construct and empty engineered pET-24 plasmid was used for transformation of 100 µl BL21 cells similar to the protocol described in (3.4). The transformed cells were spread on LB-kanamycin (50 µg/l) medium and kept at 37°C overnight. From the grown colonies 10-15 were selected for colony PCR analysis using the primers designed for the *UDPG* ORF (Table 3.) and for heterologous-expression (3.4.10.2). After confirming the positive results, 3 colonies from each transformation were subjected for an overnight liquid culture and stored in fresh liquid LB-kanamycin (50 µg/l) medium containing 30% glycerol at -70°C.

3.4.10.6. Expression induction and SDS-PAGE

From each positive colony and also from non-transformed BL21 bacteria strain, 5 ml liquid culture was started and incubated at 37°C overnight. Using 1% of each overnight culture, 4 (20 ml) fresh liquid cultures were prepared on a desk shaker with 120 rpm at 37°C. After 2-3 h when the OD_{600} was about 0.8-l, 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added to the exponentially growing cells to induce the gene expression.

From each construct, 1 culture flask remained un-induced (minus IPTG) as control. Samples (2 ml) were taken and frozen in liquid nitrogen from each culture right before and 2, 3 and 4 h after IPTG induction. The harvested culture samples were centrifuged at 10,000 rpm for 5 min at 4°C and the pellets were frozen in liquid nitrogen. The cells were re-suspended in 3 ml of extraction buffer (50 mM Tris-HCl, pH-8.0, 10 mM $MgCl_2$, 20% glycerol, 5 mM β-mercaptoethanol) and disrupted by sonication on ice cold water for 15–20 s for 6 times and 5 min intervals. The lysate was further centrifuged at 10,000 rpm for 20 min at 4°C. 25 µl of each sample supernatant was loaded on 10 % SDS-PAGE gel after heating (denaturing) the sample with 8 µl of 4 x Laemmli SDS loading buffer at 98°C for 5 min and cooling down on ice for another 5 minutes. The gel run at 120 V in 1 x SDS running buffer, at room temperature until the bromophenol blue loading dye reached the bottom of the resolving gel. The SDS gel was stained by Coomassie Brilliant Blue for 30 minutes and destined in 20:10:70 mixture of methanol: glacial acetic acid: distilled H_2O overnight.

3.4.10.7. Affinity chromatography

Gravity flow affinity chromatography was applied to purify the recombinant protein from transgenic BL21 strains. *E. coli* lysate was thoroughly centrifuged (10,000 rpm for 10 min at 4°C) and 1 ml of the supernatant was collected for chromatography. The lysate pH was adjusted to be 7.5-8. A 10 ml plastic chromatography column (Bio-Rad) was set up in a cold room (4°C) in which 1 ml amylose resin (New England BioLabs) was poured in. All the washing, equilibrating and eluting buffers were filter sterilized using 0.22 µm pore size (Millex GP filter unit from Millipore). From each lysate, 200 µl samples were stored at -20°C. The column was washed with 10 column volume (CV) of washing/equilibrating buffer (20 mM HEPES, pH 7.4; 500 mM NaCl; 1 mM EDTA). The lysate (1ml) was applied onto the column and flow-through lysate was collected and stored at -20°C. After washing the column with 10 CV of washing/equilibrating buffer, 3 CV of maltose containing elution buffer (20 mM HEPES, pH 7.4; 500 mM NaCl; 1 mM EDTA; 10 mM maltose) was loaded and 200 µl samples of flow-through fractions were collected and stored at -20°C. The column was washed with 10 CV of washing/equilibrating buffer for the next lysate sample or stored in storage/regenerating buffer (6 M guanidine hydrochloride). The lysate sample before chromatography, a flow-through sample and the eluted recombinant polypeptide (after spectrophotometry of each collected fraction for protein quantity prediction with NanoDrop ND 1000) was prepared for SDS PAGE analysis. A silver salt staining protocol (Sammons *et al.*, 1981; Schumacher *et al.*, 1983) was followed for the gel visualization.

3.4.10.8. 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 (2, 3 and 4 h) purified with affinity chromatography) were loaded in duplicates in 10% polyacrylamide gels by SDS-polyacrylamide gel electrophoresis (Bio-Rad) and run under reducing condition (3.4.10.6.). The separated proteins of the first gel were stained with Coomassie Brilliant Blue and fixed as described earlier (3.4.10.6.). The fractionated proteins of the other gel were transferred to a nitrocellulose membrane (Protran® BA85) by using an electrophoretic transfer cell (Bio-Rad Mini Trans-Blot®) at 4ºC overnight. On the next day the membrane was blocked with 5% skimmed milk powder in TBST buffer (10 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 8.0) at room temperature for 3 hours, and further incubated with the same blocking buffer containing monoclonal Anti-polyHistidine antibody (1:3000, Sigma) produced in mouse at room temperature for another 3 hours. The membrane was then rinsed three times with TBST buffer for 10 minutes. For the immunoblots this was followed by reaction with alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc. USA) in the same blocking buffer (diluted 1:3000) for 2 hours at room temperature (Schonborn *et al.*, 1991). Bound proteins were visualized by incubating with BCIP-T (5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt) and NBT (nitro blue tetrazolium) substrate solution (Thermo Sientific) after five minutes (Sambrook *et al.*, 1989).

3.4.10.9. *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 (3.2.4). For qPCR analysis the normalized cDNAs from oligo dT primed reactions as outlined earlier (3.4.3) were diluted (Table 5.) with nuclease free water and stored at -70°C.

Table 5. Dilution rows for cDNAs used in qPCR experiment

Dilution	Ratio	Final volume for storage
18 µl cDNA + 54 H_2O	1:4	62 µl
10 µl cDNA from (1:4) + 30 H_2O	1:16	32 µl
8 µl cDNA from (1:16) + 24 H_2O	1:64	32 µl

The 1:16 diluted cDNA was assumed as standard quantity in all qPCR reactions in this experiment. Primer pairs were designed to have a melting temperature (T_m) of 60°C and to amplify short amplicons of 110-150 bp long. All of the primers were diluted to 10 µM (100 ng/µl)

concentration. The qPCR reactions were carried out in 0.1 ml strip tubes with caps (Qiagen) in 12 µl reaction volume using 3 µl cDNA, 5 µl of ready-to-use "hot start" SensiMix™ SYBR® No-ROX (Bioline, Csertex, Hungary) mix, 1 µl of each forward and reverse primers (10 µM) and 2 µl of DNase-free water. All samples were amplified in a minimum of triplicates and no-template-control (NTC) and calibrators were also included in each reaction.

Amplification initiated by a denaturation phase of 10 min at 95°C, followed by a two-step cycle pattern with a combined annealing and primer extension phase at 60°C for 30 s and a short denaturation at 95°C for 10 s. Thirty cycles of amplification, data acquisition, and data analysis were carried out on Rotor-Gene 6000 (Qiagen) thermocycler. Melting curve analysis was performed from 70–99°C to assess amplification specificity. Amplification efficiency (E) of qPCR was determined for each reaction. The Cq (quantitation cycle) values were determined using a set threshold by the Rotor-Gene 6000 software (version 6), and were defined as the number of cycles needed to reach a specific fluorescent signal threshold of detection. Dilution rows (1:4, 1:16, 1:64) of cDNAs were used to generate a standard curve.

Amplification efficiency (E) was determined based on the slope (M) of the log-linear portion of each standard curve [$E=10^{(-1/M)}$ -1]. A Two Standard Curve method (Applied Biosystems) was applied for relative gene expression analysis. The method relies on having a standard curve for each gene. The standard curves are used to quantify the concentration of each gene according to its standard curve. The expression of the gene of interest (eg. *UDPG*) is then normalized with the reference gene (*actin*). Prior to qPCR analyses we investigated different normalizing (reference) genes behavior and selected the one, which statistically proved to be the most stable (*R. rosea* specific *actin*). A two-way MANOVA model with treatment (C or T) and elapsed time effect (1 h, 6 h, 12 h, 24 h, 24 h, 48 h, 96 h) as fixed factors were applied to study the actin stability. Given the significance of the overall test, the univariate main effects were examined. Normality of the residuals was proved by Shapiro-Wilk's test ($p>0.05$). Homogeneity of variances was checked by Levene's test ($p > 0.05$). In case the between-subject effect showed significant differences, treatment means were separated by Tukey's post hoc test. The statistical procedures were conducted using the software IBM SPSS22.

The obtained raw data using the Rotor-Gene 6000 software version 1.7.87 was exported to Microsoft Excel for further analysis. Statistical analyses were performed by a two-way ANOVA model with fixed factors treatment (Treated and control) and elapsed time (1 h; 6 h; 12 h; 24 h; 48 h). Normality of the residuals was proved by Shapiro-Wilk's test ($p>0.05$).

Homogeneity of variances was checked by Levene's test. In case homogeneity assumption was violated (Levene's test $p<0.05$), means after different elapsed time intervals were separated by Games-Howel's post-hoc test, in other cases, by Tukey's post hoc test. All statistical procedures were conducted using the software IBM SPSS 22.

3.4.11. Gene expression analysis in rosavins biosynthesis pathway

3.4.11.1. Primer design and qPCR

The collected plant materials from the Austrian *R. rosea* population (Hochkar, Göstling Alps) 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. Part of the mentioned genes was PCR amplified, cloned, sequenced and identified according to the strategy described before (3.4).

The roseroot specific primers for real-time PCR (Table 6.) were designed to have approximately equal melting temperatures (T_m). The products of each primer pairs were cloned and sequenced at least six times to verify primer specificity. All runs were followed by a melting curve analysis from 55 to 95°C. 72 PCRs were performed in each run covering the samples from one plant, 3 phenological stages and from two tissues (leaf and rhizome) in duplicates to analyze the relative expression of all 4 genes. Negative control (No-template control) and an internal positive control (*actin* expression) were also included in each run. The linear range of template concentration to threshold cycle value (Ct value) was determined by performing a series of six fold dilutions (one fold to 1,296-fold) using cDNA from three independent RNA extractions analyzed in three technical replicates.

Table 6. Nucleotide primer sequences used for gene expression analysis in rosavins biosynthesis pathway

Transcript	Primer Name	Primer Sequence	Fragment Size (bp)	Reference
PAL	PALRT-F	5′-GCTCATGTTTGCTCAATTCTCC-3′	104	This study
	PALRT-R	5′-TGAACCCGTAATCCAAGCTG-3′		
4CL	4CLRT-F	5′-GGATCGTGTCAGTAGAGGAATC-3′	127	This study
	4CLRT-R	5′-ATCTTATTCGGTGGCTTAGGC-3′		
CCR	CCRRT-F	5′-GGAGTTAGCCAAGGAGAAAGG-3′	108	This study
	CCRRT-R	5′-TTCTGGATGTGGACAATGCTAG-3′		
CAD	CADRT-F	5′-CTCAGACGCAGCTAGGATG-3′	102	This study
	CADRT-R	5′-TTCAAAAGCGAAATGTACGACTC-3′		
Actin	ACT-F	5′-CTCCGTGTTGCTCCAGAAG-3′	112	GenBank: JX431891
	ACT-R	5′-CAGGCACGTTGAAGGTTTC3′		

3.4.11.2. 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 $C_k^l(i) = \left(c_k^l(i)_1, c_k^l(i)_2\ c_k^l(i)_3\right), k = 4\text{CL, CAD, CCR or PAL,} \quad l = \text{rhizome or leaf}, i = \text{U, M, L, G}$.

The way of calculation was as follows:

dimension 1 was for the change from phenological stage 1 to 2

dimension 2 was for the one from phenological stage 2 to 3

dimension 3 was to express the changes from phenological stage 1 to 3.

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. The value of a code is equal to 0 if the change of gene expression is below the 10% of the mean (Figure 6.).

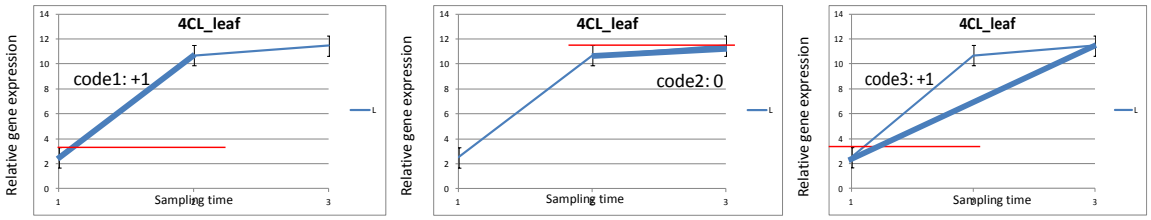

Figure 7. Calculation of the values of three-dimensional codes. An example with gene expression, 10% range of the mean and code values

We calculate the squared Euclidean distances of all pairs of curves:

$$D_k^l(j,i) = D_k^l(i,j) = \sum_{s=1}^{3}\left(c_k^l(i)_s - c_k^l(j)_s\right)^2$$

($k = 4\text{CL, CAD,CCR or PAL}$, $l = \text{rhizome or leaf}$, $i = \text{U, M, L, G}$)

and summarize them for all the individuals $i = \text{U, M, L, G}$: $I_k^l(i) = \sum_{j \neq i} D_k^l(i,j)$.

Let us denote by $\overline{I_k^l}$ the mean of $I_k^l(i)$ values taken over all plants: $\overline{I_k^l} = \frac{1}{4}\sum_{i=1}^{4} I_k^l(i)$.

If we represent 16 points $P_k(i) = \left(I_k^{leaf}(i); I_k^{rhizome}(i)\right)$ for $k = 4CL,\ CAD,\ CCR$ and PAL and $i = 1, 2, 3,$ or 4 points $k = 4CL,\ CAD,\ CCR$ and PAL, we can state that the closer a point is to the origin, the $P_k = \left(\overline{I_k^{leaf}}; \overline{I_k^{rhizome}}\right)$ for more similar set of curves it represents. Moreover, if a point is under the identity line, the curves for rhizome are more similar while if it is above the identity line, the similarity is more expressed in leaf.

Linear regression models with one or two independent variables were fitted to the dependent glycosides in rhizome of all phonological stages (rosavin, rosarin, rosin, salidroside). Explaining gene expression variables of the same or the previous phonological stages in rhizome or leaf were chosen by stepwise method. The explained variances (R-square), the F values of the ANOVA tests of the models and their significance levels were calculated. The normality of the residuals was shown by Kolmogorov-Smirnov's test ($p < 0.05$) and the significance of the parameter estimates were checked by Student's t test ($p < 0.05$).

4. RESULTS

4. 1. *In vitro* experiments of roseroot

4.1.1. *In vitro* cultures

The *in vitro* culture was initiated from seeds of *R. rosea* which were aseptically germinated and grown in controlled environment. The steps of the experiment from germination until established callus culture are shown in Figure 8; a-f. Seed germination capacity of 70% was obtained after 8 days without any pretreatment. 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, but the morphology of the developed callus lines and their induction rate were different in subculturing. The effects of the different phytohormone combinations on callogenesis rate and quality are given in Table 7. The calli grown on the medium with 2iP and IAA were friable and sensitive to sub-culturing, ending with getting colorless and finally brown after 2 weeks. The calli grown on the medium with 2iP and NAA were compact but mostly yellowish. Whereas the calli from the medium with 2iP and IAA were les compact, fleshy and more suitable for sub-culturing. Callus initiation was noticeably faster from the leaves (10 days after culture) comparing to stems (20 days) and their subsequent growth rate was significantly higher. The leaf explants gave the best growth rate and high quality callus which was used in further sub-cultures and experiments. In contrast with leaf explants the stem explants resulted in more colorless and watery callus which was ignored for the further experiments.

Table 7. Effects of different phytohormones combination on callus induction of *R. rosea* leaves

No	Culture Medium Type	Callus Induction Rate (%)*	Callus Colour and Quality
1	MS + 1mg/l NAA + 0.5 mg/l BAP	80	Green & Compact
2	MS + 0.5mg/l NAA + 0.5mg/l BAP	60	Light Green & Compact
3	MS + 0.5mg/l NAA + 1.5mg/l BAP	50	Yellowish Green & Compact
4	MS + 3mg/l 2iP + 0.3mg/l IAA	80	Green & Friable
5	MS + 0.6mg/l NAA + 3mg/l 2-iP	80	Yellowish Green & compact
6	MS + 0.1mg/l BAP + 1mg/l NAA	36	Green & Friable
7	MS + 1mg/l BAP + 0.1mg/l IBA	40	Green & Friable
8	MS + 1mg/l BAP + 0.1mg/l NAA	20	Green & Compact

All culture media contained 30g/l of sucrose and 4.4g/l of MS salt

*Induction rate is the mean of 10 replicates in each: 20 leaf explant were cultured

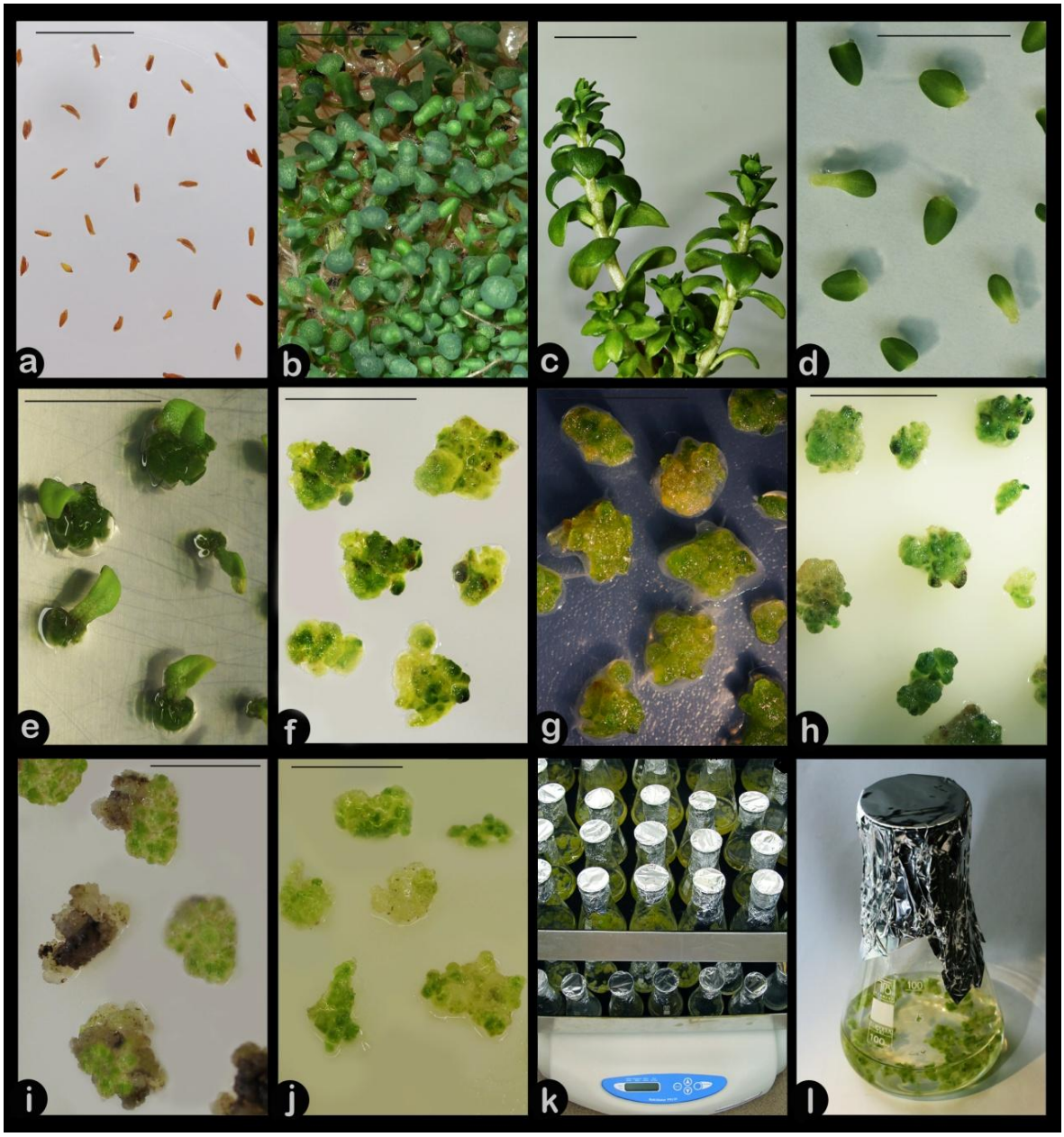

Figure 8. Callus induction and transformation of *Rhodiola rosea* callus cells

a: Decontaminated roseroot seeds on germination medium. **b:** 4 week old roseroot seedlings. **c:** 4 month old roseroot seedlings ready for callus induction from the leaves. **d:** Leaves of roseroot on callus-inducing solid MS medium enriched with 30 g/l sucrose and solidified with 4.5 g/l agar supplemented with 1 mg/l NAA and 0.5 mg/l BAP in glass Petri dishes. **e:** Roseroot callus formation after 6 weeks. **f:** Sub-culturing the vigorous roseroot callus on the fresh medium of the same composition. **g:** Co-cultivation of roseroot calli with agrobacteria on phytohormone free MS medium in dark (48 h). **h:** Callus on the selection medium containing kanamycin, carbenicillin, claforan and anti-oxidants. **i:** Callus on selection medium after 1 month**.** The green parts survived the agorobacteria co-culture and contain the transgenic cells. **j:** Further selection of transgenic sells by sub-culturing on the same fresh medium. **k:** The final selection procedure by liquid culture of transgenic calli in kanamycin containing medium. **l:** 100% transformed calli ready for mass production or any further application (Bars = 1 cm)

During the callus sub-culturing in our experiment, two types of leaf originated calli were distinguishable. Type 1 was mostly opaque with white to yellowish color and the type 2 was characterized as more fleshy and friable with sharp light green color which was chosen for sub-culturing and further experiments (Figure 9.). It should be noticed that the mentioned characteristics which led to choosing the second calli type for further experiments was in accordance to bioreactor culture circumstances like high stability in liquid culture and high growth rate. The selected callus type showed a biomass gain of two fold in every 2 weeks.

Figure 9. The favorable roseroot callus appearance

4.1.2. Transgenic roseroot callus culture establishment

To test the capacity of roseroot callus for genetic alteration, the selected calli grown in the No. 1 medium (Table 7.), were co-cultivated with Agrobacterium (Figure 8. g). More than 50% of the calli survived the infection after 2 weeks of culture on selection medium for the first time (Figure 8. h-i). In the second and further selection sub-cultures in each 2 weeks, the cell death rate decreased exponentially until the 100% stable antibiotic resistant callus has been obtained after 5 sub-cultures on solid medium (Figure 8. j) and 2 sub-cultures in liquid medium (Figure 8. l). Liquid culture in selection medium was found to be essential to eliminate the non-transformed cells that were growing on the upper parts of the callus which were not in direct contact with the medium. The transgenic callus has been growing normally in both solid and liquid culture containing 20-50 mg/l kanamycin. No morphological changes have been observed during sub-culturing. DNA isolation and polymerase chain reaction (PCR), showed the 700 bp amplified fragment of the inserted neomycin

phosphotransferase II (*npt*II) gene in all of the samples from transformed callus pieces (Figure 10. 1-20) whereas, no such a fragment was amplified in case of non-transgenic callus (Figure 10. N). The PCR for positive control by using the bacterial plasmid DNA as template amplified the same 700 bp of expected size (Figure 10. P) as were scored in transgenic callus samples. To verify the absence of *Agrobacterium* in putatively transformed calli which may lead to a false PCR amplification product, DNA samples were also tested with *virC* gene primers which amplify a 730 bp fragment of the virulence region located on the separate helper Ti plasmid. No PCR product was observed on the agarose gel using the above mentioned primers indicating the absence of any residual *Agrobacterium* in the medium or on the calli (Figure 11.).

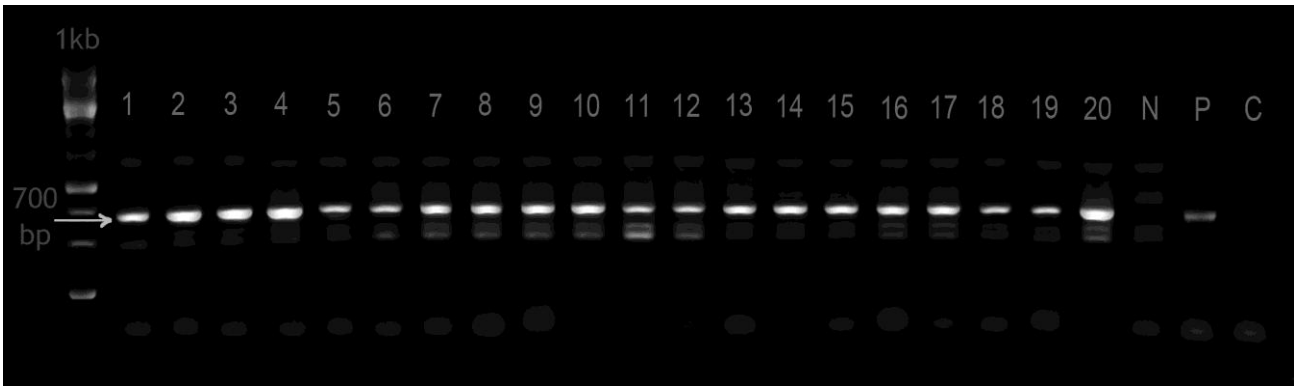

Figure 10. Molecular analysis of transgenic roseroot callus pieces using primers for a 700 bp fragment of neomycin phosphotransferase II (*npt*II) gene, **1-20**: PCR amplicons from individual lines of transgenic callus; **N:** Negative control representative of Non-transgenic callus; **P:** positive control amplified from *Agrobacterium* plasmid; **C:** PCR reaction mix without DNA template.

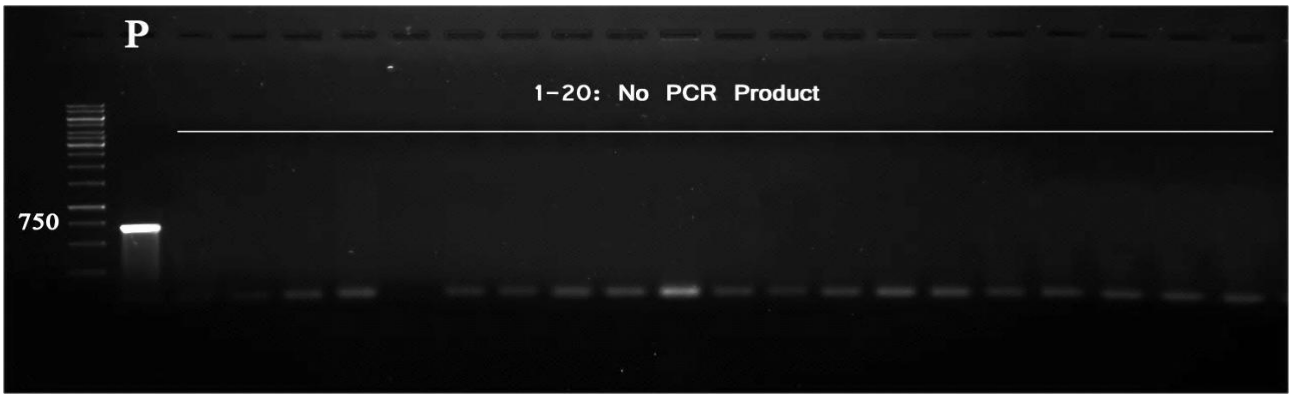

Figure 11. Agarose gel electrophoresis of transgenic roseroot calli DNA tested for the presence of *virC* gene amplicon (730 bp) from virulence region of *Agrobacterium* helper Ti plasmid. P: *virC* gene fragment amplified from *Agrobacterium* DNA;1-20: No amplification from transgenic calli samples.

As a more quantitative expression indicator, GUS test was performed twice. First, 5 days after the transformation and cultivation on selection solid medium (Figure 12. b) and second, 2 weeks after transformation and selection in liquid cultures (Figure 12. c). The latter test clearly showed

positive results by expressing blue color indicating the inserted reporter gene which was visible in all parts of the callus. After 4 weeks of selective liquid culture hardly any green callus part was visible in course of the test and the blue color was dominant. The same result has been obtained after 6 months and several sub-culturing.

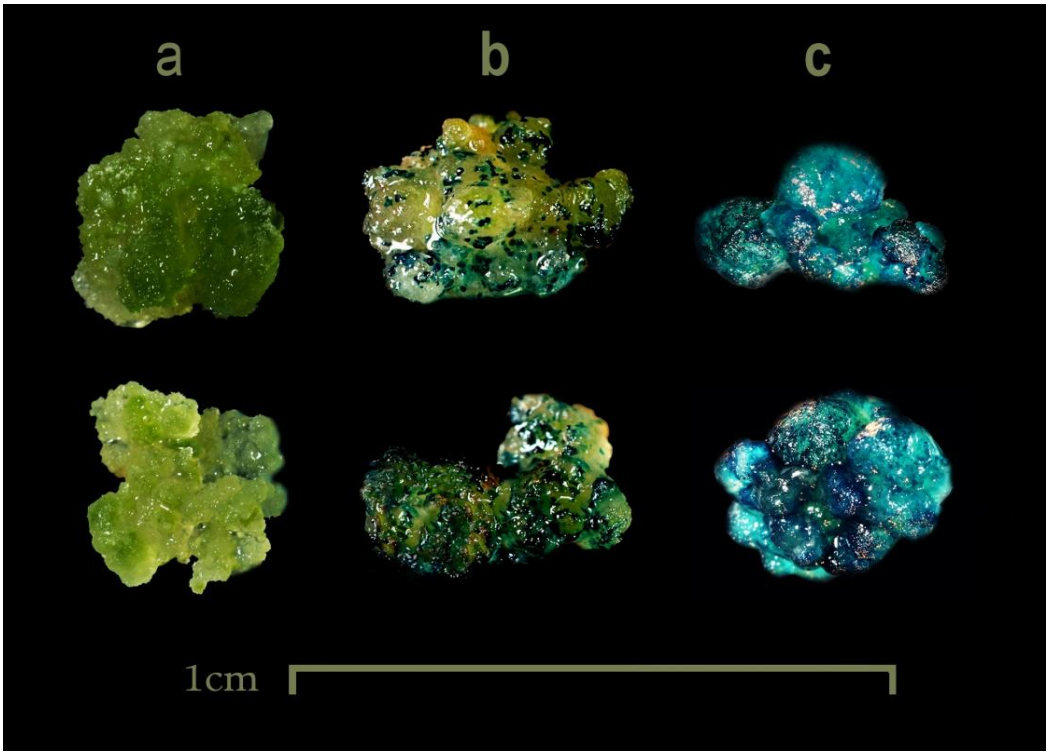

Figure 12. GUS reporting expression pattern in transgenic *Rhodiola rosea* callus. **a:** Before transformation. **b:** Calli 5 days after transformation on solid medium. **c:** GUS expression in transgenic calli cultured in liquid selection medium after 2 weeks.

4.1.3. Hormonal effects of antibiotics on *R. rosea* callus culture

An attempt was done to optimise the callus culture condition to obtain a better *in vitro* yield. Having optimising the liquid culture medium (Table 7, Medium No.1), carbenicillin and cefotaxime (claforan), two antibiotics commonly used for elimination of *Agrobacterium tumefaciens* during plant transformation, were tested for their additional effects on culture of roseroot leaf originated calli. Different concentration of carbenicillin and claforan (300, 400, 450, 500 and 550 mg/l) separately and in equal combination were added to the culture medium for 1 month subculturing in the same media every 10 days.

No inhibitory effects were observed in any of the treatments in comparison with the control culture. The positive effects of antibiotics (carbenicilline + claforan, 450 mg/l) on growth rate can be clearly seen in Figure 13; the media with the antibiotics resulted in much vigorous callus growth. Results of this experiment are presented in Figure 14.

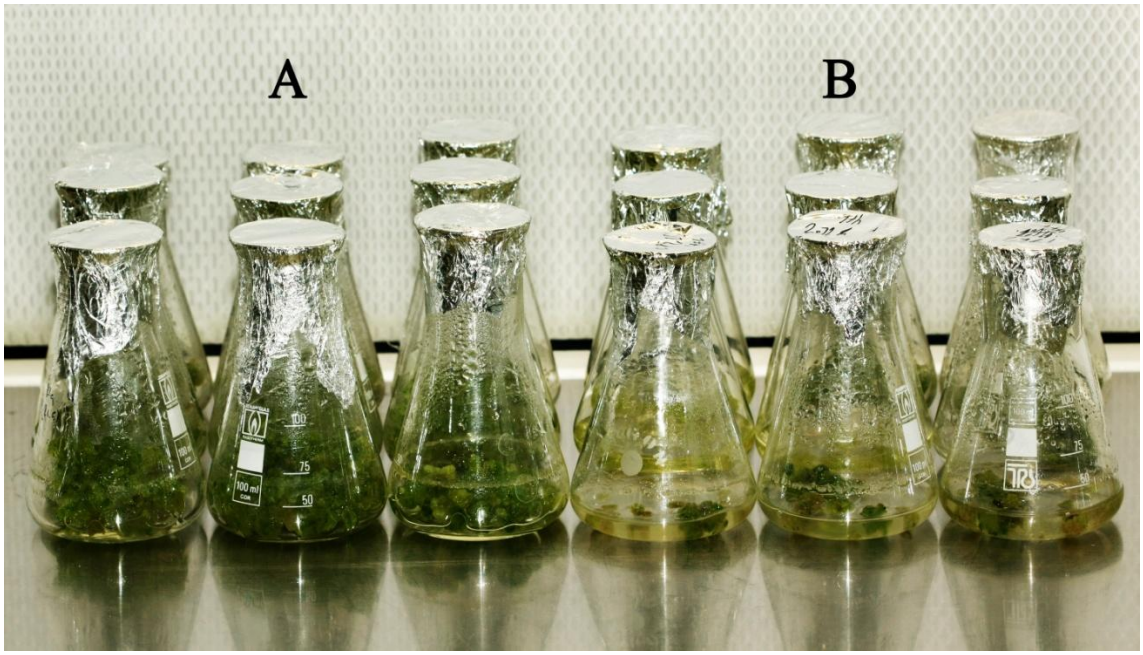

Figure 13. Roseroot callus culture. **A:** Treated (carbenicilline + claforan, 450 mg/l). **B**: Control

The highest fresh and dry weight was measured in the medium supplemented with 450 mg/l of both carbenicilline and claforan with about 20 fold higher growth rate compared with the control. After one month of culture 28 g of fresh callus was gained from 1 g of starting material. Whereas, when antibiotics were added individually, the highest callus yield was 10 g for calaforan (450 mg/l) and 20 g for carbenicillin (500 mg/l), respectively when compared with the control (8 g).

The addition of antibiotics decreased the pH of the medium to 4.8 during the culture period. To exclude the possible effect of lower pH on callus growth, another control was included with lower pH. But the result was negative (Figure 14.) as expected. This remarkable increase in callus growth rate is very significant from an *in vitro* production point of view which seems to be the most promising method to produce roseroot phytopharmaceuticals in the future. This result, if applied for engineered roseroot cell lines, would lead to a justifiable *in vitro* production in a large scale.

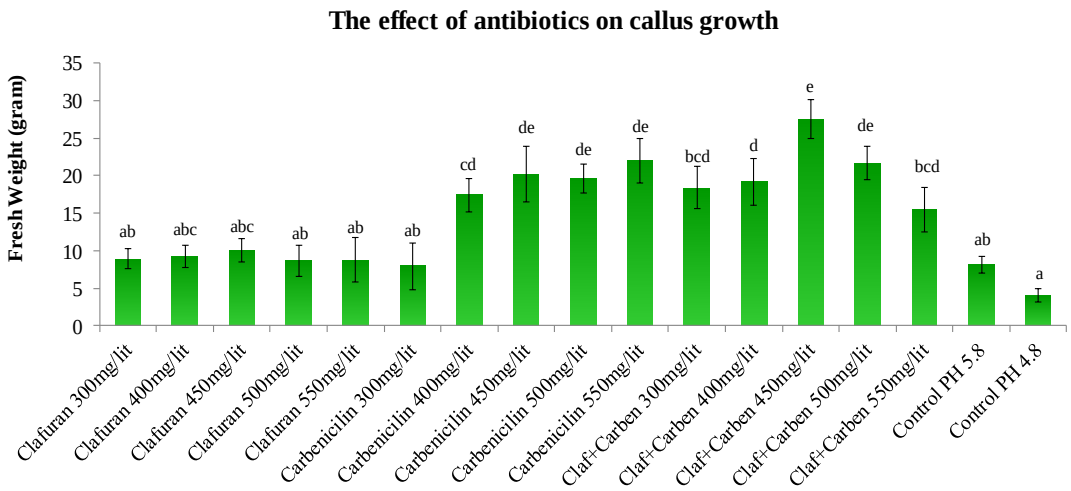

Figure 14. Effect of carbenicillin and cefotaxime (claforan) on roseroot callus growth. Different letters are for significantly different treatments effect (Tukey's post hoc test, $p<0.05$). Error bars represent standard deviation of the mean among 3 biological replicates.

4.1.4. Precursor feeding and biotransformation

Plant *in vitro* systems for production of high-value phytochemicals have been remarkably endorsed and appreciated from sustainability perspective. In such systems, phytocompounds are being produced under ecofriendly environment and according to the GMP, thus ultimately providing continuous production of biosafe, bioactive and stable natural products. This approach eliminates the influence of environmental and seasonal factors and does not threaten the natural populations of rare and endangered plant species. At present there are a limited number of reports concerning secondary metabolite production from callus and suspension cultures of *R. rosea*. As it was stated earlier, roseroot *in vitro* culture is not efficient enough to compete with the wild growing plants from the active constituent's point of view. Almost all of the earlier reports admitted the lack or only traces of roseroot important compounds in their *in vitro* trials (György *et al.*, 2004; Ma *et al.*, 2008; György and Hohtola, 2009; Martin *et al.*, 2010). We conducted an *in vitro* experiment in which 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. The position of the mentioned compounds can be seen in the proposed salidroside biosynthesis pathway (Figure 15 and 19.).

The precursors were dissolved in the roseroot liquid culture media for a final concentration of 2 mM and the callus samples were harvested after 1, 6, 12, 24, 48 and 96 h. A set of controls were included to cover the possible actions or reactions of the given compounds in the media without any callus and normally the main set of controls which were the callus cultures without any precursors during the course of this experiment. The HPLC analysis showed no significant changes in the content of desolved compounds in the controls without the roseroot cells.

Based on these results we are confident that any change in the content of the compounds was the result of biotransformation or any kind of chemical conversion that has happened due to the presence of roseroot cells. The result of precursors feeding is presented in Figures 16-26.

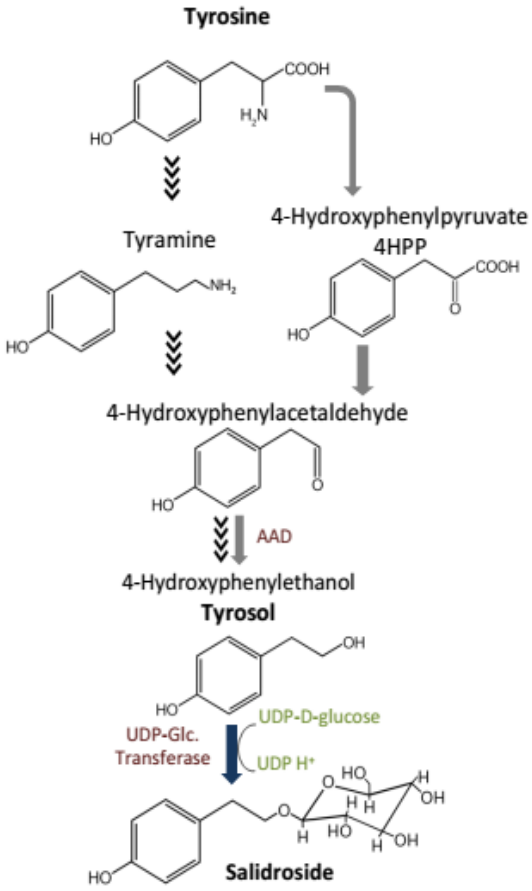

Figure 15.: Proposed biosynthesis pathway for salidroside in *R. rosea*

4.1.4.1. Precursor feeding in salidroside pathway

Based on our findings, none of the precursors in salidroside pathway was released into the medium from the plant cells. The results of tyrosol treatment are shown in Figure 16. Tyrosol is the last compound before salidroside formation in its proposed pathway. Tyrosol treatment did not have any significant effect on tyramine content. But it significantly increased the salidroside content for about 17 fold comparing to the control in a measurement after 24 hours. The salidroside formation was significantly increased from 24 hours to 48 hours. The tyrosol content in the treated samples were also significantly higher than in the controls and showed a constant content during the experiment. A clear tendency can be seen where the tyrosol content is declined in the media (lower part of the graph) and salidroside is getting formed during the experimental time.

The effect of tyramine treatment is shown in Figure 17. After 24 h, no tyramine was found in the media. A significantly higher content for tyramine was recorded after 24 h compared with the controls. This higher proportion of tyramine in treated samples was constant during the experiment. Tyramine increased the salidroside content by 3 fold after 96 h when compared with controls. Tyramine had no significant effect on tyrosol content in this experiment.

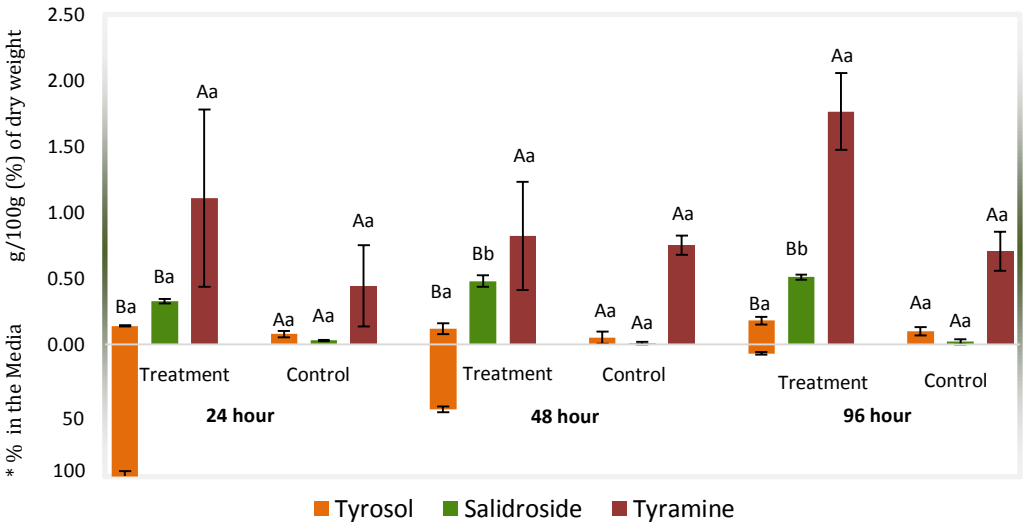

Figure 16. Effect of 2 mM tyrosol treatment on tyrosol, tyramine and salidroside content in roseroot callus culture. Upper case letters: different letters are for significantly different treatments assumed a fixed interval of elapsed time ($p<0.05$). Lower case letters: different letters are for significantly 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%.

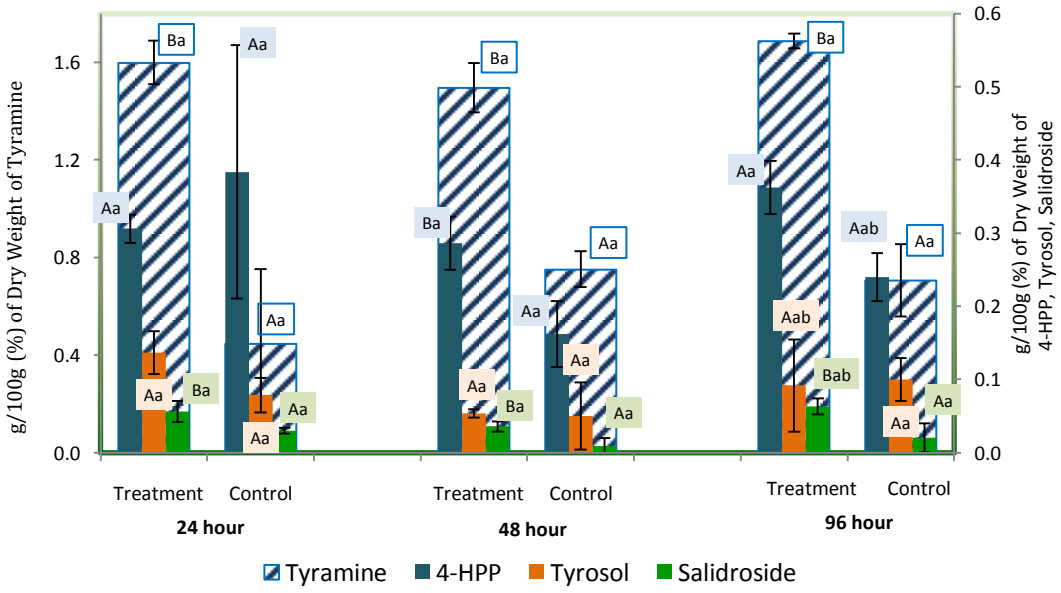

Figure 17. Effect of 2 mM tyramine on 4-HPP, tyrosol and salidroside content in roseroot callus culture. Upper case letters: different letters are for significantly different treatments assumed a fixed interval of elapsed time ($p<0.05$). Lower case letters: different letters are for significantly different 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 results of 2 mM 4-hydroxyphenylpyruvate (4-HPP) feeding on the content of tyramine, tyrosol and salidroside are presented in Figure 18. 4-HPP didn't have any significant effect of tyramine content during this experiment. 4-HPP had significant effect only on salidroside content after 48 h when compared with the control.

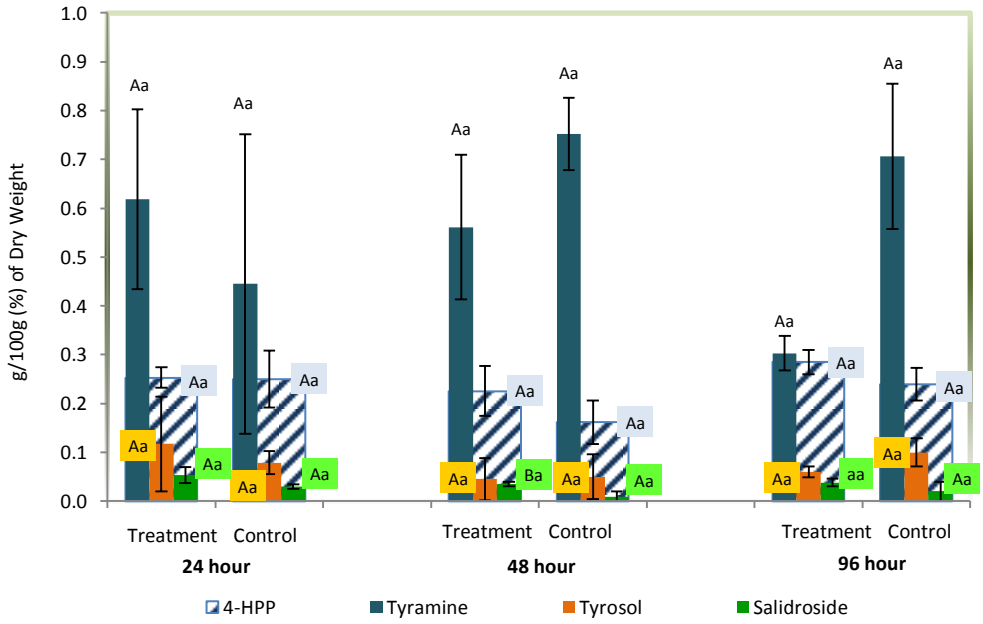

Figure 18. Effect of 2 mM 4-HPP on tyramine, tyrosol and salidroside content in roseroot callus culture. Upper case letters: different letters are for significantly different treatments assumed a fixed interval of elapsed time ($p<0.05$). Lower case letters: different letters are for significantly different 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.

4.1.4.2. Precursor feeding in cinnamyl alcohol glycosides (CAGs) pathway

The effect of feeding precursors of the CAGs biosynthesis pathway (Figure 19.) is presented in the coming figures. The only compound in this pathway which was released into the media from plant roseroot cells was rosin and only after cinnamyl alcohol treatment. 2 mM phenylalanine (Phe); the first aromatic amino acid in the proposed pathway showed no effect on CAGs biosynthesis even after 96 h (Figure 20.). The treatment with Phe significantly increased its content in the treated samples during the experiment when compared with the controls.

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.

Trans-Cinnamic acid (*t*-C.A) which was not detected in the control samples at all increased the overall content of rosavins (Figure 21.). 2 mM *t*-C.A significantly increased the rosin content by more than 75 fold after 24 h of cultures. Coumaric acid content also significantly increased as a result of t-C.A treatment during the experiment compared to the controls. The content of rosavin, which was missing from the controls, showed a gradual increase during the experiment after *t*-C.A treatment. As the rosin content declined, the rosavin content increased inversely. The rosarin content did not differ significantly among the samples.

The presence of t-C.A in the media affected the compounds in the salidroside proposed pathway as it is presented in Figure 22. The tyrosine, 4-HPP and tyrosol content were significantly higher in t-C.A fed samples when compared to the controls. Amongst these three compounds only tyramine showed a significant increase during the experiment in each sampling times. Although the tyramine content was also higher in t-C.A fed samples but the high standard deviation did not allow proving significance difference. No cinnamldehyde was found nor in the media neither in the cells after 24 h of treatment (Figure 23.). It was not present in the controls either. But cinnamyl alcohol was synthesized and detected in the treated samples with its content being declined during the experiment. 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 23.). The rosin content elevation was significant between 48 h to 96 h.

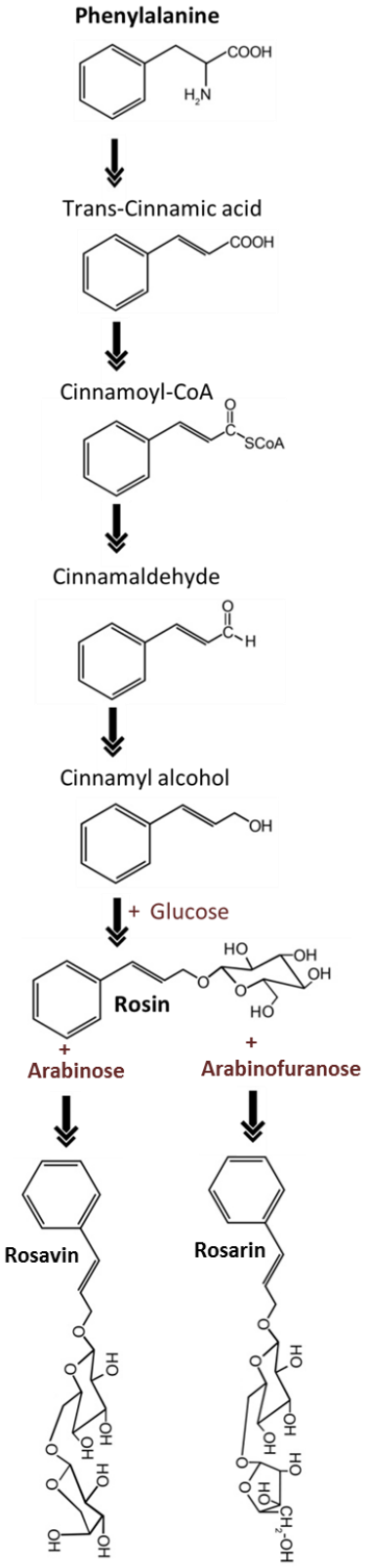

Figure 19.: The proposed biosynthetic pathway for CAGs in *R. rosea* L.

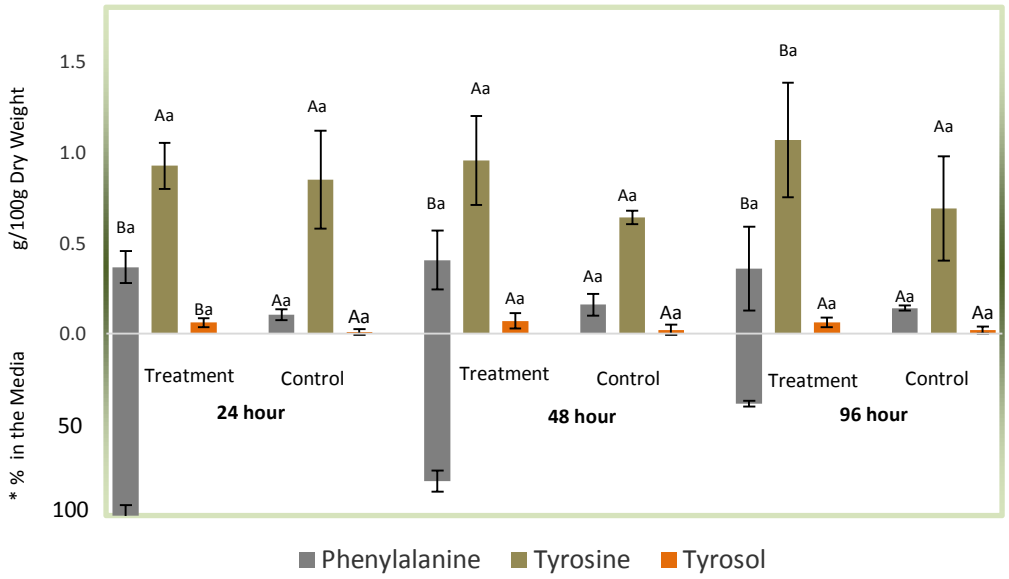

Figure 20. Effect of 2 mM phenylalanine treatment on phenylalanine, tyrosine and tyrosol content in roseroot callus culture. Upper case letters: different letters are for significantly different treatments assumed a fixed interval of elapsed time ($p<0.05$). Lower case letters: different letters are 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%.

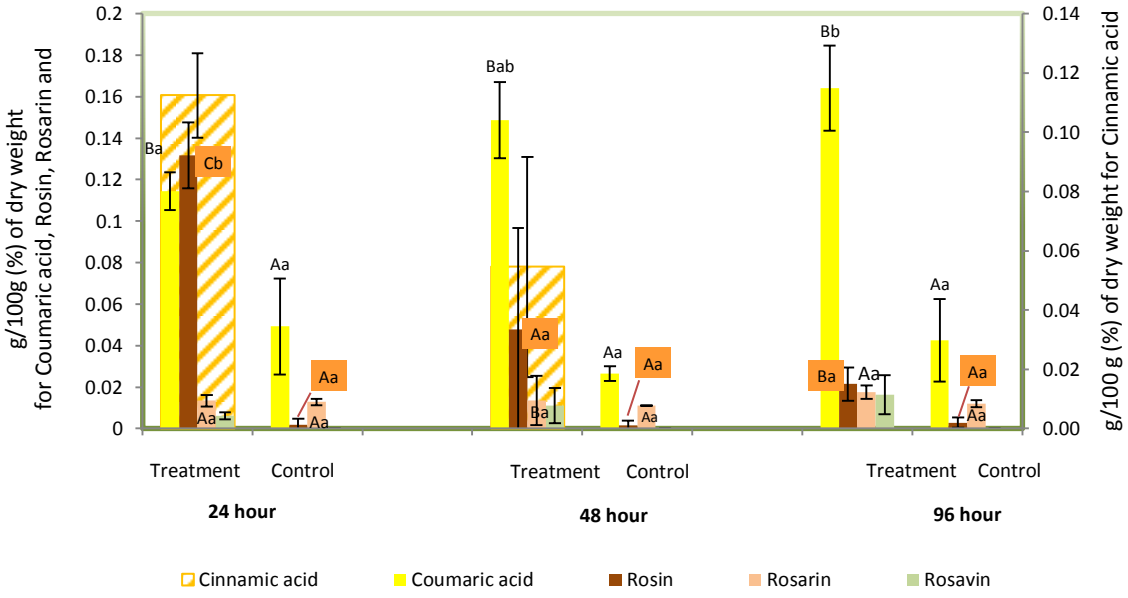

Figure 21. Effect of 2 mM *trans*-cinnamic acid treatment on cinnamic acid, rosin, rosarin and rosavin content in roseroot callus culture. Upper case letters: different letters are for significantly different treatments assumed a fixed interval of elapsed time ($p<0.05$). Lower case letters: different 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.

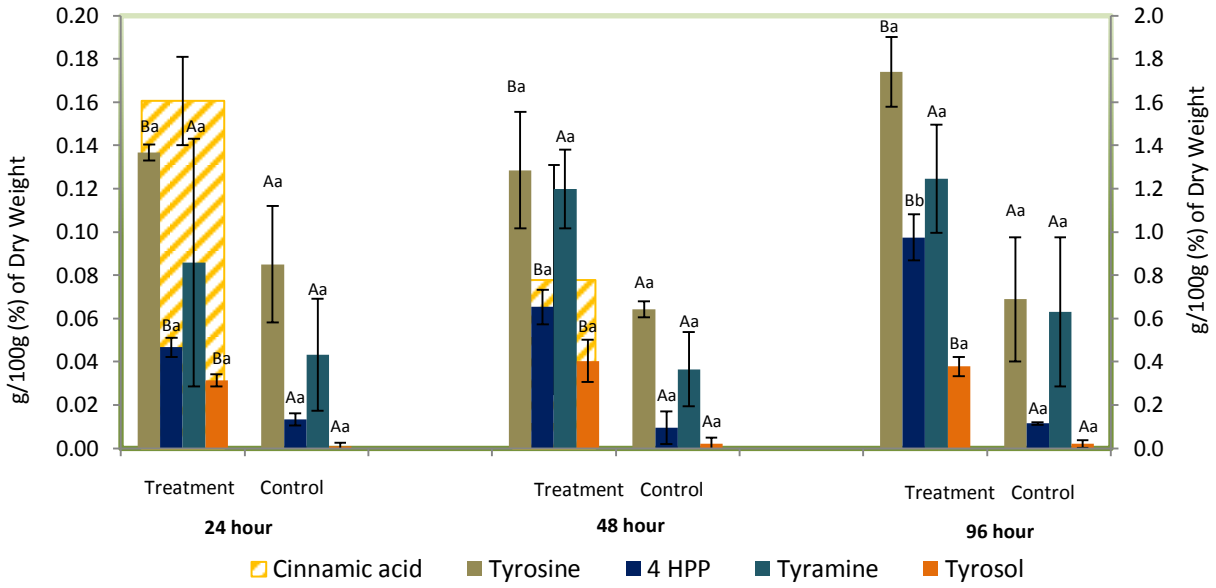

Figure 22. Effect of 2 mM *trans*-cinnamic acid treatment on cinnamic acid, tyrosine, 4-HPP, tyramine and tyrosol content in roseroot callus culture. Upper case letters: different letters are for significantly different treatments assumed a fixed interval of elapsed time ($p<0.05$). Lower case letters: 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.

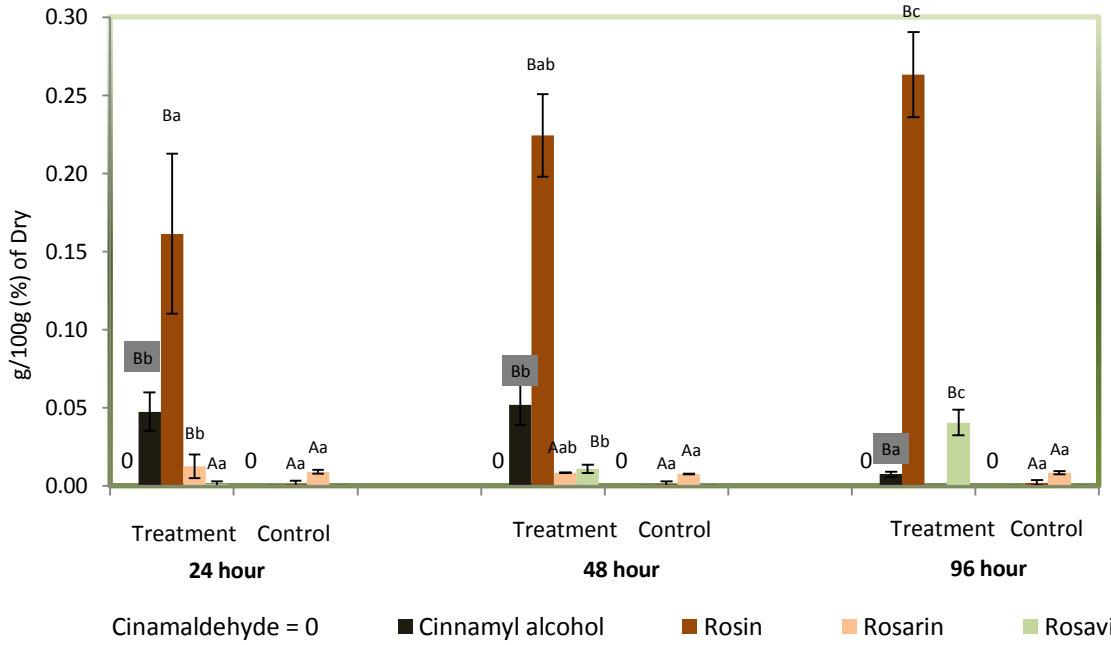

Figure 23. Effect of 2 mM cinnamaldehyde treatment on cinnamyl alcohol, rosin, rosarin and rosavin content in roseroot callus culture. Upper case: different letters are for significantly different treatments assumed a fixed interval of elapsed time ($p<0.05$). Lower case: different letters are for significantly 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.

The rosavin also showed a gradual increase as a result of cinnamaldehyed treatment during the sampling time. The rosarin content didn't change significantly in this experiment. Although cinnamaldehyde was not detected after 24 h in the media, its treatment significantly increased the tyrosine content after 48 h (Figure 24.). It also had significant effect on coumaric acid, 4-HPP and tyrosol formation during the experiment comparing to their sample counterpart. The 4-HPP and coumaric acid content significantly increased from 48 h to 96 h when cinnamaldehyde was present in the media.

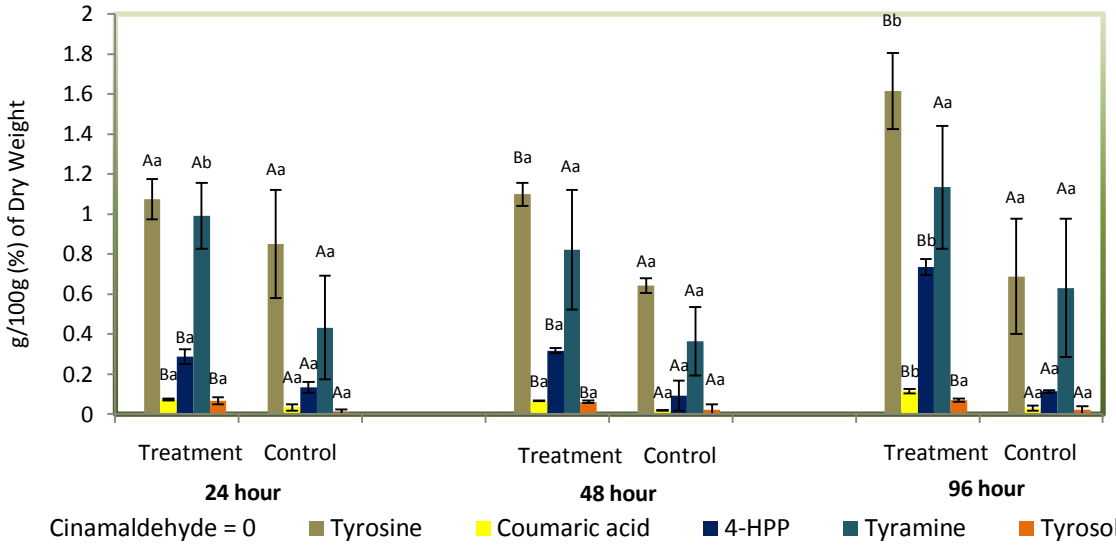

Figure 24. Effect of 2 mM Cinnamaldehyde treatment on tyrosine, coumaric acid, 4-HPP, tyramine and tyrosol content in roseroot callus culture. Upper case: different letters are for significantly different treatments assumed a fixed interval of elapsed time ($p<0.05$). Lower case: different letters are 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 results of 2 mM cinnamyl alcohol treatment on the content of rosin, rosarian and rosavin are presented in Figure 25. 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. Rosin was the only compound which was released into the media from the callus cells during the experiment and only from cinnamyl alcohol fed samples. 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 25, 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.

The cinnamyl alcohol significantly increased to content of tyrosine, 4-HPP and tyrosol; the compound which are believed to be to be involved in salidroside biosynthesis pathway and rosavins biosynthesis pathway (date are not shown).

The results of precursors feeding proved the possibility of roseroot bioactive compounds enhancement in in vitro cultures.

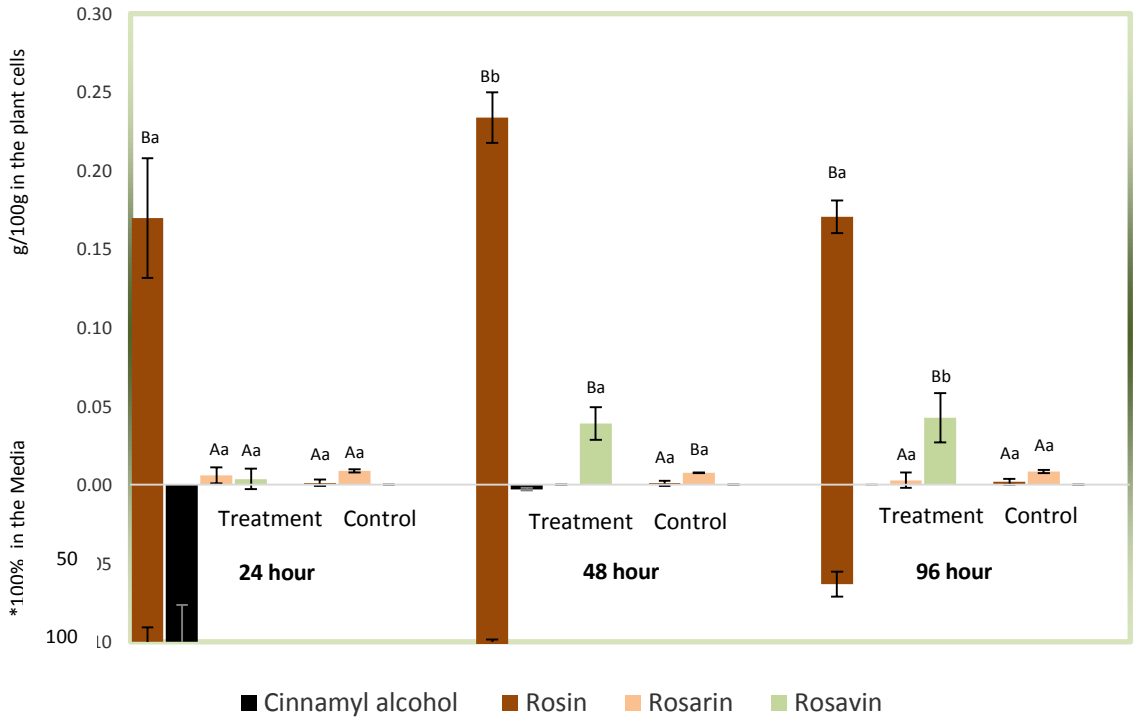

Figure 25. Effect of 2 mM cinnamyl alcohol treatment on rosin, rosarian and rosavin content in roseroot callus culture. Upper case: different letters are for significantly different treatments assumed a fixed interval of elapsed time ($p<0.05$). Lower case: different letters are for significantly different 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%.

4.2. Roseroot phytochemical analysis during the vegetation period

Our first HPLC analysis of roseroot phytochemical constituents was conducted to compare the individual specimens in an Austrian population (Hochkar, Göstling Alps; Austria: 47°48′N, 14°56′E) and one individual from our roseroot genebank in Budapest originated from Finland. Leaf and rhizome samples were analyzed during the vegetation period to detect the important roseroot phytochemicals. The HPLC results are presented in table 10 (Page 93), from which no significant similarity could have been detected among the 4 studied plants. Based on these results we could not get a clear tendency or correlation in the accumulation of the phytochemicals during the vegetation period.

To eliminate the possible environmental stresses which maybe the cause of uneven accumulation pattern detected in the first analysis, a modified experiment was attempted. Seven individuals were carefully selected, cut with their rhizomes, transported to a controlled environment (growth chamber) and let grow in pots. The results of HPLC analysis of root and rhizome samples (harvested during the vegetation period) are presented in table 8 and 9 from rhizomes and roots, respectively.

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. Rosarin content was remarkably higher in the rhizome, than in the roots being of the same order of magnitude as that of salidroside. In the rhizome samples, an intense increase was detected from the first sampling time to the 2nd in case of salidroside, tyrosol, rosavin and rosarin content whilst the cinnamyl alcohol content was more or less stable. Significant differences were hard to detect, because of the big variation in the amount of the metabolites.

Table 8. Phytochemical analysis of *R. rosea* rhizomes during the vegetation period (% of dry weight) [a]

Compound	Sampling 1	Sampling 2	Sampling 3	Sampling 4	Sampling 5
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± S D
Cinnamyl alcohol	0.68 ± 0.11	0.88 ± 0.31	0.76 ± 0.11	0.72 ± 0.19	0.77 ± 0.10
Rosin	0.15 ± 0.11	0.06 ± 0.04	0.10 ± 0.06	0.08 ± 0.04	0.07 ± 0.03
Rosavin	0.10 ± 0.08	0.27 ± 0.20	0.15 ± 0.14	0.07 ± 0.07	0.06 ± 0.05
Rosarin	1.27 ± 1.05	2.64 ± 1.76	1.52 ± 1.49	1.00 ± 1.49	0.82 ± 0.65
Tyrosol	1.20 ± 0.75	1.84 ± 0.89	0.78 ± 0.26	0.83 ± 0.63	0.83 ± 0.44
Salidroside	1.67 ± 0.74	2.73 ± 0.94	1.33 ± 0.91	0.95 ±0.90	0.93 ± 0.63

[a]) The values are mean ± SD (n = 7)

A trace of all compounds were detected by HPLC in the leaves (data not shown) indicating that the formation of these compounds can possibly take place in the areal parts of the plant or somehow they are transported back up to the leaves.

Based on the HPLC analysis no clear trends could be observed among the individuals in each sampling intervals. The seven roseroot individuals showed a very high deviation in their chemical content in each sampling time.

Table 9. Phytochemical analysis of *R. rosea* roots during the vegetation period (% of dry weight) [a]

Compound	Sampling 1	Sampling 2	Sampling 3	Sampling 4	Sampling 5
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Cinnamyl alcohol	0.30 ± 0.06	0.29 ± 0.09	0.16 ± 0.04	0.23 ± 0.11	0.22 ± 0.10
Rosin	0.08 ± 0.05	0.05 ± 0.05	0.05 ± 0.03	0.03 ± 0.02	0.04 ± 0.01
Rosavin	0.03 ± 0.03	0.01 ± 0.02	0.01 ± 0.01	0.02 ± 0.02	0.02 ± 0.01
Rosarin	0.28 ± 0.10	0.20 ± 0.11	0.13 ± 0.03	0.16 ± 0.09	0.15 ± 0.08
Tyrosol	0.70 ± 0.47	0.72 ± 0.62	0.30 ± 0.18	0.37 ± 0.24	0.37 ± 0.18
Salidroside	0.94 ± 0.64	0.88 ± 0.78	0.54 ± 0.23	0.73 ± 0.25	0.73 ± 0.32

[a]) The values are mean ± SD (n = 7)

Salidroside content varied between 0.11 % and 2.37 % in the roots and between 0.25 to 3.78 percent in rhizomes while the tyrosol content varied from 0.14-1.64 % in roots and from 0.21 to 3.32 percent in rhizomes of the 7 specimens during the sampling time. The same high fluctuation was observed in regard with cinnamyl alcohol and its glycosides too: cinnamyl alcohol varied from 0.08-0.42% (root) and from 0.51-1.47% (rhizome); rosin varied from 0.0002-0.18% (root) and from 0.015-0.31% (rhizome); rosavin varied from 0.004-0.066% (root) and from 0.005-0.7% (rhizome); rosarin varied from 0.03-0.44% (root) and from 0.24-5.38% (rhizome).

To achieve a better understanding of the metabolites formation pattern, we examined the direction of the changes of the content of each compound. The tyrosol content along with salidroside in our rhizome samples, showed a sudden increase from the 1st to the 2nd sampling time followed by the same sudden decrease in the 3rd and a gradual decrease to the 4th and the 5th sampling time. However in the root samples a different accumulation (opposite) pattern has been observed in which a mild decrease was seen from the first to the 2nd and 3rd; and a mild increase from the 3rd to the 4th and 5th stage which is very similar to a pattern reported by Bozhilova (2011). Not surprisingly the tyrosol and its aglycon (salidroside) content were always in correlation during the vegetation period.

Rosarin and rosavin content in the rhizome also increased from the 1st to the 2nd sampling time and gradually decreased till the end of the sampling period but no such pattern was observed in regard with cinnamyl alcohol and rosin neither in roots nor in the rhizomes.

The changes of the rosin content was very versatile, in the rhizome and no common trends could be detected, while in the roots the only regularity was that the content slightly decreased during the vegetation time. The content of cinnamyl alcohol in the roots decreased from the 1st to 3rd and slightly increased from the 3rd to the 5th stage which again was not similar with its pattern in the rhizome. In the rhizomes most of the compounds had their maximum in the second sampling time which was the beginning of shoot elongation and rapid mass production.

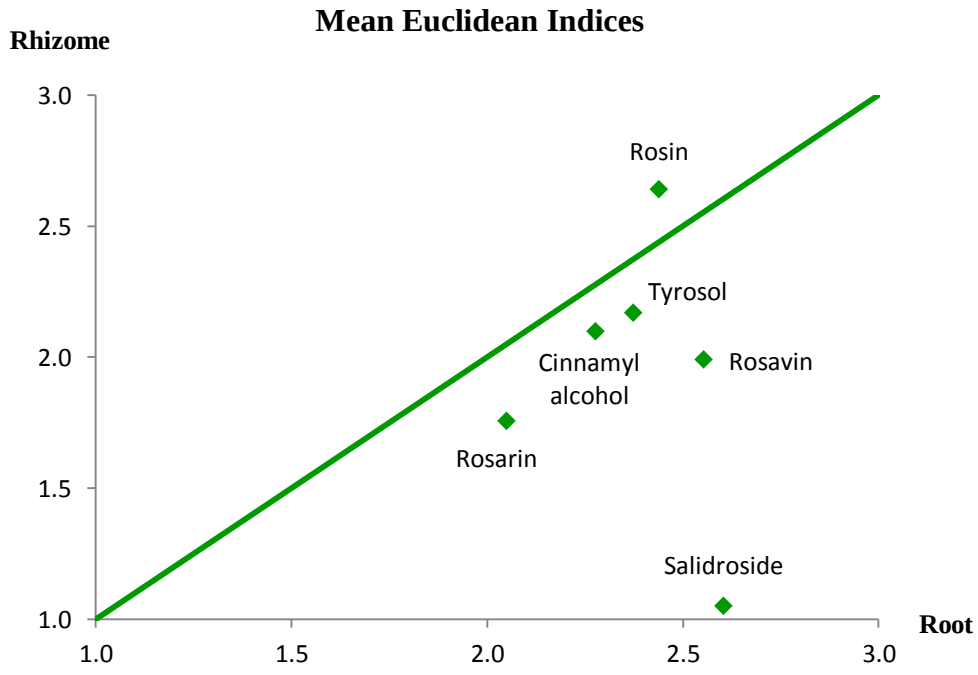

Figure 26. Pairs of dissimilarity indices of studied compounds. $P_k = \left(\overline{I_k^{root}}; \overline{I_k^{rhizome}}\right)$ (k = rosin, rosavin, rosarin, cinnamic alcohol, salidroside and tyrosol). The indices are calculated using the distances between the characteristic codes of the accumulation 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.

The similarity of the plants with regard to the accumulation of a given compound was examined with the method described in the (3.3.2). Repeated measures using an ANOVA method resulted in only slightly significant differences between the contents measured at five points of time merely in cases of rosarin (F_{root} (2.6; 15.8) = 5.02, $F_{rhizome}$ (1.8; 10.9) = 7.07) and salidroside in rhizome ($F_{rhizome}$ (2.2; 12.9) = 26.4), so we focused not on the nominal values of the contents but the characteristics of the accumulation, i.e. whether the glycosides have increased, decreased or been invariant from time to time.

Figure 26, shows that the salidroside accumulation pattern in the rhizome is very similar in all studied plants. All studied plants had the same character curve for this compound and also the similarity index is very close to its origin at Y axis. The figure also shows that the similarity is more pronounced in the rhizome than in the root as the index point is under the diagonal line. Generally, we can say that the accumulation pattern of all compounds (except rosin) was also slightly more similar in the rhizome than in the roots.

4. 3. Molecular biology experiments

To investigate the biosynthesis of the medicinally important phytochemicals of roseroot, the following approach was followed. After collecting all the possible compounds for biosynthesis of roseroot active constituents, a hypothetical pathway was drawn. The pathway then was shortened based on scientific reports on presence of each compound and the activity of corresponding enzymes in the plant kingdom. Finally, the proposed pathway (Figure 3.) for salidroside and rosavins (rosavin, rosarin and rosin) has a multi-channel possibility for the formation of each glycosides. Due to the lack of molecular knowledge about the genes and the enzymes that may play a role in the mentioned pathway in *R. rosea*, we started to search for the hypothesized genes by homology search in the databases.

4.3.1. Isolation of *R. rosea* UDP- glycosyltransferase gene

We isolated a full length *UDP- glycosyltransferase* gene for the first time from *R. rosea*. *UDPG* is the enzyme believed to be responsible for the formation of salidroside by glycosylation of its aglycon; tyrosol. The NCBI data base search brought 525 hits for *UDPG*s with complete cds tags from which 55 were selected and imported to Mega 6 software for similarity analysis through alignment. Degenerate primers were designed based on conserved regions in alignment and PCR was carried out with roseroot cDNA as template. A 510 bp long fragment was PCR amplified and visualized on agarose gele. The PCR product was sequenced and its significant similarity with other *UDPG* sequences was studied and confirmed. Specific primers were designed and subjected for a new PCR round to obtain a 273 bp long roseroot-specific product.

The nucleotide sequence analysis showed that the 273 bp cDNA fragment belonged to the glycosyltransferase GTB type super family in which the plant UDP- glycosyltransferase are included. The 88 letters long deduced amino acid sequence of the cDNA fragment was similar to the amino acid sequence of UDP glycosyltransferase from *Rhodiola sachalinensis* (66% similarity), *Populus trichocarpa* (63% similarity) and UDP-glucose flavonoid 3-O-glucosyltransferase from *Morus notabilis* (62% similarity). This suggested that the core fragment of *R.rosea UDPG* (*RrUDPG*) was obtained and could be used to isolate the full-length cDNA by RACE technology. With the nested 5′ and 3′ RACE, a 754 bp 5′ cDNA end and a 613 bp 3′ cDNA end with a poly $A_{(20)}$ tail was specially amplified and scored on agarose gel (Figure 27.). The complete open reading frame (ORF) was finally amplified and confirmed with newly designed primer (UDORF-F and UDORF-R) pairs (Figure 28.).

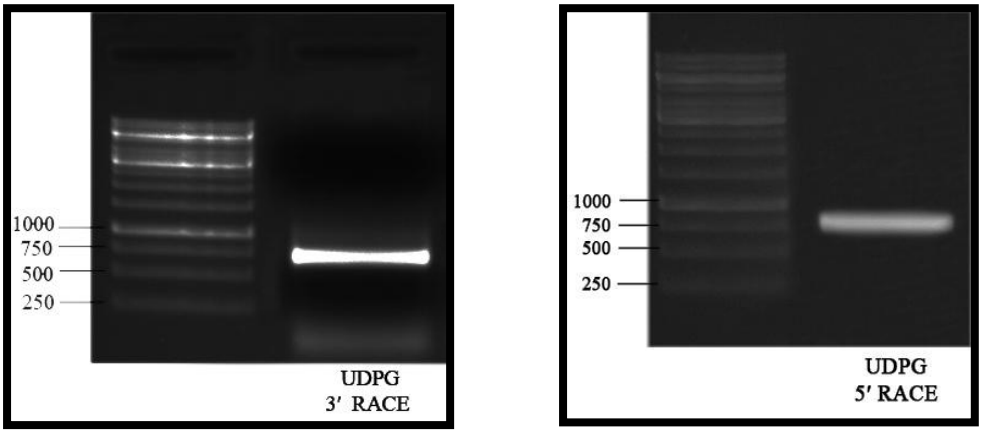

Figure 27. PCR amplified 3′ cDNA end and 5′ cDNA end using the RACE technique

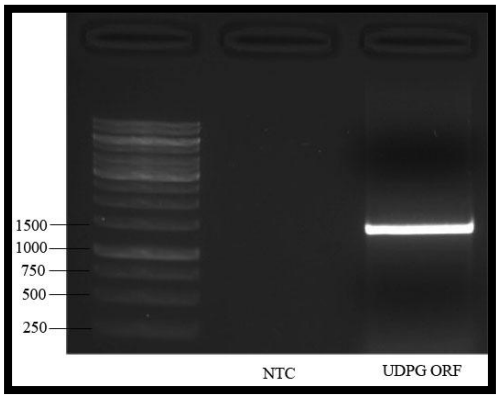

Figure 28. PCR amplification of complete ORF of *RrUDPG* (KM396888)

The full-length cDNA contains a 1425 bp long coding sequence (Figure 28 and 29.) to encode a 474 deduced amino-acid polypeptide residues with a calculated molecular mass of 53.05 kDa and a predicted isoelectric point (pI) of 5.90 (data are not shown).

The PCR amplified ORF was transferred to *E. coli*, cloned and sequenced (from 3 independent transformation events). The full length *RrUDPG* transcript contains 53 nucleotides in its 5′ (5′UTR) and 119 nucleotides in its 3′ (3′UTR) untranslated regions (Figure 30.). For the naming of the new gene, recommended nomenclature by Mackenzie *et al.*, 1997, was considered and accordingly 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).

5' UTR CDS 3' UTR

5′ACAAAAATCCGACACAACCATCGAAACAGAGGAATTCAATTCACAGAGCGATAATGGGTTCT
GATTCACGGCCTCTACGCGTCTTCTTCTTTCCCTTCATGGCTCACGGCCATCTGATTCCGATGGTC
GACATCGCCAGACTCTTCTCTTCTCAAGGAGTCCACTCCACCATCATCACCACCCCACTAAACGC
CAATTACATCTCCAAAACGACGTCTCTATCCATCAAAACGATACCGTTTCCTGCTGCGGAAGTTG
GGCTTCCGGACGGCTGCGAGAATATCGACATGCTTCCTTCGCCCGATCTCTTCTTCAAATTTTTCC
AAGCCGCCAATTTACTCCAAGCGCCGTTCGAGAACCTTCTAGAACTCGAAAGGCCCGATTGCTT
AATCTCCGACATCTTCTTCCCCTGGTCAGTCGACTCCGCCGAGAAATTCAACATCCCGAGACTCG
TTTTCCACGGCACGAGCTTCTTCGCCATGTGCGCCATGGAGAGCTTGAAGACCCACAAGCCCTAT
AAATCGGTAAGCACCGACTCTGAACCGTTCTTAATCCCGAATCTCCCTGATGAAATCAAAATGA
CTAAAAGTCAGTTCACGGTTGACGCTTGGGAAGACACCGAAAAGGGCCTTGGGAAGCTGTTGGC
TGATGCGAGAGCTTCAGGGCTGAGGAGCTTCGGCATGATCGTAAACAGCTTCCACGAGCTCGAA
CCGGCTTACGCGGATTATTACAAGAATGTGTTGAACATGAAAGCGTGGTGTGTCGGGCCTGTTTC
GGTATGTAACCGAAACGTTGAGGAGAAAATTGCGAGAGGAAAAAAATCAGCTATTGATGATCA
CGAGTGCTTAAAATGGCTGGAGGGAAAGCAGCCAGACTCCGTCGTGTACGTTTGTTTCGGGAGC
AGCGCGAGCTTCCCTGATGAGCAGTTGCGCGATATCGCATTGGGGCTGGAAGAATCTGGAGTAA
ATTTCATCTGGGTGATCAGGAGAAGTTCCGAGTCAGGATCAAAAGATTACTTGCCGGAGGGGTT
TGAGGACCGGGTGAAGGACAGAGGGATCGTGATCCGAGGTTGGGCGCCACAGGTACTGATTTTG
GACCATCCGTCGGTTGGGGGATTTGTGACTCACTGCGGATGGAATTCGGCATTGGAGGGGATTT
CAGCTGGCTTGCCGATGGTGACTTGGCCATTGTTCGCAGAGCAGTTTTTCAACCAGAAATTGATT
AAGGATGTGTTGAAAGTTGGGGTTGAGGTTGGAGTGCAGAAATGGTCTCGGAACGGGGAGGAT
CGCGTGACGAAGGAGAAGGTTGAGAAGGCGGTGAGGGCTGTTATGGTTGGGGAGGAGGCTGAG
GAGAGGCGTGGCAGAGCTCGTCAGCTTGGGAAATTGGCAAAGAAAGCTGTGGCGAAAGATGGG
TCTTCGTACATTGATCTCCACAATTTGCTTGATGAATTAAAGTTGAGAAGAGAGACTTTGTCCTA
GGTTGGATGCTTCTATATATTTTCTTGTGATGCAAATGCAAGGTAACTTATTGGAATGCTCTGTG
TTCCATTGTTCTCCGGATGCAAAAGAACTTTATTGAAAAAAAAAAAAAAAAAAAA3′

Figure 29. Complete sequence of *UDP- glucosyltransferase* transcript (cDNA) from *R. rosea* L. The Open Reading Frame (ORF) is highlighted in green and the 3′ and 5′ untranslated regions (UTR) are in gray.
Plant Secondary Products Glycosyltransferase (PSPG) BOX is underlined.
GenBank accession number: KM396888

MGSDSRPLRVFFFPFMAHGHLIPMVDIARLFSSQGVHSTIITTPLNANYISKTTSLSIKTIPFPAAEVGLPDG
CENIDMLPSPDLFFKFFQAANLLQAPFENLLELERPDCLISDIFFPWSVDSAEKFNIPRLVFHGTSFFAMCAM
ESLKTHKPYKSVSTDSEPFLIPNLPDEIKMTKSQFTVDAWEDTEKGLGKLLADARASGLRSFGMIVNSFHELE
PAYADYYKNVLNMKAWCVGPVSVCNRNVEEKIARGKKSAIDDHECLKWLEGKQPDSVVYVCFGSSASFPDEQL
RDIALGLEESGVNFIWVIRRSSESGSKDYLPEGFEDRVKDRGIVIRGWAPQVLILDHPSVGGFVTHCGWNSAL
EGISAGLPMVTWPLFAEQFFNQKLIKDVLKVGVEVGVQKWSRNGEDRVTKEKVEKAVRAVMVGEEAEERRGRA
RQLGKLAKKAVAKDGSSYIDLHNLLDELKLRRETLS

Figure 30. Deduced amino acid sequence of *Rhodiola rosea UDP- glucosyltransferase*.
Plant Secondary Products Glycosyltransferase (PSPG) motif is underlined

Deduced amino acid sequence of *RrUDPG* gene, is shown in Figure 30. The sequence showed significant similarity with other plant *UDPG*s such as 63% similarity with *Rhodiola sachalinensis* UDP-glucosyltransferase (Ma *et al.*, 2007) and 56% similarity with *Theobroma cacao UDP-glycosyltransferase* (Motamayor *et al.*, 2013).

The PSPG-box (plant secondary product glycosyltransferases-box) showed 75% identity and 81.82% similarity with the average plant PSPG-box sequences (Vogt and Jones, 2000). Within this motif, the peptide sequence of HCGWNS, which has been detected in 95% of all glycosyltransferases (Vogt and Jones, 2000), is located at position of 357. By using genomic DNA as template and the *UDPG* ORF primers for PCR, the same length PCR product was amplified and sequenced 3 times. The sequencing results revealed 100% identity to the ORF sequence of RNA transcript (cDNA) proving the absence of any intron or any transcriptional modifications in our studied gene.

4.3.2. Phylogenetic tree for *R. rosea UDPG* gene

The presented phylogenetic tree (Figure 31.) is showing the positions of *R. rosea* L. *UDPG* among 22 plant glucosyltransferases with an estimated amino acid sequence similarity of BLASTP *e* value with 0.0. The tree was inferred using the neighbor-joining statistical method (Saitou and Nei, 1987) in program MEGA version 6 (Tamura *et al.*, 2011; 2013). The tree analysis showed the highest similarity between *R. rosea* UDP- glucosyltransferase with a flavonoid glycosyltransferase protein from *Withania somnifera* (Singh *et al.*, 2013). A relatively remarkable distance can be seen between *R. rosea* and one of *R. sachalinensis* identified UDP-glucosyltransferase (Ma *et al.*, 2007) amino acid sequences (Figure 31.).

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

We isolated a full length *AAD* gene sequence from *R. rosea*. AAD (RrAAD) 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. Similar to the earlier strategy described for *UDPG*, degenerated primers were designed and subjected for PCR using *R. rosea* cDNA as template. A 197 bp long fragment was amplified and visualized on agarose gel. The PCR product was cloned and sequenced. Significant similarity (>80%) with other plant partial *AAD* sequences was found.

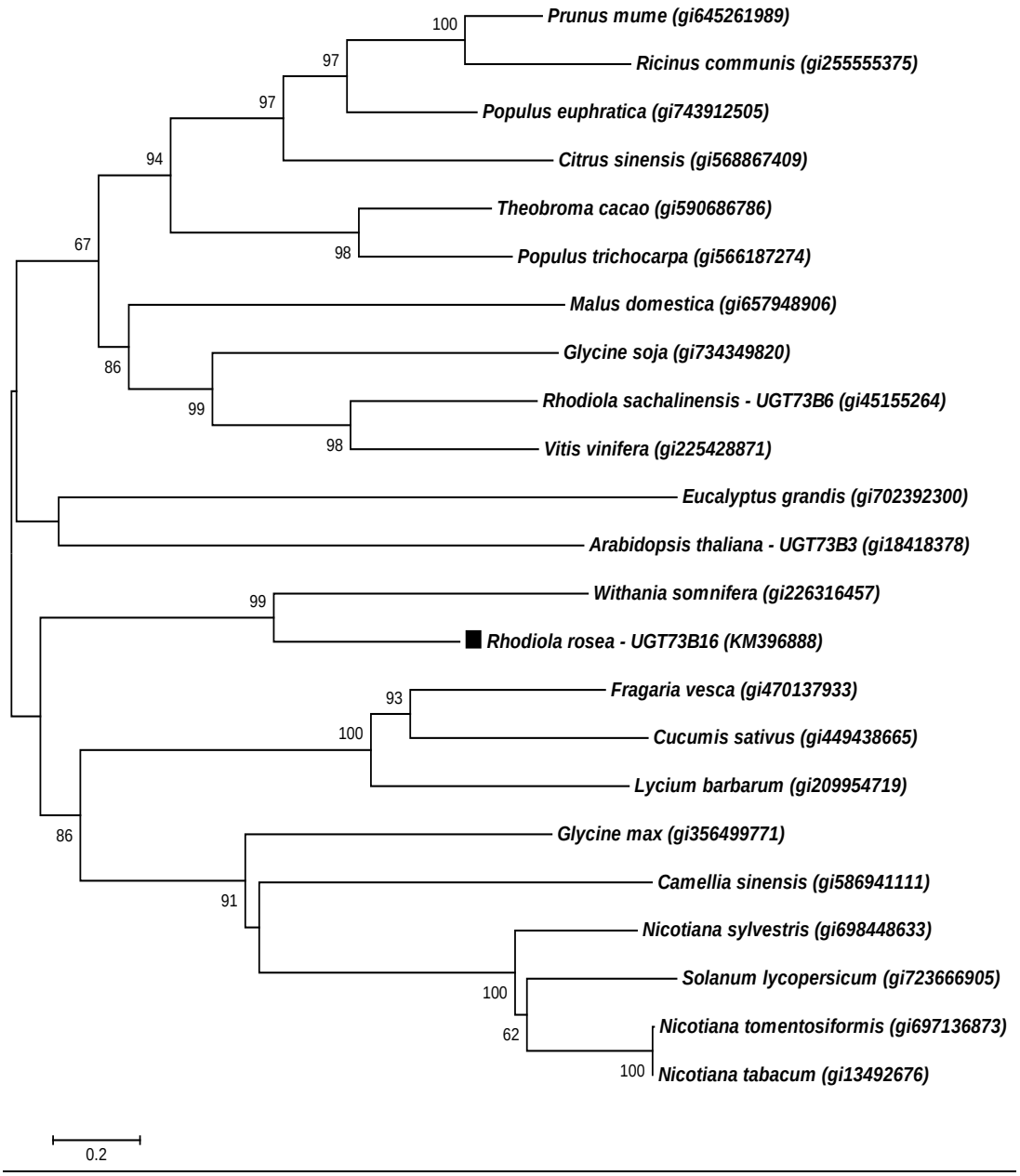

Figure 31. Phylogenetic tree showing the positions of *R. rosea* L. UDP- glucosyltransferase (marked with a black filled box) among 22 alignable plant secondary metabolites glucosyltransferase with an estimated amino acid sequence similarity (BLASTP e value) of 0.0. Numbers represent support values after 500 bootstrap replicates and the values higher than 60% are indicated above the nodes. The NCBI GenBank accession number for each UGT is shown in parentheses. The scale bar denotes the number of substitutions per site.

Using the 5′ and 3′ RACE technique, a 734 bp 5′ cDNA end and a 477 bp 3′ cDNA end with a poly $A_{(37)}$ tail was specially amplified and scored on agarose gel. The ORF was finally amplified and confirmed when new primer pairs (AAORF-F and AAORF-R) were applied. The *AAD* full-length cDNA contains 1155 bp long coding sequence (Figure 32.) 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 (data are not shown).

The PCR amplified ORF was cloned and sequenced similarly to the *UDPG*. The full length *RrAAD* transcript contains 34 nucleotides in its 5′ (5′UTR) and 259 nucleotides in its 3′ (3′UTR) untranslated regions (Figure 32.). The full-length cDNA of *RrAAD* was submitted to NCBI database (GenBank accession number: KP686072).

Deduced amino acid sequence of *RrAAD* gene, is shown in Figure 33. The sequence showed a significant similarity with the members of aldo-keto reductases (AKRs) superfamily. There is more than 80% similarity between *RrAAD* and around 50 predicted plant *AAD*s.

When genomic DNA was used as template with *AAD* ORF primers in PCR, a new and larger PCR product (~3100 bp) was amplified and scored on agarose gel. Due to the nucleotide size limitation of our cloning system which was working the best for amplicons smaller than 1.2 kb, three different primer pairs were utilized to amplify the smaller fragment from roseroot genomic DNA. After the PCR, each different product were transferred to *E. coli*, cloned and sequenced at least three times. The three DNA fragment sequences were then carefully assembled to generate full-length genomic nucleotide sequence of *AAD* gene. Using the online Splign computing tool (Kapustin *et al.*, 2008) on NCBI database, 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) which can be seen in Figure 34 and 35.

4.3.4. Phylogenetic tree for *R. rosea AAD* gene

The presented phylogenetic tree (Figure 36.) is showing the positions of *R. rosea* L. *AAD* among the 14 plant aryl alcohol dehydrogenases with estimated amino acid sequence similarity of BLASTP e value with 0.0. The tree analysis showed the highest similarity of *RrAAD* with a putative aryl-alcohol dehydrogenase from *Vitis vinifera* (gi 225436289).

5' UTR CDS 3' UTR

5′AAAGTTCAGCTTCTCAACCAGACGCAGAGCCACCATGTCGCTCCACGTCTACGCCTCCTCTGC
CACCAATCTTAAACTCCTCGCTTCTTCCACCGGCCGGGTTACGACGCCGTCACGGAGGTTTTTTT
CGAGCTCGGTCAGGTGCGAGGTGTCAACGGCTGACAACCGAGTCACGACAGTAAAGAATGGCA
GTGATGAGCTGGAAATTTGCAGAGTCATTAACGGAATGTGGCAGACGAGCGGTGGGTGGGGGA
GAATCGACCGAGACGATGCCGTAGATGCAATGCTTCGCTATGCTGATGCTGGACTGTCCACGTT
TGACATGGCGGATCACTATGGGCCTGCTGAAGATCTGTATGGACTCTTCATCAACCGAGTCCGT
AGGGAGAGACCACCCGACTACGTAGAAAAGGTCAGAGGTCTCACAAAATGGGTGCCTCCGCCT
GTTAAAATGACAAGTACCTTTGTGAGGAACAACATTGATATTTCACGTAAGAGAATGGATGTTG
CATCTTTGGACATGCTACAGTTCCACTGGTGGGATTACGCCAACACTGGCTATCTTGATGCTTTG
AAACACCTAACTGATTTGAAAGGAGAAGGTAAAATCAAGACTATTGCTTTGACAAATTTTGATA
CAGAGAGGTTACAGATAATATTGGAACATGAGATTCCCATTGTCAGCAACCAGGTTCAACATTC
CATAGTAGACATGCGCCCCCAACAAAAGATGGCGGAACTTTGCCAACTTACGGGAGTAAAATT
GATAACATATGGAACAGTAATGGGTGGTCTGCTGTCTGAGAAATTCCTCGACACCAACTTGTCC
ATTCCCTTTGCTGGCCCCCCTTTAAACACTCCCTCACTTCAAAAGTACAAAAGGATGGTCGACGC
ATGGGGAGGATGGAGTGTTTTCCAGACTCTTCTGAAGACACTAAAAACAGTAGCTGTTAAGCAT
GGGGTTTCAATCCCAACAGTTGCTGTTAGATACATTCTAGACCAGCCCTCCGTGGCAGGATCAA
TGGTGGGTGTTAGACTTGGATTATCAGAACACATCCAAGACTGCAATGCAATCTTTTCCCTCACT
CTGGATGAGGAAGATGTAAACAGCATTCAAGCAGTCACAAAGAAGGGGAAAGATCTGATGAGA
ATCATCGGTGATTGTGGGGATGAGTACAGACGCGCATTAAGCCCCCGACTATGTTGTTATATTAT
AATCTTTGAGAATAATCATGATTACAGCCTCTGATCATCGATAGGCAACTGGTTGATCGTATTTA
TACAAAGCCTTGTGATATGGGCATCAGGTATTTATTGAATATGTAAGTGTTAGATTACAACAGA
TGGTTAACGAATTGATCGATGGAGCCTTTTTGCCTTTTTGTTAATTGAATTGTATGCTCCTTTAGA
GTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA3′

Figure 32. Complete sequence of *Aryl Alcohol Dehydrogenase* (*AAD*) transcript (cDNA) from *R. rosea* L. The Open Reading Frame (ORF) is highlighted in green and the 3′ and 5′ untranslated regions (UTR) are in gray. GenBank accession number: KP686072

MSLHVYASSATNLKLLASSTGRVTTPSRRFFSSSVRCEVSTADNRVTTVKNGSDELEICRVINGMWQTSGGWGRIDRDDA
VDAMLRYADAGLSTFDMADHYGPAEDLYGLFINRVRRERPPDYVEKVRGLTKWVPPPVKMTSTFVRNNIDISRKRMDVAS
LDMLQFHWWDYANTGYLDALKHLTDLKGEGKIKTIALTNFDTERLQIILEHEIPIVSNQVQHSIVDMRPQQKMAELCQLT
GVKLITYGTVMGGLLSEKFLDTNLSIPFAGPPLNTPSLQKYKRMVDAWGGWSVFQTLLKTLKTVAVKHGVSIPTVAVRYI
LDQPSVAGSMVGVRLGLSEHIQDCNAIFSLTLDEEDVNSIQAVTKKGKDLMRIIGDCGDEYRRA

Figure 33. Deduced amino acid sequence of *Rhodiola rosea Aryl alcohol dehydrogenase* (*AAD*).

5' UTR CDS 3' UTR

5′AAAGTTCAGCTTCTCAACCAGACGCAGAGCCACCATGTCGCTCCACGTCTACGCCTCCTCTGCCACCAATCTTAAACT
CCTCGCTTCTTCCACCGGCCGGGTTACGACGCCGTCACGGAGGTTTTTTTCGAGCTCGGTCAGGTGCGAGGTGTCAACG
GCTGACAACCGAGTCACGACAGTAAAGAATGGCAGTGATGAGCTGGAAATTTGCAGAGTCATTAACGGAATGTGGCA
GACGAGCGGTGGGTGGGGGAGAATCGACCGAGACGATGCCGTAGATGCAATGCTTCGCTATGCTGATGCTGGACTGTC
CACGTTTGACATGGCGGATCACTGTAATCTCATCTTATCTCTCTCGATTTAAAACCCTAGGTTTGACTGGATGAAGACA
AATGATGGATGACGTAGTCTTATTAGCTTGCAATATTTTGAATTTCAAATAAATAATAAACATGAGGATTTTAGAATTA
TCAAATGGTAGGGACTGGAGAGGTGTAGAACCTCCCTGAAAAGGATAGAGCTCCACTTCACTGTTTTGCTCCTTAGTGT
GGCTTGTACTTAGTTATCTTATCTGTCTGAAGATGGCTGAGTTGTTGATGTATTGTTCGGTCAGCAAGTTTCTCTTCAGC
TTGAGTCAAGTGGTTGTTCTTGAGAGGTGCAATGGGTTTCTGAGGATTAGGAGGGGAAGGGGTGTGGCTAGGTGTGGG
GTGGTGGGTGATGCGGAGGTGCGGGCTGTGGTCAAGAATGGGACAGATTCGTTGGATATTTGCAGGGTTGTTAATGGA
ATGTGGCAGACTAGTGGGGGTTGGGGGAAGATTGAGAGAGATAGTGCAGTGGATTCCATGCTTAGATATGCTGATGCT
GGGCTGAATACATTTGATATGGCAGATATATGTAAAGAACTATGAAATAAGCATTTCCTTGTTACTTGCAAACTCTTGC
TGCACGAATGAATGGTTTAATTTTGAAGAATCATTGAGTACATGATTGGTTTCATGTATCCATTTATTTCCGCCTTAGTT
TTATTATAGTTAGTTCACAGAACTCTATGTTCACATCTCTTCAGTATACTGATCGCCCAACTGTGTCTTACTGACGTTAT
TGTTTCATTGTATATTGCTTGCTATTTCTTGTTGTGTCCTTGTTATTAATTTGTTAAATGTTTGCCAATTTCAGATGGGCC
TGCTGAAGATCTGTATGGACTCTTCATCAACCGAGTCCGTAGGGAGAGACCACCCGACTACGTAGAAAAGGTCAGAGG
GTGAGTTATCATCTCTCGACACAATATTTTATTTCATTTTACTTAGATTGTATGAGTAGCTGCTAGCACTTGTCTATTTTT
AACGTCTCTGGATATAAATGATGAATGGTATGTGAACCATGATATACCTATCGAGTAAGGTATGGCTCACGTTTGTCTC
ATGGGATGCAGTCTCACAAAATGGGTGCCTCCGCCTGTTAAAATGACAAGTACCTTTGTGAGGAACAACATTGATATTT
CACGTAAGAGAATGGATGTTGCATCTTTGGACATGCTACAGTTCCACTGGTATATTTTCTGTTTCCTCTTAACTCATATG
TACAACCAGGCTGTAATTACAAACACTATGTAATTGTGGTCTAGAAGGACCACTTTAGTTGAGTGCATAACAAGTGTTC
AGACAGATACTTTGGTCATTATGTTTTGCTTAACTAGGTGACTGTAGGGACAATACAAGAATAATTAAAACAAGTAGTT
CAAATGAATGCTTTCAGCTCTGATTACCTTCTATTATTGGCACATGGTACATAGACCAGCGAATTTGCAAATAGATATA
TTAATTTATCTTTCATTTGCACAAAACTGATAAGAATATTCTATAGTTTTACCTTAATATTGATTTTTCGCTTTTACTTGC
ACTAGGTGGGATTACGCCAACACTGGCTATCTTGATGCTTTGAAACACCTAACTGATTTGAAAGGAGAAGGTTAACTT
ATATCCATCAAATGTATGACATGAATCAATTCGTCTTGAATTTTCCAAGATATGATGGGATTACTTCAGCTGTAATGTG
CAGGTAAAATCAAGACTATTGCTTTGACAAATTTTGATACAGAGAGGTTACAGATAATATTGGAACATGAGATTCCCA
TTGTCAGCAACCAGGTAATTTTTTTTTTGCCATCCTTGTGGATGTCTGCTTAGTTCAATAATCACTGTAATTTTTGCTTTC
CAGGTTCAACATTCCATAGTAGACATGCGCCCCCAACAAAAGATGGCGGAACTTTGCCAACTTACGGGAGTAAAATTG
ATAACGTATTATTCTCATGTCTCTCAGTTTTACTTGGCTGTCTGGTTGGCAGCATTGCATTTTTTCCTTTTTCTTCTCCTA
ATTGAAATATGGTTTAATTTGCTTATTGGTGATGCAGATATGGAACAGTAATGGGTGGTCTGCTGTCTGAGAAATTCCT
CGACACCAACTTGTCCATTCCCTTTGCTGGCCCCCCTTTAAACACTCCCTCACTTCAAAAGTACAAAAGGGTATGCCTTT
TCAAATAAGCTAGATGTGCTTGCTAGTATGGTATCAAAGATAGAATCGAAATCTGCCAAATGAATTAAGCCTTTTTGTA
AAAAAGATAGTTGTTGGCCTGGGAGAATATTTCATCCTTGTTATTACATGCAATCCCTATAAAACATCTATTACTTGAA
GAGTTTAATAACAGAAATGTGATGCAAGTATCTAGCAAATTAAAATTGATGGCATATGTATATATGGAAGATGGTCGA
CGCATGGGGAGGATGGAGTGTTTTCCAGACTCTTCTGAAGACACTAAAAACAGTAGCTGTTAAGCATGGGGTTTCAAT
CCCAACAGTTGCtGTTAGATACATTCTAGACCAGGTCAGTCTGTTACCTCTTCTCAAATAAATGAATCTGCGAATGCATC
TAATGGTACAGTCATTTTTTCCAGCCCTCCGTGGCAGGATCAATGGTGGGTGTTAGACTTGGATTATCAGAACACATCC
AAGACTGCAATGCAATCTTTTCCCTCACTCTGGATGAGGAAGATGTAAACAGCATTCAAGCAGTCACAAAGAAGGGGA
AAGATCTGATGAGAATCATCGGTGATTGTGGGGATGAGTACAGACGCGCATAAGCCCCCGACTATGTTGTTATATTAT
AATCTTTGAGAATAATCATGATTACAGCCTCTGATCATCGATAGGCAACTGGTTGATCGTATTTATACAAAGCCTTGTG
ATATGGGCATCAGGTATTTATTGAATATGTAAGTGTTAGATTACAACAGATGGTTAACGAATTGATCGATGGAGCCTTT
TTGCCTTTTTGTTAATTGAATTGTATGCTCCTTTAGAGT3′

Figure 34. Nucleotide sequence of 3358bp long Aryl Alcohol Dehydrogenase (*AAD*) from *R. rosea* L. GeneBank Accession Number (KP686071)

Figure 35. Green boxes and black lines represent exons and introns, respectively (*Box sizes of exons and lines of introns are drawn to scale to their length in nucleotide content*).

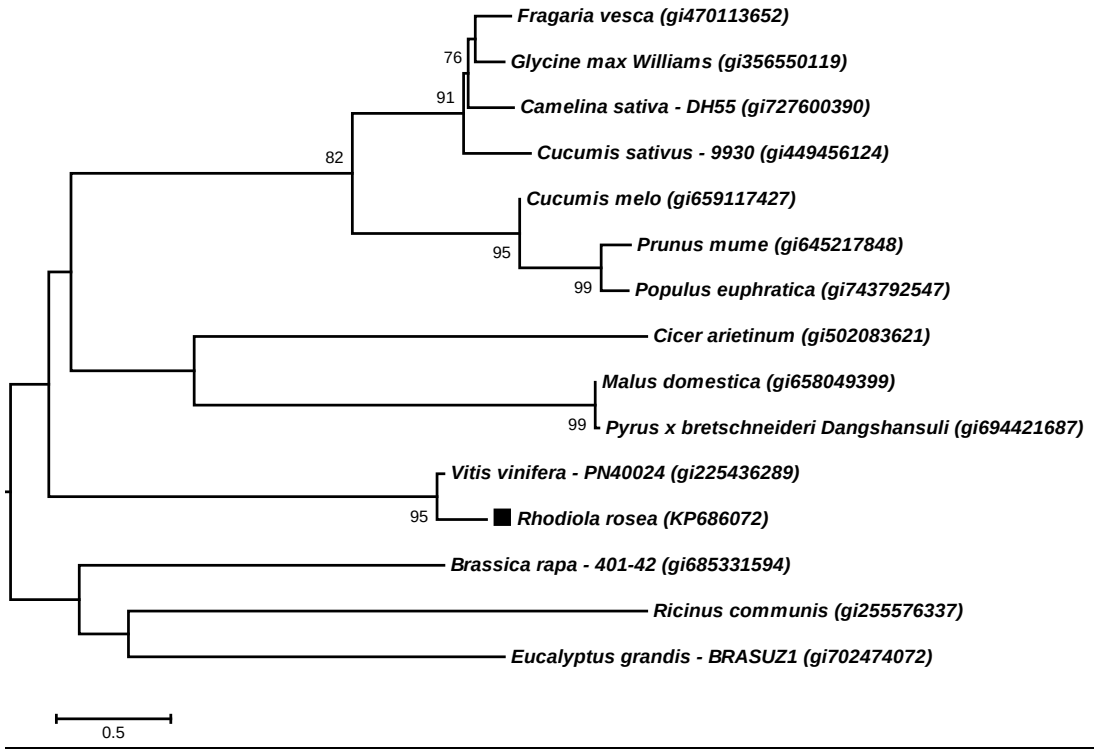

Figure 36. Phylogenetic tree showing the positions of *Rhodiola rosea* L. *Aryl alcohol dehydrogenase* (marked with a black filled box) among 14 alignable *AAD*s with an estimated amino acid sequence similarity (BLASTP e value) of 0.0. Numbers represent support values after 500 bootstrap replicates and the values higher than 60% are indicated above the nodes. The NCBI GenBank accession number for each *AAD* is shown in parentheses. The scale bar denotes the number of substitutions per site.

4.3.5. 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. Time course study revealed that the recombinant UGT73B16 was expressed after 1 h of IPTG induction and its highest expression was obtained after 4 h of induction at 37°C (Figure 37.). To evaluate the gene construction preparation the bacteria was transformed with the empty vector containing a maltose binding protein (MBP) and it's expression was induced similar to that of our final constructs. A new band comparing to the non-transformed sample can be seen in the lane E (Figure 37.) that shows the corresponding MBP (~ 42.5 kDa) protein.

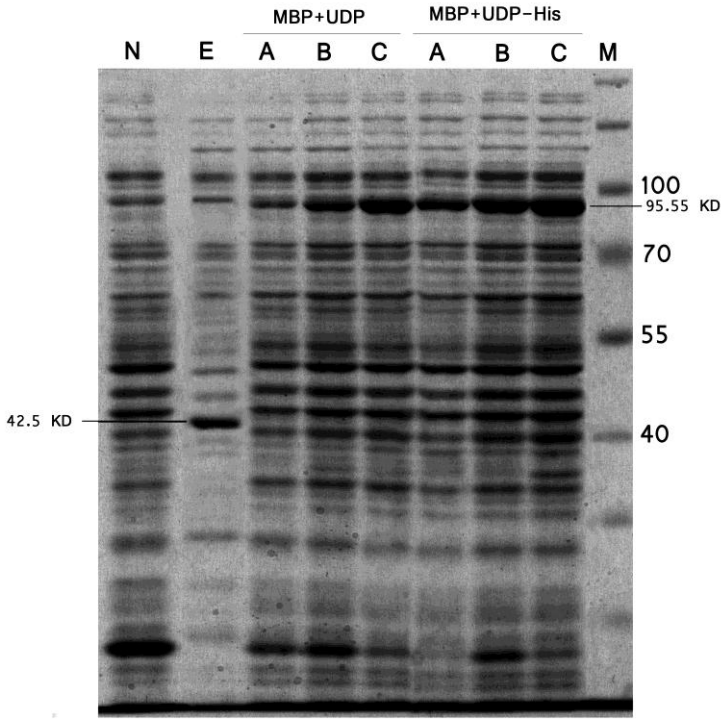

Figure 37. Heterologous expression of UGT73B16 in *E. coli*. Total protein extracts of *E. coli* cells were used for SDS-PAGE analysis (stained with coomassie blue). Lane N: non-transformed BL 21; Lane E: Transformed with empty vector (control); A, B and C: expression of recombinant protein (MBP+*UDPG* 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 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 38.). In the SDS-PAGE analysis a trace of MBP expression is visible (Figure 38, lane C) which shows its native expression level in non-transformed *E.coli*. The protein bands corresponding to the expected molecular weight for MBP and MBP+UDPH-His construct were scored after staining the gel. The objective proteins are clearly missing from the flow-through samples and are present in collected samples after elution with maltose containing buffer (Figure 38.).

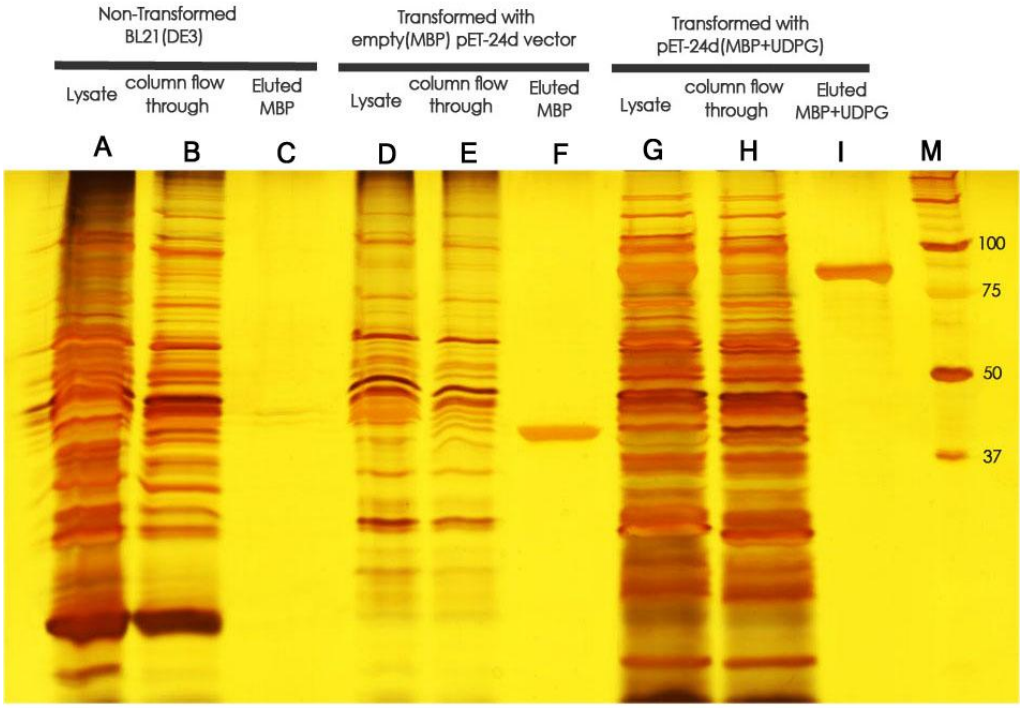

Figure 38. Purification of recombinant proteins with affinity chromatography (Expression induced by IPTG and for 4 hours and the SDS gel was stained with silver salt). A: total protein extract from non-transformed BL 21 before chromatography, B: Amylose resin column flow trough of A, C: Purified MBP 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 39 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 40, 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. The results are also indicating that a remarkable portion of the over-expressed proteins are soluble and a simple cell disruption with sonication and centrifugation was enough to fraction and score the recombinant proteins.

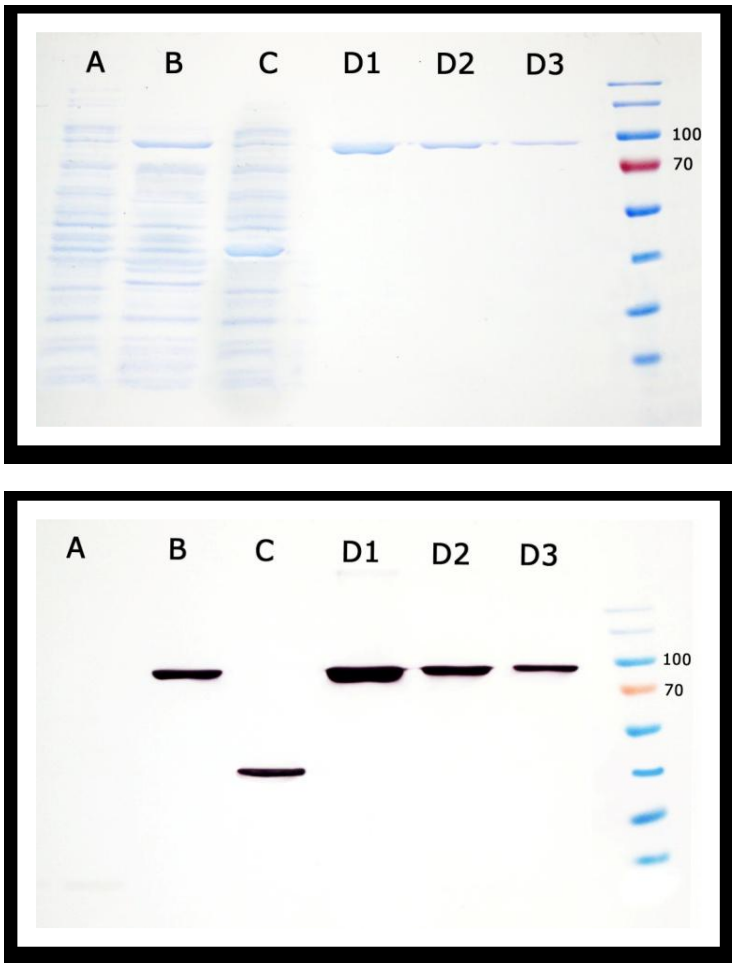

Figure 39. SDS-PAGE analysis (upper) and western blot analysis (lower) of MBP and MBP+*UDPG* proteins. **A**: Non-Transformed BL21 *E.coli* cells lysate, **B**: MBP+*UDPG*-His transformed cell lysate, **C**: Empty vector (with MBP~42.5 kDa) transformed cell lysate, **D1-D3**: Purified MBP+*UDPG*-His recombinant protein with affinity chromatography after 4, 2 and 1h IPTG induction time.

4.3.6. *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. Both genes are hypothesized to be involved in the proposed salidroside formation pathway (Figure 3.). Total RNA was extracted from the harvested samples during the experiment. The quality of all extracted RNAs was validated before quantification and normalization. Figure 40 shows the 28S and 18S major RNA band after agarose gel electrophoresis which is an indication for RNAs integrity after extraction process.

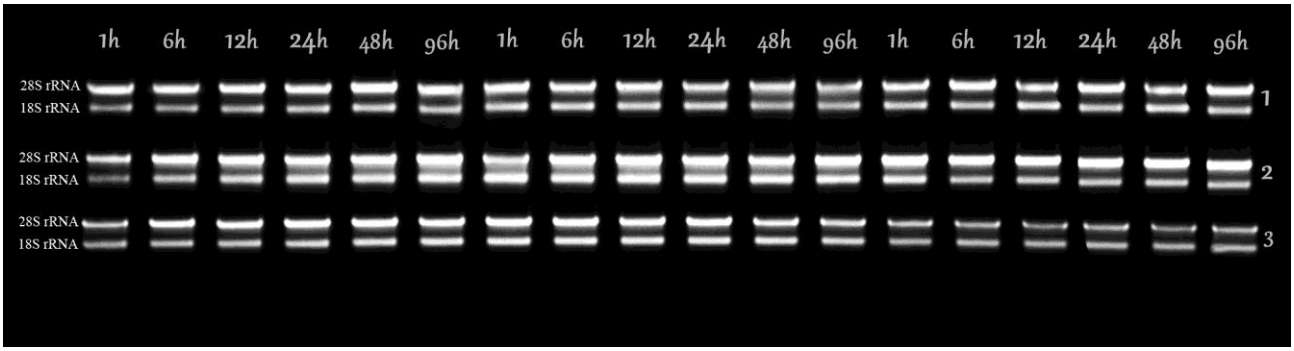

Figure 40. Total RNAs integrity assessment on agarose gel. Lanes are showing the RNA samples extracted from calli during the feeding of precursors (Tyrosol in this figure) in triplicates.

The RNAs were normalized for equal quantity (0.5 µg/µl) and subjected for cDNA synthesis. A set of minus reverse transcription reaction (False positive control) was also conducted along with each cDNA synthesis reaction to validate the absence of any genomic DNA contamination during the reaction. Figure 41 shows an example of cDNA test using the qPCR primers and the minus reverse transcriptase samples as template. The test was done for all of the cDNA samples with corresponding primer pairs. This is the most reliable method to be sure that all real time PCR products are amplified from transcribed messenger RNAs and not from genomic DNA contaminations.

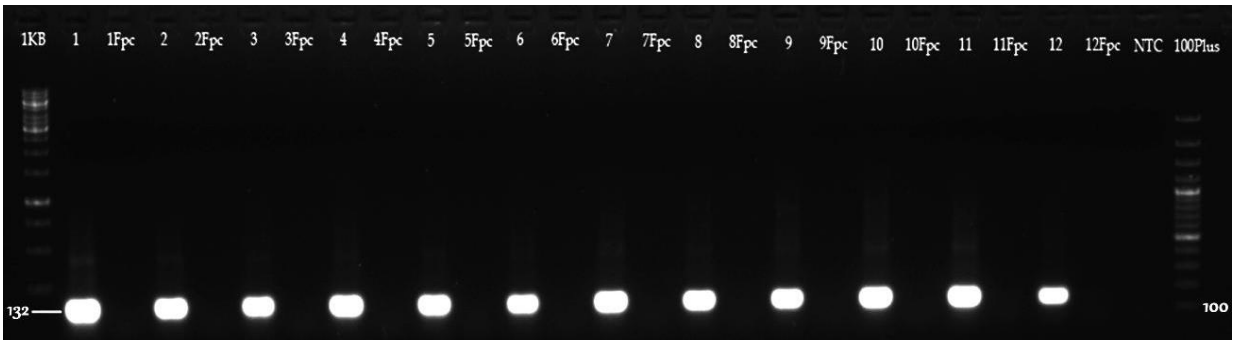

Figure 41. False positive control gel electrophoresis result for *AAD* transcript fragment (132 pp) to be amplified with qPCR primers. No amplification accrued from minus RT controls (Fpc).

The 1:16 diluted cDNA was used as the standard concentration for gene expression analysis and tested for different standard genes (housekeeping genes) expression stability. Among the tested standard genes, actin gene showed the highest stability during the biotransformation and precursor feedings. The stability of actin is statistically demonstrated and presented in figure 42. The two-way MANOVA revealed a significant multivariate main effect for elapsed time effect as well as for time*treatment interaction (unexplained variance = Wilk's λ (time) = 0.043, $p < 0.001$; Wilk's λ (interaction) = 0.122, $p < 0.001$).

However, treatment effect was not significant with Wilk's λ (treatment) = 0.791, p > 0.05). For different precursor feeding, significant univariate time effect as well as two-level interaction effect were obtained (FTyrosol(5;42)=36.401; p<0,001; FTyramin(5;42)=44.481; p<0,001; F4HPP(5;42)=32.442; p<0,001; Fint (5;42)>8.4; p<0,001).

Nevertheless, the stability of *actin* gene expression in all of the treatments showed no significant difference when compared with its corresponding control at each point of inspected time in biotransformation measurements (FTyrosol(1;42)=0.595; p=0,444; FTyramin(1;42)=2.077; p=0,156; and F4HPP(1;42)=0.322; p=0.5723).

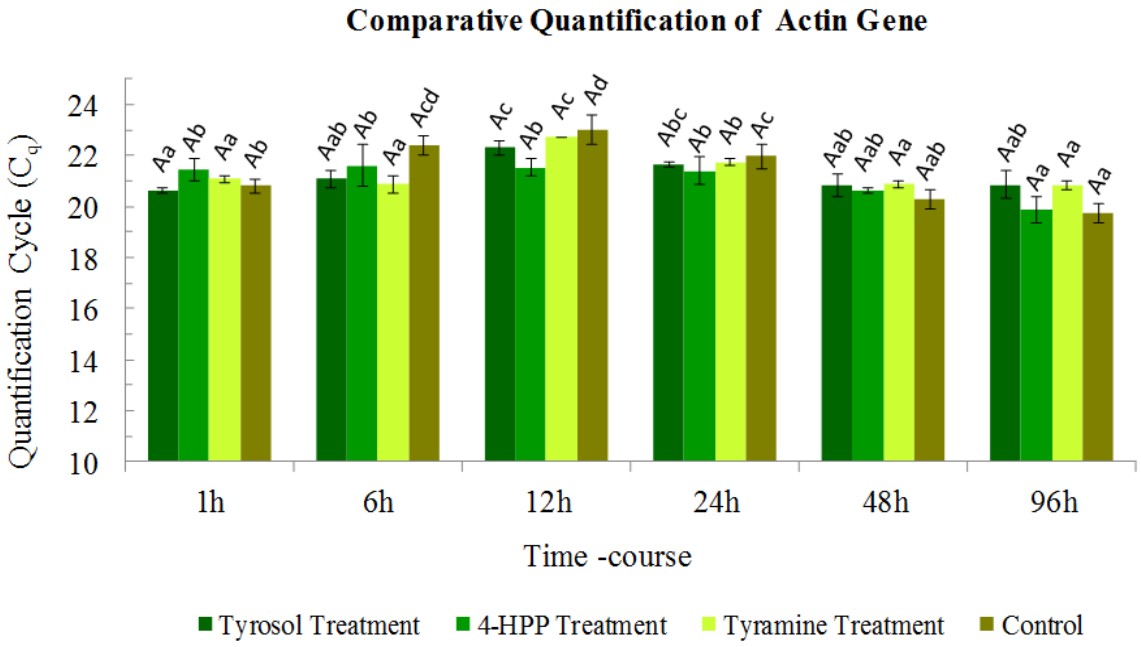

Figure 42. Comparative quantification of actin gene during the biotransformation experiment. Lower case letters: Different letters are for significantly different time effect assuming fixed treatment (Control, Tyrosol, 4HPP, Tyramin). Upper case letters: Different letters are for significantly different treatment effect compared to control at a fixed point of time (Tukey's post hoc test, p<0.05).

The relative gene expression analysis was studied for *UDPG* and *AAD* transcripts in response to biotransformation of possible substrates in salidroside formation. The qPCR reactions were checked for product specificity by running a melting curve analysis for each run. Figure 43 is showing an example from the mentioned curves generated by Rotor-Gene 6000 (Qiagen) thermocycler to assure the PCR products specificity.

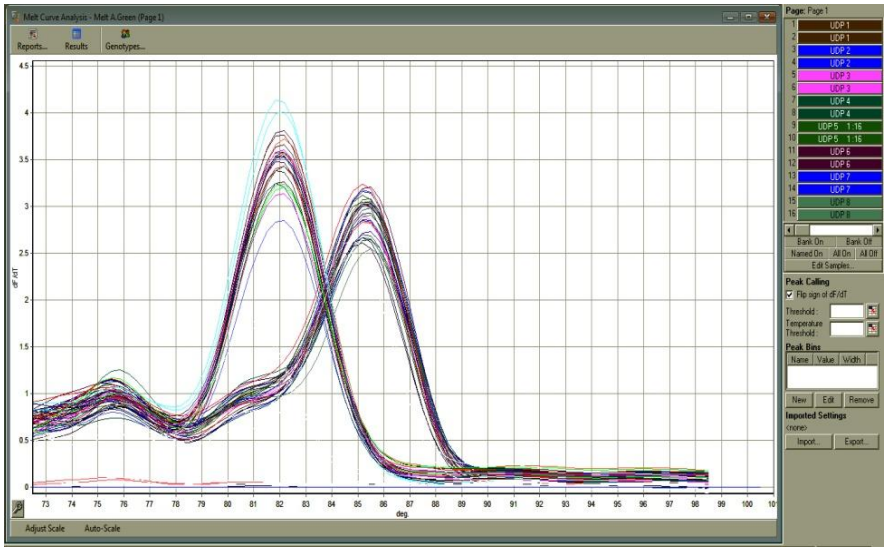

Figure 43. The melting curve analysis of *UDPG* and *Actin* qPCR amplicons. All of the PCR products from different reaction tubes showed the same melting temperature for the corresponding gene fragment.

The expression level of the studied genes (*UDPG*, *AAD*) is presented in figures 44-49. 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 44.). A logical tendency in gene expression level has been observed during this experiment. Shortly after refreshing the culture media, the gene expression started to rise and reached to the highest level after 12 h and decreased gradually to its minimum level in 96 h in both control and treated samples. The significantly higher expression for *UDPG* after 12 hours of tyrosol treatment is in a nice correlation with the results of HPLC analysis after tyrosol biotransformation. The salidroside formation was significantly increased when tyrosol was present in the media and was available for callus cells.

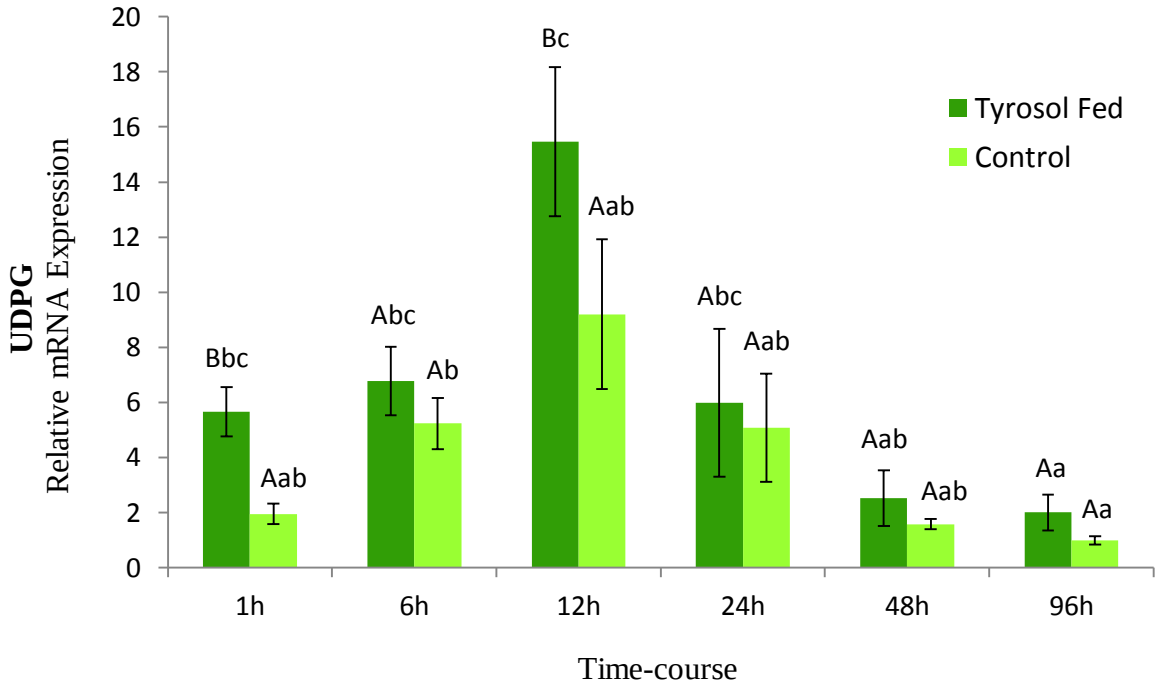

Figure 44. Upper case: different letters are for significantly different treatments assumed a fixed interval of elapsed time ($p<0.05$). Lower case: different letters are for significantly different intervals of elapsed time assumed a fixed treatment, according to Games-Howell's post hoc test ($p<0.05$). 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 45.). The *UDPG* expression pattern after tyramine feeding was very similar to the expression pattern after tyrosol feeding. When the time effect considered as evaluation factor, a significantly higher expression was revealed from 1 h to 6 h and also from 6 h to 12 h in the treated samples themselves. A sharp decline in gene expression was in common for both treated and control samples after 24 hours. This is showing a clear time frame for *UDPG* and an approximate life span of tyramine in the culture media. Based on the HPLC analysis the salidroside content was slightly increased when tyramine was given to the calluses but the effect was not as pronounced as tyrosol feeding. The *UDPG* expression significantly increased almost 6 fold in the samples fed by 2 mM 4HPP comparing to controls just after 1 h but declined suddenly in the second sampling after 6 h (Figure 46.). During the sampling times, the expression pattern of *UDPG* in 4HPP fed cultures was almost similar to controls, tyrosol and tyramine fed samples. The time effect in *UDPG* expression in 4HPP fed samples was also significant.

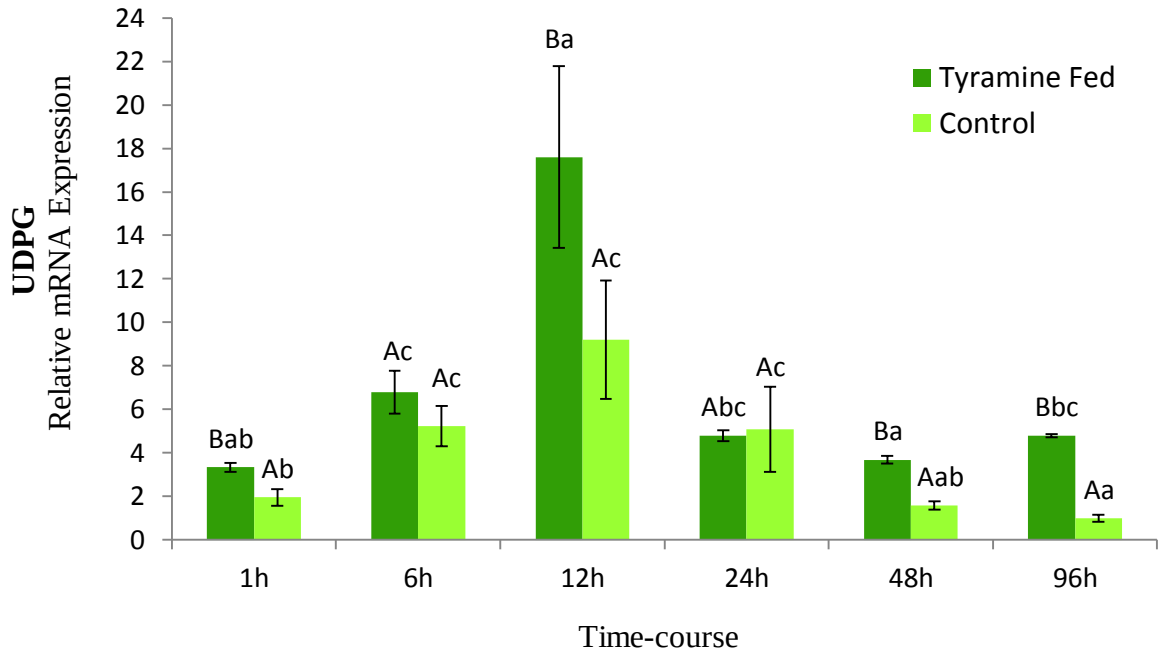

Figure 45. Upper case: different letters are for significantly different treatments assumed a fixed interval of elapsed time ($p<0.05$).Lower case: different letters are for significantly different intervals of elapsed time assumed a fixed treatment, according to Games-Howell's post hoc test ($p<0.05$). Error bars represent standard deviation of the mean among 3 biological replicates.

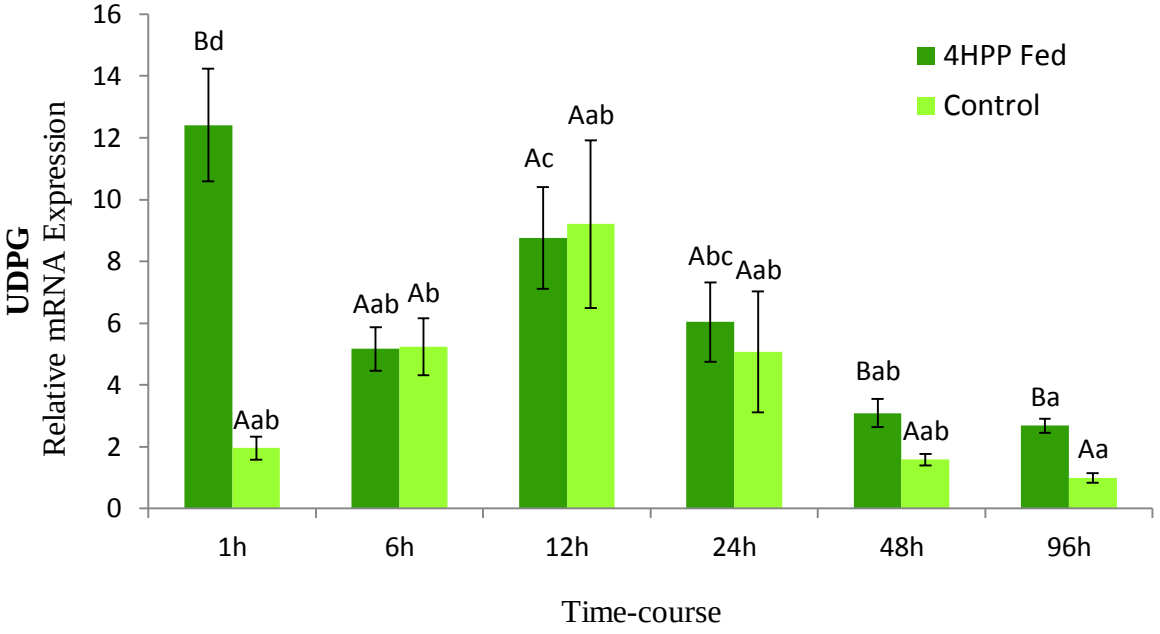

Figure 46. Upper case: different letters are for significantly different treatments assumed a fixed interval of elapsed time ($p<0.05$). Lower case: different letters are for significantly different intervals of elapsed time assumed a fixed treatment, according to Games-Howell's post hoc test ($p<0.05$). 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. The results of gene expression analysis are presented in Figures 47-49. 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 (Figure 47 and 48.). Tyramine also suppressed the expression of *AAD* in a similar manner to tyrosol and 4HPP but in a lower degree (Figure 49.). In case of time effect, the tyrosol treated samples showed a significant increase only after 12 h during the 6 sampling times but still significantly lower than its level in control at the same sampling time. The expression of *AAD* was significantly stable during the experiment with very slight changes. The *AAD* expression was significantly higher 1 hour after 4-HPP treatment comparing to the next samplings after 6 and 12 h and significantly higher again after 24 h. After 48 and 96 h the expression declined again and stayed at the same level as at 6 and 12h.

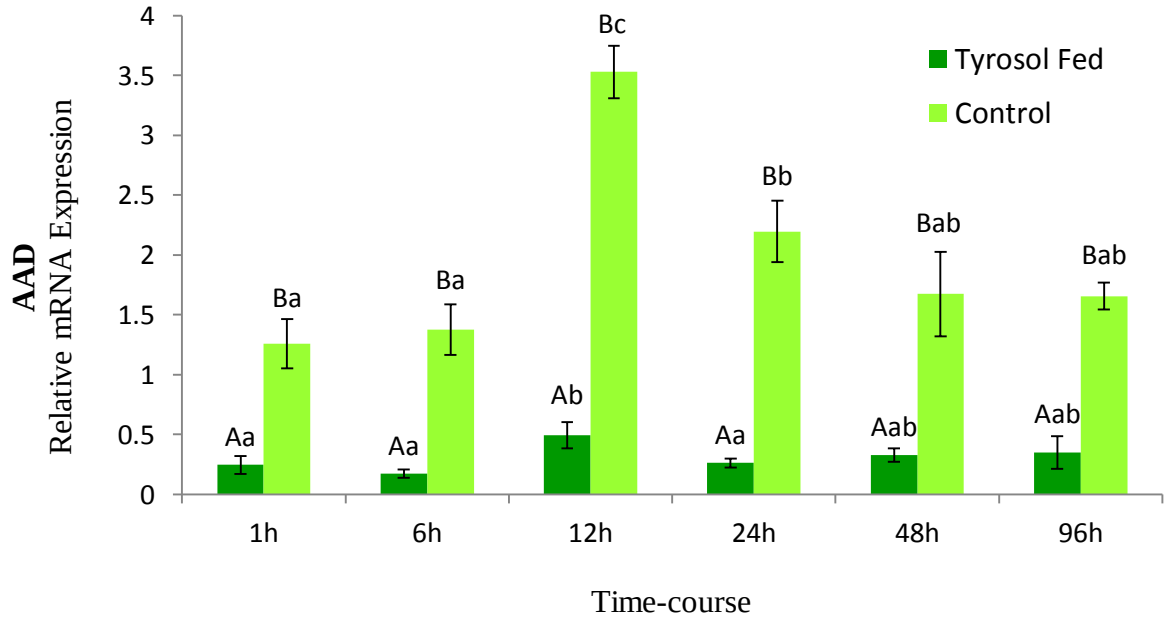

Figure 47. Upper case: different letters are for significantly different treatments assumed a fixed interval of elapsed time ($p<0.05$). Lower case: different letters are for significantly different intervals of elapsed time assumed a fixed treatment, according to Games-Howell's post hoc test ($p<0.05$). Error bars represent standard deviation of the mean among 3 biological replicates.

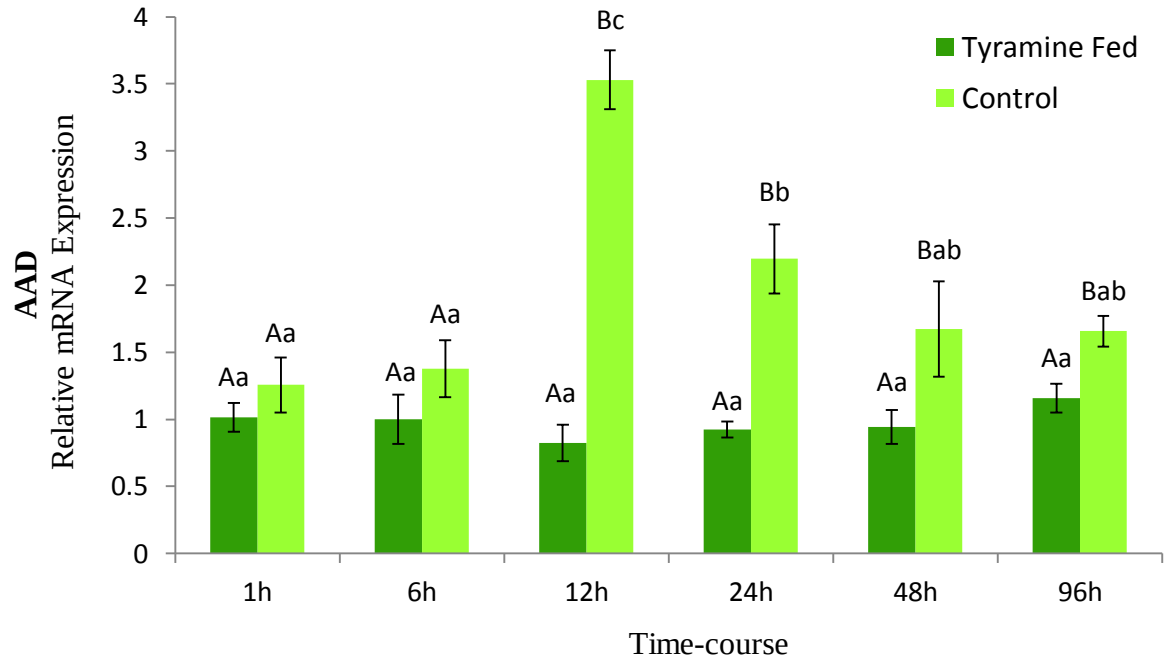

Figure 48. Upper case: different letters are for significantly different treatments assumed a fixed interval of elapsed time ($p<0.05$). Lower case: different letters are for significantly different intervals of elapsed time assumed a fixed treatment, according to Games-Howell's post hoc test ($p<0.05$). Error bars represent standard deviation of the mean among 3 biological replicates.

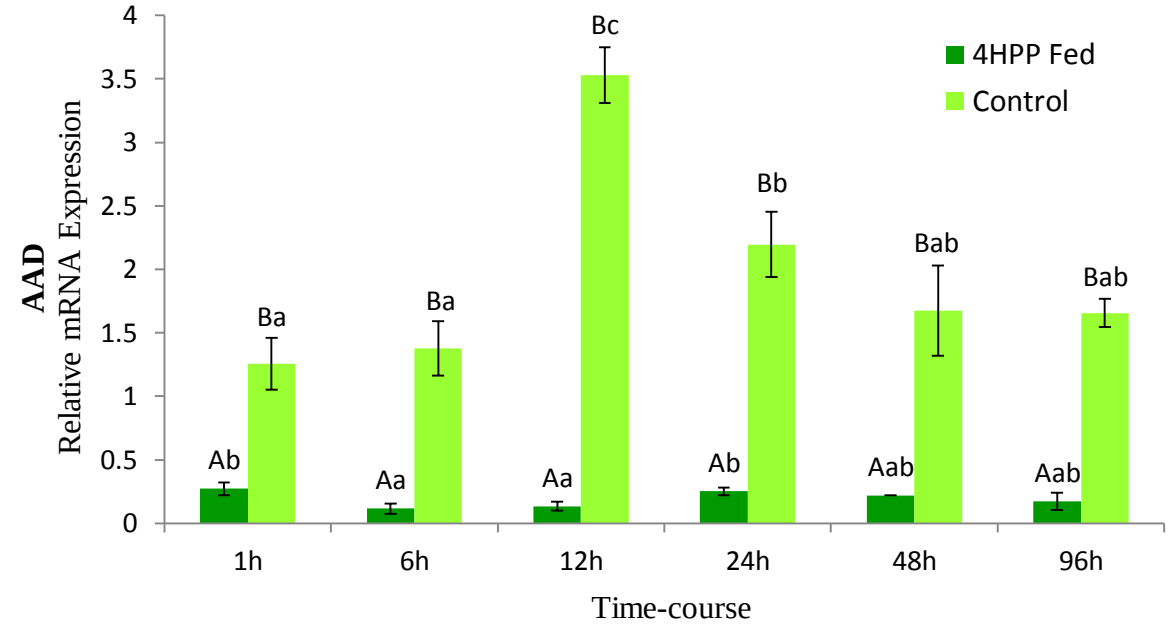

Figure 49. Upper case: different letters are for significantly different treatments assumed a fixed interval of elapsed time ($p<0.05$). Lower case: different letters are for significantly different intervals of elapsed time assumed a fixed treatment, according to Games-Howell's post hoc test ($p<0.05$). Error bars represent standard deviation of the mean among 3 biological replicates.

4.3.7. Gene expression analysis *in vivo*

Four genes from the proposed cinnamylalcohol glycosides biosynthesis pathway (Figure 3.) were studied for their expression pattern during the vegetation period. Three individual roseroot plants in their natural habitat and one individual in our gene bank were harvested at the beginning of shoot elongation, before flowering, full flowering and subjected for phytochemical analysis. Similar to the *UDPG* and *AAD* molecular experiment, part of the proposed genes were obtained, sequenced (Figure 50.) and confirmed for significant similarity with their identified counterparts in plant kingdom. 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. The melting curve analysis confirmed the PCR product specificity and no amplification were detected in non-template control reaction. Relative gene expression values were calculated with the REST software. These relative values are shown in Figure 51. The four observed plants showed great variability both in the rate of the relative expression values, and in the character of the curves. The similarity of these curves was examined with the method described in the "Material and methods" (3.4.11.2). The expression of 4CL was the most similar in the four roseroot individuals and more-over, the expression in the rhizome was almost equal, while in the leaves it showed some differences. The expression of CAD and PAL showed slightly greater variability in the rhizome, than in the leaves, while the expression of CCR showed the greatest variability both in the leaves and in the rhizome. The results of HPLC analysis from the leaf and rhizome samples are presented in Table 10. Interestingly a trace of the glycosides was present also in the leaves. The content of roseroot phytochemicals were very diverse ranging from 0.5-4.5 % of dry weigh for salidroside, 0.01-0.1% for rosin, 0.02-2.37% for rosavin and 0.1-0.55% for rosarian in the rhizome samples. Regression analysis showed that CCR at all sampling times had an effect on all of the cinnamyl alcohol glycosides, while 4CL, CAD and PAL affected only in a lower extent (Table 11). Interestingly the regression analysis showed that 4CL and CAD had an effect on the salidroside content, although salidroside is thought to be formed through a different pathway. The explained variances (R-square), the F values of the ANOVA tests of the models and their significance levels are presented in Table 11. Squared Euclidean distances of all pairs of curves for leaf and rhizome and for 4CL, CAD, CCR and PAL were calculated and represented by points as curve characteristic codes (Figure 52, left panel). To show the expression pattern of the studied genes, the graphs were then summarized to mean values for all plants (Figure 52, right panel). The HPLC diagram of detected compounds in leaf and rhizome samples along with standards mix is presented in Figure 53.

5' UTR CDS 3' UTR

5′TGAGGCTGCCGCTATAATGGAACATATCTTGGATGGGAGTGCTTATGTCAAGGCTGCCAAGAAGTTACACGAGACTG
ATCCGTTACAGAAGCCGAAGCAAGACAGGTATGCGTTGAGGACTTCTCCACAATGGTTGGGGCCTCAGATTGAAGTCA
TTAGATTTTCTACCAAGTCGATCGAGAGGGAGATCAACTCCGTCAACGACAACCCGTTGATCGACGTCTCGAGGAACA
AGGCCATTCACGGAGGCAATTTCCAAGGCACTCCGATCGGTGTGTCTATGGACAACACCAGATTAGCCATTGCGGCCA
TTGGGAA**GCTCATGTTTGCTCAATTCTCC**GAACTCGTTAATGACTTTTACAACAGTGGGCTTCCGTCGAATCTCTCAG
GCAGCAGGAACCC**CAGCTTGGATTACGGGTTCA**AAGGAGCTGAGATCGCGATGGCTTCATATTGCTCCGAGCTTCAG
TTTTTGGGCAACCCGGTGACGAACCATGTCCAGAGCGCGGAACAGCACAACCAGGACGTCAACTCATTGGGACTCATT
TCGTCAAGAAAGACTGCGGAAGCAGTTGATATCTTGAAACTCATGTCATCGACTTACTTGGTGGCACTTTGCCAAGCAA
TAGACCTCAGGCATATGGAAGAGAATCTGAGGAGCACTGTCAAAAACACGGTGAGCCAAGTCGCGAAAAGGACTCTG
ACCACAGGAGCTAACGGAGAGCTTCACGCGTCGAGGTTCTGCGAGAAAGACCTGCTCAAAGTCGTCGACAGAGAGTA
CTTGTTCGCCTACATCGATGACCCTTGCCTGGCCACTTACCCACTGATGCAGAAACTGAGGCAAGTACTCGTCGAGCAC
GCCCTCACCAACGGCGAAAACGAGAAGACCCCGAACTCTTCCATCTTCCAAAAGATTGGAGCTTTTGAAGTTGAGCTC
AAGACCCTGTTACCTAAAGAAGTCGAAAACATGAGGGTTGTCATCGAAAGCGGGAATGCTCCATTGCCGAACCAGATC
AAGGATTGCCGATCATACCCGCTGTACCGATTCGTAAGGGAGGAGCTCGGAACAGCATTGCTGACCGGAGAGAAGGT
GAGATCACCGGGAGAGGAATTTGACAAGGTGTTCACAGCCATGTGCGAAGGTAAAATCATTGATCCAATGCTCGAGTG
TCTCCAGGAGTGGAACGGCGCTCCACT…3′

Partial (1194pb) sequence of phenylalanine-ammonia lyase (PAL) transcript from *R. rosea* L.

5′…GGTCAGGGATACGGAATGACAGAAACAACTG**GGATCGTGTCAGTAGAGGAATC**CAGGTATGGAAAGCGGC
ATACAGGGTCTGCTGGAAGACTTGTATATGGAGTGGAAGCTCAGATTGTTAGCGTGGAGACAATGAA**GCCTAAGCCA**
CCGAATAAGATGGGTGAAATATGGGTCAGGGGCCCAAACATGATGAAAGGCTATTTGAATAACCCACAGGCCACCAG
AGAAACTATAGATAAAAATGGTTGGCTGCATACTGGGGACCTTGGGTACTTTGACGACGAAGGAAGGCTTTTTGTTGT
GGATCGGATTAAAGAGCTCATCAAGGTCAAAGGTTACCAGGTTGCCCCCGCAGAGCTAGAAGGCATTCTTGTTTCGCA
CCCTGAAATCCTCGATGCTGTAGTCATACCATTTCCAGATGCTGAAGCTGGTGAGGTGCCCATTGCCTATGTCGTTCGA
TCACCCAACAGCTCACTTACTGAAGAGTCTGTCAAGGAATACGTTGCAACACAGGTAGCACCATTCAAGCGGCTGCGT
AGAGTTGAATTCATCGGCAGTGTCCCCAAATCTGCTGCTGGAAAAATTTTCAGACGAGAGCTCATTGCAAAAGTCAGG
TCCAAAATT**TGA**CAGGATCATCATTTATGTGGTAATGAGGGAACTCGGTACACAAGCCTATTATATTTTAACCAAATAT
TGAATTATGATATTTGATAAGGAATTAGATTTCAATAAATTTCCCCATTTGTTATGAAAAAAAAAAAAAAAAAA3′

Partial (768pb) sequence of 4-coumarate: CoA ligase (4CL) transcript from *R. rosea* L.

5′CTTCTCCTGTTACTGATGATCCAGAACAAATGGTGGAGCCTGCTGTTAATGGAACCAAGAATGTGATCATAGCTGCTG
CTGAAGCAAAAGTTAAGCGCATGGTTTTCACTTCATCAATTGGGGCGGTTTACATGGACCCTAACAGATCTCCTGATGC
AGTGGTTGATGAGAGTTGTTGGAGTGATCTAGAGTTCTGCAAGAACACCAGGAACTGGTATTGCTATGGAAAGACAGT
GGCAGAGCAAGCAGCAAT**GGAGTTAGCCAAGGAGAAAGG**AGTGGACTTAGTAGTGATCAATCCGGTGTTGGTGTTA
GGCCCATTGTTACAGTCCACTGTAAATG**CTAGCATTGTCCACATCCAGAA**GTATTTAACTGGTTCGGCGAAAACATAT
GCTAATTCAGTCCAGGCTTATGTCCATGTCAAGGATGTTGCACTAGCTCACATCCTTGTGTATGAAACACCCTCTGCGT
CTGGCCGTTACCTTTGTGCAGAAAATGTTCTTCACCGA…3′

Partial (506 pb) sequence of cinnamoyl-CoA reductase (CCR) transcript from *R. rosea* L.

5′…**CTCAGACGCAGCTAGGATG**CAAGAAGCAGCGGATTCGCTTGATTACATTATTGACACTGTCCCGGTCAATCACCC
ACTC**GAGTCGTACATTTCGCTTTTGAA**GCTTGATGGGAAGATCATCTTGTTGGGTGTGATCAACACTCCTATGCAGTT
CATCACTCCCATGCTCATGCTTGGGAGGAAGATGATAACAGGGAGCTTCATAGGAAGCATTAAGGAGACAGAGGAGG
TCTTGGAGTTCTGCAAGGAGAAGGGGTTGAGCTCCACCATTGAA**ATG**ATCAAGCTGGATTACATCAACACCGCGCTGG
AGCGGCTAGAGAAAAACGATGTGCGTTACAGGTTTGTTGTGGACGTTGCAGAAAGCGAGTTTGAAGAGTAAAACCCCC
AAGCCAAAAGGACCAATTTCAATATGTGAAATGTTGATTGCTACTTTTCTTCTGTTCATTGTAGACAAAGGTAATACAA
ATGTATGGTGACAATATGACCCTTGTCTTTGACAGCTGCTTGAAAATGAAGGATTTCAAATTTAAAAAAAAAAAAAAA
AAAA

Partial (547 pb) sequence of cinnamyl alcohol dehydrogenase (CAD) transcript from *R. rosea* L.

Figure 50. Partial sequence of *R. rosea* studied genes for their relative expression analysis.

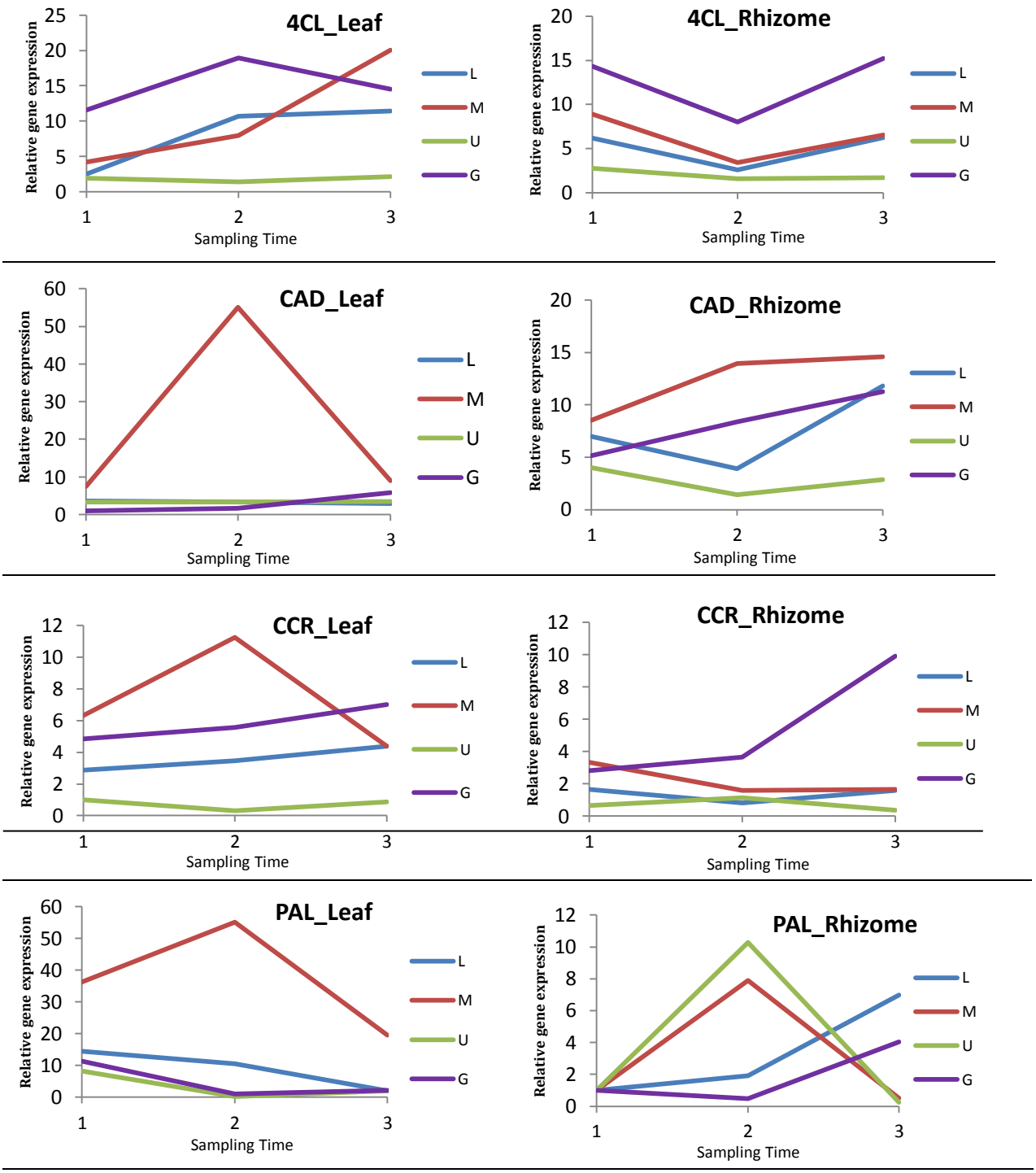

Figure 51. Relative expression values at three times (Beginning, middle and end of vegetation period) from leaves and rhizome samples of 4 roseroot individuals (L, M, U, G) of 4 genes (*PAL, 4CL, CCR, CAD*).

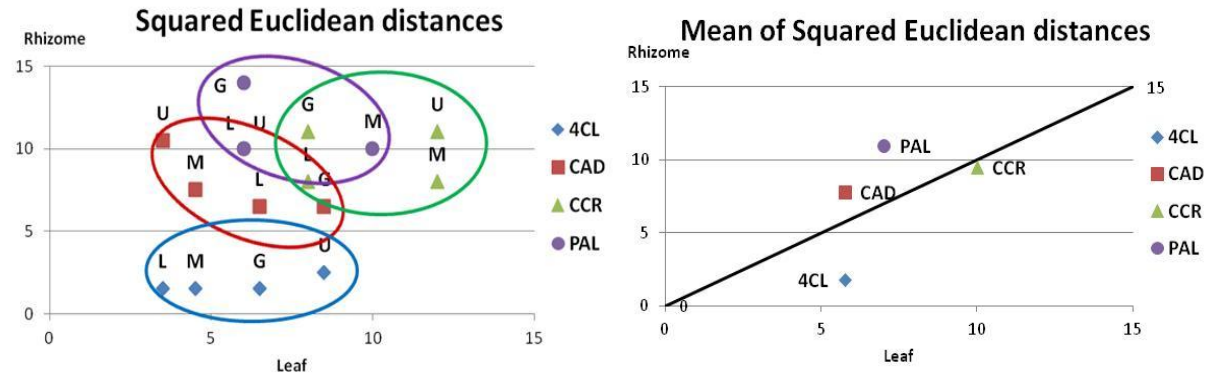

Figure 52. Squared Euclidean distances of plants i ($i = \mathrm{U, M, L, G}$) of curve characteristic codes represented as points $P_k(i) = \left(I_k^{leaf}(i); I_k^{rhizome}(i)\right)$, $k = 4\mathrm{CL, CAD, CCR}$ and PAL (left panel) and their means $P_k = \left(\overline{I_k^{leaf}}; \overline{I_k^{rhizome}}\right)$, $k = 4\mathrm{CL, CAD, CCR}$ and PAL calculated for all plants (right panel).

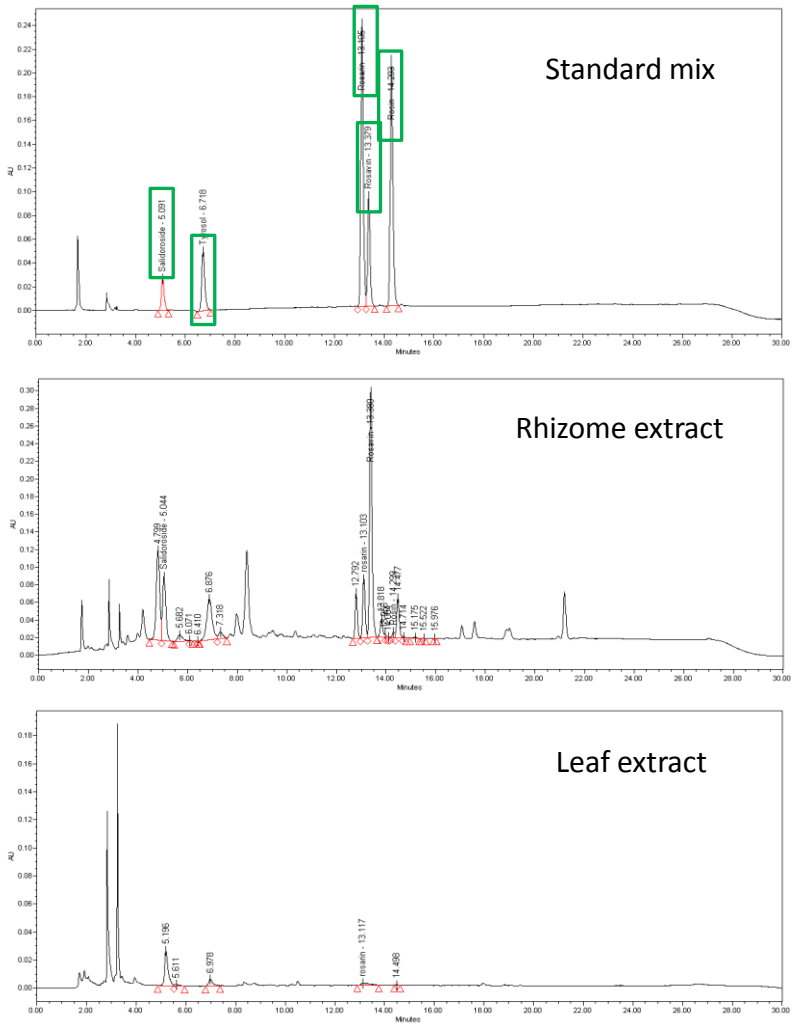

Figure 53. The HPLC diagram for roseroot phytochemicals detected in leaf and rhizome samples.

Table 10. Rresults of HPLC analysis from *R. rosea* leaves and rhizomes (g/100gdw)

Sample type	Sample plant	Rosarin	Rosavin	Rosin	Salidroside
Leaf	1 L	0.09	0.02	0.02	0.78
Leaf	1 M	0.02	0.02	0.02	0.20
Leaf	1 U	0.02	0.02	0.02	0.05
Leaf	1 G	0.07	0.02	0.02	0.05
Rhizome	1 L	0.29	1.71	0.05	1.78
Rhizome	1 M	0.55	2.34	0.06	1.12
Rhizome	1 U	0.00	0.00	0.00	0.05
Rhizome	1 G	0.29	0.05	0.02	4.20
Leaf	2 L	0.02	0.02	0.02	0.05
Leaf	2 M	0.13	0.02	0.05	0.19
Leaf	2 U	0.00	0.02	0.02	0.05
Leaf	2 G	0.00	0.00	0.00	0.00
Rhizome	2 L	0.12	0.48	0.02	0.34
Rhizome	2 M	0.16	0.05	0.09	0.52
Rhizome	2 U	0.27	0.66	0.03	0.21
Rhizome	2 G	0.33	0.15	0.03	4.30
Leaf	3 L	0.03	0.02	0.02	0.05
Leaf	3 M	0.07	0.02	0.00	0.17
Leaf	3 U	0.02	0.02	0.02	0.05
Leaf	3 G	0.02	0.02	0.02	0.10
Rhizome	3 L	0.16	0.93	0.02	1.13
Rhizome	3 M	0.06	0.06	0.02	0.78
Rhizome	3 U	0.23	0.43	0.04	0.54
Rhizome	3 G	0.13	0.02	0.09	0.87

Table 11. Results of regression analysis:

The dependent glycosides in rhizome of all phenological stages and the explaining gene expression variables with the explained variances (R-square), the F values of the ANOVA tests of the models and their significance levels.

Dependent	Sampl. time	Predictor	Sampl. time	R^2	F	P
Rosarin	1	CCR Leaf	1	0.957	67.045	0.004
Rosarin	2	CCR Rhiz.	2	0.875	20.974	0.020
Rosarin	3	4CL Leaf (C)*	3	0.986	140.62	0.007
Rosarin	3	4CL CCR (C)*	3 2	0.999	6445.6	0.009
Rosavin	1	PAL Leaf	1	0.839	15.637	0.029
Rosavin	2	CCR Rhiz. (C)*	1	0.992	262.35	0.004
Rosin	1	CAD Rhiz	1	0.887	23.579	0.017
Rosin	2	PAL Leaf	1	0.961	73.579	0.003
Rosin	3	CCR Rhiz	2	0.960	72.922	0.003
Rosin	3	CCR Rhiz. CAD Rhiz.	2 2	0.997	349.02	0.003
Salidroside	1	4CL Leaf	1	0.939	46.254	0.007
Salidroside	2	4CL Leaf	1	0.926	37.579	0.009
Salidroside	2	4CL CAD	1 1	0.999	2194.0	0,000
Salidroside	3	CAD Rhiz.	1	0.931	40.681	0.008
Salidroside	3	CAD Rhiz. CAD Leaf	1 2	0.997	372.762	0.003

* There is a constant in the linear model

DISCUSSION

5.1. Roseroot *in vitro* experiments

5.1.1. *In vitro* cultures

R. rosea is growing very slowly as the cultivation of this species aiming high levels of biologically active substances is quite long and takes between 5 to 7 years (Furmanowa *et al.*, 1999; Galambosi 2006). The salidroside content is usually low, between 0.13-1.6% of the DW (Furmanowa *et al.*, 1999; Linh *et al.*, 2000; Platikanov and Evstatieva, 2008), while that of rosavins is between 0.1-3.0% of the DW (Furmanowa *et al.*, 1999; Saunders *et al.*, 2013). In different specimens high deviation in phytochemical content and composition is observed due to heterozygosis, morphological and chemical variabilities (Weglarz *et al.*, 2008; Mirmazloum and György, 2012). Along with that, these substances are found in lower amounts in the field-cultivated *R. rosea* than the naturally growing plants (Ma *et al.*, 2008). To meet the demands for roseroot metabolites, there have been many attempts for chemical synthesis of salidroside and rosavins, but all were ineffective due to high production costs (Ma *et al.*, 2007).

These challenges could be overcome by developing biotechnological methods to improve the production of *R. rosea* secondary metabolites through *in vitro* culture systems (Lan *et al.*, 2013; Mirmazloum *et al.*, 2014). However, the *in vitro* produced metabolites are in lower amounts (György *et al.*, 2004; György and Hohtola, 2009), and in some cases have even failed to be produced by *in vitro* cultures (Martin *et al.*, 2010) or needed specific requirements for their biosynthesis, such as addition of precursors (Grech-Baran *et al.*, 2013).

In vitro cultures are dependent on endogenous levels of and exogenously applied plant growth regulators. Growth and morphogenesis of plant tissues under *in vitro* conditions are indeed influenced by the culture medium composition and culture condition. Different morphogenesis patterns have been reported from different *Rhodiola* species *in vitro* culture (Liu *et al.*, 2006; Debnath, 2009; Tasheva and Kosturkova, 2012a). We also detected different responses to plant growth regulators in different *Rhodiola* ecotypes in our experiments. Callogenesis rate of 55% have been obtained by Tasheva and Kosturkova (2010) from a Bulgarian originated *Rhodiola* explants in MS media containing three mg/l 2iP, 6 mg/l NAA and 150 mg/l glutamic acid in contrast with 3 of our supplementation which led to 80% of callus induction. It can be stated that the *in vitro* culture and micropropagation of roseroot plant is not complicated and can be readily optimized for the purpose of different experiments from different explants organs.

Different callus types are formed depending on culture environment and explant's origin from roseroot which can be taken as an advantage for final production aim. The explant type can influence not only the callus morphology, but also its ability to produce secondary metabolites. Axillary bud originated callus line produced approximately 2-fold more rosavin than the hypocotyl originated callus line, the main feature of which was the high level of rosin: 1.2-fold higher than the axillary bud originated callus (Krajewska-Patan *et al.*, 2007a). Different callus type formation from the same explant has been also reported by Furmanowa *et al.*, (1995) in roseroot *in vitro* cultures.

5.1.2. Transgenic roseroot callus culture establishment

Genetic engineering is a promising and effective approach to enhance the secondary metabolite production of *R. rosea in vitro* cultures for regulating the biosynthetic pathways through the enhancement of the activities of enzymes involved by introducing the corresponding genes' DNA into the plant genome. A few reports about genetic transformation of *R. rosea* are available in the scientific literature and all of them are dealing with hairy roots; induced by *Agrobacterium* strains (Tasheva and Kosturkova, 2012b) to obtain higher quantity of roseroot pharmaceuticals. In case of *R. sachalinensis* the *Agrobacterium tumefaciens* mediated transformation have been reported from China by overexpressing the functional genes in salidroside biosynthesis pathway (Ma *et al.*, 2007; Ma *et al.*, 2008) but to the best of our knowledge no report has been ever published about transgenic *R. rosea* plant.

From the various methods developed to introduce DNA into the plant cells so far, most include a transformation step that is mediated by *Agrobacterium tumefaciens* (Gelvin 2000; Zupan *et al.*, 2000) with an antibiotic resistance marker gene which is typically used as an indicator of gene insertion. As a reporter for transformation, GUS is frequently utilized because it offers several advantages, such as a high stability in translational fusion with other proteins and a fine resolution in histochemical staining that allows detection of signals even in single cells (Jefferson *et al.*, 1987; Lindsey *et al.*, 1993). This method was successfully applied in our genetic transformation experiment with roseroot callus. One of the weak points of callus transformation and transgenic callus is how to keep stable the transformed line for a long term. With this regard a transgenic plant would be a more stable source to deal with for *in vitro* culture establishment. The plant regeneration from *R. rosea* callus has been reported (Tasheva and Kosturkova, 2013) but the frequency of regenerated plants from callus cultures in their study was very low, not exceeding 5-6 %. Even though we also think that the plants should be used as starting material for *in vitro* cultures to assure the genetic identity of the developing callus lines, but the transformation in callus level for

establishing transgenic roseroot callus lines as we presented in this work, is fast, stable and didn't affect the normal growth rate and morphology of the callus in liquid culture. This gives the possibility for bioengineering the glycosides' pathway of *Rhodiola rosea* by inserting the functional genes and hence facilitating an effective production system in large scale bioreactors for the production of the most valuable pharmaceutically important secondary metabolites of this adaptogenic plant.

5.1.3. Precursor feeding and biotransformation

In vitro cultures from *R. rosea* are extensively used for biotransformation procedures in order to enhance the secondary metabolite production or for micropropagation of plants for the restoration of exhausted habitats (Tasheva and Kosturkova, 2010). There are many factors affecting the *in vitro* culture induction from *R. rosea*, including plant ecotype, type of explant, nutrient medium composition (plant growth regulators, carbon source and inorganic salts), light, temperature, and presence of precursors and elicitors (Tasheva and Kosturkova, 2010; 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. In our experiment the optimum culture condition was achieved for the specific callus line that we worked with and different precursors of salidroside and CAGs biosynthesis pathway were added to the optimized culture medium.

Since secondary metabolites are considered as products linked with differentiation, in the case of *R. rosea* most authors prefer to work with compact callus aggregates (CCA) instead of homogenous cell suspension. Compact callus aggregates are spherical, smooth surfaced clumps displaying some level of cellular differentiation and no dispersed cells are observed when transferred in liquid media (György *et al.*, 2004). Along with the type of precursor, the optimum amount and time of addition are also importante. Cinnamyl alcohol is one of the most effective precursors that increase or induce the biosynthesis of cinnamyl alcohol glycosides (CAGs). When added at concentrations of 2 or 2.5 mM at the beginning of the cultivation of CCAs, the rosin content was induced and reached 0.72% of the DW (György *et al.*, 2004) and the content of rosavin reached 1.01% (Furmanowa *et al.*, 1999), while in the control samples, none of these metabolites were produced (Furmanowa *et al.*, 1999; György *et al.*, 2004). The addition of 2 mM cinnamyl alcohol to our culture media also enhanced the formation of studied metabolites. After 48 h of biotransformation approximately 200 fold increases was recorded in rosin biosynthesis comparing to the control. Rosin was the only compound that naturally released to the media during the experiment after cinnamyl alcohol

treatment. After feeding *R. rosea* callus culture with cinnamyl alcohol, several new glycosides as products of biotransformation were identified (György *et al.*, 2004; Tolonen *et al.*, 2004). With addition of 2.5 mM cinnamyl alcohol, a remarkable increase in the content of rosin in the hypocotyl originated callus was observed, while the axillary bud originated callus line produced 1.2-fold less rosin and 2-fold more rosavin compared to the first line. The content of salidroside in hypocotyl originated callus increased to concentration similar to the intact plant, while the amount of tyrosol and chlorogenic acid were 20-fold higher (Krajewska-Patan *et al.*, 2007a). The content of salidroside can be enhanced by exogenous addition of tyrosol as a precursor. Its addition (5 mM) in solid and liquid CAA culture increased the level of salidroside reaching up to 4.3% of the DW after 7 days (Krajewska-Patan *et al.*, 2007b). The 2 mM Tyrosol, tyramine and 4-HPP had significant enhancing effect in our experiment as well. To the best of our knowledge no experiment with such wide range of angles has been carried out for roseroot biotransformation of related phytochemicals. We studied the effect of each precursor on the content of 13 proposed phytochemicals in salidroside and CAGs biosynthesis pathway. The results gave a broader picture of how the content of each given compound was changing during the sampling time and at the same time which other compounds were synthesized as a result of biotransformation. It is now clear that most of the precursors (2 mM) can be taken up from the media in as short time as 24 h. This timing information is an interesting indicator of the biotransformation capacity which can be optimized to its highest possible level. According to our results except phenylalanine, none of the given (2 mM) precursors was remained in the media after 96 h. This in combination with our controls without any plant cells is a certain evidence that the disappearance of the given compounds are just due the plant cell uptake and not a spontaneous conversion to other chemicals. The overall findings from this experiment showed a huge potential in this technique which can be used in many different fields from molecular studies to bioreactor production of such desired phytochemicals.

5.2. Roseroot phytochemical analysis during the vegetation period

Based on the HPLC analysis of roseroot phytochemicals, no clear trends could be observed among the individuals in each sampling intervals. The seven roseroot individuals showed a very high deviation in their chemical content in each samples. It's well-known that the underground parts of roseroot (roots & rhizomes) contain the active constituents. The presence of roseroot phytochemicals in the leaf samples detected by HPLC shows that the compounds can possibly be synthesized in the areal parts of the plant and then transported and stored in the underground organs or somehow they are moved back up to the leaves for different purposes.

The phytochemical variation among different populations of roseroot have already been reported (Altantsetseg *et al.*, 2007; Filion *et al.*, 2008) and also the significant intrapopulation variation is not new as it was measured for salidroside and cinnamyl alcohol content (Węglarz *et al.*, 2008). Despite the roseroot quantitative phytochemical variation in different vegetation sites and from the plants in different ages under different cultivation techniques which is already reported (Węglarz *et al.*, 2008; Koáodziej and Sugier, 2013; Adamczak *et al.*, 2014), our results showed that even individuals form the same origin in the same growing environment and the same soil condition are still behaving very differently with regard to the metabolites production (Mirmazloum *et al.*, 2015).

According to our findings, in the rhizomes most of the compounds had their maximum content in the second sampling time which was the beginning of shoot elongation and rapid mass production. This is in contrast with Platikanov and Evstatieva (2008) where they recorded the minimum quantity of the studied compounds in fructification stage. All these high variations and fluctuations indicates that the harvesting time should be carefully chosen to obtain the plant materials which contain the minimum required content of roseroot pharmaceutical compounds (0.8-1% of salidroside and 3% of total rosavins) according to Russian Pharmacopoeia.

So far the dynamics of roseroot glycosides accumulation has not been fully explored and only the mentioned studies have been published in this regard. Considering the results of those reports no clear tendency can be obtained in general to recommend the best harvesting time to obtain the maximum content of the active substances. On the other hand different populations of roseroots may have different pattern for their metabolites content and composition which should be examined and prescribed for each local producer or wild plants collectors. Even though our results of *in vivo* phytochemical analysis could not provide a clear accumulation pattern for roseroot phytochemicals (due to the very high deviation) but it supports our general idea and recommended strategy which is the alternative *in vitro* techniques to ultimately produce important phytopharmaceuticals.

5. 3. Molecular biology experiments

Considering the enormous number of scientific studies concerning the versatile aspects of *R. rosea* and its natural products, it is quite indisputable that the knowledge about biosynthetic pathway of roseroot bioactive metabolites is still in its infancy. There are only a few molecular study results available which are mostly cover one enzyme (TyrDC) connected to salidroside biosynthesis. An overview of the results emerged from the different areas of the enzymatic activities in higher plants as the first step towards exploring the biosynthesis of salidroside and cinnamyl alcohol glycosides.

Our survey in this area clearly demonstrates the necessity of studying the proposed pathways for both salidroside and cinnamyl alcohol glycosides at the molecular level (Mirmazloum and György, 2012). Efforts to clarify the molecular genetic background of biosynthetic pathways intensively tend to target to identify the structural genes which encode enzymes that catalyze the conversion of intermediates in a specific pathway. This is somehow related to the relatively high expression level of the structural genes, which results in abundant representation in expressed sequence tag (EST) libraries, or high probability of identification with cDNA subtraction methods or other methods that rely on differential expression (Vermerris and Nicholson, 2006). To achieve a better understanding of biosynthetic pathway of the natural products, it is also important to identify the regulatory mechanisms that control both the timing (developmental stage, environmental conditions) and the location (cells, tissues or organs) in which a specific compound or a group of metabolites are biosynthesized. The identification of such regulatory genes is generally much more difficult, due to the expression level of regulatory genes which tends to be lower than the expression of the structural genes. The remarkable significance of saldiroside and cinnamyl alcohol glycosides (rosin, rosavin and rosarin), from pharmacological point of view makes them excellent candidates to apply all the state of art molecular techniques to recognize their biosynthesis pattern to provide the possibility of producing these valuable glycosides in large scale bioreactors, and so get around the problems of collecting or growing the good quality plant raw material.

5.3.1. *R. rosea UDP-glycosyltransferase (UDPG) gene*

We isolated a full length *UDP-glycosyltransferase* (*UDPG*) gene for the first time from *R. rosea*. *UDPG* is the last enzyme believed to be responsible for the formation of salidroside by glycosylation of its aglycon; tyrosol. One of the highlighted reasons for the low salidroside biosynthesis in roseroot *in vitro* cultures thought to be the low efficiency of glycosylation and the non-synchronization of *UDP-glycosyltransferase* activity with tyrosol accumulation (Xu *et al.*, 1998a; Grech-Baran *et al.*, 2013). A large number of *UDPG*s from plant species have been identified and their functions were studied. The closest plant species to *R. rosea*, from which three *UDPG*s (UGT72B14, UGT74R1 and UGT73B6) have been identified, is *Rhodiola sachalinensis* (Ma *et al.*, 2007; Yu *et al.*, 2011). The BLAST result of GenBank search showed only 63% similarity between our *UDPG* (UGT73B16) and UGT73B6 but no significant similarity with the two other. The expression of *R. sachalinensis UDPG*s were tissue specific and UGT73B6 isozyme was highly expressed in the roots. Among the identified *R. sachalinensis UDPG*s, the UGT73B6 showed a higher level of activity for salidroside production.

Despite the high similarity in nucleotide sequences, in the phylogenetic tree, the UGT73B16 and UGT73B6 were classified in different clusters with a remarkable distance. The *R. rosea UDPG* was expressed also in leaves, rhizomes and calli. In our tyrosol biotransformation experiment the significant increase in UGT73B16 expression level was in a logical correlation with salidroside synthesis with a meaningful tendency. During the experiment as the tyrosol content was decreasing in the media the expression of *UDPG* was increasing and consequently the salidroside formation was also significantly higher compared to the controls. The heterologous expression of UGT73B16 in *E. coli*, also showed the validity of transcript sequence for translation and protein (enzyme) synthesis. The UGT73B16 enzyme role in tyrosol glycosylation and salidroside formation in roseroot callus culture is strongly supported, even if this is not the only gene involved in facilitating the salidroside biosynthesis. The identified gene can be used in metabolic engineering applications in roseroot plants and even in microorganisms for *in vitro* production of salidroside. The UGT73B6 from *R. sachalinensis* was used to engineer an *E. coli* strain for the same purpose by which they could produce 56.9 mg/l of salidroside in bacterial culture (Bai *et al*., 2014).

5.3.2. *R. rosea Aryl Alcohol Dehydrogenase* (*AAD*) gene

AAD; an enzyme believed to catalyze the conversion of 4-hydroxyphenylacetaldehyde to tyrosol. The gene (nucleotide/amino acid sequence) was reported from many plant species in the Gene Banks and data bases. But all of the reports are tagged with “putative”, “like” or “predicted” names. Almost all of the reports for *AAD* from plant kingdom are generated by automated computational analysis of genome projects and using the gene prediction methods. In a report on an *AAD* from white-rot fungus (*Phanerochaete chrysosporium*), the authors reported the existence of 8 *AAD* homologues that consist of six to nine exons which encode proteins from 240 to 398 amino acids (Yang *et al*., 2012). The *R. rosea AAD* that we report also is made up of 9 exons which encode 384 deduced amino-acid from its transcript. This is the first time that we reported a full length cDNA and genomic sequence of *AAD* from a plant species. Despite the very high similarity between the amino acid sequence of our *AAD* with the other *AAD*s in the data bases; we cannot predict the functionality of the isolated enzyme based on the homology alignment. *AAD* is thought to be involved in the one to the last enzymatic reaction in salidroside biosynthesis pathway. Therefore it has a high potential to study the heterologous expression of the isolated gene and conduct an enzyme assay to evaluate its real activity and analyze the substrate specificity.

5.3.3. Gene expression analysis *in vitro*

The expression of 2 genes (*UDPG* and *AAD*) was studied in callus culture of roseroot in response to treatment with 2mM tyramine, 4-HPP and tyrosol during their biotransformation. This is the first attempt for such analysis for *R. rosea*. The expression of a *UDPG* (UGT73B6) in *R. sachalinensis* was studied by Ma et al. (2007) where they could detect the transcript in roots, calli and stems, but not in the leaves. The authors stated that this was the first reported glucosyltransferase gene involved in salidroside biosynthesis (Ma et al., 2007). In another study by the same group two more *UDPGs* (UGT72B14 and UGT74R1) were isolated from *R. sachalinensis* (Yu et al., 2011) where the UGT72B14 transcripts were more abundant in roots, and UGT74R1 was more expressed in the calli, but not in the roots. The expression of our *UDPG* (UGT73B16) was detected in leaves, rhizomes, roots and callus of *R. rosea*. Based on the real time PCR analysis the expression of UGT73B16 was significantly increased as early as 1 hour after the precursors was given to the cultures. After 12 hours of tyrosol treatment the expression of *UDPG* was in its highest level and consequently the salidroside biosynthesis rate started to grow until 48 hours. This is the first timing report for parallel *UDPG* gene expression and salidroside formation in roseroot. It is also giving indirect information about the life span of *UDPG* transcripts and the role of tyrosol in up-regulation of *UDPG* expression. It is possible that even more *UDPG* genes are present in *R. rosea* genome but we are confident that the studied version is indeed a key player in this pathway. The tyramine and 4-HPP also significantly elevated the *UDPG* expression but in lower extent as the salidroside synthesis was also lower as a result of these treatments. It also makes sense if considering the position of the latter compounds in the proposed pathway.

The *AAD* gene from plants has not been studied and therefore its expression pattern is unknown. The *AAD* is thought to be active in reducing the 4-hydroxyphenylacetaldehyde to tyrosol. Our results showed a significant down-regulation of *AAD* expression when tyrosol and 4-HPP was given to the cultures. Due to the lack of molecular studies for *AAD* activity in higher organisms, it's hard to speculate on its role in salidroside pathway.

5.3.4. Gene expression analysis *in vivo*

This is the first attempt to analyse gene expression of roseroot in connection to the accumulation of cinnamyl alcohol glycosides from wild growing roseroot plants. Fragments of four genes were isolated, and sequenced: phenylalanine ammonia-lyase, 4-coumarate:CoA ligase, cinnamoyl-CoA oxidoreductase and cinnamyl alcohol dehydrogenase, which all proposed to have a role in the biosynthesis of rosavins.

The expression of these genes was examined along with chemical analysis of the same samples. Earlier only one paper was published about the accumulation dynamics of salidroside examined within one vegetation period. Bozhilova (2011) found that the salidroside content had two maximums, one in the beginning and one at the end of the vegetation period, while it decreased directly after the blossoming. Concerning the rosavins such study has not been conducted yet. Interpretation of our results is not easy, because the biological repetitions showed big deviations. It's also possible as it has been proved in many medicinal plant species (Xie *et al.*, 2008, Grausgruber-Gröger *et al.*, 2012) that the formation of these enzymes' substrates are not completely regulated by their gene expression level and there may be a post-transcriptional or post-translational regulation for these metabolites to be finalized. Nevertheless possible overlap in case of the salidroside content can be discovered with the results of Bozhilova (2011) as the salidroside content was similar in 50% of our samples. 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.

A more comprehensive study like transcriptome analysis especially from *in vitro* cultured roseroots may shed more insights when many genes are thought to be involved in such a complicated biosynthesis pathway.

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* by inserting the functional genes and hence facilitating an effective production system in large scale bioreactors.

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

   A comprehensive study was conducted to analyze the content of 13 proposed compounds of salidroside and CAGs biosynthesis pathway in response to biotransformation of the proposed precursors (tyrosol, tyramine, 4-hydroxyphenylpyruvate, phenylalanine, trans-cinnamic acid, and cinnamyl alcohol). Cinnamic acid, cinnamaldehyde and cinnamyl alcohol (2 mM) significantly increased the rosin content by more than 75, 130 and 200 fold, respectively, compared to the controls. 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 confirmed.

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. Our gene expression analysis showed that the *AAD* is constantly down-regulated if tyramine, 4-HPP and tyrosol were present in the culture.

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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