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Zsuzsanna György

GLYCOSIDE PRODUCTION BY IN VITRO *RHODIOLA ROSEA* CULTURES

*FACULTY OF TECHNOLOGY,
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ZSUZSANNA GYÖRGY

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Academic Dissertation to be presented with the assent of the Faculty of Technology, University of Oulu, for public discussion in Kuusamonsali (Auditorium YB210), Linnanmaa, on May 30th, 2006, at 12 noon

OULUN YLIOPISTO, OULU 2006

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Acta Univ. Oul. C 244, 2006

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ISBN 951-42-8079-2 (Paperback)
ISBN 951-42-8080-6 (PDF) http://herkules.oulu.fi/isbn9514280806/
ISSN 0355-3213 (Printed)
ISSN 1796-2226 (Online) http://herkules.oulu.fi/issn03553213/

Cover design
Raimo Ahonen

OULU UNIVERSITY PRESS
OULU 2006

György, Zsuzsanna, Glycoside production by in vitro *Rhodiola rosea* cultures
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Acta Univ. Oul. C 244, 2006
Oulu, Finland

Abstract

Rhodiola rosea is a medicinal plant, mainly used in Asia and Scandinavia. It is characterized as an adaptogen and is reported to have many pharmacological properties, which are ascribed to the glycosides of cinnamyl alcohol and tyrosol. As natural habitats are already overharvested and the cultivation of this plant needs 4–6 years, the production of the pharmacologically important compounds in *in vitro* cultures could be an alternative. In the work presented here, the production of these glycosides in compact callus aggregate cultures of roseroot was addressed.

Biotransformation of exogenously added cinnamylalcohol and tyrosol was studied. Glucosylation of the precursors yielded high amounts of rosin and salidroside and low amounts of rosavin. During the course of this work, four new glycosides of cinnamyl alcohol were found and identified. The optimal concentration of the precursors and the time needed for the biotransformation was also determined. For enhancing the biotransformation rate, glucose was added to the culture medium alongside with sucrose, which doubled the production of cinnamyl alcohol glycosides but did not affect the production of salidroside. A pilot experiment using air-lift bioreactor was performed.

A cDNA fragment encoding tyrosine decarboxylase was isolated and described. The expression of this gene was analysed in the leaves and roots of two chemotypes. The results demonstrate the important role of tyrosine decarboxylase in the production of salidroside.

The results revealed production of the pharmacologically important glycosides of *Rhodiola rosea*; however the successful pilot bioreactor experiment remains to be scaled-up. New information was obtained on the biosynthesis of salidroside, which substantiate the metabolic engineering of roseroot.

Keywords: biotransformation, compact callus aggregate, gene expression, glycosylation, Rhodiola rosea, rosavin, rosin, salidroside, tyrosine decarboxylase

Acknowledgements

This work was carried out at the Department of Process and Environmental Engineering and at the Department of Biology, University of Oulu. It was financially supported by CIMO Foundation, Biocenter Oulu, Finnish Academy (SUNARE project, grant no. 52741) and the Graduate School in Chemical Engineering.

I am most grateful to my supervisors, who have guided and supported me in the scientific world. Both are warmly acknowledged; prof. Peter Neubauer for giving me the possibility to work in his group regardless of the fact that at first it seemed I had nothing to do there and provided me a friendly and international community to work in and prof. Anja Hohtola for permitting me to work in her lab and adopting me into her group, among her "sisters".

Also, prof. Ulrike Lindequist and prof. Eva Čellárová are appreciated for critically reviewing this thesis and their valuable comments on this work. I owe many thanks to Regina Casteleijn-Osorno for carefully revising the language.

I sincerely thank Liisa Myllykoski for taking care of all the official documentation and guiding me through the Finnish administration. Antti Vasala is acknowledged for helping me with all kinds of technical problem. I warmly thank all my office-mates during the years: Tiffy Breitenstein, Beatrix Fahnert, Johanna Veijola, Stephan Riemschneider and Monika Bollok for sharing much tea with me and spending many precious moments with both Imre and myself. Beatrix is specially thanked for revising the language of my manuscripts and for always being interested in my research.

As mentioned already in the first paragraph I was a real dioecious, like roseroot itself. My office was in Bioprocess Laboratory, but I completed most of the lab work in Dept. of Biology. I express my warm thanks to my lab-mates: Laura Jaakola for guiding and advising me in the molecular work, Katja Karppinen for changing ideas in the practical work and accompanying me to the conferences we took part in, Terttu Kämäräinen, Anna Maria Mattila, Soile Jokipii and Jaana Vuosku for assistance and the cheerful atmosphere. I give my thanks to Minna Pakonen for introducing me to the work with roseroot. Also Taina Uusitalo and Hanna Liisa Suvilampi are thanked orientating me in the lab.

I also express my thanks to Ari Tolonen and also Aleksanteri Petsalo for the friendly collaboration with the chemical analysis. I specially thank Ari for being able to turn to him at any time with any kind of chemical questions.

Bertalan Galambosi is warmly acknowledged for his assistance and his inquiries concerning my work.

I am grateful to doc. György Bisztray and prof. István Velich for hosting me in the Dept. of Genetics and Plant Breeding, Corvinus University, Budapest. Also Tamás Deák, Ágnes Millei and Anna Papp are acknowledged for their help in that laboratory.

The years in Oulu were dedicated to our research, but we found true friends as well. I wish to thank Jenő and Betti for introducing us into the miracle and tweaks of Finnish life. I will never forget the excursions and nice evenings to Pannu with them and also with Pisti, Balázs and Réka. I also have nice memories about Eszter and the Petőfi kör. Our little bridge club with Beatrix, Monika and Gábor is also memorable. It was always nice to meet compatriots and have nice time together; I thank Marcsi, Nóra, Dóra, Miklós and Magdy, Zoli, Mátyás, Andrea, Krisztián and Renáta for the nice times. I became attached to Finnish people through our Finnish friends in the Salonpää family: Pekka, Eija, Justus, Julia and Petrus, whom I am grateful to them for letting us into their life.

At last I warmly acknowledge my family; my mother and father who always incited me to learn and gave me the possibility and my sister who forgave me for living abroad leaving her alone, and also my family-in-law for their love and support. Probably I wouldn't have started post-graduate studies without my dear husband. I wouldn't have even come to Oulu if not for him and there are no words to thank him his support, encouragement and sincere interest. Also for sitting into the laminar box with me or trying to find the way in the labyrinth of nucleotide sequences. Lastly I can only hope that my little daughter won't remember the times I stole from her to write this thesis, but one day she will be proud of me.

Budapest, March 2006 Zsuzsanna György

Abbreviations

4CL	hydroxycinnamate:CoA ligase
BA	benzyladenine
CAD	cinnamyl alcohol dehydrogenase
CCA	compact callus aggregate
CCR	cinnamyl-CoA reductase
GMP	Good Manufacturing Practice
GPD	glyceraldehyde-3-phosphate dehydrogenase
h	hour
HPLC	high-performance liquid chromatography
mM	milli Mol
µS	micro Siemens
MS medium	Murashige and Skoog medium
m/z	mass to charge-ratio
NAA	naphthalene acetic acid
NCBI	National Center for Biotechnology Information
PAL	phenylalanine ammonia-lyase
PCR	polymerase chain reaction
TyrDC	tyrosine decarboxylase

List of original papers

This thesis is based on the following publications, which are referred to by their Roman numerals in the text:

I György Z, Tolonen A, Pakonen M, Neubauer P & Hohtola A (2004) Enhancing the production of cinnamyl glycosides in compact callus aggregate cultures of *Rhodiola rosea* by biotransformation of cinnamyl alcohol. Plant Science, 166(1): 229-236.

II Tolonen A, György Z, Jalonen J, Neubauer P & Hohtola A (2004) LC/MS/MS identification of glycosides produced by biotransformation of cinnamyl alcohol in *Rhodiola rosea* compact callus aggregates. Biomedical Chromatography, 18: 550-558.

III György Z, Tolonen A, Neubauer P & Hohtola A (2005) Enhanced biotransformation capacity of *Rhodiola rosea* callus cultures for glycosid production. Plant Cell, Tissue and Organ Culture, 83: 129-135.

Supplementary material:

IV György Z, Tolonen A, Neubauer P & Hohtola A (2004) Biotransformation by *Rhodiola rosea* compact callus aggregates for producing salidroside. Manuscript

V György Z, Jaakola L, Neubauer P & Hohtola A (2005) Isolating and describing a cDNA fragment encoding tyrosine decarboxylase from *Rhodiola rosea*. Manuscript

Contribution to paper II: ZG designed the experiment, carried out the establishment of the callus cultures and the biotransformation experiments, analysis of the data and partly drafted the manuscript. AT carried out the chemical analysis, identification of the compounds and drafted the manuscript. JJ, PN and AH coordinated the study and critically reviewed the manuscript.

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1 Introduction

1.1 Roseroot

Roseroot (*Rhodiola rosea L.)*, also known as golden root or arctic root, has been used in the traditional medicine for centuries in Asia, Scandinavia and Eastern Europe. There are legends about its positive effects and how it increases physical and mental performance, longevity, provides resistance to high altitude sickness eliminates fatigue, treats depression and impotence (Ramazanov 1999, Kelly 2001, Brown *et al.* 2002).

Rhodiola rosea belongs to the family *Crassulaceae*. The genus *Rhodiola* probably originated in the mountainous regions of Southwest China and the Himalayas. The various *Rhodiola* species display a circumpolar distribution in the higher latitudes and elevations of the Northern hemisphere mainly in Asia and Europe (Furmanova *et al.* 1995, Brown *et al.* 2002). Roseroot was found in the Ukrainian Carpathians (Lovelius and Stoiko, 1990), in the Low Tatras (Hrouda *et al.* 1990), in the Urals (Morozova *et al.* 1997), in Japan (Ohba and Midorikawa 1991), and in Altai (Nekratova *et al.* 1992). Nearly 200 species were identified in the genus (Gemano and Ramazanov 1999).

Rhodiola rosea is a dioecious, perennial plant, reaching a height of 70 cm (Brown *et al.* 2002). It has a thick rhizome, bears flowering stems with alternate leaves and yellow to red flowers in terminal cymes (Furmanova *et al.* 1995).

Intensive research has been performed on roseroot in the former Soviet Union concerning its secondary metabolites (detailed later) and pharmacological effects (detailed later). A number of studies have dealt with its developmental biology (Polozhii and Revyakina 1976, Nukhimovskii 1976, Sviridova 1978, Nukhimovskii *et al.* 1987, Morozova *et al.* 1997, Ishmuratova and Satsyperova 1998), introduction into cultivation (Kazarinova and Opanasenko 1973, Dneprovskii et al 1975, Kim 1976, Revina *et al.* 1976 and 1977, Kheintalu 1986, Kurkin *et al.* 1988 and 1990, Kiryanov *et al.* 1989, Satsyperova *et al.* 1993, Galambosi 1999), germination (Tikhonova et al 1997, Kozlowski and Szczyglewska 2001), quality of raw material (Kurkin *et al.* 1990), tissue culture (Kurkin *et al.* 1991), and micropropagation (Kirichenko *et al.* 1993, Ishmuratova, 1998). Several studies have been conducted on this plant about its morphology (Ilyinska 1990, Lavrinenko *et al.* 1998), nodal structure (Hart 1994), chromosome numbers

(Kochjarova and Bernatova 1995, Amano *et al.* 1995, Likhonos and Kalashnik 1999), seed-coat (Knapp 1994), and taxonomy (Gontcharova 2000).

The "western world" only discovered this plant in the 90´s, which is partly due to the fact that most of the research has been published only in Slavic languages.

1.2 Secondary metabolites of *Rhodiola rosea*

The rhizome and roots accumulate many pharmaceutically active secondary metabolites which belong to different chemical groups (Table 1).

Table 1. Secondary metabolites of Rhodiola rosea.

Chemical group	Reference
phenylpropanoid glycosides	Zapesochnaya and Kurkin 1983, Brown *et al.* 2002, Tolonen *et al.* 2003
phenylethanol derivates	Troshchenko and Kutikova 1967, Brown *et al.* 2002
flavonoids	Kurkin *et al.* 1983, 1984, Brown *et al.* 2002
terpenes	Kurkin *et al.* 1986. Beloy *et al.* 1994, Brown *et al.* 2002, Rohloff 2002
phenolic acids	Brown *et al.* 2002
coumarins	Furmanova *et al.* 1995
lactones	Furmanova *et al.* 1995

Kurkin *et al.* investigated the flavonoid compounds of *Rhodiola rosea*. From the rhizomes they isolated first rhodionin, rhodiosin and rhodiolin (Kurkin *et al.* 1983) and later 8-methylherbacetin, acetylrhodalgin, kaempferol 7-O-α-L-rhamnopyranoside and methyl gallate (Kurkin *et al.* 1984). They identified 7 flavonoid compounds (rhodionin, rhodionidin, rhodiolgin, rhodiolgidin, rhodalin, rhodalidin and caffeic acid) from the aerial parts of *Rhodiola rosea* (Kurkin *et al.* 1985) and later the structures of those compounds were identified (Zapesochnaya *et al.* 1986).

Kurkin *et al.* (1986) while studying the flavonoids of roseroot, obtained terpenoid-like fractions, which were identified as rosiridol, rosiridin daucosterol and β-sitosterol. According to Rohloff (2002) the dried rhizome contains 0.05% essential oil. He identified 75 compounds, mainly from the chemical classes: monoterpene hydrocarbons (25.40%), monoterpene alcohols (23.61%) and straight chain aliphatic alcohols (37.54%). The most abundant compounds were found to be decanol (30.38%), geraniol (12.49%) being the most important rose-like odour compound, and 1,4-p-menthadien-7-ol (5.10%). Earlier Belov *et al.* (1994) proposed octadecadienoic acid, heptanol derivates and hexadecanoic acid to be the main constituents. However, the detected composition can depend on the plant origin, the chosen extraction method, and analytical method (Rohloff, 2002).

Several types of glycosides were found in *Rhodiola rosea* (Figure 1). The flavonoid glycosides, rhodionin and rhodiosin were already mentioned. Troshchenko and Kutikova (1967) isolated tyrosol and its glycoside rhodioloside from the roots of the plant. Rhodioloside was later identified as salidroside (Thieme 1969), which was found first from *Salix triandra,* even the term salidroside is derived from the *Salix* name (Brigel and

Beguin 1926). Later it was also isolated from *Olea europea* (Ryan and Robards 1988), *Vaccinium vitis-idaea* (Thieme and Winkler 1966), *Rhododendron ponticum x catawbiense* (Thieme *et al.* 1969), *Syringa vulgaris* (Kurkin *et al.* 1990), *Betula platyphylla* (Shen *et al.* 1999), *Phillyrea latifolia* (Lanza *et al.* 2001). Zapesochnaya and Kurkin (1983) isolated phenylpropanoid glycosides from the rhizome, identified them as glycosides of cinnamyl alcohol and named them rosin, rosavin and rosarin. Later Kurkin (2003) systematized phenylpropanoids and proposed their classification. Tolonen *et al.* (2003) isolated further glycosides of cinnamyl alcohol: cinnamyl-(6′-O-β-xylopyranosyl)-O-β-glucopyranoside, 4-methoxy-cinnamyl-(6′-O-α-arabinopyranosyl)-O-β-glucopyranoside, picein, and benzyl-O-β-glucopyranoside from the rhizome.

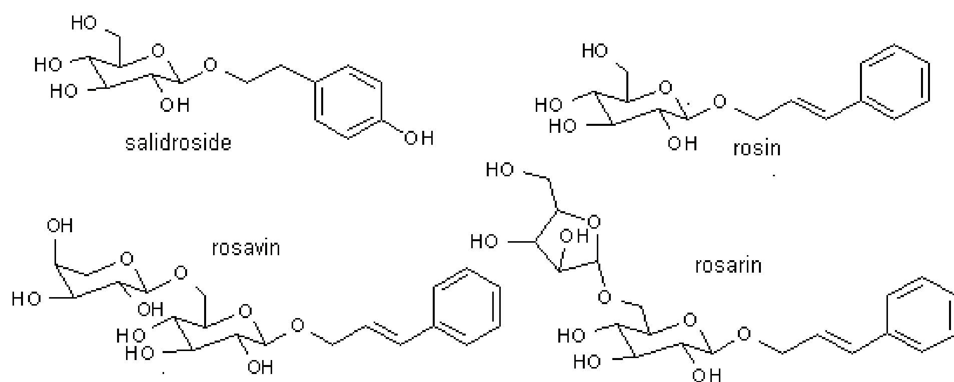

Fig. 1. The glycosides of *Rhodiola rosea.*

Besides the above mentioned compounds, also coumarins, lactones (Furmanova *et al.* 1995) and phenolic acids: chlorogenic-, hydroxycinnamic- and gallic acid (Brown *et al.* 2002) were found from *Rhodiola rosea*. Recently lotaustralin was isolated from its roots as well (Akgul *et al.* 2004).

Kurkin *et al.* (1986) compared the chemical composition of 21 *Rhodiola* species and found that the cinnamyl alcohol glycosides occurred only in *Rhodiola rosea*, distinguishing it from all other species. Kiryanov *et al.* (1988, 1989) concluded that the rosavin and salidroside contents increased as the plants got older. Kurkin *et al.* (2000) found that the amount of the cinnamyl alcohol glycosides depended on the place of origin of the plant, while the content of salidroside is not dependent on it.

Today rosin, rosavin, rosarin and salidroside are considered to be the most important components of *Rhodiola rosea*, all demonstrating adaptogenic activity (Furmanova *et al.* 1995, Germano *et al.* 1999, Panossian and Wagner 2005). Extracts used in most clinical trials are standardized to minimum 3% cinnamyl alcohol glycosides 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 *et al.* (2004) using appleseed meal as a source of β–glucosides obtaining 15.8% salidroside yield and by Zhang *et al.* (2005), who isolated the enzyme from *Aspergillas 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.

1.3 Biosynthetic pathway of salidroside and cinnamyl alcohol glycosides

Both salidroside and cinnamyl alcohol glycosides are products of phenylpropanoid metabolism, derived from phenylalanine, which is a derivative of the shikimic-chorismic acid pathway. The enzyme that directs carbon to the synthesis of phenylpropanoid metabolites is known as phenylalanine ammonia lyase (PAL). PAL is the most extensively studied enzyme in the phenylpropanoid pathway, if not in all secondary metabolism. It converts phenylalanine to cinnamic acid. At this point the pathway leaves the main phenylpropanoid biosynthesis way, which would lead to coumarins, flavonoids or lignins and lignans. However, the same types of enzymes take part in the further biosynthesis of the cinnamyl alcohol glycosides. From cinnamic acid cinnamyl-CoA ester is formed through hydroxycinnamate:CoA ligase (4CL). This CoA ester is reduced to cinnamaldehyde by cinnamyl-CoA reductase (CCR). The cinnamaldehyde is further reduced by cinnamyl alcohol dehydrogenase (CAD) to cinnamyl alcohol. The enzymes that take part in the formation of the glycosides of cinnamyl alcohol are not yet described. By one glucose transfer rosin is formed, which is the simplest glycoside of roseroot. From rosin by the connection of an arabinose rosavin and by the connection of an arabinofuranose rosarin is formed. Depending on the sugar type and the site it is connected to, further glycosides may be formed.

The salidroside biosynthesis starts from tyrosine. Tyrosine is converted into tyramine by tyrosine decarboxylase (TyrDC). Tyramine is oxidised by tyramine-oxydase to 4-hydroxy-phenylacetaldehyde, which is then reduced to 4-hydroxy-phenylacetalcohol, also known as tyrosol. Salidroside is a simple glycoside of tyrosol.

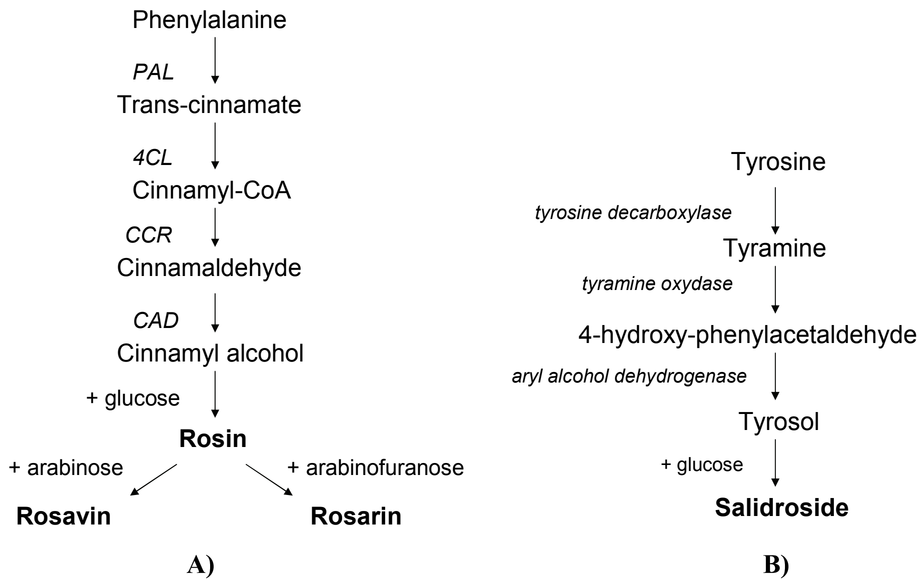

Fig. 2. Biosynthetic pathway of A) rosin, rosavin, rosarin and B) salidroside.

1.4 Pharmacological effects of *Rhodiola rosea*

Rhodiola rosea has been categorized as adaptogen by Russian researchers. The term "adaptogen" was created by Lazarev in 1947 and later Brekhman and Dardymov proposed the following specific criteria a plant must conform to be considered an adaptogen:

- The plant must be non-toxic and totally harmless to the body allowing it to continue normal physiological functioning of the individual;
- The action it exerts must be non-specific and should maintain normal body functions despite a wide range of onslaughts to the body;
- It should normalize the body functions irrespective of existing pathological conditions.

(Germano *et al.* 1999, Kelly 2001).

The clinical trials with animals and humans both using the alcohol-aqueous extract of roots and rhizome of *Rhodiola rosea* revealed various effects, listed in Table 2 (reviewed by Kucinskaite *et al.* 2004).

Table 2. Pharmacological effects of the alcohol-aqueous extract of Rhodiola rosea.

Effect	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
	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 well-being	*in vivo*	Spasov *et al.* 2000 a,b
antihyperglycaemic and insulin stimulating activity	*in vivo*	Molokovskij *et al.* 2002
hepatoprotective effect	*in vivo*	Yaremii and Grigoreva 2002
antifatigue effect	*in vivo*	Darbinyan *et al.* 2000
	in vivo	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*	Sanctis *et al*. 2004
expedites the recovery after acute non-specific pneumonia	*in vivo*	Narimanian *et al*. 2005

1.5 *In vitro* production of pharmaceutically important plant derived compounds

Plant biotechnology is getting more and more important, even more important than medical biotechnology. The world population is expected to reach 10 billion in 2050, while agricultural production is growing at a much slower rate. Today more people die from famine and malnutrition, than from the “modern” diseases. Nowadays traditional agriculture has several limitations: international trade and polices regulate the market; climatic changes, urbanization and industrialization have reduced the land and water availability; and classical breeding is now too slow to cope with the high demands of today. The potential solutions for the increasing food demands are searching for alternative food sources and more efficient plant breeding, which can later be achieved through biotechnology and recombinant DNA techniques. Agriculture now targets not only the production of low-priced food, but also high-priced specialized plant derived products, which can be of plant origin or non-plant compounds (Altman 1999).

1.5.1 Plant cell cultures

Plant secondary metabolites are in commercial use as drug, dye, flavour, fragrance, insecticide, etc. However, production by plants is not always satisfactory. Usually a compound is restricted to a few species or genus, a particular organ; might be produced only during special growth or developmental stage or under specific seasonal, stress or nutrient conditions (Verpoorte *et al.* 2002). Some plants are difficult to cultivate or have not been introduced yet, necessitating collection in the wild and thus increases the risk of the plant becoming extinct. In these cases the quality can not be assured. Others grow very slowly like *Taxus brevifolia* (Kieran *et al.* 1997). Nowadays the production needs to meet GMP-rules as well. Because of all these considerations, plant cell cultures became a possible production method for these compounds. The advantages of this approach are the more rapid biomass production, the controllable nutritional and environmental factors, avoidance of the over-collection of endangered species and the elimination of geopolitical and import price fluctuations (Oksman-Caldentey and Hiltunen 1996). Despite the extensive research so far only a few industrially feasible processes exist for these compounds, such as the production of shikonin, taxol and berberine. This might be due to the fact that production of secondary metabolites is controlled in a tissue-specific manner and so dedifferentiation results in loss of production (Verpoorte *et al.* 2002). Another main problem is that many times the basic knowledge of the biosynthetic routes and the mechanisms behind the production is missing (Buitelaar and Tramper 1992). However, there are some techniques that have beneficial effects on the production in many cases. Considerable amount of research has been done on this issue; in the following only few examples are given.

1.5.1.1 Screening

The most simple and common approach is the screening and selection for high producing cell lines. This approach was successful in case of *Coptis japonica* cell cultures for berberine (Sato and Yamada 1984) or *Lithospermum erythrorhizon* for shikonin production (Fujita 1988). This method is very simple in cases where such compounds are perceptible to the eye e.g. anthocyanins. But in case, where no product is found in the initial cell cultures, this approach will not be successful.

1.5.1.2 Environmental factors

Composition of the media, including the types and amounts of plant growth regulators, mineral salts, carbon sources, as well as culture conditions, including temperature, light and gas composition during culture may affect the production of secondary metabolites (Stafford *et al.* 1986). Much research has been done on the composition of the media (content of carbon, nitrogen, phosphorus and hormones) for the growing of ginseng cell cultures and the production of saponins (Wu and Zhong 1999). Choi *et al.* (1994) found the optimal concentration of sucrose to be between 30 and 50 g l^{-1} for cell growth, while

the saponin content increased with increasing sucrose concentration up to 60 g l^{-1}. Zhang *et al*. (1996) found that the saponin production increased as the ratio of NH_4^+/NO_3^- decreased. The effect of temperature on the production of indole alkaloids of *Catharanthus roseus* has been studied by ten Hoopen *et al.* (2002) and found that both for the biomass growth and for the alkaloid production 27.5°C was the optimal.

1.5.1.3 Elicitation

Molecules that stimulate secondary metabolism are called elicitors. Depending on their origin they are classified as either biotic or abiotic. Abiotic elicitors are environmental stress factors such as osmotic shock, presence of heavy metal ions or other chemicals and UV radiation. Treating hairy root cultures of *Beta vulgaris* with up to 10-fold calcium of that is present in the medium increased the production of betalains by 3-fold (Savitha *et al*. 2005). The addition of 4 g l^{-1} potassium chloride increased the ajmalicine production of *Catharanthus roseus* four-fold (Zhao *et al.* 2001). Biotic elicitors include polysaccharides derived from plant cell walls or microorganisms, glycoproteins and low-molecular weight organic acids (Dörnenburg and Knorr 1995). A crude extract from *Fusarium oxysporum* increased the taxol production in suspension cultures of *Taxus chinensis* var. *mairei* 3-times (Yuan *et al*. 2002). Extracts of *Aspergillus niger* and *Rhizopus oryzae* had positive effect on shikonin production in suspension cultures of *Arnebia euchroma* (Fu and Lu 1999).

1.5.1.4 Biotransformation

Biotransformation can be the solution when the formation of a compound does not occur in the cultured plant cells and neither can be induced by elicitation, furthermore when the chemical synthesis is complicated and expensive. The cultured cells have the ability to stereo- and regiospecifically convert the exogenously added substrates into the desired, or into new end products. The substrate does not need to be a natural intermedier and it also can be of synthetic origin (Pras *et al.* 1995, Giri *et al.* 2001). Hydroxylation, oxidation, reduction, hydrogenation, glycosylation, esterification, methylation, acetylation, and isomerisations can be such reactions (Stöckigt *et al.* 1995, Giri *et al.* 2001, Ishihara *et al.* 2003). *Catharanthus roseus* cell cultures can oxidize the phenylsulphonyl group from the synthetic compound 1,5-diphenylsulphinyl-3-methyl-3-nitropentane (Bourgogne *et al*. 1989). Mevalonic acid and farnesol, which are intermediates of saponin biosynthesis increased the saponin production of ginseng callus cultures by 20% (Furuya et al. 1983). Biotransformation has been reported e.g. about digitoxigenin into digitoxigenone, epidigitoxigenin and epidigitoxigenin glucoside by *Digitalis purpurea* (Hirotani and Furyuya 1980), ajmaline into raumacline and methylraumacline by *Rauwolfia serpentina* (Polz *et al.* 1990), and limonene into carvone by *Solanum aviculare* and *Dioscorea deltoidea* cells (Vanek *et al.* 1999).

1.5.1.5 Organ cultures

Secondary metabolites are often accumulated in special types of cells or organs; i.e. the biosynthesis of a special compound is often coupled to a certain morphological differentiation (Alfermann and Petersen 1995). As expected, organ cultures produce similar secondary metabolites as the plant itself. Mostly two types of organ cultures are used for such purposes: hairy root and shoot cultures. Hairy root cultures can be obtained by transformation of *Agrobacterium rhizogenes* and grown without plant growth hormones. These cultures are genetically very stable and grow much faster compared to normal root cultures. Also culturing them in bioreactor is possible (Toivonen 1993, Giri and Narasu 2000). Hairy root cultures can be used for producing ginsenosides by a *Panax* hybrid (Washida *et al.* 1998), or by *Panax ginseng* (Yoshikawa and Furuya 1987) or indole alkaloids by *Catharanthus roseus* (Parr *et al.* 1988). Shoot cultures are less spread, but still there are several examples: artemisinin production by *Artemisia annua* (Liu *et al.* 1998), vindoline and catharanthine production by *Catharanthus roseus* (Hirata *et al.* 1990) or tropane alkaloid production by *Duboisia myoporoides* (Khanam *et al.* 2001).

1.5.1.6 Metabolic engineering

Besides improving resistance to various factors or increasing biomass and grain size, one main aim of genetic transformation is improving or facilitating the production of pharmaceuticals in plants or in cell cultures. (Oksman-Caldentey and Hiltunen 1996).
This so called metabolic engineering was enabled when more and more biosynthetic pathways have been explored in the last decade. Usually this approach is based on the identification of limiting enzyme activities, which are then improved by genetic transformation. Most of the strategies developed so far play with the introduction of gene(s) isolated from more efficient organisms or promoters that enhance the expression of the target gene and even antisense and co-suppression techniques. Plants can also be used as hosts for the production of recombinant proteins; this so called molecular farming uses however field-grown plants and not *in vitro* ones (Bourgaud *et al.* 2001). Metabolic engineering of alkaloid producing plants probably received more attention because of their pharmacological importance. This strategy has been extensively studied in *Catharanthus roseus* (reviewed by Verpoorte *et al.* 2002).

1.5.2 Large scale in vitro production of plant derived compounds

Currently, plant cell cultures are not widely used to make products of commercial interest, because the development of large-scale cultivation methods are hampered by low productivity, cell line instability and difficulties in the scale-up process, such as shear sensitivity, low oxygen requirements and slow growth (Taticek *et al.* 1994, Pan *et al.* 2000, Ramachandra Rao and Ravishankar 2002). Plant cell cultures are only economically viable if the metabolites are high-value, low-volume products in the plant, not synthesized by microorganisms and too complex for chemical synthesis (Zhong *et al.*

1995, Sajc *et al.* 2000). Today only shikonin, ginsenosides, berberine and taxol are produced on a large scale (Wu and Zhong 1999, Sajc *et al.* 2000, Verpoorte *et al*. 2002).

In the beginning the large-scale cultivation of plant cells was carried out using stirred-tank bioreactors, in which the main advantages were the existing industrial capacity and proven performance (Zhong *et al.* 1999). However, over the years it became clear that the properties of plant cell suspensions are different from microbial cultures. Problems like shear sensitivity and gas composition in the reactor affected cultures of different species at a different level (Zhong *et al.* 1995).

Various types of bioreactors have been developed for cultivating plant cells or organ cultures, such as modified stirred tank with different impeller designs, air-lift reactor, rotating drum, balloon type reactor and membrane reactors (reviewed by Panda et al 1989, Doran 1993, Sajc *et al.* 2000). There are however reports on large-scale cultures of e.g. *Catharanthus roseus* using conventional stirred tank reactor with flat-blade impellers (Schiel and Berlin 1987), *Digitalis lanata* cells in air-lift reactor (Reinhard *et al*. 1988), ginseng cells in a centrifugal impeller bioreactor (Zhong *et al*. 1999) and cell cultures of *Taxus cuspidata* in ballon type bubble reactor (Son *et al*. 2000).

2 Aims of the study

The main objective of the study was to produce the glycosides of *Rhodiola rosea* in callus cultures. As natural habitats are already overharvested and the cultivation of this plant needs 4-6 years, the production of these pharmacologically important compounds in *in vitro* cultures could supersede the extraction from plants either from wild populations or from cultivation. To open the door for the large scale production in a bioreactor system, research was carried out:

1. to establish suitable cultures for the experiments (I),
2. to investigate the possibility of producing the glycosides by biotransformation (I, II, III, IV),
3. to substantiate the metabolic engineering of roseroot (V).

3 Material and methods

3.1 Initiation of compact callus aggregate cultures

Callus used in the experiments, derived from the leaves of *in vitro* grown *Rhodiola rosea* originated from a wild Austrian population (I). In order to establish a suspension culture of compact callus aggregates (CCA) calli from the solid media were freed from media pieces and gently broken using forceps. About 10 g (fresh weight) of callus was transferred into 100 ml liquid MS medium (Murashige and Skoog 1962) supplemented with 0.5 mg l^{-1} BA and 1 mg l^{-1} NAA (this composition is called MS-Rh later) in 250 ml Erlenmeyer flasks and shaken at 135 rpm. Subcultures were carried out in every 8-10 days by decanting all of the medium from the flask and adding fresh medium. Cultures were kept at 23°C and the light cycle was 16 h light and 8 h dark.

The growth of the CCA cultures was followed for 30 days in 50 ml Erlenmeyer shake flasks containing 20 ml MS-Rh media, inoculated with one gram fresh weight CCA.

Fresh and dry weight measurements and the chemical analysis were performed as described in papers I, II, III, and IV.

3.2 Biotransformation experiments

All experiments (I, II, III, IV) were performed in 50 ml Erlenmeyer flasks containing 20 ml MS-Rh media, in case of paper III, ten grams of glucose was incorporated into the media besides twenty grams of sucrose, and as control 30 g sucrose was used as in the other experiments. One gram fresh weight CCA was inoculated into each flask. For the production of the cinnamyl alcohol glycosides, cinnamyl alcohol and for the production of salidroside, tyrosol were added to the media in final concentrations as specified in the original papers (I, II, III, IV). Both precursors were dissolved in 70% ethanol (0.1 g in 1 ml EtOH), and sterilized by passing through a 0.2 nm filter. The CCAs were cultured in MS-Rh media supplemented with precursors for one week or as specified in the original

papers. The experiments were made in triplicate. Three flasks of each composition were harvested for the determination of fresh and dry weight, viability and for the chemical analysis.

3.3 Bioreactor experiment (unpublished data)

After preliminary studies to find the suitable reactor type, a pilot experiment was performed in a 2 l glass air-lift reactor with 1.5 l working volume. Ninety grams of CCAs were inoculated in the reactor. The mixing and the aeration were done by pressurised air driven through a flow meter and an air filter. The aeration was kept at 15 l h^{-1} during the first week, and later as the CCAs grew the aeration was increased. On the last day aeration was 100 l h^{-1}. The growth of the culture was followed by measuring the conductivity of the media. A calibration curve was previously done in shake flasks. Cinnamyl alcohol (2 mM) was added when the growth approached the stationary phase according to the conductivity of the media. Samples were taken before the addition of cinnamyl alcohol, and 1, 2, 3 days after the cinnamyl alcohol addition.

3.4 Gene walking and gene expression analysis

Nucleic acids were extracted and purified from deep-frozen plant material, which was collected from the Botanical Garden's test field at the University of Oulu, Finland. Total RNA was isolated according to Jaakola *et al.* (2001) and genomic DNA was extracted according to Pirttilä *et al.* (2001). The quality of the isolated RNA and DNA was verified from the absorbance measurements at wavelengths 230, 260 and 280 nm and on a 1% (w/v) ethidium bromide-stained agarose gel.

RNA was reverse-transcribed into cDNA as detailed in paper V. A fragment of the tyrosine decarboxylase gene was amplified from the cDNA by PCR. Several partially degenerated primer pairs were designed based on homologies found in the TyrDC genes that were isolated earlier from *Arabidopsis thaliana*, *Papaver somniferum*, *Petroselinum crispum* and *Thalictrum flavum* subsp. *glaucum*.

Dynazyme DNA polymerase (Finnzymes, Espoo, Finland) was used for the PCR amplification in a final volume of 25 µl. PCR was performed using "touch down" strategy as detailed in the original paper (V). The PCR products were run on a 1% (w/v) ethidium bromide-stained agarose gel. The band of expected size (680 bp) was sequenced as described in paper V.

After the PCR product proved to be similar to the corresponding fragments of the already known TyrDC genes, new primers were designed for genome walking (Table 3), which was performed according to the manufacturer's instructions (Universal GenomeWalker™ Kit, BD Biosciences Clonetech, Palo Alto, USA). Analysis of the sequences was done by Sci Ed Central´s Align Plus 4 program.

For the gene expression analysis RNA was extracted from leaves and roots of roseroot plants. High (V4) and low (V15) salidroside producer genotypes were used in the

experiment. Both were from Hortus Botanicus Arcto-Alpinus, Kirovsk, Russia. The salidroside content of the high producer line was 18.23 mg g^{-1} and of the low producer line was 3.85 mg g^{-1} (Minna Pakonen, personal communication). The RNA was transcribed into cDNA and contaminating DNA was removed with a gel extraction method (Jaakola *et al.* 2004). The gene expression analysis was performed by Real-Time PCR using LightCycler instrument (Roche Molecular Biochemicals, Mannheim, Germany) and DyNAmoTM SYBR® Green qPCR kit (Finnzymes, Espoo, Finland). For the quantification of PCR products, glyceraldehyde-3-phosphate dehydrogenase (GPD) was used as a control gene. The ratio of TyrDC expression to GPD signal was defined as relative expression. The primers for Real-Time PCR were designed based on the obtained sequence (Table 1). These primers gave a 202 bp fragment for GPD and a 246 bp fragment for TyrDC.

Table 3. The primers used in the genome walking for isolating a cDNA fragment encoding TyrDC gene form Rhodiola rosea.

		Primers 5’- → 3’	Reference
upstream	outer	AAACTGGTCGTCTACTGCTCCGACCAA	V
	nested	ACATTTCACAATCCACAAGGGCGCAAA	
	outer	TGGTGGATTACAAGGACTGGCAGATA	V
	nested	GTTGAGTCGTCGATTTAGAGCGATCAA	
	outer	GGTGGCAAGAGAGTTTGATTTGTGGTT	V
	nested	CATGTTGATGGAACTTATGGTGGCAGT	
downstream	nested	GATGGTAGTACTGATTATGAAATCTACG	V
	outer	AACCTGGCTTGACACGTGGCTGAACT	
	nested	GACAACCCTCTCAATGATACGAGGCCT	V
	outer	ACAGTAACCCAAGTGTACCTCGAGGAG	

Table 4. The roseroot specific primers, used in the Real Time PCR for the expression of TyrDC gene in Rhodiola rosea plants.

Gene	Primers 5’- → 3’	Reference
TyrDC	TGTCATTGGGTTCACATGGAG	V
	GTTTGGTCGGAGCAGTAGAC	
GPD	ACTGTTTTGCCCCACTTGCTA	V
	ACAGGCAACACCTTACCAACA	

4 Results

4.1 Initiation of compact callus aggregate cultures

During the first weeks after transferring the callus to liquid media some smaller pieces fell off from the bigger callus clumps. The compact callus aggregates started to form in the liquid culture three to five weeks later. The established CCA culture was composed of green or light green, spherical, smooth surfaced callus aggregates. The medium was totally clear. No dispersed cells or cell debris was observed in the flasks. The CCAs grew slowly, doubling the fresh weight within 8 days and continuing to increase in size. CCAs with a diameter above 1 cm were cut into small pieces along the naturally occurring ruptures. Four to five weeks later new CCA cultures were obtained. Cavities were formed in the centre of large aggregates (I).

4.2 Growth of the CCA cultures

The growth of CCA cultures was relatively slow. The callus cultures just started to enter the stationary phase on the last day of the experiment, which was the 29th day. The maximum biomass obtained was 5.8 g (fresh weight) that meant a nearly six-fold increase. The chemical analyses of the samples showed that neither salidroside, nor rosin, rosarin and rosavin were produced in the callus (I).

4.3 Effect of the precursors on the biomass growth

When cinnamyl alcohol was added up to 0.1 mM concentration to the media, it showed no significant deviation compared to that of the control (0 mM cinnamyl alcohol) in neither dry nor fresh weight. If cinnamyl alcohol was added to the media in concentrations of 2 to 5 mM the fresh weight decreased to the half of the control and also

the dry weight decreased. There was a slight change of the callus colour from dark green to light green at 4 and 5 mM cinnamyl alcohol concentrations (I).

When tyrosol was added to the media in concentrations between 0.05 and 2 mM no significant effect compared to the control (0 mM tyrosol) was observed on the biomass production, neither in the fresh nor in the dry weight. If tyrosol was added to the media at concentrations up to 9 mM the biomass production decreased with concentrations higher than 3 mM (IV).

4.4 Effect of the precursors on the production of the selected compounds

Biotransformation was a successful tool to enable the production of the desired compounds of roseroot in callus cultures. The optimal cinnamyl alcohol concentration to be transformed was 2 mM. Although the callus can tolerate even higher concentrations, there is no use to add more, since no more could be transformed. The main product of the cinnamyl alcohol biotransformation was rosin, the highest concentration obtained was 1.25% of the dry weight, and the lowest was 0.4% (I). Low amount of rosavin was produced and the highest concentration obtained was 0.083% of the dry weight (I).

Since the consumption rate of cinnamyl alcohol was found to be much higher than the production of the known glycosides, a detailed study of the samples was performed (II). Four new products (Figure 3) were identified and named as compound 337, 481, 483 and 321 after their m/z-ratio of the sodium adducts (M+Na = M+23), i.e. their molecular peaks seen in the MS-spectra.

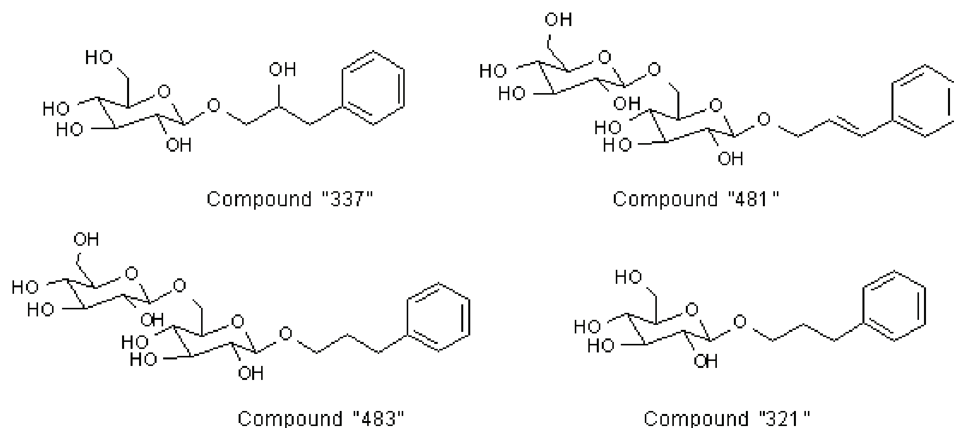

Fig. 3. Four new compounds of *Rhodiola rosea* resulted from the biotransformation of cinnamyl alcohol, found by LCT analyses.

For producing salidroside, the optimal tyrosol concentration was also found to be 2 mM (IV). At higher concentrations the tyrosol was detected in the media, indicating that not all tyrosol was used in the biotransformation. The highest concentration of salidroside achieved was 2.72% of the dry weight.

4.5 The necessary time for the biotransformation

During the first days the rosin content increased but then it started to decrease, still being much higher than the concentration in the control samples. The cinnamyl alcohol content decreased continuously during the 5 days of the experiment (I).

After the first two days tyrosol was not detected in the media, which means that it was completely utilized in the biotransformation reaction. In the first 3 days the salidroside content rapidly increased in the cells, after which the levels did not change (IV).

4.6 Enhancing the biotransformation

The addition of glucose proved to be beneficial concerning the production of the cinnamyl alcohol glycosides. In this case, the production of rosin and compounds 337, 481, 483, 321 nearly doubled. Rosavin was not produced at all when only sucrose was used, just if glucose was added as well (III).

However, the addition of glucose to the medium did not show any positive effect on the accumulation of salidroside. The accumulation pattern was essentially the same with or without addition of glucose (III).

4.7 Production in bioreactor (unpublished data)

The bioreactor experiment was run for a total of 32 days in a 2 l volume air-lift reactor (unpublished data). The growth of the culture was followed by measuring the conductivity of the media (Figure 4). When the conductivity reached 2 $\mu S\ cm^{-1}$ and the biomass was at the maximum, cinnamyl alcohol was added to the medium. The CCAs were harvested 4 days after the precursor addition. The accumulation pattern (Figure 5) of the expected compounds was similar to that in the shake flasks.

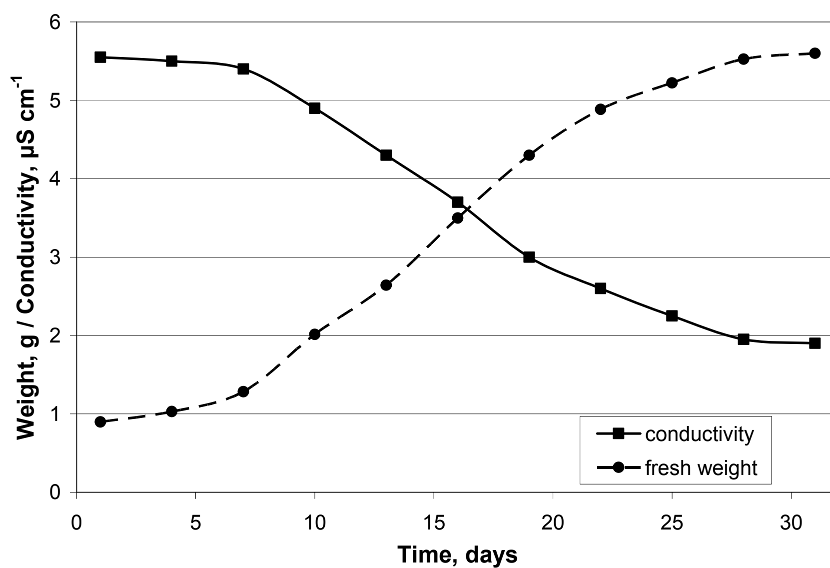

Fig. 4. The correlation between the conductivity of the media and the growth of the CCAs.

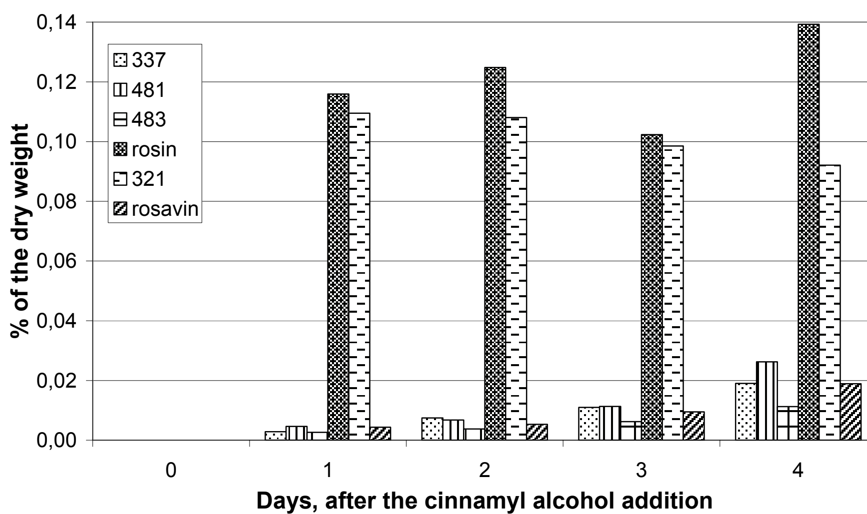

Fig. 5. The content of the biotransformation products in CCAs cultured in air-lift reactor.

4.8 Gene walking and gene expression analysis

Out of the several primers designed for roseroot, the successful ones are listed in Table 2. They gave a fragment of 680 bp for TyrDC and of 330 bp for GPD (V). The Blast service of NCBI revealed homology to the corresponding genes from other species.

Table 5. The primers used in amplifying fragments for sequencing.

Gene	Primers 5’- → 3’	Reference
TyrDC	TC/AACA/TCATTGGCAAAGTC′	V
	AAA/GAACCAT/CTTG/ATGTGCATT	
GPD	GCTCCCAGCAAGGATGCCCC	Jaakola *et al.*
	CGGAAGGCCATTCCAGTCAACT	2002

This 680 bp fragment was used as the basis for the gene walking. Finally 2025 bases were sequenced, of which 1520 bases proved to be of TyrDC gene. Aligning this sequence with the previously reported TyrDC sequences of other species similarity was found. Comparing it to the parsley gene, it showed 53% identity, while the others showed 55-62% identity to the sequence of parsley.

The TyrDC gene was shown to be expressed in both leaves and roots. However, difference was found in the expression depending on the organ and also on the genotype. The expression in roots was higher than in leaves. In case of the high salidroside producer line, the expression of the gene was considerably higher than in the low salidroside producer line concerning the roots (V).

5 Discussion

5.1 In vitro cultures of *Rhodiola rosea*

Tissue culture of *Rhodiola rosea* has been investigated since the 1980s. In 1981 Aleksandrova *et al.* (cited by Furmanova *et al.* 1995) patented the method for root regeneration from callus, but did not provide information on the callus induction and its maintenance. Several studies investigated the pharmacological properties and effects of alcoholic extracts of tissue culture derived material (Krendal 1989, Barilyak and Dugan 1994, Krendal *et al.* 1995). Kurkin et al (1991) described 13 compounds isolated from callus of which the main was triandrine.

In the 90s subsequent papers were published on micropropagation (Kirichenko *et al.* 1993 and Ishmuratova 1998) and on callus induction and plant regeneration of *Rhodiola rosea* (Furmanova *et al.* 1995). Research was also carried out on searching for the pharmaceutically important glycosides in callus and in *in vitro* plants beside intact plants. While Kurkin *et al.* (1991) found neither salidroside nor cinnamyl alcohol glycosides in callus, Furmanova *et al.* (1995 and 1998) reported traces of these glycosides in addition to triandrine and caffeic acid. Kurkin *et al.* (1991) suggested that rosin was transformed to hydroxyrosine (triandrine) in callus.

The use of CCAs instead of cell suspensions has been a successful tool in some cases aiming at secondary metabolite production. The use of such cultures improved the taxol production of *Taxus cuspidata* (Xu *et al*. 1998a), indole alkaloid production of *Catharanthus roseus* (Zhao *et al*. 2001), flavonoid production of *Saussurea medusa* (Fu *et al*. 2005) and peroxidase production by *Daucus carota* (Xu *et al*. 1998b). However, the application of CCAs has been investigated mostly in connection with production of salidroside by *Rhodiola sachalinensis* (Jianfeng *et al*. 1998 a and b, Xu *et al*. 1999). In itself culturing CCAs raised the salidroside content six-fold according to Jianfeng *et al* (1998a) and three fold according to Xu *et al*. (1999).

In the beginning of this study the aim was to produce the glycosides in cell suspensions, cultivated in bioreactor. However, establishing a cell suspension culture failed due to several reasons. The available callus of roseroot was hard and compact. Even the most friable callus did not disperse in shake flasks. It is known that plant cells

have a natural tendency to adhere together (George and Sherrington 1984). Although after mechanical comminution cell suspension was achieved, the culture was rapidly overgrown by bacteria, which were found to be of endophyte origin (unpublished, preliminary result). Hence culturing CCAs was promising and even including the possibility of producing the desired secondary metabolites.

The results presented in this study confirm that there is no production of the pharmaceutically important glycosides in callus cultures of *Rhodiola rosea*, even in compact callus aggregates (I).

5.2 Biotransformation

Among the biotransformation reactions glycosylation was of special interest since it facilitates the conversion of water-insoluble compounds into water-soluble compounds, which was difficult to achieve by microorganisms or by chemical synthesis (Giri *et al*. 2001, Ishihara *et al*. 2003). Besides altering the hydrophilicity, glycosylation also resulted in increased stability, changes in the cellular localization of the compound and amends the bioactivity of it (Bowles et al. 2005, Gachon et al. 2005).

Xu *et al*. (1998c) studied the biotransformation by *Rhodiola sachalinensis* cell cultures for producing salidroside. Three possible precursors: phenylalanine, tyrosine and tyrosol were added to the cultures in final concentrations of 0.05, 0.1, 0.5 and 1 mM. All three compounds had adverse effects on the biomass growth in proportion to the concentration (phenylalanine only to a lower extent). 1 mM of tyrosine and tyrosol set it back to one third of the control. On the other hand both tyrosine and tyrosol improved the salidroside content. The concentration of 1 mM tyrosine gave 1% and 1 mM tyrosol gave 1.44% salidroside, which was nine-times higher than in the control. Afterwards Xu *et al* (1998d) studied the tyrosol glucosylation in more detail. They determined the activity of the tyrosol glucosyltransferase over the cell growth cycle and found that it was the highest during the exponential growth phase while the intracellular tyrosol accumulated in large amounts at the stationary growth phase. They suggest that this non-synchronization is responsible for the low amounts of salidroside obtained in cell suspensions. Based on this result 1 mM tyrosol was added at the beginning of the exponential growth phase, which was transformed in 95% after 24 h. When 0.5, 1, 2, 3, 4, 5 mM tyrosol was added to the cultures, the amount of salidroside formed was proportional to the amount of tyrosol added up to 3 mM. Concentrations higher than 3 mM caused a sharp decrease in the salidroside content. By the repeated addition of 3 mM tyrosol at 24 h intervals a 36 fold (516 μmol g^{-1}) increase of the salidroside content was achieved in the cells. Salidroside however, was not released into the medium.

Wu *et al*. (2003) explored the culturing conditions of *R. sachalinensis* CCAs for a high yield of salidroside. They found that acidic medium and faster shaking speed favourably influenced the salidroside production. Among the several auxins and cytokinins tested, 2.4-D stimulated the salidroside production but it inhibited the biomass growth. The addition of salicylic acid, phenylalanine or tyrosine up to 0.5 mM increased the salidroside production slightly, but 4 mM tyrosol boosted the production to 5.77%, which is the highest content ever detected.

Furmanova *et al* (1999a and 2002) studied the same reaction with *Rhodiola rosea* cell cultures. 2.5 mM tyrosol was added of which 50-67% was transformed after 72 h, and 1.2 to 2.3% salidroside yields were obtained. Residual unconverted tyrosol was detected both in the medium and in the cells.

The results presented in this study (IV) are similar to those with *R. sachalinensis*. However, up to 3 mM tyrosol did not have such a serious adverse effect as described by Xu *et al*. (1998c); the biomass was set back by only 10%. Concentration of 2 and 3 mM tyrosol resulted in the highest salidroside yield of 2.62 and 2.72% respectively. Salidroside was not released into the medium, like in the case of *R. sachalinensis*. Already after 48 h 100% of the tyrosol was transformed when 2 mM tyrosol was added. At this concentration tyrosol was neither detected in the medium nor in the cells at the end of the experiments.

The production of the cinnamyl alcohol glycosides by biotransformation is much less studied, which can be explained by the fact that these compounds are specific for *Rhodiola rosea*. Furmanova *et al*. (1999b) 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 in this present work are more detailed. Beside rosin, also rosavin and four new products were detected and identified (I, II, III). The optimal cinnamyl alcohol concentration was found to be 2 mM (I), since the resulting rosin concentration was the highest at that concentration. This amount of cinnamyl alcohol did not have an adverse effect on the biomass growth, as seen with higher concentrations, and all cinnamyl alcohol was converted at this concentration, 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 if the cells were further cultivated. The maximum achieved rosin contents 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 *et al* (1998d); but it even inhibited the production (I).

The four new compounds identified (II) are all closely related to rosin and rosavin. Compound “321” differs from rosin by opening the double bond in the middle of the propyl chain of the aglycone, while “337” differs by an extra hydroxyl group at the C-8 position. Compound “481” has an extra hydroxyl group on the 3rd carbon of the second glucose compared to rosavin, and compound “483” is very similar, but again the double bond in the middle of the propyl chain of the aglycone is opened. The presence of the many closely related products after the biotransformation indicates that either several enzymes take part in the glucosylation of cinnamyl alcohol or at least some of the products form spontaneously.

For increasing the glucosylation of cinnamyl alcohol and tyrosol, a simple trick was applied (III). Since the MS medium contains only sucrose as a sugar source, glucose was added into the medium to be directly used in the glucosylation reaction. This approach was very effective and beneficial for the production of the cinnamyl alcohol glycosides; yields were doubled compared to the control. Rosavin was only produced in the glucose containing media. However, the salidroside production was not affected at all. This ambiguous effect of sugars on secondary metabolite production was unexpected, but it is

not uncommon. Several studies have shown, that higher levels of sugars, usually sucrose, may result in higher levels of secondary metabolites like in the case of *Catharanthus roseus* for the production of arbutin (Yokoyama and Yanagi 1991). In the case of *Coleus blumei* for the rosmarinic acid production (Misawa 1985), or in the case of *Eschscholtzia californica* for the production of benzophenanthridine alkaloids (Ramachandra Rao and Ravishankar 2002) this also occured. However, there are also reported studies where higher sugar concentrations reduced the production, like in the case of *Dioscorea doryophora* where more diosgenin was produced at a lower sucrose level (Vanisree *et al*. 2004) or in the case of *Aralia cordata* where the lower sucrose level also favoured the production of anthocyanin (Ramachandra Rao and Ravishankar 2002). In addition, the optimal type of the sugar source depends on the plant species. Usually sucrose is used, but in the case of *Taxus brevifolia* fructose was found to be the optimal for paclitaxel production or in the case of *Angelica dahurica* var. *formosana* glucose was found to be the best carbon source for imperatorin production (Vanisree *et al*. 2004). As one can see from these varying inconsistent results, the optimal sugar source should be found for each plant species, even to each secondary compound to be produced.

In the course of the work, one of the main problems of plant cell cultures was observed. Namely the instability of cell lines, which affected the biotransformation capacity as well.

5.3 Expression of TyrDC

The biosynthesis of salidroside begins with decarboxylation of tyrosine yielding tyramine. Tyramine serves as precursor to various amines and amides and to isoquinoline alkaloids beside salidroside. These alkaloids (for example morphine, codeine or thebaine) are very important in modern medicine, hence the biosynthesis of plant isoquinoline alkaloids is under extensive research. Tyramine has also been found to be incorporated into the plant cell wall (Negrel and Jeandet 1987). Tyrosine decarboxylase (TyrDC) was shown to be transcriptionally activated upon fungal infection or elicitor treatment in *Petroselinum crispum* (Kawalleck *et al*. 1993), *Arabidopsis thaliana* (Trezzini *et al*. 1993) and *Solanum tuberosum* (Schmidt *et al*. 1998). When potato is infected with *Phytophtora infestans,* phenolic compounds such as hydroxycinnamic acid tyramine amides accumulate in the course of the defence reactions (Schmidt *et al*. 1998), which are believed to play role in the defence by decreasing the digestibility of the cell wall. Landtag *et al*. (2002) transformed potato to express parsley TYDC for the study of the role of tyramine in the response to the infection with *P. infestans.* They wanted to see if the expression leads to higher tyramine-derived compounds. However, the expected reaction was not observed, rather it led to the accumulation of another compound, which had not been shown from potato previously and was identified as tyrosol glycoside, i.e. salidroside.

In the present work (V) the expression of TyrDC was studied and its relation to the salidroside biosynthesis in *Rhodiola rosea*. Based on the results of Landtag *et al*. (2002) TyrDC was supposed to be a key enzyme in the biosynthetic pathway of salidroside. The

gene expression analysis supported this hypothesis. The expression of TyrDC was higher in roots than in leaves. This is the result that was expected as salidroside accumulates only in the underground parts of the plant (Troshchenko and Kutikova 1967). The other result obtained, i.e. the expression was higher in the high salidroside producer line, was also in accordance with the assumption.

In the course of the work a cDNA fragment encoding TyrDC was isolated and analysed. According to RT-PCR reactions it seems that in *Rhodiola rosea* it is a single copy gene. In poppy TyrDC was found to be encoded by a family of 10 to 15 genes that can be categorized into two subgroups based on sequence identity (Facchini and De Luca 1994, Facchini et al. 1998). Each subgroup consists of approximately six members that share approximately 90% identity at the nucleotide and amino acid levels. In contrast, a comparison of subgroup members (represented by TYDC1 and TYDC2) revealed sequence identities of less than 75%. The TYDC gene family exhibits differential and organ- and temporal-specific expressions (Facchini *et al*. 1998). Meanwhile TyrDC was found to be encoded by 4 genes in parsley (Kawalleck *et al*. 1993), while in *Arabidopsis thaliana* (Trezzini *et al.* 1993) and in *Catharantus roseus* (Goddijn *et al*. 1994) by single copy gene.

6 Conclusions and future prospects

This thesis and the original papers describe the production of the pharmacologically important glycosides in compact callus aggregate cultures of *Rhodiola rosea* by glucosylation of the exogenously added aglycones. The achieved salidroside and rosin contents were significantly higher than in wild growing plants. Beside the expected products four new glycosides of cinnamyl alcohol were identified, of which the pharmacological importance remains to be unravelled. The optimal concentration of the precursors and the optimal harvest time were determined. The incorporation of glucose into the culture medium doubled the glucosylation rate of cinnamyl alcohol and even rosavin was produced; however the glucosylation of salidroside was not dependent on the presence of glucose.

A pilot experiment using a 2 l volume air-lift reactor was performed. Compact callus aggregates of *Rhodiola rosea* could be cultured in such a system. Production of the desired glycosides could be performed in two phases; first culturing the CCAs reaching the highest biomass possible, and then adding the precursor to be converted.

The first step towards genetic engineering of the salidroside pathway was taken. A cDNA fragment encoding tyrosine decarboxylase was isolated and described. This fragment is the first nucleotide sequence published of roseroot. The expression of this gene was analysed in leaves and roots of two chemotypes. These results point out the important role of tyrosine decarboxylase in the production of salidroside. Based on this knowledge overexpression of this gene might give a possibility for salidroside formation in callus without biotransformation and/or generate a high salidroside producer roseroot line.

Establishing hairy root cultures of *Rhodiola rosea* would be also a promising solution for the production of these glycosides.

These days administration of prophylactic products is very popular. *Rhodiola rosea* is becoming widely known and used more and more. As the natural habitats are already overharvested and are threatened by extinction on one hand, and cultivation takes several years on the other hand, production in *in vitro* systems may be alternative source for these valuable compounds.

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Supplementary material:

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ISBN 951-42-8079-2 (Paperback)
ISBN 951-42-8080-6 (PDF)
ISSN 0355-3213 (Print)
ISSN 1796-2226 (Online)