Chemical investigations of biotransformed *Rhodiola rosea* callus tissue

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Summary

The main aim of this study was to search the influence of exogenous addition of rosavin precursor: cinnamyl alcohol on the enhancing of rosavins content in callus culture of *R. rosea*, cultured on solid and liquid media (CCA). This is the first report – according to available literature – which concerns its biotransformation on solid medium conditions. The two strains of *R. rosea* tissue cultures showed different ability of cinnamyl alcohol glycosides production: both of them produced rosin (with or without supplementation), but the obtained level of rosavin production was notable higher in case of supplementation of the strain induced from axially buds of *R. rosea*. The exogenous cinnamyl alcohol was added into medium at concentration of 2.5 mM/L or 5 mM/L in the day of the inoculation. The application of 2.5 mM cinnamyl alcohol resulted in the increase of rosin content in the callus started from hypocotyle to very high levels: 1056.183 mg/100 g on solid medium and 776.330 mg/100 g in liquid medium. The content of rosavin showed the same growing tendency, but the final concentration of this phenylopropanoid in the supplemented callus tissue was about 7 times lower as compared to the roots of intact plant (63.603 mg/100 g). Addition of cinnamyl alcohol also enhanced rosarin biosynthesis but only in small amount: to 4.896 mg/100 g on solid medium. Callus tissue obtained from axially buds and treated by cinnamyl alcohol (2.5 mM) produced rosavin in a higher concentration: 92.801 mg/100 g and reached one fifth part of the amount produced by roots. The process of supplementation with cinnamyl alcohol influenced the enhanced biosynthesis of another bioactive substances as well (salidroside, tyrosol, chlorogenic acid). The obtained results confirmed that

even on a solid medium the callus tissue can produce the characteristic active substances and the concentration of some of them, mainly rosin and rosavin, can be significantly improved by addition of the precursors to the medium.

Key words: *Rhodiola rosea L., biotransformation, callus cultures, Compact Callus Aggregate (CCA), salidroside, rosavins, cinnamyl alcohol, precursors*

INTRODUCTION

Roseroot (*Rhodiola rosea* L., Crassulaceae), a herbaceous perennial plant, grows in the Arctic and in the mountain regions of Asia, North America and Europe. *R. rosea* is a popular plant in traditional Russian and Asian medicine, used for enhancing the physical and mental abilities of human body [1].

The roots of *R. rosea* contain a range of biologically active compounds: phenylpropanoids – rosavin, rosarin, rosin [2, 3] (rosavins are compounds specific for this species of *Rosea* genus), phenolic compounds – salidroside, tyrosol [3, 4], flavonoids – rodionin, rodiolin, rhodiosin, acetylrodalgin and tricin [5], phenolic acids – gallic acid, chlorogenic acid, hydroxycinnamic acid [1, 6], monoterpenes [1], β-sitosterol, daukosterol [7], tannins [1], fatty acids [8], cyanogenic glucoside – lotaustralin [9] and essential oils – n-decanol, geraniol [10].

Rosavins were isolated from the rhizomes of *R. rosea* by Zapesochnaya and Kurkin in 1983 [11]. Later Kurkin et al. [12] concluded that the cinnamyl alcohol glucosides – rosin, rosavin rosarin (fig. 1) – occurred only in this species of *Rhodiola* genus. Rosavins are formed from phenylalanine *via* cinnamyl alcohol [13]. Rosavin (aglicon + glucose) is the simplest glycoside of *R. rosea* (fig. 2) and by connection with other sugars rosarin or rosin can be formed [14].

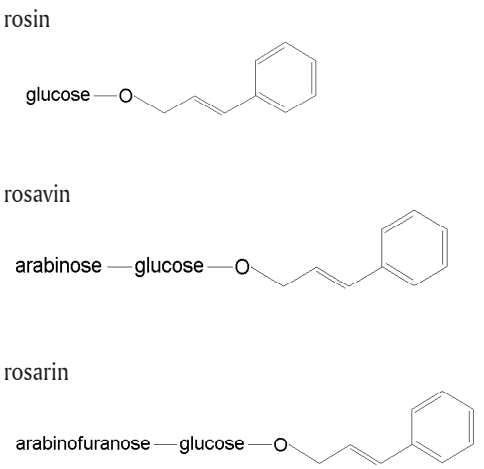

Figure 1. Rosavins from *Rhodiola rosea* L.

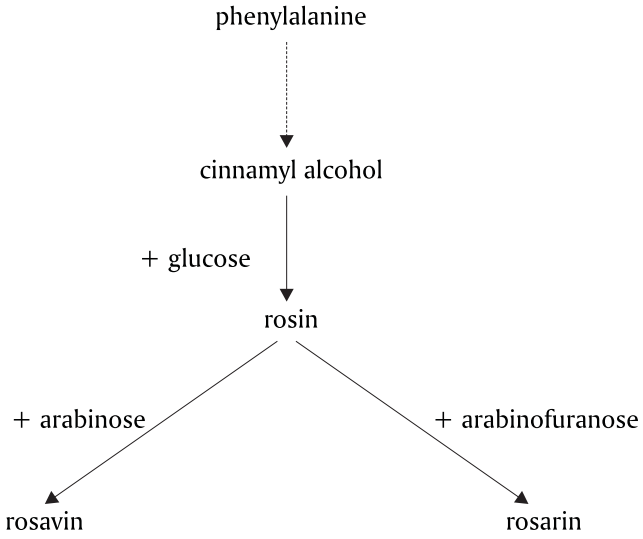

Figure 2. The pathway of cinnamyl glycosides biosynthesis (according to György, 2006)

This plant is attributed to physiological and pharmacological activities influencing central nervous system (CNS): stimulating CNS activity [15-17], enhancing physical and mental work performance [18-20], eliminating fatigue and adaptogenic activity [21-23]. Some of these activities have been proved in pharmacological and clinical studies [19, 20]. Salidroside, tyrosol and phenylopropanoids (rosavins) are pointed to be responsible for the CNS-stimulating and adaptogenic properties. This kind of psychostimulating activity is the most interesting aspect of the plant and arouse the growing interest in pharmaceutical industry. Investigations on the other activities like cardioprotective [25], anticancer [26, 27], antioxidant [4, 28, 29] and antimicrobial properties [30, 31] were carried out in more cases only in animals.

The biotechnological investigations on *R. rosea* biotransformation *in vitro*, one of the most effective methods of enhancing the content of biological active substances, have been carried out in Poland by Furmanowa et al. [32-35] and by Krajewska-Patan et al. [36, 37] as well as in Finland by György et al. [13, 14, 38, 39]. Their investigations confirmed that addition of the precursors of biosynthesis (cinnamyl alcohol, p-tyrosol) might significantly increase the content of rosavins [13, 14, 32, 34, 38, 39] or salidroside [14, 35, 36] in callus tissue and compact callus aggregates cultures of *R. rosea*.

The main goal of this research, as the continuation of previously published investigations on supplementation tissue cultures with p-tyrosol [36, 37], was to search the influence of exogenous addition of rosavin precursors, cinnamyl alcohol as the aglicon and glucose, on the enhancing of rosavins content in the tissue culture of *R. rosea*, cultured on solid and liquid media.

MATERIAL AND METHODS

The callus tissue cultured on solid medium and compact callus agregate (CCA) were used in the experiments. The plant material was originated from the Garden of Medicinal Plants of the Research Institute of Medicinal Plants in Poznań. The callus was obtained from hypocotyls of the seedlings or axially buds of intact plant and cultured on Murashige-Skoog (MS) medium [40] supplemented with α-naphtaleneacetic acid (NAA), benzyladenine (BA) and adenine chloride (solid media and CCA on the shaker).

The culture of CCA was initiated from callus cultured on solid media. The callus clumps (about 20 g, age of culture: 4 weeks) were transferred into 50 ml of liquid medium (MS supplemented with α-naphtaleneacetic acid (NAA), benzyladenine (BA) and adenine chloride) of 250 ml flask and shaken at 110 rpm on rotary shaker. The liquid culture was subcultured every 14 days.

The exogenous trans cinnamyl alcohol (Fluka Sigma) was added to callus culture to enhance the production of rosavins via biotransformation. The sterile filtred cinnamyl alcohol (in methanol solution) was added to medium at a concentration of 2.5 mM/L or 5 mM/L into solid and liquid medium in the day of the inoculation.

The callus was collected and air-dried (25°C) on the 7th day following biotransformation. The fresh and dry biomass was measured. The content of active compounds was determined in callus tissue after biotransformation, in callus without addition of precursor and in the roots of plants cultured *in vivo* in the Garden of Medicinal Plants of the Research Institute of Medicinal Plants in Poznań (RIMP). The obtained results were calculated as the average value of three repetitions of each trial.

CHROMATOGRAPHICAL PROCEDURES

The contents of rosavins (rosarin, rosavin, rosin), salidroside, p-tyrosol, chlorogenic and gallic acids were determined in the callus treated with cinnamyl alcohol, in the control tissue (with no addition of cinnamyl alcohol) and in the root of intact plant. In this study HPLC method was used. For HPLC analysis the samples (1 g each) were extracted with 20 ml of 70% methanol at boiling point for 15 min. Extraction was repeated three times with 70% aqueous methanol. HPLC analysis was performed on Hewlett Packard Agilent 1100 HPLC system, equipped with photodiode array detector. For all separation a Lichrospher 100 RP 18 column (250 x 4 mm, Merck) was used. The mobile phase consisted of 0.2% phosphoric acid in water (A) and acetonitrile (B), applied in the gradient elution (tab. 1).

Table 1.

Scheme of gradient elution of extracts from *R. rosea*

time [min]	0.2% H_3PO_4 aq [%]	CH_3CN [%]
0.00	95.0	5.0
30.00	80.0	20.0
35.00	80.0	20.0
40.00	20.0	80.0
56.00	20.0	80.0
60.00	95.0	5.0
70.00	95.0	5.0

The following rate was adjusted to 1 ml/min., the wavelength detection set to DAD at λ=205.5 nm, 220.4 nm and 254.4 nm. Then 20 μL of each sample was injected. All separations were performed at the temperature of 24°C. Peaks were assigned by spiking the samples with standard compounds (from Chromadex) and compared with the UV-spectra and retention times.

RESULTS

The content of active substances in callus tissue of *R. rosea* with and without precursor addition was searched in comparison to root extract (table 2, 3). Cinnamyl alcohol was added to culture medium in concentration of 2.5 and 5.0 mM, but only the supplementation with lower quantity of precursor resulted in enhancing of rosavins production (table 2.).

Table 2.

Influence of cinnamyl alcohol addition on the active substances contents in the callus tissue of *R. rosea* (hypocotyle strain) cultured on solid medium and liquid medium on shaker

R. rosea tissue	content of active substances (mg/100 g of dry weight)		
	rosarin	rosavin	rosin
on solid medium – control	0.400	1.413	16.198
in solid medium + CA* 2.5 mM	4.896	48.662	1056.183
in solid medium + CA* 5 mM	0.811	12.307	4.510
in shaker – control	2.761	1.726	8.933
in shaker + CA* 2.5 mM	1.243	63.603	776.330
intact plant - roots	82.052	456.650	33.631

* – cinnamyl alcohol

The application of 2.5 mM cinnamyl alcohol resulted in the increase of rosin content in the callus, starting from hypocotyle to very high levels: of 1056.183 mg/100 g on solid medium and of 776.330 mg/100 g in liquid medium, which is 30 times higher than in the root of intact plant of *R. rosea*, culturated in Polish climatic conditions. The content of rosavin showed the same growing tendency,

but the final concentration of this phenylopropanoid in the supplemented callus tissue was about 7 times lower in comparison to roots (63.603 mg/100 g). Addition of cinnamyl alcohol has also enhanced rosarin biosynthesis but only in small amount – up to 4.896 mg/100 g on solid medium.

Callus tissue obtained from axially buds of *R. rosea* plants, supplemented with cinnamyl alcohol showed similar tendency in rosavins biosynthesis. However, the rosin content did not achieve the above mentioned level (tab. 3). It is worth emphasizing that rosavin production was higher in this callus tissue then in the strain mentioned as the first one: after 7 days of biotransformation reached 92.801 mg/100 g –one fifth part of the amount produced by roots.

Table 3.

Influence of cinnamyl alcohol addition on the active substances contents in the callus tissue of *R. rosea* – axially buds strain – cultured on solid medium and liquid medium on the shaker

R. rosea tissue	content of active substances (mg/100 g of dry weight)		
	rosarin	rosavin	rosin
on solid medium – control	0.443	4.606	7.934
on solid medium + CA* 2.5 mM	3.650	92.801	850.076
intact plant – roots	82.052	456.650	33.631

* – cinnamyl alcohol

The process of supplementation with cinnamyl alcohol influenced the biosynthesis of another bioactive substances as well. The concentration of salidroside reached the level of 112.246 mg/100 g (hypocotyle strain solid medium), similarly to intact plant. Likewise, p-tyrosol content was up to 14 times higher than in the intact plant (tab. 4). Chlorogenic acid content grown rapidly, especially in the callus from axially buds in solid medium, to about 50 times higher level (49.895 mg/100 g) as compared to the intact plant (tab. 5). The influence on the other active ingredients contents was not noticed.

Table 4.

Influence of cinnamyl alcohol addition on the active substances contents in the callus tissue of *R. rosea* – hypocotyle strain – cultured on solid medium and liquid medium in the shaker

R. rosea tissue	content of active substances (mg/100 g of dry weight)			
	salidroside	tyrosol	gallic acid	chlorogenic acid
on solid medium – control	13.812	42.225	5.623	1.101
on solid medium + CA* 2.5 mM	112.246	85.353	3.708	24.493
on solid medium + CA* 5 mM	1.805	6.961	8.279	2.078
in shaker – control	5.814	69.237	2.761	0.695
in shaker + CA* 2.5 mM	69.621	141.982	1.243	17.207
intact plant – roots	128.312	5.894	82.052	0.945

* – cinnamyl alcohol

Table 5.

Influence of cinnamyl alcohol addition on the active substances contents in the callus tissue of *R. rosea* – axially buds strain – cultured on solid medium and liquid medium on the shaker

R. rosea tissue	content of active substances (mg/100 g of dry weight)			
	salidroside	tyrosol	gallic acid	chlorogenic acid
on solid medium – control	10.488	42.611	1.673	1.360
on solid medium + CA* 2.5 mM	57.969	29.175	4.028	49.895
intact plant – roots	128.312	5.894	42.177	0.945

* – cinnamyl alcohol

DISCUSSION

This article reports the investigations on *R. rosea* biotransformation in solid and liquid media. According to authors' knowledge, presented results on *R. rosea* supplementation is the first report about the biotransformation on solid medium conditions. Previously the problem of biotransformation capacity of *R. rosea* tissue *in vitro* (with use of cinnamyl alcohol) was investigated by some scientific groups only in liquid medium [13, 14, 34, 38]. The addition of trans cinnamyl alcohol to cell suspension cultures resulted with rosavin production enhanced to 1.01 % of dry weight– previously no rosavins were detected in the tissues [34]. The cultures of compact callus aggregates (CCA), studied by Finnish group, produced mainly rosin. When cinnamyl alcohol was added to the liquid medium the highest rosin content in dry weight was 1.25%; the rosavin content was only 0.083% of dry weight [13, 36]. None secondary metabolites were formed in non-treated cultures [38]. Moreover, the beneficial effect on cinnamyl alcohol derivatives were detected in CCA cultures supplemented with glucose only (instead of sucrose) [39].

It is worth pointing that in the presented trials on solid medium the callus tissue of *R. rosea* produced all spectrum of active compounds of the intact plant – salidroside, p-tyrosol, rosavins, chlorogenic and gallic acids. This ability of rosavins production without artificial supplementation was detected in CCA cultures as well, opposite to the cultures performed by György et al. [14], where rosavins were not produced without supplementation. First of all, the process of exogenous addition of cinnamyl alcohol on solid medium enhanced the rosin production up to 1.06% of dry weight (in comparison to 0.03% produced by intact plant). Rosin production was also observed in CCA cultures but the reached level was lower. Another glycoside, rosavin, was produced in considerable less amount. This tendency to synthesize mainly the rosin, the simplest glycoside, was also noticed by György et al. [14] in CCA cultures. This is on the contrary to supplemented suspension cultures of *R. rosea* obtained by Furmanowa et al. [34], where the tissues produced rosavin in high amount: 1.1% of dry weight. Finnish authors [14] suggested, that this phenomenon is probably connected with glucose and other sugars presences.

The ability to produce cinnamyl alcohol glycosides of obtained two strains of *R. rosea* tissue cultures differed. Both produced rosin, with or without supplementation, but the obtained level of rosavin was notable higher in the case of supplementation of the strain induced from axially buds of *R. rosea*.

The obtained results confirmed that even on a solid medium the callus tissue can produce the characteristic active substances and the concentration of some of them, mainly rosin and rosavin, can be significantly improved by addition of the precursors to the medium.

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BADANIA CHEMICZNE TKANEK KALUSOWYCH RÓŻEŃCA GÓRSKIEGO (*RHODIOLA ROSEA* L.) PODDANYCH BIOTRANSFORMACJI

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S t r e s z c z e n i e

Celem prowadzonych prac było określenie wpływu alkoholu cynamonowego (prekursor rozawin) dodanego do pożywki na produkcję rozawin w kulturach kalusowych *Rhodiola rosea* L. na pożywkach stałych i płynnych (agregaty CCA). Jest to pierwsze – według dostępnej literatury – doniesienie dotyczące biotransformacji na pożywce stałej. W badaniach posłużono się dwiema liniami różeńca górskiego, które wykazywały różną zdolność do produkcji glikozydów alkoholu cynamonowego. Obie linie produkowały rozynę (niezależnie od suplementacji), lecz w wyniku biotransformacji linia otrzymana z pączków bocznych syntetyzowała rozawinę w znacznie większym stopniu. Alkohol cynamonowy dodawano do pożywek w stężeniach: 2,5 mM i 5 mM w pierwszym dniu hodowli. Po dodaniu 2,5 mM alkoholu cynamonowego do pożywki stwierdzono bardzo wysoki wzrost zawartości rozyny w kalusie pochodzącym z hypokotyla (1056,183 mg/100 g) na pożywce stałej i płynnej (776,330 mg/100 g). Zaobserwowano również wzrost stężenia rozawiny, ale ostateczne stężenie tego fenylopropanoidu w tkance kalusowej było około 7 razy niższe niż w korzeniach roślin gruntowych (63,603 mg/100 g). Dodatek alkoholu cynamonowego zwiększał również zawartość rozaryny, lecz w niewielkim stopniu: 4,896 mg/100 g. Największe stężenie rozawiny otrzymano w kalusie z linii hypokotyl (92,801 mg/100 g) traktowanym 2,5 mM alkoholem cyna-

monowym, co odpowiada jednej piątej zawartości rozawiny produkowanej w korzeniach roślin gruntowych. Biotransformacja alkoholem cynamonowym wpłynęła na wzmożoną biosyntezę innych związków czynnych, takich jak salidrozyd, tyrozol i kwas chlorogenowy. Otrzymane wyniki potwierdzają, że kalus rosnący na pożywce stałej jest zdolny do produkcji związków czynnych charakterystycznych dla gatunku, a większe stężenie niektórych z nich, głównie rozyny i rozawiny, można uzyskać poprzez dodanie prekursorów do pożywki.

Słowa kluczowe: *Rhodiola rosea L., biotransformacja, kultury kalusowe, kultury kalusowe agregatów (CCA), salidrozyd, rozawiny, alkohol cynamonowy, prekursory*