STUDY OF BIOTRANSFORMATION COMPOUNDS IN CALLUSAR CULTURE OF *RHODIOLA ROSEA* SPECIE

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Abstract

Rhodiola rosea L. is a well known species of plants, which has been used medicinally for decades, but study of its pharmacological effects and the compounds responsible for it use still continues. We present data about induced accumulation of secondary metabolites and as well the results of biotransformation of cinamic alcohol in callus culture of *R. rosea* of Carpathian origin under the influence of same stress factors. The presence of secondary metabolites was investigated by HPLC-MS analysis. The obtained results can be used for selection of valuable genotypes and their future cultivation in artificial conditions.

Key words: *Rhodiola rosea* L., callus culture, cinnamyl alcohol, HPLC-MS analysis.

Plants, due to the content of nutritive and medicinal compounds, are widely used in the food industry, pharmaceutics and cosmetics. They represent important sources of secondary metabolites which are used in medicine. It should be mentioned, that often the content level of secondary compounds in plants is low and dependent on the plant growth stage and pedo-climatic conditions. Uncontrolled plant collection from spontaneous flora for medicinal purposes damages the ecological situation and put under risk the existence of some precious species. One of the ways to solve this problem can be utilization of *in vitro* method for cultivation and propagation of medicinal plant cells and organs.

Golden root (*Rhodiola rosea* L.) represents a perennial herbaceous plant, which due to its precious properties, is under the risk of disappearance in many places of the World. *R. rosea* is dioic specie and grow in spontaneous flora in the Northern Asia, Northern and Central Europe, inclusive Carpathian Mountains from Romania.

The active components, typically for *R. rosea* specie, are, mostly, accumulated in rhizomes. In extracts obtained from rhizomes active compounds were indentified, belonging to different classes of chemical compounds: phenylpropanoids, phenylethanoids, proantocyanidine, monoterpens, flavones, tannins and organic acids (Kurkin VA, Zapesochanaya GG., Rohloff J., 2002). It was established that curative properties of the mentioned specie are due to the presence and contents of compounds as phenylpropanods (rosavin, rosin, rosarin), rosiridin, salidrozid and p-tirosol. Remarkable that only *R. rosea* specie of about 200 spaces of *Rhodiola* genus contains rosavin and rosarin. The quality of *R. rosea* extracts are standardized by content of rosavin and salidrozid (Kurkin VA, Zapesochanaya GG, 1990). Salidrozid can be found in other species of *Rhodiola* genus and for this reason it can't be used as a quality marker of *R. rosea* specie (Kiryanov A.A., 1991).

The contents of secondary metabolites, isolated from rhizomes collected in Carpathian Mountains (Romania) and offered by individual cultivators from Ukraine, were analyzed under the international project (Toma S., 2003-2005). The comparison of extracts obtained from both sources showed that rhizomes form Carpathian Mountains contain about 6% of extractive compounds reported to their dry mass (Dascaliuc A., Calugaru-Spataru T., Ciocarlan A. *et al.*, 2006, 2008), and these data are in accordance with those obtained for rhizomes collected in Altai Mountains (Kurkin VA., 1990). On contrary extracts obtained from rhizomes grown in artificial conditions didn't contain specific active compounds for *R. rosea* specie.

Thought the information on Golden root cultivation exists for about 100 years, nowadays *R. rosea* plantations are practically absent and natural

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resources can't satisfy demands of the market. Taking into consideration that *R. rosea* rhizomes accumulate the maximal contents of secondary metabolites at 4th year of the cultivation, and during of this period their harvest can be compromised by different factors (including absent of well elaborated agrotechnical conditions), we proposed to accelerate the process of secondary metabolites manufacturing using biotechnological method. Investigations on biotechnological transformation of metabolites in callous culture of *in vitro R. rosea* are important for determination of factors that can influence the accumulation of active compounds in spontaneous plants. Such kinds of research for *R. rosea* specie were carrying out in Finland (György et al. 2004, György et al. 2005) and Poland (Krajewska-Patan A., 2007). Their studies established that supply of culture media with some precursors conducts to the increase of active compound accumulations in cultivated *in vitro* cells.

Samples of dry callus and rhizomes (2 g) were extracted with methanol in 1:20 ratio, at room temperature over 24 hours, extracts were filtered and solvent removed at the reduced pressure. The separation and identification of secondary metabolites from callus and rhizomes of *R. rosea* was effectuated by HPLC-ESI-MS method, using Agilent 6500 Series Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS system.

MATERIAL AND METHOD

Biologic material was obtained from seeds collected from plants growing in spontaneous flora of the Carpathians massive Ineu (Romania) and Svidovet (Ucraina) in August, 2005.

Leaves from sterile plants of *R. rosea*, grown up from seeds were used for callus induction. Foliar segments of 0,3-0,5 cm length were inoculated on MS nutritive medium (Murashige T., Skoog F., 1962). Authors (Furmanowa, M., György Z., Krajewska-Patan A.) mentioned that *in vitro* conditions of biosynthesis and accumulation of secondary metabolites are more intensive in cell culture aggregates cultivated in liquid media. Taking in account this fact, we decided to induce and maintain callus culture aggregates (CCA) in liquid medium, using callus obtained before from sterile plants of *R. rosea* (Calugaru T., Malinoc A., 2004). Callus fragments of 0,3-1 cm were inoculated in MS medium supplemented with 1,5 mg l^{-1} BA, 0,5 mg l^{-1} ANA, 30g l^{-1} sucrose, without agar. Before sterilization, the pH value was adjusted to 5,8 with HCl (1N) solution. The cultivation vessels were placed in a shaker at 120 R/min. The callus was grown at 26^0C and a photoperiodic period of 16 h of illumination (1000 lux intensity) and 8 h of darkness. Aliquots of cellular suspension were transferred on fresh MS medium every 10 days of cultivation for maintenance the cell suspension viability.

Taking in account natural conditions that *R. rosea* plants are subjected to us tested the influence of ultraviolet (UV) radiation, low temperature and elicitor (cinnamyl alcohol) on secondary metabolites accumulation according to the following scheme:

1. The combination UV light (280-320 nm) and cinnamyl alcohol, starting with 12th day after cultivation by 30 min irradiation during 20 days.
2. The combination of low temperature and cinnamyl alcohol, CCA were kept starting with 12th day after cultivation at $+4^0$C for 30 min during 20 days.
3. The influence of elicitor (cinnamyl alcohol), CCA was grown in common nutritive medium, supplemented with cinnamyl alcohol at 2 mM concentration. Its biochemical analysis was carrying out in 2 weeks.
4. The combination of cinnamyl alcohol, low temperature and UV light in according with method 3, applying supplementary methods 1 and 2.

The extracts from callus and rhizomes were redissolved in methanol and analyzed by HPLC method, using an *Agilent 1200 Series* system chipped with a solvent degases, a binary pump, an automatic injection system, an revere phase (RP) chromatographic column (Agilent 300 Extended C_{18}, 4.6 x 150 mm, 5 µm) and a diode array detector (UV-VIS DAD). The optimal conditions for a well separation were: room temperature, 10 µl injection volume, 1mL/min solvent debate (with a splitter 9:1 for ESI-MS), elution in gradient: 0-5% B, 0-10 min; 5-40% B, 10-32 min; 40-100%, 32-45 min; then 100% B till 55 min, came-back to 0% B in 5 min and column equilibration at 0% B in 10 min, where (A) is a mixture of 89% water, 10% acetonitrile and 1% of formic acid; (B) 100% methanol. The solvents were filtered and degassed before use. The separation process was monitored by UV-VIS DAD detector at 254 and 280 nm; The LC system was connected directly to ionization source *via* mass spectrometer electro spray (ESI). The Q/TOF MS selected conditions were: ESI in a negative mod, drying gas debit (N_2) 7L/min, gaz temperature 250ºC; nebulizer pressure 35 psig, capillary voltage 4200 V; fragmentation voltage 200 V; compounds were investigated in a field of *m/z* 100–1500. Data were registered and processed using Mass Hunter Workstation software.

RESULTS AND DISCUSSIONS

The present paper present the results of qualitative analysis of the secondary metabolites content in samples of callus and rhizomes of *R. rosea* collected from spontaneous flora of

Carpathian Mountings (Ineu mountain, Romania) using HPLC-MS method.

Initially, 5 compounds typical for *R. rosea* specie (salidrozid, tyrosol, rosavin, rosin and rosaridin) were studied by HPLC-ESI-MS method, to established retention times, structure elucidation and molecular mass evaluation.

The chromatogram of extract, obtained from Carpathian rhizomes, registered at λ = 254 nm, is presented in figure 1 and includes 13 well defined signals.

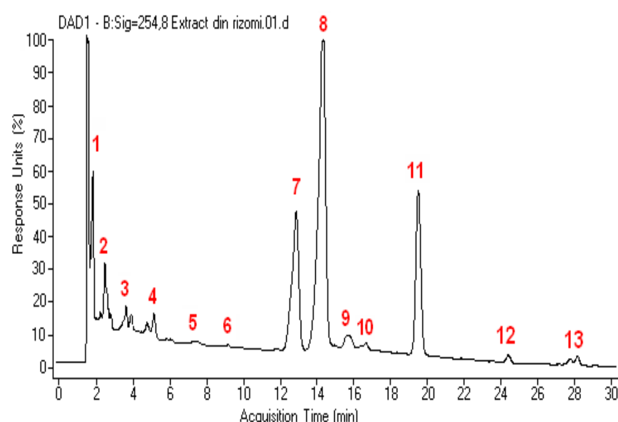

Figure 1 **The HPLC chromatogram of extract from rhizomes.**

The comparison of retention times of extract obtained from rhizomes with those of the marker and their mass spectra (MS) analysis gave the possibility to attribute the signals as follow: **1** - gallic acid (Rt=1.8 min, m/z=169, $(M-H)^-$), **2** - salidrozid (Rt=2.5 min, m/z=299 $(M-H)^-$, m/z=345 $(M-HCOO)^-$, m/z=599 $(2M-H)^-$), **3,4** - 4-methoxy cinnamyl-(6´-O-α-arabinopyranosyl)-O-βglucopy-ranoside (Rt=4.2 and 5.1 min, m/z=457 $(M-H)^-$, m/z=915 $(2M-H)^-$), **5,6** – derivatives of compounds **3,4** type (Rt=8.9 min, m/z=491 and 10.2 min m/z=269, 711), **7** – rosarin (Rt=12.8 min, m/z=473 $(M-HCOO)^-$, m/z=855 $(2M-H)^-$), **8** – rosavin (Rt=14.2, m/z=427 $(M-H)^-$, m/z=473 $(M-HCOO)^-$ m/z=855 $(2M-H)^-$), **9** - cinnamyl-(6´-O-β-xylopyranosyl)-O-β-glucopyranoside (Rt=14.6 min, m/z=427 $(M-H)^-$, m/z=473 $(M-HCOO)^-$, m/z=855 $(2M-H)^-$), **10** – rosiridin (Rt=16.2 , m/z=377 $(M-HCOO)^-$, m/z=663 $(2M-H)^-$), **11** – derivative of compounds **3,4** type (Rt=19.3 min, m/z=469 $(M-H)^-$, m/z=939 $(2M-H)^-$), **12, 13** – derivatives of compounds **3,4** type (Rt=24.3 and 28.2 min, m/z=173, 287, 455)**.**

The chromatograms of extracts obtained from samples of *R. rosea* callus, which were treated by UV irradiation, low temperature and elicitor are presented in figure 2 (chromatograms A, B, C and D).

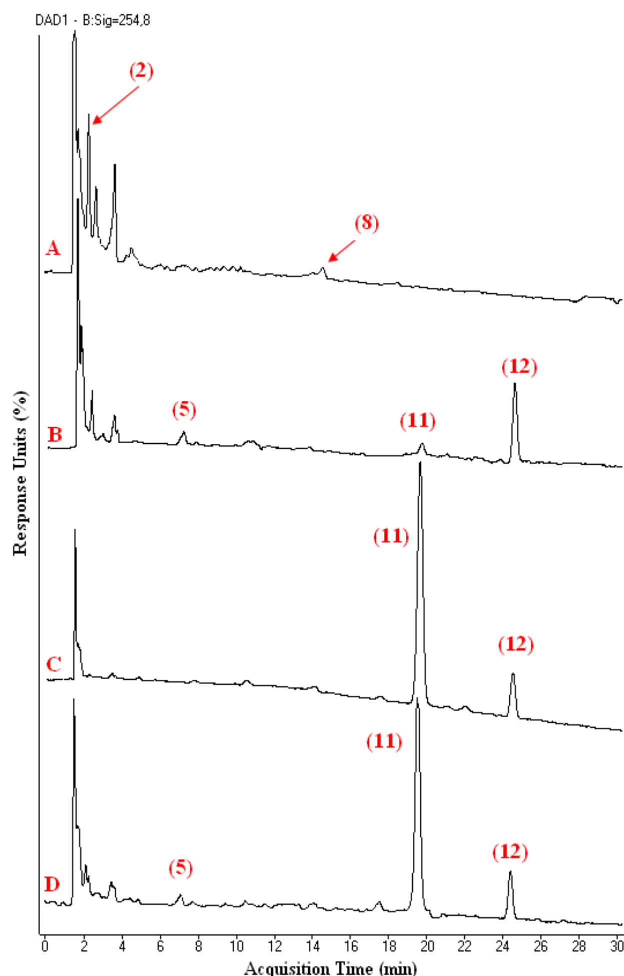

Figure 2 **The chromatograms of extracts from callus, where A - marker, B – sample after UV irradiation, C – sample under low temperature, D – sample treated with UV irradiation, low temperature and elicitors.**

It is well seen that in the chromatogram of the CCA extract – marker *(fig.2A)*, there are a lower number of signals of small intensity in comparison with sample obtained from rhizomes. It is important the presence of salidrozid **1** and rosavin **8,** typically for *R. rosea* specie compounds. The low temperature application and presence of cinnamyl alcohol in CCA *(fig.2C)*, favorites synthesis as of salidrozid and rosavin, and appearance of new compounds **11**, **12** with m/z=469 and 455, which, according to mass spectra can be 4-methoxy-cinnamyl-(6´-O-α-arabinopyranosyl)-O-β-glucopyranoside type derivatives.

The influence of UV light and presence of cinnamyl alcohol changed substantially the chemical composition of callus culture *(fig.2B)*. On chromatogram appeared a new compound **5** with m/z= 491, a derivative from the same series with compounds **11** and **12**. The combination of three factors: cinnamyl alcohol, UV radiation and low temperature *(fig.2D)* also lead to the synthesis of mentioned for all samples compounds. It is

necessary to mention high intensity of component **11** in comparison with others.

CONCLUSIONS

So, for the first time it was experimentally demonstrated that low temperatures and ultraviolet irradiation are important factors that cause the accumulation of secondary metabolites in cells of *R. rosea*. Utilization of these factors, in combination with elicitors, increases the content of secondary metabolites in artificial culture of *R. rosea.*

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