

PRODUCTION OF THE PHENOLS SALIDROSIDE AND
ROSAVINS IN *RHODIOLA ROSEA* REGENERANTS EX
VITRO ADAPTED TO NATURAL CONDITIONS

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Received on April 12, 2022

Presented by I. Ivanov, Member of BAS, on June 21, 2022

Abstract

Rhodiola rosea L. is a high-altitude medicinal plant with various health-beneficial effects, associated mainly with the phenolic substances salidroside and rosavins (rosavin, rosin and rosarin). The intensive collection of *R. rosea* rhizomes and roots to be used in folk medicine and pharmacy has led to a decrease of the species natural resources. New opportunities for species conservation and accelerated synthesis of biologically active substances have been provided by various systems for in vitro and ex vitro cultivation. In this study, the salidroside and rosavins content was determined using HPLC analysis in in vitro cultures of *R. rosea* and further monitored in adapted regenerants grown ex vitro. The targeted secondary metabolites were not detected in the calli and in the roots of 1-month-old regenerants obtained on different nutrient media. The roots/rhizomes of adapted regenerants grown ex vitro in a greenhouse for 1 year and in the mountain for 1, 2 and 3 years produced increasing amounts of salidroside (from 0.4 to 1.2%) and rosavins (from 4.3 to 4.5%). The salidroside to rosavins ratio (1:3.75) in 3-year-old regenerants grown in the mountain was close to the pharmacopoeial standard ratio of 1:3. These results show that *R. rosea* plants, obtained by in vitro micropropagation and grown ex vitro are promising for drug production.

Key words: ex vitro, HPLC, in vitro, medicinal plant, *Rhodiola rosea*

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DOI:10.7546/CRABS.2023.09.06

Abbreviations: BAP – 6-Benzylaminopurine, BAS – Biologically active substances, CAS – Casein hydrolysate, GA₃ – Gibberellic acid, IAA – Indole-3-acetic acid, IBA – Indole-3-butyric acid, 2,4-D – 2,4-Dichlorophenoxyacetic acid.

Introduction. *Rhodiola rosea* (Golden root, Roseroot) is a perennial high mountain medicinal plant, endangered and protected by law in many countries. Golden root is valuable for the adaptogenic, antidepressant, anticancer, cardio protective and neuroprotective effects of its valuable secondary metabolites [1]. The Golden root constituents currently selected for standardization of extracts for therapeutic activity are the phenolic substances salidroside (rhodioloside) and rosavins (a collective name for rosavin, rosin and rosarin), mostly accumulated in the roots and rhizome [2].

Due to overharvesting and poor reproduction in nature, artificial cultivation has been attempted to promote the production of Golden root. However, a real obstacle are the low propagation rates, the late start of synthesis of the biologically active substances (BAS) (at 5th to 7th year), low biosynthetic levels and slow accumulation of metabolites [3–6]. Moreover, these substances are less synthesized in field-cultivated plants compared to those grown naturally at high altitude [2].

Plant biotechnology provides new opportunities for faster cloning and conservation of endangered species, as well as for alternative synthesis of their BAS [7]. In particular, for *R. rosea* various systems for in vitro cultivation and manipulations, and secondary metabolites production in callus and cell cultures have been developed [2,3,8–10]. Complete protocols for callus induction, plantlet micropropagation and adaptation of the regenerants ex vitro and in wild conditions in *R. rosea* were established [11,12].

The potential of plant biotechnology for secondary metabolites production could be assessed by accurate determination of the BAS in in vitro obtained cultures of *R. rosea*. Complete and precise phytochemical studies of wild grown *R. rosea* plants has been performed using high performance liquid chromatography (HPLC)-, gas chromatography-mass spectrometry (GC-MS)- and nuclear magnetic resonance (NMR)-based metabolomics [1]. To the best of our knowledge, there are no reports analyzing changes in the accumulation of BAS in in vitro obtained *R. rosea* plants grown ex vitro and introduced in nature.

The goal of this study was to determine the salidroside and rosavins content in in vitro obtained cultures of *R. rosea* (calli and plantlets), in regenerants cultivated in greenhouse, and in those introduced in the mountain using HPLC technique.

Materials and methods. Plant material and in vitro cultivation. Plant material (seeds in the year of their formation) was collected from 2–3-years old plants naturally grown in the Rhodope Mountains, Bulgaria and used for establishment of in vitro cultures using the previously described protocol [11]. Plant propagation was achieved on two MURASHIGE and SKOOG (MS) [13] nutrient

media: P1 – 1.0 mg/l zeatin, 0.1 mg/l IAA, 0.4 mg/l GA3, 400 CAS; P2 – 2.0 mg/l zeatin, 0.2 mg/l IAA and rooting on MS media containing 0.2 mg/l IAA, 2.0 IBA, 0.4 mg/l CAS. The regenerants were adapted and cultivated in a greenhouse (at 560 m altitude) and in the experimental fields in the Rhodope Mountains, Bulgaria (at 1600 m altitude).

Calli were obtained from leaves excised from in vitro propagated plants using the protocol developed by us [11] and cultivated on 5 selected MS nutrient media: C1 – 1.0 mg/l BAP, 0.5 mg/l 2,4-D; C2 – 0.5 mg/l BAP, 1.0 mg/l 2,4-D; C3 – 1.0 mg/l BAP, 1.0 mg/l 2,4-D, 1000 mg/l CAS; C4 – 1.0 mg/l BAP, 0.5 mg/l 2,4-D, 1000 mg/l CAS; C5 – 0.1 mg/l 2,4-D, 0.2 mg/l IAA, 1000 mg/l CAS.

The sucrose was 30 g/l for propagation media and 20 g/l for callus and for rooting media. Content of agar-agar was 6.0 g/l. The culture media were autoclaved at 1.1 kg.cm⁻², 121 °C for 20 min. The cultivation conditions, morphological description and measurements followed the protocol published in our previous studies [11,12].

Phytochemical analysis. *Plant samples* Salidroside and rosavins (rosavin, rosin and rosarin) were quantitatively determined in: (i) calli cultivated on C1, C2, C3, C4, and C5 media; (ii) roots from 1 month-old in vitro regenerated plants cultivated on P1 and P2 media, and transferred for root formation on R media; (iii) roots and rhizomes from in vitro regenerated plants grown for 1 year in a greenhouse (R1yG); (iv) roots and rhizomes from in vitro regenerated plants grown for 1, 2 or 3 years in the mountain experimental field (R1yM, R2yM, R3yM, respectively). Roots and rhizomes from regenerated plants grown in a greenhouse and in the mountain were collected at the end of vegetation period as mixed samples from six male and six female plants. All types of samples were dried at room temperature.

Callus extraction. One hundred milligrams of fine powdered calli was extracted three times with 1 ml methanol. After 10 min centrifugation at 3000 rpm, the supernatants were combined and adjusted to the final volume of 1 ml with methanol.

Rhizome and root extraction. One gram of fine powdered root/rhizome was extracted three times with 3 ml of methanol by sonication for 10 min. After centrifugation at 3000 rpm for 10 min, the supernatants were combined in a 10 ml volumetric flask and adjusted to the final volume with methanol.

Analyses were performed on an Agilent 1100 Series HPLC system, equipped with a MWD UV-Vis detector. The column used was Chromsep SS (250 × 4.6 mm ID), precolumn Intertsil 5 ODS 2. The mobile phase was water (A) and acetonitrile (B) applied in the following gradient: from 92.5A:7.5B (v/v) in 45 min to 80A:20B (5 min isocratic elution). Each run was followed by a 15 min equilibration period. The flow rate was 1 ml/min, 10.0 µl of sample was injected. Salidroside was detected at 220 nm, while rosin, rosin and rosarin – at 254 nm. The reference standards of salidroside, rosin, and rosarin were purchased from Sigma-

Aldrich (MO, USA). We used these four compounds to a final concentration of 400 mg/ml to prepare calibration curves. Each calibration curve concentration was performed in triplicate. The limits of detection (LOD) and quantification (LOQ) were separately determined under the same chromatographic conditions at a signal-to-noise ratio (S/N) of 3 and 10, respectively.

Statistics. Statistical analyses for in vitro experiments were performed using Sigma Plot 12.5 (Systat Software Inc., SSI) and the data were expressed as means $\pm$ standard errors (SE). For phytochemical analysis every sample solution was injected in triplicate. The results are presented in percentage of dry weight (% DW) of the plant underground parts as an average of three replicates.

Results. BAS in in vitro cultures. Obtained calli cultures had different characteristics (texture and structure) and growth rate depending on plant growth regulators added to the media: C1 – fast growth, grain structure, compact, pale in colour; C2 – medium growth, 62% more compact calli, 32% looser tissue; C3 – medium growth, compact, grain, pale-yellowish-beige; C4 – fast growth, compact, grain, beige-green in colour; C5 – medium growth, grain, liquidy, pale beige in colour (Fig. 1). The callus induction ability ranged from 50 to 85%, and growth rate ranged from 3 to 5 folds (callus weight varied from 306 to 481 mg). HPLC analysis did not detect neither salidroside, nor rosavins in the calli produced on the all tested nutrient media, despite its different characteristics.

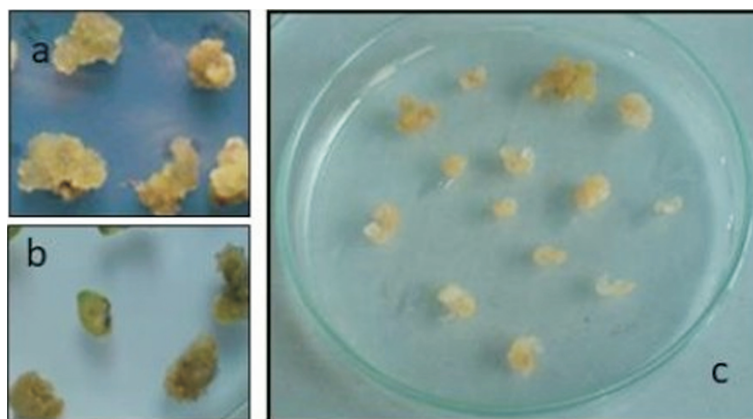


Fig. 1. Callus culture on different media (a) C1, (b) C4, (c) C5

The targeted secondary metabolites were not found also in the roots from regenerants obtained on different media predisposing vigorous formation of roots (Fig. 2), where mean number plantlets per explant was 4.53 ± 0.18 for P1 media and 6.83 ± 0.14 for P2 and root length was 2.58 ± 0.08 cm and 1.93 ± 0.05 cm, respectively.

BAS in regenerants grown ex vitro. Adapted regenerants grown ex vitro in a greenhouse for one year (R1yG) had well-developed root and rhizomes (Fig. 3) and produced the basic *Rhodiola* complex of substances (Table 1). The total



Fig. 2. In vitro propagated plants on P1 media



Fig. 3. Root and rhizomes of 1-year-old in vitro obtained plant, cultivated in greenhouse

amount of phenols was 4.7%, and the ratio salidroside to rosavins was estimated as 1:10.75.

In regenerants introduced and grown at high altitude in the mountain for one and two years (R1yM and R2yM), the total amount of phenols was higher (4.9%) due to a slight increase in the salidroside content compared to those cultivated in a greenhouse, while the amount of produced rosavins was the same, and their

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Content (% dry weight) of salidroside and rosavins in roots and rhizomes of *Rhodiola rosea* L. plants regenerated in vitro and adapted in a greenhouse and in the mountain

Biologically active substances	R1yG	R1yM	R2yM	R3yM
Salidroside	0.4	0.6	0.6	1.2
Rosarin	0.9	0.9	0.9	1.0
Rosavin	3.2	3.2	3.2	3.3
Rosin	0.2	0.2	0.2	0.2
Total amount of rosavins	4.3	4.3	4.3	4.5
Total amount of phenols	4.7	4.9	4.9	5.7
Ratio of salidroside to rosavins	1:10.75	1:7.17	1:7.17	1:3.75

R1yG – regenerants grown in a greenhouse for 1 year

R1yM, R2yM, and R3yM – regenerants grown in the mountain for 1, 2 and 3 years, respectively

ratio was estimated as 1:7.17 (Table 1). However, in the micropropagated plants grown for three years in the mountain (R3yM), a considerable increase in the accumulation of salidroside was recorded, coupled with a slight increase in the rosarin and rosavin content, so the total amount of phenols was 5.7%, and the ratio salidroside to rosavins was 1:3.75 (Table 1).

Discussion. Plant in vitro cultures can be an excellent technological platform for both species conservation and production of pharmaceutically valuable plant secondary metabolites [2].

The current study did not detect the *R. rosea* basic phenolic complex in the calli and plantlets obtained in in vitro cultures using the listed nutrient media. Earlier studies demonstrated production of rosavin (1.1% DW) in suspension calli cultures [3] and in calli from seedling hypocotyls and axillary buds with or without medium supplements [8]; compact callus aggregates produced mainly rosin [9].

However, the presented here HPLC analysis detected increasing amounts of phenolic substances in the ex vitro cultivated regenerants, the most considerable accumulation of salidroside being found in 3-year-old rhizomes/roots of plants introduced and grown at high altitude in the mountain (1.2% DW). The rosavins content in rhizomes of both the greenhouse grown and the mountain grown regenerants was high (4.3–4.5% DW), and the ratio of salidroside to rosavins changed from 1:10.75 in the greenhouse-cultivated regenerants to 1:3.75 in those grown in the mountain for three years. The salidroside content in 1- and 2-year-old regenerants grown in experimental field in the mountain recorded by the HPLC analysis in the current study confirmed the earlier reported results obtained spectrophotometrically for other ex vitro grown regenerants of *R. rosea* [11].

The reported here amounts of active compounds in the regenerants cultivated in the mountain are similar to those in plants propagated by the conventional method [4]. The authors showed that in vegetatively propagated plants, the salidroside content varied within the range of 0.2–1.5% DW in dependence of the duration of cultivation, plant sex, type of underground part (roots or rhizomes), and the phenological stage, while the total rosavins content varied from 1.5% in female rhizomes to 2.4% in male rhizomes. The detected in the current study salidroside amount of 1.2% in the mixed rhizomes/roots samples of 3-year-old plants (male and female) is close to the maximum content of 1.5% in 2–3-year-old rhizomes of male conventionally cloned plants, collected at the end of the vegetation period [4]. These results are promising and suggestive for even higher salidroside amounts if samples are collected from male rhizomes.

Generally, our data are in line with data on other wild ecotypes of *R. rosea*, originated from different countries, although large variation in the quantities of these active compounds was observed due to genetic differences, geographic origin, plant age, etc. [14]. However, the detected by us levels of salidroside in 1-to-3-year-old rhizomes/roots of the introduced regenerants grown in the Rhodope Mountains (0.6–1.2% DW) are less than the reported amounts in the rhizomes

(2.67%) and much higher than the amounts in roots (0.31%) of wild plants collected in Bulgarian Pirin mountain [1]. On the contrary, the total rosavins levels are considerably higher in the introduced plants (4.3–4.5% DW) in comparison with the wild rhizomes (2.32%) or roots (0.46%) [1].

The extracts from Golden root, used in most clinical trials have been standardized to a minimum 0.8–1% salidroside and 3.0% of rosavins, the ratio of these compounds should be between 1:3 and 1:3.75 in favour of rosavins, similarly to their naturally occurring ratio [15]. Therefore, the obtained ratio of 1:3.75 suggested that in vitro propagated and transferred to their natural habitat plants are likely to accumulate phenolic compounds in their underground parts after three years of cultivation in amounts that are promising for pharmaceutical use.

Conclusions. The applied HPLC analysis detected salidroside and rosavins in roots and rhizomes of in vitro regenerated plants grown ex vitro in a greenhouse and in the mountain at high altitude. Highest amounts of both substances were determined in 3-year-old regenerants grown in the mountain, and the salidroside to rosavins ratio was very close to the pharmacopoeial standard ratio of 1:3. These pioneer results show that *R. rosea* plants, obtained by micropropagation in vitro and grown ex vitro are promising for drug production.

REFERENCES

- [1] MARCHEV A. S., I. Y. ANEVA, I. KOYCHEVA, M. I. GEORGIEV (2017) Phytochemical variations of *Rhodiola rosea* L. wild-grown in Bulgaria, *Phytochemistry Letters*, **20**, 386–390.
- [2] MARCHEV A. S., A. T. DINKOVA-KOSTOVA, Z. GYÖRGY, I. MIRMAZLOUM, I. Y. ANEVA et al. (2016) *Rhodiola rosea* L.: from Golden root to green cell factories, *Phytochem. Rev.*, **15**(4), 515–536.
- [3] FURMANOWA M., M. HARTWICH, A. W. ALFERMAN, W. KOŹMIŃSKI, M. OLEJNIK (1999) Rosavins as a product of glucosylation by *Rhodiola rosea* (roseroot) cell cultures, *Plant Cell Tissue Organ Cultures*, **56**, 105–110.
- [4] PLATIKANOV S., L. EVSTATIEVA (2008) Introduction of wild Golden root (*Rhodiola rosea* L.) as a potential economic crop in Bulgaria, *Economic Bot.*, **62**(4), 621–627.
- [5] SAUNDERS D., D. POPPLETON, A. STRUCHKOV, R. IRELAND (2014) Analysis of five bioactive compounds from naturally occurring *Rhodiola rosea* in eastern Canada, *Can. J. Plant Sci.*, **94**, 741–748.
- [6] ERST A. A., A. S. ERST, A. I. SHMAKOV (2018) In vitro propagation of rare species *Rhodiola rosea* from Altai Mountains, *Turczaninowia*, **21**(4), 78–86.
- [7] TASHEVA K., G. KOSTURKOVA (2013) Role of Biotechnology for Protection of Endangered Medicinal Plants. In: *Environmental Biotechnology – New Approaches and Perspective Applications* (ed. Petre M.), UK, In Tech, 235–286.
- [8] KRAJEWSKA-PATAN A., M. DREGER, A. LOWICKA, M. GÓRSKA-PAUKSZTA, A. MŚCISZ et al (2007) Chemical investigations of biotransformed *Rhodiola rosea* callus tissue, *Herba Polonica*, **53**(4), 77–87.

- [⁹] MIRMAZLOUM I., Z. GYÖRGY (2012) Review of the molecular genetics in higher plants towards salidroside and cinnamyl alcohol glycosides biosynthesis in *Rhodiola rosea* L., Acta Alim., **41**(1), 133–146.
- [¹⁰] MARTÍNEZ M. I., G. BARBA-ESPÍN, B. F. TREVENZOLI, H. LÜTKEN (2020) *Rhizobium rhizogenes*-mediated transformation of *Rhodiola rosea* leaf explants, Bragantia, **79**(2), 213–223.
- [¹¹] TASHEVA K., G. KOSTURKOVA (2010) Bulgarian Golden root *in vitro* cultures for micropropagation and reintroduction, Cent. Eur. J. Biol., **5**(6), 853–863.
- [¹²] TASHEVA K., G. KOSTURKOVA (2012) Establishment of callus cultures of *Rhodiola rosea* Bulgarian ecotype, Acta Horticulturae, **955**, 129–135.
- [¹³] MURASHIGE T., F. SKOOG (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures, Physiol. Plant., **15**, 473–497.
- [¹⁴] ELAMEEN A., V. M. KOSMAN, M. THOMSEN, O. N. POZHARITSKAYA, A. N. SHIKOV (2020) Variability of Major Phenyletanes and Phenylpropanoids in 16-Year-Old *Rhodiola rosea* L. Clones in Norway, Molecules, **25**(15), 1–12.
- [¹⁵] BROWN R., P. GERBARG, Z. RAMAZANOV (2002) *Rhodiola rosea*: a Phytomedicinal overview, HerbalGram, **56**, 40–52.

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