

Callus tissues of *Rhodiola Kirilowii* (Regel) Maxim. – dynamic of growth and active compounds production

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Summary

Rhodiola Kirilowii (Regel) Maxim. (Crassulaceae) is a traditional medicinal plant used in North Asia and China, especially in the cardiopulmonary disorders in the hypoxic conditions induced by high altitude. The presented results are the part of the investigations carried out in the Branch of Medicinal Plants of the Institute of Natural Fibres and Medicinal Plants in cooperation with the Department of Biology and Pharmaceutical Botany, Medical University in Warsaw on *R. Kirilowii* plants and tissue cultures. The aim of recent study was to determine the growth dynamics and active compounds production during the cultivation of callus tissues of *R. Kirilowii* on solid/liquid media. Tissue cultures of *R. Kirilowii* shown the ability to produce all the active compounds determined in the roots of plants of Polish origin. It is worth emphasizing, that rosavins, according to known literature, were not detected in roots of plants growing in Asia. The best time for collection the tissues from solid medium was

fifth or sixth week of the culture – the tissues were growing dynamically and the contents of the main active compounds was high. The material from suspension should be collected in 12–15 days after inoculation. The obtained results will be applied in future investigations on the use of *R. Kirilowii* extracts in the experimental hypoxia in rats.

Key words: *Rhodiola Kirilowii*, roots of plant, in vitro cultivation, callus tissues, suspension cultures, hypoxia, salidroside, rosavins, phenolic acids, tannins, growth dynamics

INTRODUCTION

Rhodiola Kirilowii (Regel) Maxim. (Crassulaceae) is a traditional medicinal plant used in North Asia and China. It is a perennial plant that grows in Thien Shan, Altaj and Pamir Mountains at 2000–5600 m above sea level [1, 2]. The studies on animals showed significant protective effect of *R. Kirilowii* root extract especially in the cardiopulmonary disorders in hypoxic conditions induced by high altitude [3]. Not numerous carried out clinical investigations confirmed the activity of plant extracts in hypoxia preservation [4]. Till now, over 30 active compounds have been isolated and identified from root and callus extracts of *R. Kirilowii*: salidroside, p-tyrosol [5], phenylpropanoids – rosavins [6], flavonoids [7-11], hydroxycoumarins [5] and isocoumarins [12], gallic acid [5, 8], phytosterols [13], tannins [7], cyanogenic glucosides [11], oligoglycosides [11] and terpenoids [13]. Salidroside and rosavins are supposed to be the main biological active compounds of *Rhodiola* genus. The aim of this study was the determining the growth dynamics and active compounds production during the cultivation of callus tissues of *R. Kirilowii* on solid/liquid media.

MATERIAL AND METHODS

Plant material

The roots of the intact plant (collected in October 2008) and the seeds were obtained from Medicinal Plants Garden (in Plewiska near Poznań), Branch of the Medicinal Plants of the Institute of Natural Fibres and Medicinal Plants in Poznań. The callus tissues of *R. Kirilowii* were initiated from cotyledons (line II) and from hypocotyle (line H) of the sterilised seedlings.

Biotechnological methods

The seeds of *R. Kirilowii* were sterilized by soaking in 70% ethyl alcohol with addition of several drops of Tween (2 min), 4% sodium hypochlorite (10-15 min) and washed with sterile water for 5 times. The seeds were placed in the Petri

dishes with wet paper and kept in breeding chamber under light. The callus tissue started from cotyledons on MS media [14] supplemented with BA (2.0 mg/ml), adenine chloride (1.0 mg/ml), NAA (2.0 mg/ml) was incubated under fotoperiod (light for 16 h, 2500 lux). and temp. $23^{\circ}\text{C} \pm 1^{\circ}\text{C}$. The callus started from hypocotyle on MS medium supplemented with 2,4-D was cultivated in dark in temp. 24°C . Cultures were subcultured every four weeks. The suspension cultures were initiated from callus line H and cultivated in Erlenmayer flasks on the rotary shaker (80 r.p.m.) in dark. The samples collected for the chemical investigations were dried in temperature of $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

Growth rate determination

Studies of growth dynamics were performed at 7 days intervals (on solid medium) or at 3 days intervals (suspension cultures). The size of the sample from solid medium was established as the 10 cultures (10 jars) and three flasks in the case of suspension. The fresh and dried weights was determined with use of moisture analyser (HR73 Metler Toledo, temperature of drying 105°C). The increase of fresh/dry weight was calculated as a difference between weight in the day of measurement and the weight in the day of the inoculation. The determination of growth parameters was measured in two or three passages.

Chromatographic procedures

In this study HPTLC, HPLC and spectrophotometric methods were used for chemical analyses. Samples for TLC and HPLC analysis were extracted with 70% methanol in $60\text{--}70^{\circ}\text{C}$. HPLC analysis was performed on Agilent 1100 HPLC system equipped with photodiode array detector. For all separations a Lichrospher 100 RP18 column (250x4 mm, $5\mu\text{m}$) from Merck was used. The mobile phase consisted of 0.2% phosphoric acid in water (A) and acetonitrile (B), applied in the following gradient elution: from 95A/5B in 30 min to 80A/20B isomatic elution for 5 min, then from 80A/20B in 5 min to 20A/80B and an isocratic elution in 20 min to the end. Each run was followed by an equilibration period lasting 10 min The follow rate was adjusted to 1 ml/min, the detection wavelength set to DAD at $\lambda=205$ nm, 254 nm, 330 nm and $20\mu\text{L}$ of samples was injected. All separations were performed at the temperature of 25°C . Peaks were assigned by spiking the samples with standard compounds and comparison of the UV-spectra and retention times.

Spectrophotometric analysis of tannins expressed as pyrogallol were performed according to the methods of European Pharmacopoeia [15] on UV-Visible Spectrometer *Cintra 20 GBC 9*.

The statistic data were expressed as the average value, standard deviation and standard error.

RESULTS

Tissue cultures of *R. Kirilowii* obtained from cotyledons (line II) and from hypocotyle (line H) of seedlings were investigated on growth dynamics on the solid medium and in the suspension culture, respectively.

The growth dynamics

Results of the growth dynamics of culture line II on solid MS medium (supplemented with BA, NAA and adenine chloride) published in *Herba Polonica* in 2006 [6] shown, that the maximum dried weight was attained between 21 and 35 days of cultivation, reached ca. 0.77 g/culture (fig. 1). The increase of dry weight of the callus in the sixth week was ca. 0.53 g/culture (fig. 2).

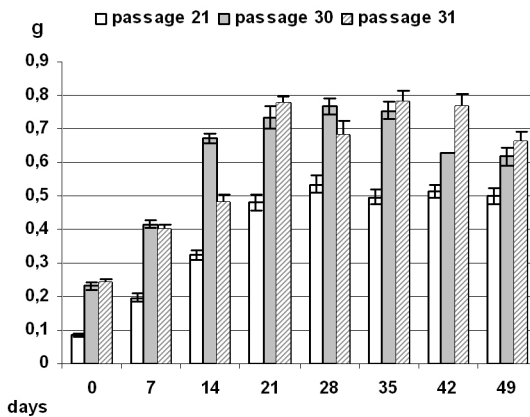


Figure 1. The dry weights of *R. Kirilowii* callus culture (line II) – solid medium

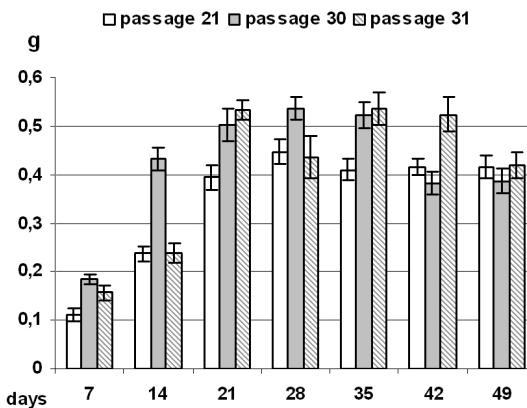


Figure 2. The increase of dry weights of *R. Kirilowii* callus culture (line II) – solid medium

The suspension culture of line H cultivated in MS medium supplemented with 2,4-D allowed to reach callus with 0.43g of d.w./culture after fifteen days of cultivation (fig. 3). It means that the increase of dry weight was 0.26g/culture (fig. 4).

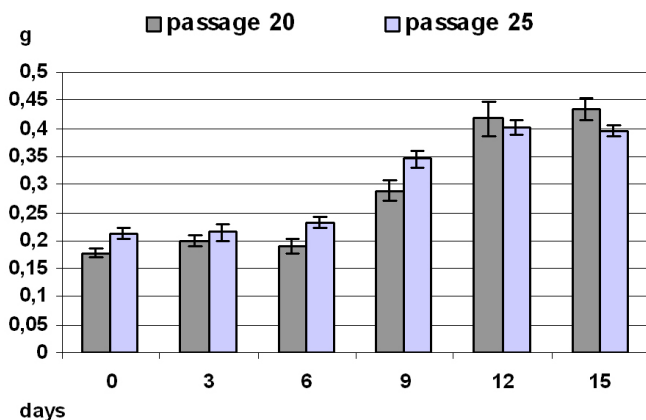


Figure 3. The dry weights of *R. Kirilowii* callus suspension culture (line H)

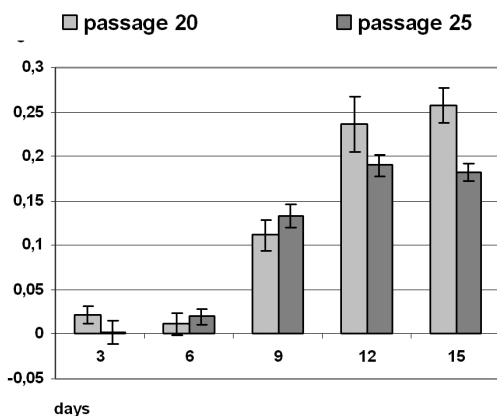


Figure 4. The increase of dried weights of *R. Kirilowii* callus suspension culture (line H)

Chemical compounds production during vegetation process

Callus tissues of *R. Kirilowii* cultivated on solid medium produced all spectrum of active compounds characteristic for the roots of plant. The content of tannins was well correlated with the age of cultures and were grown during the vegetation process (tab. 1). In the period of intensive growth (between 21 and 42 days of cultivation), the content of rosavins was higher and the content of searched phenolic acids was growing up reaching the maximum at the end of cultivation (tab. 1, 2).

Table 1.

The content of salidroside, p-tyrosol, tannins and rosavins in *R. Kirilowii* callus tissues (line II) during cultivation on MS medium with BA, NAA and adenine chloride (vs. roots of plant) – the average values

day	content of salidroside [mg/100 g of dry weight]	content of tyrosol [mg/100 g of dry weight]	content of tannins expressed as pirogalol [%]	content of rosavins [mg/100 g of dry weight]
0	1.39	0.09	–	0.45
7	0.99	0.11	0.20	0.29
14	1.56	0.12	0.24	0.29
21	1.26	0.14	0.43	0.56
28	0.13	0.16	0.44	0.72
35	0.14	0.13	0.61	0.39
42	0.08	0.06	0.73	0.82
49	0.33	0.17	0.65	0.39
roots of plant	5.43	2.57	2.26	29.56

Table 2.

The content of phenolic acids in *R. Kirilowii* callus tissues (line II) during cultivation on MS medium with BA, NAA and adenine chloride (vs. roots of plant) – the average values

day	content of gallic acid [mg/100 g of dry weight]	content of chlorogenic acid [mg/100 g of dry weight]	content of caffeic acid [mg/100 g of dry weight]
0	28.43	0.54	6.65
7	20.17	0.83	7.21
14	28.96	1.01	7.01
21	34.92	1.16	6.09
28	33.12	1.02	6.82
35	36.01	1.40	9.40
42	38.18	2.80	9.94
roots of plant	181.41	1.59	15.04

CONCLUSIONS

The presented results are the part of the investigations carried out in the Branch of Medicinal Plants of the Institute of Natural Fibres and Medicinal Plants in cooperation with the Department of Biology and Pharmaceutical Botany, Medical University in Warsaw on *R. Kirilowii* plants [8-11, 16, 17], tissue cultures [6, 18, 19], micropropagation [20] and transformation *in vitro* [21]. Tissue cultures of *R. Kirilowii* are able to produce all the active compounds determined in the plants of Polish origin. It is worth emphasizing that rosavins, according to known literature, were not detected in roots of plants growing in Asia [5]. The investigations

allowed to determine the growth and active compounds production during *in vitro* cultivation. The best time to collect the tissues from solid medium is fifth or sixth week of the culture – the tissues are growing dynamically and the contents of main active compounds is high. The material from suspension should be collected in 12–15 days after inoculation. The obtained results will be applied in the future investigations on the use of *R. Kirilowii* extracts in the experimental hypoxia in rats.

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KULTURY KALUSOWE *RHODIOLA KIRILOWII* (REGEL) MAXIM. – DYNAMIKA WZROSTU I GROMADZENIA SIĘ ZWIĄZKÓW CZYNNYCH

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Streszczenie

Rhodiola Kirilowii (Regel) Maxim. (Crassulaceae) to tradycyjna roślina lecznicza stosowana w Północnej Azji i Chinach w zaburzeniach krążenia na tle hipoksji wywołanej przebywaniem na dużych wysokościach. Prezentowane wyniki są częścią wspólnie prowadzonych badań nad różencem Kiryłowa, w tym kulturami *in vitro*, przez Oddział Roślin Zielarskich Instytutu Włókien Naturalnych i Roślin Zielarskich oraz Katedrę Biologii i Botaniki Farmaceutycznej Warszawskiego Uniwersytetu Medycznego. Celem prezentowanych badań było określenie parametrów dynamiki wzrostu oraz dynamiki wytwarzania związków biologicznie czynnych przez tkanki kalusa *R. Kirilowii* hodowanego na pożywce stałej i płynnej. Tkanki kalusowe produkowały wszystkie aktywne związki uprzednio zidentyfikowane w roślinach z gruntu uprawianych w Polsce. Warto podkreślić, że (według dostępnej literatury) nie potwierdzono obecności rozawin w surowcu pochodzącym z Azji. W badaniach stwierdzono, że najlepszym okresem do zbioru tkanki hodowanej na pożywce stałej był 5-ty – 6-ty tydzień hodowli, wtedy kalus osiągał wysoką zawartość związków biologicznie czynnych oraz korzystne parametry wzrostu. Materiał z hodowli zawieszinowych winien być zbierany po 12–15 dniach hodowli. Otrzymane wyniki posłużą do dalszych badań farmakologicznych dotyczących wpływu ekstraktów z *Rhodiola Kirilowii* na szczury w warunkach doświadczalnej hipoksji.

Słowa kluczowe: *Rhodiola Kirilowii*, korzenie rośliny gruntowej, hodowla *in vitro*, kalus, hodowla zawieszinowa, hipoksja, salidrozyd, rozawiny, kwasy fenolowe, garbniki, dynamika wzrostu