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THE EFFECT OF SUCROSE CONCENTRATION ON *IN VITRO* CALLOGENESIS OF GOLDEN ROOT – ENDANGERED MEDICINAL PLANT

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Abstract

Rhodiola rosea is a medicinal plant, containing a range of antioxidant compounds, including p-tyrosol, organic acids (gallic acid, caffeic acid, and chlorogenic acid), and flavonoids (catechins and proanthocyanidins). The stimulating and adaptogenic properties of Rhodiola rosea are attributed to p-tyrosol, salidroside, rhodioniside, rhodiolin, rosin, rosavin, rosarin, and rosiridin. The recent investigations showed that sucrose influences secondary metabolism in cell, tissue and organogenic cultures. Higher sucrose levels in certain limits lead to increased production of alkaloids in in vitro cultures of various plants. The aim of this work was to study the influence of different sucrose concentrations on the process of callus formation. The nutrient medium containing different percentage of sucrose (20, 30 and 40 %) were used. Leaves excised from in vitro propagated golden root plants were plated for callus induction and maintenance. The results showed that the callogenesis of golden root plants was more effective (2 - 3 folds) when sucrose was 20 g/l compared to higher concentrations of sucrose at the background of the same combinations of plant growth regulators. The obtained calli differed in structure and color. HPLC analysis of the calli was performed.

Keywords: *endangered plant, Rhodiola rosea, golden root, in vitro, calli, sucrose, HPLC*

Abbreviations
BAP – N6-benzylaminopurine;
IAA – Indolyl-3-acetic acid;
Kin – Kinetin;
MS – Murashige and Skoog medium, 1962;
NAA – a- naphthyl acetic acid;
2-iP – 6-(y,y-dimethylallyl amino) purine;
2,4-D – 2,4- dichlorophenoxyacetic acid.
CAS – casein hydrolysate
HPLC – High-performance liquid chromatography

INTRODUCTION

Rhodiola rosea L. (Golden root, Rose root, *Sedum roseum* (L.) Scop., *S. rhodiola* DC.) is a valuable species for Bulgarian medicinal plants genfund. The restoration abilities of the wild plants are defined as relatively limited due to both, low germination and very low coefficient of vegetative propagation, traditionally used (Platikanov and Evstatieva 2008). The extracts obtained from the plants have antidepressant, anticancer, cardio protective and central nervous system stimulation effects (Kelly, 2001; Brown et al., 2002). The plant root, rhizomes and calli produce pharmaceutically important compounds referring to 6 different groups:(1) phenylpropanoids; (2) phenylethanol derivaties (3) flavonoids; (4) monoterpenes; (5) triterpenes; (6) phenolic acid (Ganzera et al., 2000; Linh et al., 2002). The antioxidant activity in different tissues and organs in wild and in *in vitro* cultivated plants and calli of *Rh. rosea* 4 due to four phenolic component, including flavonoids (salidroside, rosarin, rosavin and rosin), identified by HPLC (Furmanowa et al., 1998).

Plant *in vitro* cultures offer possibilities for the production of secondary metabolites in bioreactors, manipulation of metabolic pathways and metabolic engineering. Studies showed that the sucrose can influence secondary metabolites production in cell and organ cultures (Fowler, 1983; Paiva and Janick, 1983). The results of many investigations have concluded that the increase of sucrose within certain limits leads to increased production of alkaloids in tissue cultures of various plants

(Wijnsma et al., 1986; Duraz et al., 1994 Vázquez-Flota et al., 1994). Less data are available for the effect of other carbon sources. According to Paiva and Janick (1983) increasing the concentration of sucrose but not that of the glucose influences the content of lipids, alkaloids and anthocyanins in the zygotic and somatic embryos of *Theobroma cacao* cultivated *in vitro*. There are data showing that the presence of maltose in nutrient medium also increases accumulation of secondary metabolites (Kinnersle and Henderson, 1988; Rao and Narasu, 1999).

There is not a great number of experiments investigating the possibilities for callus formation in different species of *Rhodiola* and following by the chemical analysis of the biologically active substances and determination of the parameters for their optimal synthesis in *in vitro* condition.

Sheng et al. (2005) investigated the induction and maintenance of callus cultures in *Rhodiola quadrifida* (plant used in traditional Chinese medicine over 1000 years). Callus cultures were induced using Murashige and Skoog (1962) MS medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) (1.0 mg/l) , α- naphthyl acetic acid (NAA) (2.0 mg/l), N^6-benzylaminopurine (BA) (0.5 mg/l) and kinetin (0.1 mg/l). For maintaining the calli MS medium was supplemented with 2,4-D (1.0 mg/l), BA (0.1 mg/l) and kinetin (0.5 mg/l). After 30 days of cultivation the content of salidroside in calli cultures was measured and it was found out that callus tissue was able to produce biologically active substances. Calli induced from the stem and leaves of *Rhodiola kirilowii* had potential to produce salidroside. The latter amount is influenced from the type of the explant, temperature and period of cultivation (Li Wei et al., 2005). Soo Jung Kim et al. (2004) found that calli obtained from *Rhodiola sachalinensis* cultivated on Gamborg's B5 nutreint media enriched with 0.5 mg/l NAA, 1 mg/l BA and 5 % sucrose can produce high level of salidroside (0.41 % on the basis of dry weight) compared to intact root (0.17 %). György et al. (2004) and György (2006) obtained and cultivated for a long time callus from *Rh. rosea* in liquid medium supplemented with precursors of biologically active substances with the aim to increase the synthesis of substances of the basic biologically active complex. In these cultures, rosin content was elevated to 1.25% dry weight while rosavin was 0.083% dry weight.

The aim of this investigation was to study the possibility of callus induction from *Rhodiola rosea* Bulgarian ecotype and secondary metabolites production by calli using different concentrations of sucrose.

MATERIALS AND METHODS

Callus cultures. Leaves excised from *in vitro* propagated *Rhodiola rosea* plants were used for callus induction. Leaves were plated on solidified culture media containing different combinations and concentrations of phytoregulators (Tasheva and Kosturkova, 2012a; Tasheva and Kosturkova, 2012b, Tasheva and Kosturkova, 2013c).

Composition of culture media for callus induction in vitro. Different variants of nutritious media containing Murashige and Skoog (1962) basic medium (MS) supplement with phytoregulators in various combinations and concentrations were used (Table 1). However, content of sucrose was different (20 g/l, 30 g/l and 40 g/l) while the content of agar-agar was constant (6.0 g/l). Culture media were autoclaved at 1.1 $kg.cm^{-2}$, 121° C for 20 min. The experiments were in two replicates.

In vitro cultivation condition. Callus cultures were induced and maintained in cultivation room at temperature of 23-24° C and dim light (20 $\mu Mm^{-2}s^{-1}$). Response of 30 to 50 explants was examined for each medium variant and Sigma Plot 11.1 applied for statistics.

Table 1. Culture media composition [mg/l] for induction and maintaining of callus cultures of *Rhodiola rosea*.

Media variant	BAP	2,4-D	IAA	NAA	CAS*
1. C-MS Control					
C-1	1.0	1.0			
C-2	1.0	0.5			
C-3	0.5	1.0			
C-4	1.0	1.0			1000
C-5	1.0	0.5			1000
C-6		0.1	0.2		1000
C-7	1.0			0.5	1000

*Casein hydrolysate

Phytochemical analysis. For quantity determination of the main polyphenols: salidroside, rosavin, rosin and rosarin in *in vitro* obtained calli was used HPLC.

Extract Preparation. The calli from nutrient media C-1, C-2, C-3, C-4, C-5, C-6 and C-7 were dried at room temperature.

Callus extraction: Hundred milligrams of the finally powdered calli was extracted three times with 1 ml methanol. After centrifugation at 3000 rpm for 10 min, the supernatants were combined and adjusted to the final volume of 1 ml with methanol. The solution was injected in triplicate.

Analytical methods (HPLC): HPLC analyses were performed on an Agilent 1100 Series HPLC system, equipped with a MWD UV-Vis detector. The column used was Chromsep SS (250 x 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. The effluent was monitored at wave length of 205 nm and 254 nm.

RESULTS AND DISSCUSIONS

Rhodiola rosea leaf explants placed on MS medium supplemented with various concentrations of auxins and cytokinins formed callus within 4 weeks.

In our previous experiments induction of callogenesis was successful from leaf explants, isolated from *in vitro* propagated plants, on MS media enriched with BAP in concentration from 0.5 mg/l to 2.0 mg/l; 6-(y,y-dimethylallyl amino) purine (2-iP) - 0.3 and 3.0 mg/l; 2,4-D - from 0.1 to 2.0 mg/l; IAA - 0.2, 0.3 and 1.0 mg/l; NAA - 0.5, 1.0, 1.5 mg/l and casein hydrolysate 1000 mg/l (Tasheva and Kosturkova, 2012a). The highest response to formation of callus (62.85% and 73.17%) was observed on two media-containing 1 mg/l BAP and either 1 mg/l or 0.5 mg/l 2,4-D (Table 2) . Calli formation was less effective or calli was not formed when sucrose was 30 g/l and 40 g/l in the culture medium (0.5 mg/l BAP as compared to 1 mg/l 2,4-D in variant C-3 for instance).

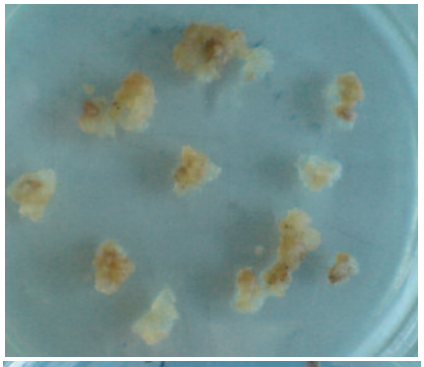
Table 2. Callogenesis induction and characteristics of the calli tissues on 30-th day of cultivation.

Nutrient Medium №	Callus induction [%] on different concentrations of sucrose			Characteristics of the callus tissue growth on different concentration sucrose		
	20 g/l	30 g/l	40 g/l	20 g/l	30 g/l	40 g/l
MS.	0	0		Explants whitened and died		
C-1.	62.85	33.00	4.00	½ loosy calli with beige color; 12 % brownish and loosy calli	33 % compact calli like structures	Calli like structures
C-2.	73.17	26.00	-	Compact, grainy calli, pale yellow color	Pale yellow color	Necrotic yellowish explants
C-3.	43.90	8.00	-	Loosy, liquidy calli, pale, yellow color	Pale yellow color	Necrotic yellowish explants
C-4.	54.17	53.00	12.00	Compact, solid calli, grainy, yellow-beige color	53% liquidy, loosy calli like structures; 47 %-brown, necrotic	liqued calli like formation
C-5.	79.2	36.5	8.2	Soft, grain calli, beige in color	Soft calli, yellow-beige in color	Yellow calli like structures, necrotic
C-6	48	10.2	-	liquidy calli; pale beige in color	liquidy calli;	Necrosis
C-7.	51.78	11.00	-	Fresh calli, yellow-green color	20 % bud induction	Necrotic yellowish explants

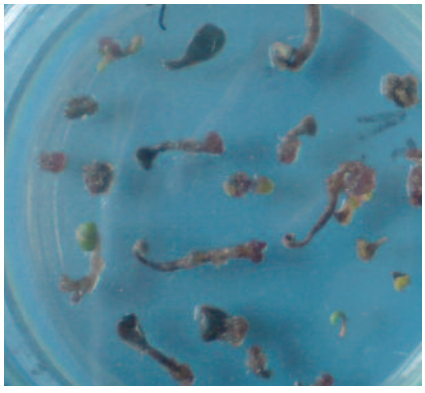
Induction of callogenesis on the same variants of phytoregulators but with a higher concentration of sucrose (3% or 4%) was twice, three times lower percentage. For example the percentage of induced calli was 62.85% and 33%, respectively in culture medium variant C-1 containing 1.0 mg/l BAP and 1.0 mg/l 2 4-D and 2 % or 3 % sucrose (Figure 1) . Callus induction rate was 73.17 % and 26 %, respectively, in variant C-2 containing 1.0 mg/l BAP, 0.5 mg/l 2,4-D and 2 % or 3 % sucrose. Variant C-4 containing BAP (1.0 mg/l), 2,4-D (1.0 mg/l) and casein hydrolysate (1000 mg/l), and 2 % or 3 % of sucrose stimulated formation of callus by 54.17% and 53.00 %, respectively. Variant C-7 (BAP – 1.0 mg/l, NAA –0.5mg/l and casein hydrolysate – 1000 mg/l), and 2 % and 3% of sucrose induced calli up to 51.78% and 11.00 %, respectively.

Obtained calli had different characteristics – morphology, color etc.

Growth and Characteristics of the Callus. Callus growth rate and tissue characteristics varied depending on the culture media composition.



A.



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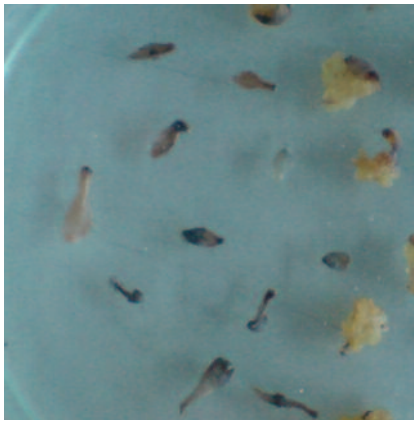

C.

Figure 1. Callus cultures on variant C-1containing different concentrations of sucrose A: 2 %, B: 3%, C: 4%

Culture medium composition was important for callus structure and texture which varied among variants and within one medium variant. BAP in concentration of 1 mg/l favored fast growing compact tissue with grain structure (var. C-2, C-4, C-5 and C-7) which was easy to be maintained. Its color varied from greenish to yellowish pale. Calli in other variants were predominantly liquidy and loosy with beige-brownish color that was not suitable for further use.

HPLC analysis. The HPLC analyses of the callus cultures induced and grown at various concentrations of sucrose in the medium (samples C-1, C-2, C-3, C-4, C-5, C-6 and C-7 passage II) did not show presence of basic compounds responsible for the pharmacological effect of the species or the produced biologically active substances were in negligible quantities.

Soo Jung Kim et al., (2004) found that *Rhodiola sachalinensis* calli cultivated on B5 nutrient medium enriched whit 5% sucrose can produced high level of salidroside (0.41% on the basis of dry wt) compared to the intact root (0.17%). In the other valuable species, like *Leucojum aestivum* Georgieva (2012) observed negative correlation between the levels of carbon source in the medium and the accumulation of galantamine in organogenic cultures, what was in contradiction to the results obtained by Sellés et al. (1997) in a similar experiment using *in vitro* cultures of narcissus. The greatest amount of galantamine is measured in explants grown without carbon source. In all other cases the

amount is much less. In variants with glucose, quantity of the galantamine decreases when increasing glucose concentration. In variants with maltose and with sucrose were observed some increase in their highest concentrations

CONCLUSIONS

The present study contributed to determine the proper concentrations of sucrose in culture media for the development of callus cultures of Bulgarian Golden Root. The optimal concentration of sucrose (2 %) was determined for efficient induction and maintaining of callus with grain structure suitable for long-term cultivation. The callogenesis efficiency expressed by the percentage of explants forming undifferentiated tissue, their growth and characteristics depended significantly on the type and concentrations of the phytoregulators.

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