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REGULAR ARTICLE

IN VITRO CULTURE ESTABLISHMENT OF *SCHIZANDRA CHINENSIS* (TURZ.) BAILL. AND *RHODIOLA ROSEA* L., TWO ADAPTOGENIC COMPOUNDS PRODUCING PLANTS

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SUMMARY

Schizandra chinensis and *Rhodiola rosea* are known to contain immune-stimulating compounds. Additionally, these compounds are able to increase the ability of an organism to adapt to environmental factors and to avoid the damage caused by such factors. A possible explanation of the mechanism behind this so called adaptogenic effect, is their influence on the level and activity of monoamines and opioid peptides such as beta-endorphins. The aim of our work was to develop and optimize the hormonal composition of a growth medium for cultures of these plants. The production of lignans by *S. chinensis* calli and biosynthesis of phenolic glycoside rhodioloside by *R. rosea* cells was also evaluated.

The highest stimulating effect on growth in the *Schizandra chinensis* culture was found with a nutrient medium containing 2,4-D 0.1 mg l^{-1} with KT 1.0 mg l^{-1} or NAA 1.0 mg l^{-1} with KT 1.0 mg l^{-1}. For *Rhodiola rosea* culture, the best callus production was obtained with nutrient media containing 2,4-D 1.0 mg l^{-1} and IBA 1.0 mg l^{-1}; 2,4-D 0.1 mg l^{-1}; 2,4-D 1.0 mg l^{-1} or IAA 0.1 mg l^{-1}. Plants of *Rhodiola rosea* were regenerated in two media, one containing IAA 1.0 mg l^{-1} and another with IBA 10.0 mg l^{-1} and KT 1.0 mg l^{-1}. Root development was monitored after the addition of NAA 0.5 mg l^{-1}. However, in spite of increased growth, HPLC analysis did not reveal the presence of any lignans in the callus culture of *Schizandra chinensis* nor of the glycoside rhodiolosid in the callus culture of *Rhodiola rosea*. On the other hand, rhodiolosid was detected in *Rhodiola rosea* plants regenerated from calli. It is suggested that the phytohormone systems mentioned above are a major starting point for a further investigation focused on the induction of higher levels of secondary metabolite production in these callus cultures.

Key words: Phytohormones, *Rhodiola rosea*, *Schizandra chinensis*, Rhodiolosid

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1. Introduction

Schizandra chinensis (Turcz.) Baill. (*Schizandraceae*) is a perennial woody and deciduous creeping vine. This plant is native to East Asia, Russia, Japan, Korea and Southern China [1]. Its main active substances are lignans, which occur in roots, stems and especially in seeds [2, 3, 4]. *Schizandra* lignans are known to be stimulants [5, 6], hepatoprotectives [7, 8, 9] and relievers of stress factors [10].

Rhodiola rosea L., (*Crassulaceae*) is a perennial herb and medicinal plant used mainly in Asia and Eastern Europe. It can be found in all mountainous areas of Europe. The major compounds occurring in *R. rosea* are flavonoids, 2,6-octadiene derivatives, glycosides of cinnamyl alcohol and 2-(4-

hydroxyphenyl)ethanol. One of the compounds belonging to the last group, represented by *p*-tyrosol [2-(4-hydroxyphenyl) ethanol] and its glycoside rhodiolosid, is considered to be the most important component.

Rhodiola rosea and *Schizandra chinensis* are natural sources of substances which increase non-specific immunity in organisms [5, 6, 11, 12]. Other plants studied for their ability to offer generalized resistance to physical, chemical and biological stress are, for example, *Eleutherococcus senticosus* (Siberian ginseng) and *Panax ginseng* (Korean ginseng). Unlike those two "ginseng" plants, which are thought to exert their adaptogenic activity primarily at the level of the Hypothalamic-Pituitary-Adrenal axis (HPA) function [13, 14, 15], *Rhodiola rosea* appears to exercise its adaptogenic effect by working centrally and peripherally on monoamine and opioid synthesis, transport, and receptor activity [16]. The adaptogenic effect of *R. rosea* seems to be advantageous in circumstances of acute stress, depression and schizophrenia, and among patients with a high risk of cardiovascular disease [16]. There are also reports of positive effect against cancer and HIV-1 protease [16, 17]. The efficacy of routine anticancer agents has been observed to increase if they are combined with the mentioned adaptogen.

Schizandra chinensis is traditionally used for its tonic activity. Apart from its traditional and popular use, previous investigations have proven *Schizandra* to be medicinally useful for the treatment of many disorders (such as coronary heart disease and menopause-related symptoms relief) but none as interesting as its use as an adaptogen [18, 19]. It is believed that these dibenzo[a,c]cyclooctadiene lignans are responsible for the beneficial effect of *Schizandra* extracts: wuweizisu [9, 20], gomisin N [9] and gomisin A [21, 22]. Antioxidant effect [23, 24] and enhanced hepatic glutathione status in rats [7] has been confirmed. In addition, schizandrin B - a dibenzocyclooctadiene compound isolated from *Schizandra chinensis* - exhibited an inhibitory effect on P-glycoprotein, the membrane transporter which mediates drug efflux and is responsible for multidrug resistance (MDR) phenomena in cancer cells [25]. Sheng Mai San (SMS), a multicomponent preparation in traditional Chinese medicine comprising *Panax ginseng, Schizandra chinensis* and *Ophiopogon japonicus*, is used for the treatment of ischemic heart disease [24].

Although *Schizandra chinensis* [26] and *Rodiola rosea* can be successfully grown in central Europe, there is a tendency to develop *in vitro* systems as an alternative to field production of plants used as a source of compounds of interest. Such a system is not dependent on predators, pathogens, limited sources of nutrients, climatic changes, or political influences and can thus be better controlled. Additionally, each plant, commercially exploited as a source of a drug, becomes a potential member of the group of 4 000-10 000 worldwide endangered plant species [27, 28]. This provides a very negative repercussion of the ever-increasing demand for medicinal drugs. Moreover, in practice, any study dealing with prospective use of a pharmaceutically valuable plant involves derivation of its culture *in vitro* in order to obtain standard natural material for the multiplication, breeding and production of therapeutically important metabolites [29].

Secondary metabolite production by *Rhodiola sachalinensis* has been successfully achieved using compact callus aggregates (CCA) instead of cell suspensions [30]. CCA are spherical, smooth-surfaced clumps displaying some levels of cellular or tissue differentiation which are a prerequisite for the production of metabolites [31, 32]. For effective induction of CCA, both auxin and cytokinin are required [30, 33]. A higher cytokinin/auxin ratio has been reported to favour the growth of CCA and the production of salidroside (rhodioloside) in *Rhodiola sachalinensis* [31]. The presence of 2,4-dichlorophenoxyacetic acid (2,4-D) enhances the production and accumulation of salidroside in CCA of *R. sachalinensis* in spite of exerting an inhibitory effect on the callus growth itself [33]. Moreover, the high biotransformation potential of these aggregates is represented by the increased

production of cinnamyl glucosides after cinnamyl alcohol feeding [34].

In this study, the optimal method for the establishment of the callus cultures of *Schizandra chinensis* and *Rhodiola rosea* was determined, reported by evaluating the mass growth in different hormonal compositions of the growing medium. The effects of phytohormones on the growth of callus cultures and the production of secondary metabolites were investigated.

2. Materials and Methods

Plant materials and culture establishment

Tissue cultures of *S. chinensis* were derived from aseptic closed flower buds of outdoor plants grown in botanic garden of the Faculty of Pharmacy in Hradec Králové, Czech Republic. The flower buds were sterilized by treatment with chlorinated lime (10%, 20 min) followed by sodium hypochlorite (10%, 20 min). Seedlings of *R. rosea*, used as a source of the material for tissue culture, were grown from seeds which had been previously sterilized with ethanol (70%, 3 min), chlorinated lime (10%, 20 min) and sodium hypochlorite (10%, 20 min). Epicotyls obtained from one month old seedlings were used as initial explants for *R. rosea* culture. For induction of calli, flower buds (*S. chinensis*) and epicotyls (*R. rosea*) were transferred into Erlenmeyer flasks (100 ml) containing 25 ml of Murashige and Skoog (MS) medium [35] supplemented with benzyladenine (BA 1.0 mg l-1), 0.9% agar and 3% sucrose. MS medium supplemented with 0.9% agar and 3% sucrose without growth regulators was used to obtain regenerated young seedlings. Prior to the supplementation, MS medium was adjusted to pH 5.7 and sterilized at 121 °C for 20 min.

Growth regulators

The MS media supplemented with different growth regulators were used for the callus formation and growth of *S. chinensis* and *R. rosea*. The pH of MS medium was adjusted to 5.7 before autoclaving. After sterilization, the medium was distributed into 100 ml Erlenmeyer flasks and supplemented with different phytohormones. The callus cultures were cultivated at 25 ± 2 °C in darkness and transferred to the same fresh medium after 28 to 30 days. Each experiment was performed in triplicate for each condition.

Statistical analysis

The overall growth of callus cultures was evaluated after 4 weeks of cultivation in the presence of individual phytohormones or their combinations, as a percentage increase of the fresh weight of the callus culture. An inoculum of the same fresh weight (2 g) was used for all experiments. ANOVA was used for the statistical evaluation except for the groups of *R. rosea* - indole-3-acetic acid (IAA) and 2,4-dichlorophenoxyacetic acid (2,4-D). For the groups without random differences of means ($p<0.05$) indicated by ANOVA, t-tests were used for the evaluation of differences between the means. The software employed was a part of the STATISTICA package (StatSoft Inc., Tulsa, USA; Czech version produced by StatSoft CR Ltd., Prague, Czech Republic).

Regeneration of *R. rosea* plant

Callus cultures of *R. rosea* were initially pre-cultivated (60 days) in MS medium with IAA (1.0 mg l^{-1}) after which they were transferred to the medium containing indole-3-butyric acid (IBA 10.0 mg l^{-1}) with kinetin (KT 1.0 mg l^{-1}) and left for 30 days. Both cultures were performed in the dark and then submitted to 24 h continuous illumination (55 µmol m^{-2} s^{-1}, cold light) in order to stimulate the growth of buds and shoots. Induction of adventitious roots IAA was achieved by addition of α-naphthaleneacetic acid (NAA) and IBA to the MS medium at concentration levels of 0.1, 0.5 and 1.0 mg l^{-1}, respectively.

Analysis of metabolites

The calli of *S. chinensis, R. rosea* and regenerated plants of *R. rosea* were dried at room temperature and pulverized. An aliquot of 250 mg of the resulting powders were extracted by constant stirring with 20 ml of 95% ethanol during 20 min (*S. chinensis*) or with 10 ml of 99.9% methanol (*R. rosea*) in a water bath at 65°C in both cases.

TLC analysis of *R. rosea* extracts

Methanolic extracts of calli and *R. rosea* regenerated plants were analyzed by TLC

along with reference standard of rhodiolosid (dissolved in chloroform – methanol (3:2)). Samples applied on Silica gel 60 F254 (Merck, Darmstadt, Germany) TLC plates (20 x 20 cm) were eluted with chloroform:methanol:water (26:14:3). Plates were air-dried and sprayed with Pauly´s reagent [36] and with 1% $FeCl_3$ in ethanol - 1% aqueous K_3FeCN_6 (1:1) for visualization. UV detection at λ = 254 and 366 nm were also used.

HPLC analysis

HPLC analysis was performed on a Philips PU4100 Liquid Chromatograph (Philips, Cambridge, England) equipped with a PU 4100 pump (Philips, Cambridge, England), a PU 4110 UV-VIS detector (Philips, Cambridge, England) and a Pye Unicam PU 4021 multichannel detector (Philips, Cambridge, England).

Separation was carried out using a slightly modified method developed originally by Bartlova et al [37], adjusted for each sample. *S. chinensis* samples were analyzed using a C_{18} (SGX, 4x250 mm, 7 µm) (TESSEK, Prague, Czech Republic) column with a C_{18} precolumn, (CGC SGX C_{18}, 30x3 mm, 10 µm) (TESSEK, Prague, Czech Republic) at 25 °C, a flow rate of 0.5 ml min^{-1} and eluted with a gradient of water - acetonitrile 50:50 (v/v) to 40:60 (v/v) in 60 min, followed by 30 minutes isocratic elution with water – acetonitrile 70:30 (v/v); detection was UV 254 nm. *R. rosea* samples were separated on the same column as mentioned for *S. chinensis* but with a flow rate of 1.0 ml min^{-1} and a gradient of water - methanol 95:5 (v/v) to 5:95 (v/v) in 15 min and UV detection at 285 nm. Sample volume was 20 µl in both cases.

Solutions of reference compounds of schizandrin, gomisin A, deoxyschizandrin, γ-schizandrin, gomisin N and wuweizisu C (lignans) and rhodiolosid were used as external standards for qualitative and quantitative analysis.

3. Results

Fig. 1: *Schizandra chinensis,* individual hormonal treatment

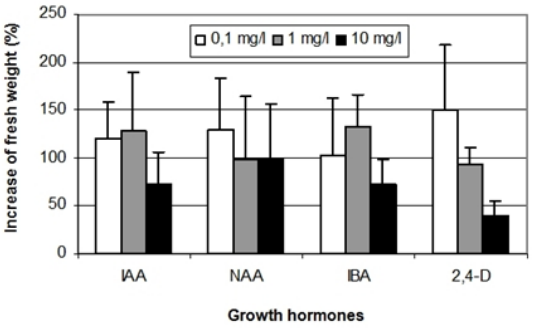


Fig. 2: *Schizandra chinensis,* hormonal combination treatment

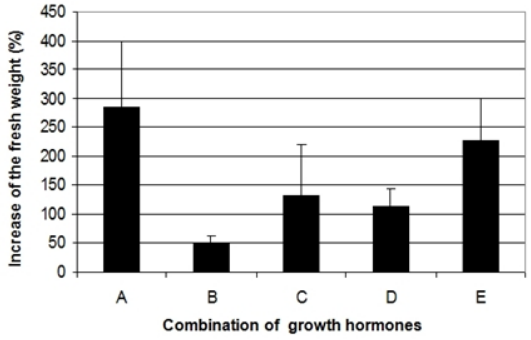


Figure 1 and 2: The influence of various phytohormones and different concentrations of these on the growth of callus cultures of *Schizandra chinensis* (% w/w relative to original inoculum). Fig. 1: individual hormonal treatment, Fig. 2: Combined hormonal treatment: (A) KT 1.0 $mg.l^{-1}$ + 2,4-D 0.1 $mg.l^{-1}$; (B) KT 1.0 $mg.l^{-1}$ + 2,4-D 1.0 $mg.l^{-1}$; (C) KT 1.0 $mg.l^{-1}$ + IAA 0.1 $mg.l^{-1}$; (D) KT 1.0 $mg.l^{-1}$ + IAA 1.0 $mg.l^{-1}$; (E) KT 1.0 $mg.l^{-1}$ + NAA 1.0 $mg.l^{-1}$.

Fig. 3: *Rhodiola rosea,* individual hormonal treatment

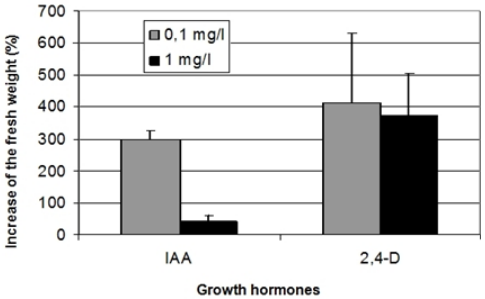


Fig. 4: *Rhodiola rosea,* hormonal combination treatment

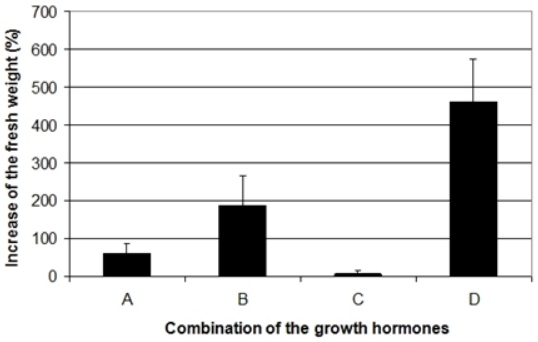
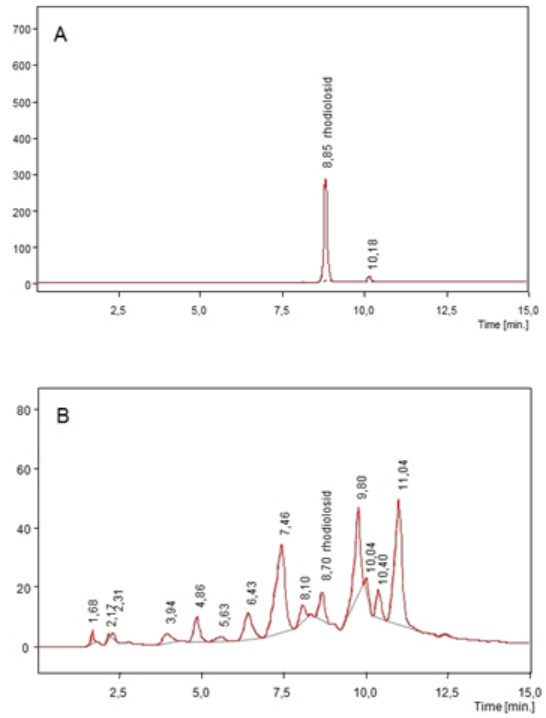


Figure 3 and 4: The influence of various phytohormones and different concentrations on these on the growth of callus cultures of *Rhodiola rosea,* (% w/w relative to original inoculum). Fig. 3: individual hormonal treatment, Fig. 4: Combination of hormones: (A) 2,4-D 0.1 mg.l^{-1} + KT 1.0 mg.l^{-1}; (B) 2,4-D 0.1 mg.l^{-1} + CM (20%); (C) 2,4-D 1.0 mg.l^{-1} + KT 1.0 mg.l^{-1}; (D) 2,4-D 1.0 mg.l^{-1} + IBA 1.0 mg.l^{-1}.

Figure 5: HPLC chromatograms of standard rhodiolosid (**A**) and an extract from regenerated plants of *Rhodiola rosea* (**B**). Column: C_{18}; gradient elution water: acetonitril 50: 50 (v/v) to 40: 60 (v/v) in 60 min; flow-rate: 0.5 ml.min^{-1}; UV detection: 254 nm; column temperature: 25° C



4. Discussion

Effect of phytohormones on callus culture growths

When selecting the initial explants for the derivation of the producing cultures, the localization of biosynthesis and accumulation of secondary metabolites should be considered. Secondary metabolites are accumulated in the pericarps and seeds of *S. chinensis* [38] and in the rhizomes of *R. rosea* [39]. Based on the previous reports, the flower buds and shoots with auxiliary buds were established for the callus cultures of *S. chinensis* and *R. rosea*, respectively.

The increase in *S. chinensis* fresh weight (as compared to the initial inoculum) achieved by addition of the individual growth regulators was between 127.6% and 150.7% (Fig. 1), while the highest increase was attained with culture media containing mixtures of 2,4-D with KT (285.5%) and NAA with KT (227.4%) (Fig. 2). A significantly lower increase was observed with 2,4-D, IAA and IBA at concentrations of 10.0 mg l^{-1} (Fig. 1). The increase of weight was noted to be inversely proportional to the concentration of 2,4-D, for example, additions of 10.0 mg l^{-1}, 1.0 mg l^{-1} and 0.1 mg l^{-1} produced a weight increase of 39.9%, 93.4% and 150.7% respectively. The callus culture grew in the form of a brown, disorganized callus after the treatment with all phytohormones.

The effects of kinetin, coconut milk (CM) and auxins such as IBA, IAA and 2,4-D on the growth of *R. rosea* callus were investigated. Previous studies showed that CCA cultures required both auxin and cytokinine for their formation and growth [30, 31, 33]. All tested auxins had a positive effect on callus growth (Fig. 3). Interestingly, callus growth was inhibited when KT 1.0 mg l^{-1} was added together with 2,4-D. It was also observed that the combination of 2,4-D with CM, a liquid endosperm with cytokinine activity, had no effect on the growth (Fig. 4). The highest increase (3-4.5 fold) was observed for MS media supplemented with 2,4-D 1.0 mg l^{-1} combined with IBA 1.0 mg l^{-1}, 2,4-D 0.1 mg l^{-1} or 2,4-D 1.0 alone and IAA 0.1 mg l^{-1} (Fig. 3, 4). The average biomass output for these media was 462.1% compared to the initial inoculum. Previously,

Wu et al. [33] had reported a negative effect of 2,4-D on the callus growth of *Rhodiola sachalinensis*. On the contrary, 2,4-D induced the growth in our system though independently of its concentration. In the case of IAA, however, a clearly distinguishable dependence on the concentration of the phytohormone was observed (Fig. 3). The discrepancies between the effects of phytohormones on the growth of calli reported for *Rhodiola sachalinensis* [30, 33] and ours (*R. rosea*) may be explained by differences between *Rhodiola* species. No shoot was observed on the calli in the presence of phytohormones and their combination.

Regeneration of *R. rosea* plants

The callus cultures, as irregular clumps, showed a low level of differentiation after 30 days when transferred into the MS medium containing IBA 10.0 mg l^{-1} with KT 1.0 mg l^{-1}. Adventitious buds and subsequent shoots were induced as a consequence of the exposure of the callus cultures to the light (55 µmol m^{-2} s^{-1}, cold light) for 24 h. Under these conditions, the shoot formation was occurred with very high frequency (100%, with 3-6 shoots per callus).

All used auxines induced root formation, the highest effect (100%) being observed after NAA 0.5 mg l^{-1} treatment. Rooted (majority) plantlets were later transferred and acclimatized in the greenhouse, and resulted to be morphologically identical to initial plants.

Analysis of secondary metabolites in callus cultures and regenerated plants

The HPLC analysis of the callus culture of *S. chinensis* showed no lignans. Nor callus culture of *R. rosea* revealed any rhodiolosid. This might be because their concentration was below the detection limit of our analytical method (0.01 mg.g^{-1} DW) or because they were not produced, due to a lack of cell differentiation. As is known from other studies (Pasquali et al., 2006; Lindsey and Yeoman, 1983; Kutchan et al., 1983; Kutchan, 2005), biosynthesis pathways in most cases require cooperation between cells, tissues or organs on the intra- and intercellular level. Thus, for some steps of the biosynthesis, a certain degree of differentiation is crucial. The whole system is strictly regulated and very susceptible to several external (various forms of stress) or internal (e.g. maturity of the plant) stimuli.

Rhodiolosid (exclusively) was detected in our regenerated plants of *R. rosea* by TLC and HPLC at a concentration of 0.12% (Fig. 5). In order to get enough biomass, whole plants (shoots and roots) were used for the preparation of the extracts. The isolation of rhodiolosid from rhizomes (0.5-1.2%) and aerial parts (0.08-0.21%) of intact plants has been previously reported (Kurkin et al., 1986). These results indicated that the production of secondary metabolites by *S. chinensis* or *R. rosea* callus cultures *in vitro* is not directly correlated with the optimal growth ratio and thus the hormonal composition has to be directed in favor of both growth and secondary metabolite production.

This preliminary study successfully established optimized conditions for the *in vitro* growth of cultures of the mentioned plants, but unfortunately no desirable metabolites were detected. Based on the present findings, this study will be extended in the future in order to reach the ultimate goal – the increase of production of targeted secondary metabolites by *in vitro* cultures.

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