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ORIGINAL ARTICLE

Influence of Spectral Light Composition on Flavones Formation in Callus Culture of *Scutellaria baicalensis* Georgi

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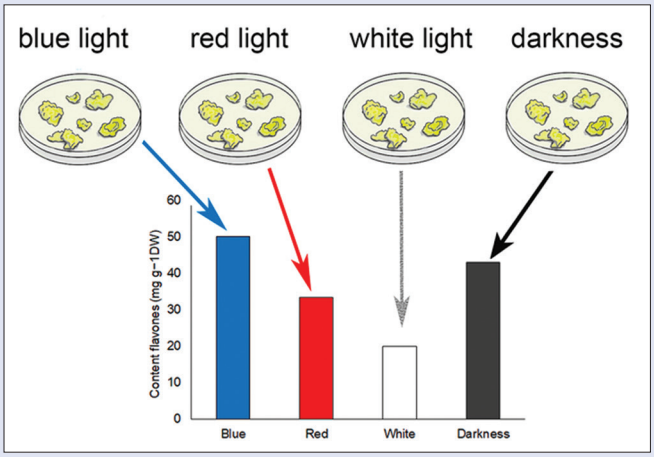
ABSTRACT

Background: *Scutellaria baicalensis* is one of the most popular medicinal plants, which roots extracts are widespread in medicine and cosmetology. The area of growth of the *S. baicalensis* is rapidly declining; therefore, the involvement of biotechnological approaches to obtain its biomass is relevant. Since the content of flavones in cultured *in vitro* cells is usually much lower than in intact plants, there is a need to strengthen the synthesis of target substances. **Objectives:** The objective of this study is to investigate the effect of blue, white, and red light for the growth and content of the four main flavones (baicalin, wogonoside, baicalein, and wogonin) in the callus tissue of the *S. baicalensis*. **Materials and Methods:** Calluses in the experiments were continuously illuminated for a month with blue (420–480 nm), red (600–650 nm), warm white (400–800 nm) using LEDs with illumination of 1 μmol/m²/s. High-performance liquid chromatography was used to determine the content of the main flavones. **Results:** The presence of the light of all studied parts of the spectrum contributed to the elongation of the stationary phase against the background of callus growth suppression, in addition, the content of flavones increased, mainly due to baicalin. The maximum number of flavones was formed in blue light (5%). **Conclusion:** The blue light is an important factor to the accumulation of the main flavones in the calluses of *S. baicalensis*. The results obtained can be used not only in fundamental research but also in practice.

Key words: Baicalin, callus tissue, flavones, light, *Scutellaria baicalensis*

SUMMARY

- The content of the flavones in calluses, first, of the main flavone-glucuronide – baicalin of *Scutellaria baicalensis* increased in all studied variants of the light spectrum. However, the highest baicalin content was observed in blue light significantly. These results may helpful for pharmaceutical biotechnology.



Abbreviations used: 2,4-D: 2,4: Dichloro acetic acid; HPLC: High pressure liquid chromatography.

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INTRODUCTION

Scutellaria baicalensis (Baikal skullcap) is a plant widespread in Chinese, Tibetan, far Eastern medicine. Currently, more than 100 phenolic compounds have been identified in *S. baicalensis*.[1] However, the medicinal properties of the plant are due to the presence in it of four main flavones-baicalin and wogonoside and their aglycones-baicalein and wogonin, localized mainly in the underground part of the plant [Figure 1].[2]

Because the natural growth area of *S. baicalensis* is rapidly decreasing, it is advisable to use biotechnological approaches to obtain its biomass, in particular, the cultivation of *in vitro* cell cultures.

The ability to synthesize them retain in *in vitro* cultured cells; although the aforesaid flavones are root specific.[3,4] However, the content of the flavones in the cultured cells is significantly lower than in intact plants.[5-7]

The intensity of their biosynthesis can be changed using physiological, physical, and genetic methods. The genetic approach involves enhancing the formation of flavonoids by introducing genes that control key enzymes of the metabolic pathway.[8,9] Currently, it is the most common, but it has a number of disadvantages and despite its laboriousness, does not always lead to a positive result.[10] The physiological methods include a change in mineral composition of the medium, ratio of phytohormones, and introduction of precursors of the metabolic pathway into the medium,[11,12] they are less expensive and simpler in execution. Recent studies include studies of the influence of physical factors, including light, on the biosynthesis of secondary metabolites.[13,14] Light is one of the most significant physical factors for a plant. Its main role is to ensure the process of photosynthesis; in addition, it plays a key role in the process of plant morphogenesis and can also participate in the regulation

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of the synthesis of secondary metabolites. Some studies have shown that changes in light intensity and its spectral composition increased the yield of biologically active substances in medicinal plants;[14-16] although, the results may be ambiguous.[14]

The aim of our work was to study the effect of light of different spectral composition on the content and ratio of flavones.

MATERIALS AND METHODS

Plant material and cultivation conditions

The object of this study was a stably growing callus culture of *S. baicalensis* obtained in 2016 from "hairy roots" (collection strain IPP RAS Sc. Baic-1).[17]

Callus were grown on solid nutrient medium B5 with the addition of hormones – 1 mg/l kinetin and 1 mg/l 2.4-D.[18] Callus were subcultured every 28 days, transferring 300 mg inoculums to Petri dishes with a diameter of 9 cm. Cultivation was carried out at 24°C. Callus in the experiments were continuously illuminated for a month with blue (420–480 nm), red (600–650 nm), warm white (400–800 nm) using LEDs with illumination of 1 μmol/m^2/s. Callus grown in the dark served as control. To characterize the growth of callus cell cultures were determined biomass growth and the growth index. Biomass growth (P_i) was evaluated every 7 days by the formula:

$P_i = (m_i - m_0)/m_0$,

where m_i is the raw weight of the i-th day of cultivation (g);

m_0 is the starting raw weight of the culture (g).

The growth index (I) for each variant of the experiment was calculated by the formula:

$I = (m_{max} - m_0)/m_0$,

where m_0 and m_{max} are the starting and maximum raw weight (g).

Extraction and sample preparation for the high-pressure liquid chromatography analysis of flavones

Sampling of biomass for the determination of flavones was carried out during the entire cycle of cultivation. Extraction from samples of freeze-dried biomass was performed with methanol (1:100 biomass: extractant ratio) in an FS14H ultrasonic bath (Fisher Scientific, USA), within 180 min, then 1 ml of the extract was taken and centrifuged for 10 min at 8000 rpm. A volume of 0.85 ml of supernatant was taken, diluted 3–4.8 times with 96% ethanol and used for high-pressure liquid chromatography (HPLC).[17]

The flavones separation was carried out on a Shimadzu LC-20 Prominence chromatograph with a Shimadzu SPD20MA diode array detector and a Zorbax C_{18} column (150 mm × 4.6 mm, particle size of a phase 5 μm). A mixture of solvents – acetonitrile (solvent A) and 0.1% trifluoroacetic acid (solvent B) was used as the mobile phase. During the separation, the regime with gradient and isocratic components was used: 0 min – 20% A, 4 min – 55% A, 14 min – 55% A, and 16 min – 20% A. The flow rate was 1 ml/min; the column temperature was 24°C; the sample volume was 20 μl. Detection was performed at λ =276 nm. The peaks of flavonoids were identified by comparing their UV spectra and retention times with the corresponding parameters of chromatographically pure baicalin, wogonoside, baicalein, and wogonin standards from AppliChem (Germany). Chromatograms were processed in the program "LabSolutions." The flavones content was determined using calibration curves constructed in the concentration range of 2–235 μg/ml [Table 1].

The equation of the calibration curves had the form $y = a \times x$, where x – the mass of the standard (μg), y – the corresponding peak area according to the results of HPLC (cu), a – the proportionality coefficient.

The absolute content of the studied flavones in terms of a gram of dry root weight was determined by the following formula:

$C = S/(a \times m \times 1000)$,

Where C is the flavone content in the dry material sample (mg/g), m is the dry material weight (g), S – peak area for the i-th flavone on the chromatogram, a is the proportionality coefficient from the calibration curve equation.

Statistical analysis

The statistical processing of the data was carried out using the Microsoft® Excel software. The text shows the average arithmetic values of the parameters. The bars on the diagram correspond to the maximum values of confidence intervals at the 95% probability level according to the Student's t-criterion. All experiments were performed at least three-fold.

RESULTS AND DISCUSSION

Growth parameters and the intensity of biomass accumulation of cells cultured *in vitro* may change under the action of light of different spectral composition; therefore, at the first stage of the study, we estimated the growth of callus grown in white, blue, and red light. Based on the data on biomass growth, for all variants of the experiments, growth curves were constructed that had a standard S-shape [Figure 2].

At the same time, the cells entered the stationary phase at different periods: for the control variant – on the 21st day of cultivation, in the red and blue light – on the 14th day of cultivation. In the white light, there was an abrupt increase of growth from 21 to 28 days of cultivation, after which they entered the stationary phase. The maximum growth index (10.1) had callus cultivated in the dark. In callus grown in white and red light, the growth index was not significantly different and was 8.4 and 8.8, respectively, this indicator was slightly lower in blue light – 7.5.

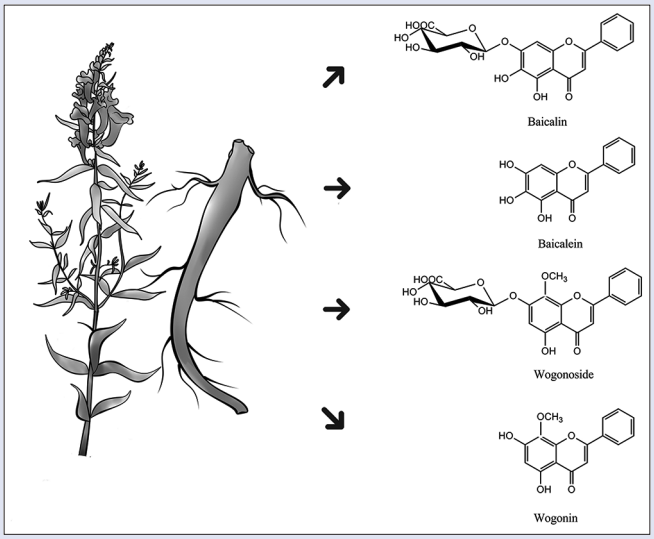

Figure 1: The main flavones of *Scutellaria baicalensis*

Table 1: Calibration curve data

Flavone	Concentration range (μg/μl)	Calibration curve equation	R^2
Baicalein	7.8-125.0	$y = 71401x$	0.9799
Baicalin	9.4-235.0	$y = 39616x$	0.9977
Wogonin	7.0-112.5	$y = 95119x$	0.9993
Wogonoside	2.0-31.3	$y = 38367x$	0.9992

Thus, in our study, placing the callus in the light in all variants led to an elongation of the stationary phase and some suppression of their growth [Figure 2]. Published data on the effect of light of different spectral composition on the biomass accumulation of plant cell cultures are contradictory. In some studies, the usage of light of different spectrum parts increased callus biomass from 1.5 to 5 times, compared to controls grown in the dark.[14,19] In other works carried out on *Stevia rebaudiana* callus, *Artemisia absinthium*, and *Eleutherococcus senticosus* suspensions, on the contrary, a decrease in biomass accumulation in the light was showed.[20-22] Probably, the ambiguity of the results is related to the fact that the reaction of plant cells to light depends on the kind of plant and the intensity of light used.

It is known that the content and ratio of basic flavones depend on the growth stage of the culture. The accumulation of secondary metabolites tends to correlate with an increase in biomass and reaches its maximum in the stationary phase of growth.[23-25] This may be because a decrease in growth in this phase promotes the use of aromatic amino acids in the secondary metabolism and consequently, enhances the flavonoids formation.[23] Therefore, in studying the content of the major flavones-glucuronides (baicalin and wogonoside) and their aglycones (baicalein and wogonin) in cell cultures grown under different light used callus located at the stationary growth phase. In our study, no correlation between the intensity of biomass accumulation and the content of the studied flavones was found, although it was noted in other authors' works on different kinds of skullcap.[3,14] In the study by Kawka *et al.* it was shown a relationship between these parameters on *Scutellaia lateriflora* callus cultured in blue light, but it was absent in the red light.[14] In the same work, it was shown that at a concentration of 1 mg/l NAA and 1 mg/l BAP, the flavone content was higher in all variants with the light of different spectra, except darkness. For callus grown in the dark, the best ratio of hormones for the formation of flavones was 3 mg/l NAA and 1 mg/l BAP, as well as 1 mg/l and 0.5 mg/l, respectively. At a concentration of 3 mg/l NAA, the flavones content was the minimum in blue and red light. It follows that there are complex relationships between the spectral composition of light, the ratio of hormones, primarily auxins since flavonoids can regulate their transport and the flavones content.[26] Changes in the hormonal level of the environment (first, the content of auxins) probably are more significant factor than light.

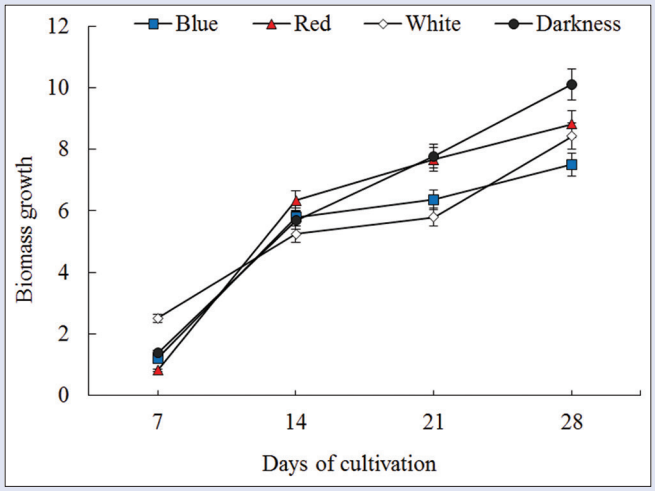

Figure 2: The dynamics of growth of calluses cultivated in different light

As a result of the HPLC analysis, it was shown that the total amount of flavones was higher in callus grown on white, blue, and red light than in darkness [Figures 3 and 4]. By the end of the callus cultivation cycle, the maximum content of basic flavones was noted in the blue light variant (up to 5%). This is probably due to the fact that blue light causes the development of oxidative stress and the formation of active oxygen forms to a much greater degree than other spectra, as shown earlier.[27] Probably, formed under oxidative stress-free radicals are the mechanism that triggers the response in the form of the formation of antioxidants, which include flavones. In addition, in the study of Shieh

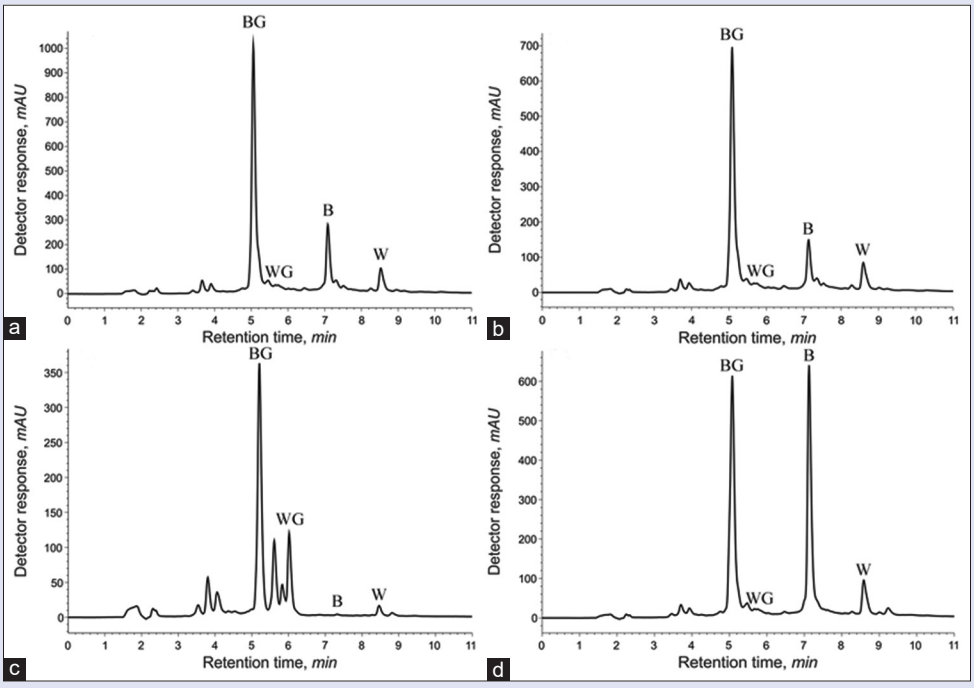

Figure 3: Results of the high pressure liquid chromatography analysis. BG: Baicalin; WG: Wogonoside; B: Baicalein; W: Wogonin. (a) Blue light. (b) Red light. (c) White light. (d) Darkness

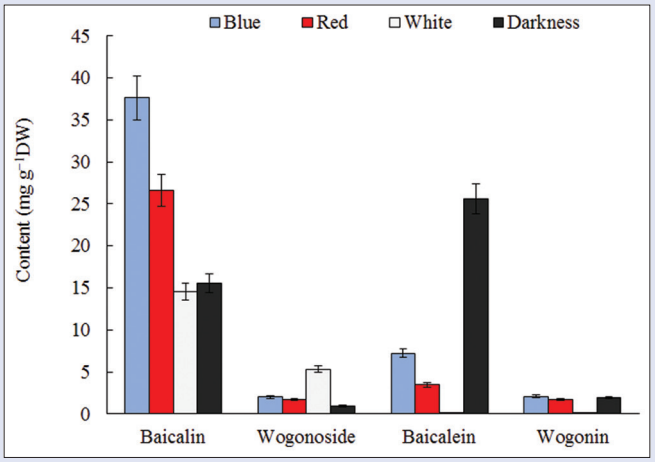

Figure 4: Influence of different light spectra on the content of the main flavones in calluses of the *Scutellaria baicalensis*

et al. it is shown that the activity of about 90% of the genes included in the phenylpropanoid pathway increases under the action of blue light, the same applies to the genes of flavonoid biosynthesis (about 77% of genes), which include flavones.[28] Thus, due to increased activity of genes of because enzymes, an increase in the accumulation of flavonoids in blue light in arabidopsis,[29] Norway spruce,[30,31] and Japanese kelp[32] was noted.

The dominant flavone in the studied callus culture was glucuronide – baicalin (60%–80% of the total number of flavones), as well as in the roots of intact plants, but its content varied in different versions of the experiment. However, in the presence of the light of the studied parts of the spectrum, the amount of baicalin, in general, was 1.8–2.6 times higher than in the dark. The highest content was observed in callus grown in blue light, it was 37 mg/g dry weight. The baicalin content in callus was comparable to its content in hairy roots cultures, on which the attention of most researchers has recently been focused.[32] This is probably due to the influence of *rol*-genes, since the calluses for our study were obtained from the hairy roots of the skullcap. The content of the second glucuronide– methylated flavone wogonoside was much lower than the baicalin and ranged from 2.1% to 26% of the total content of flavones in the callus. The highest content of wogonoside was observed in callus cultured in the dark and accounted for 26% of all flavones. It should be noted that baicalin is a much more effective antioxidant than wogonoside; perhaps, it is linked to the increase of its content in the light of different spectral composition.[28,33]

CONCLUSION

Thus, in our work, the effect of light of different spectral composition (red, blue, and white) on the formation of basic flavones in Baikal skullcap calluses obtained from the culture of hairy roots was studied. It was shown that the level of content of flavones, first of all, of the main flavone-glucuronide – baicalin, increased in all studied variants of the light spectrum. However, the highest baicalin content was observed in blue light, probably due to a higher level of oxidative stress and the release of reactive oxygen species.

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Conflicts of interest

There are no conflicts of interest.

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