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ORIGINAL ARTICLE

Update on the role of different lighting parameters in the regulation of secondary metabolism in *Lithospermum erythrorhizon* Sieb. et Zucc. calli

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Abstract

Currently, global challenges require the search for alternative sources of phytochemicals used by Mankind throughout its history. Plant cell culture technology can provide efficient and sustainable sources of phytochemicals with reduced energy and carbon footprints. The first patented industrial process of cultivating calluses as sources of phytochemicals was based on the calli of *Lithospermum erythrorhizon* Siebold & Zucc (Boraginaceae). The high pharmacological activity of *L. eryrthrorhizon* is due to the content of the naphthoquinone shikonin. Despite the development of LED lighting technology, in-depth studies of the effects of light on the biosynthesis of shikonin in cell cultures have not been carried out. In the present work, the impact of artificial monochromatic and bichromatic LED light at wide ranges of intensities (50, 100, and 300 µmol $m^{-2}s^{-1}$) on the growth and biosynthesis of caffeic acid derivatives (CADs) as well as shikonin and shikonofurans in long-term continuously cultivated *L. erythrorhizon* calli was investigated for the first time. Red light has the greatest growth-stimulating effect regardless of intensity. The most effective treatment for CAD production is red/blue and green light treatment. The most effective way to produce shikonin in long-term cultivated *L. erythrorhizon* calli is to use red light with an intensity of 50 µmol $m^{-2}s^{-1}$ and increase the inductor concentration. The blue and green components of white light may also have a negative effect on shikonin biosynthesis because of the light-dependent shift in the biosynthesis of CADs.

Key message

Low-intensity red LED light can improve shikonin production in L. erythrorhizon calli, whereas the blue and green components of white light have a negative effect on shikonin biosynthesis because of the light-dependent shift in the biosynthesis of CADs.

Keywords Artificial light · Long-term cultured calli · Purple gromel · Rabdosiin · Rosmarinic acid · Shikonin derivatives

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Introduction

Throughout history, plants have been the main source of phytochemical compounds used by mankind for food, medicinal, and cosmetic purposes (Jamshidi-Kia et al. 2018). Currently, global climate change, limited fresh water and food supplies, achievements in sustainable development and increasing energy needs are among the most important global challenges facing humanity. Plant cell culture technology can address some of these issues by providing efficient and sustainable sources of phytochemicals with reduced energy and carbon footprints (Krasteva et al. 2021). In vitro plant cultures have been successfully used for the production of metabolites as well as for the biotransformation of organic compounds. These pioneering studies served as a fundamental basis for subsequent studies on the large-scale cultivation of plant cells as bioreactor systems with ever-increasing working volumes (Pantchev et al. 2018). Notably, the first patented industrial process of cultivating cell culture as a source of phytochemicals was based on the callus line of Purple Gromwel, also known as Zicao, *Lithospermum erythrorhizon* Siebold & Zucc, which belongs to the Boraginaceae family (Curtin 1983). *L. eryrthrorhizon* is one of the main plants in traditional Chinese medicine and has been actively studied in recent decades (Sun et al. 2022). The high pharmacological activity of *L. eryrthrorhizon* is due to the content of specific polyphenols and mainly derivatives of the naphthoquinone shikonin.

Shikonin has a variety of therapeutic effects, including anti-inflammatory, anticancer, cardiovascular, antimicrobiome, analgesic, antiobesity, and neuroprotective effects. These effects are caused primarily by the regulation of intracellular signal transduction cascades. The pharmacokinetics of shikonin include unfavourable oral bioavailability, a plasma protein binding rate of 64.6%, and an increase in the levels of several metabolic enzymes, especially cytochrome P450. With respect to toxicological effects, shikonin has the potential to produce skin allergies and nephrotoxicity (Sun et al. 2022; Yadav et al. 2022). The main source of shikonin in wild-grown *L. erythrorhizon* has faced the risk of extinction in recent years. Alternative approaches such as cultivation or chemical biosynthesis do not provide cost-effective sources for the industrial production of this compound (Yazaki 2017). Since the 1970s, *L. erythrorhizon* cell cultures have been actively studied as sources of shikonin. The results of numerous studies have made it possible to develop and optimize a two-stage system for the industrial production of shikonin on the basis of cell culture. Compared with farm-based production, cell culture-based shikonin production increases yield by up to 800 times (Yazaki 2017).

Over decades of active research into optimizing *L. erythrorhizon* cell culture productivity, a list of shikonin inducers and repressors has been compiled (Sun et al. 2022). Various components of the nutrient medium, including agar, plant growth regulators, and various elicitors, are capable of both inducing or increasing, as well as completely blocking, the biosynthesis of shikonin. One of the least studied effectors is light. In 2002, it was shown that exposure to light has a negative effect on the biosynthesis of shikonin. The experiment was conducted as follows: the culture flask was covered with a commercially available colored cellophane sheet and irradiated with white light (10000 lx) from fluorescent lamps. The light intensities in the blue, red, and green cellophane-covered flasks were 600, 1000, and 900 lx, respectively. White and blue light have been shown to have strongly negative effects, whereas red and green light slightly reduce shikonin accumulation (Yamamoto et al. 2002). However, despite the development of lighting technology and the possibility of regulating the intensity and spectral composition of light, studies on the effects of light on the biosynthesis of shikonin in cell cultures have not been carried out. However, such studies are of at least general interest, since in addition to shikonin, a number of other compounds are synthesized in cell cultures that accompany the shikonin biosynthetic pathway.

A detailed definition of the shikonin biosynthesis pathway allows for a more informed approach to the issue of its regulation. While free shikonin cannot be detected, it is present in living plant cells as esters of low-molecular-weight fatty acids such as acetate. Shikonin biosynthesis occurs through the phenylpropanoid and mevalonate pathways. The byproduct geranylhydroquinone is a precursor to both shikonin and shikonofurans. In addition to shikonin esters and shikonofurans, phenylpropanoid pathway products such as caffeic acid derivatives have been found in large quantities in *L. erythrorhizon* cell cultures. Moreover, their biosynthesis competes with shikonin biosynthesis at the coumaroyl-CoA stage (Sun et al. 2022). Thus, in addition to shikonin esters, *L. erythrorhizon* cell cultures contain shikonofurans and caffeic acid derivatives (CAD), such as lithospermic acid B (LAB), rosmarinic acid (RA), and rabdosiin. The approach to the activation of shikonin biosynthesis must be based on differential regulation of the biosynthesis of the entire spectrum of compounds. Modern methods for modulating light exposure allow reproducible experiments on the influence of light factors on the secondary metabolism of plants to be conducted (Cavallaro and Rosario Muleo 2022). The effects of light exposure on the growth parameters of plant cell cultures are no less interesting. Thus, red light stimulates the growth of undifferentiated cultures without damaging secondary metabolism (Sobhani Najafabadi et al. 2019; Veremeichik et al. 2024a, 2025).

Therefore, in the present work, we investigated the effects of fine-tuning parameters such as the spectrum and

intensity on the growth and phytochemical content of long-term continuously cultivated calli of *L. eryrthrorhizon*. The Callus line BK-39 of *L. eryrthrorhizon* was obtained in 1993, while the first phytochemical analysis of this callus was performed in 2001 (Bulgakov et al. 2001). At that time, the 8-year-old *L. eryrthrorhizon* callus line contained 7 shikonin derivatives: acetylshikonin, propionylshikonin, isobutyrylshikonin, dimethyacrylshikonin, isovalerylshikonin, hydroxyisovalerylshikonin, and methylbutyrylshikonin. There are no published data about any other phytochemical compounds produced by the callus line BK-39 of *L. eryrthrorhizon* until 2005. In 2005, the 12-year-old callus line BK-39 of *L. eryrthrorhizon* produced approximately 1% DW of both rabdosiin and RA (Bulgakov et al. 2005). A more detailed analysis performed in 2021 (Shkryl et al. 2021) revealed the presence of caffeic acid derivatives (rabdosiin and RA), shikonofuran derivatives (shikonofuran D, E, and C), and shikonin derivatives (hydroxyisovalerylshikonin, acetylshikonin, isobutyrylshikonin, and isovalerylshikonin) in the 28-year-old callus line BK-39 of *L. eryrthrorhizon*. However, quantitative analysis of these compounds in the 28-year-old callus line BK-39 of *L. eryrthrorhizon* has not been performed. In the present work, we conducted a detailed qualitative and quantitative analysis of phytochemical compounds produced by the 32-year-old callus line BK-39 of *L. eryrthrorhizon*. The effects of fine-tuning the LED spectrum and intensity on the growth and content of individual compounds in long-term continuously cultivated calli of *L. eryrthrorhizon* were also studied. The design features of the lighting equipment used and the availability of measuring instruments made it possible to conduct repeatable experiments under the same conditions. Thus, these studies were conducted for the first time and allowed us to expand our understanding of the role of light factors in the regulation of secondary metabolism in the callus culture of *L. eryrthrorhizon*.

Materials and methods

Plant materials, growth conditions, and experimental design

The callus line BK-39 of *L. erythrorhizon* was obtained previously, in 1993, from stem explants (Bulgakov et al. 2001). The modified W media (ammonium nitrate content was reduced to 400 mg/L according to White 1939) was supplemented with the following components (mg/L): nicotinic acid (0.5), thiamine-HCl (0.2), mesoinositol (100), peptone (100), pyridoxine-HCl (0.5), sucrose (25000), agar (6000), kinetin (2), indole-3-acetic acid (0.2), and CuSO4 (0.3); pH 5.5–5.8. *L. erythrorhizon* calli were aseptically cultivated continuously in the same sterilized solid medium in the dark at 24 °C for more than 30 years and subcultured once every 30 days. The study employs specimens (strains) deposited in the Bioresource Collection of the Federal Scientific Centre of East Asia Terrestrial Biodiversity of the Far East Branch of the Russian Academy of Sciences (reg. number 2797657).

For the experiments, after inoculation (2 g of inoculant per 50 mL of solid medium in Erlenmeyer flasks), the calli were immediately transferred under different light treatments for 30 days. As a control, we used calli growing in the dark. Thirty-day-old *L. erythrorhizon* calli growing under different light conditions were photographed, harvested, weighed, and dried for chemical analysis. For a technical replication, 10 jars containing 2 g of inoculant were exposed to each variant of LED light. Three separate experiments were conducted as biological replicates.

The four-section growing chambers (100 × 50 × 50 cm) with light sources and a photoperiod (light/dark) of 16–8 h were designed and manufactured at the IACP FEB RAS. The light source matrices were made up of 24 three-watt LEDs (CHANZON, China) of various colours, creating one integrated light source as described previously (Veremeichik et al. 2024a). The reflective aluminum foil was used to diffuse light. The temperature (24 °C) and air humidity (70%) were supported *via* an FFB1212SH 12,025 exhaust fan. The spectrum and intensities in each divided section were modulated according to the experimental design. In this study, in addition to warm white light (designated "W"), red (660 nm, designated "R") and green (520 nm, designated "G") monochromatic light as well as bichromatic red and blue (designated "RB") monochromatic light were used. Each section of the chamber was equipped with LED lamps with different light intensities: 50, 100, or 300 µmol $m^{-2}s^{-1}$. Each segment had 1–10 light-emitting matrices, which produced the requisite level of photosynthetic photon flux density (PPFD). The intensity of the light in each portion of the chamber was adjusted by altering the supply current for each matrix. The spectra were measured with a PG200N spectrophotometer (UPRtek, Taiwan). Currents in the driver supply system were managed by a UT61A digital multimeter (Uni-T, China).

Quantitative and qualitative analysis of phytochemicals

Chemicals

The analytical standard of rosmarinic acid was obtained from Aldrich (Germany). An analytical standard of rabdosiin with a purity of 98.5% was obtained previously from a callus culture of *Eritrichium sericeum* (Fedoreyev et

al. 2005). A standard sample of shikonin with a purity of 98.5% was obtained previously from the roots of the *L. erythorhizons plant* collected in Primorsky Krai (Fedoreev et al. 1979). All the eluents and extraction solutions were prepared with ultrapure water (Millipore, Bedford, MA, USA). All solvents were of analytical grade.

Sample preparation

For analysis, the calli were harvested after 30 days of cultivation and dried (45 °C for 12–16 h). For analytical chromatography, extraction was performed according to a previously published protocol (Veremeichik et al. 2024a). Briefly, 50 mg of powdered callus material was extracted in 1 ml of 80% aqueous methanol *via* ultrasonic treatment at 45 °C for 20 min and incubated for 12 h on a vortex (45 °C, 800 rpm). For spectrophotometric analysis of total shikonin esters in the callus material, 50 mg of powdered callus material was extracted in 2.0 ml of ethanol *via* ultrasonic treatment at 45 °C for 10 min and incubated for 2 h at room temperature. The resulting extract was filtered through a 0.45 µm syringe filter (Millipore, Bedford, MA, USA) before analysis.

Analytical chromatography, mass spectrometry, and spectrophotometry

The polyphenol extracts were studied at the Instrumental Centre of Biotechnology and Gene Engineering of IBSS FEB RAS *via* a 1260 Infinity analytical HPLC system (Agilent Technologies, Santa Clara, California, USA) interfaced with an ion trap mass spectrometer (Bruker HCT ultra PTM Discovery System, Bruker Daltonik GmbH, Bremen, Germany). An analytical Zorbax C18 column (150 mm, 2.1 mm i.d., 3.5 µm, Agilent Technologies, USA) for polyphenol separation was applied at 40 °C. The mobile phase consisted of a gradient elution of ultrapure water (A) and acetonitrile (B) with 0.1% formic acid added in both cases. The following linear gradient at a flow rate of 0.2 mL/min was used: 0 min, 5% B; 20 min, 30% B; and 30 min, 100% B. A photodiode array detector was employed in the range between 200 and 600 nm to obtain UV–Vis spectra. Chromatograms for quantification were recorded at a wavelength of 325 nm. The MS instrument was operated in electrospray ionization (ESI) mode, and negative ions were detected. The following settings were used: the range of *m/z* detection was 100–650, the drying gas (N_2) flow rate was 8.0 L/min, the nebulizer gas (N_2) pressure was 25 psi, the ion source potential was −3.8 kV, and the drying gas temperature was 325 °C. Tandem mass spectra were acquired in Auto-MS^2 mode (smart fragmentation) by increasing the collision energy. The fragmentation amplitude was set to 1 V.

The total content of shikonin esters in the plant material was determined *via* a UV-1800 spectrophotometer (Shimadzu USA Manufacturing Inc., Oregon, USA). The optical density of the extracts was measured at a wavelength of 526 nm. The content of shikonin derivatives in the sample was calculated *via* a calibration curve created from a solution of shikonin standard in ethanol ($y = 0.0199577x - 0.0035186$, $R^2 = 0.9994$).

The productivity of the polyphenols was calculated as follows:

Productivity (mg/l) = Content × DW,

where Content denotes the content of an individual compound (mg/g DW) and DW denotes the dry weight (g) of the callus biomass per liter of medium (g/l).

Statistical analysis

The STATISTICA software package (StatSoft, Inc., USA) was used for the statistical analysis. All values are presented as the mean ± standard error (SE). Student's t test was employed for the statistical assessment to compare two independent groups. Analysis of variance (ANOVA) was used, together with a multiple comparison approach, to compare several datasets. The cut-off point for statistical significance was fixed at $p < 0.05$.

Results

Phytochemical composition of the 32-year-old callus line BK-39 of *L. eryrthrorhizon*

In the present work, we first analysed phytochemical compounds in the 32-year-old *L. eryrthrorhizon* callus line BK-39 and compared the obtained results with known data to determine the influence of continuous long-term cultivation on the productivity of the *L. eryrthrorhizon* callus line BK-39. The HPLC–PDA-ESI-HR-MS/MS2 method was used to determine the phenolic compounds in the crude aqueous-metabololic extracts of *L. erythrorhizon* cells. First, we ensured that all previously identified components (Shkryl et al.,2021) were preserved in the studied cell culture. The chromatographic profile (Fig. 1) of the control sample demonstrated the presence of several peaks divided into three groups. The two major peaks were identified as caffeic acid derivatives because of their full similarity with available standards: rabdosiin (**1**) and rosmarinic acid (**2**) (Fig. 1, a). We were also able to identify minor components *via* identification carried out earlier (Shkryl et al.,2021). The next three peaks were assigned as shikonofurans: shikonofuran D (**3**), shikonofuran E (**4**) and shikonofuran C (**5**) (Fig. 1, a). Red naphthoquinone pigments (shikonin derivatives) were

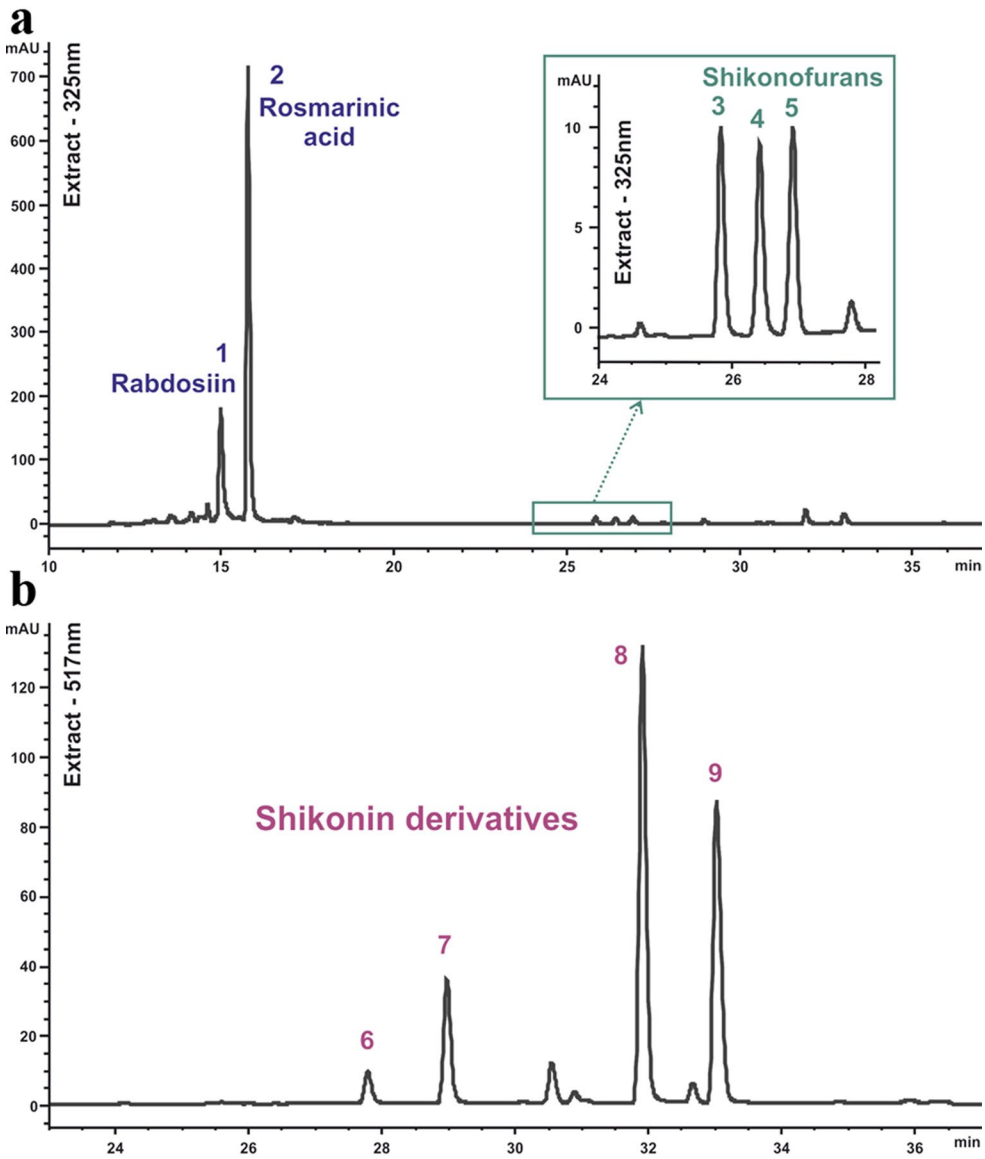

Fig. 1 HPLC profiling of phenolic compounds detected in the crude extract obtained from *L. erythrorhizon* calli grown under control conditions in the dark. Chromatograms were recorded at 325 nm (**a**) and 517 nm (**b**). The peak numbers correspond to those listed in Table 1

also detected: hydroxyisovalerylshikonin (**6**), acetylshikonin (**7**), isobutyrylshikonin (**8**) and isovalerylshikonin (**9**) (Fig. 1, b). All the information about the determined phenolic compounds is summarized in Table 1. The quantitative measurement of rabdosiin (**1**) was performed *via* the external standard method with a research-grade standard sample of previously isolated rabdosiin. Rosmarinic acid (**2**) and shikonofurans (**3–5**) were quantified on the basis of four-point regression curves built with the reference commercial standard of rosmarinic acid.

The results of the quantitative analysis (Table 1) revealed that the contents of the major compounds, rabdosiin and RA, did not change beginning in 2005 during continuous long-term cultivation and reached 1% DW. However, the content of shikonin derivatives was significantly lower than that in 2001. Only four of the seven compounds were detected in the 32-year-old callus line BK-39 of *L. eryrthrorhizon*. Interestingly, these four shikonin derivatives were predominant in the 8-year-old callus line BK-39 of *L. eryrthrorhizon*. While minor compounds were completely absent, the compositions of these four major shikonin derivatives changed. In 2025, the major compounds were isobutyrylshikonin, isovalerylshikonin, and hydroxyisovalerylshikonin (48, 35, and 12% of shikonin derivatives, respectively), while the content of acetylshikonin was reduced to 4% of that of shikonin derivatives. The total content of shikonofurans in the 32-year-old callus line BK-39 was not greater than 0.06% DW.

Table 1 List of phenolic compounds produced by *L. erythrorhizon* calli grown under control dark conditions

Peak number	Compound	Rt, min	UV max, nm	ESI-MS, $[M-H]^-$, m/z	Content, % DW
1	Rabdosiin	15.0	253, 285, 346	717	1.061 ± 0.003
2	Rosmarinic acid	15.8	287, 327	359	1.042 ± 0.138
3	Shikonofuran D	25.8	269, 323	343	0.023 ± 0.003
4	Shikonofuran E	26.4	271, 328	355	0.021 ± 0.002
5	Shikonofuran C	26.9	270, 325	357	0.018 ± 0.003
6	Hydroxyisovaleryl-shikonin	27.8	272, 518	387	0.124 ± 0.002
7	Acetylshikonin	29.0	274, 516	329	0.039 ± 0.003
8	Isobutyrylshikonin	31.9	273, 517	357	0.451 ± 0.037
9	Isovalerylshikonin	33.0	273, 517	371	0.341 ± 0.024

The growth of *L. eryrthrorhizon* calli cultivated under different light treatments

Previously, we showed that different LED treatments can improve the productivity of long-term cultivated calli (Veremeichik et al. 2024a). On the basis of previous studies, we can also conclude that the most appropriate intensities of LED light exposure are 100 and 300 µmol $m^{-2}s^{-1}$. In 2002, light exposure was shown to block shikonin biosynthesis (Yamamoto et al. 2002). However, despite significant advances in photobiology, similar studies using finely tuned lighting have not been carried out. In the present study, we investigated the phytochemical content of the 32-year-old callus line BK-39 of *L. eryrthrorhizon* grown for 30 days under monochromatic red, green, bichromatic red and blue LED light at intensities of 100 and 300 µmol $m^{-2}s^{-1}$: R100 and R300; G100 and G300; and RB100 and RB300. Dark conditions (D) were used as positive controls, and warm white LED light with an intensity of 100 µmol $m^{-2}s^{-1}$ (W100) was used as a negative control.

First, we investigated the effects of different light conditions on the growth of *L. eryrthrorhizon* callus cultures. *L. eryrthrorhizon* calli were cultivated once under different light condition for 30 days. In our work, we investigated the effects of two main variables of LED lighting on the productivity of *L. eryrthrorhizon* cell cultures: spectral composition and illumination intensity, expressed in PPFD. The four-section chambers are equipped with adjustable lamps combined into one matrix (Fig. 2, a). Warm white (W) and monochromatic, red (R) and green (R), and bichromatic red and blue (RB) lighting options (Fig. 2, b-e) with different characteristics were used (Fig. 2, f). The light intensities chosen for the experiments were 100 and 300 µmol $m^{-2}s^{-1}$. Calli grown in the dark were used as a control.

We analysed the effects of warm white light, red, green and a combination of red and blue light with intensities of 100 and 300 µmol $m^{-2}s^{-1}$. Compared with the dark-grown control, all the light treatments had no negative effect on the growth of the *L. eryrthrorhizon* callus culture. However, warm white, green and a combination of red and blue light resulted in the loss of color in the culture. While *L. eryrthrorhizon* calli grown in the dark are rich in crimson color, the calli grown under light conditions are devoid of color. However, the calli grown under red light remained colored (Fig. 3).

Phytochemical contents of *L. eryrthrorhizon* calli cultivated under different light treatments

First, we were interested in how different LED light treatments affect the contents of shikonin derivatives. As shown in Table 2, all the light treatments completely blocked the biosynthesis of the shikonin derivatives in *L. eryrthrorhizon* calli, despite red light of both intensities (100 and 300 µmol $m^{2}s^{1}$). Red light treatment led to a 20-fold reduction in the contents of the major compounds isobutyrylshikonin and isovalerylshikonin. Biosynthesis of minor compounds (hydroxyisovalerylshikonin and acetylshikonin) as well as the biosynthesis of shikonofurans were blocked in red light-treated *L. eryrthrorhizon* calli.

We analysed the effects of different light treatments on the contents of the major polyphenolic compounds, rabdosiin and RA. Warm white light with an intensity of 100 µmol $m^{-2}s^{-1}$ did not affect the biosynthesis or growth of either caffeic acid derivative compared with the control dark-grown *L. eryrthrorhizon* calli (Fig. 4). Interestingly, whole green and red–blue light treatments with an intensity of 100 µmol $m^{-2}s^{-1}$ led to a 1.5- and 2-fold increase in the rabdosiin content, respectively, whereas red light treatment resulted in an almost twofold increase in the rabdosiin content compared with that of the control dark-grown *L. eryrthrorhizon* calli (Fig. 4, a). Increasing the light treatment intensity to 300 µmol $m^{-2}s^{-1}$ did not have positive effects on the rabdosiin content. Considering the impact of light treatments on the growth of *L. eryrthrorhizon* calli, the productivity of rabdosiin in G100 and RB100 light-treated *L. eryrthrorhizon* calli was greater than 1.5 times greater than that in the control dark-grown *L. eryrthrorhizon* calli (Fig. 4, a). At that time, no light treatment had any positive effect on the biosynthesis or productivity of RA (Fig. 4, b).

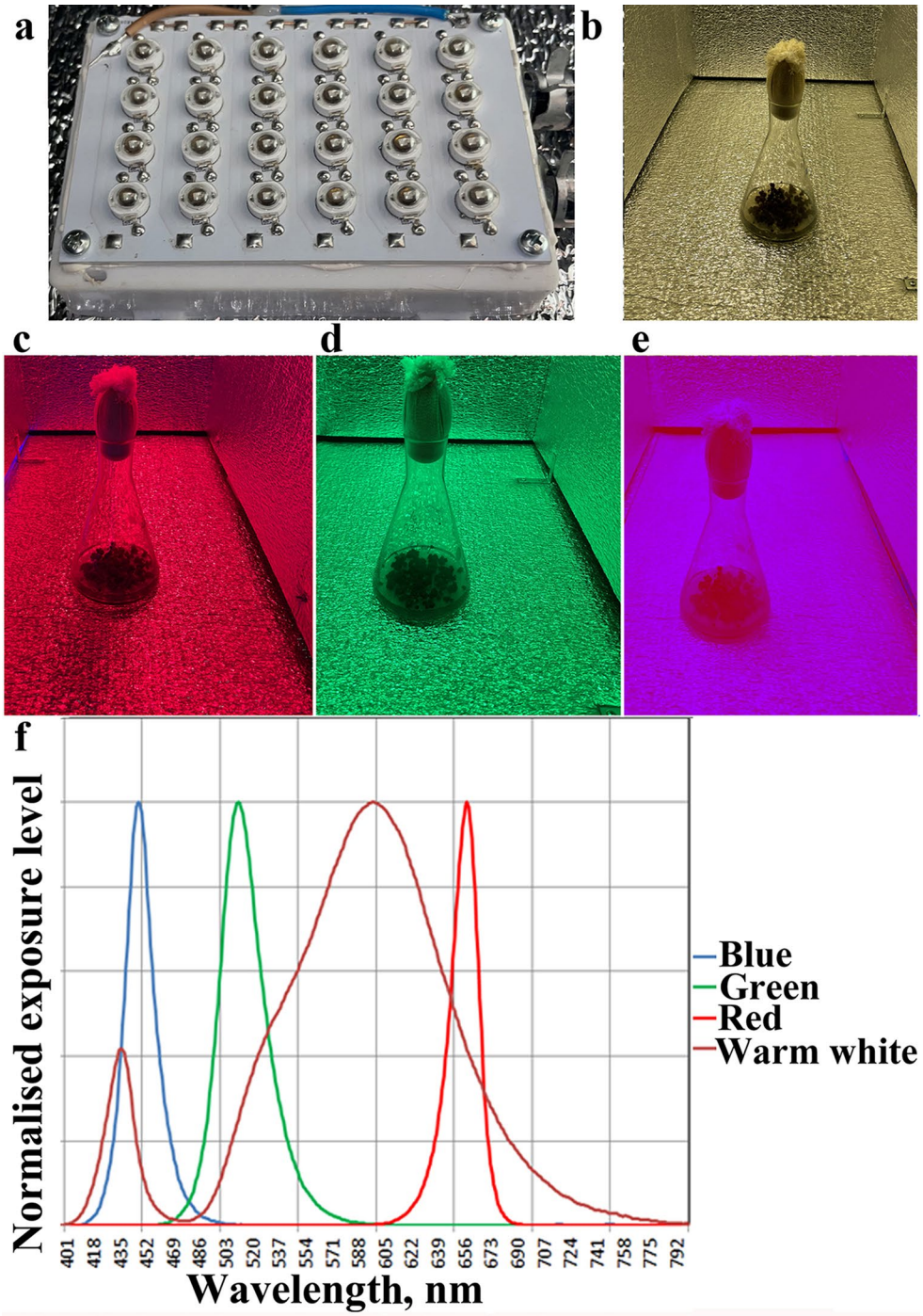

Fig. 2 Growth chambers and characteristics of light. The appearance of the lamp, consisting of 24 LEDs (**a**); growing chambers (**b-e**) with artificial lighting variations (left to right): warm white and monochromatic sources such as red and green, and bichromatic sources combining red and blue. The normalized spectral characteristics of the light emission levels of warm white and monochromatic light sources as a function of wavelength (nm) are presented (**f**)

The growth and phytochemical content of *L. eryrthrorhizon* calli cultivated under low-intensity red and blue light treatments

Since exposure to red light did not block shikonin biosynthesis in *L. eryrthrorhizon* calli, we next tested the effect of red light at a reduced intensity of up to 50 µmol $m^{-2}s^{-1}$ (R50). In addition, we studied the effects of supplementation with 1/5 (R40B10) or half (R25B25) blue light on the overall intensity of 50 µmol $m^{-2}s^{-1}$. All three light treatments had a strongly positive effect on callus growth, with an increase of more than 15% (Fig. 5, a). However, when the calli were exposed to pure red light, the color of the culture was no less intense than that of the dark-grown control. When blue light was supplemented, the color of the calli visually became less saturated, which indicates a decrease in the accumulation of shikonin (Fig. 5, b).

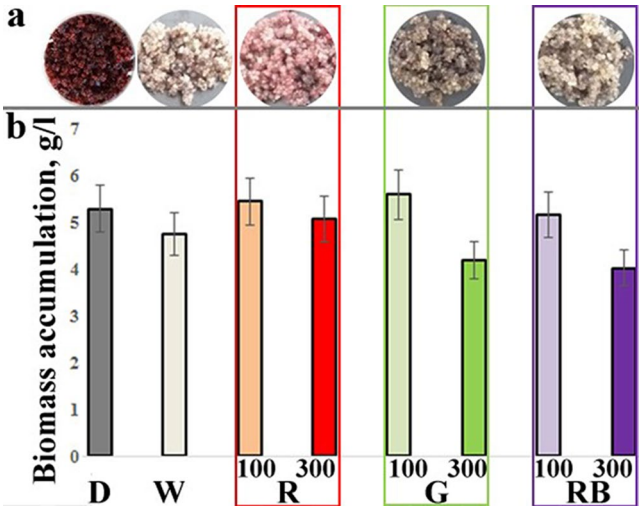

Fig. 3 Development of a callus culture of *L. eryrthrorhizon* grown under various light conditions. Morphology (**a**) and biomass accumulation (**b**, g/L) of 30-day-old *L. eryrthrorhizon* calli (2 g FW inoculants per 50 ml of solid medium) were cultivated for 30 days under various light treatments: D, darkness; warm white, monochromatic red and green, and bichromatic sources of combinations of red and blue light treatments were designated W, R, G, and RB, respectively. Listed light variants were used with intensities of 100 and 300 µmol $m^{-2}s^{-1}$. The mean ± standard error of the mean is used to show the data from three separate studies with ten biological replicates

Surprisingly, R50 treatment led to a significant increase in shikonin biosynthesis. As shown by HPLC analysis, the contents of hydroxyisovalerylshikonin and isovalerylshikonin were increased approximately 2- and 1.4-fold, respectively, compared with those in the control dark-grown *L. eryrthrorhizon* calli (Table 3). The contents of isobutyrylshikonin and acetylshikonin did not change. At that time, the content of shikonofurane was reduced approximately 2-fold in R50-treated *L. eryrthrorhizon* calli compared with that in the control dark-grown *L. eryrthrorhizon* calli (Table 3). Supplementation of red light with blue light led to a dramatic decrease in the shikonin derivative content and total blockade of shikonofurane biosynthesis. Interestingly, none of these light treatments led to significant changes in the rabdosiin content; however, R50 treatment led to a significant decrease in the RA content (Fig. 6, a). However, considering the impact of these light treatments on growth, the productivity of both rabdosiin and RA was more than 20% greater in R40B10-treated *L. eryrthrorhizon* calli than in control dark-grown calli (Fig. 6, b).

The impact of inductors and low-intensity red light treatment on the production of shikonin in *L. eryrthrorhizon* calli

Copper ions are the most effective inducers of shikonin biosynthesis in the callus cultures of *L. eryrthrorhizon* (Sun et al. 2022). We investigated the combined effects of low-intensity red LED light (50 µmol $m^{-2}s^{-1}$) and increased concentrations of inducers (1.2 and 2.4 mg/L) on shikonin production in *L. eryrthrorhizon* calli. Increasing the copper glycerate concentration to 1.2 mg/L did not suppress growth and increased the content and production of shikonin esters by 20% when the samples were grown in the dark (Fig. 7). When grown in the dark, increasing the copper glycerate concentration to 2.4 mg/L had an inhibitory effect on growth (Fig. 7, a). However, the content of shikonin esters did not increase compared with that in the 1.2 cultivar (Fig. 7, b). Moreover, the production of shikonin increased by 20% with the addition of 1.2 and insignificantly with the addition of 2.4 (Fig. 7, c). When *L. eryrthrorhizon* calli were grown under red light with an intensity of 50 µmol $m^{-2}s^{-1}$, we did not find a negative effect of increasing the concentration of copper glycerate on the growth of the culture. Increasing the concentration of copper glycerate to 1.2 mg/L and growing *L. eryrthrorhizon* calli under red light with an intensity of 50

Table 2 HPLC analysis of Shikonin derivatives in *L. eryrthrorhizon* calli cultivated for 30 days under various light treatments: D, darkness; warm white, monochromatic, and bichromatic sources designated W, R, G, and RB, respectively, with intensities of 100 and 300 µmol $m^{-2}s^{-1}$

	Light treatment, µmol $m^{-2}s^{-1}$							
	D	W100	R100	R300	G100	G300	RB100	RB300
Shikonins, mg/g DW								
Hydroxyisovalerylshikonin	1.18 ± 0.02	ND	ND	ND	ND	ND	ND	ND
Acetylshikonin	0.39 ± 0.03	ND	ND	ND	ND	ND	ND	ND
Isobutyrylshikonin	4.53 ± 0.37*	ND	0.193 ± 0.001	0.235 ± 0.003	ND	ND	ND	ND
Isovalerylshikonin	3.35 ± 0.24*	ND	0.225 ± 0.001	0.215 ± 0.002	ND	ND	ND	ND
Shikonofurans, mg/g DW								
Shikonofuran D	0.17 ± 0.03	ND	ND	ND	ND	ND	ND	ND
Shikonofuran E	0.16 ± 0.02	ND	ND	ND	ND	ND	ND	ND
Shikonofuran C	0.18 ± 0.03	ND	ND	ND	ND	ND	ND	ND

ND, not detected

The mean ± standard error of the mean is used to show the data from three separate studies with ten biological replicates

* above the error indicates statistically significant differences (ANOVA, $p < 0.05$)

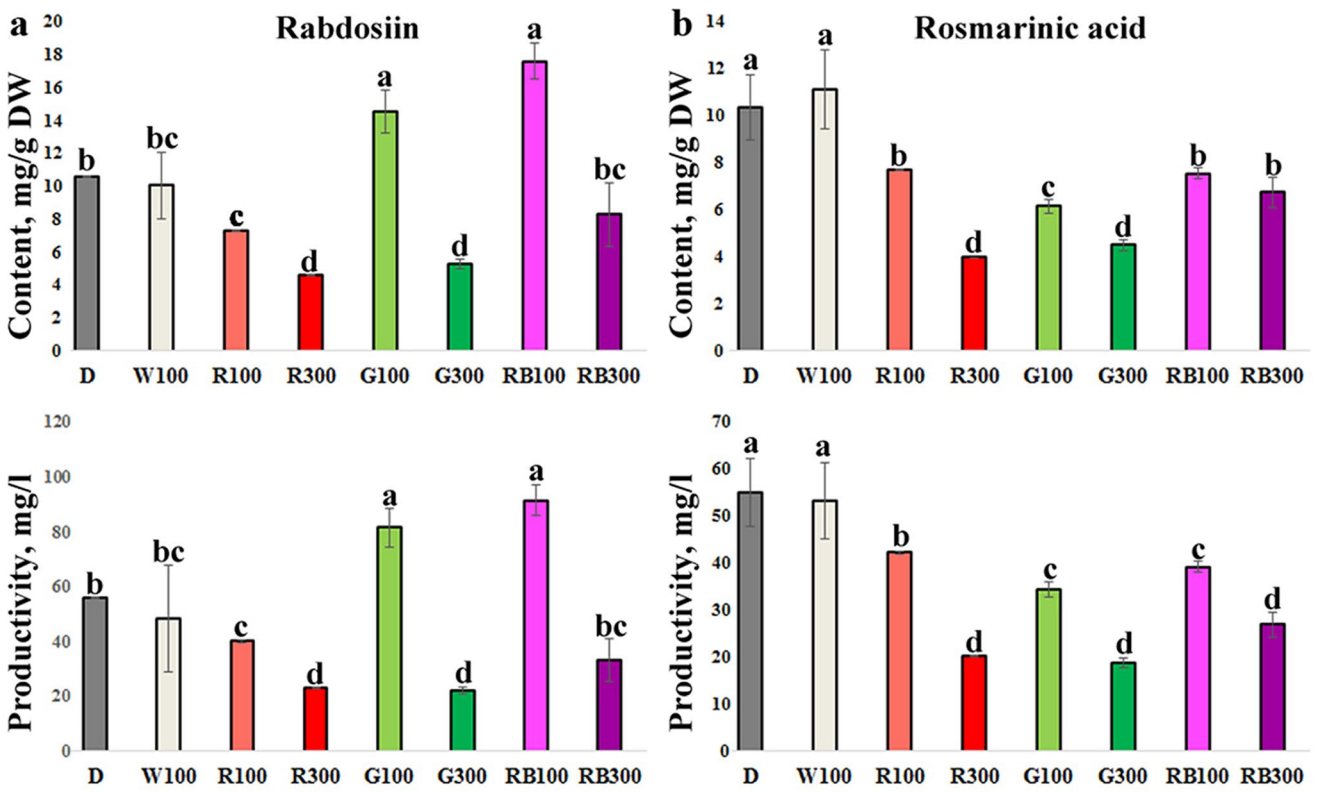

Fig. 4 Contents and productivity of rabdosiin and rosmarinic acid in *L. eryrthrorhizon* calli grown under different lighting conditions. Contents (**upper panel**) and productivity (**lower panel**) of rabdosiin (**a**) and rosmarinic acid (**b**) in *L. eryrthrorhizon* calli (mg/g DW) cultivated for 30 days under various light treatments: D, darkness; warm white, monochromatic red and green, and bichromatic sources of combinations of red and blue light treatments are designated W, R, G, and RB, respectively. Listed light variants were used with intensities of 100 and 300 µmol $m^{-2}s^{-1}$. The data obtained from three independent experiments with ten biological replicates are presented as the mean ± standard error of the mean, and different letters above the error bars indicate statistically significant differences (ANOVA, $p < 0.05$)

µmol $m^{-2}s^{-1}$ allowed us to increase the productivity of the culture by more than 2 times (Fig. 7).

Discussion

It is believed that with long-term perennial cultivation, the ability of calli not only to regenerate but also to produce secondary metabolites decreases (Liu et al. 2009). In the present work, we analysed the growth and biosynthetic characteristics of the *L. eryrthrorhizon* callus line BK-39 obtained in 1993 after 32 years of cultivation (more than 380 passages). Thus, the growth of the callus line BK-39 was approximately 19 g/L DW in 2001, after 8 years of continuous cultivation. As we showed in the present work, after 32 years of continuous cultivation, the growth of the *L. eryrthrorhizon* callus line BK-39 was approximately 5 g/L DW. We suggest that long-term cultivation may be the reason for this significant (approximately fourfold) decrease in growth. The effects of over a decade of continuous cultivation on the growth of a callus culture are poorly understood. In a recent study, we demonstrated that long-term cultured calli did not experience a decline in growth when their secondary metabolite biosynthesis decreased (Veremeichik et al. 2024a, b, 2025). We assumed that the youthful culture was characterized by explosive growth, which eventually levelled off to a comfortable level.

In this study, we also examined phytochemicals in the 32-year-old *L. eryrthrorhizon* callus line BK-39. To determine the impact of continuous long-term cultivation on the production of the *L. eryrthrorhizon* callus line BK-39, we compared the results with existing data. The chromatographic profile of the *L. eryrthrorhizon* callus line BK-39 demonstrated the presence of two major peaks, rabdosiin and RA, and minor components such as shikonofurans and shikonin derivatives. The Callus line BK-39 of *L. eryrthrorhizon* was obtained in 1993, while the first phytochemical analysis of this callus was performed in 2001 (Bulgakov et al. 2001). In 2005, the 12-year-old callus line BK-39 of *L. eryrthrorhizon* produced approximately 1% DW of both rabdosiin and RA (Bulgakov et al. 2005). Quantitative analysis revealed that the contents of the major compounds, rabdosiin and RA, did not change beginning in 2005 during continuous long-term cultivation and reached

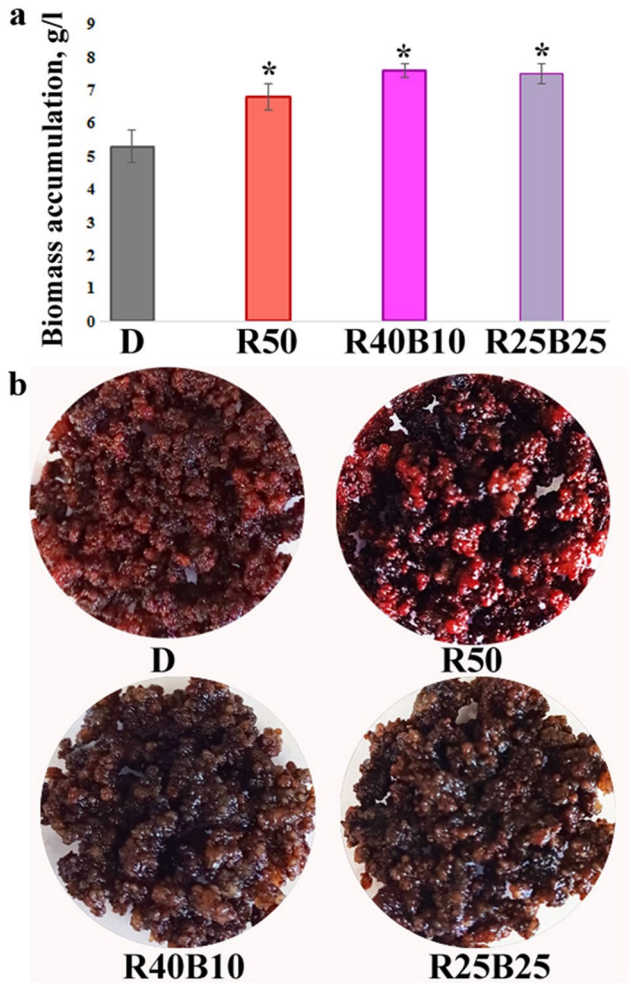

Fig. 5 Development of a callus culture of *L. eryrthrorhizon* grown under various light conditions. Biomass accumulation (**a**, g/L) and morphology (**b**) of 30-day-old *L. eryrthrorhizon* calli (2 g FW inoculants per 50 ml of solid medium) cultivated for 30 days under various light treatments: D, darkness; R, red light with intensities of 50 µmol $m^{-2}s^{-1}$, and red light supplemented with blue light with intensities of 40 and 10 µmol $m^{-2}s^{-1}$ (R40B10) and with intensities of 25 and 25 µmol $m^{-2}s^{-1}$ (R25B25). The mean ± standard error of the mean is used to show the data from three separate studies with ten biological replicates. Asterisks above the error bars indicate statistically significant differences (ANOVA, $p < 0.05$)

1% DW. However, the content of shikonin derivatives was significantly lower than that in 2001. Only four of the seven compounds were detected in the 32-year-old callus line BK-39 of *L. eryrthrorhizon*. Interestingly, these four shikonin derivatives were predominant in the 8-year-old callus line BK-39 of *L. eryrthrorhizon*. In 2001, the major compounds were isobutyrylshikonin, acetylshikonin, isovalerylshikonin, and hydroxyisovalerylshikonin (up to 38, 27, 12, and 9% of shikonin derivatives, respectively). In 2025, the major compounds were isobutyrylshikonin, isovalerylshikonin, and hydroxyisovalerylshikonin, while the content of acetylshikonin was reduced to 4% of that of shikonin derivatives. Unfortunately, we cannot assess changes in the content of shikonofurans since there are no earlier data. The total content of shikonofurans in the 32-year-old callus line BK-39 was not greater than 0.06% DW.

We previously demonstrated that various LED treatments can increase the productivity of *Mertensia maritima* calli cultivated for a long period of time (Veremeichik et al. 2024a). We may infer from earlier research that the ideal range for LED light exposure intensity is between 100 and 300 µmol $m^{-2}s^{-1}$. Shikonin production was demonstrated to be blocked by light exposure in 2002 (Yamamoto et al. 2002). However, comparable investigations with precisely calibrated lighting have not been conducted, despite notable advancements in photobiology. First, we compared the phytochemical content of a 32-year-old *L. eryrthrorhizon* callus line grown for 30 days under monochromatic red and green light and bichromatic red and blue LED light treatment to that of calli grown under warm white light as a negative control and dark conditions as a positive control. Compared with that of the dark-grown control, the growth of the *L. eryrthrorhizon* callus culture was unaffected by any of the light treatments. However, the color of the culture was lost as a result of warm white, green, and a mix of red and blue light. The calli of *L. eryrthrorhizon* that are grown in the dark have a deep crimson hue, but those that are grown in light have no colour at all. The color of the calli that were exposed to red light, however, was maintained. Therefore, regardless of intensity, red light has the strongest growth-stimulating effect. Growth is not adversely affected by the impacts of green light. This pattern is typical of cell cultures in general. The information gathered for this study is consistent with earlier findings for plants and callus cultivation. Accordingly, cultivation of *Hypericum perforatum* callus cultures under red light and dark conditions resulted in much greater biomass accumulation, whereas cultivation of the cultures under blue light had the opposite effect (Najafabadi et al. 2019). Notably, cardoon seedlings grow 60–100% faster under red light than they do in a greenhouse, whereas blue light inhibits their growth (Rabara et al. 2017). According to our earlier findings, blue light at an intensity of 100 µmol $m^{-2}s^{-1}$ completely inhibited the growth of *M. maritima*, whereas red and green light had no detrimental effects on cell culture growth (Veremeichik et al. 2024b).

First, we wanted to determine how the content of shikonin derivatives was affected by various LED light treatments. Despite red light, all light treatments completely prevented the production of shikonin compounds in *L. eryrthrorhizon* calli. The amount of the main shikonin derivatives was reduced by 20 times when the samples were exposed to red light at intensities of 100 and 300 µmol $m^{-2}s^{-1}$. We examined how the various light treatments affected the levels of rabdosiin and RA, two important polyphenolic chemicals.

Table 3 HPLC analysis of Shikonin derivatives in *L. eryrthrorhizon* calli cultivated for 30 days under various light treatments: D, darkness; monochromatic, and bichromatic sources designated R and RB, respectively, with intensities of 50 µmol $m^{-2}s^{-1}$

	Light treatment, µmol $m^{-2}s^{-1}$			
	D	R50	R25B25	R40B10
Shikonins, mg/g DW				
Hydroxyisovalerylshikonin	1.18 ± 0.02^{b}	2.23 ± 0.64^{a}	0.132 ± 0.003^{c}	0.114 ± 0.001^{c}
Acetylshikonin	0.39 ± 0.03^{a}	0.46 ± 0.04^{a}	ND	ND
Isobutyrylshikonin	4.53 ± 0.37^{a}	4.45 ± 0.37^{a}	0.244 ± 0.098^{b}	0.362 ± 0.001^{b}
Isovalerylshikonin	3.35 ± 0.24^{b}	4.17 ± 0.46^{a}	0.284 ± 0.136^{c}	0.485 ± 0.003^{d}
Shikonofurans, mg/g DW				
Shikonofuran D	0.17 ± 0.03^{a}	0.071 ± 0.006^{b}	ND	ND
Shikonofuran E	0.16 ± 0.02^{a}	0.114 ± 0.005^{b}	ND	ND
Shikonofuran C	0.18 ± 0.03^{a}	0.102 ± 0.004^{b}	ND	ND

ND, not detected

The mean ± standard error of the mean is used to show the data from three separate studies with ten biological replicates

The different letters above the error indicate statistically significant differences (ANOVA, $p < 0.05$)

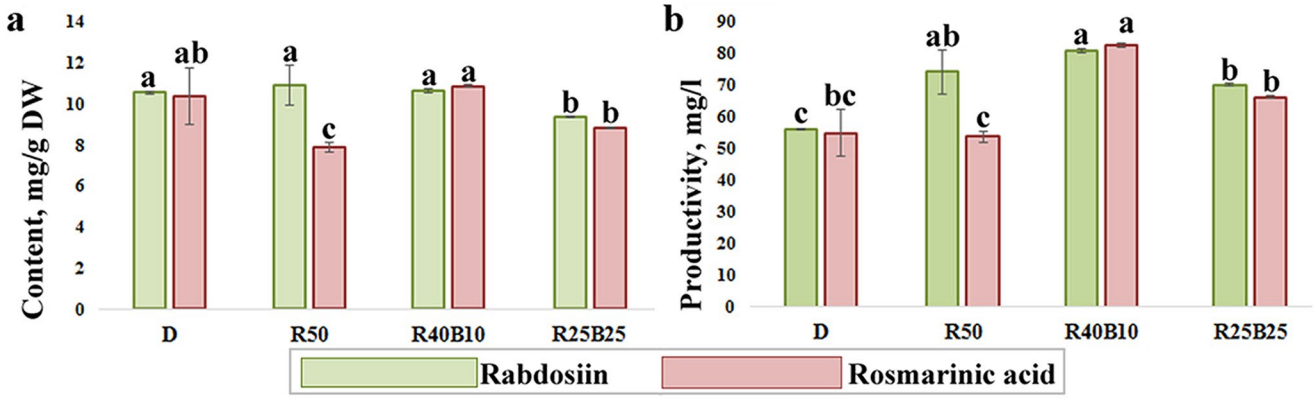

Fig. 6 Contents and productivity of rabdosiin and rosmarinic acid in *L. eryrthrorhizon* calli grown under different lighting conditions. Contents (**a**) and productivity (**b**) of rabdosiin and rosmarinic acid acids in *L. eryrthrorhizon* calli (mg/g DW) cultivated for 30 days under various light treatments: D, darkness; R, red light with intensities of 50 µmol $m^{-2}s^{-1}$, and red light supplemented with blue light with intensities of 40 and 10 µmol $m^{-2}s^{-1}$ (R40B10) and with intensities of 25 and 25 µmol $m^{-2}s^{-1}$ (R25B25). The mean ± standard error of the mean is used to show the data from three separate studies with ten biological replicates. Different letters above the error bars indicate statistically significant differences (ANOVA, $p < 0.05$)

Compared with that of the control dark-grown *L. eryrthrorhizon* calli, the production of both caffeic acid derivatives was unaffected by warm white light. Intriguingly, the rabdosiin content increased 1.5- and 2-fold in response to the green and red–blue light treatments, but it decreased nearly twofold in response to the red light treatment compared with that in the control dark-grown *L. eryrthrorhizon* calli. Raising the light treatment intensities to 300 µmol $m^{-2}s^{-1}$ did not have the same beneficial effect on the amount of rabdosiin. Compared with that in the control dark-grown *L. eryrthrorhizon* calli, the productivity of rabdosiin in the G100 and RB100 light-treated *L. eryrthrorhizon* calli was more than 1.5 times greater, considering the effects of the light treatments on growth. None of the light treatments had any beneficial effects on RA biosynthesis or output.

It was previously demonstrated that blue light suppressed the formation of shikonin while increasing the level of RA (Gaisser and Heide 1996). These findings imply that Lithospermum cells have two routes, one that leads to the biosynthesis of polyphenols and the other to the creation of shikonin, both of which share an early biosynthetic sequence. Importantly, the pattern of RA accumulation in the aerial and underground sections of the whole plant differed; that is, shikonin was found only in the underground tissues, whereas RA was essentially undetectable in the root tissues. Nevertheless, it is unknown whether light regulates the biosynthesis of these caffeic acid oligomers in the same way that it governs the biosynthesis of shikonin (Yamamoto et al. 2000). However, effective approaches for the regulation of RA biosynthesis include the use of RA, which is one of the most frequently occurring caffeic acid esters in the plant kingdom in addition to chlorogenic acid. RA has numerous biological and pharmacological activities (Petersen 2013). In plants, RA is believed to serve as a preformed defence compound against pathogens and herbivores (Petersen and Simmonds, 2003). Moreover, caffeic acid esters can act as UV protectants (Cle´ et al., 2008). Numerous pharmacological and biological activities, such as anti-inflammatory,

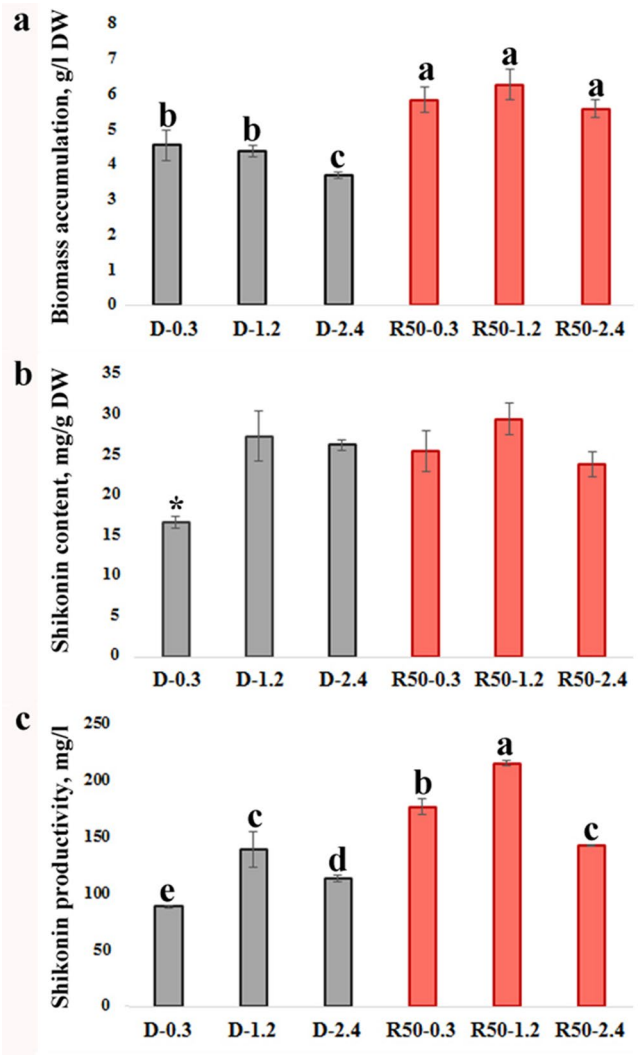

Fig. 7 Combined effect of red light and copper glycerate on growth and shikonin content in *L. eryrthrorhizon* calli. Biomass accumulation (**a**, g/l DW) and shikonin content (**b**) and productivity (**c**) were calculated for *L. eryrthrorhizon* calli grown under control and experimental conditions. Spectrophotometric analysis of shikonin derivatives in *L. eryrthrorhizon* calli cultivated for 30 days under D, darkness and monochromatic R with intensities of 50 µmol $m^{-2}s^{-1}$. Calli of *L. eryrthrorhizon* were grown on culture media supplemented with copper glycerate at a standard concentration (0.3 mg/L) and increased in concentration to 1.2 and 2.4 mg/L. The mean ± standard error of the mean is used to show the data from three separate studies with ten biological replicates. Asterisks or different letters above the error bars indicate statistically significant differences (ANOVA, $p < 0.05$)

antioxidative, antidiabetes, antivirus, antitumour, neuroprotective, and hepatoprotection effects, of RA and related compounds have been described (Guan et al. 2022). Antiviral activity was shown for extracts from *Melissa officinalis* against *herpes simplex* infections (Astani et al. 2012). Owing to their high productivity, plant cell cultures are a potential source of RA. Thus, approximately 36% of *Salvia officinalis* suspension cultures are produced (Hippolyte et al. 1992). The regulation of rhabdosin biosynthesis is of no less interest. The RA dimer rabdosiin belongs to the lignans, constituting an abundant class of phenylpropanoids and having a number of medically important biological activities, such as antitumour, antimitotic, and antiviral properties (Umezawa 2003). Rabdosiin was detected in *Rabdosia japonica* (Lamiaceae) (Agata et al. 1988)d *erythrorhizon* (Boraginaceae) (Yamamoto et al. 2000). Recently, it was shown that rabdosiin has anticancer (Flegkas et al. 2019), antiallergic (Ito et al. 1998), and nefroprotective activities (Inyushkina et al. 2007).

Since exposure to red light did not block shikonin biosynthesis in *L. eryrthrorhizon* calli, we tested the effect of red light at a reduced intensity of up to 50 µmol $m^{-2}s^{-1}$. In addition, we studied the effect of supplementation with 1/5 and half blue light on the overall intensity of 50 µmol $m^{-2}s^{-1}$. These light treatments had a strongly positive effect on callus growth, with an increase of more than 15%. R50 treatment led to a significant increase in shikonin biosynthesis, whereas blue light supplementation led to a decrease in the accumulation of shikonin and total blockade of shikonin biosynthesis. Interestingly, none of these light treatments led to significant changes in the rabdosiin content; however, the R50 treatment led to a significant decrease in the RA content. However, considering the impact of these light treatments on growth, the productivity of both rabdosiin and RA was more than 20% greater in R40B10-treated *L. eryrthrorhizon* calli than in the dark-grown control. Copper ions are the most effective inducers of shikonin biosynthesis in the callus cultures of *L. eryrthrorhizon* (Sun et al. 2022). We investigated the combined effects of low-intensity red LED light and increased concentrations of inducers on shikonin productivity in *L. eryrthrorhizon* calli. Increasing the copper glycerate concentration to 1.2 mg/L did not suppress growth and increased the content and production of shikonin esters by 20% when the samples were grown in the dark. When the mixture was grown in the dark, the production of shikonin increased by 20% with the addition of 1.2%, but the increase was not significant with the addition of 2.4. When *L. eryrthrorhizon* calli were grown under R50, we did not find a negative effect of increasing the concentration of copper glycerate on the growth of the culture. Increasing the concentration of copper glycerate to 1.2 mg/l allowed us to increase the productivity of the culture by more than 2 times.

In summary, we propose the following scheme for the light-dependent differential regulation of the biosynthesis of shikonin and CADs in *L. erythrorhizon* calli (Fig. 8). Shikonin and shikonofurans are byproducts of geranylhydroquinone. Two key precursors of geranylhydroquinone, geranyl diphosphate (GPP), are derived *via* the mevalonate pathway, and *p*-hydroxybenzoic acid (PHB) is derived *via*

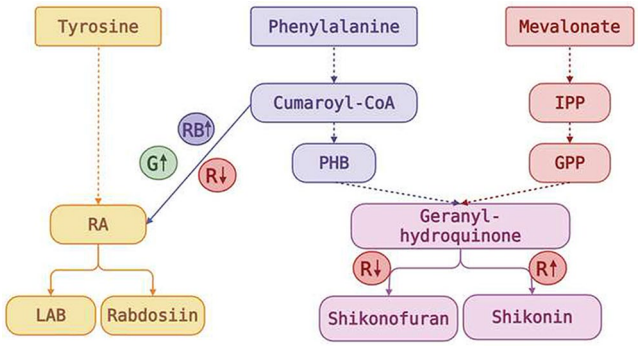

Fig. 8 Effects of monochromatic and bichromatic LED light treatments on secondary metabolism in *L. erythrorhizon* calli. Shikonin and shikonofurans are byproducts of geranylhydroquinone. Two key precursors of geranylhydroquinone, geranyl diphosphate (GPP), are derived *via* the mevalonate pathway, and *p*-hydroxybenzoic acid (PHB) is derived *via* the phenylpropanoid pathway. Biosynthesis of the aromatic intermediate PHB derived from coumaroyl-CoA, which is a key precursor for RA biosynthesis derived from tyrosine. The main derivatives of RA in *L. erythrorhizon* are lithospermic acid B (LAB) and rabdosiin (Sun et al. 2022). As we showed in the present work, monochromatic green and bichromatic red and blue LED light treatments shifted biosynthesis from the coumaroyl-CoA stage to the RA stage, whereas low-intensity red light treatments, in contrast, shifted biosynthesis to the shikonin stage. Moreover, low-intensity red light treatment shifted the biosynthesis to the side of shikonin in the geranylhydroquinone stage

the phenylpropanoid pathway. Biosynthesis of the aromatic intermediate PHB derived from coumaroyl-CoA, which is a key precursor for RA biosynthesis derived from tyrosine. The main derivatives of RA in *L. erythrorhizon* are lithospermic acid B (LAB) and rabdosiin (Sun et al. 2022). As we showed in the present work, monochromatic green and bichromatic red and blue LED light treatments shifted biosynthesis from the coumaroyl-CoA stage to the RA stage, whereas low-intensity red light treatments, in contrast, shifted biosynthesis to the shikonin stage. Moreover, low-intensity red light treatment shifted the biosynthesis to the side of shikonin in the geranylhydroquinone stage.

Conclusion

Research on light sources and environmental factors that can increase the sustainability and profitability of PFALs has become increasingly important in recent years (Orsini et al. 2020). In this study, the impact of artificial monochromatic and bichromatic LED light at wide ranges of intensities (50, 100, and 300 µmol $m^{-2}s^{-1}$) on the growth and biosynthesis of caffeic acid derivatives as well as shikonin and shikonofurans in long-term cultivated *L. erythrorhizon* callus cultures was investigated for the first time. In general, the following conclusions can be drawn: (i) In long-term cultivated *L. erythrorhizon* callus cultures, the content of shikonin decreased after more than 30 years of cultivation, whereas the content of CADs did not significantly change. (ii) Red light has the greatest growth-stimulating effect regardless of intensity. It can also be assumed, on the basis of literary data, that this pattern is characteristic of cell cultures in general. (iii) The most effective treatment for CAD productivity (both RA and rabdosiin) is red/blue and green light treatment. (iv) The most effective way to produce shikonin in long-term cultivated *L. erythrorhizon* calli is to use red light with an intensity of 50 µmol $m^{-2}s^{-1}$ and increase the copper glycerate concentration. (v) The blue and green components of white light may also have a negative effect on shikonin biosynthesis because of the light-dependent shift in the biosynthesis of CADs.

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Author contribution G.N. Veremeichik: Conceptualization, Data curation, Project administration, Supervision, Validation, Visualization, Writing – original draft. G.N. Veremeichik; V.P. Grigorchuk; S.A. Silantieva; E.P. Subbotin; E.V. Brodovskaya, G.K. Tchernoded; O.A. Tikhonova; S.A. Fedoreyev; N.P. Mishchenko; E. A. Vasileva; A.A. Khopta; S.O. Kozhanov: Investigation, Methodology, Formal Analysis. V.P. Bulgakov; Y.N. Kulchin; Siberev A.S.: Resources, Funding acquisition.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

Code availability Not applicable.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication All the authors whose names appeared on the submission approved the version to be published and agreed to be accountable for all aspects of the work in ensuring that the questions related to the accuracy of integrity of any part of the work were appropriately investigated and resolved.

Conflict of interest The authors declare that they have no competing interests.

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