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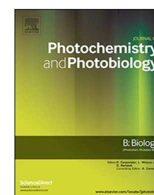
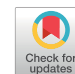


The influence of light quality on the production of bioactive metabolites – verbascoside, isoverbascoside and phenolic acids and the content of photosynthetic pigments in biomass of *Verbena officinalis* L. cultured *in vitro*



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A R T I C L E I N F O

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A B S T R A C T

In vitro callus cultures of *Verbena officinalis* L. were maintained on solid Murashige and Skoog medium, enriched with 1 mg dm^{-3} BA and 1 mg dm^{-3} IBA under LED lights (red, blue, red/blue 70%/30%), in darkness and under control fluorescent lamps. The measurements of 2 phenylpropanoid glycosides (verbascoside and isoverbascoside) and 23 phenolic acids were performed in methanolic extracts from the biomass collected after 2-, 3- and 4-week growth cycles using the HPLC-DAD method. The presence of verbascoside, isoverbascoside and additionaly 7 phenolic acids (protocatechuic, chlorogenic, vanillic, caffeic, ferulic, o-coumaric and m-coumaric acids) was confirmed in all extracts. Blue and red/blue lights stimulated the accumulation of verbascoside (max. of 6716 and 6023 mg 100 g^{-1} DW after a 4-week growth cycle) and isoverbascoside (max. 333 and 379 mg 100 g^{-1} DW also after 4 weeks). The maximum amounts of verbascoside and isoverbascoside were respectively 1.8- and 7.0-fold higher than under the control conditions. Phenolic acids were accumulated in different amounts, and the maximum total amounts ranged from 36 to 65 mg 100 g^{-1} DW. LED lights also stimulated their accumulation in comparison with darkness and control. The main phenolic acids included: m-coumaric acid (max. 39 mg 100 g^{-1} DW), ferulic acid (max. 12 mg 100 g^{-1} DW), and protocatechuic acid (max. 13 mg 100 g^{-1} DW). Additionally, the quantities of photosynthetic pigments (chlorophyll *a*, b and carotenoids) were estimated in acetonic extracts using spectrophotometry. Red/blue light stimulated the biosynthesis of pigments (max. total content 287 µg g^{-1} FW after 4-week growth cycles). This is the first study describing the effect of LED lights on the production of phenylpropanoid glycosides and phenolic acids in *V. officinalis* callus cultures. Very high amounts of verbascoside and isoverbascoside are interesting from a practical point of view.

1. Introduction

Verbena officinalis L. (*Verbena sororia* D. Don*, V. spuria* L.) – vervain – is a species belonging to the genus Verbena and *Verbenaceae* family (*Verbeneae* subfamily) [1]. It is a herbal, medicinal plant wildly distributed around the world in a temperate climate zone. Its medicinal properties are well known in traditional and modern European, Asian and North American phytotherapies. *V. officinalis herba* has been listed in official European pharmaceutical documents from 2008 as the "Verbena herb" monograph which was introduced into the 6th European Pharmacopoeia. Moreover, the "*Verbenae herba"* monograph has been included in the 8th Chinese Pharmacopoeia since 2005. This plant species has long been known in the USA as a source of raw materials for allopathic and homeopathic purposes. Its description can be found in the Pharmacopoeia of the American Institute of Homeopathy from 1897.

V. officinalis herb is primarily used as an antimicrobial, secretolytic and expectorant remedy. It is used in the treatment of upper respiratory tract diseases, affecting mostly throat and sinuses [2,3]. In skin diseases, this raw material is used as a softening, anti-inflammatory and antibacterial agent, especially to treat wounds difficult to heal or gingivitis [2,4–6]. It is also known for its diuretic effect and is used in kidney and urinary bladder diseases. In women, this plant raw material is applied in menstrual disorders and in breast-feeding mothers to

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stimulate lactation [2,4]. It is traditionally used in nervous system disorders - in depression, insomnia and anxiety disorder treatments [4]. Research data indicate that *V. officinalis* is applied in the treatment of liver diseases, hepatitis and cholecystitis [2]. Herb extracts of this plant are administered to treat fever accompanying colds and as supportive agents in malaria and rheumatism [4,7,8].

The main constituents of *V. officinalis* include iridoid glycosides: verbenalin (verbenaloside), aucubin (verbenin) and hastatoside, and phenylpropanoid glycosides: verbascoside (acteoside, eucovoside) and isoverbascoside (isoacteoside). Moreover, many flavonoids have been identified in vervain herb extracts, including popular compounds, such as kempherol, luteolin, apigenin, and less common ones: scutellarein and pedalitin. The presence of the following phenolic acids is also worth noting: chlorogenic, ferulic, protocatechuic and rosmarinic acids and dicavoiloquinic acid derivatives.

Our earlier studies on *V. officinalis in vitro* cultures proved that the biomass obtained by using biotechnological methods could be a plausible alternative for the soil-grown plant material. Well established callus cultures in our laboratory have been proven to produce outstanding amounts of verbascoside and considerable amounts of phenolic acids [9].

In vitro cultured biomass has many advantages in relation to *in vivo* cultivated plant material. Well-known plant biotechnology strategies have been often applied in order to increase the production of secondary metabolites [10–12]. Culture conditions, such as basal medium composition, plant growth regulators or light conditions are very important factors in the production of secondary metabolites.

Light, its intensity and wavelength, photoperiod and lack of light belong to physical conditions that can be easily manipulated to increase product yield in plant biomass maintained *in vitro* [13]. Light is known to affect the production of several groups of secondary metabolites, including anthocyanins [14], polyamines [15], flavonoids [16] as well as individual compounds used in medicine as herb-derived drugs, such as artemisinin, digoxin, taxol or vinblastine [17].

Our previous studies have demonstrated a positive effect, especially of blue light, on the production of phenolic acids and linear furanocoumarins in shoot cultures of *Ruta graveolens* [18,19], and phenolic acids and flavonoids in shoot cultures of *Aronia* sp. [20] as well as dibenzocyclooctadiene lignans in *Schisandra chinensis* shoot-differentiated cultures [21].

Encouraged by our earlier results, we have currently designed a study on the significance of electroluminescent LED light with different wavelengths: red, blue, red/blue (70%/30%) as well as darkness and control fluorescent lamps (Figs. 1 and 2), for the accumulation of phenylpropanoid glycosides: verbascoside and isoverbascoside as well as phenolic acids in *V. officinalis* callus cultures. Moreover, we also tested in this study the influence of different lighting conditions on photosynthetic pigments contents in cultured biomass and we look for its correlation with secondary metabolites production.

2. Materials and methods

2.1. Initiation of in vitro cultures

Verbena officinalis L. callus cultures were established from leaf buds and stem fragments in 2014 from parent plants growing in the Garden of Medicinal Plants, Faculty of Pharmacy, Jagiellonian University, Medical College, Kraków (Poland). Initial cultures were established on Murashige and Skoog (MS) medium [22] solidified with agar supplemented with the addition of two plant growth and development regulators (PGRs): 1 mg dm^{-3} of 6-benzyladenine (BA) and 0.5 mg dm^{-3} of 1-naphthaleneacetic acid (NAA). The cultures were grown under continuous artificial lighting conditions (photosynthetic photon flux density (PPFD) of 40 μmol m^{-2} s^{-1}) at a temperature of 25 ± 2 °C. Vigorous callus cultures were obtained after several successive passages.

2.2. Experimental in vitro cultures

Experimental cultures were grown on MS agar medium supplemented with 1 mg dm^{-3} BA and 1 mg dm^{-3} IBA (indole-3-butyric acid) and 7.2 g l^{-1} of Phytoagar (Duchefa Biochemie B.V., Nederlands). This medium PGRs composition was selected as a growth medium and also a production medium based on our previous studies [9]. Different light-emitting diode (LED) variants were applied: red light (λ = 670 nm), blue light (λ = 430 nm), a combination of red and blue lights (70%/30%), darkness (no light) and control fluorescent lamps (Philips TL-D 36 W/54 cool fluorescent lamps) (λ = 390–760 nm) (Fig. 1).

LED lighting consists of panels and a central controller. On the panels were groups of LEDs with four different light spectra: blue light (430 nm), red light (670 nm), far-red light (730 nm), and white light (430–730 nm). For good mixing each group contains four different LED's on 6 × 6 mm area. The central controller regulates the light intensity for each type of LED (radiation composition) and the real-time clock gives ability to program photoperiod. Controller connected to the computer after initial configuration operates independently [23].

Spectral characterization of the tested LEDs and fluorescent lamp were made using a BTS256-LED Tester (Gigahertz-Optik, Germany).

Experimental cultures were maintained under a 16/8 h photoperiod, at temperature 25/23 ± 1 °C (day/night), and 70% relative humidity in the phytotron.

Growth cycles lasted 2, 3 and 4 weeks (3 culture series). In the present experiment, a maximum of 4-week culture cycle was used, because a distinct biomass die-off was observed in longer cycles [23].

2.3. Extraction

2.3.1. Verbascoside and isoverbascoside

Lyophilized biomass (0.30 g samples) collected after 2-, 3- and 4-week growth cycles was extracted five times with 3 ml portions of a water/methanol mixture (3:7 *v*/v) (total volume equal 15 ml) in an ultrasonic bath. The extract was centrifuged each time. The combined supernatants were filtered through a syringe filter (Millex®GP, Millipore, 0.22 μm, Filter Unit) and analyzed by HPLC.

2.3.2. Phenolic acids

The biomass harvested from cultures after 2-, 3- and 4-week growth cycles was lyophilized and extracted with methanol. Samples (0.50 g each) were extracted under reflux condenser twice with 50-ml methanol portions for 2 h. Extracts, after evaporation to dryness, were dissolved in 2 ml of methanol and filtered through a syringe filters (Millex®GP, Millipore, 0.22 μm, Filter Unit).

2.4. DAD-HPLC analysis of metabolites

2.4.1. Verbascoside and isoverbascoside

Chromatographic analysis of phenylpropanoid glycosides (verbascoside and isoverbascoside) was carried out by DAD-HPLC method [3] on the Merck-Hitachi system and a Kinetex C-18 analytical column (150 × 4.6 mm, 2.7 μm; Phenomenex). The mobile phase consisted of: A – water: 0.1% trifluoroacetic acid (1:4 *v*/v); B – acetonitrile. The gradient program was as follows: 0–4 min, 11% B; 4–5 min, 11–12% B; 5–10 min, 12–16% B; 10–17 min, 16–19% B; 17–28 min, 19–27% B, 28–33 min, 27–98% B, 33–45 min, 98–100% B, 45–50 min, 100–11% B. The flow rate was 1 ml/min and temperature was 25 °C. Compounds were determined using a DAD detector. Injection volume was 5 μl. Detection wavelength was set at 330 nm. The confirmation of detected compounds was performed using an internal standard method and compared with UV-DAD spectra and retention time (t_R) of commercial standards. The quantification was carried out by comparing with verbascoside and isoverbascoside standards (ChromaDex®).

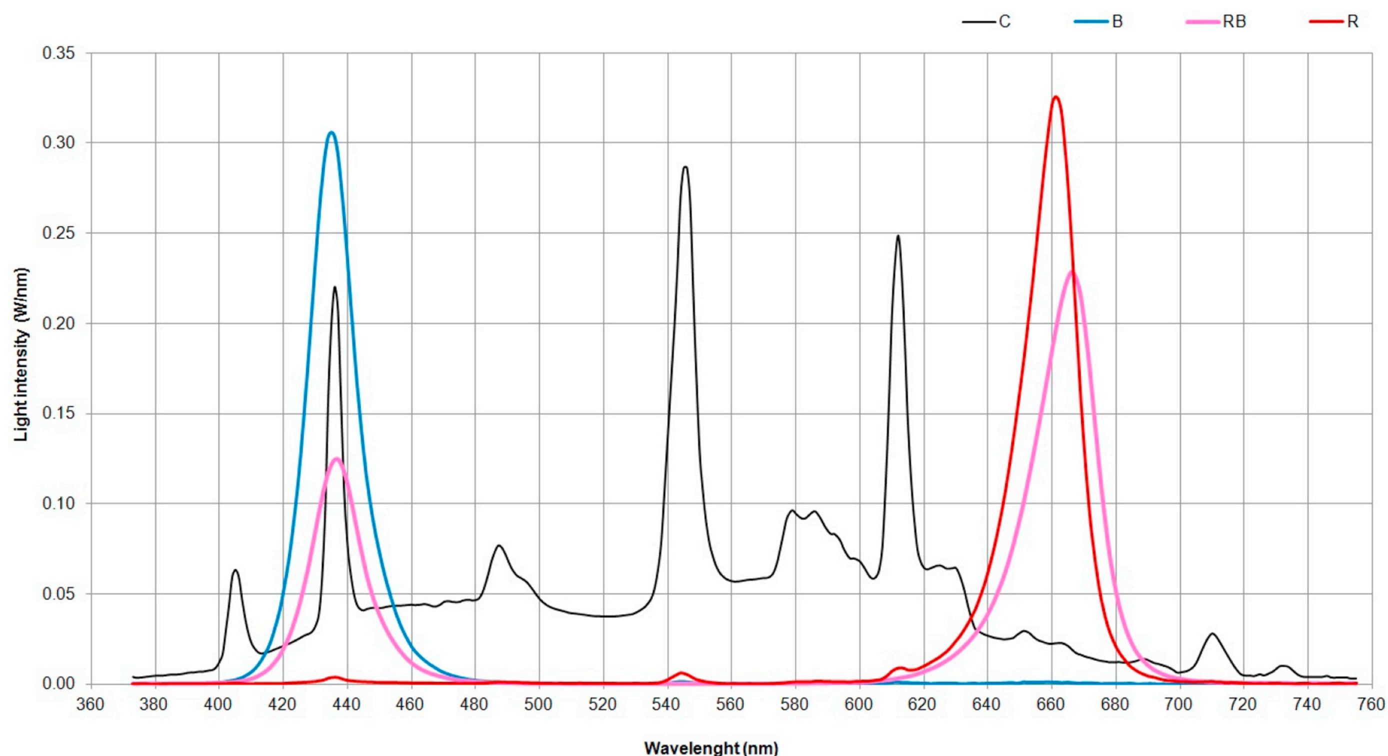


Fig. 1. Tested light spectra during the experiment: C - control fluorescent light, B – 100% blue LED, RB - red/blue LED (70%/30%), R – 100% red LED (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

2.4.2. Phenolic acids

Chromatographic analysis of phenolic acids was carried out using DAD-HPLC [24,25]. DAD-HPLC-system (Merck-Hitachi) and a Purospher RP-18e analytical column (4 × 250 mm, 5 μm; Merck) were used. The mobile phase consisted of: A – methanol: 0.5% acetic acid (1:4 *v*/v); B – methanol. Gradient program was as follows: 0–20 min, 0% B; 20–35 min, 0–20% B; 35–45 min, 20–30% B; 45–55 min, 30–40% B; 55–60 min, 40–50% B, 60–65 min, 50–75% B; 65–70 min, 75–100% B, with a hold time of 15 min, at 25 °C. The flow rate was 1 ml/min. Compounds were determined using a DAD detector. Injection volume was 20 μl. Detection wavelength was set at 254 nm. The confirmation of detected compounds was performed by an internal standard method and comparison with UV-DAD spectra and the t_R values of commercial standards. The quantification was carried out by comparison with precursor standards of subgroups of phenolic acids, cinnamic acid and benzoic acid and the following phenolic acids: caffeic, caftaric, chlorogenic, m-coumaric, o-coumaric, p-coumaric, cryptochlorogenic, 3,4-dihydroxyphenylacetic, ellagic, ferulic, gallic, gentisic, p-hydroxybenzoic, hydroxycaffeic, isochlorogenic, isoferulic, neochlorogenic, 3-phenylacetic, protocatechuic, rosmarinic, salicylic, syringic and vanillic acids (Sigma-Aldrich Co.).

2.5. Spectrophotometric analysis of photosynthetic pigments

Fresh biomass from cultures (0.20 g) was homogenized in a mortar with the addition of a small amount of calcium carbonate, quartz sand and 80% acetone (15 ml) added in portions. After filtration, the solution was filled up with acetone to 20 ml. The content of chlorophyll *a* and b and carotenoids was determined spectrophotometrically using a UV/VIS Helios Alpha spectrophotometer (Unicam Ltd., Cambridge, Great Britain). Absorbance was tested at the following wavelengths: chlorophyll *a* – λ = 663.2 nm, chlorophyll *b* – λ = 646.8 nm, carotenoids – λ = 470 nm [26].

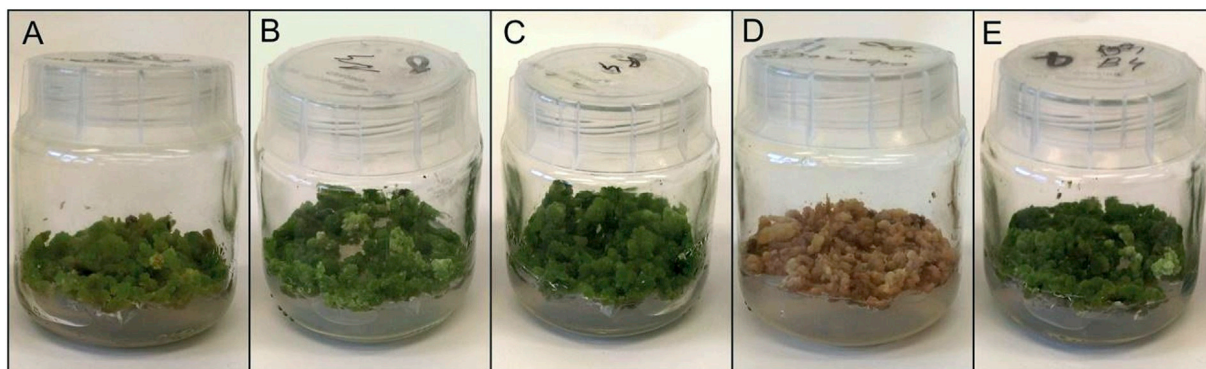


Fig. 2. Four-week *V. officinalis* callus cultures (MS, 1 mg dm^{-3} BA and 1 mg dm^{-3} IBA) grown under different light conditions: A – red light, B – blue light, C – red/blue light (70%/30%), D – darkness, E – control (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

2.6. Statistical analysis

Principal Component Analysis (PCA) and ANOVA (ANalysis Of VAriance) were performed inside the free GNU R computational environment (version 3.5.1) using built-in “lm” and “prcomp” functions. PCA was performed in a scaled way and all results were combined into a single matrix (dimensions: 15 rows (5 light conditions x 3 time points) and 15 columns (15 measured parameters). To perform ANOVA, each parameter was regressed against the design matrix containing four independent variables: week number (as a continuous time variable) and the presence of red, green and blue light (factor variable). Control light was treated as containing all three colors. Linear models were fitted to time, including interactions of the slope with three light colors. This allowed to estimate the significance of slope changes caused by the presence of each wavelength range; 90% confidence level was treated as significant.

3. Results

3.1. Dry biomass increases

The increase of dry biomass was variable and differed depending on the light conditions and the length of the culture cycle (Fig. 3A).

Under blue light, the fold increase in dry biomass was 13.01 (2 weeks), 19.19 (3 weeks) and 17.21 (4 weeks), respectively, depending on the duration of the culture cycle; the fold increase in the combination of red with blue light was as follows: 15.27 (2 weeks), 18.42 (3 weeks) and 17.37 (4 weeks), and in the presence of red light: 13.45 (2 weeks), 15.58 (3 weeks) and 14.49 (4 weeks).

Dry biomass fold increases under control conditions (control fluorescent lamps) were similar to those obtained in blue light and in the combination of red and blue light and amounted to 13.32, 18.78 and 16.55 fold, respectively.

The increase in dry biomass obtained in cultures grown in the darkness was considerably lower than in the presence of LED lights and control conditions, depending on the duration of the culture cycle. They were 9.22, 12.25 and 10.69 folds (2-, 3- and 4-week cultures), respectively (Fig. 3A).

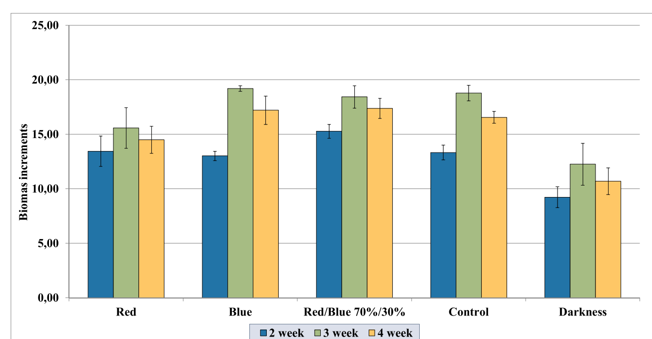
3.2. Dynamics of phenylpropanoid glycosides accumulation

All studied extracts were confirmed to contain verbascoside and isoverbascoside (Fig. 3 B,C; Table 1). Verbascoside was definitely the dominant metabolite. Its contents in biomass cultivated under the tested LED light conditions ranged from 2277 to 6716 mg 100 g^{-1} DW (dry weight). The content of verbascoside in the parent plant was 8.9-fold lower than the maximal content in the biomass cultured *in vitro* [9]. Under blue light, verbascoside contents increased gradually during 4-week growth cycles from 4057to 6716 mg 100 g^{-1} DW, while when red light was applied, verbascoside amounts varied from 2277 to 5202 mg 100 g^{-1} DW. Illumination of the cultures by a mixture of red and blue light (70%/30%) resulted in an increase of this metabolite from 4004 to 6023 mg 100 g^{-1} DW.

The dynamics of compound accumulation was observed, and the maximum contents of verbascoside were confirmed in biomass cultured under blue and red/blue lights during a 4-week growth cycle. The changes in verbascoside accumulation were different when red light was applied. The amounts of this glycoside increased 2.28-fold, from 2277 to 5202 mg 100 g^{-1} DW, between 2 and 3 weeks of culture and remained at the same level up to 4 weeks of culture (5166 mg 100 g^{-1} DW).

Verbascoside contents in the biomass cultivated in the dark differed from LED light conditions and ranged from 3072 to 5061 mg 100 g^{-1} DW. The maximum content was recorded after 3 weeks of culture, followed by a slow decline after 4 weeks (4689 mg 100 g^{-1} DW).

The dynamics of verbascoside accumulation in control conditions was entirely different. After a conspicuous increase of its production between the 2nd and 3rd week (the content increased from 3684 to



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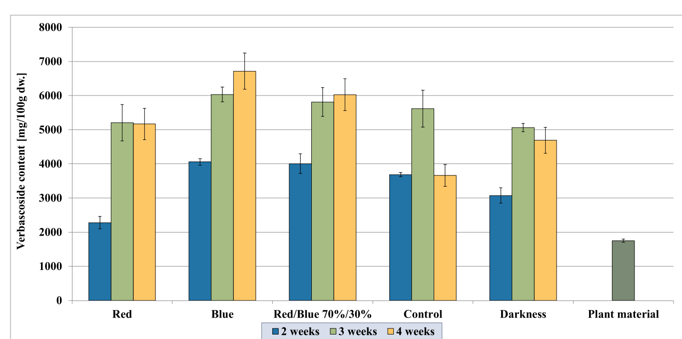

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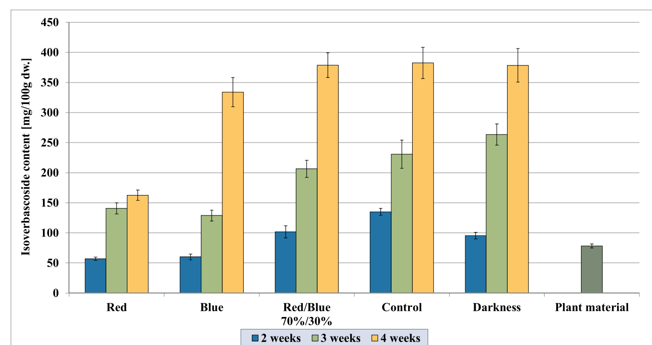

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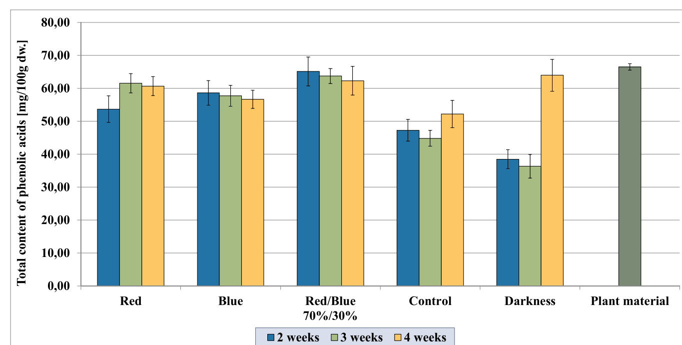

D

Fig. 3. Comparison of dry biomass increases (A), and metabolite contents [mg 100 g^{-1} DW ± SD] of: verbascoside (B), isoverbascoside (C) and total phenolic acids (D).

Table 1
Verbascoside and isoverbascoside contents [mg 100 g^{-1} DW ± SD].

Duration of growth cycle	Phenylpropanoid glycosides	Tested light conditions				
		Red	Blue	Red + Blue	Control*	Darkness
2 weeks	Verbascoside	2277 ± 184	4057 ± 95	4004 ± 289	3684 ± 62.	3072 ± 223
	Isoverbascoside	57 ± 3	60 ± 5	102 ± 10	135 ± 6	95 ± 5
	Total	2334 ± 187	4117 ± 100	4106 ± 299	3819 ± 68	3167 ± 228
3 weeks	Verbascoside	5202 ± 534	6032 ± 220	5812 ± 420	5615 ± 540	5061 ± 119
	Isoverbascoside	141 ± 9	129 ± 9	207 ± 14	231 ± 23	264 ± 17
	Total	5343 ± 543	6161 ± 229	6019 ± 434	5846 ± 563	5325 ± 137
4 weeks	Verbascoside	5166 ± 459	6716 ± 528	6023 ± 466	3658 ± 321	4689 ± 378
	Isoverbascoside	163 ± 9	334 ± 24	379 ± 21	383 ± 26	378 ± 28
	Total	5328 ± 468	7049 ± 552	6402 ± 487	4041 ± 347	5067 ± 406

* Control fluorescent lamps.

5615 mg 100 g^{-1} DW), a marked reduction to 3658 mg 100 g^{-1} DW was observed in the next week of culture.

The second of the analyzed phenylpropanoid glycosides - isoverbascoside was accumulated in the biomass cultured *in vitro* in much lower quantities. Its content ranged from 57 to 383 mg 100 g^{-1} DW. Under blue light, isoverbascoside amounts increased from 60 to 334 mg 100 g^{-1} DW, and from 102 to 379 mg 100 g^{-1} DW under red/blue light between the 2nd and 4th week cultures grow.

Red light clearly was not beneficial for isoverbascoside accumulation. The confirmed isoverbascoside contents were significantly reduced compared to blue and red/blue light and fluctuated during the growth cycles from 57 mg 100 g^{-1} DW (at 2 weeks), through 141 mg 100 g^{-1} DW (at 3 weeks) to 163 mg 100 g^{-1} DW at 4 weeks.

The content of isoverbascoside in the darkness varied from 95 mg 100 g^{-1} DW, through 264 mg 100 g^{-1} DW to 378 mg 100 g^{-1} DW (at 4 weeks of culture).

Under control conditions, isoverbascoside ranged from 135 mg 100 g^{-1} DW, through 231 mg 100 g^{-1} DW to 383 mg 100 g^{-1} DW (at the end of growth cycles).

Thus, the analyses performed in the present study proved that blue and red/blue LED light were beneficial for the accumulation of phenylpropanoid glycosides when compared to red LED light and comparable with darkness and control conditions. Maximum contents of these pharmaceutically important compounds could be obtained after 4-week growth cycles.

3.3. Dynamics of phenolic acids accumulation

All extracts of callus cultures cultivated under the tested light conditions were confirmed to contain 7 of 23 examined phenolic acids: protocatechuic, chlorogenic, vanillic, caffeic, ferulic, o-coumaric and m-coumaric acids (Table 2). The total contents of phenolic acids under all tested LED light conditions were comparable and ranged from 54 to 65 mg 100 g^{-1} DW. The total content of phenolic acids widely varied in the darkness (from 36 to 64 mg 100 g^{-1} DW). In control conditions, the total content of phenolic acids ranged from 45 to 52 mg 100 g^{-1} DW. Lack of light and control conditions were less favorable for the production of majority of phenolic acids in comparison to LED lights (Fig. 3 D, Table 2).

The m-coumaric acid was the quantitatively dominant metabolite and its content slightly fluctuated in the extracts of biomass grown under the tested light conditions (*ca.* 32 mg 100 g^{-1} DW to 39 mg 100 g^{-1} DW). In the biomass cultivated in the dark, m-coumaric acid content remained virtually unchanged throughout the growth cycles (32–33 mg 100 g^{-1} DW). The content of this compound was also very similar under control conditions (33–34 mg 100 g^{-1} DW).

Two other compounds: ferulic acid and protocatechuic acid were the next quantitatively dominant compounds in the studied extracts, however, their maximum contents were of different order of magnitude, ranging from 7 to 12 mg 100 g^{-1} DW and from 3 to 5 mg 100 g^{-1} DW under LED lights, respectively. In the dark, ferulic acid contents were lower (1–7 mg 100 g^{-1} DW) than under LED lights. Control fluorescent lamps also did not favor the accumulation of this compound (up to 3 mg 100 g^{-1} DW). Protocatechuic acid content was relatively low in the dark after a 2- and 3-week growth cycle (3 mg 100 g^{-1} DW), but significantly inreased in the following week (13 mg 100 g^{-1} DW). Protocatechuic acid contents under control conditions changed in the consecutive weeks of the growth cycle from 3 to 6 mg 100 g^{-1} DW.

The maximum contents of the remaining four phenolic acids: chlorogenic, vanillic, caffeic and o-coumaric acids were very low under LED lights (3, 2, 4, 6 mg 100 g^{-1} DW), in the dark (1, 2, 3, 4 mg 100 g^{-1} DW) and under control conditions (1, 2, 3, 5 mg 100 g^{-1} DW).

The current analyses proved that LED lights, especially blue and red/blue lights, guaranteed the highest total production of phenolic acids under study (max. Respectively 59 and 65 mg 100 g^{-1} DW). Changes in the accumulation of phenolic acids in the darkness were surprising, because their content was found to increase from 36 mg 100 g^{-1} DW (3 weeks) to 64 mg 100 g^{-1} DW (4 weeks). Phenolic acids accumulated in the biomass in much lower quantities than verbascoside and isoverbascoside.

3.4. Photosynthetic pigment content analysis

The quality of light had an impact on the physiological response of plants, which was reflected in the level of photosynthetic pigments in the analyzed biomass.

The total content of photosynthetic pigments varied depending on light conditions and the length of the culture cycle and ranged from 90 μg g^{-1} FW (fresh weight) to 287 μg g^{-1} FW (Table 3, Fig. 4). The highest total pigment production ranged from 179 to 287 μg g^{-1} FW was found for all tested LED lights after 4-week growth periods. The total contents of pigments were lower under control conditions (from 105 to 245 μg g^{-1} FW). The production of pigments was the lowest in cultures grown without access to light (max. 112 μg g^{-1} FW).

Chlorophyll a was produced in the amount from 42 to 125 μg g^{-1} FW depending on the LED light used; these quantities in control conditions ranged from 53 to 114 μg g^{-1} FW and from 10 to 32 μg g^{-1} FW in the darkness. The highest content of this pigment under red light, control conditions and without the light were documented in the 3rd week of the growth cycle. The highest quantities under blue light and combination of red and blue light were observed in the 4th week of culture.

The quantities of chlorophyll *b* produced under LED light fluctuated from 24 to 93 μg g^{-1} FW, from 23 to 71 μg g^{-1} FW under control conditions and from 15 to 54 μg g^{-1} FW in cultures grown without the light. The highest amounts of chlorophyll *b* in all LED light variants were observed in the 4th week of biomass growth and in the 3rd week of growth under control conditions and absence of light.

Carotenoids were produced in the lowest quantities and depending on light conditions, their contents were as follows: 24–69 μg g^{-1} FW

Table 2
Phenolic acid contents [mg 100 g^{-1} DW ± SD].

Duration of growth cycle	Phenolic acids	Tested light conditions				
		Red	Blue	Red + Blue	Control*	Darkness
2 weeks	Protocatechuic acid	3 ± 0.2	5 ± 0.3	4 ± 0.3	3 ± 0.1	3 ± 0.2
	Chlorogenic acid	2 ± 0.1	1 ± 0.0	1 ± 0.1	1 ± 0.1	traces
	Vanillic acid	1 ± 0.1	1 ± 0.0	1 ± 0.0	1 ± 0.1	traces
	Caffeic acid	2 ± 0.1	3 ± 0.1	2 ± 0.1	3 ± 0.2	traces
	Ferulic acid	7 ± 0.2	12 ± 0.8	12 ± 0.4	3 ± 0.3	3 ± 0.1
	o-Coumaric acid	2 ± 0.1	2 ± 0.1	6 ± 0.6	2 ± 0.1	traces
	m-Coumaric acid	37 ± 3.3	35 ± 2.4	38 ± 2.8	34 ± 2.5	33 ± 2.6
	Total	54 ± 4.0	59 ± 3.7	65 ± 4.4	47 ± 3.3	38 ± 2.9
3 weeks	Protocatechuic acid	5 ± 0.4	4 ± 0.1	4 ± 0.2	4 ± 0.3	3 ± 0.2
	Chlorogenic acid	2 ± 0.0	2 ± 0.1	3 ± 0.1	1 ± 0.1	traces
	Vanillic acid	2 ± 0.2	2 ± 0.1	2 ± 0.1	2 ± 0.1	traces
	Caffeic acid	2 ± 0.1	4 ± 0.1	4 ± 0.2	3 ± 0.2	traces
	Ferulic acid	10 ± 0.3	8 ± 0.4	10 ± 0.3	traces	1 ± 0.1
	o-Coumaric acid	5 ± 0.1	4 ± 0.2	5 ± 0.2	1 ± 0.0	traces
	m-Coumaric acid	37 ± 1.8	34 ± 2.2	37 ± 1.2	33 ± 1.7	32 ± 3.3
	Total	62 ± 2.9	58 ± 3.2	64 ± 2.3	45 ± 2.4	36 ± 3.6
4 weeks	Protocatechuic acid	5 ± 0.1	5 ± 0.4	4 ± 0.3	6 ± 0.3	13 ± 0.4
	Chlorogenic acid	3 ± 0.1	2 ± 0.2	1 ± 0.1	1 ± 0.1	1 ± 0.1
	Vanillic acid	2 ± 0.1	1 ± 0.1	1 ± 0.1	1 ± 0.0	2 ± 0.2
	Caffeic acid	3 ± 0.2	4 ± 0.3	3 ± 0.3	2 ± 0.1	3 ± 0.3
	Ferulic acid	9 ± 0.4	7 ± 0.5	8 ± 0.4	2 ± 0.2	7 ± 0.6
	o-Coumaric acid	6 ± 0.4	4 ± 0.2	6 ± 0.6	5 ± 0.3	4 ± 0.3
	m-Coumaric acid	33 ± 1.5	34 ± 1.1	39 ± 2.7	34 ± 3.2	33 ± 3.0
	Total	61 ± 2.9	57 ± 2.8	62 ± 4.4	52 ± 4.2	64 ± 4.9

* Control fluorescent lamps.

(LED lights), 30–60 μg g^{-1} FW (control conditions) and 11–26 μg g^{-1} FW (darkness). The highest carotenoid contents under red light, control conditions and in the dark were found in the 3rd week and in the presence of blue light and the combination of red and blue light in the 4th week of culture.

The combination of red and blue light was the most favorable condition for the production of chlorophyll *a* and b as well as carotenoids.

3.5. Linear regression analysis

The significance of the parameter change in time was estimated with ordinary least squares regression. First, each respective parameter was regressed against time variable under each light conditions (15 parameters, 5 light conditions). The statistical significances of the slope was used as measures of the significance. The results are presented in the left section of Table 4. It is observed that IVE increases regardless of the conditions, increase of VAN is stimulated with the red light, whereas CLB significantly increases with blue light. FER, MKU and TPH are parameters which decrease significantly in time in the presence of red and/or blue light only.

The next step was the analysis of each wavelength range (red, green, blue) on the slope of the regression. It was done by decomposing the used light sources into the separate factors and by regression of the parameters against the factor matrix, using interaction term between slope and three respective colors of the light. It was observed that red and blue light region significantly increases the slope, whereas green light significantly decreases the slope in part of parameters (the right section of Table 4). These changes are indicated by different arrows to allow deeper understanding: they present the significance and direction of *slope change*, not slope itself. Therefore, there is no correlation between direction of the arrows and significance between these two sections of the table. For example the blue light increases the slope of VER significantly comparing to the light without the blue region, however

Table 3
Content [μg g^{-1} FW ± SD] of individual photosynthetic pigments depending on light conditions and culture cycle duration.

Duration of growth cycle	Photosynthetic pigments	Tested light conditions				
		Red	Blue	Red + Blue	Control*	Darkness
2 weeks	Chlorophyll A	42 ± 7	57 ± 6	108 ± 18	53 ± 3	26 ± 4
	Chlorophyll B	24 ± 3	28 ± 3	79 ± 2	23 ± 5	41 ± 7
	Carotenoids	24 ± 4	31 ± 8	45 ± 2	30 ± 3	23 ± 2
	Total	90 ± 14	116 ± 16	232 ± 22	105 ± 11	91 ± 13
3 weeks	Chlorophyll A	90 ± 17	58 ± 4	121 ± 15	114 ± 13	32 ± 1
	Chlorophyll B	37 ± 2	46 ± 7	91 ± 8	71 ± 3	54 ± 2
	Carotenoids	45 ± 7	32 ± 2	58 ± 2	60 ± 5	26 ± 6
	Total	172 ± 26	136 ± 12	271 ± 25	245 ± 21	112 ± 9
4 weeks	Chlorophyll A	74 ± 5	89 ± 5	125 ± 10	86 ± 6	10 ± 1
	Chlorophyll B	62 ± 3	58 ± 4	93 ± 2	43 ± 2	15 ± 2
	Carotenoids	43 ± 3	47 ± 4	69 ± 3	44 ± 3	11 ± 1
	Total	179 ± 11	194 ± 12	287 ± 15	173 ± 11	35 ± 3

* Control fluorescent lamps.

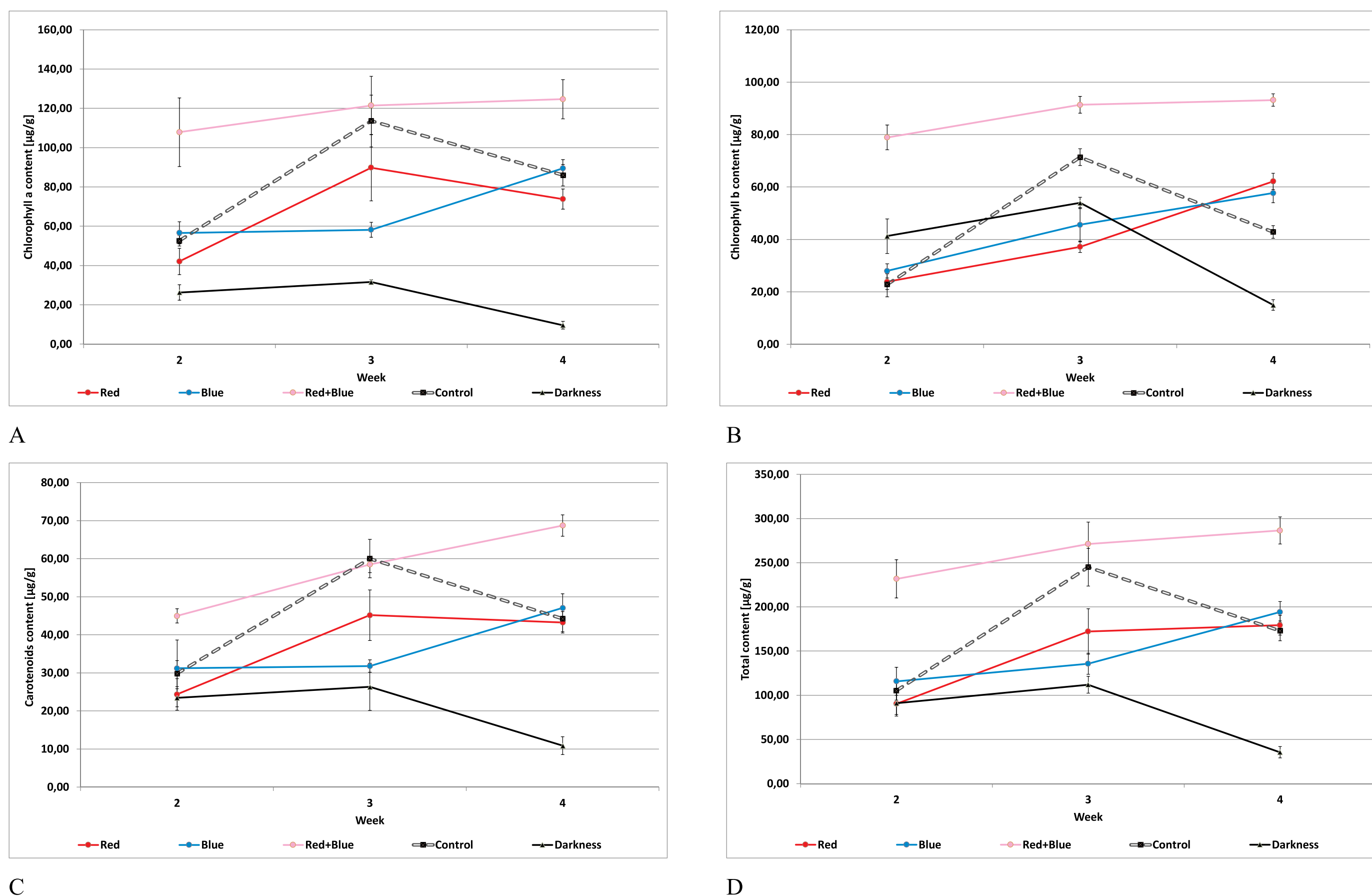

Fig. 4. Comparison of the content [µg g^{-1} FW] of chlorophyll *a* (A), chlorophyll *b* (B), carotenoids (C) and total photosynthetic pigments (D).

this slope is still insignificant when analyzing significance of the trend.

3.6. Principal component analysis (PCA) results

All time points (3 time points x 5 light conditions) were projected to a reduced space to interpret the main trends in time. It was revealed that 84% of the total information could be preserved by projection onto a four-dimensional space, thus there were four main trends in this dataset (Fig. 5). The main trend (PC1) represented 41.9% of the total variance and was associated with an average increase of all investigated factors (besides protocatechuic acid), where all parameters increased over time and the highest increase was observed in the first two weeks. A slight change in subsequent two weeks was observed for red with blue light, whereas dark sample represented the lowest values and growth. The main trend in parameter differences was represented by PC2 (20.2% of variance).

Chlorophyll b, chlorophyll *a*, total photosynthetic pigments and caffeic acid pointed in the opposite directions than protocatechuic acid (in particular), vanillic acid and total phenolic acids, therefore the increase in chlorophyll was negatively correlated with the rise of phenols

Table 4
Significance of the changes (slope of linear regression) in time of measured parameters. Arrow up – significantly increases, arrow down – significantly decreases, no arrow – no significant change, together with relative significances of the *slope changes* caused by different wavelengths (denoted with analogous skewed arrows respectively).

	Red	Blue	Red/Blue (70%/30%)	Control*	Darkness	Red	Green	Blue
Protocatechuic acid (PRO)								
Chlorogenic acid (CHL)								
Vanillic acid (VAN)	↑							
Caffeic acid (CAF)								↗
Ferulic acid (FER)			↓				↘	
o-Coumaric acid (OKU)						↗	↘	
m-Coumaric acid (MKU)		↓				↗	↘	↗
Total phenolic acids (TPH)		↓	↓				↘	
Chlorophyll A (CLA)						↗	↘	↗
Chlorophyll B (CLB)		↑				↗	↘	↗
Carotenoids (CAR)						↗		↗
Total photosynthetic pigments (TCH)						↗	↘	↗
Total biomass							↘	
Verbascoside (VER)							↘	↗
Isoverbascoside (IVE)			↑	↑	↑			

* Control fluorescent lamps.

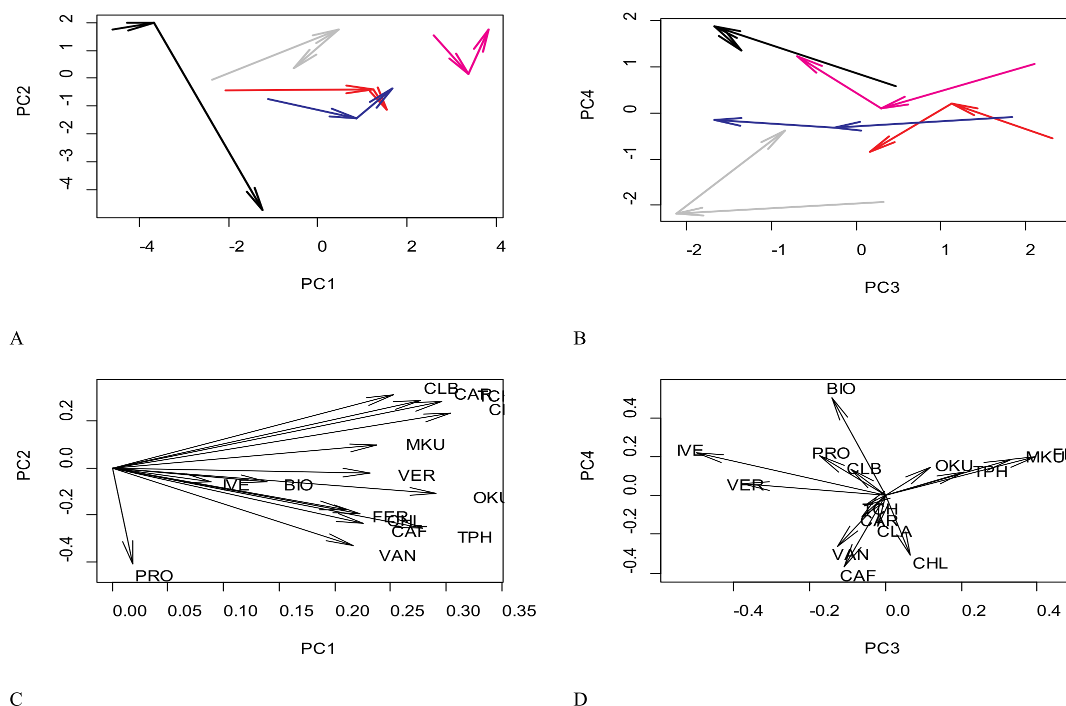

Fig. 5. Time trends in reduced PCA space of PC1-PC2 (A) and PC3-PC4 (B) with the corresponding loadings (C, D). Arrow colors: red – red light, blue – blue light, magenta – red and blue light, grey – control, black – darkness. Each light condition is represented by two arrows, connecting 2 - > 3 - > 4 weeks time points. (PRO; Protocatechuic acid, CHL; Chlorogenic acid, VAN; Vanillic acid, CAF; Caffeic acid, FER; Ferulic acid, OKU; o-Coumaric acid, MKU; m-Coumaric acid, TPH; Total phenolic acids, CLA; Chlorophyll A, CLB; Chlorophyll B, CAR; Carotenoids, TCH; Total photosynthetic pigments, BIO; Total biomass, VER; Verbascoside, IVE; Isoverbascoside). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

content. This phenomenon was stimulated by blue light, whereas darkness represented the highest opposite trend. The third trend, PC3 (13.4% of variance), represented a visibly higher growth rate of verbascoside, isoverbascoside and protocatechuic acid than o-coumaric acid, m-coumaric acid, ferulic acid and total phenolic acids. The last, fourth trend (8.7% of variance) represented differences between the light spectra. Control light induced the highest production of chlorogenic acid, caffeic acid, vanillic acid and chlorophyll A and lower total biomass. This trend was reversed in the case of samples from dark conditions.

4. Discussion

The experiments proved the effect of different wavelengths of LED light and the duration of the culture cycle on dry biomass increases and the accumulation of secondary metabolites in *V. officinalis* callus cultures (Fig. 3A). Fold increases of dry biomass were high in the studied culture conditions. Depending on LED conditions used, they fluctuated from 13.01 to 19.19 and were similar to those obtained under control conditions (from 13.32 to 18.78 folds); they were also higher than fold increases obtained in cultures grown without light (from 9.22 to 12.25 folds). The highest (19.19 folds) increases were found in the presence of blue light after 3 weeks of culture. High (18.42 folds) increases were obtained in cultures grown in a combination of red and blue light (70%/30%), also after a 3-week culture period. The highest biomass increases (4.4-folds) in *Schisandra chinensis* (Schisandraceae) shoot-differentiating callus cultures, grown under different fluorescent light conditions, were also obtained under blue light in 30-day growth cycles [21]. A similar effect of light conditions was found for *Aronia* species (Rosaceae) shoot cultures cultivated under different fluorescent light conditions; dry biomass increases for blue light fluctuated depending on the species from 2.86 to 8.06 folds in 4-week growth cycles [20]. Similarly, *Myrtus communis* (Myrtaceae) shoot cultures cultivated under LED lights showed the highest biomass increases after a 6-week growth cycle under blue and red/blue (70%/30%) LED lights [27]. Both red and blue light was the most favorable for the increase of dry biomass in the cultures of *Zantedeschia albomaculata* (*Araceae*), [28]. The highest biomass increases in *Dendranthema grandiflora* (Asteraceae) cultures were found under red/blue (50%/50%) LED lights [29]. The largest biomass increases in *Tripterospermum japonicum* (*Gentianaceae*) shoot cultures were also obtained for a combination of red and blue LED lights (70%/30%) [30].

Factors, such as light wavelength, its intensity, photoperiod, time of culture, temperature or culture medium composition affect the endogenous accumulation of secondary metabolites in *in vitro* cultured biomass. Some studies showed that light quality is one of the most important factors influences the production of different bioactive metabolites, molecularly or physiologically. In the last three decades, the extensive research linking: plant molecular biology, genetic and physiology, facilitated the isolation of the genes encoding the red/far-red light absorbing phytochromes and the blue light absorbing cryptochromes and phototropins. Studies provided a powerful experimental system to tackle some of the central questions in photobiology research, including the exploration of the molecular mechanism by which photoreceptors regulate gene expression and thereby plant growth and development [31]. Light signals are transmitted from photoreceptors along specific pathways and induce gene expression, which finally results in the physiological response. It was proposed that phytohormones were involved in the plant responses to light action [32]. Light is the major energy source for photosynthesis and regulation of plant morphogenic [33] and gene expression [34]. Another study employing monochromatic light had shown that blue light promoted gene expression of CHS (chalcone synthase) and DFR (dihydroflavonol-4-reductase) which regulate the anthocyanins pathway [35]. According to Tattini et al. (2006) [36] and Agati et al. (2011) [37], the content of polyphenols, particularly flavonoids, may be related to the reaction of plants to particular environmental conditions, such as light conditions. Additionally, Bian et al. (2015) [38] showed that biosynthesis,

metabolism and accumulation of plant secondary metabolites in vegetables is affected by environmental factors. Light condition (light quality, light intensity and photoperiod) is one of the most important environmental variables in regulating vegetable growth, development and phytochemical accumulation, particularly for vegetables produced in controlled environments [38].

Our research has focused on the analysis of LED light effect on *in vitro* cultures growing on optimal medium in terms of biomass increases and accumulation of secondary metabolites selected in previous experiments [9]. The obtained results confirm the rationale of optimizing the conditions of *in vitro* cultures and prove how significantly they influence the accumulation of secondary metabolites in cultivated cells. The change in light conditions, conducted as part of the experiment, allowed to increase the production of secondary metabolites, especially of highly accumulated verbascoside: from 3658 mg 100 g^{-1} DW (control, 4-week growth cycle) to 6716 mg 100 g^{-1} DW (blue LED light, 4-week growth cycle). The highest maximum content of verbascoside founded in cultures grown for 4 weeks under blue light was over 1.8-fold higher than in control conditions and over 1.4-fold higher compared to culture growing without light (Fig. 3 B, Table 1).

Isoverbascoside was accumulated in the largest quantities after a 4-week growth cycle (379 mg 100 g^{-1} DW) in the combination of red and blue light. The maximum quantity of this compound was almost 7-fold higher compared to control conditions and > 13-fold higher than in cultures carried out in the absence of light (Fig. 3 C, Table 1).

The use of blue light and red and blue lights combination in this experiment caused a significant increase in the production of verbascoside and isoverbascoside, respectively, in *V. officinalis* callus cultures. Blue light often has a stimulating effect on the accumulation of secondary metabolites in *in vitro* plant cultures, as proven for verbascoside in *in vitro* cultures of *Scutellaria lateriflora* (Lamiaceae). The influence of fluorescent light conditions (monochromatic lights) on the content of verbascoside was analyzed in these cultures, and its largest quantity was also found in the presence of blue fluorescent light (max. 169 mg 100 g^{-1} DW). Its content was 1.86-fold higher than in control conditions [39]. In addition, 1.31-fold higher amounts of schisandra lignans were found in *S. chinensis* shoot-differentiating callus cultures under blue fluorescent light than under control light [21].

Numerous studies demonstrate the necessity of empirical selection of light conditions both, *in vivo* and *in vitro* conditions to optimize the production of secondary metabolites in the biomass. It has been proven that blue light and UV radiation *in vivo* stimulates the production of anthocyanins [40]. Periodic irradiation with red light also stimulated the production of steviol in *Stevia rebaudiana* plants (Asteraceae) [41]. A stimulating effect of red light has been also demonstrated in somatic embryo proliferation of *Eleutherococcus senticosus* (Araliaceae) cultured in a balloon-type bubble bioreactor [42]. Our research has shown a significant influence of different LED light wavelengths on the accumulation of bioactive compounds in *V. officinalis* callus cultures. The experiments also tested the influence of the lack of light on callus cultures, because, according to some authors, such conditions may stimulate the production of certain metabolites, *e.g.* alkaloids or naphthoquinones [43,44]. This correlation was also confirmed by previous studies of our team where the highest production of bergapten (linear furanocoumarin) was found for *Ammi majus* (Apiaceae) [45] callus cultures and the highest production of phenolic acids and furanocoumarins for shoot-differentiating callus cultures of *Ruta graveolens* ssp. *divaricata* (Rutaceae) [19] cultivated in the darkness. Additionally our previous studies on the biosynthetic potential of *V. officinalis* also demonstrated a beneficial effect of the lack of light on the production of phenolic acids [9].

The present studies also showed the stimulating effect of light conditions on the production of phenolic acids in *V. officinalis in vitro* cultures (Fig. 3 D, Table 2); however, the amounts of these compounds were significantly lower than the obtained content of phenylpropanoid glycosides. The combination of red and blue LED light was most favorable for the accumulation of phenolic acids in the studied cultures, as the total phenolic acid content, regardless of culture duration, ranged from 62 to 65 mg 100 g^{-1} DW and was 1.4-fold higher than in control conditions. The m-coumaric acid was the most significantly accumulated phenolic acid in terms of quantity in the biomass of *V. officinalis* callus cultures (max. 39 mg 100 g^{-1} DW) (Table 2).

The production of phenolic acids was stimulated by blue fluorescent light in both stationary liquid shoot cultures of *Ruta graveolens* and stationary liquid shoot-differentiating callus cultures of *R. graveolens* ssp. *divaricata* and the total content of phenolic acids was 83 and 100 mg 100 g^{-1} DW, respectively [19]. From among the tested LED light variants in *in vitro* cultures of *Myrtus communis*, control fluorescent lamps light was the most conducive to the accumulation of gallic acid (155 mg 100 g^{-1} DW), while under blue light, this compound was produced in the lowest quantities (36 mg 100 g^{-1} DW). Red light favored the most the production of p-hydroxybenzoic acid, and the combination of red and blue lights (70%/30%) stimulated protocatechuic acid accumulation [27].

The influence of light conditions on the content of photosynthetic pigments in *V. officinalis in vitro* cultures was also examined as part of the conducted studies (Fig. 4, Table 3). Their concentrations were dependent on the tested light conditions. The highest content of pigments was obtained in the combination of red and blue light (70%/30%) after a 4-week culture cycle. The lowest results were obtained in the absence of light and under red light. Studies conducted with other plant species also confirmed the stimulating effect of red and blue LED lights combination on chlorophyll content in *Chrysanthemum* sp. *(Asteraceae)* sp. [29]and chlorophylls a, b and carotenoids in orchid (*Dendrobium officinale* (Orchidaceae)) – *in vitro* cultures [46]. Blue fluorescent light was the best for chlorophylls (a and b) production for the following species: *Zantedeschia jucunda* (Araceae) [47] and *Gossypium hirsutum* (Malvaceae) [48].

Stimulation of secondary metabolite production by different light conditions of *in vitro* cultures is one of the simpler methods to increase the production of important bioactive compounds. Further optimization of *V. officinalis in vitro* culture conditions provides the opportunity to use them as a potential biotechnological source of obtaining mainly phenylpropanoid glycosides. These compounds exhibit, among others, antioxidant, anti-inflammatory, cytoprotective, antiviral, and antibacterial properties.

Research on the possibility of obtaining verbascoside from *in vitro* cultures was also carried out in other research centers. These studies proved a high accumulation of this compound in other models of *in vitro* cultures, *Salvia vulgaris* (Lamiaceae) (max content 16 g% DW) and *Cistanche salsa* (Orobanchaceae) (max. Content 689 mg dm^{-3}) suspension cultures, carried out with the addition of precursors, and in *Harpagophytum procumbens* (Pedaliaceae) cultures conducted in bioreactors (max. 165 mg dm^{-3} day^{-1}) [49], as well as in „hairy roots" cultures of *Paulownia tomentosa* (Paulowniaceae) (max. 9 g% DW) [50] or *Rehmannia glutinosa* (Orobanchaceae) (max. 1690 mg 100 g^{-1} DW) [51].

The attractive results obtained by us in stationary *V. officinalis* cultures encourage testing other culture models in the next stage, including suspension and bioreactor cultures.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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