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The regulating effect of light on the content of flavan-3-ols and derivatives of hydroxybenzoic acids in the callus culture of the tea plant, *Camellia sinensis* L

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ABSTRACT

The objective was the study of the regulating effect of light on the composition and contents of phenolic compounds in the callus culture of the tea plant (*Camellia sinensis*) grown for 28 days in the light (16-h photoperiod) or in the dark. For the analyses of phenolic compounds, an ultra-performance liquid chromatography in combination with a photodiode array detector and a high-resolution Q Exactive Orbitrap mass spectrometry (UPLC-PDA-HRMS/MS) was used. Nineteen phenolic compounds were characterized. The main phenolic compounds were flavan-3-ols (mono- and oligomers of flavan-3-ols) and derivatives of hydroxybenzoic acids (mono- and digalloyl glucoses, hexosides of 2,5- and 2,3-dihydroxybenzoic and salicylic acids). It was shown that the growth of the tea callus in the light or in the dark does not affect the composition of phenolic compounds, but differently affects the content of flavan-3-ols and derivatives of hydroxybenzoic acids. In the callus grown in the light, the content of flavan-3-ols was about 2-fold higher than in the dark. On the contrary, the content of derivatives of hydroxybenzoic acids in the light was at average 3-fold lower. The regulating role of light in the biosynthesis of phenolic compounds, as well as light control of the distribution of carbon flux between the branches of the shikimate pathway, which are associated with the biosynthesis of flavan-3-ols and hydroxybenzoic acids, are discussed.

1. Introduction

Tea from *Camellia sinensis* leaves is a very popular beverage all over the world. Its daily consumption supports the human healthiness due to the presence of biologically active secondary compounds: alkaloids, terpenoids and phenolics (Zhang and Tsao, 2016). Many of these compounds have proved antioxidant, antimutagenic, anticarcinogenic, antidiabetic, antibacterial, anti-inflammatory, antihypertensive, and anticardiovascular disease activities (Rothenberg and Zhang, 2019).

The biochemical specificity of the tea plant is the accumulation of large amounts of phenolic compounds. Their total content in the leaves reaches 36% of dry mass, with flavan-3-ols and flavonols accounting for more than 50% (Jiang et al., 2013; Zhu et al., 2021). Flavan-3-ols are mainly represented by (+)-catechin, (−)-epicatechin, (+)-gallocatechin and (−)-epigallocatechin, their galloylated and methylated forms, glycosides, as well as oligo- and polymers (procyanidins) (Fraser et al., 2012; Jiang et al., 2013; Zhu et al., 2021). Along with flavan-3-ols, the tea plant accumulates different flavonol glycosides, galloyl glucoses, galloyl quinic and caffeoyl quinic acids (Ku et al., 2010; Zhu et al., 2021).

There are many studies of the phenolic compounds in tissue cultures of tea plant (Muthaiya et al., 2013; Dias et al., 2016; Shi et al., 2020). It was shown that compared to the intact plant, the cultures often had lower contents of the phenolic compounds with simpler molecular structures (Wang et al., 2012 a; Zubova et al., 2020). Optimization of the growing conditions: light, humidity, temperature, and the chemical characteristics of the nutrient medium, allow to activate and modify their biosynthesis (Muthaiya et al., 2013; Dias et al., 2016; Nechaeva

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et al., 2020; Shi et al., 2020).

Light is one of the environmental factors that stimulates the biosynthesis and accumulation of flavonoids, proanthocyanidins, lignins, and phenylpropanoids in tea plants, including *in vitro* cultures (Ku et al., 2010; Wang et al., 2012 b; Hong et al., 2014; Shi et al., 2020; Jin et al., 2021). However, the biosynthesis of various classes of phenolic compounds reacts differently to light. For example, full sunlight activated the synthesis and accumulation of flavonoids in the leaves of the tea plant, but decreased content of gallic and galloyl quinic acids (Wang et al., 2012 b). Illumination of etiolated seedlings also rapidly reduced the content of gallic acid and galloylated catechins with a simultaneous increase of the flavan-3-ols content (Lu et al., 2014).

Earlier, it was found that the initially high content of total phenolic compounds in tea callus culture, which was growing in the dark, decreased after several days of illumination, and then began to increase again (Zagoskina et al., 2005). However, the composition of phenolic compounds in the callus culture was not studied, and it is not known which phytochemical classes were responsible for these changes. Therefore, the objective of the work was the comparison of the content of various classes of phenolic compounds in the tea callus cultures that were growing in the light or in the dark. To study the composition of the phenolic compounds an ultra-performance liquid chromatography in combination with a photodiode array detector and a high-resolution mass spectrometry (UPLC-PDA-HRMS/MS) was used.

2. Materials and methods

2.1. Plant material

The object of the study was the culture of tea callus (strain IPP ChS-2) obtained from the stem of *Camellia sinensis* (L.) O. Kuntze. Callus was cultured for 42 days in the dark on Heller's medium containing 5 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D), 25 g/L of glucose, and 7 g/L of agar at 26 °C (Nechaeva et al., 2020). Then, portions of the callus of about 5 g were transferred to fresh nutrient medium. There were used two groups of callus samples in the experiment. The first group of callus continued to grow in the dark and was used as a control (three biological repeats) (Fig. 1A). The second group grew in the light (16-h photoperiod, three biological replicates) (Fig. 1B). The culture was illuminated with white light with an intensity of 5000 lux (40 W fluorescent lamps, Osram, Germany). For the analysis of phenolic compounds, the callus samples were taken on the 28th day of growth in the middle of the linear growth phase, which was determined by the callus biomass growth index (Zubova et al., 2020).

2.2. Chemicals and reagents

The acetonitrile was from LiChrosolv® hypergrade for LC-MS (Merck KGaA, Darmstadt, Germany). Analytical grade formic acid, (+)-catechin, (−)-epicatechin, and (1*R*)-(−)-10-camphorsulfonic acid purchased from Sigma-Aldrich (Steinheim, Germany). Ethanol (99.5%, v/v) was from Primalco (Rajamäki, Finland) and acetone – from VWR Chemicals (EC). Pure water was obtained with the Elgastat UHQ-PS purification system (Elga, Kaarst, Germany).

2.3. Extraction of phenolic compounds

The tea callus samples were frozen in liquid nitrogen, freeze dried for 72 h, and reduced to a powder using a MM 301 vibrating mill (Retsch GmbH & Co. KG) at 30 Hz for 2 min. Samples of 30.0 ± 1.0 mg were extracted with 1 ml of 70% aqueous acetone containing the internal standard (1*R*)-(−)-10-camphorsulfonic acid (5 mg/ml). Extraction of phenolic compounds was carried out at room temperature and under continuous stirring (Vortex, Genie 2) for 30 min. The extracts were separated from insoluble residue by centrifugation at 20,000×*g* for 10 min and evaporated to dryness on a rotary concentrator at 40 °C (Concentrator 5301, Eppendorf AG, Germany). The dry extracts were dissolved in 1 ml of water, filtered (4 mm, 0.2 μm PTFE, Thermo Fisher Scientific Inc., Waltham, USA) and diluted with water at 5 times.

2.4. UPLC–PDA–HRMS/MS analysis

The UPLC system (Acquity UPLC® 2.9.0, Waters Corporation, Milford, USA) consisted of a sample manager, a binary solvent manager, and a photodiode array detector (PDA). The column Acquity UPLC® BEH Phenyl (2.1 × 100 mm, 1.7 μm, Waters Corporation, Wexford, Ireland) was used. Two eluents, 0.1% formic acid (A) and acetonitrile (B), were used in a gradient program: 0–0.5 min, 0.1% B in A; 0.5–5.0 min, 0.1–30.0% B in A (linear gradient); 5.0–6.0 min, 30.0–35.0% B in A (linear gradient); 6.0–6.1 min, 35.0–95.0% B in A (isocratic); 6.1–8.1 min, 95.0% B in A. The flow rate was 0.5 ml/min and the injection volume was 5 μl. The PDA operated in the range of 190–500 nm.

The UPLC system was combined with a Thermo Fisher Scientific high resolution Q Exactive Orbitrap mass spectrometer 2.5, equipped with heated electrospray ionization (HESI) source. The mass spectrometer operated in the negative ionization mode and ions were scanned in the range of *m/z* 150–2250. The HESI conditions were as follows: sheath gas flow rate was set at 60, the auxiliary gas flow rate at 20, arbitrary units as set by Tune software: spray voltage at −3 kV, capillary temperature at 380 °C, and S-lens RF level at 60.0. The settings for full scan mode were: microscans 1, resolution 140,000 FWHM and 34,599 FWHM (data dependent MS^2), AGC target 3×10^6 and maximum IT 200 ms. PierceTM

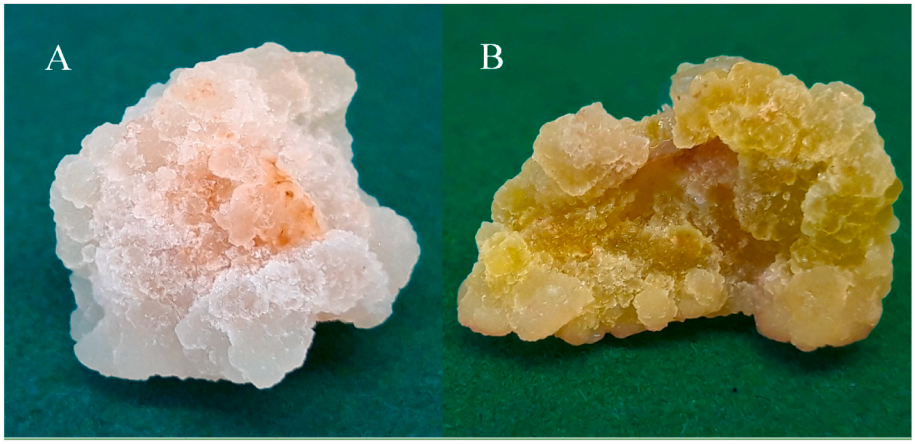

Fig. 1. Photo of the callus samples of *Camellia sinensis* grown in the dark (A) and in the light (B, 16-h photoperiod) for 28 days.

ESI negative ion calibration solutions (Thermo Fischer Scientific Inc., Waltham, MA, USA) was used to calibrate the mass-spectrometer. The instrument operated by Xcalibur software (Version 3.0.63, Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.5. MS data processing

The raw MS data were converted into NetCDF format and processed with MetAlign program (De Vos et al., 2007). MetAlign parameters were set up according to the specific requirements of scalability and chromatographic and mass spectrometry conditions used in the experiment. The program was corrected automatically the baseline of chromatograms, accurately calculated the mass spectra of metabolites, estimated intensity of the *m/z* ions and aligned their position on the chromatograms. The MetAlign processed MS data were open with Excel software as a matrix, in which the first row contained the code of sample, and the first three columns were number, retention time (min) and *m/z* value of ions. The other columns contained relative intensity of the recorded *m/z* ions of all samples.

2.6. Data analysis

The data matrix was exported into the SIMCA-15 software package for multivariate analysis (SIMCA, Sartorius, Sweden). Principal component analysis (PCA) was used as the first step of the callus samples classification (Wiklund et al., 2008). However, it is known that the PCA results can be affected by many factors: biodiversity, pathological variations, instrumental drift, artifacts, and other experimental conditions (Wiklund et al., 2008). Therefore, to determine the differences in the contents of phenolic compounds between samples of the tea callus grown in the light and in the dark, the data were analyzed using a controlled classification method - orthogonal partial least squares to latent structures (OPLS). The statistical significance of the obtained OPLS model was tested by ANOVA of cross-validated predictive residuals (CV-ANOVA). OPLS model results were visualized using S-plot (Wiklund et al., 2008).

The relative content of phenolic compounds was calculated by the intensity of the ion, the *m/z* value of which corresponds to the *m/z* value of the deprotonated ion $[M\text{-}H]^-$ or multiply charged ions, normalized per internal standard (*m/z* 231) and 1 g of dry mass of callus sample. The significance of differences in the content of phenolic compounds was determined by the correlation of their content with the orthogonal component of the OPLS model, and checked also using paired Student's *t*-tests (Wiklund et al., 2008).

2.7. Identification of phenolic compounds

The UV, MS and MS/MS data of phenolic compounds were used for their identification or tentative characterization with application of mass spectrometry databases "Metlin" (Guijas et al., 2018), "The Human

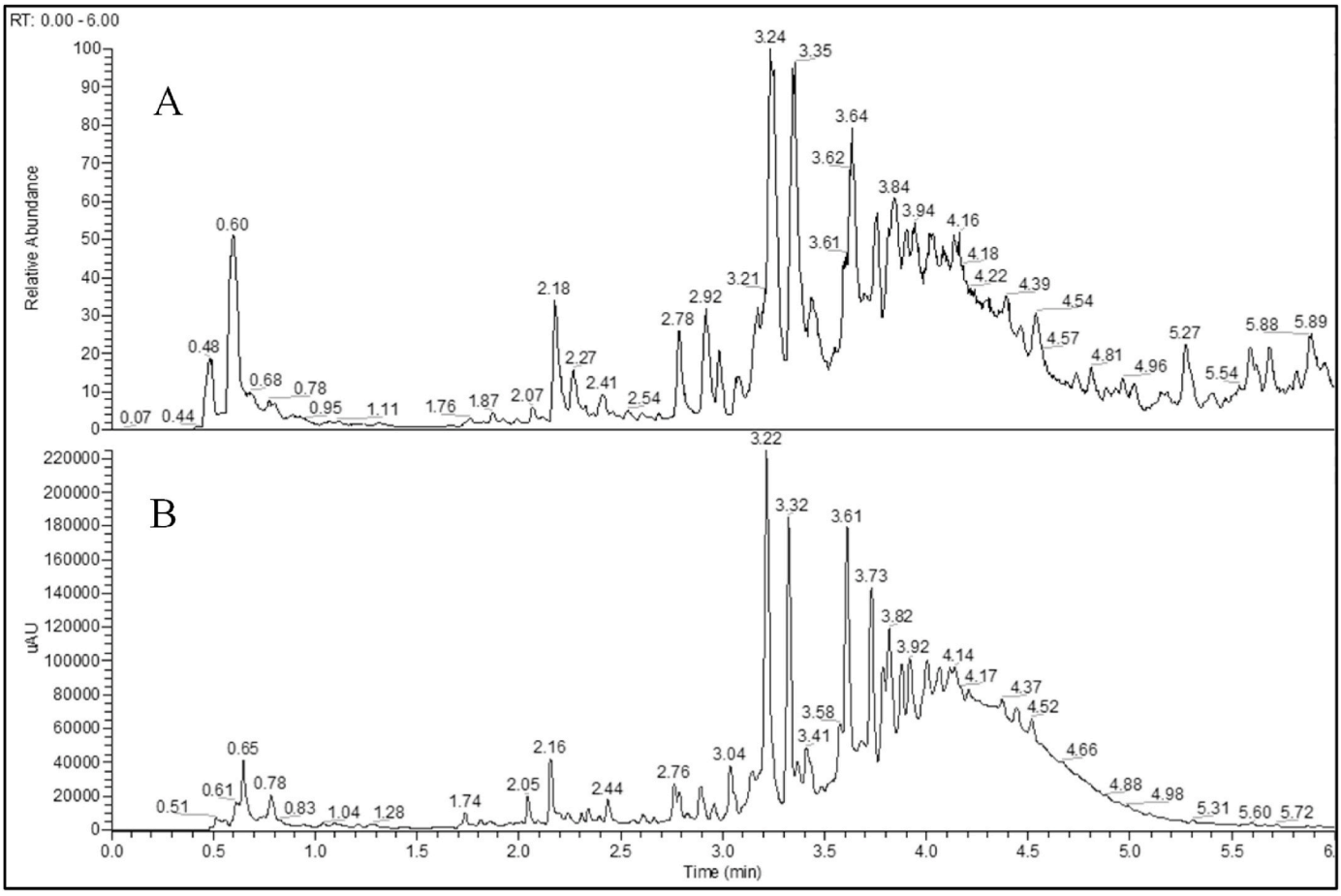

Fig. 2. UPLC-UV-HRMS profiles of phenolic compounds of the tea callus culture, *C. sinensis*. A - MS profile, TIC in negative mode, range *m/z* 150–2250; B–UV profile, range 240–400 nm. Characterization of the phenolic compounds (retention time on MS profile): 2.18 min, 2,5-dihydroxybenzoic acid hexoside; 2.27 min, galloyl glucose; 2.41 min, 2,3-dihydroxybenzoic acid hexoside; 2.78 and 3.24 min, procyanidin dimers, B-type, isomers 1 and 2; 2.82 min, salicylic acid hexoside; 2.92 min, (+)-catechin; 2.94 min, digalloyl glucose; 2.99 min, epicatechin glucoside; 3.09, 3.17 and 3.64 min, procyanidin trimers, B-type, isomers 1, 2 and 3; 3.35 min, (−)-epicatechin; 3.43, 3.50 and 3.76 min, procyanidin tetramers, B-type, isomers 1, 2 and 3; 3.52 min, kaempferol dihexosyl-methylpentoside; 3.61 and 3.84 min, procyanidin pentamers, B-type, isomers 1 and 2; the range of ~4.0–5.0 min, procyanidin polymers with degree polymerization more than 5 (see Table 3, Fig. S2).

Metabolome Database" (Wishart et al., 2018), and data published in the literature (Yang et al., 2012; Li et al., 2014, 2020; Lin et al., 2014; Gu et al., 2015; Elsadig Karar and Kuhnert, 2016; Huang et al., 2018; Leppä et al., 2018; Rue et al., 2018; Yuzuak et al., 2018; Lee et al., 2019).

3. Results

3.1. Identification of phenolic compounds

Analyzing the UV and MS profiles of the tea callus extracts, there were detected nineteen phenolic compounds: mono- and oligomers of flavan-3-ols, galloyl and digalloyl glucoses, hexosides of two dihydroxybenzoic acids and hexoside of salicylic acid, epicatechin hexoside and kaempferol triglycoside (Fig. 2, Table 1). The identification of phenolic compounds was based on their UV spectral features, comparison of the measured and theoretical monoisotopic masses within $< \pm$ 1.5-ppm error and the presence of diagnostic MS/MS fragments of the parent ion (Table 1).

Flavan-3-ols had UV spectra with a single absorption maximum at 275–280 nm and characteristic MS ions (Table 1). The main *m/z* value for compounds (6) and (12) was 289.0717, which corresponds to the $[M-H]^-$ ion of flavan-3-ol monomers. MS/MS of the parent ion *m/z* 289.0717 showed major fragments *m/z* 245.08, 205.05, 151.04, 137.02, 123.05 and 109.033 (Table 1), which are characteristic for the fragmentation of catechin and epicatechin in Orbitrap-MS system (Yuzuak et al., 2018). Based on MS and MS/MS data, and retention times of the standards, these two flavan-3-ol monomers were identified as (+)-catechin (6) and (−)-epicatechin (12) (Table 1). There was found also the only flavan-3-ol glycoside (8), which MS showed $[M-H]^-$ ion at *m/z* 451.1246 and major MS/MS fragment ions *m/z* 289.02, 123.05 and 109.033. According to MS and MS/MS data, this compound was identified as epicatechin hexoside (Yuzuak et al., 2018) (Table 1).

Along with the flavan-3-ol monomers, the tea callus culture contained 10 flavan-3-ol oligomers that composed of multiple monomer subunits with interflavanoid C–C linkages (Table 1). Based on the *m/z* values of $[M-H]^-$ ions at *m/z* 577.1359, 865.1981, 1153.2622 and 1441.3258, these compounds were identified as B-type procyanidin dimers (4, 11), trimers (9, 10, 17), tetramers (13, 14, 18), and pentamers (16, 19), respectively (Leppä et al., 2018; Rue et al., 2018; Yuzuak et al., 2018; Li et al., 2020) (Table 1, Fig. S1).

MS/MS fragmentation of the parent ion of procyanidin oligomers includes three main pathways: quinone methide cleavage of the interflavanoid bond (QM), heterocyclic ring fission (HRF) and retro Diels-Alder fission of the heterocyclic ring system (RDA) (Karonen et al., 2004; Rue et al., 2018; Yuzuak et al., 2018). Procyanidin dimers from callus culture were fragmented onto monomers of catechin or epicatechin ions with *m/z* 289.07 (Table 1) that is characteristic to the QM cleavage pathway (Sui et al., 2016). The RDA fission of the heterocyclic ring also is a common fragmentation pathway for B-type procyanidin (Rue et al., 2018; Yuzuak et al., 2018). This pathway produces a fragment ions of *m/z* 425.09 and 407.08 [$-H_2O$] (Table 1). QM cleavage of B-type trimers produces ions at *m/z* 287.06 and 575.10 (extension units) and *m/z* 289.07 and 577.14 (terminal units). For B-type tetramers and pentamers, fragment ions for QM cleavage were observed at *m/z* 287.06, 289.07, 575.10 and 577.14 (Table 1). Therefore, results of MS/MS fragmentation of procyanidin oligomers from the tea callus culture supported their identification as B-type procyanidins.

The UPLC-UV-MS profiles of phenolic compounds of the tea callus have the shape of an elongated hump (Fig. 2 A, B). This means that, in addition to the flavan-3-ol oligomers, the callus contains also procyanidin polymers that have degree of polymerization (DP) more than 5 (Li et al., 2020). The presence of these polymers in plants can be detected by their multiply charged ions (Leppä et al., 2018; Li et al., 2020). The use of this approach made it possible to detect seven procyanidin polymers of B-type that formed doubly (DP 6–8) and triply charged ions (DP 10, 11, 13 and 14) in the electrospray ionization source (Fig. S2, Table 3).

In addition to flavan-3-ols, the tea callus contained five phenolic compounds (1, 2, 3, 5 and 7), which belong to the class of hydroxybenzoic acids (Table 1). Compounds (2) and (7) had monoisotopic masses, respectively, 332.0747 and 484.0850 Da (Table 1). MS/MS fragmentation of their parent ions $[M-H]^-$ showed the presence of ions *m/z* 169.0087 [gallic acid-H]$^-$ and *m/z* 125.0230 [gallic acid–CO_2–H]$^-$ (Yang et al., 2012; Singh et al., 2016). Compound (7) also gave a fragment ion with *m/z* 313.0565, which corresponds to the [M-galloyl group-H]$^-$ ion (Table 1). Thus, compounds (2) and (7) were identified, respectively, as galloyl glucose and digalloyl glucose (Table 1) (Yang et al., 2012; Singh et al., 2016).

The tea callus contained also three more derivatives of hydroxybenzoic acids (1, 3, 5) (Table 1). Compound (5) had a monoisotopic mass of 300.0844 Da. MS/MS fragmentation of its parent ion $[M-H]^-$ gave two major ions with *m/z* 137.0244 [M-hexosyl-H]$^-$ and 93.0346 [M-hexosyl–CO_2–H]$^-$ (Table 1), which characteristic for hexoside of hydroxybenzoic acid. According to UV and MS data compound (5) was identified as salicylic acid hexoside (Table 1) (Elsadig Karar and Kuhnert, 2016).

Compounds (1) and (3) had identical monoisotopic masses 316.0674 Da ($C_{13}H_{16}O_9$) and were tentatively identified as isomers of dihydroxybenzoic acid hexosides (Table 1). Some differences between the two isomers were found in MS/MS fragmentation of the parent ion *m/z* 315.0674 (Table 1). The main fragments of isomer (3) were *m/z* 153.0179 (26%) and 109.0238 (100%), which correspond to [M-hexosyl-H]$^-$ and [M-hexosyl–CO_2–H]$^-$ ions. In contrast, the relative intensity of these *m/z* fragments of isomer (1) was low, but the major fragments were *m/z* 152.0101 (25%) [M-hexosyl-2H]$^-$ and 108.0200 (100%) [M-hexosyl–CO_2–2H]$^-$ (Table 1). Analogous results of MS/MS fragmentation of the isomers of dihydroxybenzoic acid glucosides were found in the study of Gu et al. (2015) and Formato et al. (2021). Formato et al. (2021) explained these differences in fragmentation of deprotonated glucosides by the result of homolytic and heterolytic cleavage of the hexose fragment. It is possible that the differences in the fragmentation of hexosides of dihydroxybenzoic acids (1) and (3) were are associated with different positions of the hydroxyl groups. According to UV, MS and MS/MS data, these two compounds were identified, respectively, as hexosides of 2, 5-dihydroxybenzoic acid (1) and 2,3-dihydroxybenzoic acid (3) (Table 1) (Gu et al., 2015; Li et al., 2014; Huang et al., 2018). Free forms of salicylic, 2,5- and 2,3-dihydroxybenzoic acids were not found in the tea callus culture.

Flavonols are characteristic for phenolic compounds of the tea plant, but in the tea callus they were represented only by a single compound (15) (Table 1). Analysis of UV, MS and MS/MS showed that this is a triglycoside of kaempferol. According to the studies of flavonoids in the leaves of *C. sinensis* (Lee et al., 2019), compound (16) was identified as kaempferol dihexosyl-methylpentoside (Table 1).

3.2. Effect of light on the content of phenolic compounds

To detect the regulating effect of light on the content of phenolic compounds in *C. sinensis* callus culture, PCA was used as an initial step evaluating the UPLC-HRMS data (Wiklund et al., 2008). The result showed that the 1st component of the PCA model clearly separated the two groups of the tea callus samples, grown in the light and in the dark (Fig. 3 A).

The supervised orthogonal partial least squares to latent structures (OPLS) model divided the two groups of the tea callus samples in one predictive component and one orthogonal component (1 + 1) with high values of cumulative (R2Y cum - 0.97) and cumulative predictive (Q2 cum - 0.99) parameters of variation (Fig. 3 B). Very high values of the model variations indicated that the groups' separation was highly stable and reliable. Evaluation the OPLS model with application of CV-ANOVA also supported its significance ($F = 180.9$, $P = 0.005$). Therefore, the obtained results indicate that the phenolic compounds determining a strong and significant difference between the two compared groups of

Table 1
UPLC-PDA-HRMS/MS characterization of phenolic compounds in the callus culture of *C. sinensis*.

Number	Retention time (min)	UV maxima (nm)	Mass spectrum		Values of parent ion MS/MS fragments (*m/z*) and intensity (%)	Observed Monoisotopic mass (Da)	Molecular formula	Calculated Monoisotopic mass (Da)	Error (ppm)	Compound characterization
			$[M - H]^-$ (*m/z*)	others (*m/z*)						
1	2.18	314	315.0719	631.1520 $[2M-H]^-$	152.01 (25), 153.02 (5.5), 108.02 (100), 109.02 (3.1) 109.03 (100)	316.0792	$C_{13}H_{16}O_9$	316.0794	−0.72	2,5-Dihydroxybenzoic acid hexoside
2	2.27	277	331.0674		331.07 (100), 169.01 (71), 125.02 (98)	332.0747	$C_{13}H_{16}O_{10}$	332.0743	0.35	Galloyl glucose
3	2.41	309	315.0722		152.01 (7.2), 153.02 (26), 108.02 (18), 109.03 (100)	316.0795	$C_{13}H_{16}O_9$	316.0794	0.23	2,3-Dihydroxybenzoic acid hexoside
4	2.78	277–280	577.1349	1155.2769 $[2M-H]^-$	425.09 (14), 407.08 (30), 289.07 (40), 161.02 (33)	578.1422	$C_{30}H_{26}O_{12}$	578.1424	1.21	Procyanidin dimer, B-type, isomer 1
5	2.82	288	299.0771	599.1624 $[2M-H]^-$	137.02 (37), 93.03 (100)	300.0844	$C_{13}H_{16}O_8$	300.0845	−0.33	Salicylic acid hexoside
6	2.92	277–280	289.0716		289.07 (91), 245.08 (25), 205.05 (11), 151.04 (15), 137.02 (28), 123.05 (85), 109.03 (100)	290.0789	$C_{15}H_{14}O_6$	290.0790	−0.34	(+)-Catechin
7	2.94	277–280	483.0783		483.08 (100), 313.06, 169.01 (77), 125.02 (87)	484.085	$C_{20}H_{20}O_{14}$	484.0853	−0.62	Digalloyl glucose
8	2.99	277–280	451.1246		289.02 (100), 123.04 (44), 109.03 (60)	452.1319	$C_{21}H_{24}O_{11}$	452.1319	0.00	Epicatechin hexoside
9	3.09	277–280	865.1981		577.1 (14), 287.06 (18), 243.03 (17), 175.04 (23), 161.02 (41), 125.02 (100)	866.2054	$C_{45}H_{38}O_{18}$	866.2058	−0.46	Procyanidin trimer, B-type, isomer 1
10	3.17	277–280	865.1985		577.1 (12), 287.06 (24), 243.03 (21), 175.04 (22), 161.02 (40), 125.02 (100)	866.2058	$C_{45}H_{38}O_{18}$	866.2058	0.00	Procyanidin trimer, B-type, isomer 2
11	3.24	277–280	577.1351	1155.2765 $[2M-H]^-$	425.09 (13), 407.08 (38), 289.07 (51), 161.02 (33), 125.02 (100)	578.1424	$C_{30}H_{26}O_{12}$	578.1424	0.00	Procyanidin dimer, B-type, isomer 2
12	3.35	277–280	289.0717		289.07 (78), 245.08 (24), 205.05 (10), 151.04 (16), 137.02 (22), 123.05 (67), 109.03 (100)	290.079	$C_{15}H_{14}O_6$	290.0790	0.00	(−)-Epicatechin
13	3.43	277–280	1153.261	576.1288 $[M-2H]^{2-}$	577.13 (20), 287.06 (22), 243.03 (24), 175.04 (23), 161.02 (42), 125.02 (100)	1154.2683	$C_{60}H_{50}O_{24}$	1154.2692	−0.78	Procyanidin tetramer, B-type, isomer 1
14	3.5	277–280	1153.2612	576.1287 $[M-2H]^{2-}$	577.13 (13), 287.06 (19), 243.03 (26), 175.04 (23), 161.02 (39), 125.02 (100)	1154.2685	$C_{60}H_{50}O_{24}$	1154.2692	−0.61	Procyanidin tetramer, B-type, isomer 2
15	3.52	278, 330, 344	755.2049		755.20 (100), 285.04 (95), 255.03 (97), 227.04 (84)	756.2122	$C_{33}H_{40}O_{20}$	756.2113	1.19	Kaempferol dihexosyl-methylpentoside

(*continued on next page*)

Table 1 (*continued*)

Number	Retention time (min)	UV maxima (nm)	Mass spectrum		Values of parent ion MS/MS fragments (*m/z*) and intensity (%)	Observed Monoisotopic mass (Da)	Molecular formula	Calculated Monoisotopic mass (Da)	Error (ppm)	Compound characterization
			$[M - H]^-$ (*m/z*)	others (*m/z*)						
16	3.61	277–280	1441.3252	720.1611 $[M-2H]^{2-}$	575.12 (17), 287.06 (20), 243.03 (31), 175.03 (28), 161.02 (49), 125.02 (100)	1442.3325	$C_{75}H_{62}O_{30}$	1442.3326	−0.07	Procyanidin pentamer, B-type, isomer 1
17	3.64	277–280	865.1979		577.1 (11), 243.03 (20), 175.04 (22), 161.02 (41), 125.02 (100)	866.2052	$C_{45}H_{38}O_{18}$	866.2058	−0.69	Procyanidin trimer, B-type, isomer 3
18	3.76	277–280	1153.2616	576.1288 $[M-2H]^{2-}$	577.13 (10), 287.06 (23), 243.03 (23), 175.04 (25), 161.02 (43), 125.02 (100)	1154.2689	$C_{30}H_{26}O_{12}$	1154.2692	−0.26	Procyanidin tetramer, B-type, isomer 3
19	3.84	277–280	1441.3255	720.1610 $[M-2H]^{2-}$	575.12 (16), 287.06 (19), 243.03 (28), 175.03 (27), 161.02 (47), 125.02 (100)	1442.3328	$C_{75}H_{62}O_{30}$	1442.3326	0.14	Procyanidin pentamer, B-type, isomer 2

References for identification of phenolic compounds: dihydroxybenzoic acid hexosides (Li et al., 2014; Gu et al., 2015; Huang et al., 2018), salicyllic acid hexoside (Elsadig Karar and Kuhnert, 2016), galloyl glucoses (Yang et al., 2012; Singh et al., 2015; Liu et al., 2020), procyanidin oligomers and polymers (Lin et al., 2014; Leppä et al., 2018; Rue et al., 2018; Yuzuak et al., 2018; Li et al., 2020), epicatechin hexoside (Yuzuak et al., 2018), kaempferol dihexosyl-methylpentoside (Lee et al., 2019).

the *C. sinensis* callus cultures grown in the light and in the dark.

The S-plot of the OPLS model was applied to identify phenolic compounds that are affected by light (Wiklund et al., 2008). Phenolic compounds with high value of correlation with orthogonal component of OPLS model (>0.8 and <-0.8), were considered as potentially important compounds, which discriminate two groups of callus cultures, the grown in the light and in the dark (Table 2). It was shown that illumination did not affect the composition of phenolic compounds, but had strong positive or negative effect on the content of 16 out of 19 phenolic compounds (Table 2).

The strongest positive effect of light was found on the content of mono- and oligomeric flavan-3-ols (Table 2). Their levels in the callus grown in the light were at 1.4–2.4 fold higher than in the dark. The only flavan-3-ol, which content did not change under the light, was epicatechin hexoside (8). The increase in the contents of kaempferol dihexosyl-methylpentoside (15) and procyanidin B-type pentamer (16) in the light by 4.8 and 1.6 fold, respectively, were statistically insignificant (Table 2).

To assess the effect of light on the relative content of procyanidin polymers, the intensity of ions whose *m/z* values correspond to the *m/z* values of double and triple charged ions of procyanidin polymers was used (Li et al., 2020). The positive effect of light was found on the content of procyanidin polymers with DP 6–8 only (Table 3). Changes in the content of polymers with a higher DP were not statistically significant.

In contrast to flavan-3-ols, the relative content of the hydroxybenzoic acids derivatives was decreased under the light at average 2.4 fold (Table 2). For example, levels of galloyl glucose (2) and digalloyl glucose (7) were decreased, respectively, at 4 and 2 fold. Contents of salicylic acid hexoside (5), 2,5-hydroxybenzoic acid hexoside (1) and 2,3-dihydroxybenzoic acid hexoside (3) were decreased, respectively, at 3.5, 1.8 and 3.4 fold (Table 2).

4. Discussion

Light is a source of energy for a plant and an important factor in regulating its growth, development and adaptation to the environment (Shi et al., 2020; Jin et al., 2021). One of the manifestations of the regulating function of light in plants is its effect on the biosynthesis and accumulation of phenolic compounds. The main phenolic compounds of the studied callus culture of *C. sinensis* were various mono-, oligo- and polymers of flavan-3-ol. Derivatives of hydroxybenzoic acids were represented by galloyl and digalloyl glucoses, as well as hexosides of the salicylic acid, 2,5- and 2,3-dihydroxybenzoic acids. In addition, the tea callus contained epicatechin hexoside and kaempferol dihexosyl-methylpentoside.

Growing the *C. sinensis* callus in the light or in the dark for 28 days did not affect the composition of phenolic compounds, but differently influenced the content of flavan-3-ols and derivatives of hydroxybenzoic acids. The content of flavan-3-ols in the callus growing in the light was about 2-fold higher than in the dark, but the content of galloyl glucose and digalloyl glucoses, hexosides of salicylic, 2,5- and 2,3-dihydroxybenzoic acids, in contrast, was about 3-fold less. Earlier, similar negative effect of the light on the content of hydroxybenzoic acids was found in the leaves of the *C. sinensis* (Wang et al., 2012 b). Illuminating the leaves by the full intensity sunlight decreased the content of gallic and galloyl quinic acids, compared to the leaves grown in the shade. This was explained by the competitive biosynthetic pathways for gallic acid derivatives and flavonoids for the common precursor (Wang et al., 2012 b). However, the observed differences of the light effect on the content of flavan-3-ols and derivatives of hydroxybenzoic acids are probably more complex and were due to the peculiarities of the biosynthetic pathways, their regulation, and differences in the biological functions of these groups of phenolic compounds.

Flavan-3-ols are synthesized in *C. sinensis* from the end product of the shikimate pathway, phenylalanine (Fig. 4) (Jiang et al., 2013). On the contrary, gallic acid and its derivatives are formed directly from shikimic acid. The first compound of the gallic acid metabolism in plants is the galloyl glucose that is an ester of gallic acid and glucose (Widhalm and Dudareva, 2015). Further, the galloyl glucose is converted into structurally more complex hydrolysable tannins (gallo- and ellagitannins) (Jiang et al., 2013; Wei et al., 2019). Hydrolysable tannins in the different *Camellia* species are represented by various isomers of di-, tri- and tetragalloyl glucoses, and ellagitannins: gemin D, tellimagrandin

Table 2
The difference in the relative content of phenolic compounds between two groups of the callus cultures of *C. sinensis* grown for 28 days in the light (16-h photoperiod) or in the dark conditions.

Number	Retention time (min)	Phenolic compound	Difference in content		Correlation[b] from S-plot data
			Light/Dark[a] (fold)	*t*-test *p*-value	
1	2.18	2,5-Dihydroxybenzoic acid hexoside	−1.79	0.000	−1.00
2	2.27	Galloyl glucose	−4.04	0.000	−0.99
3	2.41	2,3-Dihydroxybenzoic acid hexoside	−3.35	0.000	−0.99
4	2.78	Procyanidin dimer, B-type, isomer 1	2.46	0.030	−0.89
5	2.82	Salicylic acid hexoside	−3.49	0.000	−0.99
6	2.92	(+)-Catechin	2.74	0.020	0.94
7	2.94	Digalloyl glucose	−2.00	0.005	0.94
8	2.99	Epicatechin hexoside	1.10	0.198	0.59
9	3.09	Procyanidin trimer, B-type, isomer 1	1.63	0.048	0.91
10	3.17	Procyanidin trimer, B-type, isomer 2	1.41	0.018	0.90
11	3.24	Procyanidin dimer, B-type, isomer 2	2.51	0.035	0.88
12	3.35	(−)-Epicatechin	1.57	0.012	0.97
13	3.43	Procyanidin tetramer, B-type, isomer 1	1.50	0.104	0.82
14	3.50	Procyanidin tetramer, B-type, isomer 2	1.61	0.043	0.91
15	3.52	Kaempferol dihexosyl-methylpentoside	1.76	0.004	0.99
16	3.61	Procyanidin pentamer, B-type, isomer 1	1.56	0.064	0.81
17	3.64	Procyanidin trimer, B-type, isomer 3	1.62	0.016	0.99
18	3.76	Procyanidin tetramer, B-type, isomer 3	1.71	0.034	0.91
19	3.84	Procyanidin pentamer, B-type, isomer 2	1.66	0.001	0.88

[a] Ratio was calculated with the intensity of *m/z* ion that corresponds to value of ion $[M\text{-}H]^-$ (see Table 1).

[b] Correlation of phenolic compounds content with orthogonal component of OPLS model was obtained from S-plot data.

I, pedunculagin, casuariin, strictinin, camellin A and B (Jiang et al., 2013). In contrast to the leaves, the *C. sinensis* callus culture contained the galloyl glucose and digalloyl glucose only. The more complex galloyl glucoses and ellagitannins were not found. Another pathway of the gallic acid metabolism in *C. sinensis* plants is the esterification of flavan-3-ols (Jiang et al., 2013). The formation of galloylated catechins in *C. sinensis* callus cultures was found in a study of Shi et al. (2020), but in our callus culture they were not defined.

Light activates and coordinates the expression of genes encoding many enzymes of the shikimate, phenylpropanoid and flavonoid pathways (Fig. 4) (Wang et al., 2012 a; Yang et al., 2012; Widhalm and Dudareva, 2015). However, active biosynthesis and accumulation of flavan-3-ols (proanthocyanidins) and gallic acid derivatives (hydrolyzable tannins) can be associated with different stages of plant growth and development (Riipi et al., 2004). It was shown that the maximum content of gallic acid derivatives and high expression of genes encoding enzymes associated with their biosynthesis were observed in buds and young leaves of *C sinensis*, and then rapidly decrease (Jiang et al., 2013). In contrast, the content of flavonoids in the buds and young leaves was the lowest and increased with the growth and development of the leaves.

In addition, the highest levels of galloyl glucose and galloylated catechins were found in etiolated seedlings of *C. sinensis* (Lu et al., 2014). Illumination of seedlings quickly reduced their content and stimulated the accumulation of flavan-3-ols. Thus, in the process of growth and development of the *C. sinensis* leaves, a sequential activation of the biosynthesis of gallic acid derivatives and flavan-3-ols is observed. It has also been shown that light has negative effect on the content of gallic acid derivatives and positive on flavan-3-ols (Lu et al., 2014). Therefore, it can be assumed that the higher content of flavan-3-ols in the *C. sinensis* callus culture growing in the light is mainly the result of activation of callus cells differentiation and formation of a photomixotrophic type of metabolism (Shi et al., 2020; Zubova et al., 2020). Whereas the higher content of gallic acid derivatives in the callus growing in the dark, on the contrary, is the result of its more active growth and formation of poorly differentiated cells, which are characterized by a relatively higher activity of the gallic acid pathway (Fig. 4).

In addition to the regulation of the biosynthesis of phenolic compounds through the genes expression, there are post-genomic mechanisms, which control the activity and direction of the carbon flux for the biosynthesis of certain compounds required at a given time and in accordance with the present state of the plant metabolism. However, in plants, there is no regulation of DAHP synthase activity by intermediate or end products of the shikimate pathway (Francenia Santos-Sánchez et al., 2019). This may be due to the branched character of the shikimate pathway in plants, which, in addition to aromatic amino acids, is the source of many secondary metabolites (Widhalm and Dudareva, 2015) (Fig. 4). Therefore, in the higher plants, the mechanisms controlling the distribution of carbon flux between the branches of the shikimate pathway should play an important role in the regulation of the biosynthesis of various classes of phenolic compounds.

One of these mechanisms is the control of the shikimic acid distribution between the main trunk of the shikimate pathway and the branch reaction of the gallic acid synthesis in according to the state of the energy metabolism in chloroplasts (plastids) (Francenia Santos-Sánchez et al., 2019). This control is carried out by the shikimate kinase, which activity showed a strong dependence on the ATP concentration (Francenia Santos-Sánchez et al., 2019). Therefore, the increased content of flavan-3-ols in the *C. sinensis* callus culture grown in the light may be associated with the formation of photomixotrophic type of metabolism and, as a consequence, with a more efficient synthesis of ATP in forming chloroplasts, where the shikimate pathway localized. In the dark grown callus, the heterotrophic metabolism of plastids is different, probably, by the lower level of ATP and low ratio of the reducing potential ($NADPH/NADP^+$). Because of this, the activity of shikimate kinase in the dark will be less than in the light, and a larger amount of the formed shikimic acid will be redirected to the oxidative and energetically less costly pathway of gallic acid synthesis (Fig. 4).

Along with the gallic acid derivatives, the content of hexosides of salicylic, 2,5- and 2,3-hydroxybenzoic acids in the *C. sinensis* callus growing in the dark was also higher than in the light. In spite on the similar effect, the reason for the negative influence of the light on the content of these hydroxybenzoic acids is different and due to their different pathway of biosynthesis and metabolic functions. For many years, it was believed that these hydroxybenzoic acids are formed from phenylalanine via hydroxycinnamic acids (Janda et al., 2020). However, the pathway from intermediate compound of the shikimate pathway, from chorismate, was found (Janda et al., 2020) (Fig. 4). Two steps of this more direct pathway were catalyzed by isochorismate synthase and pyruvate lyase, which convert the chorismate to isochorismate and further to salicylic acid (Widhalm and Dudareva, 2015). 2,5- and 2,3-dihydroxybenzoic acids are formed from salicylic acid by its

Table 3
The difference in the relative content of procyanidin polymers B-type between two groups of the callus cultures of *C. sinensis* grown for 28 days in the light (16-h photoperiod) or in the dark conditions.

Number of polymer	Retention time (min)	Ion (*m/z*)	Charge state	Calculated mass (Da)	Degree of polymerization (DP)	Difference in content		Correlation[b] from S-plot data
						Light/Dark[a] (folds)	*t*-test *p*-value	
1	3.94	864.1909	2	1730.3974	DP6	1.8	0.038	−0.925
2	4.03	1008.2280	2	2018.4716	DP7	1.8	0.025	−0.914
3	4.10	767.8334	3	2306.5236	DP8	2.2	0.011	−0.927
4	4.22	959.8774	3	2882.6556	DP10	1.7	0.098	−0.811
5	4.27	1055.8990	3	3170.7204	DP11	1.5	0.060	−0.843
6	4.31	1247.9412	3	3746.8470	DP13	−1.2	0.180	0.600
7	4.44	1343.9606	3	4034.9052	DP14	1.2	0.264	−0.603

[a] Ratio was calculated with the intensity of *m/z* ion that corresponds to value of ions $[M\text{-}2H]^{2-}$ or $[M\text{-}3H]^{3-}$ in the light and dark samples.
[b] Correlation of procyanidin polymer content with orthogonal component of OPLS model was obtained from S-plot data.

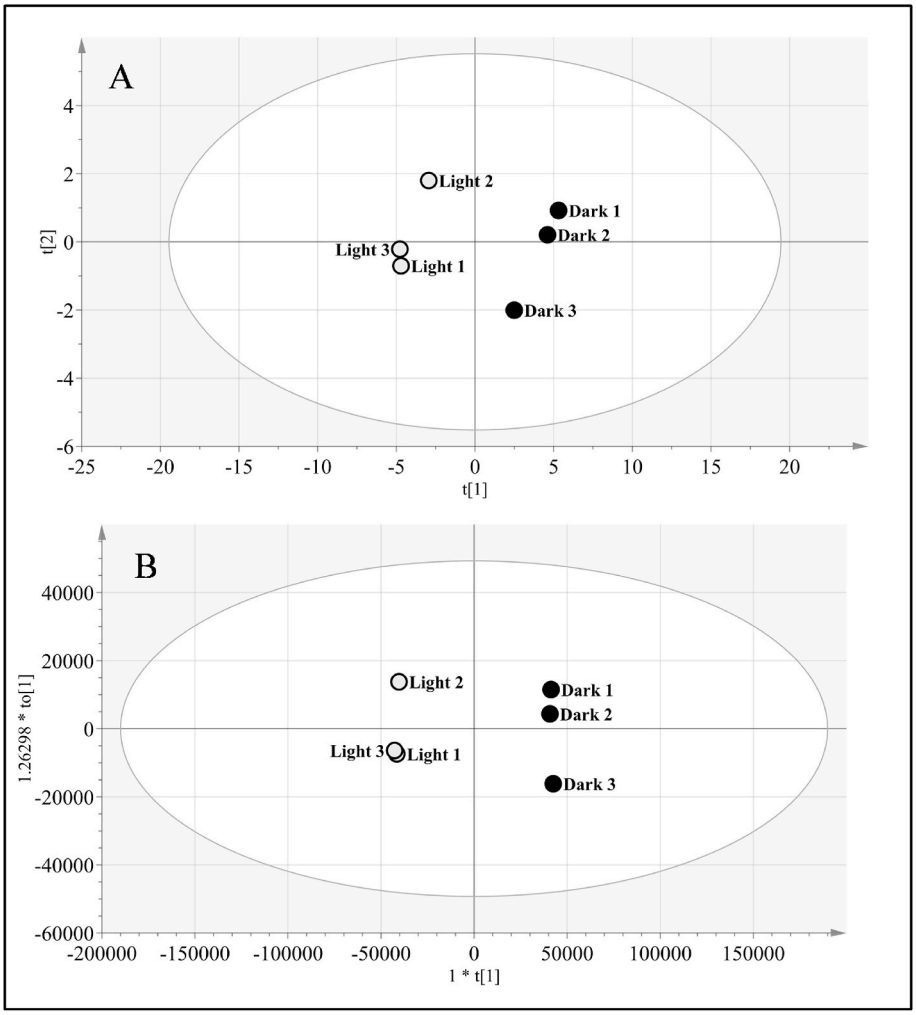

Fig. 3. Score plots of principal component analysis model (A) and orthogonal projections to latent structures analysis model (B) obtained for polar metabolites from *C. sinensis* callus cultures grown in the light (16-h photoperiod) or in the dark for 28 days (6 samples, 59014 masses).

hydroxylation (Zhang et al., 2017). The relative activities of the pathways for the salicylic acid biosynthesis from phenylalanine or chorismic acid were not studied yet. However, it has been shown that under stress Arabidopsis plant synthesized salicylic acid mainly through the isochorismate pathway, the pathway via phenylalanine was much less active (Dempsey et al., 2011).

Unlike hydrolyzable tannins and proanthocyanidins, which are chemical factors that ensure the plants resistance to various environmental stresses, the salicylic acid is a signaling metabolite regulating many metabolic processes in plants, including the biosynthesis of phenolic compounds (Janda et al., 2020). It is assumed that 2,5- and 2, 3-dihydroxybenzoic acids can also play the role of signaling compounds that regulate plant response to environmental stress (Zhang et al., 2017). Stimulation of the accumulation of phenolic compounds by salicylic acid

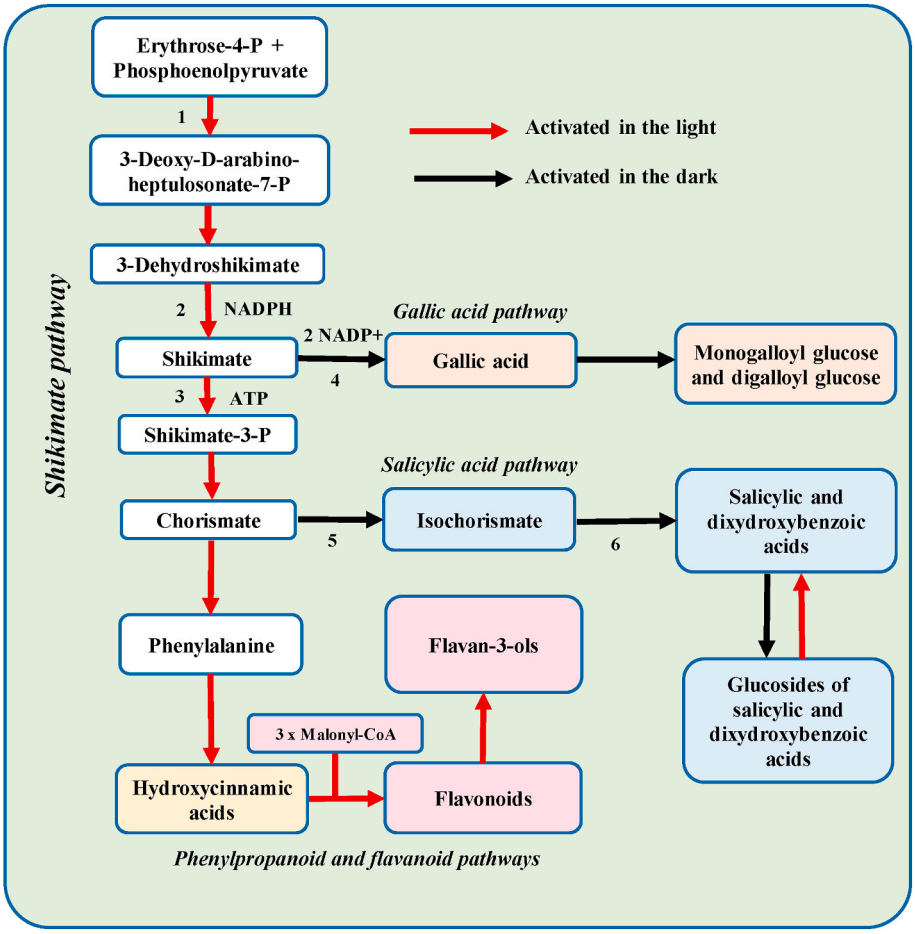

Fig. 4. Scheme of the regulating effect of light and dark on the total activity of the shikimate pathway and the distribution of carbon flux between the branches related with the biosynthesis of flavan-3-ols and derivatives of hydroxybenzoic acids in the *C. sinensis* callus culture. Abbreviations: ATP, adenosine triphosphate; NADPH and NADP+, reduced and oxidized forms of nicotinamide adenine dinucleotide phosphate; P, phosphate. Enzymes: 1, 3-Deoxy-D-arabinoheptulosonate-7-phosphate synthase; 2, Shikimate dehydrogenase; 3, Shikimate-kinase; 4, Isoenzyme of shikimate dehydrogenase, which catalyzes two consecutive reactions: conversion of shikimate to 3-dehydroshikimate and then 3-dehydroshikimate to gallic acid; 5, Isochorismate synthase; 6, Isochorismate pyruvate lyase.

was found in many plants and correlated with the activity of phenylalanine ammonia-lyase, 4-coumaroyl-CoA ligase, flavonol synthase, and chalcone-flavanone isomerase, which play an important role in the biosynthesis of phenylpropanoids and flavonoids (Chen et al., 2006). Similar regulatory effect of salicylic acid on the phenolic compounds was found also in the plant tissue cultures (Dias et al., 2016; Nechaeva et al., 2020).

Free forms of salicylic acid and 2,5- and 2,3-dihydroxybenzoic acids are present in plants at low concentrations due to their rapid metabolic modifications: hydroxylation, glycosylation, methylation, amino acid conjugation and protein binding (Wang et al., 2012 a; Widhalm and Dudareva, 2015; Zhang et al., 2017; Huang et al., 2018). For this reason, these phenolic compounds were found in the tea callus in the form of glucosides, which are considered as inactive and reserve forms localized in the vacuolar pool (Bartsch et al., 2010).

In the tea callus growing in the dark, the expression of the protein that binds salicylic acid was approximately 3-fold lower than in the light (Wang et al., 2012 a). This means that in the dark, the signaling function of salicylic acid is suppressed. It has also been shown that the shading of tea plant was stimulated the salicylic acid biosynthesis (Li et al., 2020). Thus, the reason for the higher content of hexosides of salicylic, 2,5- and 2,3-dihydroxybenzoic acids in the tea callus grown in the dark may be the more intensive formation of their free forms. However, due to the low efficiency of use, these free forms are converted into inactive reserve forms of glucosides that accumulated in the vacuole.

5. Conclusions

The main phenolic compounds of the *C. sinensis* callus culture are mono-, oligo- and polymers of flavan-3-ol. Their synthesis and accumulation is a constitutive process that takes place in the dark, but is stimulated by light about 2 fold. In addition, there were found derivatives of hydroxybenzoic acids: galloyl glucose and digalloyl glucose, hexosides of salicylic acid, and 2,5- and 2,3-dihydroxybenzoic acids. In contrast to flavan-3-ols, content of these phenolic compounds in the dark grown callus was higher than that in the light. The observed positive effect of light on the content of flavan-3-ols and negative effect on the derivatives of hydroxybenzoic acids may be associated with a change the total activity of the shikimate pathway and the regulation of the carbon flux distribution between the branches of the shikimate pathway related with the biosynthesis of flavan-3-ols and hydroxybenzoic acids.

Author contributions

Vladimir Ossipov: Conceptualization; Methodology; Investigation; Data curation; Formal analysis; Writing - Original draft; Visualization. **Maria Zubova**: Resources; Investigation; Methodology. **Tatiana Nechaeva**: Resources; Investigation; Methodology. **Natalia Zagoskina**: Resources; Project administration. **Juha-Pekka Salminen**: Supervision; Review and Editing.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bse.2022.104383.

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