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Light-Responsive Quantitative Changes in Major Flavonoids in Spinach (*Spinacia oleracea* L.) Leaves

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Highlights

What are the main findings?

- White LED increased both the shoot and root biomass of spinach.
- Nine major flavonoids were identified in spinach leaves and mostly tended to decrease for 4-week artificial lights.
- Strong blue light was better to maintain P3g, S3a, TMMG, DDMG, and DMMG content.

What is the implication of the main finding?

- The flavonoids exhibited four distinct accumulation patterns depending on light wavelength, intensity, and exposure time.
- S3g dramatically decreased after all artificial light application.

Abstract

Artificial lighting offers a practical method for regulating plant growth and nutritional content. This study examined how LED light quality, intensity, and exposure duration affect *S. oleracea* growth and individual flavonoid accumulation. After 4 weeks of juvenile growth, *S. oleracea* plants were cultivated for another 4 weeks under red, blue, red + blue (5:5), or white LEDs at intensities of 50, 100, and 200 μmol m^{-2} s^{-1}. Growth responses differed among lighting conditions: At week 4, white light at 200 μmol m^{-2} s^{-1} produced the greatest observed shoot dry weight (4.33 g) and root dry weight (0.80 g). The nine identified flavonoids exhibited distinct accumulation profiles across light treatments and sampling times. Five compounds generally accumulated during the first week and then declined, with lower irradiance favoring their accumulation. Axillarin-4′-glucuronide (A4g) and jaceidin 4′-*O*-glucuronide (J4g) levels declined over time, although this decrease was less pronounced under high-intensity blue light. Under 200 μmol m^{-2} s^{-1} blue light at week 4, A4g and J4g contents were 2.38 and 1.03 (100 mg kg^{-1} d.w.), respectively, significantly exceeding those observed under the other light treatments. Conversely, 5,4′-dihydroxy-3-methoxy-6,7-methylenedioxyflavone 4′-glucuronide content increased with light intensity, whereas spinacetin 3-*O*-gentiobioside levels declined after the first week. These findings show that light quality, intensity, and exposure duration selectively influence individual flavonoids rather than uniformly enhancing all nine identified compounds. Thus, tailoring

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LED regimes to specific target compounds may help improve the nutritional quality of *S. oleracea* grown in controlled environments.

Keywords: vegetable; LED light; growth regulation; metabilite regulation

1. Introduction

Plant factories enable year-round production of vegetables with consistent quality. *S. oleracea* is an important leafy vegetable well suited to plant-factory production, where precise control of environmental parameters can improve crop productivity and nutritional quality [1].

S. oleracea leaves are valued for their high nutritional and functional value. Along with well-known carotenoids, vitamins, and minerals, *S. oleracea* contains a range of flavonoids [2,3], which are important components of plant antioxidant systems and contribute to leafy vegetables' functional properties. The flavonoid profile of *S. oleracea* is relatively complex, comprising glycosylated and glucuronidated derivatives with distinct structural characteristics [4]. These structural modifications can influence flavonoid solubility, stability, and metabolism, potentially contributing to compound-specific responses to environmental conditions [5]. Understanding how these individual compounds respond to environmental conditions is therefore important for developing cultivation strategies aimed at improving *S. oleracea* quality.

Light is a major environmental factor affecting plant growth and secondary metabolism [6–8]. Its effects on flavonoid accumulation depend on spectral composition, irradiance, plant species, and the metabolite examined. UV-B irradiation and blue light have been associated with increased phenolic compound and flavonoid accumulation [9–11], although response levels vary among plant systems. Flavonoid accumulation also changes during plant development and may depend on the duration of light exposure [12–14]. Thus, measurements based solely on total flavonoid content or a single sampling time may not adequately capture changes in individual flavonoids.

Identifying how individual flavonoids respond to various combinations of light quality, intensity, and duration could clarify how light management affects *S. oleracea*'s functional quality. It could also help distinguish light conditions that favor biomass production from those that promote specific health-related metabolite accumulation. However, the effects of these three factors on the temporal accumulation patterns of individual flavonoids in *S. oleracea* remain insufficiently characterized.

In the present study, *S. oleracea* plants were grown under four LED light qualities, namely red, blue, red + blue (5:5), and white light, at three light intensities: 50, 100, and 200 μmol m^{-2} s^{-1}. Plants were sampled after 0, 1, 2, and 4 weeks of treatment. The study aimed to determine how these lighting conditions affected *S. oleracea* growth and individual flavonoids' temporal accumulation profiles.

2. Materials and Methods

2.1. Plant Materials

Seeds of *S. oleracea* cv. 'Fresco' were soaked in water for 24 h and then sown in plastic pots filled with commercial horticultural soil (Baroker, Seoulbio Co., Eumseong, Republic of Korea). Seedlings were grown for around 4 weeks in a greenhouse at Kyung Hee University (Yongin, Republic of Korea), until they had developed at least four true leaves.

2.2. Light Treatment

At 32 days after sowing, seedlings of uniform size and developmental stage were transferred to an LED-based growth chamber. They were grown under red, blue, red + blue (5:5), or white LEDs. The spectral distributions of these LEDs were measured using a UV–Vis spectrometer (SV2100; K-MAC, Daejeon, Republic of Korea; Figure S1). Photosynthetic photon flux density (PPFD) was adjusted to 50, 100, and 200 μmol m^{-2} s^{-1} by varying the distance between the LEDs and plants, and was measured using a photoradiometer (HD 2302.0; Delta OHM SRL, Marconi, Italy). A 12/12-h light/dark photoperiod was used, with cultivation temperature maintained at 20 ± 1 °C. Plants were grown under each light condition for 4 weeks. During cultivation, they received a 1000-fold diluted Hyponex nutrient solution (N-P_2O_2-K_2O = 6-10-5; Hyponex Japan Corp., Ltd., Osaka, Japan). The calculated final concentrations of N, P, K, Ca, Mg, Mn, B, and Mo in the diluted nutrient solution were 72.60, 52.81, 50.22, 0.173, 0.146, 0.010, 0.015, and 0.002 mg L^{-1}, respectively.

2.3. Sample Collection

Aboveground tissues were harvested at 0, 1, 2, and 4 weeks after LED treatment. At each time point, four plants were randomly selected from each treatment as independent biological replicates. Samples were immediately frozen in liquid N_2 and stored at −80 °C until further analysis.

2.4. Growth Rate Measurements

Leaf length, leaf width, petiole length, shoot dry weight, and root dry weight were measured to assess *S. oleracea* growth responses to different light treatments. At each sampling time, leaf length, leaf width, and petiole length were measured on the most developed leaf of each individual *S. oleracea* plant. Shoots and roots were washed with water, freeze-dried to constant dryness, and weighed.

2.5. Extraction

Flavonoids were extracted using a previously reported procedure with modifications [14]. Freeze-dried samples were ground into a fine powder, and 100 mg was extracted in 2 mL of 80% (*v*/*v*) ethanol. The mixture was sonicated for 1 h at <40 °C, centrifuged, and the supernatant collected. This was passed through a 0.45-μm membrane filter before LC-MS/MS analysis.

2.6. Flavonoid Identifications

Flavonoids were identified using a Thermo Vanquish UHPLC system (Thermo Fisher Scientific, Germering, Germany) coupled to a Thermo Q Exactive mass spectrometer with a heated electrospray ionization source. Chromatographic separation was performed on a Waters Cortecs T3 column (2.1 × 150 mm, 1.6 μm, Waters Corporation, Milford, MA, USA) maintained at 45 °C. The mobile phases were (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid, with a flow rate of 0.25 mL/min. The gradient elution program was as follows: 0–0.1 min, 3% B; 0.1–15 min, 3–15% B; 15–50 min, 15–100% B; 50–55 min, 100% B; 55–55.1 min, 100–3% B; and 55.1–60 min, 3% B.

Mass spectrometric data were acquired in positive and negative ionization modes, with spray voltages of 3500 and 3000 V, respectively. Sheath, auxiliary, and sweep gas settings were 50, 10, and 1 arbitrary units, respectively, and the ion transfer tube was maintained at 320 °C. MS1 and MS2 resolutions were set to 35,000 and 17,500, respectively, over a scan range of m/z 100–1500. Data-dependent MS/MS acquisition was performed using a TopN setting of 10 and stepped normalized collision energies of 10, 30, and 50.

2.7. Flavonoid Quantifications

Flavonoids were quantified using a Waters 2695 Alliance HPLC system equipped with a Waters 996 photodiode array detector (Waters Corporation, Milford, MA, USA). The analysis followed a previously reported HPLC method, with modifications [4]. Separation was performed on a PRONTOSIL 120-5-C18-SH column (BISCHOFF Chromatography, Leonberg, Germany) maintained at 27 °C. The mobile phases were (A) water containing 0.1% formic acid and (B) acetonitrile. The gradient elution program was as follows: 8% B at 0 min, increased to 23% B at 5 min and 35% B at 34 min, followed by an increase to 75% B at 36 min. The proportion of B was held at 75% until 40 min, decreased to 8% at 42 min, and remained at 8% until 44 min to re-equilibrate the column. The flow rate was 1.0 mL/min, the injection volume was 10 µL, and the detection wavelength was set at 350 nm. Flavonoid analysis was conducted in triplicate. For quantification, a calibration curve constructed using authentic spinacetin (ChemFaces, Wuhan, China) as an external reference standard was used to determine the concentrations of the identified flavonoids. The limits of detection (LOD) and quantification (LOQ) for spinacetin were estimated from signal-to-noise (S/N) ratios calculated using the United States Pharmacopeia (USP) method. The measured S/N ratios of spinacetin calibration standards were used to estimate the concentrations corresponding to S/N ratios of 3 and 10 for the LOD and LOQ, respectively. The calibration parameters and estimated LOD and LOQ values are presented in Supplementary Table S1. To evaluate extraction completeness, leaf samples were subjected to three successive extractions under the same conditions. The first-extraction efficiency (%) of each identified flavonoid was calculated as the amount recovered in the first extraction relative to the cumulative amount recovered across all three extractions. The results are presented in Supplementary Table S2.

2.8. Data Analysis

Data were analyzed using SAS software (Enterprise Guide 9.4 version, SAS Institute Inc., Cary, NC, USA). Analysis of variance was used to assess differences in growth characteristics and flavonoid levels among treatments. Mean comparisons were conducted using Fisher's least significant difference at $p < 0.05$. Results are presented as means ± standard errors. Principal component analysis (PCA) was performed separately for each treatment time using Z-score-standardized growth parameters and flavonoid levels. PCA biplots were used to visualize treatment distributions and relationships among variables at each treatment time.

3. Results and Discussion

3.1. Growth and Morphological Responses to Light Quality and Intensity

S. oleracea plants showed distinct growth responses to LED treatments over the 4-week cultivation period (Figure 1 and Table 1). After 4 weeks, the longest leaf length was recorded under white light at 50 µmol m^{-2} s^{-1} (4.38 cm), whereas the greatest leaf width occurred under white light at 200 µmol m^{-2} s^{-1} (2.88 cm). Blue light at 100 µmol m^{-2} s^{-1} promoted petiole elongation, which reached 5.33 cm at 4 weeks. Shoot dry weight was highest under white light at 200 µmol m^{-2} s^{-1} (4.33 g), followed numerically by blue light at 100 µmol m^{-2} s^{-1} (4.00 g) and red + blue light at 200 µmol m^{-2} s^{-1} (3.31 g), with no significant differences observed. Root dry weight was also highest under white light at 200 µmol m^{-2} s^{-1} (0.80 g).

Leaf elongation responses to increasing light intensity varied with spectral composition. After 4 weeks, leaf length under monochromatic blue light was 60.8% shorter at 200 µmol m^{-2} s^{-1} (1.60 cm) than at 100 µmol m^{-2} s^{-1} (4.08 cm), a reduction not observed under red + blue and white LEDs. These responses may reflect differences in photosyn-

thetic acclimation under monochromatic versus combined-light conditions. Prior research reported that combining red and blue light improved *S. oleracea* growth and photosynthetic performance compared with monochromatic light [15]. In particular, combined light maintained stable photosystem II quantum yield and carbon and nitrogen assimilation, whereas monochromatic blue light significantly increased photooxidative stress indicators, including superoxide and malondialdehyde levels. Collectively, these findings indicate that spectral composition may induce distinct developmental responses even at the same light intensity. Meanwhile, the greatest observed leaf length under red light at 50 μmol m^{-2} s^{-1} and blue light at 50 and 200 μmol m^{-2} s^{-1} was lower than the initial value. Because the largest developed leaf was measured from independent plants at each time point, these changes do not indicate leaf shrinkage. Restricted development of newly formed leaves, senescence, or loss of previously developed leaves may have contributed to the observed patterns.

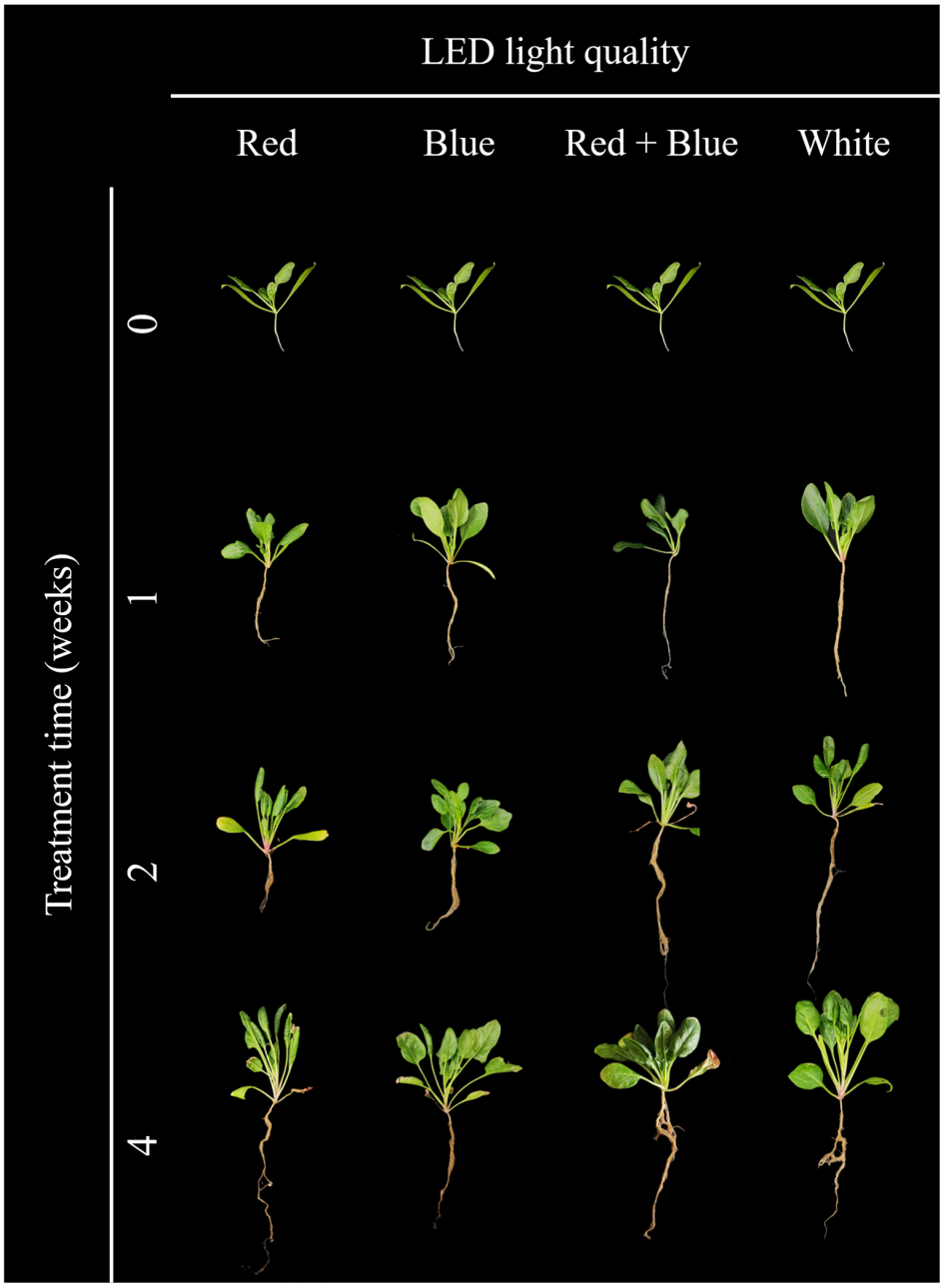


Figure 1. Typical morphology of *S. oleracea* grown under various LED light qualities at 100 μmol m^{-2} s^{-1}.

Table 1. Growth characteristics of *S. oleracea* grown under various LED light conditions.

	Treatment Time (Weeks)	Red			Blue			Red + Blue			White			
		50	100	200	50	100	200	50	100	200	50	100	200	*LSD*
Leaf length (cm)	0	3.14 ^{A}	3.14 ^{A}	3.14 ^{A}	3.14 ^{A}	3.14 ^{B}	3.14 ^{A}	3.14 ^{A}	3.14 ^{A}	3.14 ^{A}	3.14 ^{C}	3.14 ^{B}	3.14 ^{B}	
	1	3.26 ^{abA}	3.16 ^{abA}	3.11 ^{bA}	3.44 ^{abA}	3.78 ^{aAB}	3.34 ^{abA}	2.93 ^{bA}	3.08 ^{bA}	3.46 ^{abA}	3.36 ^{abC}	3.15 ^{abB}	3.55 ^{abAB}	0.64
	2	2.98 ^{dA}	3.35 ^{bcdA}	3.21 ^{cdA}	3.33 ^{bcdA}	4.18 ^{aA}	3.09 ^{dA}	2.94 ^{dA}	3.16 ^{dA}	3.51 ^{bcdA}	3.89 ^{abB}	3.36 ^{bcdB}	3.83 ^{abcA}	0.63
	4	1.34 ^{fA}	2.50 ^{cdefA}	3.05 ^{defA}	1.69 ^{cdefA}	4.08 ^{abcA}	1.60 ^{defA}	2.93 ^{efA}	3.33 ^{cdefA}	3.48 ^{bcdeA}	4.38 ^{aA}	3.81 ^{abcdA}	4.16 ^{abA}	0.76
	LSD	0.76	0.32	0.86	0.92	0.85	0.56	0.67	0.63	0.57	0.36	0.41	0.67	
Leaf width (cm)	0	1.95 ^{A}	1.95 ^{AB}	1.95 ^{A}	1.95 ^{A}	1.95 ^{B}	1.95 ^{A}	1.95 ^{A}	1.95 ^{AB}	1.95 ^{A}	1.95 ^{B}	1.95 ^{B}	1.95 ^{B}	
	1	1.70 ^{dAB}	1.86 ^{bcdAB}	1.83 ^{bcdA}	1.75 ^{cdA}	2.41 ^{aAB}	1.94 ^{bcdA}	1.63 ^{dB}	1.74 ^{cdC}	2.08 ^{abcA}	1.91 ^{bcdB}	1.79 ^{bcdB}	2.15 ^{abB}	0.36
	2	1.65 ^{cdAB}	1.65 ^{cdB}	1.84 ^{bcdA}	1.78 ^{cdA}	2.94 ^{aA}	1.85 ^{bcdA}	1.53 ^{dB}	1.95 ^{bcdBC}	2.29 ^{bA}	2.03 ^{bcB}	1.96 ^{bcdB}	2.30 ^{bAB}	0.50
	4	1.40 ^{fB}	2.17 ^{deA}	1.84 ^{efA}	2.05 ^{deA}	2.79 ^{abA}	2.25 ^{cdeA}	1.55 ^{fB}	2.18 ^{cdeA}	2.24 ^{cdeA}	2.36 ^{bcdA}	2.59 ^{abcA}	2.88 ^{aA}	0.48
	LSD	0.36	0.35	0.46	0.68	0.54	0.45	0.35	0.22	0.41	0.19	0.30	0.58	
Petiole length (cm)	0	2.19 ^{B}	2.19 ^{B}	2.19 ^{A}	2.19 ^{B}	2.19 ^{C}	2.19 ^{B}	2.19 ^{A}	2.19 ^{A}	2.19 ^{A}	2.19 ^{C}	2.19 ^{C}	2.19 ^{B}	
	1	2.63 ^{bcdB}	2.63 ^{bcdB}	2.23 ^{cdeA}	3.13 ^{bAB}	3.81 ^{aB}	2.76 ^{bcB}	2.24 ^{cdeA}	2.09 ^{deA}	1.75 ^{eB}	3.09 ^{bB}	3.08 ^{bB}	2.60 ^{bcdAB}	0.55
	2	3.38 ^{bcdA}	2.79 ^{defAB}	2.44 ^{efA}	3.71 ^{bcA}	5.29 ^{aA}	3.10 ^{cdeA}	2.55 ^{efA}	2.19 ^{fA}	2.24 ^{fA}	3.69 ^{bcA}	3.91 ^{bA}	2.84 ^{defA}	0.71
	4	3.65 ^{cdeA}	3.52 ^{bcdA}	2.53 ^{fgA}	3.98 ^{bcA}	5.33 ^{aA}	3.20 ^{cdefA}	2.76 ^{efgA}	2.49 ^{fgA}	2.29 ^{gA}	3.94 ^{bcdA}	4.60 ^{abA}	3.05 ^{defgA}	0.90
	LSD	0.61	1.02	0.46	1.00	0.88	0.48	0.76	0.40	0.43	0.38	0.83	0.64	
Shoot dry weight (mg)	0	0.02 ^{C}	0.02 ^{C}	0.02 ^{B}	0.02 ^{C}	0.02 ^{D}	0.02 ^{B}	0.02 ^{B}	0.02 ^{B}	0.02 ^{D}	0.02 ^{D}	0.02 ^{D}	0.02 ^{C}	
	1	0.54 ^{dAB}	0.67 ^{cdBC}	0.70 ^{bcdB}	0.68 ^{bcdB}	1.29 ^{abcC}	0.83 ^{bcdAB}	1.19 ^{bcdA}	0.89 ^{abcdA}	1.22 ^{aC}	0.89 ^{bcdC}	0.89 ^{bcdC}	1.26 ^{abB}	0.45
	2	0.91 ^{deA}	1.07 ^{cdeAB}	0.70 ^{eB}	1.28 ^{bcdeA}	2.31 ^{aB}	1.17 ^{bcdeA}	1.09 ^{bcdeA}	1.05 ^{A}	1.78 ^{abB}	1.46 ^{bcdB}	1.63 ^{abcB}	2.32 ^{aB}	0.69
	4	0.39 ^{gBC}	1.64 ^{cdefA}	2.01 ^{cdeA}	1.10 ^{efgAB}	4.00 ^{aA}	1.37 ^{defgA}	0.68 ^{fgA}	1.33 ^{defgA}	3.31 ^{abA}	2.43 ^{bcdA}	2.70 ^{bcA}	4.33 ^{aA}	1.24
	LSD	0.41	0.80	1.14	0.59	0.74	1.02	0.57	0.62	0.91	0.52	0.59	1.17	
Root dry weight (mg)	0	0.00 ^{B}	0.00 ^{C}	0.00 ^{B}	0.00 ^{C}	0.00 ^{C}	0.00 ^{B}	0.00 ^{B}	0.00 ^{B}	0.00 ^{B}	0.00 ^{B}	0.00 ^{C}	0.00 ^{B}	
	1	0.06 ^{dAB}	0.08 ^{cdBC}	0.13 ^{bcdB}	0.10 ^{bcdB}	0.17 ^{abcB}	0.12 ^{bcdAB}	0.11 ^{bcdA}	0.15 ^{abcdA}	0.25 ^{aB}	0.12 ^{bcdA}	0.14 ^{bcdB}	0.18 ^{abB}	0.10
	2	0.14 ^{cdA}	0.10 ^{dAB}	0.17 ^{cdB}	0.15 ^{cdA}	0.36 ^{bcA}	0.32 ^{bcdA}	0.12 ^{cdA}	0.19 ^{cdA}	0.51 ^{abA}	0.19 ^{cdA}	0.52 ^{abA}	0.61 ^{aA}	0.25
	4	0.04 ^{fB}	0.18 ^{defA}	0.45 ^{bcA}	0.06 ^{efB}	0.27 ^{cdAB}	0.26 ^{cdeA}	0.05 ^{fB}	0.18 ^{defA}	0.55 ^{bA}	0.20 ^{defA}	0.22 ^{defB}	0.80 ^{aA}	0.20
	LSD	0.08	0.09	0.21	0.05	0.14	0.22	0.08	0.14	0.25	0.10	0.09	0.38	

Different lowercase letters indicate significant differences among light treatments, whereas different uppercase letters indicate significant differences among treatment times within the same light-treatment group ($p < 0.05$).

3.2. Flavonoid Identification in *S. oleracea* Leaves

By combining chromatographic and mass spectrometric data, including UV absorption, molecular masses, and MS/MS fragmentation patterns, nine flavonoids were identified in *S. oleracea* leaves (Table 2). Negative-ion mode yielded $[M-H]^-$ ions from m/z 503.08 to 801.21. Patuletin derivatives exhibited characteristic fragment ions at m/z 330.04 and 315.02, whereas spinacetin derivatives produced ions at m/z 344–345 and 329, aligned with previous reports on *S. oleracea* flavonoids [4,16,17]. These patterns distinguished flavonoids with different glycosyl or glucuronide substituents.

Table 2. Flavonoids identified in *S. oleracea* leaves via LC-MS/MS and HPLC.

No.	Compound	Abbr.	λmax (nm)	MW	MH (Frag. MS^2 m/z)
1	Patuletin 3-(2″-apiosylgentiobioside)	P3a	333.8, 244.7, 216.4	788.20	787.19 (330.04, 315.02)
2	Patuletin-3-*O*-gentiobioside	P3g	354.1, 256.5, 211.7	656.16	655.15 (330.04, 315.02)
3	Spinacetin 3-(2″-apiosylgentiobioside)	S3a	338.5, 250.6	802.22	801.21 (344.05, 329.03)
4	Spinacetin 3-*O*-gentiobioside	S3g	338.5, 249.4, 211.7	670.17	669.17 (345.06, 329.03)
5	Axillarin-4′-glucuronide	A4g	337.3, 271.9	522.10	521.09 (330.04, 315.01)
6	Jaceidin 4′-*O*-glucuronide	J4g	335.0, 271.9, 251.8	536.12	535.11 (344.05, 329.03)
7	5,3′,4′-Trihydroxy-3-methoxy-6,7-methylenedioxyflavone 4′-glucuronide	TMMG	357.5, 277.8, 256.5	520.08	519.08 (345.05, 328.02)
8	5,4′-Dihydroxy-3-methoxy-6,7-methylenedioxyflavone 4′-glucuronide	DMMG	339.7, 277.8, 251.8	504.09	503.08 (327.05, 312.03)
9	5,4′-Dihydroxy-3,3′-dimethoxy-6,7-methylenedioxyflavone 4′-glucuronide	DDMG	331.4, 279.0	534.10	533.09 (342.04, 327.01)

MS data were acquired in negative-ion mode.

The glycosides identified were patuletin 3-(2″-apiosylgentiobioside) (P3a), patuletin-3-*O*-gentiobioside (P3g), spinacetin 3-(2″-apiosylgentiobioside) (S3a), and spinacetin 3-*O*-gentiobioside (S3g). The other five were axillarin-4′-glucuronide (A4g), jaceidin 4′-*O*-glucuronide (J4g), and three methylenedioxyflavone glucuronide derivatives [5,3′,4′-trihydroxy-3-methoxy-6,7-methylenedioxyflavone 4′-glucuronide (TMMG); 5,4′-dihydroxy-3-methoxy-6,7-methylenedioxyflavone 4′-glucuronide (DMMG); 5,4′-dihydroxy-3,3′-dimethoxy-6,7-methylenedioxyflavone 4′-glucuronide (DDMG)] (Table 2). These compounds illustrate *S. oleracea* flavonoid structural diversity and provide a basis for assessing how LED light quality, intensity, and exposure duration affect individual flavonoid accumulation.

3.3. Quantitative Profiles of Individual Flavonoids

The nine flavonoids exhibited distinct responses to LED light quality, intensity, and treatment duration (Figures 2–5). P3a, P3g, S3a, TMMG, and DDMG exhibited similar accumulation patterns across the tested light treatments: their levels rose rapidly during the first week, then declined gradually with prolonged exposure. Lower light intensities generally favored the accumulation of these flavonoids, although several compounds exhibited relatively higher accumulation under high-intensity blue light at specific sampling time points. P3a and P3g showed higher accumulation under blue light at 200 μmol m^{-2} s^{-1} during the first week of treatment, while S3a, TMMG, and DDMG exhibited higher contents under blue light at 200 μmol m^{-2} s^{-1} than at 50 μmol m^{-2} s^{-1} at subsequent sampling time points. Thus, irradiance responses depended on both spectral composition and the flavonoid

examined. Light can regulate the expression of genes involved in the phenylpropanoid and flavonoid pathways [9,10]. The initial increase may reflect flavonoid biosynthesis activation in *S. oleracea* due to changes in the light environment. During prolonged treatment, shifts in biosynthetic activity, metabolite turnover, and plant developmental status may have contributed to the subsequent decline in flavonoid content. These findings suggest flavonoid responses were influenced not only by light intensity but also by the spectral characteristics and duration of illumination.

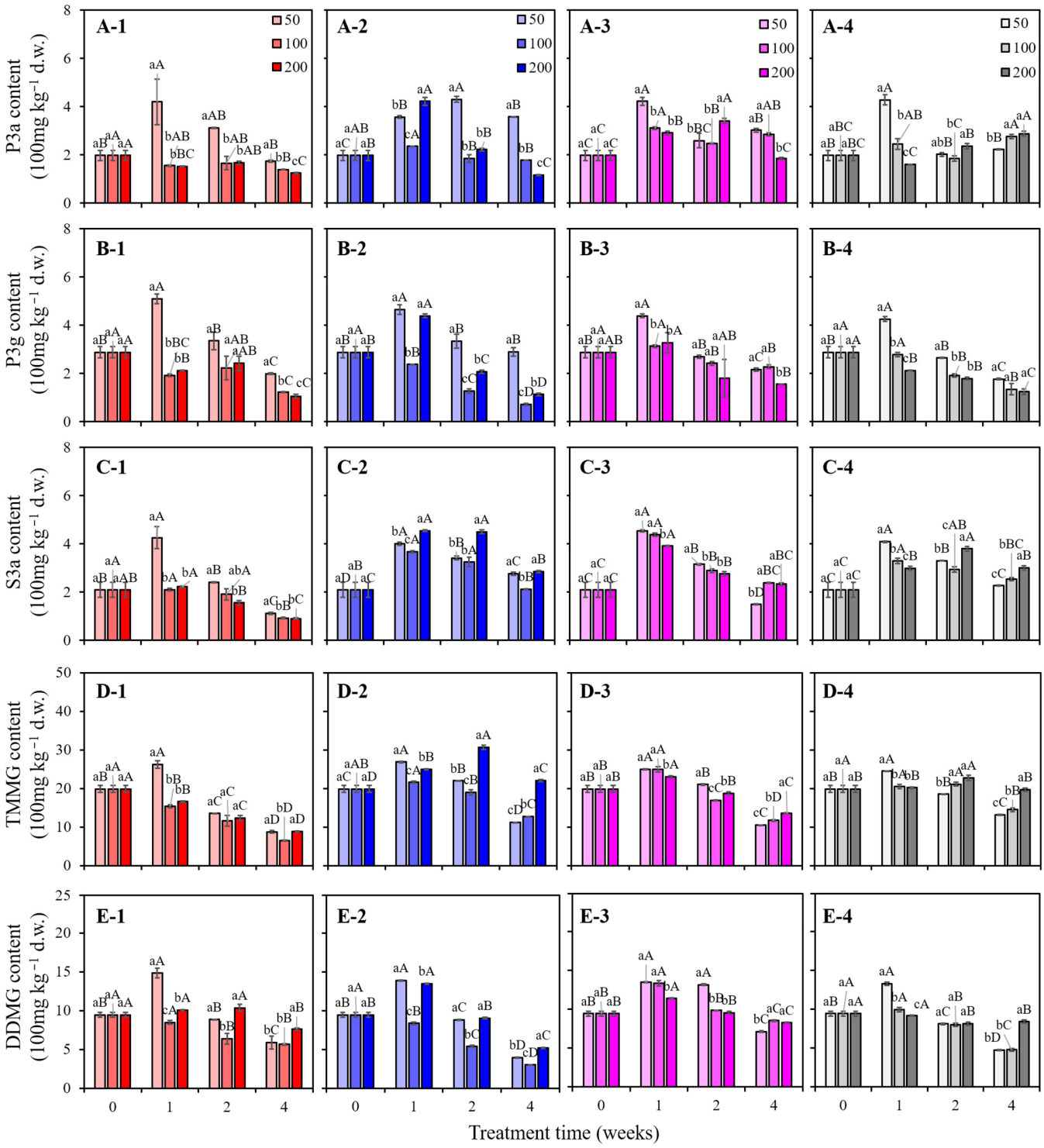


Figure 2. Effects of LED light quality, intensity, and treatment duration on five flavonoids in *S. oleracea* leaves. (**A**) Patuletin 3-(2″-apiosylgentiobioside) (P3a); (**B**) patuletin-3-O-

gentiobioside (P3g); (**C**) spinacetin 3-(2″-apiosylgentiobioside) (S3a); (**D**) 5,3′,4′-trihydroxy-3-methoxy-6,7-methylenedioxyflavone 4′-glucuronide (TMMG); and (**E**) 5,4′-dihydroxy-3,3′-dimethoxy-6,7-methylenedioxyflavone 4′-glucuronide (DDMG). Numbers adjacent to panel labels indicate LED light quality: (**1**) red, (**2**) blue, (**3**) red + blue (5:5), and (**4**) white. The values 50, 100, and 200 denote light intensity (μmol m^{-2} s^{-1}). Different lowercase letters indicate significant differences among light intensities within the same light quality; uppercase letters indicate significant differences among treatment times at the same intensity ($p < 0.05$).

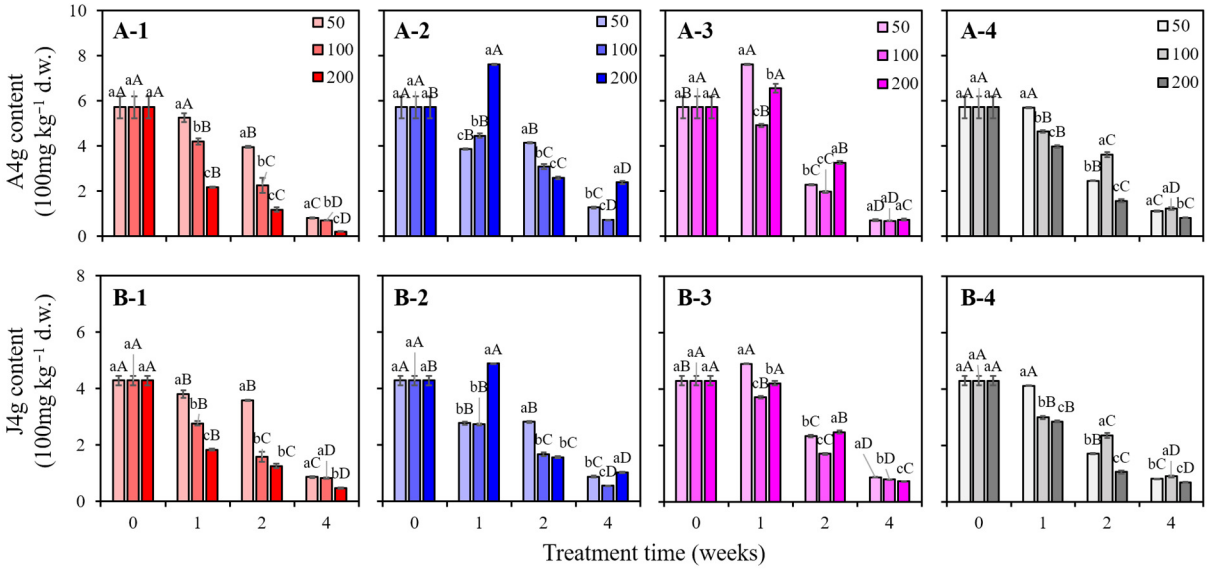


Figure 3. Effects of LED light quality, intensity, and treatment duration on two flavonoids in *S. oleracea* leaves. (**A**) Axillarin-4′-glucuronide (A4g); and (**B**) jaceidin 4′-O-glucuronide (J4g). Numbers adjacent to panel labels indicate LED light quality: (**1**) red, (**2**) blue, (**3**) red + blue (5:5), and (**4**) white. The values 50, 100, and 200 denote light intensity (μmol m^{-2} s^{-1}). Different lowercase letters indicate significant differences among intensities within the same light quality; uppercase letters indicate significant differences among treatment times with the same intensity ($p < 0.05$).

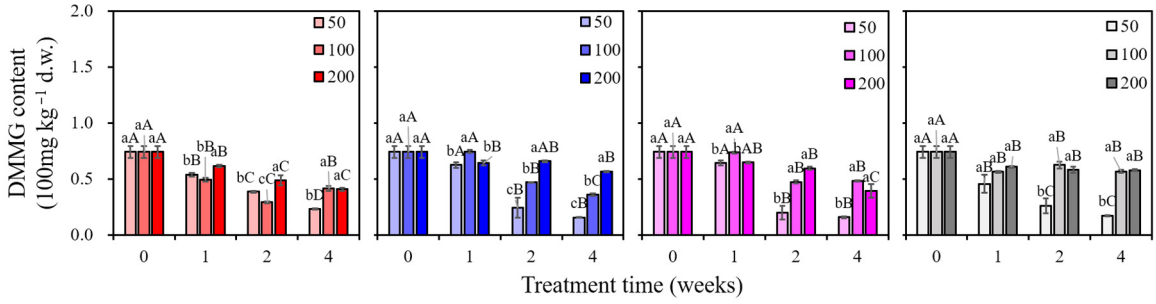


Figure 4. Effects of LED light quality, intensity, and duration on *S. oleracea* leaf 5,4′-dihydroxy-3-methoxy-6,7-methylenedioxyflavone 4′-glucuronide (DMMG) content. Panels from left to right represent red, blue, red + blue (5:5), and white LED light. The values 50, 100, and 200 denote light intensity (μmol m^{-2} s^{-1}). Different lowercase letters indicate significant differences among light intensities within the same light quality; uppercase letters indicate significant differences among treatment times at the same intensity ($p < 0.05$).

A4g and J4g exhibited a different temporal pattern from the five compounds described above (Figure 3). Their contents generally decreased as treatment continued, although the magnitude of the decline varied among lighting conditions. Under high-intensity blue light, the decrease appeared less pronounced, suggesting that this treatment may help maintain the levels of these two glucuronides over time. This response differed from the pattern

observed from Figure 2, in which accumulation was generally highest during the early stage of treatment.

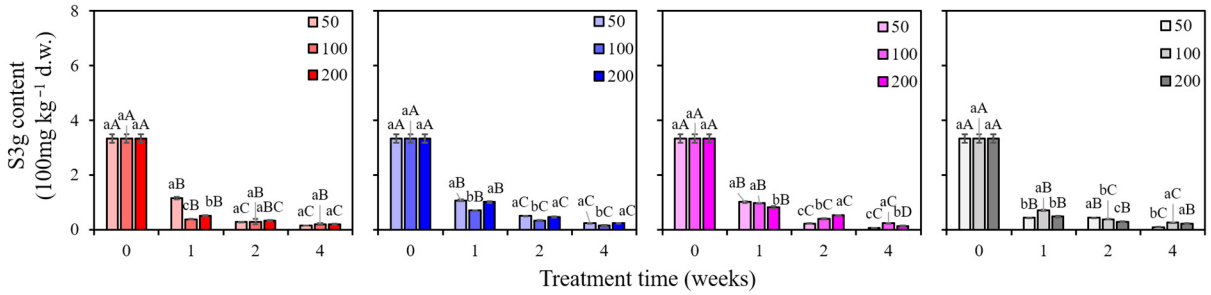


Figure 5. Effects of LED quality, intensity, and duration on the flavonoid spinacetin 3-O-gentiobioside (S3g) in *S. oleracea* leaves. Panels from left to right represent red, blue, red + blue (5:5), and white LED light. The values 50, 100, and 200 denote light intensity (μmol m^{-2} s^{-1}). Different lowercase letters indicate significant differences among light intensities within the same light quality; uppercase letters indicate significant differences among treatment times at the same intensity ($p < 0.05$).

Figure 4 shows a different response pattern for DMMG compared with the above flavonoids, which generally exhibited higher levels under high-intensity light than under lower-intensity treatments. This trend contrasted with the responses of several flavonoids in Figure 2, suggesting compound-specific effects of light intensity. In Figure 5, S3g decreased markedly after the first week under most lighting conditions, indicating that its initial content was not maintained during prolonged exposure. Overall, the results demonstrate that flavonoid accumulation in *S. oleracea* depended on the interaction of light quality, intensity, and treatment duration.

The nine flavonoids showed distinct time- and irradiance-dependent responses (Table 3). P3a, P3g, S3a, TMMG, and DDMG levels generally increased during the first week before declining, with lower irradiance often promoting their accumulation, whereas high-intensity blue light enhanced some responses. A4g and J4g generally decreased, although the decline was less pronounced under high-intensity blue light. DMMG content increased with increasing light intensity, whereas S3g declined markedly after the first week. Thus, light quality, intensity, and exposure duration collectively shape flavonoid accumulation.

Table 3. Characteristics of flavonoid groups in response to LED light treatments.

Group	Compounds	Characteristics
1	P3a; P3g; S3a; TMMG; DDMG	• Increased in response to low light intensity, regardless of wavelength • Increased rapidly in the first week, followed by gradual decrease • Increased in response to blue light at intensities of 200 μmol m^{-2} s^{-1}
2	A4g J4g	• Increased in response only to high-intensity blue light during the first week • Decreased overall during the treatment period, but the extent of the decrease was mitigated under high-intensity blue light
3	DMMG	• Increased in response to high light intensity, regardless of wavelength.
4	S3g	• Decreased significantly after the first week of treatment

Although the photoprotective roles of flavonoids under excessive light are well established [18], P3a, P3g, S3a, TMMG, and DDMG tended to accumulate most at the lowest light intensity in the present study. Previous research reported found that individual flavonoids respond individually differentially to changes in light intensity [19]. Consistent

with this, DMMG accumulation increased as light intensity rose, unlike the abovementioned flavonoids. Such contrasting accumulation patterns may reflect redistribution of phenylpropanoid-derived compounds. However, further studies on metabolic flux and flavonoid degradation are needed to clarify the underlying mechanisms.

The different responses of nine flavonoids suggest that light intensity and exposure time regulate their accumulation in distinct ways. High-intensity light may trigger compound-specific changes in flavonoid biosynthesis, as light-responsive transcriptional networks differentially regulate flavonoid pathway genes, leading to different accumulation patterns [20,21]. Light signaling and transcriptional regulators control flavonoid biosynthesis [22]. Plants perceive red/far-red and blue light through photoreceptors, including phytochromes and cryptochromes, respectively. These signals regulate photomorphogenesis and flavonoid biosynthetic gene expression. Photoreceptor-mediated light perception suppresses COP1–SPA complex activity, promoting HY5 stabilization and accumulation. Stabilized HY5 can regulate flavonoid biosynthesis by directly controlling structural gene expression or by interacting with regulatory networks involving MYB transcription factors. Previous studies have reported that blue light regulates flavonoid accumulation through HY5-mediated signaling pathways [14,23]. In the present study, P3a, P3g, S3a, TMMG, and DDMG accumulated at relatively high levels under certain high-intensity blue light conditions. In addition, decreases in A4g and J4g levels over the treatment period were less pronounced under high-intensity blue light. These findings indicate that blue light–mediated signaling may also contribute to flavonoid accumulation in *S. oleracea*.

S. oleracea flavonoids occur mainly as glucuronide derivatives, although other glycosylated forms are also present [16]. In plants, UDP-dependent glycosyltransferases, including glucosyltransferases and glucuronosyltransferases, catalyze flavonoid glycosylation, transferring glucose or glucuronic acid residues, respectively [24]. Although flavonoid glycosyltransferase activity has been demonstrated in *S. oleracea* [25], the specific enzyme responsible for the biosynthesis of its diverse flavonoid glycosides and glucuronides remains largely uncharacterized. In the current study, A4g, J4g, TMMG, DMMG, and DDMG were classified as flavonoid glucuronides, whereas P3a, P3g, S3a, and S3g were classified as flavonoid glycosides. Thus, structurally similar flavonoids showed distinct accumulation patterns across light conditions, suggesting that flavonoid metabolism is not governed solely by uniform activation of shared pathways. The contrasting accumulation patterns of structurally related flavonoids may reflect differential regulation of late biosynthetic steps, including glycosylation and glucuronidation, as well as variation in compound stability, metabolic conversion, and degradation. Further molecular studies, including transcriptional analyses, are needed to clarify the underlying regulatory mechanisms.

3.4. PCA Analysis

PCA revealed temporal shifts in the relationships between *S. oleracea* growth characteristics and individual flavonoid accumulation under different LED treatments (Figure 6). The principal components (PC1 + PC2) explained 61.42%, 51.73%, and 51.42% of the total variance at weeks 1, 2, and 4, respectively. At week 1, growth-related and flavonoid loadings were generally oriented in opposite directions along PC1, indicating contrasting patterns between growth characteristics and flavonoid accumulation. High-intensity white and blue light treatments tended to be associated with growth-related variables, whereas several low-intensity treatments were positioned closer to flavonoid loadings. At week 2, growth-related loadings were concentrated along the positive PC1 axis, while most flavonoid loadings were oriented toward the positive PC2 axis, except for DMMG. DMMG showed a loading directed toward negative PC2, distinguishing it from the other flavonoids and reflecting its distinct light intensity-dependent accumulation pattern. By week 4, the loadings of individ-

ual flavonoids became more widely dispersed, accompanied by greater differentiation of treatment scores according to light quality and intensity. These findings suggest that the associations between *S. oleracea* growth and individual flavonoid accumulation vary with light quality, intensity, and exposure duration.

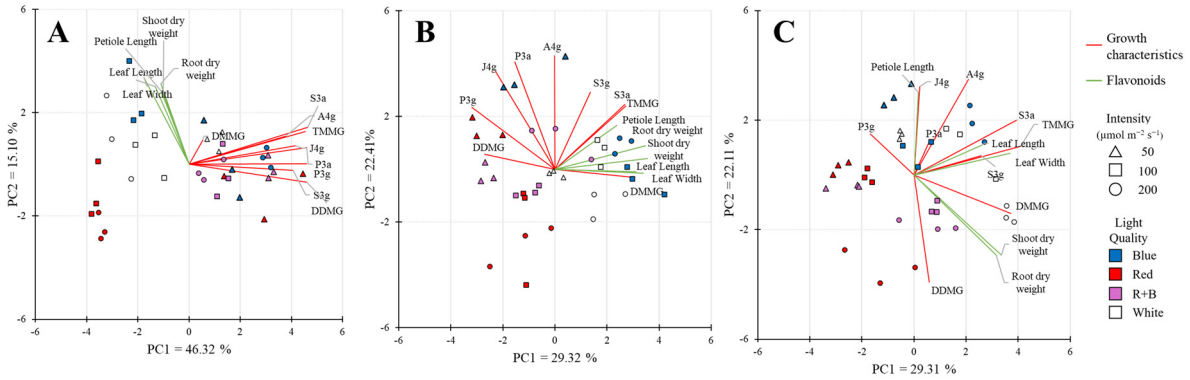


Figure 6. Principal component analysis (PCA) of growth and individual flavonoid contents in *S. oleracea* under various LED light treatments. (**A**–**C**) represent 1, 2, and 4 weeks of treatment, respectively.

4. Limitations

Prior studies have reported approximately twofold differences in total flavonoid content among *S. oleracea* genotypes [16]. However, the present study examined only one *S. oleracea* cv. 'Fresco', limiting the assessment of genotypic variation. Therefore, further studies are needed to determine whether genotype influences flavonoid accumulation in response to LED treatment. Additionally, the PPFD was limited to 50–200 μmol m^{-2} s^{-1}, which may not represent the full range used in plant-factory systems. Evaluating a broader PPFD range would help assess the generalizability of these findings. Finally, this study revealed distinct accumulation patterns of individual flavonoids in response to light quality, intensity, and treatment duration, but did not investigate the underlying molecular mechanisms. Integrating these findings with transcriptomic or targeted gene expression analyses may provide further insight into the molecular basis of these light-dependent responses.

5. Conclusions

LED light quality, intensity, and exposure duration had distinct effects on *S. oleracea* growth and flavonoid accumulation. White light at 200 μmol m^{-2} s^{-1} maximized shoot dry weight, and individual flavonoids exhibited different temporal and intensity-dependent responses. Overall lower light intensity favored the accumulation of several flavonoids, whereas high-intensity blue light enhanced specific compound levels and slowed the decline of others. This study provides compound-level evidence that *S. oleracea* flavonoid accumulation responds differently to light quality, intensity, and exposure duration, offering further insight into the distinct accumulation patterns of individual flavonoids under different LED conditions. These findings highlight the compound-specific nature of light responses and suggest that LED regimes should be tailored to the target flavonoids and exposure duration to improve *S. oleracea* quality. Further molecular studies are needed to clarify the regulatory mechanisms underlying these responses and establish a mechanistic basis to optimize LED strategies for targeted flavonoid accumulation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae12111421/s1, Figure S1: Spectral distribution of red (A), blue (B), red+blue(5:5) (C), and white (D) LEDs; Figure S2: Chromatogram of spinach extract

at 350 nm; Table S1: Analytical performance of HPLC-PDA quantification using spinacetin as an external standard. Table S2: First-extraction efficiency of nine spinach flavonoids.

Author Contributions: Conceptualization, S.H.E.; methodology, S.D. and C.K.K.; software, C.K.K.; validation, C.K.K. and C.J.; formal analysis, C.K.K., K.Y., J.B., J.C. and S.D.; investigation, C.K.K. and C.J.; resources, C.K.K.; data curation, C.K.K.; writing—original draft preparation, S.D.; writing—review and editing, S.H.E.; visualization, S.H.E.; supervision; S.H.E.; funding acquisition, S.H.E. All authors have read and agreed to the published version of the manuscript.

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