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Influence of Green Light Added with Red and Blue LEDs on the Growth, Leaf Microstructure and Quality of Spinach (*Spinacia oleracea* L.)

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Abstract: The aim of this study was to investigate the effects of green light, added with red and blue LEDs, on the growth, leaf microstructure and quality of spinach plants. Plants were transplanted and grown hydroponically for 30 days under different combinations of red:blue with a 4:1 ratio (R4B1), red:blue:green with a 5:2:3 ratio (R5B2G3) and red:blue:green with a 1:1:1 ratio (R1B1G1), at a 190 μmoL $m^{-2} \cdot s^{-1}$ photosynthetic photon flux density (PPFD). The results showed that green light, added to red and blue LEDs at a reasonable ratio, could reduce the growth, leaf microstructure and quality of spinach plants, but not the organic acid content. The highest values for the growth parameters, photosynthetic pigments, leaf structure characteristics and quality of the spinach plant were observed for the R4B1 treatment, but not for the organic acid content. Therefore, our results suggest that green light added to red and blue LEDs at a reasonable ratio is not suitable for the growth of spinach.

Keywords: growth; leaf microstructure; light quality; quality; spinach

1. Introduction

Light regulates a variety of plant development pathways, from germination to the induction of flowering and fruit development [1]. As an important part of the light spectrum for normal plant growth, red light affects plant morphogenesis by inducing transformations in phytochromes and is crucial in the development of the photosynthetic apparatus, as well as the regulation of the synthesis of phytochemicals such as phenolics and oxalate [2–4]. Blue light is effective in the stimulation of photomorphogenesis and adaptive phenomena such as the regulation of stoma opening/closing, as well as biomass accumulation and chlorophyll and anthocyanin biosynthesis [5–9].

However, it has been reported that monochromatic red or blue light cannot satisfy the requirements for normal plant growth. For example, plants under red light alone displayed an abnormal morphology and reduced net photosynthetic rates (Pn) compared to those under white light or red light supplemented with blue light [10–12]. Blue light alone, or a constant illumination with high amounts of blue light, might have negative effects, such as reduced Pn, in many species, due to chloroplast avoidance responses [13,14]

and impaired mesophyll conductance [15]. Therefore, previous studies have shown that a combination of blue and red light in the visible light spectrum is ideal for photosynthesis and the normal growth of different crops [16–18]. Additionally, the combination of red and blue light resulted in increased Pn and shoot biomass compared to monochromatic red or blue light [19–22].

Recently, it has been reported that green light plays an important role in light absorption, similar to blue light [23,24]. Green light is also known to stimulate deeper photosynthesis in the canopy and improve photosynthesis and plant growth, such as extending the stem height, inducing morphological changes, improving the leaf anatomy and enhancing the antioxidant activity, antioxidant content and aromatic compounds in leaves [16,25,26]. Green light can participate in photosynthesis through proteins that receive photosynthetic pigments, such as phytochromes and cryptochromes; therefore, it can affect plant growth and development. Green light regulates leaf expansion, stem stretching and stomatal conductance. Moreover, it has been shown that green light leads to greater dry mass accumulation and growth stimulation [27]. However, green light alone is not enough to support plant growth. However, when used in combination with red, blue and far-red light, it results in some important physiological reactions. Terashima et al. [28] stated that green light supplemented with strong white light made photosynthesis more effective than red light in sunflower leaves. As Kim et al. [29] pointed out, green light combined with red and blue LED light that can promote plant growth.

Recently, greenhouse spinach production has emerged as an alternative to traditional tomato production because it allows the production of many shorter cycles in a year and thus faster economic returns than the tomato crop [30]. Moreover, spinach has a high content of protein, vitamins, carotenoids, organic acids and alkaline mineral constituents, as well as antioxidants [31,32]. Thus, the objective of this study was to investigate whether adding green light to red and blue LEDs would affect the growth, leaf microstructure and quality of hydroponically cultivated spinach plants.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

This experiment was conducted in an indoor system at the Institute of Agrobiology, Vietnam National University of Agriculture. These methods draw upon our previous work [33,34]. The room temperature and humidity were maintained at 27 ± 0.5 °C and 65 ± 5%, respectively. Heat-treated F1 seeds of the PD512 spinach variety (*Spinacia oleracea* L.) were provided by Phu Dien Trading & Production Co. Ltd., Hanoi, Vietnam. The seeds were sown in 128-cell plug trays (Bumnong, Jeongeup, Korea) that had been filled with commercial growing substrate (Klasmann TS-2, Germany). Ten days after germination, seedlings of the same size were transplanted into plastic in the circulating hydroponic system. The experiment was conducted in 9 indoor hydroponic systems, where one system has 4 layers (three hydroponic systems for one light quality treatment). Each layer has got 5 rows and 9 plant sites per one row. Every hydroponic system was equipped with light of a separate quality (at the same PPFD = 190 μmoL $m^{-2}{\cdot}s^{-1}$). Light was supplied by red LEDs (R) with peak wavelengths of 660 nm, by blue LEDs (B) with a peak wavelength of 445 nm and by green LEDs (G) with a peak wavelength of 550 nm. The three light qualities were created from a ratio of R:B that was 4:1 (R4B1), R:B:G that was 5:2:3 (R5B2G3) and R:B:G that was 1:1:1 (R1B1G1), at the same intensity of 190 μmoL $m^{-2}{\cdot}s^{-1}$.

The LEDs were manufactured and supplied by the Rang Dong Light Source & Vacuum Flask Joint Stock Company, Hanoi, Vietnam. The plants were grown under a 12/12 h light/dark photoperiod cycle. The harvest time was 30 days after transplanting.

A solution based on Hoagland's nutrient solution was used in the experiment [35]. The pH and EC of the nutrient solutions were maintained at 6.0–6.5 and 1200 μS/cm, respectively, by changing the solutions in the hydroponic containers every 7 days.

2.2. Growth Parameters

Specific leaf area (SLA) (cm^2/g) = leaf area/leaf dry weight.

The relative growth rate (RGR) was calculated using the following equation of Hoffmann and Poorter [36]:

$$RGR = (\ln W2 - \ln W1)/(t2 - t1)$$

The net assimilation rate (NAR) was calculated using the following equation of Radford [37]:

$$NAR = [(W2 - W1)/(t2 - t1)] \times [(\ln A2 - \ln A1)/(A2 - A1)]$$

where:
ln = the natural logarithm;
t1 = time one (in days); W1 = the dry weight of the plant at time one (in grams);
t2 = time two (in days); W2 = the dry weight of the plant at time two (in grams);
A1 = the leaf area of the plant at time one; A2 = the leaf area of the plant at time two (in square meters).

2.3. Photosynthetic Pigments

The chlorophyll and carotenoid concentrations were determined by Lichtenthaler and Wellburn's method. Then, the pigment contents in the fresh leaves were converted to mg/g [38].

2.4. Anatomical Features of Leaves

The anatomical features of the mesophyll cells in the spinach leaves were determined using Clark's method [39]. Cross-sections were cut by hand. The leaf compactness was calculated using the following formula:

$$\text{Leaf compactness} = \text{Palisade tissue length}/\text{Leaf thickness}$$

The ratio of the thickness of the palisade to that of the spongy tissue (PT/ST) was calculated as follows: PT/ST = Palisade tissue length/Spongy tissue length [40].

2.5. Stomatal Traits

For epidermal studies, leaves were soaked in absolute ethanol for 24 h and then transferred to 80% acetone for 2–4 h. The leaf samples were immersed in NaOH/ethanol (1:5 mM NaOH/absolute ethanol). Next, the samples were placed on a glass slide with lactic acid and kept overnight [41].

The leaf cross-section and stomatal microphotographs were taken using an electron microscope (Nikon Eclipse 80i, Japan) coupled with a digital microscope camera and filar micrometer. The images were processed and analyzed with ImageJ software (National Institutes of Health, USA). The size and density of the stomata and epidermal cells were calculated for both the adaxial and abaxial epidermal surfaces.

2.6. Quality Parameters

The total organic acid content, fiber content and total polyphenol content were determined at the Vietnam National University of Agriculture. The total acid content was determined by the titration method, according to Horwitz [42]. The crude fiber content was measured by the digestion and gravimetric technique according to Antial et al. [43]. The total polyphenol content was determined by the colorimetric method using Folin–Ciocalteu reagent, in accordance with Singleton and Rossi [44].

2.7. Statistical Analysis

Statistical analyses were conducted with Excel and R software. Data were analyzed by analysis of variance (ANOVA), and the differences between means were tested using Duncan's test ($p \leq 0.05$).

3. Results

3.1. Effect of Light Quality on Growth Parameters of Spinach Plant

The hydroponically cultivated spinach grown under various light qualities showed significant differences in growth characteristics (Table 1). The relative growth rate (RGR), net assimilation rate (NAR), fresh weight (FW) and dry weight (DW) of the spinach plant decreased with a decrease in the ratio of red light. The highest values of RGR, NAR and FW of the whole plant were observed in the R4B1 treatment; the lowest values of the RGR, NAR and FW for the whole plant were observed for the R1B1G1 treatment. The DW for the R1B1G1 treatment was not statistically significantly different to that for the R5B2G3 treatment. However, the highest specific leaf area (SLA) was observed for the R5B2G3 treatment.

Table 1. Effects of different light qualities on the growth characteristics of hydroponically cultivated spinach (30 DAT).

Treatment	SLA (cm^2/g)	RGR (g/day)	NAR ($g/m^2/day$)	FW of Whole Plant (g/Plant)	DW of Whole Plant (g/Plant)
R4B1	2.99 c	0.140 a	5.56 a	74.69 a	4.20 a
R5B2G3	4.08 a	0.132 b	5.35 b	53.57 b	2.45 b
R1B1G1	3.57 b	0.121 c	5.22 c	36.31 c	2.19 b
CV%	4.40	2.71	1.14	2.76	6.90
LSD0.05	0.31	0.007	0.12	3.02	0.36

DAT: Days after transplanting; SLA: Specific leaf area; RGR: Relative growth rate; NAR: Net assimilation rate; FW: Fresh weight; DW: Dry weight. Different lowercase letters in the same column indicate significant differences among treatments ($p \leq 0.05$; $n = 3$).

3.2. Effect of Light Quality on Photosynthetic Pigments of Spinach Plant

The highest values of the photosynthetic pigment content were observed for the R4B1 treatment, followed by the R5B2G3 and R1B1G1 treatments, except for the Chla/chlb ratio. However, there were no statistically significant differences in the Chla content and Chla/Chlb ratio between the R5B2G3 and R1B1G1 treatments. There were no statistically significant differences in the Chlb and Chl(a + b) contents, Chla/Chlb ratio and carotenoids between the R5B2G3 and R4B1 treatments (Table 2).

Table 2. Effects of different light qualities on the photosynthetic pigments of hydroponically cultivated spinach (30 DAT).

Treatment	Chla (mg/g)	Chlb (mg/g)	Chl(a + b) (mg/g)	Chla/Chlb	Carotenoids (mg/g)
R4B1	0.290 a	0.522 a	0.812 a	0.555 a	0.183 a
R5B2G3	0.274 b	0.515 a	0.789 a	0.532 ab	0.181 a
R1B1G1	0.268 b	0.498 b	0.766 b	0.538 b	0.169 b
CV%	1.68	1.51	1.38	1.60	2.34
LSD0.05	0.009	0.015	0.022	0.017	0.008

DAT: Days after transplanting; Chl: Chlorophyll. Different lowercase letters in the same column indicate significant differences among treatments ($p \leq 0.05$; $n = 3$).

3.3. Effect of Light Quality on Leaf Structure Characteristics of Spinach Plant

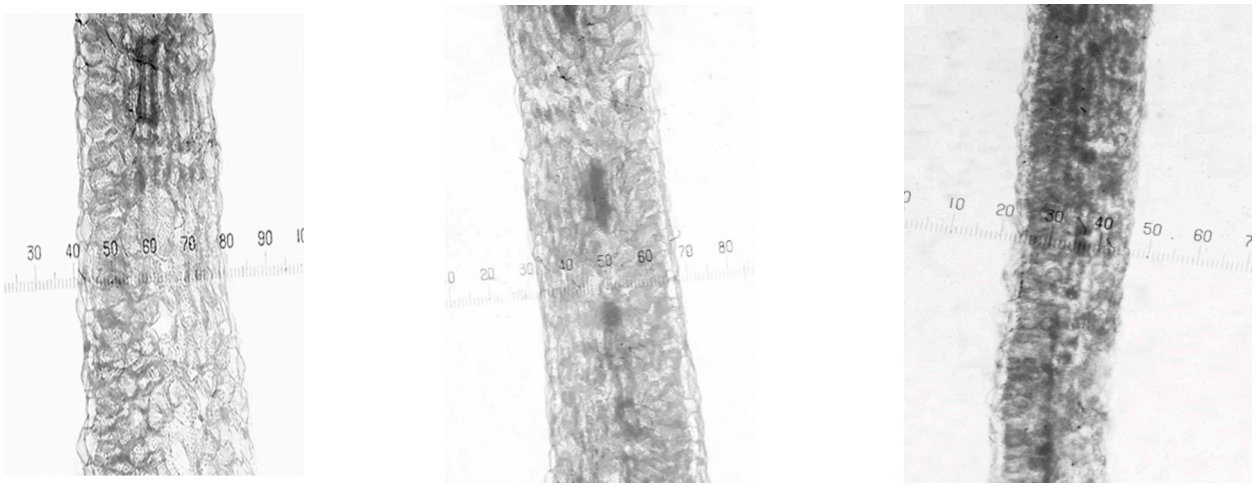
3.3.1. Anatomy of the Leaf Cross-Sections

The highest values of the palisade tissue length, spongy tissue length, leaf thickness and ratio of palisade tissue length/spongy tissue length were observed for the R4B1 treatment, followed by the R5B2G3 and the R1B1G1 treatments. There was no statistically significant difference in leaf compactness between the R4B1 and R5B2G3 treatments, but the highest value of leaf compactness was also observed for the R4B1 treatment (Table 3 and Figure 1).

Table 3. Effects of different light qualities on the anatomical structure of hydroponic spinach leaves (30 DAT).

Treatment	Palisade Tissue Length (μm)	Spongy Tissue Length (μm)	Leaf Thickness (μm)	PT/ST	Leaf Compactness
R4B1	78.31 a	253.00 a	382.43 a	0.310 a	0.205 a
R5B2G3	65.00 b	218.59 b	320.92 b	0.297 b	0.203 a
R1B1G1	46.91 c	163.48 c	251.88 c	0.287 c	0.186 b
CV%	2.34	0.71	0.59	2.51	2.15
LSD0.05	1.36	1.38	1.74	0.007	0.004

DAT: Days after transplanting; PT/ST: ratio of palisade tissue length/spongy tissue length. Different lowercase letters in the same column indicate significant differences among treatments ($p \leq 0.05$; $n = 3$).

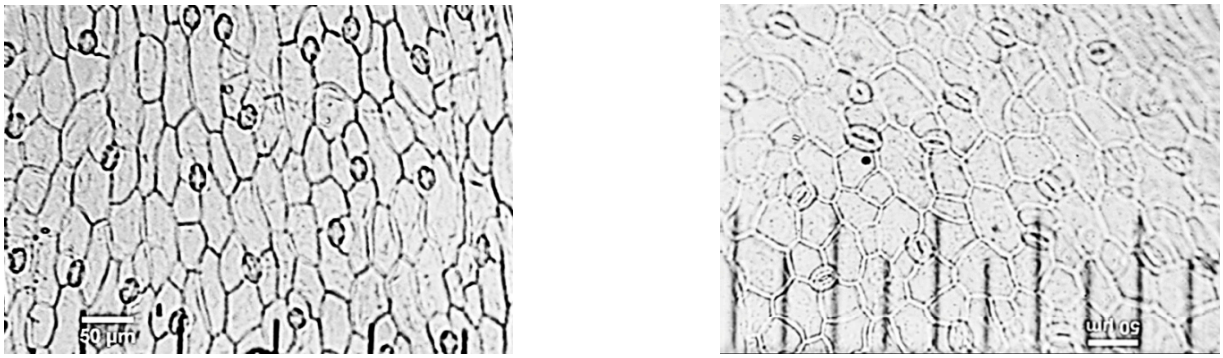


(**a**) R4B1 (**b**) R5B2G3 (**c**) R1B1G1

Figure 1. Anatomy of the spinach leaf cross-section at different light qualities: (**a**) R4B1, (**b**) R5B2G3 and (**c**) R1B1G1. Scale bar is 50 μm.

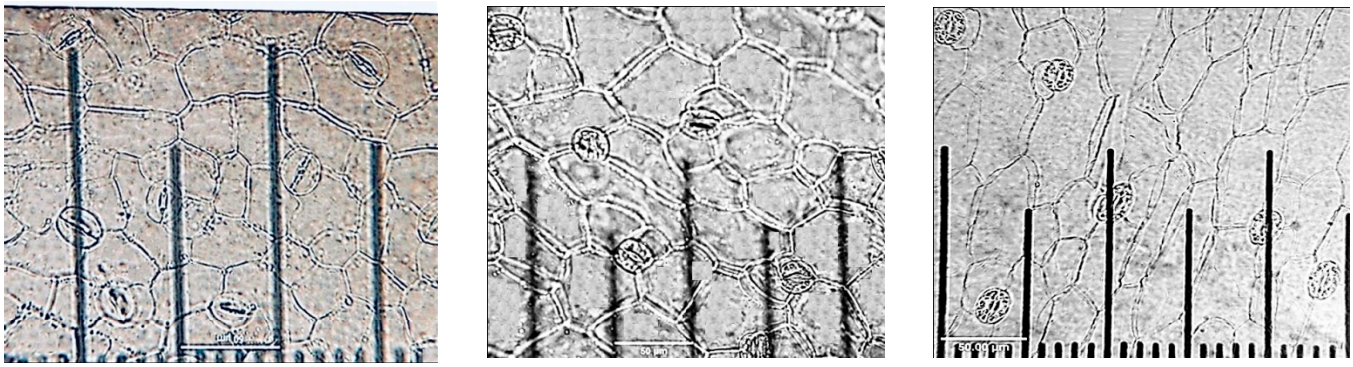
3.3.2. Characteristics of Stomata Cells

The characteristics of the responses of the stomatal cells in the adaxial epidermis to the light treatments are shown in Figures 2–4. The highest values of the stomatal length and stomatal width/length ratio were observed for the R4B1 treatment, followed by the R1B1G1 and the R5B2G3 treatments. However, there was no statistically significant difference in stomatal length between the R1B1G1 and R5B2G3 treatments. The highest values of stomatal width and stomatal density were observed for the R4B1 treatment, followed by the R5B2G3 and R1B1G1 treatments.



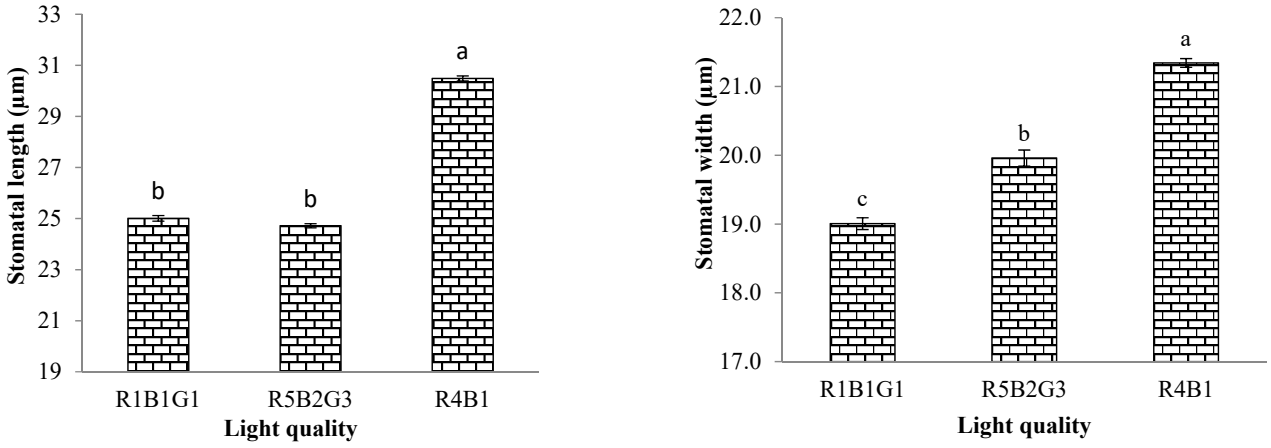
(**a**) Adaxial leaf epidermis (**b**) Abaxial leaf epidermis

Figure 2. Adaxial epidermis (**a**) and abaxial epidermis (**b**) of hydroponically cultivated spinach leaves under R5B2G3 treatment. Scale bar is 50 μm.



(**a**) R4B1 (**b**) R5B2G3 (**c**) R1B1G1

Figure 3. The adaxial epidermis of spinach leaves at different light qualities: (**a**) R4B1, (**b**) R5B2G3 and (**c**) R1B1G1. Scale bar is 50 μm.



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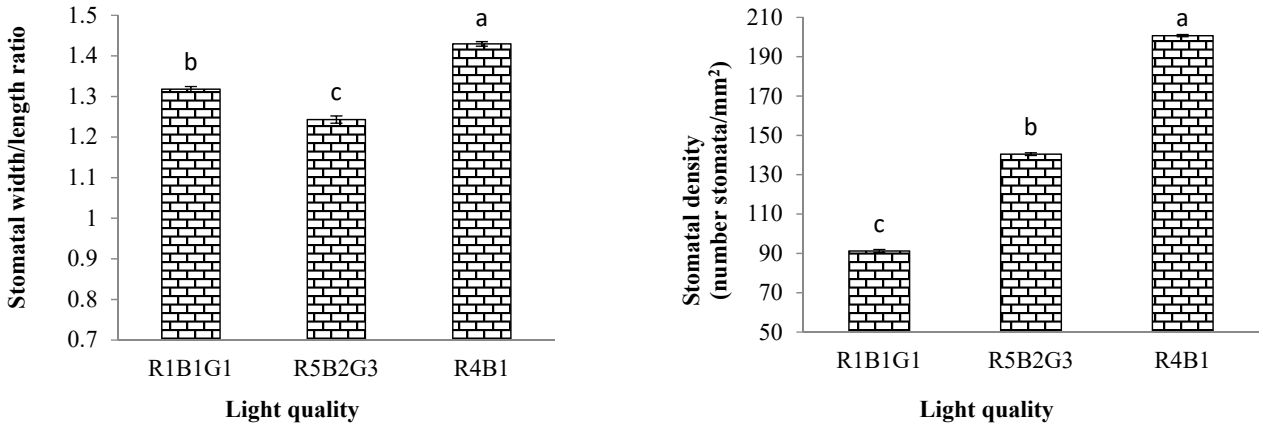
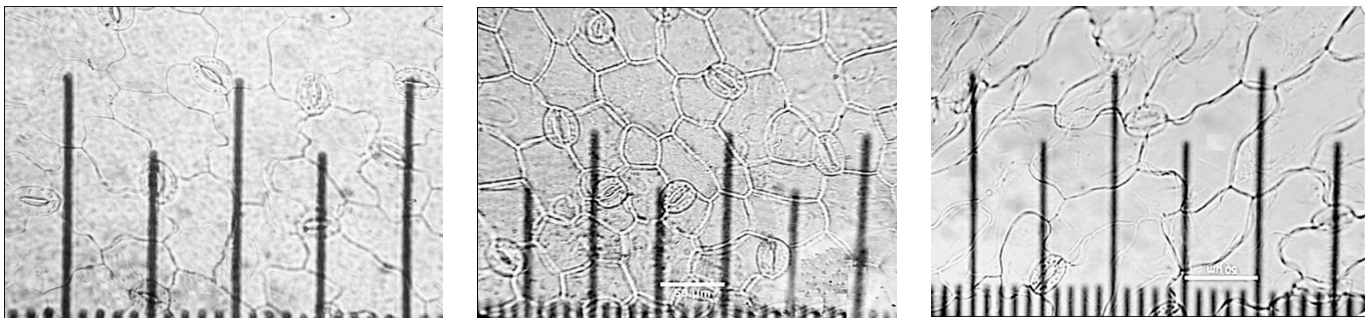


Figure 4. Characteristics of the stomatal cells in the adaxial spinach epidermis leaves for the different light qualities. The error bars show the standard errors. Data were analyzed by R software. Different letters indicate a significant difference among treatments ($p \leq 0.05$).

The highest values of the stomatal length, stomatal length/width ratio and stomatal density in the abaxial spinach epidermis leaves were observed for the R4B1 treatment, followed by the R5B2G3 and R1B1G1 treatments. However, there was no statistically significant difference in the stomatal length/width ratio between the R1B1G1 and R5B2G3 treatments. The highest value of the stomatal width was observed for the R5B2G3 treatment, followed by the R4B1 and R1B1G1 treatments (Figures 5 and 6).



(**a**) R4B1 (**b**) R5B2G3 (**c**) R1B1G1

Figure 5. The abaxial epidermis of spinach leaves at different light qualities: (**a**) R4B1, (**b**) R5B2G3 and (**c**) R1B1G1. Scale bar is 50 µm.

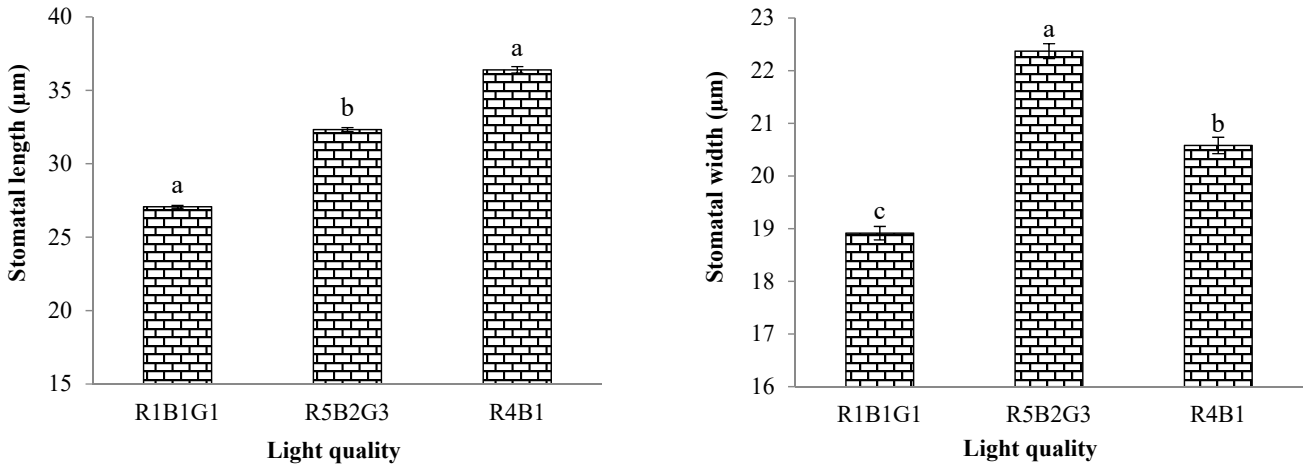


Figure 6. *Cont.*

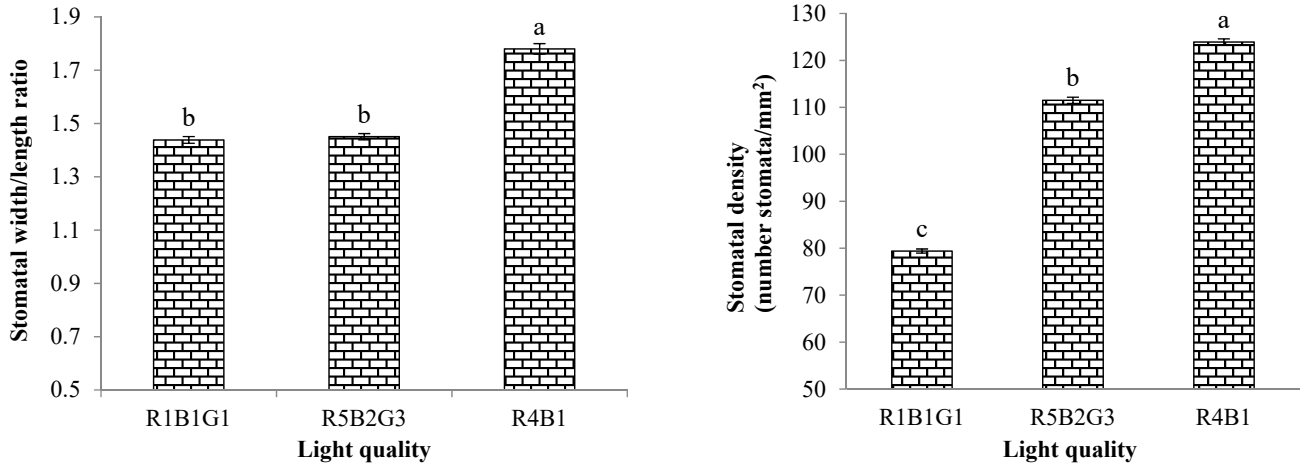


Figure 6. Characteristics of the stomatal cells in the abaxial spinach epidermis leaves for the different light qualities. The error bars show the standard errors. Data were analyzed by R software. Different letters indicate a significant difference among treatments ($p \leq 0.05$).

3.4. Effect of Light Quality on Nutrition Content and Quality of Spinach Plant

The highest values of the crude fiber content and total polyphenol content were observed for the R4B1 treatment, while the lowest values were observed for the R1B1G1 treatment. However, there was no statistically significant difference in the crude fiber content and total polyphenol content between the R1B1G1 and R5B2G3 treatments (Figure 7). The highest organic acid content was observed for the R5B2G3 treatment, followed by the R1B1G1 and R4B1 treatments. However, there was no statistically significant difference in the organic acid content between the R1B1G1 and R5B2G3 treatments, or between the R4B1 and R5B2G3 treatments (Figure 7).

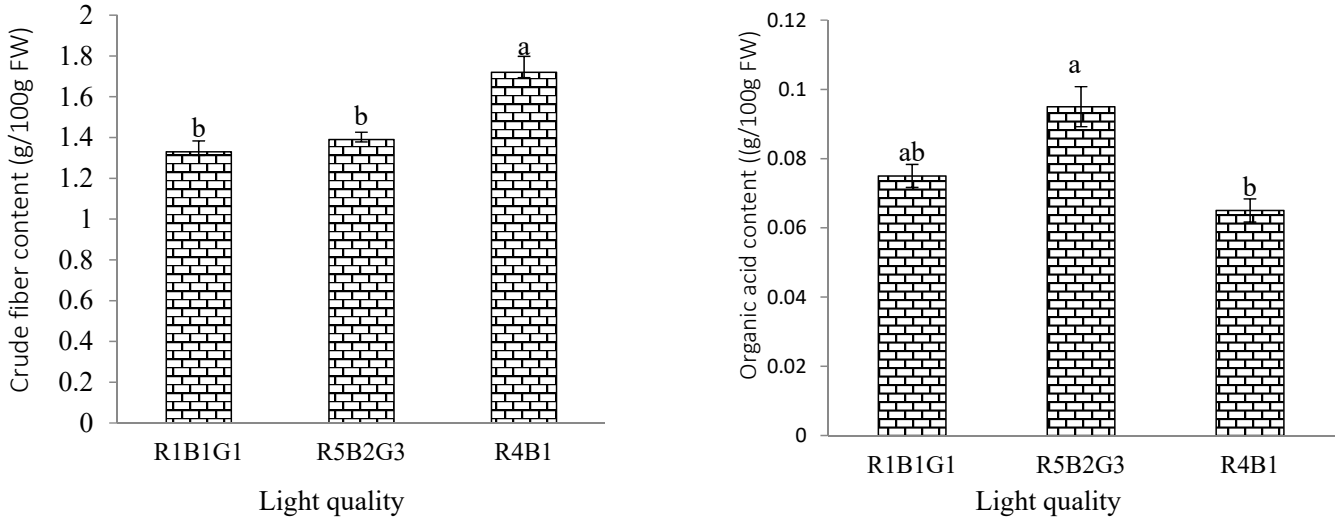


Figure 7. *Cont.*

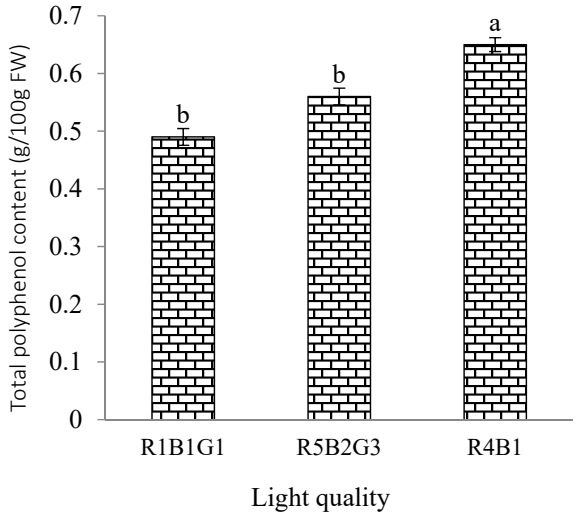


Figure 7. Effects of LEDs with a different light quality at the same intensity on some nutritional parameters of hydroponically cultivated spinach (30 DAT). The error bars show the standard errors. Data were analyzed by R software. Different letters indicate a significant difference among treatments ($p \leq 0.05$).

4. Discussion

Current LED-based artificial lights for crop cultivation consist of red and blue lights, because their spectra effectively promote leaf photosynthesis. Red and blue light are the most important light regions, necessary for plant growth and development [9]. The combination of red and blue light resulted in increased photosynthesis and biomass of the plant compared to monochromatic red or blue light [12,19–22]. However, it has recently been reported that green light plays an important role in light absorption, similar to blue light [23,24]. Green light is also known to stimulate deeper photosynthesis in the canopy, and improve photosynthesis and plant growth, such as extending the stem height, causing morphological changes, altering the leaf anatomy and enhancing the antioxidant activity, antioxidant content and aromatic compounds in leaves [16,25,26]. Green light can participate in photosynthesis through proteins that receive photosynthetic pigments, such as phytochromes and cryptochromes; therefore, it can affect plant growth and development. Kim et al. [29] grew lettuce under blue:red and blue:green:red and found that plants grown under 24% green had a greater fresh mass, dry mass and leaf area than plants grown under blue:red. However, in our study, the green light, when added with red and blue LEDs, resulted in decreased growth parameters for the spinach plant compared to the red and blue light combination. This result is similar to the result of Hernández and Kubota [45], who reported that the addition of green to the spectrum did not have any influence on cucumber plants. However, Wollaeger and Runkle [46] also found that tomato, impatiens and salvia transplants had the same shoot dry weights when grown under combination LED lights of green:red, blue:green:red and blue:red. Therefore, Hernández and Kubota [46] suggested that the effect of green light on a plant's growth rate is species-specific.

Blue and red LEDs are commonly used for plant growth, as chlorophyll a and b efficiently absorb blue and red wavelengths in the ranges of blue (maximum absorption at 430 and 453 nm) and red (maximum absorption at 663 and 642 nm) [47]. On the other hand, Wollaeger and Runkle [46] suggested that light quality is an important factor for photosynthetic pigments, and red light has a greater influence than other light spectra. Therefore, in our study, the photosynthetic pigment content decreased with a decrease in the ratio of red light. The highest values of the photosynthetic pigment content were observed in the R4B1 treatment, followed by the R5B2G3 and R1B1G1 treatments. This result is similar to the result of Terashima et al. [28], who suggested that the light in the red and blue regions of the spectrum is mainly absorbed by photosynthetic pigments.

The combination of red and blue light is effective for photosynthesis and the normal growth of different plants [16–18]. Changes in the light spectrum strongly influence plant growth and quality [48]. In our study, the anatomical structure of the spinach leaves decreased with a decrease in the ratio of red light. The highest values for the anatomical structure of the spinach leaves were observed for the R4B1 treatment, followed by the R5B2G3 and R1B1G1 treatments. The results also presented similarities with those of the study of Stryjewski et al. [49] on the anatomical structure of spinach leaves exposed to different qualities of light, which showed that the leaf thickness, palisade tissue length and spongy tissue length were larger with a 660 nm red-light-supplemented 470 nm blue light LED treatment than with others (690, 700 and 725 nm combined with 470 nm) [49].

The ratio of blue and red LEDs was important for the morphology, growth and phenolic compounds with antioxidant properties in two lettuce varieties [50]. Accordingly, in our study, the highest values for crude fiber content and total polyphenol content were observed with the R4B1 treatment. Our results for the phenolic content also slightly differ from those of Samuoliene et al. [51]. The authors demonstrated that the phenol concentration in red leaf lettuce increased by 52.7 and 14.5%, respectively, when red–blue LEDs were used 16 h before harvest. However, the highest organic acid content in our study was observed with the R5B2G3 treatment, followed by the R1B1G1 and R4B1 treatments. The change in organic acid content was also similar to the findings of Viršilė et al. [52]. Light (green 510 nm, 595 nm orange) supplementary to the LED light (435 nm blue LED, 627 nm and 660 nm and 735 nm red LEDs) at PPFD 300 μmoL m^{-2} s^{-1} did not affect the growth of lettuce; however, it had a pronounced effect on the organic acid content. Orange and green light had a different effect on the metabolism of red and green leaf lettuce, correlating with the nutritional and safety values in lettuce production. The metabolic reactions were characteristic of the plant varieties; however, green light had a reasonable impact on the levels of primary metabolites in red and green leaf lettuce [52].

5. Conclusions

The ratio of red, blue and green LEDs was an important factor in the growth, photosynthesis and biosynthesis of metabolites in spinach plants. The combination of red and blue light at a 4:1 ratio had higher values of the growth parameters, photosynthetic pigments, leaf structure characteristics and quality of the spinach plant than combinations of red:blue:green with 5:2:3 and 1:1:1 ratios. Green light, when added to red and blue LEDs at a reasonable ratio, could reduce the growth, leaf microstructure and quality of spinach plants, but not the organic acid content.

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