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Response of *Eustoma* Leaf Phenotype and Photosynthetic Performance to LED Light Quality

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Abstract: In a controlled environment, light from light-emitting diodes (LEDs) has been associated with affecting the leaf characteristics of *Eustoma*. LEDs help plant growth and development, yet little is known about photosynthetic performance and related anatomical features in the early growth stage of *Eustoma* leaves. In this study, we examined the effects of blue (B), red (R), and white (W) LEDs on the photosynthetic performance of *Eustoma* leaves, as well as leaf morphology and anatomy including epidermal layer thickness, palisade cells, and stomatal characteristics. Leaves grown under B LEDs were thicker and had a higher chlorophyll content than those grown under the R and W LEDs. Leaves under B LEDs had greater net photosynthetic rates (A), stomatal conductance (g_s), and transpiration rates (E), especially at a higher photon flux density (PPFD), that resulted in a decrease in the intercellular CO_2 concentration (C_i), than leaves under the W and R LEDs. B LEDs resulted in greater abaxial epidermal layer thickness and palisade cell length and width than the R and W LED treatments. The palisade cells also developed a more cylindrical shape in response to the B LEDs. B LED leaves also showed greater guard cell length, breadth, and area, and stomatal density, than W or R LEDs, which may contribute to increased A, g_s and E at higher PPFDs.

Keywords: adaxial and abaxial epidermal layer; *Eustoma grandiflorum*; leaf gas exchange; leaf thickness; palisade cell; photosynthetic performance; stomatal characters

1. Introduction

Light is the sole energy source for photosynthesis and an environmental trigger in a wide range of plant growth and development phenomena. In a controlled environment, in response to requirements for optimal light quality, quantity, and distribution, light-emitting diodes (LEDs) have been proposed as a promising light source for plants [1], either as a main or supplementary light source [2], and for space-based plant growth [3,4]. Compared with light intensity and photoperiod, light quality filters have much more complex effects on plant morphology and physiology. Using different color lights greatly influences the plant development cycle and physiology [5]. Hence, blue (B) and red (R) LED light absorbed by photosynthetic pigments are more effective than other wide-spectrum light [6]. B light is associated with physiological responses such as plant photo-morphogenesis, phototropism, vegetative growth, stomatal opening, leaf expansion, anatomy and photosynthetic functioning, enzyme synthesis, chloroplast movement, and gene expression [6–11]. The range of wavelengths of B light impacting growth of plants is diverse and crop-specific [10,12]. In contrast, R light produces a narrow-spectrum light that regulates root-to-shoot ratio, chlorophyll content, and the photosynthetic

apparatus [13,14]. Although R light is the main light absorbed for photosynthesis, plants cannot develop optimally without B light [15,16]. In addition, plants under white (W) LED light alone have regular leaf morphology and a higher photosynthetic rate compared with plants grown under R or B light [9,17,18].

Generally, net photosynthetic rate (A), stomatal conductance (g_s), transpiration rate (E), and intercellular CO_2 concentration (C_i) vary under different light spectrums. In particular, stomata strongly affect photosynthesis in plants, where stomatal conductance is correlated with CO_2 assimilation and limits excessive water loss in response to changing external environments [19–22]. An increasing proportion of B light stimulated A and g_s in cucumber plants [17], and components of stomatal function affecting photosynthesis were both dependent on and independent of B light, whereas stomatal function affecting photosynthesis were dependent on response to R light [23].

Eustoma grandiflorum (Raf.) Shinn originated as a wild flower from North America to South America, and has bright colorful petals and a long postharvest life compared with other cut flowers. However, conventional *Eustoma* plant production is often shortened by climatic conditions, especially in summer and winter in tropical and temperate regions, respectively. In addition, the environment (temperature, water, sunlight availability, etc.) can retard growth during the seedling stage to early developmental stage of *Eustoma*, resulting in plants with irregularly shaped leaves. Currently, plant production under controlled environmental conditions has been explored in many countries. Using artificial light for plants, in which the system controlling major environmental factors is strongly maintained, results in easier plant production regardless of border climatic conditions [24]. *Eustoma* leaves respond to different color LED lights in their physiological performance and stomatal function, which act as gateways linking the intercellular gas spaces to the external environment for photosynthesis because light significantly regulates plant response in terms of leaf phenotype and canopy architecture [25,26]. The optimal epidermal layer traits and stomatal activities of *Eustoma* leaves under B, R, and W LEDs have not been determined to date, nor have there been studies on the effects of B, R, and W LED light on leaf photosynthetic performance in the early growth stage of *Eustoma* leaves.

The objective of this study was to examine the effects of B, R, and W LEDs on leaf phenotypic and photosynthetic performance, the epidermal layer, palisade cells, and stomatal behavior in the early growth stage of *Eustoma*.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

Voyage type 2 pink *Eustoma* (*Eustoma grandiflorum*) seeds (Sakata Seed Corporation, Kanagawa, Japan) were sown in half-strength MS medium with 3% sucrose concentration for in vitro culture. During seed germination and seedling growth in vitro, the temperature and light conditions were 23 ± 2 °C under 16/8 h (light/dark) and 98 μmol m^{-2} s^{-1}, respectively. After 8 weeks, morphologically uniform, 4th fully-expanded leaf pair were removed from the culture bottle and washed carefully in running water. Afterward, the cultured seedlings were transferred to plastic pots (6 cm in diameter and 9 cm in depth) containing fertilized soil with 380, 290, and 340 mg L^{-1} of N:P:K (Tanekura No. 42; Sumirin Agricultural Industry Co., Ltd., Tokyo, Japan) and quickly transferred to a phytotron to establish the seedlings in the soil during acclimatization. After 2 days, the 30 seedlings were transferred to a walk-in-type, environmentally-controlled LED growth chamber (fabricated environment controlled growth chamber; Nikkan Co., Ltd., Tokyo, Japan); the air temperature was 22/18 °C during the light and dark period, respectively. Photoperiod, relative humidity and CO_2 concentration were 16 h day/8 h night, 65%, and 400 μmol mol^{-1} during LED light acclimation, respectively. The seedlings were watered daily and acclimated for 65 days in the growth chamber. The seedlings were subjected to B, R, and W LED lights (Tube LED light; Beamtech Co., Ltd., Tokyo, Japan, with an average photosynthetic photon flux density (PPFD) of 100 ± 25 μmol m^{-2} s^{-1} under each of the LED treatments. The quality

of irradiance of the B, R, and W LEDs was individually maintained by adjusting the DC power supply for each treatment. The LED lights were positioned 25 cm above the plants. The B, R, and W LEDs had peak wavelengths of 420–550, 580–670, and 420–750 nm, respectively (Figure S1; Light Analyzer, LA-105; NK-System, Osaka, Japan).

2.2. Leaf Morphological Measurements

At the early growth stage (65 days), leaf length and width, and fresh weight were measured on single leaves of 10 plants to determine the effect of each light treatment. Leaf thickness, relative content of chlorophyll, and leaf area were recorded at the third internode with fully-expanded leaves. Leaf thickness was measured using a Vernier caliper (Mitutoyo Corp., Kawasaki, Kanagawa, Japan), and the relative content of chlorophyll was estimated using a chlorophyll meter (SPAD-502; Minolta, Osaka, Japan). The development of leaf area was analyzed using ImagJ software (version 1.8.0; http://imagej.nih.gov/ij/) at a 1 cm scale bar.

2.3. Leaf Gas Exchange and Photosynthetic Measurements

The method for measuring leaf gas exchange parameters was reported previously [27]; a portable photosynthesis system (Li–6400XT; Li-Cor Inc., Lincoln, NE, USA) was used between 09:00 a.m. and 12:00 p.m. at 1 h intervals for each LED treatment, to avoid midday depression of photosynthesis and transpiration [28]. Leaf photosynthetic rate (A), transpiration rate (E), stomatal conductance (g_s), and intercellular CO_2 (C_i) at different photosynthetic photon flux densities (PPFD) were measured. In each LED light treatment, PPFD was obtained at representative single leaves in mid-canopy. During the experiments, the CO_2 concentration in the gas chamber and leaf temperature were set at 360 ppm and 20 °C, respectively, and the photon flux density (PPFD) was started at 250 μmol m^{-2} s^{-1}, followed by 500, 750, 1000, 1250, and 1500 μmol m^{-2} s^{-1}. Marchese et al. [28] reported that the photosynthetic performance of *Eustoma* improved under a high light condition (1441.6 μmol m^{-2} s^{-1}). Photosynthetic light response curves and photosynthetic characteristics were analyzed using the portable photosynthesis system on single fully-expanded 65-day-old leaves of 10 plants exposed to each LED light (B, R, and W) using the method of Li et al. [29], with little modification. Hence, leaf photosynthesis was recorded in the range of 250 to 1500 μmol m^{-2} s^{-1} (PPFD), with a 3–5 min equilibration time at each step of the light response curve to compare the difference in photosynthetic performance under a controlled environment using B, R, and W LED lights.

2.4. Leaf Anatomy and Stomatal Character Measurements

The 65-day-old leaf samples from the plants grown under B, R, and W LED light were collected and immediately kept in autoclaved water. Leaves were manually cut into thin transverse sections using a double-edged disposable razor blade on a rubber cutting mat [30]. Leaf segments were fixed in red stain (Acetocarmine solution; Kishida Chemicals Co. Ltd., Osaka, Japan) for 30 s. After staining, sections were mounted on a microscope slide and the orientation checked before being covered with a glass slide. To measure the thickness of the upper and lower epidermis, the leaf adaxial and abaxial layers and palisade cell size were observed under a light microscope (DX-50; Olympus, Tokyo, Japan) at a magnification of 200×.

To observe the stomata, a layer of transparent nail polish was applied to the lower epidermis of the fully expanded leaves and allowed to dry for 5–10 min. Slides were made using the leaf epidermal fingerprint with transparent nail polish method [31]. A piece of clear cellophane tape was placed over the section of nail polish, carefully peeled from the leaf, and the 'impression' was transferred to a microscope slide. Imprints were observed under a light microscope equipped with a digital microscope camera (DP-12; Olympus, Tokyo, Japan). Images were saved to a computer and analyzed in ImageJ. The abaxial layer of the leaves was examined for leaf stomatal aperture under each LED-light treatment. Guard cell length and width, and cell area were measured using the light microscope at a magnification of 400×, and stomatal density was measured at a magnification of 200×. Internal leaf anatomy was

likewise recorded in a subset of samples including ten replicates for each LED-light treatment (n = 10) because of the time needed to clear, fix, embed, section, and analyze microscopic features.

2.5. Statistical Analysis

A completely randomized design was used with ten replicates for the LED-light treatments. Significant differences among the means were determined by one-way ANOVA with Tukey's HSD test at $P < 0.05$. For all statistical analysis, KaleidaGraph-4.5.0 (Synergy Software, Reading, PA, USA) was used. Data are reported as means ± standard error (SE).

3. Results

3.1. Influence of LEDs on Leaf Morphology

The growth and morphology of the Eustoma leaves were significantly affected under the B, R, and W LED treatments (Figure 1A), particularly leaf shape and size (Figure 1C). The leaves grown under the B and W LEDs were longer than those grown under the R LEDs (Figure 1(A1)). On the other hand, the leaves grown under the W LEDs were wider than the leaves grown under the B or R LEDs (Figure 1(A2)). Leaf thickness was greater under the B LEDs than the R and W LED treatments (Figure 1(A3)). The highest values of single leaf fresh weight were for leaves grown under the B and W LEDs, but there was no significant difference between the B and W LED treatments (Figure 1(A4)).

3.2. Influence of LEDs on Leaf Area and Chlorophyll Content

The B, R, and W LED treatments significantly affected leaf area and chlorophyll content (Figure 1B). The leaves grown under the W LEDs showed a larger leaf area than the leaves grown under the other LEDs, and chlorophyll content was markedly increased by the B LEDs (Figure 1(B1,B2)).

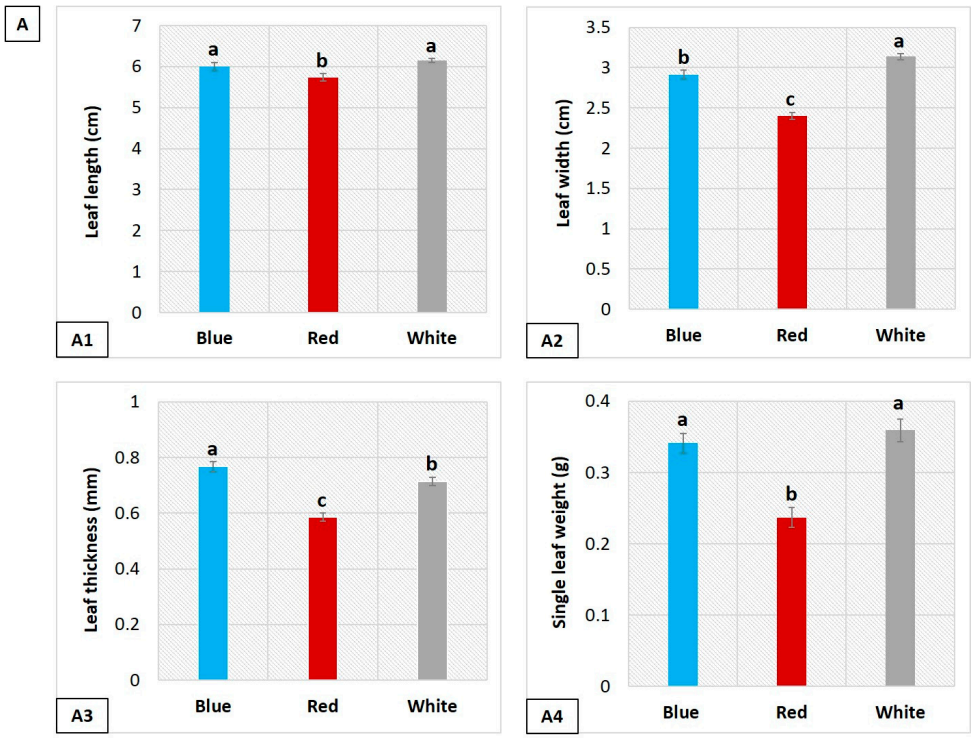

Figure 1. *Cont.*

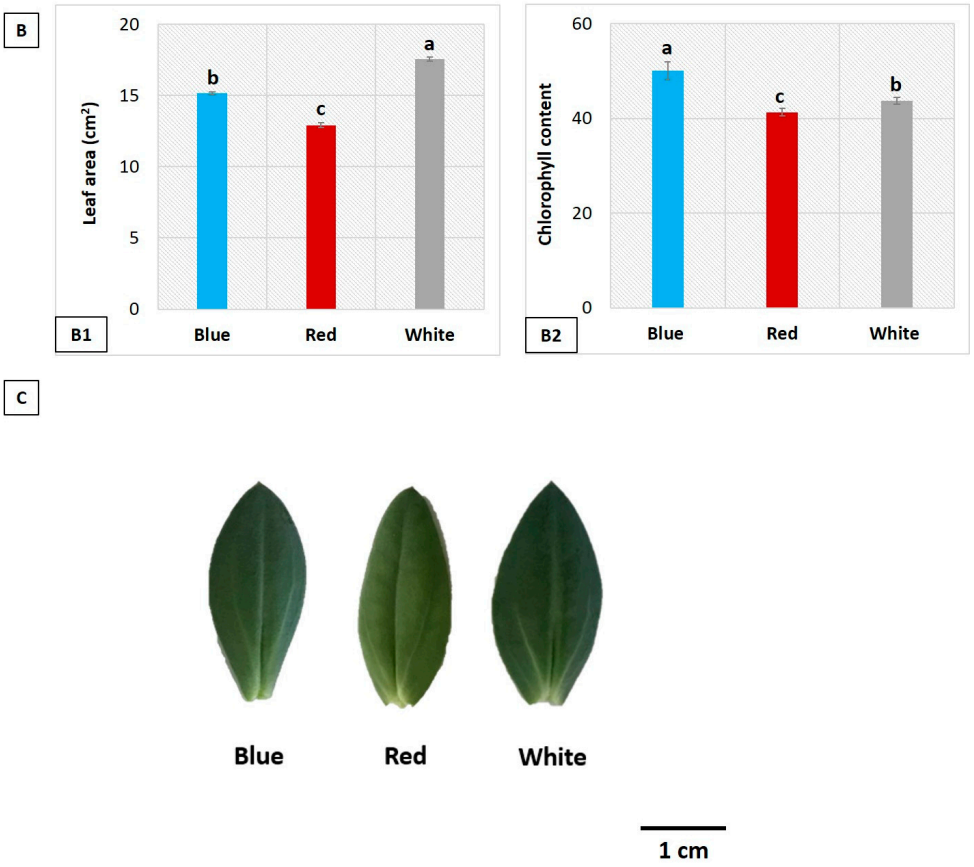

Figure 1. Effect of blue, red, and white LEDs on leaf traits of *Eustoma*. (**A1**), leaf length (cm); (**A2**), leaf width (cm); (**A3**), leaf thickness (mm); (**A4**), single leaf weight (g); (**B1**), leaf area (cm^2); (**B2**), chlorophyll content; and (**C**), leaf morphology. Data are mean values (n = 10) and the vertical bars represent ± SE (Tukey's HSD at $P < 0.05$).

3.3. Influence of LEDs on Leaf Gas Exchange and Photosynthetic Performance

Figure 2 indicates that leaf A (Figure 2A), g_s (Figure 2B), and E (Figure 2C) increased for each PPFD value from 250 μmol m^{-2} s^{-1} to 1500 μmol m^{-2} s^{-1}, and that C_i decreased (Figure 2D). For the plants grown under the B LED light, C_i; A, g_s, and E were greater at most PPFDs than the plants grown under the R and W LEDs (Figure 2A–C and Figure S1). In contrast, C_i differed significantly between the plants grown under the B, R, and W LEDs; the values were higher in the plants grown under the B LEDs than under the R LEDs at all PPFDs (Figure 2D). Therefore, B LED light at 1500 μmol m^{-2} s^{-1} PPFD resulted in increased photosynthetic function of leaves, with the greatest increase occurring for A, g_s, and E compared with the values for the R and W LEDs. The correlation between A and g_s (Figure 3) may be explained either by the effect of the B, R, and W LEDs or by the different PPFDs (Figure S1).

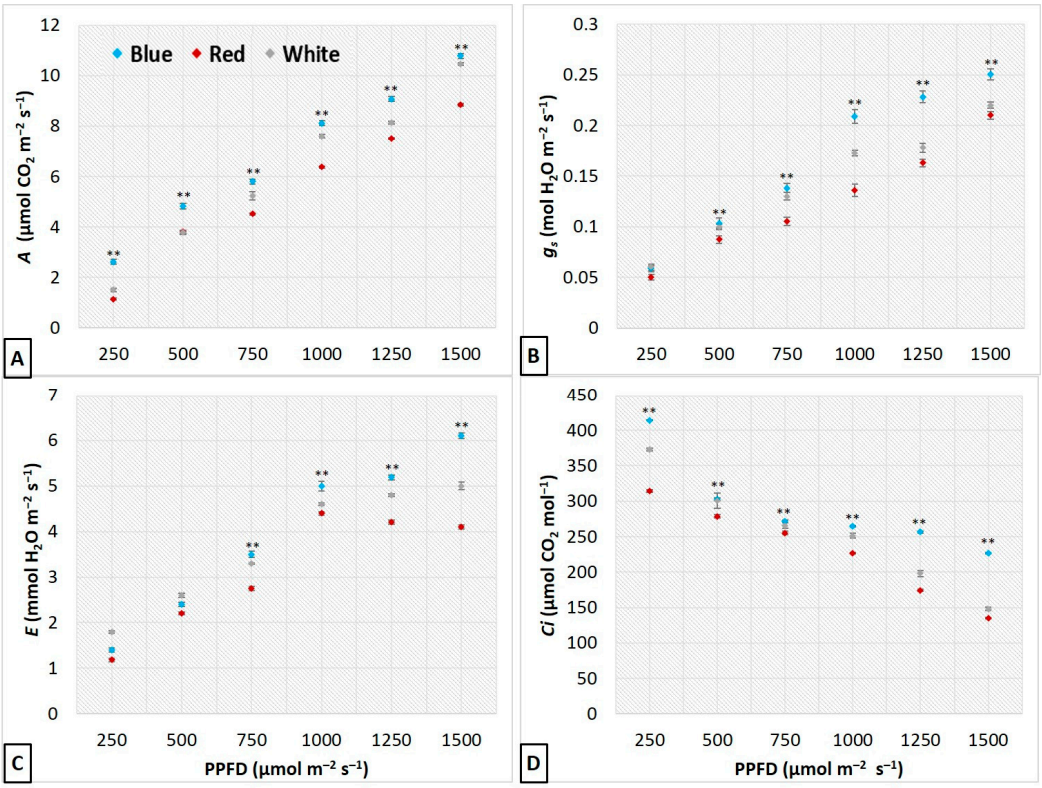

Figure 2. Effects of blue, red, and white LED light on leaf gas exchange at different photosynthetic photon flux densities (PPFD) in *Eustoma* leaves. (**A**), A (µmol CO_2 m^{-2} s^{-1}); (**B**), g_s (mol H_2O m^{-2} s^{-1}); (**C**), E (mmol H_2O m^{-2} s^{-1}), and (**D**), C_i (µmol CO_2 mol^{-1}). Data represent means ± SE (n = 10); asterisks indicate a significant difference among the LED treatments at $P < 0.01$ (**) by Tukey's HSD-test. The SE bar may not be seen on data points due their small values.

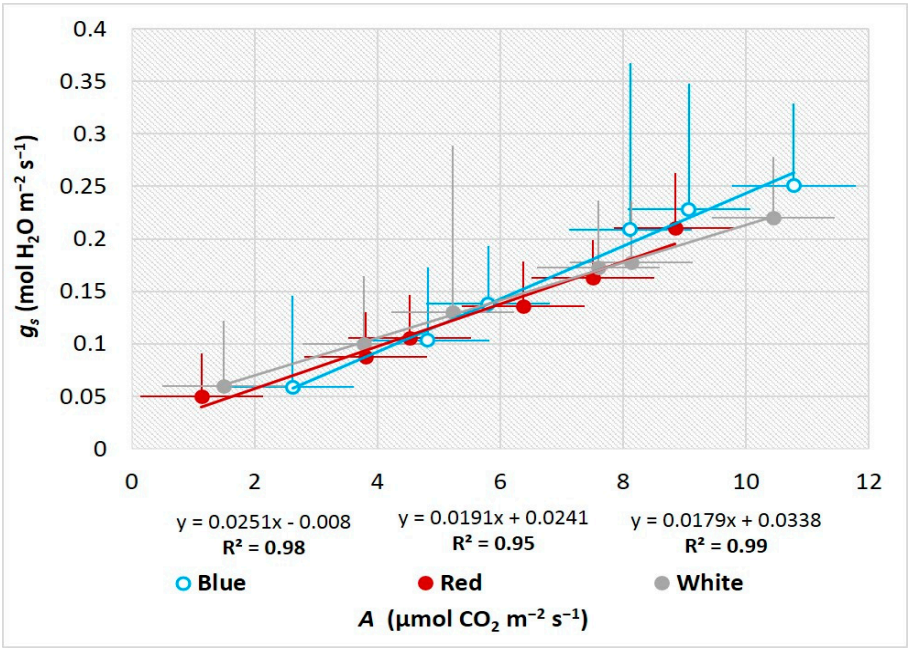

Figure 3. Correlation between photosynthetic rate (A) and stomatal conductance (g_s) in *Eustoma* leaves under blue, red, and white LEDs at different PPFDs. Data are mean values (n = 10); the vertical and horizontal bars represent ± SE. All R^2 values are statistically significant at $P < 0.01$.

3.4. Influence of LEDs on Leaf Epidermal Layer

Significant differences were found in the adaxial and abaxial epidermal layers of the leaves grown under the B, R, and W LED treatments (Figure 4). There was no significant difference between the leaf adaxial epidermal layer under the B and W LEDs, but the adaxial layer was thicker under both than those under the R LEDs. (Figure 4(A1)). Likewise, the abaxial layer was thicker in the leaves grown under the B LEDs compared with those grown under the R and W LEDs (Figure 4(A2)); however, no significant difference was observed between the R and W LED treatments. Significant differences were also observed at the microscopic level in both the adaxial and abaxial epidermal layers (Figure 4C). A transverse section of the leaves showed increased palisade cell height and width in the B LED-treated leaves compared with those treated under the R and W LEDs and the difference was significant (Figure 4(B1,B2)). The cylindrical shape of the palisade cells increased under the B LEDs, whereas the cells remained almost spherical under the R LEDs (Figure 4(C1,C2)). A cylindrical cell shape was also found in the leaves treated under the W LEDs (Figure S1 and Figure 4(C3)).

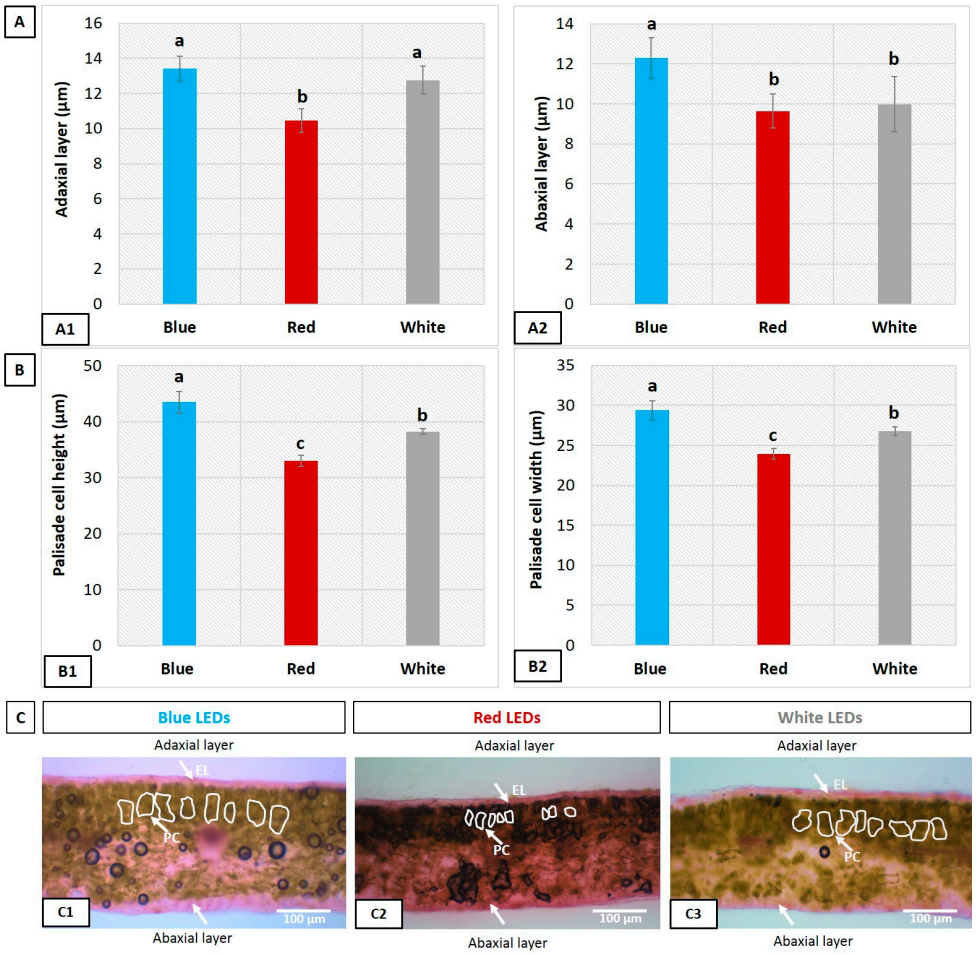

Figure 4. Effect of blue, red, and white LEDs on the leaf anatomy of *Eustoma*: (**A1**), adaxial layer (µm) and (**A2**), abaxial layer (µm); (**B1**), palisade cell height (µm) and (**B2**), palisade cell width (µm). Representative cross-sections showing anatomical parameters of the adaxial and abaxial epidermal layers in Eustoma leaves under B, R, and W LEDs: (**C1**), blue (B); (**C2**), red (R), and (**C3**), white (W) LEDs. Data are mean values (n = 10) and the vertical bars represent ± SE (Tukey's HSD at $P < 0.05$).

3.5. Influence of LEDs on Stomata

Microscopic analyses of leaf stomata indicated significant differences among the leaves grown under the B, R, and W LED treatments (Figure 5). The B LED-treated leaves showed well-organized

guard cells with open stoma, and greater guard cell length, width, and area than the R and W LED-treated leaves (Figure 5(A1–A3,B1–B3); Figure S1). Stomatal density was higher for the leaves grown under the B LEDs followed by those under the W and R LEDs (Figure 5(A4,B1–B3)). The blue (B) LED light resulted in open stomata, which helps gas exchange.

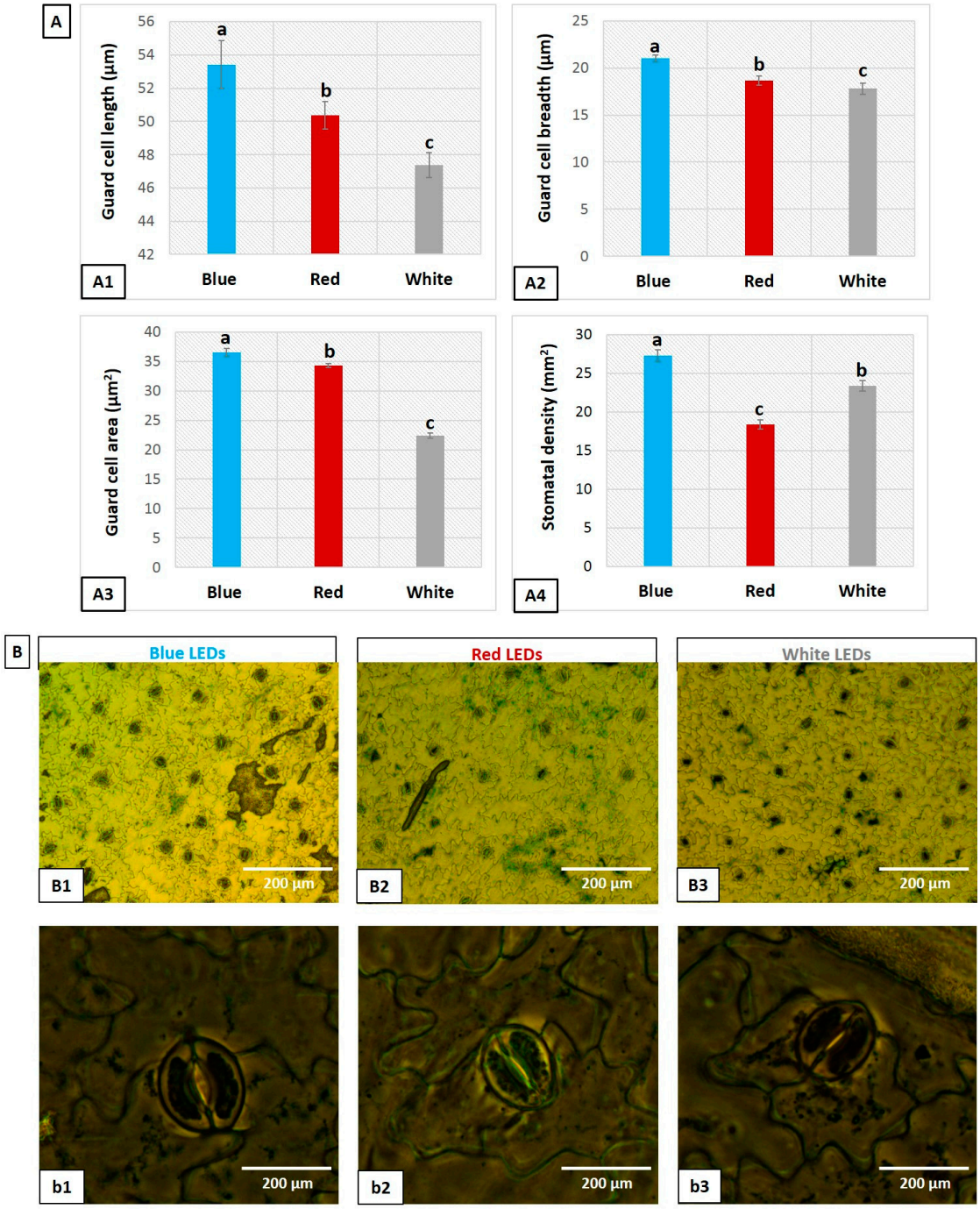

Figure 5. Effect of B, R, and W LED light on stomatal characteristics of the abaxial layer of *Eustoma* leaves. (**A1**), guard cell length (µm); (**A2**), guard cell breadth (µm); (**A3**), guard cell area (µm^2); and (**A4**), stomatal density (mm^2). Distribution and anatomical parameters of the stomata in the abaxial epidermal layer of *Eustoma* leaves using blue, red, and white LEDs from representative cross-sections. (**B1**,**b1**), blue; (**B2**,**b2**), red; and (**B3**,**b3**), white LED light. Data are mean values (n = 10), the vertical bars represent ± SE, and means are separated by Tukey's HSD at $P < 0.05$.

4. Discussion

LED lights are widely used in *Eustoma* cultivation in controlled environments. LEDs therefore provide opportunities for altering leaf structure and physiology in the early growth stages during horticultural production in either greenhouses or space. In general, light affects plant growth and development [32], and numerous studies have already been conducted with LED lights on various horticultural crops [33,34]. In this work, we explored leaf phenotypic and photosynthetic performance, and microscopically examined the leaf epidermal layers and stomata of *Eustoma* leaves, grown under B, R, and W LEDs.

Leaves varied in length and width under the LED light treatments. Leaf shape and size change as a function of plasticity, natural variation, and environmental adaptation [35]. In the early growth stage, *Eustoma* leaves grew faster because photosynthetic performance under the W LED light leads to vigorous growth [18]. In the B LED light-treated leaves, repression of gibberellin (GA) biosynthetic-related genes and induction of the GA inactivation-related genes has been reported, which constrained the elongation of rice leaves [36]. In Figure 1(A1,A2), leaves under B and W LEDs were longer compared with the leaves under R LEDs, and the leaves under W LEDs were wider compared with the leaves under B and R LEDs. The shortest and narrowest leaves were those under the R LEDs. Notably, leaf length of the leaves treated under the W and B LEDs was not significantly different, but leaf width under all LEDs was significantly different. The wavelengths of the B and W LEDs broadened leaf width compared with the R LEDs [37]. Furthermore, no leaf blade curling was observed in the B and W LED-treated leaves, but there was in the R LED-treated leaves. Leaf curling is a response to phytochrome B [38].

The structure and physiology of leaf thickness is controlled by light [39], and mesophyll cell size is also induced to increase, which in turn increases leaf thickness under different light intensities [40]. From the results, leaf thickness was significantly greater in the leaves under the B LEDs compared with the other LED treatments (Figure 1(A3)). Additionally, exposure to the R LEDs decreased the single leaf fresh biomass compared with the other LEDs, and there was no significant difference in leaf fresh weight between the B and W LEDs (Figure 1(A4)). However, partitioning of B and W LEDs has increased the fresh weight for other processes, possibly leaf thickening or the production of carbohydrates [16].

In this work, leaf growth and biomass were greatly promoted by the B and W LEDs, showing that the morphology and growth of the *Eustoma* leaves were regulated by the LED light in the early growth stages. Leaf area increases in response to the photomorphogenic function of phytochrome during photosynthesis, polyploidization, and environmental or physiological conditions [17,41]. R LEDs resulted in reduced leaf area compared with other LED light in chrysanthemum, tomato, and lettuce [42]. Moreover, lack of appropriate LED light arrangement (distribution, positioning above and below, and angle) in a growth chamber has resulted in reduced leaf area under B LEDs compared with W LEDs in lettuce [43]. Figure 1(B1) shows that leaves grown under the W LEDs had a greater leaf area than the B LED-treated leaves. LEDs may promote cell expansion or division, increasing leaf size, although the effect of B LEDs on leaf area is crop specific. Dougher and Bugbee [44] and Wang et al. [45] tested the effect of a B light on leaf area in lettuce and *Cucumis sativus*, respectively, and found the maximum leaf area in leaves subjected to B-light treatments. Chlorophyll content receives much attention because it is involved in light absorption and leaf photosynthesis. Generally, chlorophyll absorbs B and R light and additional B LEDs increased the chlorophyll content in leaves more than other LED lights because chlorophyll absorbs light from B LEDs at 440 to 470 nm [46]. Consequently, chlorophyll *a* and *b* molecules in the B LED-treated leaves may absorb light in a different ratio than the other LED treatments (Figure S1) [47,48]. Our results indicated that leaves grown under the B LEDs had a higher chlorophyll content (Figure 1(B2)), which may account for their increased photosynthetic rate.

LED light is likely a key controller of leaf photosynthetic capacity and growth in a controlled environment. In general, B and R LEDs have a combined effect on photosynthesis at different

wavelengths [17,49]. Increasing the percentage of blue light and light irradiance results in increased photosynthesis, as reported by Hogewoning et al. [17]. In this study, higher *A* occurred under the B LEDs at higher PPFDs, followed by the W LEDs and R LEDs (Figure 2A), with the greatest differences at the highest PPFDs (Figure S1, Figure 2A). A reduction in quantum efficiency (PSII) in the B LED-treated leaves may activate energy distribution in the photosystem, increase non-photochemical quenching (NPQ), and protect the leaf from LED-light injury [16,50]. In contrast, a lower rate of photosynthesis was found in R LED-treated leaves in lettuce [51], rice [52], and wheat [53]. Consequently, the lower rate of photosynthesis occurred due to low N-content of leaves under R LEDs with lower chlorophyll content [17]. At low PPFD (250 μmol m^{-2} s^{-1}) under B LEDs, a higher photosynthetic light compensation point was observed compared with the other treatments (Figure 2A). Low light may result in lower chlorophyll content and consequently low light absorption, which means fewer electrons are excited; this results in fewer electrons passing down the two electron transport chains (PSI and PSII) during photosynthesis. Consequently, it appears that in the plants grown under B LEDs, leaf morphology (Figure 1A), chlorophyll content (Figure 1(B2)), and palisade mesophyll cell size (Figure 4B) resulted in better photosynthetic functioning in response to low light conditions compared with the plants grown under R and W LEDs. The current observation is supported by a recent report by Lanoue et al. [54], who stated that low light intensity under monochromatic LED lights results in photosynthesis, but there was no significant difference among the treatments.

Stomatal conductance (g_s), a photosynthesis parameter, is a bridge for gas exchange and water transpiration, and is significantly correlated with the light spectrum (Figure 3) [53,55,56]. CO_2 diffuses through the leaf stomata to be used for photosynthesis. B LEDs increase g_s because blue light is perceived directly by blue-light photoreceptors (phototropin and cryptochrome), activating a signaling cascade that results in fast stomatal opening under background R [19,57]. Our results also showed that g_s increased in leaves grown under B LEDs compared with leaves grown under R and W LEDs (Figure 2B). In contrast, g_s in R LED-treated leaves decreased when the *A* and demand for *Ci* were high [19]. W LEDs may also affect g_s regulation. Several other studies have shown a positive correlation between the proportion of B light and g_s values [17,58]. In this study, the increase in the *A* in the leaves under B LEDs led to a decrease in C_i (Figure 4D) and to an increase in *E* and g_s (Figure 2B,C). The relationship between photosynthesis and C_i may also affect the diffusion of CO_2 in response to the light intensity under LED treatments. In the B LED-treated leaves, a low PPFD (250 μmol m^{-2} s^{-1}) led to increased C_i compared with the R and W LEDs, whereas *A* decreased at low PPFD (Figure 2A,D). We propose that increasing C_i may limit RuBP regulation under low PPFD (250 μmol m^{-2} s^{-1}) in plants treated with B LEDs. Thus, RuBisCO regulation in the mesophyll cells (Figure 4B), and stomatal shape, aperture, and density (Figure 5A), may have resulted in acclimation of g_s and *A* to high CO_2 at low PPFD under B LEDs compared with the other treatments. In contrast, RuBP may be regulated differently to decrease C_i at higher light intensity under LEDs for *Eustoma* leaves. Whiteman and Koller [59] and Lawson et al. [60] also examined stomatal and mesophyll cell responses to CO_2 and irradiance, concluding that stomata were more likely to respond to C_i. In addition, the rate of diffusion of CO_2 into or out of the leaf depends on the partial pressure gradient and the resistance of the diffusion pathway from outside the leaf to the site of fixation in the chloroplasts [61]. Notably, the leaves grown under W LEDs showed a higher transpiration at 250 μmol m^{-2} s^{-1}, but the change in transpiration rate response to light quality was small compared with the other treatments (Figure S1 and Figure 2C). In particular, the higher A observed supported the growth of *Eustoma* leaves from our results [28]. It is well established that g_s correlates with the *A* [62] and a lower *E* is also related to a reduction in g_s [63]. The higher *E* under the B and W LEDs led to an increase in fresh biomass and a change in leaf shape compared with the R LEDs at higher PPFDs (Figure 1(A4),C; Figure 2C).

In the internal leaf anatomical observations, thicker and thinner adaxial and abaxial epidermal layers were found under different light intensities; a thicker adaxial layer had thicker palisade mesophyll cells and increased cell length and thickness [64]. In addition, a higher *A* was related to thicker palisade mesophyll cells, and these palisade cells are key photosynthetic sites [65].

In Figure 4A-C, our results indicated that the higher A in the B LED-treated leaves were related to thicker leaf adaxial and abaxial layers compared with the leaves treated under the R and W LEDs. Indeed, a thicker abaxial epidermal layer resulting from treatment with B LEDs may be beneficial for increasing the photosynthetic rate and CO_2 fixation of *Eustoma* leaves.

We also observed a cylindrical shape of the palisade cells that developed in response to B LED-light treatment compared with the W and R LEDs (Figure 4(B1,B2),C, Figure 2A). Kozuka et al. [66] reported that cylindrical palisade cells under blue light leads to increased photosynthesis. Phototropin 2 under B LEDs affects the development of palisade cells [66]. Other studies have found that the development of cylindrical palisade cells was promoted by blue light in *Pelargonium zonale* [67] and *Alternanthera brasiliana* [68]. In this study, palisade cells under the W LEDs also resulted in a mostly cylindrical cell shape that had higher A compared with the R LEDs (Figure 4B and Figure S1). However, it remains unclear from our results to what extent the shape of LED-treated palisade cells contributed to A. The difference might be too small to be detected with the methods used in this study.

Figure 6 shows stomatal aperture response to light intensity and quality, the size of which is determined by the accumulation of K^+ salts and/or sugars in the guard cells resulting in reduced water potential, uptake of water into the cell via osmosis, and photosynthesis in the leaves [19,69,70]. Stomatal aperture is also larger under B LEDs than R and W LEDs [19,71]. In general, elongation of the guard cell is subject to widening of the stomatal opening [72], and loss of water from the guard cell drives stomatal closure [73]. We found that the increase in guard cell length, width, and area in the leaves treated with B LEDs relative to the other LEDs may be associated with stomatal conductance (Figure 5(A1–A3,b1–b3); Figure S1). In addition, a larger stomatal aperture was observed in the leaves grown under the B LEDs than in the leaves grown under R and W LEDs, as shown in Figure S1 and Figure 5B. Stomatal density increased in the leaves treated with the B LEDs compared with the other LEDs (Figure 5(B1–B3); Figure S1). However, stomatal density in the R LED-treated leaves was lower (Figure 5(A4,B2)), which may be related to the lower water loss during transpiration in the plants and leaf edge curling. Therefore, the higher light intensity with the B LEDs increases stomatal density [50,74] and may enhance the photosynthetic rate and stomatal conductance in the early growth stage of *Eustoma* leaves.

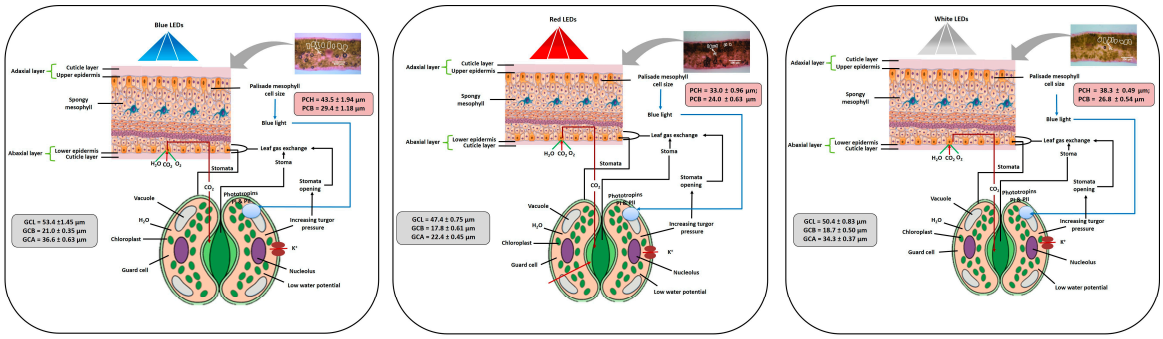

Figure 6. Hypothetical representation of leaf gas exchange, photosynthetic performance, epidermal layer thickness, palisade cell, and stomatal opening traits under blue, red and white LED light. PCH = palisade cell height, PCB = palisade cell breadth, GCL = guard cell length, GCB = guard cell breadth and GCA = guard cell area. Palisade cell image bar = 100 µm.

5. Conclusions

From a practical standpoint, B LEDs, compared with R and W LEDs, provoked both a qualitative signaling effect enabling greater photosynthetic functioning, A, g_s, E, and C_i, in *Eustoma* leaves under a high photon flux density (PPFD), and a quantitative response stimulating leaf phenotype and anatomy normally associated with acclimation to different LEDs. Leaf phenotype was influenced more by B LEDs than those grown under the R and W LEDs. However, single leaf fresh weight was higher in

the plants treated with the B and W LEDs than the R LEDs., Chlorophyll content in the B LED-treated leaves was highest. Further, we observed that the B LEDs were associated with thicker leaf abaxial epidermal layers that may be related to greater leaf A and apparent enhancement of g_s. In addition, the color and wavelength of the light altered stomatal characters such as length, width, and area of guard cells, and stomatal density (Figure 6). The B LED light also promoted more cylindrical palisade cell development than other LEDs, which may have led to increased A and higher chlorophyll content. Further studies are needed to elucidate the specific genetic mechanisms of LED responses of *Eustoma* leaves.

Supplementary Materials: Figure S1 is available online at www.mdpi.com/2311-7524/3/4/50/s1.

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Abbreviations

LED	Light-emitting diode
B	Blue
R	Red
W	White
A	Photosynthetic rate
g_s	Stomatal conductance
E	Transpiration
C_i	Intercellular CO_2 concentration
PPFD	Photosynthetic photon flux density
PSI and PSII	Photosystem I and II
NPQ	Non-photochemical quenching
RuBP	Ribulose 1,5-biphosphate
RuBisCO	Ribulose-1,5-bisphosphate carboxylase oxygenase

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