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In vitro seedlings of *Eustoma grandiflorum* in response to LED light in an acclimation environment

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Abstract: Transferring *in vitro*-cultured *Eustoma* seedlings to an *ex vitro* condition (acclimation) is a big challenge that may expose the seedlings to biotic and abiotic stresses, and affect the internal and external structure of the plants. In addition, *in vitro*-cultured seedlings of *Eustoma* are difficult to handle and phenotype and physiological traits such as survival and rosette rate may have altered in the acclimation stage. Therefore, the present study aims to examine the effects of blue, red, and white LED light on the growth and development *ex vitro* of *in vitro*-cultured seedlings of *Eustoma*. The results showed that blue LEDs resulted in greater plant height, internode length, and leaf number, increased upper and lower fresh biomass, and higher chlorophyll content compared with treatment by the other LED lights. Higher stomatal density on the abaxial leaf surface was also observed in the blue LED-treated plants, which also showed a higher survival rate and lower rosette rate. In contrast, the white LED-treated plants had the highest leaf width and internode diameter. Acclimation of the *Eustoma* plants *ex vitro* suggests that a combination of blue and white LEDs may be advantageous for better growth and development for large-scale production in a controlled environment.

Key words: *ex vitro*; internode; light-emitting diodes; stomata; rosette

1. Introduction

Acclimation of *in vitro* seedlings is a critical stage for the success of a tissue culture method. Although an *in vitro*-cultured technique is suitable for rapid production of high quality, disease-free uniform seedlings, regardless of weather and season, the transplantation stage to an acclimation environment continues to be a major constraint for the successful establishment and survival of *in vitro*-cultured seedlings. To increase growth and reduce mortality in seedlings in an acclimation environment, research has focused on a light-emitting diode (LED) system in an enclosed environment. Acclimation of plants to the LED light conditions could improve growth and reduce the energy needed for assimilation lighting through photosynthesis[1]. Acclimation under LED light may affect various aspects of plant growth, for example, plant height[2], changes in leaf size[3,4], photosynthesis[4,5], and stomatal characters[4,6], but there have been no studies on how LED light influences *in vitro*-cultured *Eustoma* seedlings in an acclimation environment in terms of growth and development, survivability, and rosette. However, light from light-emitting diodes (LEDs) has been associated with affecting the morpho-physiological characteristics of *Eustoma*[4].

LED light is an important environmental factor affecting plant development and

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growth by regulating morphological changes[7,8]. In photobiological studies, light-emitting diodes (LEDs) are now a promising narrow-band light source for space-based plant growth chambers and enclosed environments because of their small mass and size, solid-state construction, superior safety, and longevity[9,10]. Plant development is strongly influenced by light quality, which refers to the color or wavelength reaching a plant's surface[11], and a number of studies using LED lights have been performed on the effect of light spectral quality on plant growth and morphogenesis[4,12]. Blue and red LEDs have the greatest effect on plant growth because they are the major energy sources for photosynthetic CO_2 assimilation in plants[12]. Despite the increasing popularity of color LEDs as a radiation source for growing plants, information is available for only a few plant species, which directly compares growth and development in an acclimation environment. For example, blue LED light is related to 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[3,4,5]. In contrast, red LED light produces a narrow-spectrum light that regulates the root-to-shoot ratio, chlorophyll content, and photosynthetic apparatus[13,14]. In addition, plants grown under white LED light alone have regular leaf morphology and a higher photosynthetic rate compared with plants grown under red or blue light[5].

Eustoma (*Eustoma grandiflorum*) is a flowering plant originating from North America and is found in a wide range of environments. Considerable efforts have been made to optimize the conditions for the *in vitro* stages of *Eustoma* micropropagation[15,16,17], but the process of acclimation of micropropagated *Eustoma* plants to an LED light environment has not yet been fully studied. Further, the acclimation environment needs to be considered for reliable seedling growth of *Eustoma* under large-scale production using LED light to control critical parameters such as plant height, internode growth, survival percentage, and rosette rate. Plants with shortened internodes and leaf clusters, called rosettes, exhibit delayed or no flowering[18]. In addition, the concentration of sucrose in the media for *in vitro* culturing influences *ex vitro* rooting and establishment in LED light in a controlled environment[19]. In particular, LED light contributes to higher growth morphology and physiology at the acclimation stage *ex vitro*[20]. To determine the acclimation performance due to LED light, in this study we examined plant growth in a walk-in-type growth chamber with differing LEDs light quality. Therefore, the objectives of this study were to evaluate the effects of LED light on the growth, survival, and rosette rate of *in vitro*-cultured *Eustoma* seedlings in an acclimation environment *ex vitro*.

2. Materials and Methods

2.1. Plant materials and growth conditions

The experiment was conducted in the Laboratory of Floriculture and Vegetables, Kochi University, Japan, to evaluate *in vitro*-grown *Eustoma* seedlings in the acclimatization stage under different LED lights *ex vitro*. *Eustoma* (Voyage type-2 pink) seeds (*Eustoma grandiflorum*) were used in this experiment; well-matured and dry seeds were collected from Sakata Seed Cooperation, Japan. The surface-sterilized seeds were sown on Murashige and Skoog (MS) medium[21] with half-strength media of macro and micro salts and 30.0 g/L (3%) sucrose concentration[17]. After 8 weeks culturing, 30 seedlings with four pairs of true leaves were removed from the UM culture bottles (As One, Japan) and washed carefully in running water. The *in vitro*-cultured seedlings were quickly transferred to a phytotron for healing of seedlings in the hardening stage. Before transplanting the *in vitro* seedlings, the temperature was kept at 23/18°C (day/night) to maintain the growth conditions in the phytotron. Consequently, the phytotron was kept under 60–70% relative humidity and a photoperiod of 16/8h (light/dark) was maintained by using artificial fluorescent light[22]. Cultured seedlings were transferred to plastic pots (6 × 7 cm) with soil medium (Tanekura No. 42; Sumirin Agricultural Industry Co.

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Ltd., Japan). After 2 days, the 30 seedlings were transferred to a walk-in-type environment-controlled growth chamber with LED light (fabricated environment-controlled growth chamber; Nikkan Co. Ltd., Japan).

2.2 LED light in an acclimation environment

The effects of the LEDs in the acclimation stage were determined by treating seedlings cultured *in vitro* in half-strength medium in a walk-in type environment-controlled growth chamber with LED light. Seedlings were watered daily. The LED lights were positioned 25 cm above the seedlings in each LED-light growth chamber to ensure maximum irradiance from the LEDs. The seedlings were subjected to blue, red, and white LED tube lights (Tubular LED light; Beam Tech Co. Ltd., Japan). The LEDs provided blue, red, and white light with wavelengths of 420–550, 580–670, and 420–750 nm, respectively (Figure 1; Light Analyzer, LA-105; NK-System, Japan). Air temperature was 22/18°C during the photo and dark period. Photoperiod, relative humidity, and CO_2 concentration were 16/8h (day/night), 65%, and 400 µmol/mol, respectively[4]. After 45 days' *ex vitro* growth, data on plant height, fresh shoot and root weight, and survivability rate were collected from the seedlings grown under the different LED light treatments. Chlorophyll content was estimated using a chlorophyll meter (SPAD-502; Minolta, Osaka, Japan). Plants with shortened internodes and leaf clusters, called rosettes, exhibit delayed or no flowering. The rosette rate of *Eustoma* plants[18] were observed under different LED lights in an acclimation environment.

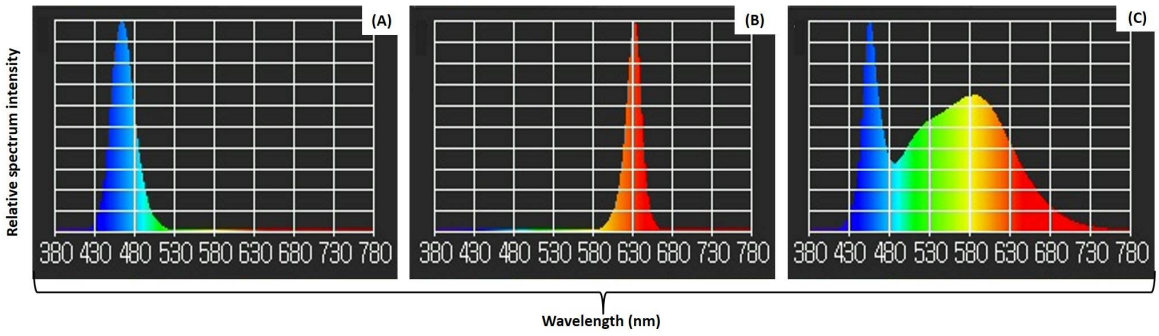

Figure 1. Distributions of relative spectrum intensity of LED light: (A) blue; (B) red; and (C) white.

2.3. Stomata observation

Mature leaf samples were collected from the 45-day-old plants grown under the blue, red, and white LEDs 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[4,23]. Leaf of independent LEDs was fixed in Toluidin Blue (Sigma Aldrich, USA) for 30 s. To observe the stomata, transparent fingernail polish was smeared on the lower epidermis of the fully expanded leaves and allowed to dry for 5–10 min. The slides were made using the leaf epidermal fingerprint with transparent nail polish method[24]. Clear cellophane tape was fixed over the section of nail polish and carefully peeled from the leaf, and the 'leaf impression' was transferred to a microscope slide. Imprints were observed under a light microscope (Olympus DX-50; Olympus, Tokyo, Japan) equipped with a digital microscope camera (Olympus DP-12; Tokyo, Japan) at a magnification of 200×.

2.4. Statistical analysis

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For each LED treatment, there were five replications and the results were expressed as mean ± standard error (SE). For all comparisons, statistical analysis was performed using one-way ANOVA followed by Tukey's test, and $p < 0.05$ was considered statistically significant. The graphs were prepared using KaleidaGraph-4.5.0 (Synergy Software, USA).

3. Results and Discussion

3.1. Effect of LEDs on seedling growth ex vitro

The different LED light qualities influenced the growth traits in the acclimation environment of *Eustoma ex vitro*. Plant height, leaf number, leaf length, and leaf width differed significantly according to the LED light treatments *ex vitro* (Figure 2). The tallest plant (11.3 ± 0.34 cm) resulted from blue LED treatment, and the mean height of the plants differed significantly among the blue, red, and white LED lights (Figure 2A and Figure 3). The highest number of leaves (15.2 ± 0.37) was found in the plants grown under the blue LEDs, and the lowest number of leaves resulted from white LED treatment (Figure 2B). Leaf length (4.3 ± 0.17 cm) and width (2.0 ± 0.08 cm) were greater in the plants grown under the white LED light compared with the other treatments, but there was no significant difference in leaf length between the blue and white LED treatments (Figure 2C–D).

Blue LED light may function to activate the cryptochromes and phytotropin that etiolated the stem length of *Eustoma ex vitro*. Shimazaki et al.[25] and Wang et al.[26] found that this wavelength activates the action of cryptochromes, so stem growth is maintained. It has also been found that exclusively using blue light induces increased stem elongation in petunia[27] and sunflower[28] compared with other narrow-band wavelengths. In contrast, the effect of red light on stem elongation depends on the presence of phytochrome[29]. As a consequence, phytochrome, red light receptor, is responsible for photomorphogenesis or plant movement, which regulates the elongation of stems in plants grown under red LED light[30]. Figure 2A shows that the seedlings treated with blue light were the tallest. Furthermore, blue and white LED light subject to develop leaf size of *Eustoma* plants in the early growth stage[4], which may be a response to normal photosynthetic function in leaves[31]. In particular, *Eustoma* leaves grow faster under white LED compared to blue and red LEDs light because photosynthetic performance under white LED light leads to vigorous growth[32]. In the blue LED light-treated leaves, suppression of gibberellin (GA) biosynthetic-related genes and induction of the GA inactivation-related genes has been reported, which constrains the elongation of rice leaves[33]. These results indicate that blue LEDs increase leaf number and leaf width of *Eustoma* under *ex vitro* acclimation; however, there was no significant difference in leaf length between the blue and white LEDs (Figure 2A–D).

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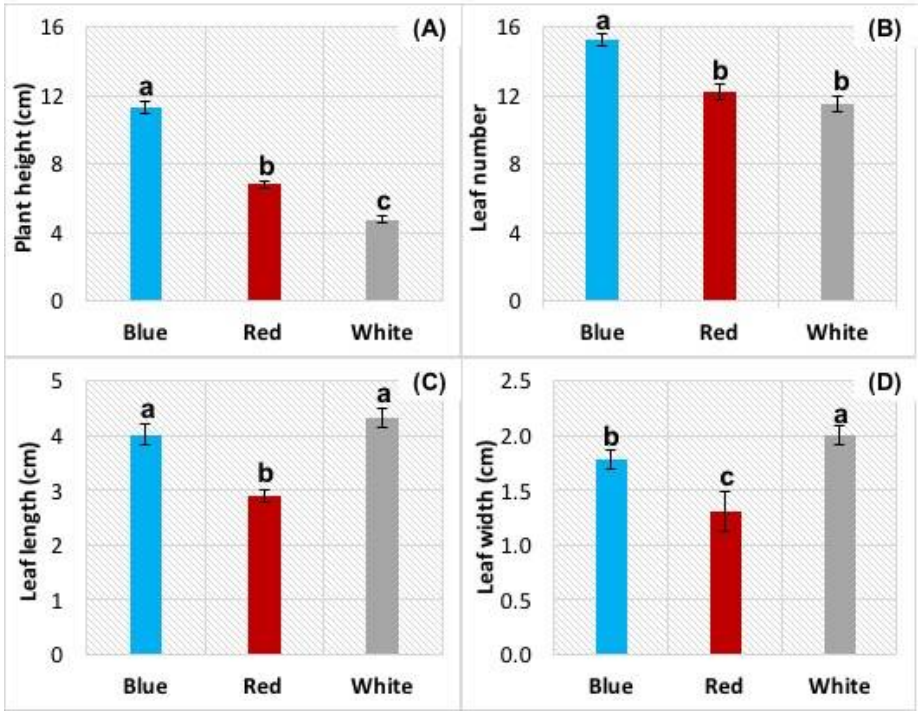

Figure 2. Effect of blue, red, and white LED lights on the growth and morphology of *Eustoma ex vitro* for acclimation. Data are mean values (n = 5) and the vertical bars represent ± SE (Tukey's HSD at $p < 0.05$).

Figure 3. Effect of LED light on the growth and morphology of *Eustoma ex vitro* for acclimation: (A) blue; (B) red; and (C) white.

3.2. Effect of LEDs on seedling growth and physiological traits ex vitro

At 45 days *ex vitro* under LED light, the influence of blue, red, and white LED light resulted in significant variation in seedling growth and physiological traits (Figure 4). Internode length (2.2 ± 0.09 cm) was higher in the plants treated with blue LEDs compared with the other treatments (Figure 4A). In contrast, the plants grown under the white LEDs showed greater internode width (2.0 ± 0.07 mm) than the plants grown under the blue and red LEDs *ex vitro* (Figure 4B). The plants grown under the blue LEDs had a higher chlorophyll content (42.2 ± 0.78) than the plants grown under the other treatments (Figure 4C). Overall, stomatal density (58.4 ± 1.32 mm^2) was higher in the blue LED-treated *Eustoma* leaves than in the plants grown under the other LED treatments (Figure 4D and 5).

Generally, plants grown in a blue light-rich environment have increased photosynthesis in response to stomatal character compared with plants grown under other conditions[4,34,35]. An elongated or shorter internode is a response to cryptochrome-mediated blue light effects[36].

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Several studies have already reported that blue light leads to elongated internodes[25,26]. However, blue light increases internode elongation in the presence of far-red light, as studied by Gautam et al.[28]. In addition, cell enlargement of *in vitro*-grown potato results in increased internode size under blue LED light compared with plants grown under red LED light[37]. Generally, supplemental blue light increases chlorophyll content in leaves more than other LED lights (Figure 4C) and shows the relationship between blue light and leaf chlorophyll content[4,38] because chlorophyll absorbs light from blue LEDs at 440 to 470 nm[39]. Consequently, chlorophyll *a* and *b* molecules in blue LED-treated leaves may absorb light in a different ratio than under other LED treatments[40].

Stomatal development is influenced by light quality, which in turn influences stomatal conductance (g_s) of air through the leaf mesophyll and stomata. The higher light intensity with the blue LEDs increases stomatal density[41] and incrementally increases the photosynthetic rate and stomatal conductance in the early growth stage of *Eustoma* leaves[4]. Increased stomatal density of chrysanthemum leaves under blue light was also observed by Kim et al.[42]. Further, we observed that stomatal density was higher in the blue-LED treated *Eustoma* seedlings at 45 days *ex vitro* (Figure 4D), which could provide better photosynthetic performance in an acclimation environment. The results show that the seedlings grown under blue LED light had enhanced internode length, chlorophyll content, and stomatal density during *ex vitro* establishment.

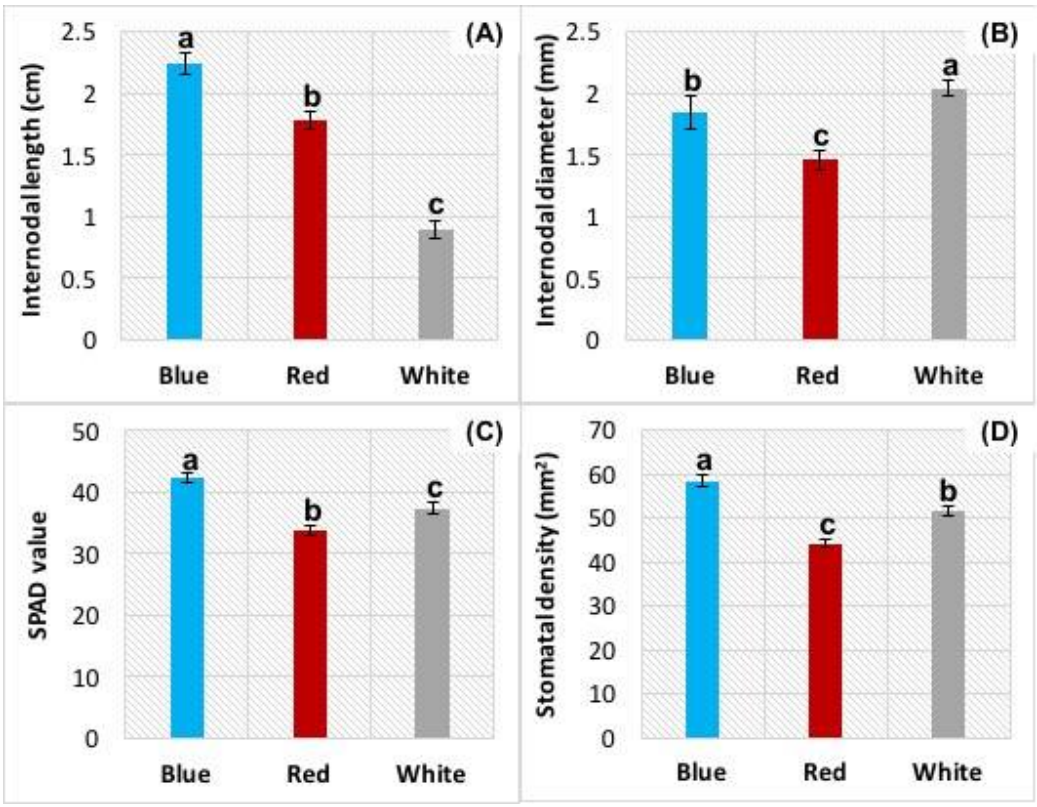

Figure 4. Effect of blue, red, and white LED lights on the growth and physiology traits of *Eustoma ex vitro* for acclimation. Data are mean values (n = 5) and the vertical bars represent ± SE (Tukey's HSD at $p < 0.05$).

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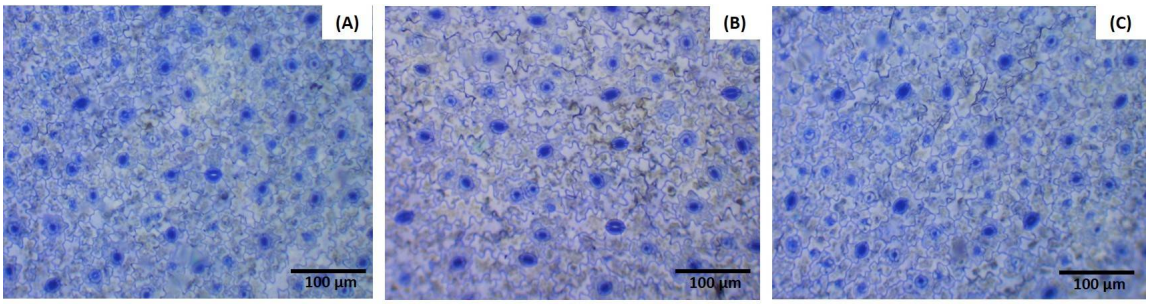

Figure 5. Differences in anatomical parameters of the abaxial layer of stomata in *Eustoma* leaves grown under different LEDs from representative cross-sections: (A) blue; (B) red; and (C) white.

3.3. Effect of LEDs on fresh biomass, survival rate, and rosette rate ex vitro

The establishment of *in vitro* seedlings *ex vitro* is related to biomass production for acclimation. The blue, red, and white LED lights significantly affected the biomass production, and rosette rate of *Eustoma* under *ex vitro* establishment; however, no significant results found in survival rate (Figure 6). The higher amount of upper (236.8 ± 3.63 mg) and lower (165.4 ± 4.38 mg) fresh biomass was found in the seedlings treated with blue LED light compared with the other LED treatments (Figure 6A–B). In other words, the root:shoot ratio (1.6 ± 0.06) was also higher in the blue LED-treated plants in the acclimation environment, but the red and white LEDs did not significantly affect the root:shoot ratio (Figure 6C). The survival rate was highest (91.8 ± 0.78%) and the rosette rate was lowest (22.7 ± 1.12%) in the plants grown under the blue LED light at 45 days after establishment *ex vitro* compared with the other treatments (Figure 6D–E). However, there was no significant variation in survival rate among the plants grown under the blue, red, and white LED lights.

The blue light determines to perceive the cryptochrome that increases the upper and lower fresh biomass and root:shoot ratio compared with the red and white LED light[11,37]. Additionally, exposure to the red LED light decreased the fresh biomass compared with the other LEDs, and there was a significant difference in fresh biomass among the LED light treatments (Figure 6A–B). However, partitioning of blue light increases the upper fresh biomass for other processes, possibly leaf size or the production of carbohydrates[43]. Therefore, our results indicate that the blue light-treated seedlings *ex vitro* showed more chlorophyll content (Figure 4C). Chlorophyll content receives much attention because it is involved in light absorption and *Eustoma* leaf photosynthesis[4], which is used for better plant stature establishment such as biomass production, and results in a higher survival rate and lower rosette rate in an acclimation environment.

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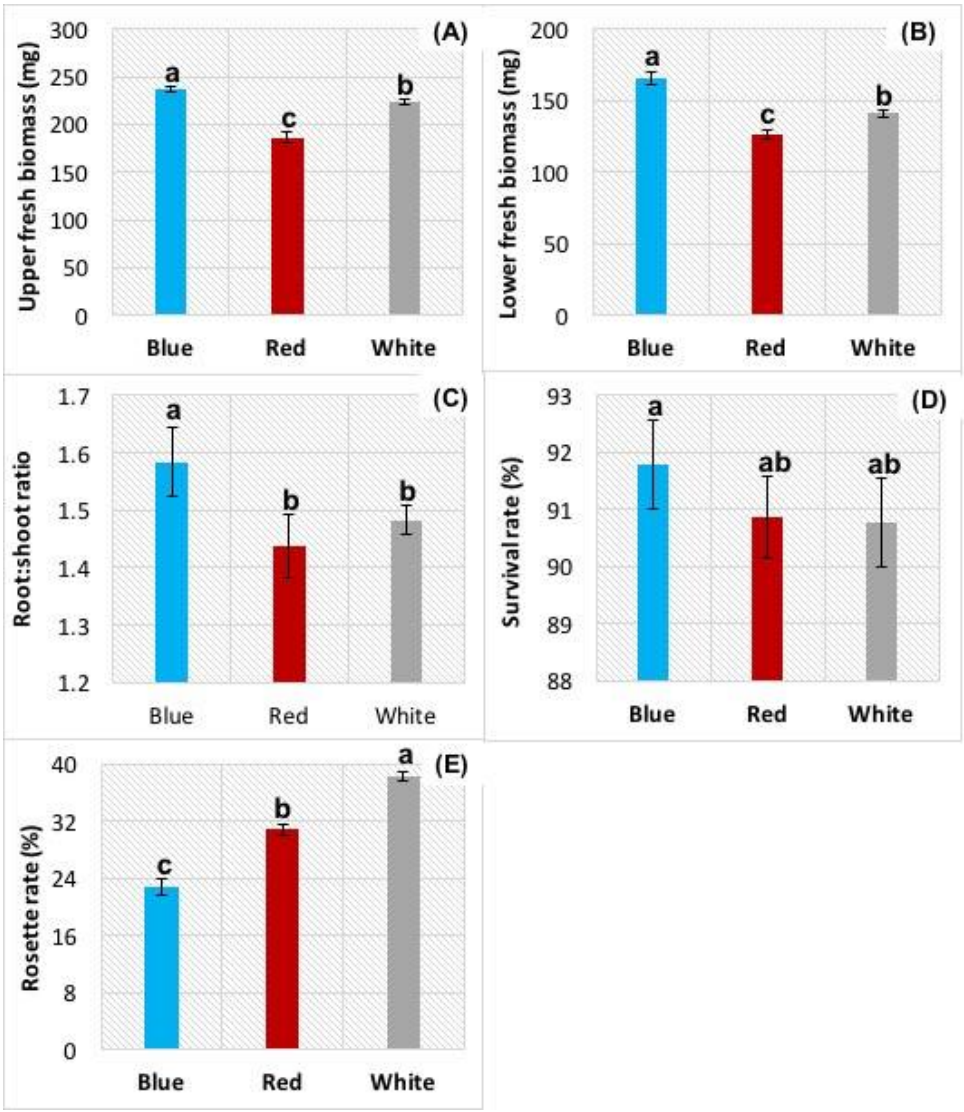

Figure 6. Effect of blue, red, and white LED lights on the growth biomass, survival, and rosette rate traits of Eustoma *ex vitro* for acclimation. Data are mean values (n = 5) and the vertical bars represent ± SE (Tukey's HSD at $p < 0.05$).

4. Conclusions

In conclusion, the results indicate that *in vitro*-developed *Eustoma* seedlings may be beneficially affected after transplanting to an acclimation environment under blue LED light *ex vitro*. *Eustoma* growth characters including improved plant stature, internode growth, fresh biomass, and lower rosette rate were found to be optimal in response to higher stomatal character and chlorophyll content under blue LED light *ex vitro*. In addition, white LED light showed better effects on leaf width and internode diameter. Therefore, our results also suggest that a combination of blue and white LED lights may positively effect on morpho-physiological performance in an acclimation environment. Moreover, these measurable features may still be amenable for detecting more subtle light source differences that will support a more direct testing of *in vitro* seedlings difference effects detected by LED light on plant growth.

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Author Contributions: I state on behalf of all the co-authors that all authors contributed equally to this work. Md Zohurul Kadir Roni conducted and designed the experiments, analyzed the data, and wrote the manuscript; Md Saiful Islam observed the experiments and revised the manuscript, and Kazuhiko Shimasaki supervised the analysis and edited the manuscript. All authors discussed the results and implications and commented on the manuscript at all stages.

Conflicts of Interest: The authors declare that there are no conflicts of interest.

Abbreviations

LED = Light-emitting diode, GA = Gibberellin acid, g_s = Stomatal conductance,

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