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Growth and Acclimation of In Vitro-Propagated M9 Apple Rootstock Plantlets under Various Visible Light Spectrums

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Abstract: This study aimed to explore the suitable light quality condition for ex vitro acclimation of M9 apple plantlets. Light quality treatments were set as followed; monochromatic LEDs (red (R), green (G), blue (B)) and polychromatic LEDs (R:B = 7:3, 8:2 and 9:1; R:G:B = 6:1:3, 7:1:2 and 8:1:1). Plant height of R, R9B1, and R8G1B1 treatments were significantly higher than the other treatments. The number of leaves and SPAD value of B were significantly higher than the other treatments. Root fresh weights of R9B1 and R7G1B2 treatments showed an increase of at least 1.7-times compared to R, G and R8B2. R8G1B1 accumulated higher starch contents than the other treatments. Photosynthetic rate of R9B1 and R8B2 were significantly higher than the other treatments. In terms of stomatal conductance and transpiration rate, treatments with high blue ratio such as B, R7B3 had higher values. Rubisco concentration was high in R and B among monochromatic treatments. In conclusion, red light was effective to increase photosynthetic rate and biomass and blue light increased chlorophyll content and stomatal conductance. Therefore, for R9B1 and R8G1B1, a mixture of high ratio of red light with a little blue light would be proper for the acclimation of in vitro-propagated apple rootstock M9 plantlets to an ex vitro environment.

Keywords: LED; light quality; ex vitro condition; photosynthetic rate; starch; biomass; Rubisco

1. Introduction

Plant tissue culture is an effective method for mass production of virus-free plantlets [1]. However, typical environmental characteristics of in vitro such as high relative humidity, low light intensity, and artificial supply of sugar and growth regulator through culture medium are major factors that are associated with low survival rates in transplanting virus-free plantlets to ex vitro [2]. Thus, acclimation process for a certain period is required for a successful survival in ex vitro conditions [3]. Acclimation refers to the manipulation of ex vitro environment to ensure that plantlets have resistance to harsher conditions compared to in vitro conditions without fatal growth impediment [4]. Factors affecting acclimation include light, humidity, and carbohydrate concentration of culture medium [5].

Among them, light is a crucial factor affecting photomorphogenesis and photosynthesis of plants, which have a significant influence on the acclimation of the in vitro-propagated plantlets. For example, in previous studies related to acclimation of in vitro-propagated plantlets, the effect of light intensities on photosynthetic capacity and the activity of enzymatic antioxidants have been examined in various crops [6–9] but the studies showing the effect of light quality are limited. In general, red light induces the accumulation of biomass and stem elongation [10], while blue light induces changes in

stomatal development, density and opening [11,12]. Green light is reported to affect plant morphology, metabolisms and photosynthesis [13,14]. Therefore, it is considered that light quality as well as light intensity affects the acclimation of in vitro-propagated plantlets. Light quality studies in in vitro conditions have been carried out [15–17], but there are few studies related to ex vitro light quality of in vitro-propagated plantlets.

Apple trees are one of the most important fruit crops (83.1 million ton/year) in the temperate regions [18]. It has self-incompatibility and a long juvenile period when reproductive development does not occur [19], so that a vegetative propagation method, layering, is used for mass propagation of apple rootstock seedlings. This conventional propagation method may be sensitive to virus infection and result in low growth rate according to season [20]. Micropropagation of apple plantlets overcame the problems of conventional propagation methods and enabled rapid growth of virus-free fruit trees on a commercial scale [21]. Webster and Jones [22] reported that direct rooting of in vitro-propagated M9 in the greenhouse showed a higher rooting rate than that of in-vitro rooting. Moreover, Modgil et al. [23] conducted a study on the acclimation of M7 and MM106 cultivars according to growing medium in greenhouses. Studies using mycorrhizal fungi have also been carried out to increase the acclimation rate of apple plantlets [24–26]. Recently, in our previous studies several environmental factors like relative humidity, growing medium and nutrient solution conditions for successful survival and acclimation of in vitro-propagated M9 apple dwarf rootstocks have been established [27,28]. However, there are still limited numbers of studies on light condition for acclimation of in vitro-propagated plantlets although there are lots of studies on the effect of light quality of leafy vegetable in indoor conditions like plant factories with artificial lighting. The effect of light period and its interaction with growth regulators on the growth and development of apple plantlets was investigated [29,30]. In addition, the studies on the light quality have only confirmed the response of plants to monochromatic light using the fluorescent lamp with polyester filter, and few studies have reported the changes of the plantlet in response to mixed light [31,32]. To the best of our knowledge, there have been no studies that investigated acclimation of in vitro-propagated apple plantlets in closed-type plant factories.

Thus, in this study, the primary objective of the present study was to determine the effect of ex vitro light quality using red, green and blue light-emitting diodes (LEDs) on the growth, photosynthesis and primary metabolism of in vitro-propagated 'M9' apple plantlets during the acclimation process. The information gained from this study would be useful to set up light conditions of in vitro-propagated apple plantlets and provide valuable insights into the research on light quality, an essential factor affecting acclimation in a closed-type plant production system. Besides, this environmental information may apply to vertical agriculture, which is future high-tech agriculture, and may help facilitate the acclimation of other virus-free seedlings, including fruit tree seedlings.

2. Materials and Methods

2.1. Plant Materials and Acclimation Conditions

Apical meristems of mature M9 apple trees, which are not infected with viruses, were cultured in MS medium containing 0.5 mg·L^{-1} IBA, 30 g·L^{-1} sucrose, and 8.4 g·L^{-1} agar for 8 weeks to multiply in vitro-propagated apple plantlets. It was then incubated for 12 weeks in the rooting medium supplemented with 0.5 mg·L^{-1} IBA, 30 g·L^{-1} sucrose and 8.4 g·L^{-1} agar on MS medium. Thereafter, the rubber stopper equipped with the ventilation filter of the culture flask was partly opened and preliminary acclimation was fulfilled at 25 °C and the relative humidity of 90%. Thirty apple plantlets per treatment were transplanted into the deep flow technique (DFT) system, which was judged to be suitable for acclimation and growth of in vitro-propagated apple plantlets and a newly developed nutrient solution for apple plantlets (pH 6.0, EC 0.5 dS·m^{-1} to 2.0 dS·m^{-1} at 4 weeks) was supplied to the apple plantlets [28]. The plantlets were grown in a closed plant production system under environmental conditions at 25 °C, 16 h in photoperiod, 100 µmol·m^{-2}·s^{-1} and reduced relative

humidity by 90% → 80% → 60% for 6 weeks after transplanting to ex vitro conditions [27]. The plantlets were rotated clockwise every two days during the experiment due to uneven light distribution.

2.2. Light Treatments

Red (R, 657 nm), green (G, 524 nm) and blue (B, 448 nm) monochromatic LEDs (Bissol led, Seoul, Korea), three RB mixed LEDs (R:B = 9:1, 8:2, 7:3) and three RGB mixed LEDs (R:G:B = 8:1:1, 7:1:2, 6:1:3) were treated (Figure 1). Light intensity was measured using a quantum sensor (LI-1400; Li-Cor, Lincoln, NE, USA) in the plot (70 m × 110 m, L × W) of each light quality treatment. The plot was also divided into 12 points and the average of point values was set to 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ using the adjustment of radiation height and LED number. The light spectrum of each treatment was measured by a spectroradiometer (Jaz-EL 200; Ocean Optics, Dunedin, FL, USA) (Table 1).

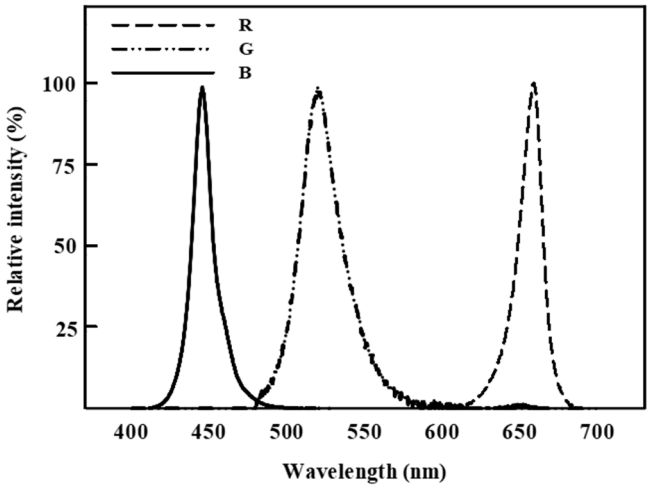

Figure 1. Relative spectral distribution of monochromatic red (R), green (G), and blue (B) LEDs.

Table 1. Spectral data for monochromatic and various combined ratios of red (R) to blue (B), and RB with green (G) light emitting diodes (LEDs).

Light Source	PPFD (400–700 nm)	Fraction(%) [z]		
		Red (600–700 nm)	Green (500–600 nm)	Blue (400–500 nm)
R	100	100	0	0
G	100	0	100	0
B	100	0	0	100
R9B1	100	90	0	10
R8B2	100	80	0	20
R7B3	100	70	0	30
R8G1B1	100	81	10	9
R7G1B2	100	70	9	21
R6G1B3	100	59	10	31

[z] Fraction of red, green, and blue wavelengths in terms of photosynthetic photon flux density (PPFD).

2.3. Growth Characteristics

The growth characteristics of in vitro-propagated apple plantlets acclimated under different light quality treatments were compared. Plant height, stem diameter, shoot and root fresh weights, SPAD value (chlorophyll content index) and survival rate were measured at 6 weeks after transplantation. Plant height and stem diameter on the basal and 4th leaf from apical meristem were measured using a ruler and a digital Vernier caliper (NA530-300S; Bluebird, Seoul, Korea). Fresh weight of shoot and root were measured with an electronic scale (SI-234; Denver Instrument, Denver, CO, USA). The SPAD

value of the 3rd or 4th leaf from an apical meristem was measured by a portable chlorophyll meter (SPAD-502; Konica Minolta, Tokyo, Japan).

2.4. Photosynthetic Rate

Photosynthetic rate, stomatal conductance, and transpiration rate of apple plantlets were measured using a portable photosynthesis device (LI-6400; Li-Cor, Lincoln, NE, USA) equipped with a clear chamber bottom (2 × 3 cm) in a standard leaf chamber (LI-6400-40; Li-Cor, Lincoln, NE, USA) at 5 weeks after transplantation. The measurement was performed from 9:00 to 13:00, two hours after the light was on. The measurement conditions were set similarly to the cultivation environment; air flow rate 400 μmol·s^{-1}, CO_2 concentration 500 μmol·mol^{-1}, relative humidity 70% and block temperature 25 °C. The measurement was conducted under each lighting source at the light intensity of 100 μmol·m^{-2}·s^{-1}.

2.5. Rubisco Concentration

Fresh leaf sample of 0.5 g was lyophilized with liquid nitrogen and stored at −70 °C until analysis. The sample was grinded using a mortar and extracted with 5 mL of 25 mM PBS (pH 7.4) solution. The extracted sample was stored at 4 °C in a 2 mL microtube for 2 h. After then, centrifugation was carried out at 12,000× *g* and 2 °C for 20 min and supernatant of the sample (1.5 mL) was used for analysis. All reagents were prepared at room temperature before analysis. The quantitative determination of Rubisco concentration was performed using a Rubisco ELISA Kit (MBS779145; MyBioSource, San Diego, CA, USA) and the optical density of final reaction solution was measured at 450 nm by a multi-mode microplate reader (Synergy HTX; BioTek, Winooski, VT, USA).

2.6. Starch Content

Freeze-dried shoot of apple plantlets was powdered using a Tube Mill control (IKA, Wilmington, NC, USA) and stored at 4 °C until analysis. A powdery sample (0.1 g) was added to a 15 mL conical tube, mixed with 80% ethanol of 10 mL, and then vortexed. After this, centrifugation was performed at 4 °C for 10 min at 3250× *g* at the centrifuge (5810R; Eppendorf, Hamburg, Germany). The pellet was stored at −80 °C until analysis.

The starch content of the pellet was analyzed using the modified dinitrosalicylic acid method [33]. The pellet was dissolved in distilled water of 2 mL and then autoclaved at 121 °C for 30 min. The solution was mixed with 0.2 M Na-acetate (pH 5.5) buffer, 1 mL 30 U amyloglucosidase (Sigma-Aldrich) and 1 mL 10 U β-amylase (Sigma-Aldrich) and centrifuged at 13,000× *g* for 10 min. The supernatant (50 μL) of the centrifuged sample was mixed with 0.5 mL of DNS (dinitrosalicylic acid reagent) and reacted in boiling water at 100 °C for 5 min. After completely cooling, 0.9 mL of distilled water and 0.1 mL of each reaction solution were mixed and measured by absorbance at 525 nm using a spectrophotometer (UV-1800; Shimadzu, Kyoto, Japan). The starch content of each sample was shown as mg glucose (Sigma-Aldrich) per dry weights of in vitro-propagated apple plantlets.

2.7. Statistical Analysis

Each measurement parameter had five replicates per light quality treatment except for photosynthetic rate and Rubisco concentration. Four replicates were used for photosynthesis parameters and Rubisco analysis. All measured data were analyzed using the SAS program (SAS 9.4; SAS Institute, Cary, NC, USA). One-way analysis of variance (ANOVA) was performed and significant comparison among treatments means were conducted by Duncan's Multiple Range Test (DMRT).

3. Results

3.1. Growth Characteristics

The plant height of apple plantlets was significantly higher values for R and R9B1 with a high ratio of red light that the others at 6 weeks after transplantation (Figure 2). These treatments significantly increased about 1.3 times more than B which had the lowest value.

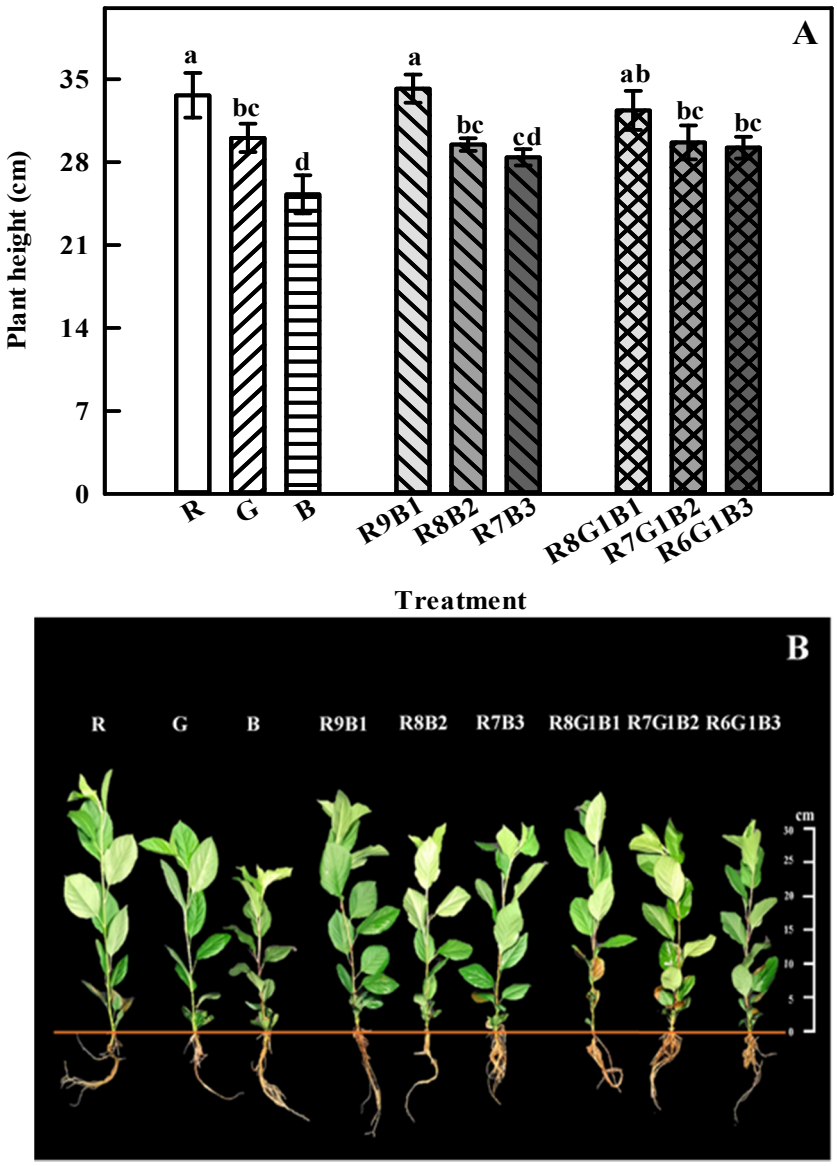

Figure 2. Plant height (**A**) and an image (**B**) of in vitro-propagated apple plantlets at 6 weeks after transplanting. Different letters indicate significant differences at p-value < 0.01 (n = 5).

In terms of stem diameter, the top indicated the part connected with the scion part and the basal part was designated at the bottom. Stem diameters at top and bottom were measured at 6 weeks after transplantation (Figure 3). Stem diameter by light quality did not show a significant difference in both parts. However, changes of stem diameter at the top tended to be similar to those of plant height. For example, R in monochromatic treatment and R9B1 and R8G1B1 in combination treatment, which were all high ratio of red light, recorded high values (Figure 3A).

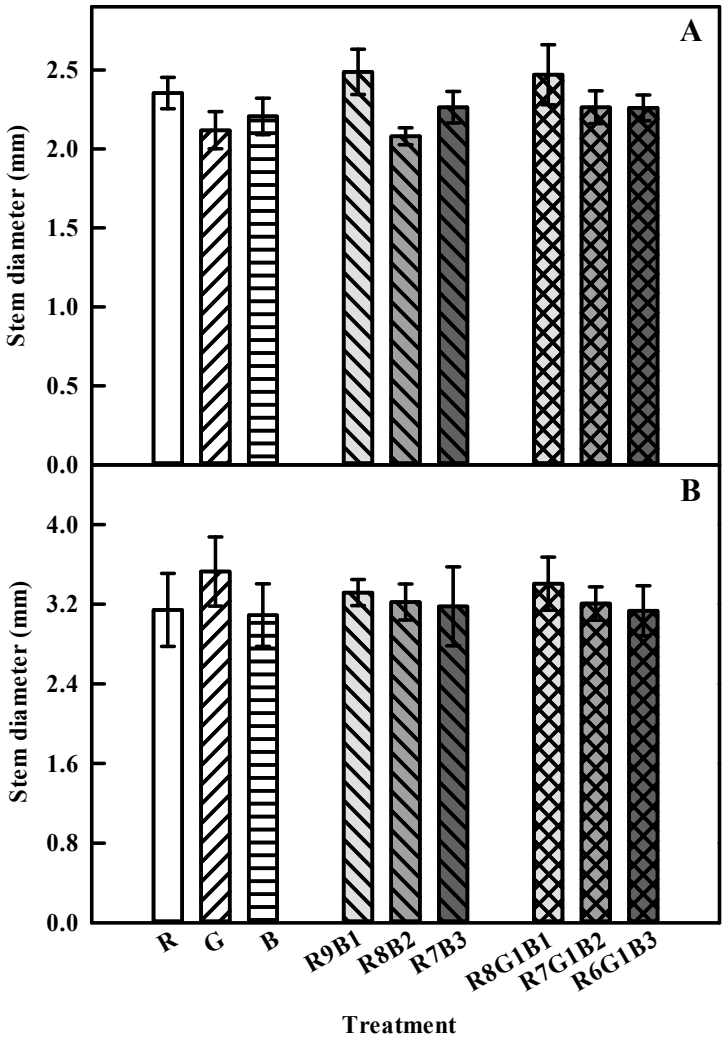

Figure 3. Stem diameter at top (**A**) and bottom (**B**) of in vitro-propagated apple plantlets acclimated under various light quality conditions at 6 weeks after transplanting. There was no statistically significant difference at p-value < 0.05 (n = 5).

The number of leaves was significantly higher in B treatment at 6 weeks after transplantation (Figure 4A). The total leaf area did not show significant difference, but R and R9B1 treatment showed high values (Figure 4B). B treatment induced the highest SPAD value and the other treatments except B showed no significant difference value at 6 weeks after transplantation (Figure 4C).

Although no significant difference between the treatments was observed in the shoot fresh weight, there was a significant difference in the shoot dry weight at $p < 0.01$. R, R9B1 and R8G1B1 showed high values, and R9B1 had a significantly 1.4-fold higher value than R8B2 (Figure 5A,C). Significant differences were also shown in root fresh and R9B1 and R7G1B2 treatments had at least 1.7 times higher values compared to R, G, and R8B2. The root dry weight also showed a similar tendency with root fresh weight and the highest values were observed in R9B1 and R7G1B2 (Figure 5B,D). Additionally, the survival rate of apple seedlings was recorded as from 83% to 96% regardless of light treatments (Figure 6).

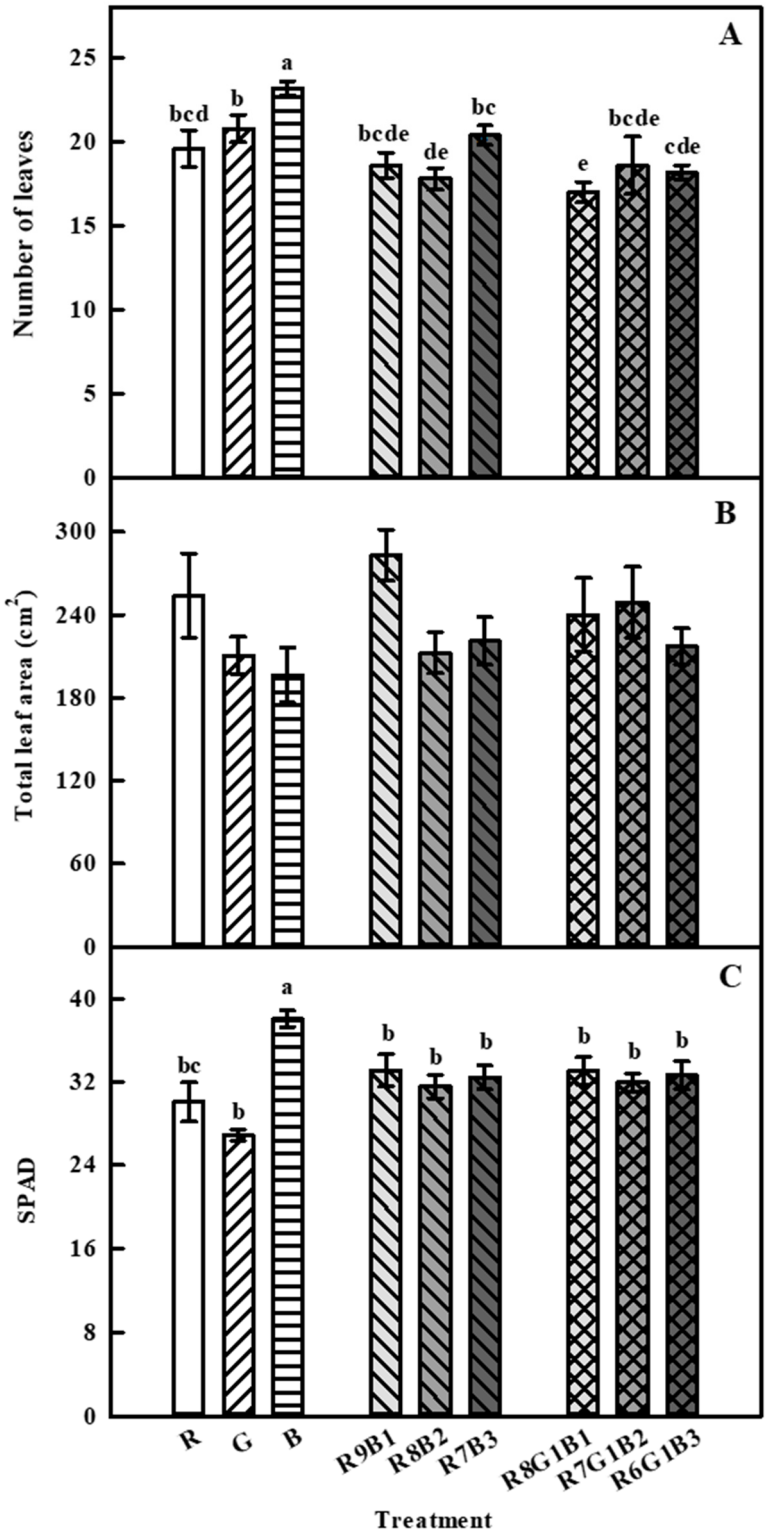

Figure 4. Number of leaves (**A**), total leaf area (**B**) and SPAD value (**C**) at 6 weeks after transplanting of in vitro-propagated apple plantlets. Different letters indicate significant differences at p-value < 0.01 (n = 5).

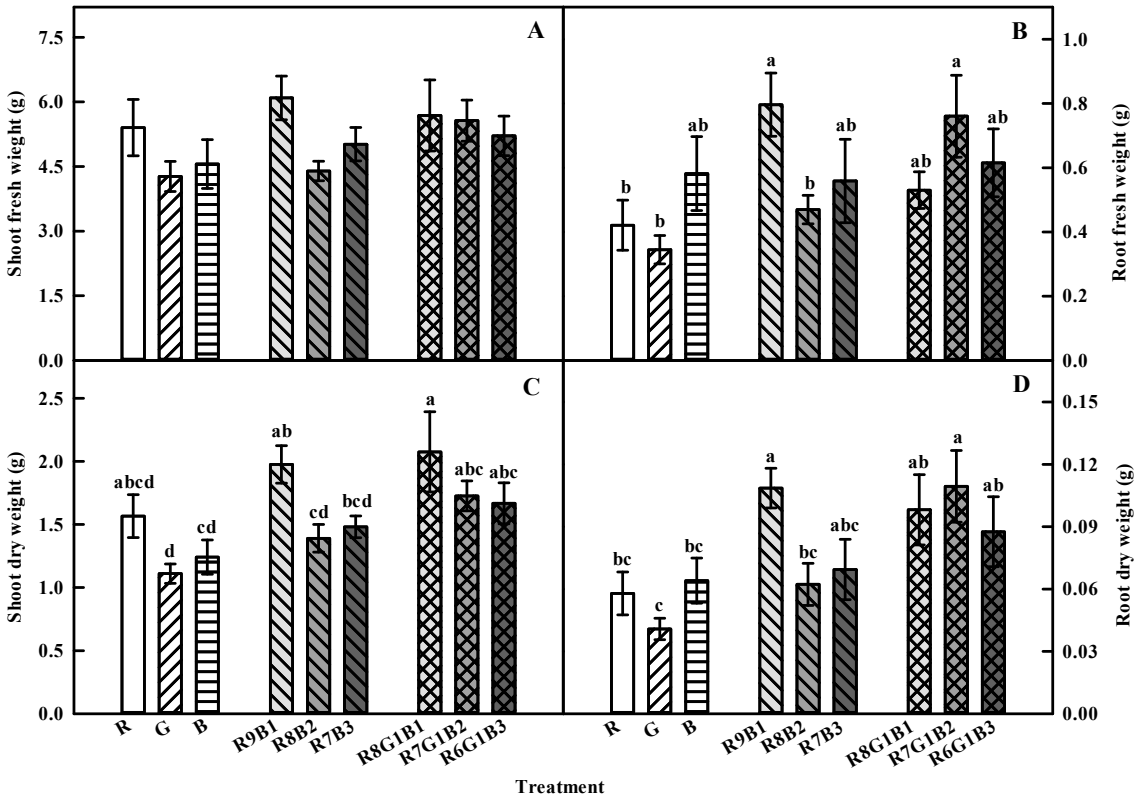

Figure 5. Shoot fresh weight (**A**), root fresh weight (**B**), shoot dry weight (**C**) and root dry weight (**D**) of in vitro-propagated apple plantlets at 6 weeks after transplanting. Different letters indicate significant differences at p-value < 0.01 (n = 5).

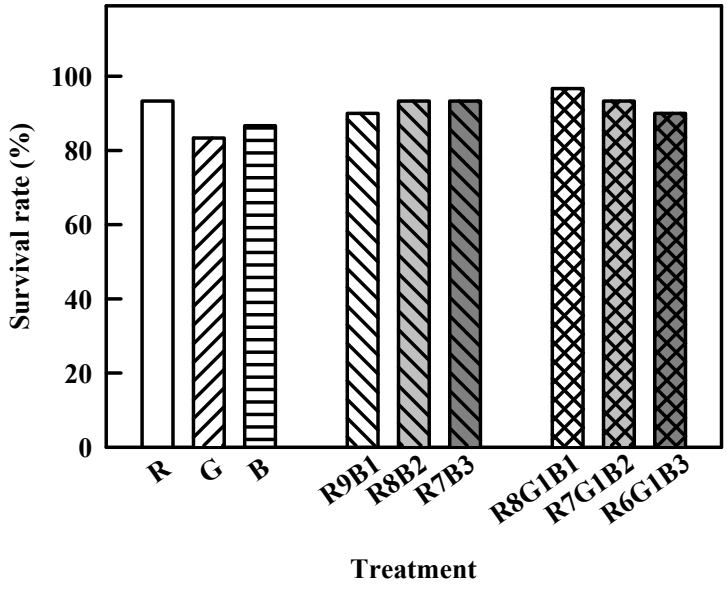

Figure 6. Survival rate of in vitro-propagated apple plantlets at 6 weeks after transplanting.

3.2. Photosynthetic Parameters

The photosynthetic rate was significantly lower in G and B treatments, and R9B1 and R8B2 treatments were significantly higher than the others except for R7G1B2 at 5 weeks after transplantation (Figure 7A). Stomatal conductance and transpiration rate for R7B3 and B showed significantly higher value compared to the other treatments (Figure 7B,C). The change of intercellular CO_2 concentration also showed a similar trend with the stomatal conductance (data not shown).

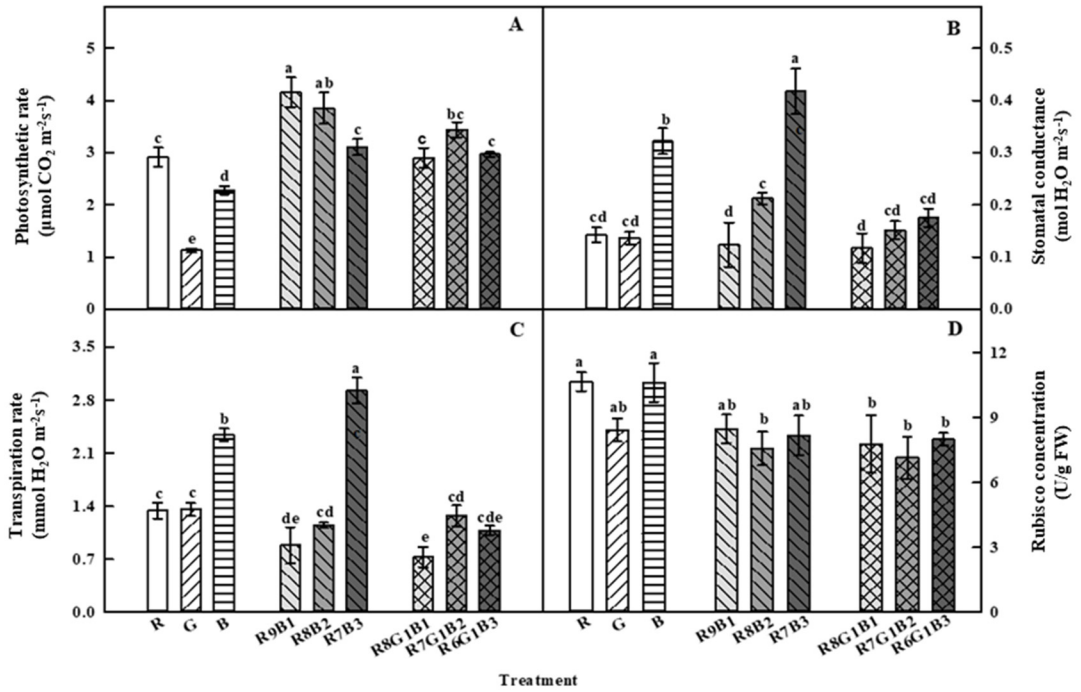

Figure 7. Photosynthetic rate (**A**), stomatal conductance (**B**), transpiration rate (**C**) and Rubisco concentration (**D**) of in vitro-propagated apple plantlets acclimated under various light quality conditions at 5 weeks after transplanting, respectively. Different letters indicated significant differences at p-value < 0.01 (n = 4).

Rubisco concentration showed high values in R and B among monochromatic treatments, which were significantly higher than those for RB and all RGB mixed treatments (Figure 7D).

3.3. Starch Content

Starch content was higher in R among monochromatic group and in R9B1 among RB combination treatments and R8G1B1 among RGB combination treatment had significantly higher starch content than those of G, B, R8B2, R7B3, R7G1B2, and R6G1B3 treatments at 6 weeks after transplantation (Figure 8).

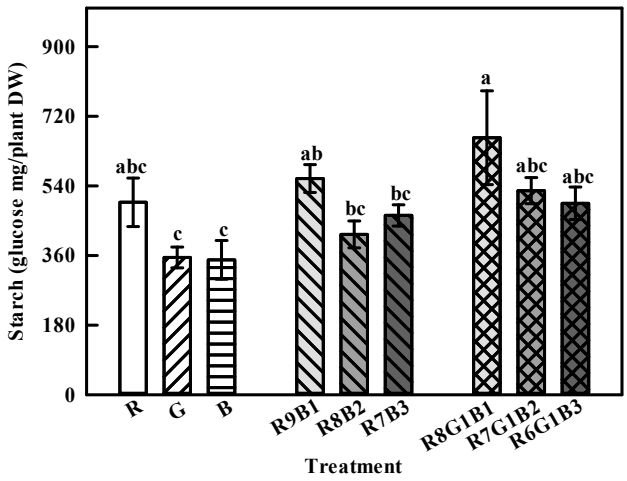

Figure 8. Starch contents of in vitro-propagated apple plantlets according to several light quality at 6 weeks. Different letters indicate significant differences at p-value < 0.01 (n = 4).

4. Discussion

The plant's characteristics controlled by the plant photoreceptors were plant height, leaf angle, shape, and size [34]. Our results showed that plant height of in vitro-propagated apple plantlets showed significantly higher values at the R, R9B1 and R8G1B1 treatments at 6 weeks after transplantation and B had significantly the lowest value (Figure 2). Blue light absorbed by phototropin in the hypocotyl cell makes the cryptochrome formation signal more efficient and inhibited the hypocotyl elongation of Arabidopsis [35]. In addition, inhibitory effects on plant growth by blue light (400–500 nm) were reported in other plant species such as pea, Arabidopsis, lettuce and soybean [36–38] and similar responses were shown in the in vitro-propagated apple plantlets. The top part of the stem diameter also showed a similar tendency to the plant height (Figure 3A), suggesting that a high ratio of R light promoted secondary growth after the primary growth of cells and tissues derived from the apical meristem. Our results were consistent with the results of Li et al. [39], who reported that stem diameter of tomato seedling was significantly higher under the 75% red light treatment in red and blue combination treatment. The M9 seedlings used in this study are one of the apple cultivars used as dwarfing rootstocks. Rootstock is the base and root portion of grafted plants. A scion (shoot), the flowering and/or fruiting part of the plant, is grafted onto a rootstock. Therefore, the stem diameter can be an important factor when the scion is grafted onto a rootstock. The observed positive effects of thickest stem diameter rootstocks were rootstock's vigorous root system which absorb water and nutrients more efficiently [40]. It was reported that grafted trees with thicker stem diameter produced significantly more fruits [41]. Thus, in this study R8B1 and R8G1B1 with high stem diameter could have a positive impact on growth and fruit after transplanting to field. In addition, Shin et al. [42] observed that leaf elongation and expansion of in vitro-cultured *Doritaenopsis* plants could be promoted by red light, which supports our result that the vigorous leaf growth was observed in R and R9B1 (Figure 4B).

SPAD value (an indirect index of chlorophyll content) of blue light was significantly higher at 6 weeks after transplantation than the others (Figure 4C). Our results are supported by previous studies where blue light had positive effects on chlorophyll formation as well as stomatal opening in other plant species [42–44]. The fast increase of leaf area and stem diameter was occurred in R and R9B1 treatments which also had high shoot fresh weights (Figure 5A). The expansive leaves intercept more light, which can remarkably increase biomass even under low light intensity. These results are in agreement with those found by Li and Kubota [45]; the biomass of baby leaf lettuces significantly increased, presumably due to enhanced light interception by enlarged leaf area under low light intensity. Meanwhile, relatively low shoot dry weight was observed in treatments with a high ratio of blue since blue light inhibited the growth of fresh and dry weight of leaves [38,45,46] (Figure 5A,C). Liu et al. [47] reported that red light induces root elongation by promoting polar IAA migration from apical meristem to root. Therefore, the values of R9B1 and R7G1B2 with a relatively high ratio of red light-induced vigorous root growth in this study (Figure 5B,D). The vigorous root growth of apple seedlings could contribute to successful transplanting to field due to its essential physiological function such as water and mineral uptake.

Blue light is important for chlorophyll synthesis and chloroplast development and red light is a major light for the development of photosynthetic apparatus [48], which means that each monochromatic light has its own role in plant development and photosynthesis. However, it has a relatively limited effect over mixed light. The photosynthetic rate showed the highest value of R9B1 in red and blue combination treatment, and the monochromatic treatments showed a lower photosynthetic rate compared to other treatments at 5 weeks after transplantation. Our results were in agreement with Matsuda et al. [49], who reported that the photosynthetic capacity of spinach leaves grown in red and blue ratio of 9:1 was higher than that of spinach cultivated in monochromatic light of red. In our study, improved carbon assimilation rate by the radiation of high ratio of red light treatment contributed to significant increases in growth parameters such as plant height, stem diameter and leaf area. Meanwhile, monochromatic green light showed the lowest photosynthetic rate (Figure 7A). This was consistent with the tomato study by Wu et al. [50], in which the photosynthetic rate of *Solanum*

Lycopersicum seedlings under the green light was significantly reduced. The green light may be effective to plant growth in addition to red and blue lights because green light can penetrate into the plant canopy and the leaves of the lower canopy can use the transmitted green light in fixing CO_2 [51,52]. Although the photosynthetic rate was low in the monochromatic green light, the polychromatic LEDs (R:G:B) showed a high level of photosynthetic rate. The dry weights of the shoot and root were higher under all RGB treatments than the monochromatic green light (Figure 5C,D). Since blue light plays a role in stomata opening, blue light showed significant high stomatal conductance and transpiration rate in monochromatic light treatments. In addition, Shimazaki et al. [53] reported that the addition of blue light to red light-based light quality activates signaling that brings fast stomata openings which supported our result of high stomatal conductance and transpiration rate at the treatment of R7B3. Photosynthetic rates showed a different trend with stomatal conductance and transpiration rate, indicating that even though the in vitro-propagated apple plantlets had less stomata opening at 5 weeks after transplantation, it did not significantly affect photosynthetic rate due to abundant CO_2. Rubisco, a key enzyme in Calvin cycle, begins carbon dioxide assimilation action through carboxylation of RuBP. Rubisco was significantly higher in R and B than the others at 5 weeks after transplantation (Figure 7D). In a previous study, proteins synthesized in red light were as active as those synthesized in blue light but a higher Rubisco concentration per unit leaf area was observed under blue light [54]. This implies that blue light was more effective in Rubisco protein synthesis than red light. However, our results of Rubisco concentration showed no correlation with the results of the photosynthetic rate which were consistent with the result of Ernstsen et al. [55], who reported that light quality had a small or no effect on Rubisco induction.

Light quality can regulate carbohydrate metabolism of higher plants [56]. In most plants, starch and sucrose are the major storage form of carbohydrates and the principal form in which carbon is transported through the plants [39]. The starch content was showed a higher value in monochromatic treatment of R, red and blue combination treatment of R9B1 and red, blue and green combination treatment of R8G1B1 at 6 weeks after transplantation (Figure 8). Maas [57] and Li et al. [39] reported that red light increased starch accumulation in rose plants and cotton plantlets. The increment in starch content by a high ratio of red light treatment might be due to the increase in carbon through possible inductions in the photosynthetic rate [39]. The photosynthetic rate also showed a similar trend to that of starch content (Figures 7A and 8). In this study, there was a significant increase in photosynthetic rate and starch content under the combination of R and B LED lights, especially R9B1, compare with the other treatments. Previous studies suggested that the spectral energy distribution of red and blue wavelength coincided with the absorption spectrum of chlorophyll, and therefore photosynthetic rate was stimulated [58]. Therefore, this combination light might be effective for the accumulation of soluble carbohydrates in the apple plantlet. Therefore, our results suggest that the increase in shoot biomass of the apple plantlets may be caused by an accumulation in starch content by a red light.

5. Conclusions

Plant tissue culture is an effective method for producing virus-free plantlets. However, due to the environmental characteristics of in vitro, the manipulation of the acclimation environment is crucial to increase the survival rate of the plantlets in indoor places such as plant factories with artificial lighting. In particular, the light environment such as light quality and light intensity is an essential factor for the successful acclimation of the in vitro-propagated plantlets. In this study, we demonstrated that a high ratio of red light was effective to increase photosynthetic rate and biomass and blue light was an adequate light source to increase chlorophyll content and stomatal conductance of virus-free apple plantlets. Therefore, our study suggested that R9B1 or R8G1B1 would be a proper lighting condition for successful acclimation of in vitro-propagated apple rootstock M9 plantlets to ex vitro conditions. Our trial for the acclimation of virus-free apple plantlets showed an applicability of environmental control technology in closed-type plant production systems to fruit seedlings, a new target crop group. Moreover, this technology can be applied to vertical farming in urban areas in the near future.

Author Contributions: Methodology, Resources, Investigation, Formal Analysis, Data Curation, Writing, G.-J.C.; Methodology, Investigation, Writing, Visualization, J.-H.L.; and Conceptualization, Methodology, Supervision, Validation, Writing, Review and Editing, Funding Acquisition, M.-M.O. All authors have read and agreed to the published version of the manuscript.

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