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EFFECTS OF BLUE AND RED LED LIGHTS ON RUBISCO ACTIVASE GENE EXPRESSION, CO_2 FIXATION EFFICIENCY AND PLANT MORPHOLOGY OF *Gerbera jamesonii*

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SUMMARY

Light quality is known to affects numerous plants' physiological and metabolic processes during development period, including photosynthesis and morphogenesis. Light properties such as light wavelength has been optimized for several cultivated crops. In this study, the effects of LED light wavelengths blue and red on both photosynthetic performance and morphology of *Gerbera jamesonii* plantlets was investigated. The results showed the photosynthesis efficiency via the photo-pigment system and mediated regulation of rubisco activase encoding gene (RCA) mechanism was significantly enhanced by blue light. The expression of RCA was approximatly 2.7 times higher than those under red light. In contrast, chlorophyll contents, CO_2 assimilation, total carbohydrate and RCA expression was reduced under red wavelength. However, both single-wavelengths caused a dramatically negative impact on *G. jamesonii*. Total chlorophyll/carotenoid value or higher carotenoid contents was lower under blue light; fragile petioles and drawrfism morphology was occurred under red and blue LED, respectively. The combination of blue and red light in the lighting spectrum significantly improved the limitations of single-wavelength. The supplement of blue light under red light background (at blue LED and red LED ratio = 1:4) improved plant photosynthesis while maintained the normal morphology of plants, although the expression of RCA and net photosynthesis of plants in BR LED was lower than in the control. Our results obviously provide the scientific evidences for requirement of LED light in micropropagation or canopy culture of *G. jamesonii*.

Keywords: CO_2 assimilation, *Gerbera jamesonii*, LED, photo-morphogenesis, RCA.

INTRODUCTION

Oxygenic photosynthesis is one of the most important light-induced processes on the Earth, which directly contributes O_2 to the oxygen-consuming organisms and occurs in plants, alga or cyanobacteria. Overall, photosynthesis includes three main stages: (1) light-harvesting from sunlight; (2) use of that energy for the production of ATP and reducing power, reduced ferredoxin, and NADPH; and (3) capture and conversion of CO_2 into carbohydrates and other

cell constituents, which is also named as the CO_2 fixation pathway. However, the only true light reactions are over when charge separation has ended at the reaction center (Mirkovic *et al.*, 2016). Recently, several enzymes of the carbon reactions discovered require light for regulation (Michelet *et al.*, 2013). These findings are strong evidences for the control of light on CO_2 fixation efficiency.

One of the key regulatory mechanisms of light on the CO_2 fixation process is based on rubisco activase (RCA). Rubisco activase belongs to the AAA family (ATPase associated with diverse cellular activities) and structured with an ATP binding domain, which plays an important role in AAA proteins. These proteins are involved in a wide variety of different functions in which the energy extracted from ATP hydrolysis is used in molecular remodeling events (Snider *et al.*, 2008). Rubisco activase takes responsibility for activating the enzyme rubisco (Ribulose-1,5-bisphosphate carboxylase/oxygenase - EC 4.1.1.39). Previous results indicated that RCA catalyzing function is affected by several environmental factors such as temperature, low ion concentration or UV exposition (Portis, 2003). However, light significantly regulates the activation efficiency of RCA. The light-mediated response of rubisco activase is based on the ATPase activity which is very sensitive to the stromal ATP/ADP ratio (Portis *et al.*, 2007). Apart from the ATP/ADP ratio regulation, RCA activity is also controlled at the transcription stage (Qu *et al.*, 2011). Chao *et al.* (2014) discovered several light-responsive *cis*-acting elements in the RCA promoter region, for example: GA motif, I-box, ATCT motif or Sp1 motif.

On the other hand, light does not only affect plant photosynthesis but also plant morphogenesis. The light wavelength, intensity, direction and the lighting duration are both signals for the development of or the transition between different stages in plants (Wada *et al.*, 2000). Compared with light intensity and photoperiod, light quality shows much more complex effects on plant morphology and physiology. Specific spectrum stimulates different morphological and physiological responses (Wang *et al.*, 2016). For developmental regulation, plants detect light of different wavelengths using several classes of photoreceptors, which are red/far-red light sensing phytochromes, blue light-sensing cryptochromes or UV-photoreceptors (Folta, Childers, 2008; Galvão, Fankhauser, 2015).

Base on the effects of light wavelengths on plant, solid-state lighting sources such as LED light, which emit monochromatic wavelength, have been applied in various fields of agriculture, recently. One of the potential applications of LED in this field is being lighting sources for plant *in vitro* micro-propagation and plant development regulation. However, different wavelengths have varied effects to plant growth. Numerous influences of monochromatic blue or red wavelengths have been recognized such as stem elongation, leaf expansion, roots length or organ anatomy (Wada *et al.*, 2000; Hogewoning *et al.*, 2010; Muneer *et al.*, 2014; Wang *et al.*, 2016). Previous researches showed that mixed lighting (blue and red wavelength) generated a significantly benefit for plant development (Nhut *et al.*, 2003; Ryu *et al.*, 2012; Wang *et al.*, 2016). Hogewoning *et al*. (2010) reported that only 7% blue light was sufficient to prevent any over dysfunctional photosynthesis, which can be considered a qualitatively blue light effect. The photosynthetic capacity (A_{max}) was twice as high for leaves grown at 7% blue compared with 0% blue and continued to increase with increasing blue percentage during growth, measuring up to 50% blue. Amongst various combined ratios, mixed blue-red LED at a ratio of 1:4 have been reported to provide the sufficient lighting requirement for the development of numerous plant species including *Codonopsis* sp. (Hung *et al.*, 2016), *Mesembryanthemum* sp. (He *et al.*, 2017) or *Chrysanthemum morifolium* (Nhut, Nam, 2009).

Therefore, in this study the effects of LED lights (blue LED, red LED and mixed blue-red LED at ratio 1:4 on photosynthetic performance and morphology of *Gerbera jamesonii*, one of

the top 10 traded cut flowers in the world (Shagufta *et al.*, 2012; Gordon, 2015), was evaluated. The obtained results are expected to be references in lighting sources designing for *Gerbera jamesonii* culturing *in vitro* or under canopy conditions.

MATERIALS AND METHODS

Plant materials and experimental establishments

In vitro Gerbera jamesonii at rooting stage were transferred to pots in green house and cultured under 45 µmol.m^{-2}.s^{-1} irradiance of mono-wavelength blue LED (450 nm) (B) or red LED (660 nm) © and mixed blue-red LED at a ratio of 1:4 (BR LED). The control was fluorescent lamp (C). Air temperature was 25°C, photoperiod and relative humidities were 16 h.d^{-1} and 80% respectively. The effects of light conditions on photosynthesis efficiency, RCA expression level and plant morphology were determined after 3 weeks. All of the treatments were conducted in three replications.

RNA extraction, cDNA biosynthesis and cloning RCA fragment

Total RNA was prepared using Trizol reagent following the producer's instructions (Thermo Scientific, USA). Contaminant DNA was eliminated by DNase I (Thermo Scientific, USA). Treated RNA was used as template for the cDNA synthesis process by RevertAidTM H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, USA).

A fragment of RCA was amplified by PCR using primer pair of *rca*-F (5'-AAGGTTGAAGTTCGTGGTGG-3') and *rca*-R (5'-TACCTCAACACCCTTGCCTC-3'), designed based on its conservative region according to reference sequences including *Medicago truncatula* (GenBank: XM_003616402.2); *Solanum pennellii* (GenBank: XM_010314058.2); *Glycine max* (GenBank: NM_001289312.1) and *Nicotiana tabacum* (GenBank: Z14980.1) and *Camellia sinensis* (GenBank: XP_028070998.1). PCR reaction program was as follow: initial denaturation at 94°C for 5 min, then 30 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 40 s; and final extension at 72°C for 5 min.

PCR products were purified using Gene Jet genomic DNA purification Kit (Thermo Scientific, USA) before being cloned into pBT vector by T/A cloning (Hoang *et al.*, 2005). The plasmid containing the target insert was sequenced using API 3500 system.

BLAST tool from NCBI was used for measurment the similarity between *G. jamesonii* RCA nucleotide sequences with references in the database. Putative amino acid sequence was used to determine the similarity of *G. jamesonii* RCA with references by using MAFFT aligment tool (https://mafft.cbrc.jp/alignment/server/index.html). All sequences were adjusted to same length with BioEdit (www.mbio.ncsu.edu) before aligment.

Evaluate RCA encoding gene via semi-quantitative PCR method

Samples of each treatment were collected in the 3rd week of cultivation after one hour exposure to a lighting source. Then, total RNA was extracted, cDNA was synthesized following the method described above. Approximately 150 ng of RNA/cDNA was used to perform multiplex PCR reaction in exactly 20 µL of total volume with RCA gene primer pairs (*rca* F/R) and reference actin gene actin-F (5'-GGTGTTATGATTGGTGCTGCTGT-3'); actin-R (5'-ATCTATCTCTCGTGGGGTGCTTT-3'), designed based on its sequence (GenBank: GACN01029707). The PCR reaction program was as follow: initial denaturation at 94°C for 5 min, then 30 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 40 s; and final extension at 72°C for 5 min. PCR products were visualized using agarose 0.8% by electrophoresis. All of the results were pictured in the same conditions and DNA band density was determined based on these figures with ImageJ and GelQuannet software (distributed by

biochemlabsolutions.com). The relative expression levels were calculated through the DNA band density of RCA and the reference actin gene as described by Al-Janabi (2015).

Plant anatomy

Gerbera petioles were sectioned into 1 - 2 µm in thickness and 7 - 10 µm in length layers. Thin layers were observed and pictured under 10 X and 40 X magnifications using light microscopy (Soukup, 2014). Cells size were calculated with ScopeImage 9.0 software.

Chlorophyll concentration, total carbohydrate, LMA evaluation and Leaf gas exchange

Photo-pigments were determined following Hager and Meyer (Hager and Meyer-Bertenrath, 1966), which was modified from Wellburn method (Wellburn, 1994). Samples were excised from the leaves of 10 plants at a similar position for each treatment. The pigments were extracted with anhydrous acetone. Chlorophyll a (Chl a), chlorophyll b (Chl b) and total carotenoid (Car) were calculated using formulas:

$$\text{Chl a (mg/g)} = \frac{(11.75 \times A_{662} - 2.35 \times A_{645}) \times V}{1000 \times W}$$

$$\text{Chl b (mg/g)} = \frac{(18.61 \times A_{645} - 3.96 \times A_{662}) \times V}{1000 \times W}$$

$$\text{Car (mg/g)} = \frac{(1000 \times A_{470} - 2.27 \times \text{Chl a} - 81.4 \times \text{Chl b}) \times V}{1000 \times 227 \times W}$$

Where: *V* is the total volume of acetone extract (mL) and *W* is fresh weight (g) of sample

The total carbohydrate concentration was determined by using the Anthrone method and measured at 630 nm (Hansen, Moller, 1975). CO_2 assimilation was evaluated by using the Portable photosynthesis system TPS-2. The leaf mass area (LMA) was calculated following Hernández and Kubota (2016).

Statistical analysis

All treatments were replicated 3 times. Data were analyzed by Microsoft excel 2010 and Statgraphic XV software using variance ANOVA, and significant differences between the means were tested using Duncan's multiple range test at 95% confidence. Nucleotide

RESULTS

Rubisco activase gene cloning

DNA product of 774 bp fragment of rubisco activase gene was amplified using RCA F/R primer pairs. NCBI BALST against similar genes revealed the high similarity to rubisco activase genes founded in other species (*M. truncatula*, XM_003616402.2; *S. pennellii*, XM_010314058.2; *G. max*, NM_001289312.1; *N. tabacum*, Z14980.1, 84%, 83%, 82% and 82% respectively). This might be the consequence of the length of input sequence, which was only 774 bp compared with over 1500 bp of reference genes. However, putative amino acid sequence translated from the fragment showed extreme similarity rate with RCA protein sequences of *G. max* (98%), *N. tabacum* (97%), *C. sinensis* (98%), *M. truncatula* (98%) and *S. pennellii* (99%) (Figure 1). In addition, the catalytic conservation region of rubisco activase was also recognized from the predicted amino acid sequence of cloned segment (data not shown). Therefore, these results indicated that a fragment of RCA was successfully cloned from *G. jamesonii* leaves. The new *G. jamesonii* leaf CDS-derived sequence was also deposited in the GenBank (NCBI database) with accession number MF097965.

Effect of LED lights on RCA expression

Overall, semi-quantitative PCR analysis indicated that LED lights reduced the expression level of RCA. Especially, the gene expression was extremely inhibited under the red LED treatment (Figure 2, 3). However, apart from the control, blue LED significantly promoted the transcription activity of RCA as compared with that in the red light and BR LED (Figure 2). The RCA relative expression level of plants grown under blue LED was about 2.7 times higher than those under red LED light and approximately 1.73 times under BR LED light.

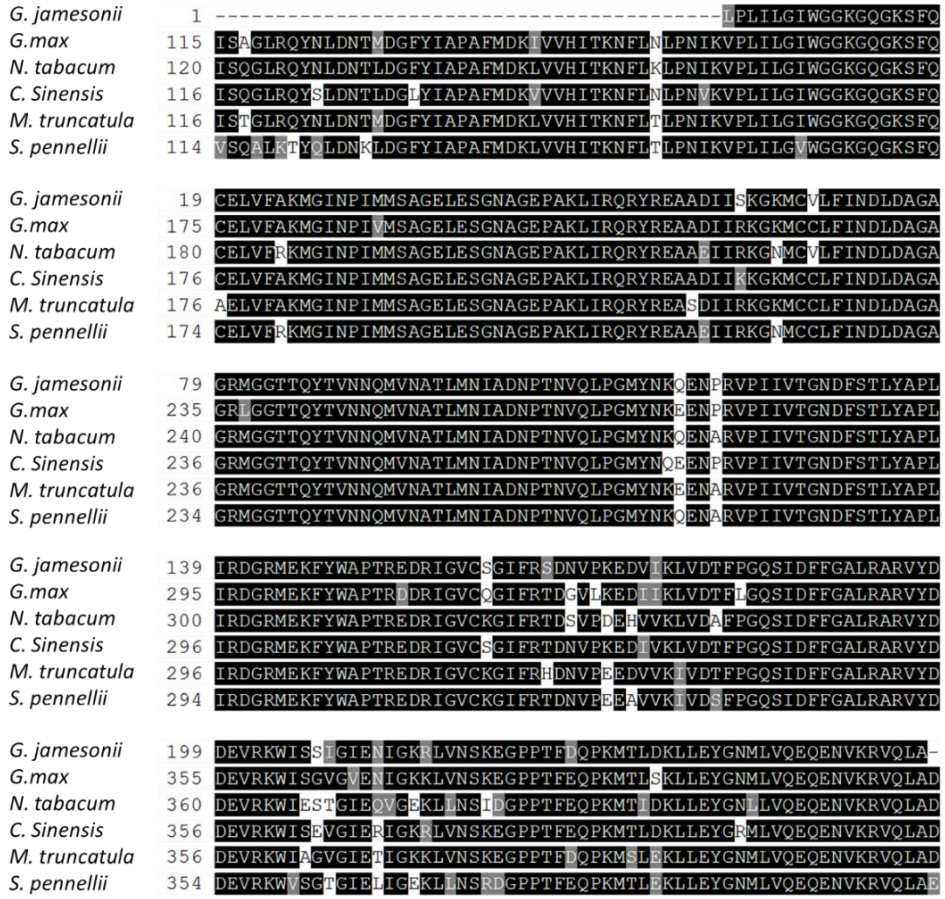

Figure 1. Multiple amino acid alignment of rubisco acivase of *G. max, N. tabacum, C. sinnesis, M. truncatula*, S*. pennellii* and G. *jamesonii* deduced sequence.

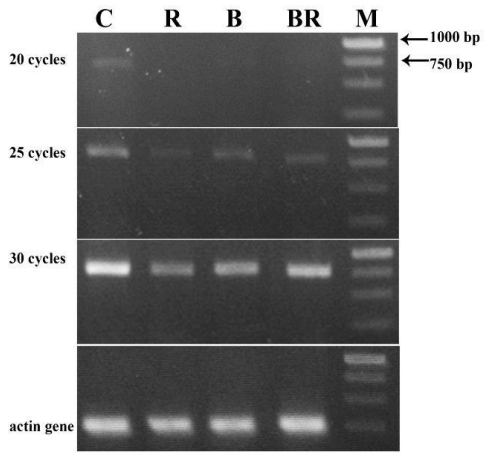

Figure 2. Electrophoresis of PCR products of RCA encoding gene after 20, 25 and 30 cycles with reference actin gene. C: Fluorescent light, R: red LED; B: blue LED; BR: BR LED. M**:** Gene Ruler 1 kb DNA Ladder.

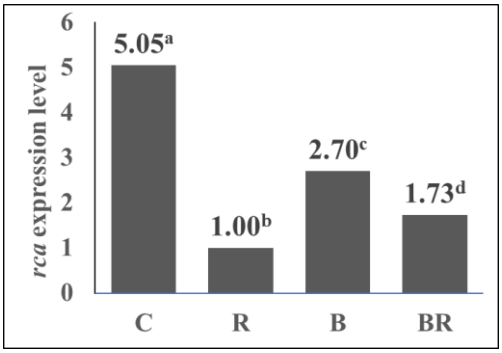

Figure 3. RCA encoding gene relative expression level in leaves of *G. jamesonii* grown under different light conditions. C: Fluorescent light, R: red LED; B: blue LED; BR: BR LED. The symbols (a, b…) illustrated the difference levels among different lighting treatments following Duncan test with α= 0.05.

Effect of light conditions on the photo-pigment accumulation

Photo-pigment accumulation analysis showed that red LED caused the dramatic reduction in chlorophyll accumulation of *G. jamesonii* at both Chl a and Chl b indexes. In comparison, plants cultured under the control, the blue LED or BR LED conditions resulted in higher chlorophyll concentrations, approximately doubling as compared with that in the red LED (Table 1). Carotenoid concentration was the lowest in red LED treatment (at about 0.09 mg/g), while the figures for the control, blue LED or BR LED was approximately more than double, at 0.25, 0.22 and 0.18 mg/g respectively. On the other hand, the total chlorophyll/car index was the highest in red LED treatment (at about 9.92), the values of blue LED and BR LED treatments were lower, at around 6.43 and 7.72 respectively. Meanwhile, the value of Chl a/b index was the lowest in the red LED treatment (Table 1).

Table 1. Effects of light conditions on photo-pigment accumulation.

	C	R	B	BR
Chl a (mg/g)	1.06^{*a}	0.54^{b}	1.10^{a}	0.99^{a}
Chl b (mg/g)	0.45^{c}	0.35^{a}	0.29^{b}	0.36^{a}
Car (mg/g)	0.25^{a}	0.09^{b}	0.22^{a}	0.18^{a}
Total chlorophyll/car	5.99^{c}	9.92^{a}	6.43^{c}	7.72^{b}
Chl a/b	2.35^{d}	1.55^{a}	3.83^{b}	2.75^{d}

*The average of replications; The symbols in the same rows (a, b…) illustrate the difference levels among four lighting treatments following Duncan test with α = 0.05. C: Fluorescent light, R: red LED; B: blue LED; BR: BR LED.

Effects of light on CO_2 gas exchange, total carbohydrate and LMA

As illustrated in figure 4a, blue and red light caused opposite effects on net photosynthesis. Under blue LED treatment, the net rate of photosynthesis in *G. jamesonii* was almost double in comparison with the red LED treatment, at 1.26 and 0.66 µmol $CO_2/cm^2/s$, respectively. In which, this photosynthetic index under BR LED treatment was the same as that in the red LED treatment. Similar effects of blue LED and red LED on *G. jamesonii* were also observed in total carbohydrate accumulation and the LMA index (Figure 4b, c). However, as compared with the control, only the blue LED treatment resulted in higher photosynthesis efficiency in *G. jamesonii*.

Effects of lighting conditions on plant morphology

Morphogenesis in *G. jamesonii* was significantly different when treated with blue LED, red LED or BR LED. The results showed that red LED and BR LED remarkably enhanced the average length of plant petioles in comparison with the control (Table 2). Meanwhile, blue LED extremely inhibited the length of *G. jamesonii* petioles. A similar trend was observed in the average length of parenchyma cells (Table 2, Figure 5a). In addition, petiole anatomical results also revealed that the density of parenchyma cells of plant treated with red LED and BR LED was higher than that of blue LED or the control (Figure 5 b), approximately 50.33 and 44.81 cell/mm2 compared to 26.75 and 18.38 cell/mm^2, respectively. However, plants delivered from the red LED treatment were much more fragile than those treated with the blue LED and the BR LED light. On the other hand, there were no statistical differences between three light conditions including the control, the blue LED and BR LED treatments in the leaf area index (Table 2). In contrast, the red LED caused a reduction in the leaf area (Figure 6).

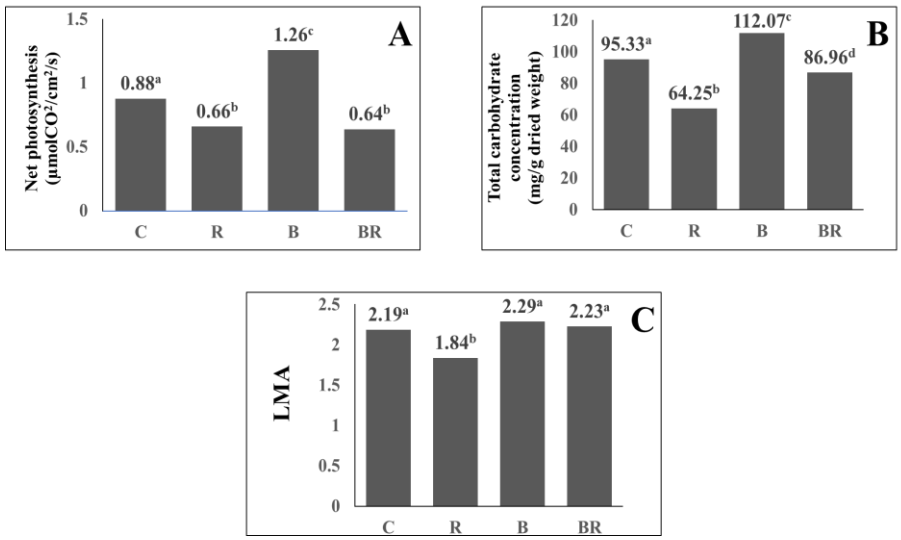

Figure 4. The effects of lighting conditions on (A) Net photosynthesis; (B) Total carbohydrate concentration and (C) the LMA index. C: Fluorescent light, R: red LED; B: blue LED; BR: BR LED.

Table 2. Effects of lighting conditions on *G. jamesonii* morphology.

Lighting conditions	The length of petioles (cm)	The length of parenchyma cell (μm)	Parenchyma cells density (cells/mm^2)	Leaf area (cm^2)
C	3.14*^{d}	14.43^{c}	28.38^{a}	3.83^{a}
R	4.60^{b}	24.43^{b}	50.33^{b}	2.15^{b}
B	2.55^{a}	11.74^{a}	26.75^{a}	3.73^{a}
BR	3.83^{c}	21.59^{b}	44.81^{c}	3.90^{a}

*The average of replications; The symbols in the same columns (a, b, c) illustrated the difference levels among three lighting treatments following Duncan test with α= 0.05. C: Fluorescent light, R: red LED; B: blue LED; BR: BR LED.

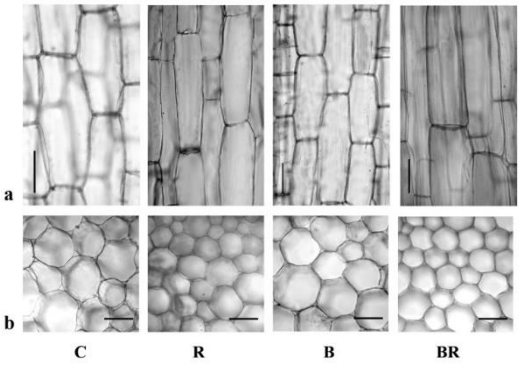

Figure 5. Anatomy of (a) longitude thin cells layer of C, R, B and BR treatments with the magnification of 10X and (b) transverse layer with parencyma cells structure of *G. jamesonii* petioles grown under C: Fluorescent light, R: red LED; B: blue LED; BR: BR LED with the magnification of 40X. bar = 5 μm.

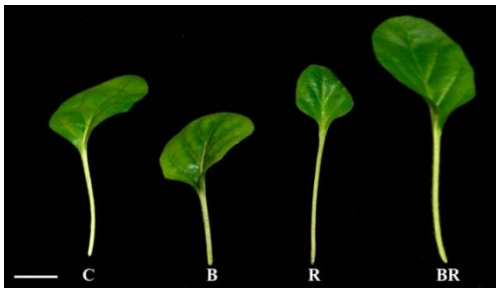

Figure 6. Morphology of *G. jamesonii* 's leaves grown under different light conditions C: Fluorescent light, R: red LED; B: blue LED; BR: BR LED. bar = 1 cm.

DISCUSSION

Our premier results showed that although plants grown under single LED lights resulted in lower photosynthesis efficiency (the red LED treatment, for an instant) or negative effects on plant morphology (both of the blue LED and red LED treatments) compared to the fluorescent, the combined LED in the BR LED treatment could sufficiently satisfy the light requirements of *G. jamesonii*. Our first result of the effects of blue/red mixture light on *G. jamesonii* showed that the supplement of blue light into red light background improved not only the photosynthetic performance (Figure 4, 5) but also the plant morphology (Table 3) compared to the single red light or blue light. Plants grown under BR LED had longer petioles than those under blue light; they presented thicker and stronger petioles and especially greater CO_2 assimilation, LMA, total carbohydrate and RCA relative expression level than those under the red light. Therefore, *G. jamesonii* requires both blue and red wavelength in the lighting spectrum for maintaining normal photosynthesis and morphogenesis. The necessity of combination of blue and red light for maintaining normal development has been reported in numerous plant species (Nhut *et al.*, 2003, 2015; Hogewoning *et al.*, 2010; OuYang *et al.*, 2015; Wang *et al.*, 2016). Furthermore, our results revealed a part of the mechanism of the effects of blue LED on the *G. jamesonii*'s photosynthesis performance.

Effects of blue LED on photosynthesis process of *G. jamesonii*

Light quality extremely influences the plant photosynthesis activity. Our results showed that blue light significantly enhanced the photosynthetic performance of *G. jamesonii*, which could be observed through the CO_2 assimilation, total carbohydrate or the LMA index (Fig. 4). These results obtained in this study are similar to previous research on the effects of blue light on plant photosynthesis (Hogewoning *et al.*, 2010; Wang *et al.*, 2016). Muneer *et al*. showed that the CO_2 assimilation, fresh weight and dry weight of lettuce were both increased when cultured under blue light, in comparison with that under red light or green LED light (Muneer *et al.*, 2014). A similar trend was also obtained in cherry tomato (Xiaoying, 2012), cucumber (Wang *et al.*, 2009) or chrysanthemum (Nhut, Nam, 2009). In addition, the results of BR treatments consolidated the enhancing effects of blue light on *G. jamesonii* photosynthetic efficiency. By supplementing blue LED under red LED background, we obtained greater results in the CO_2 gas exchange, total carbohydrate and LMA index (Fig. 4), as compared with those under mono-wavelength red LED. The efficiency of the combination of blue and red light on plant photosynthesis has been recorded in numerous plant species (Hogewoning *et al.*, 2010; Wojciechowska *et al.*, 2013; Wang *et al.*, 2016). Furthermore, the results from this study also showed that blue LED affected the photosynthesis process of *G. jamesonii* at both the photo-physic stage and gene regulation level.

The regulation of blue LED on *G. jamesonii* during photo-physic stage of photosynthesis process

At the photo-physic stage, blue LED influenced *G. jamesonii* photosynthesis mediated via photo-pigments, especially through the chlorophyll. These pigments are one of the first light reception factors. Higher chlorophyll could increase light absorption, which was a benefit for photosynthetic efficiency (Wang *et al.*, 2016). The increase in chlorophyll concentration may improve electron transportation efficiency. The increase in both chlorophyll contents and photosynthetic performance were observed in several plant species cultured under blue LED such as lettuce (*Lactuca sativa* L) (Muneer *et al.*, 2014; Wang *et al.*, 2016); cherry tomato (*S. esculentum* var. cerasiforme) (Xiaoying, 2012) or *Cucumis sativus* (Wang *et al.*, 2009).

Chl a/b index is additional evidence for the regulation of blue LED on *G. jamesonii* photosynthesis. Land plants change the

compositions of light-harvesting complexes (LHCs) and chlorophyll Chl a/b ratios in response to the variable light environments which they encounter (Tanaka, Tanaka, 2005). A higher Chl a/b indicates a high light-adapted photosynthetic apparatus with less Chl b containing light-harvesting antennae, and thereby a higher capacity for electron transportation and more Calvin cycle enzymes on a Chl basis (Evans, 1988). Our results showed higher value at both chl a and chl a/b index, CO_2 assimilation and dry maters content of plant growth under blue LED or BR LED, in comparison with those in red light treatment. These results are similar to Wang *et al.* (2016) and Wang *et al.* (2009) when *Lactuca sativa* L or *Cucumis sativus* are cultured under different blue to red ratios of LED light. These studies showed that blue light both enhanced the chl a/b index and P_n value, compared to those in monochromatic red light.

Blue light promotes photosynthesis process mediated via rubisco activase encoding gene regulation

Previous studies discovered the light-responsive *cis*-acting elements in the RCA promoter region (Orozco and Ogren, 1993; Chao *et al.*, 2014). Therefore, light completely regulates RCA through the control of its expression. In this study, the relative expression level of *rca* in plant grown under blue LED or BR LED treatments was extremely higher than that at the red LED treatment. Consequently, higher mRNA accumulation may raise the amount of RCA, and as a result, the activate efficiency may increase. The relationship between *rca* expression and P_n was reported in previous research (Jurczyk *et al.*, 2016). In addition, numerous studies on the effects of light on *rca* expression have been reported recently. However, our results were similar to Wang *et al.* (2009). In this study, the relative RCA mRNA abundance of *C. sativa* was highest when treated with blue light, at about 3.17, and the figure for red light treatment was approximately 0.98, a significantly lower value. Our achievements on photosynthetic performance and RCA expression level sharply contributed to consolidating the association between RCA and photosynthesis efficiency, although the activate activity is also based on the ADP/ATP ratios or redox state of ferredoxin (Michelet *et al.*, 2013).

CONCLUSION

In this study, our results showed that blue light could enhance *G. jamesonii* photosynthesis by both photo-physic and mediated-RCA expression level pathways. Overall, the chlorophyll contents, the CO_2 assimilation, LMA or RCA relative expression level of plants cultivated under blue light was highest amongst three examined lighting sources. However, compared with mono-wavelength blue or red light, a mixture of blue and red light was more efficient in promoting *G. jamesonii* growth and photosynthesis. These results demonstrated the application capacity of LED lights on micro-propagation or canopy culturing of gerbera plantlets.

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