Plant Physiology and Biochemistry 141 (2019) 477–486

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Leaf chlorosis, epinasty, carbohydrate contents and growth of tomato show different responses to the red/blue wavelength ratio under continuous light

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ARTICLE INFO

Keywords:
Continuous light
Tomato
Carbohydrates
Chlorosis
Epinasty
Monochromatic light

ABSTRACT

The induction of leaf injuries, including leaf chlorosis and epinasty, by continuous light in tomato plants is one of the most interesting and mysterious phenomena regarding plant interactions with light, the mechanism of which has not yet been revealed. To gain further insights into this particular response of tomato plants, we cultivated tomato seedlings (*Solanum lycopersicum* cv. Momotaro) for 14 days under continuous light with different ratios of red and blue light and compared their performance to those grown under continuous or 14/10-h photoperiodic white light using novel methods to quantitatively evaluate the level of leaf chlorosis and epinasty. Continuous monochromatic blue light induced severe chlorosis but almost completely alleviated epinasty in tomato leaf. In contrast, continuous monochromatic red light caused a lower level of leaf chlorosis but very severe epinasty. The combination of red and blue light at different ratios significantly reduced both leaf chlorosis and epinasty under continuous light condition. Carbohydrate contents showed no correlation with leaf chlorosis, while glucose and fructose contents showed correlations with the petiole and leaflet curvatures. Histochemical staining with 3,3′-diaminobenzidine and nitro blue tetrazodium chloride also did not reveal any significant buildup of hydrogen peroxide and superoxide anion in monochromatic blue light treatment. Taken together, these results suggest that chlorosis and epinasty are two distinctive leaf injuries caused by continuous light that may follow very different mechanisms, and an overaccumulation of carbohydrates in the leaf may not be the main cause of continuous light-induced leaf chlorosis in tomato.

1. Introduction

Continuous light (CL) is an effective method to increase efficiency in energy and facility utilization in protected horticulture, especially in growing facilities using artificial light. In theory, CL will provide plants with more time per day to perform photosynthesis, thus allowing an increase in the daily light integral for faster plant growth and a reduced cultivation time. For greenhouses using natural sunlight, CL can also be applied in the form of supplemental light during the night and has already been utilized in many companies with good results.

Unfortunately, not every plant species responds well to CL. Tomato plants (*Solanum lycopersicum* L.) in particular are especially sensitive to CL, suffering some very specific disorders including leaf mottled chlorosis and necrosis starting at the base of the leaflets, smaller leaves, leaf epinasty and ultimately plant death under prolonged treatment (Arthur, 1936; Hillman, 1956). This particular response of tomato toward CL was documented as early as 1930 (Arthur et al., 1930). Since then, great efforts have been made to uncover the cause and mechanism responsible for this interesting phenomenon, and based on a collection of evidence, several hypotheses have been proposed, including carbohydrate overaccumulation, circadian rhythm disorder and circadian rhythm asynchrony (for a more detailed review, see Velez-Ramirez et al., 2011). However, nearly 90 years after its discovery, a complete and satisfactory explanation regarding the true nature of CL-induced

Abbreviations: CL, Continuous light; DAB, 3,3′-diaminobenzidine; EC, electrical conductivity; LED, light-emitting diode; NBT, nitro blue tetrazodium chloride; PPF, photosynthetic photon flux; ppi, pixel per inch

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https://doi.org/10.1016/j.plaphy.2019.06.004
Received 15 January 2019; Received in revised form 30 May 2019; Accepted 4 June 2019
Available online 10 June 2019

injury in tomato remains elusive. It seems that more research aimed at carefully dissecting different aspects of light and comparing the injuries and performance of tomato plants under various settings of CL is needed to solve the mystery.

Light quality is an important aspect of light that can strongly affect many physiological and metabolic processes of plants, including photosynthesis and the photoassimilate export rate (Hogewoning et al., 2010; Lanoue et al., 2018; Miao et al., 2016), carbohydrate accumulation (Li et al., 2013; Li et al., 2017) and the circadian rhythm (Fankhauser and Staiger, 2002; Oakenfull and Davis, 2017). As all the above physiological and metabolic processes have been hypothesized to be involved in the mechanism of CL injury in tomato, it can be expected that different light wavelengths will lead to different levels of injury in tomato under CL condition, as shown in several recent reports (Matsuda et al., 2016; Velez-Ramirez et al., 2017). Furthermore, light quality has also been found to significantly alter plant morphological characteristics, many of which might be closely related to the leaf epinasty injury in tomato under CL such as petiole and stem elongation (Sasidharan et al., 2010; Nanya et al., 2012), leaf size and shape (Chitwood et al., 2015), leaf thickness (Schuerger et al., 1997), etc. This provides an opportunity to cross-check the level of CL injury with the status of these processes and characteristics in tomato plants under different light spectra to test the proposed hypotheses and narrow down the correct cause of CL injury in tomato. However, no attempt in this direction has yet been made.

In this research, we strived to examine the injury level of tomato plants under CL of warm white light or a combination of red and blue light of different ratios and compared that to the accumulation of common carbohydrates (starch, sucrose, glucose and fructose) and reactive oxygen species, particularly hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-), in the leaves to test a popular hypothesis that carbohydrate overaccumulation is the cause of CL injury in tomato.

2. Methods

2.1. Cultivation conditions

On day 13 after sowing, uniform tomato seedlings (*Solanum lycopersicum* cv. Momotaro) were transplanted into pots (diameter 11 cm, height 10 cm) with commercial soil mix (Plant world, NongwooBio Co., Ltd., South Korea) and then placed for 3 days under warm white LEDs with a PPF of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and photoperiod of 14/10 h for acclimatization. The pots were then transferred to each treatment condition, and the plants were grown for 14 days. The temperature was maintained at 25 ± 2 °C during cultivation. For irrigation, Yamazaki's tomato nutrient solution (pH 6.4, EC 1.28 $dS \cdot m^{-1}$) was used. To address the differences in water consumption due to different photoperiods, the plants treated with continuous light received twice the irrigation times per day but with half-strength solution (EC 0.73 $dS \cdot m^{-1}$) compared to the control treatment using the 14/10-h photoperiod. The pots were irrigated by drip irrigation until leaking was observed, indicating full soil capacity.

2.2. Light treatment

The experiment consisted of 7 treatments, with 8 plants × 3 replicates per treatment. PPF in all treatments was set at 200 ± 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$, measured at the plant shoot tips at the start of treatment period (35 cm distance from the lamps). In 5 treatments, continuous light was used with red and blue LEDs (model V3.5_B, Future Green Co., Ltd., South Korea, wavelength 655.0 ± 27.2 and 449.2 ± 13.4 nm, respectively, Fig. 1) at different PPF ratios: 100% red, 75% red:25% blue, 50% red:50% blue, 25% red:75% blue and 100% blue (treatment code: R200B0, R150B50, R100B100, R50B150 and R0B200, respectively). One treatment used continuous light with warm white LEDs (model v3.0_B, Future Green Co., Ltd., South Korea, treatment code: WW, Fig. 1). The last treatment, which served as a control, consisted of the same warm white LEDs as above but with a photoperiod of 14/10 h (treatment code: Control).

2.3. Evaluated parameters

After 14 days of exposure to the treatment conditions, the plants were harvested for evaluation. The measured growth parameters included fresh and dry weight, shoot and root length, number of open leaves, leaf area and stem diameter. The length of the second node was measured from the lower junction point of the stem with the first leaf to that with the second leaf.

Color analysis for chlorosis evaluation was perform following a method modified from Haque et al. (2015). The first leaflets of the sixth leaves (in order of unfolding) were cut in half along the main veins and scanned on a white background together with a ruler and a white piece of paper acting as the white standard using a commercial scanner (CanoScan LIDE 220, Canon, USA) at a resolution of 300 ppi (pixels per inch). The images were then adjusted for white balance based on the white standard with Photoshop CS6 (Adobe Systems Inc., USA), and the main veins of the leaflets as well as the backgrounds were removed to reduce noise during color analysis. The processed images were analyzed with the Java-based image processing program ImageJ (National Institutes of Health, USA) using the color threshold tool with the HSB color space option based on the hue value. Pixels with a hue value falling within 0–65 were denoted chlorotic spots. This value was selected by manually comparing 15 images of leaflets from plants grown under continuous warm white light and having clear chlorotic spots with 15 images of leaflets from plants grown under a 14/10-h photoperiod in a preliminary experiment and choosing the hue value that could best distinguish the chlorotic areas. The chosen hue value was then applied to all subsequent analyses of leaf images. The percentages of chlorotic area as separated by the ImageJ program per total leaflet area were calculated.

Epinasty evaluation was based on three parameters: percentage of twisted leaves, petiole arc angle and leaflet arc angle. The percentage of twisted leaves was calculated by dividing the number of twisted leaves by the total number of leaves on the main shoot. A leaf was counted as twisted when the first leaflet turned more than 90° compared to its normal horizontal position (Fig. 2A and B). The petiole arc angle of the sixth leaf, which expressed the curvature of the petiole, was determined by measuring the width (w) and height (h) of the arc (also known as the length of the chord and sagitta, respectively) formed by the petiole in its natural (unstretched) state (as shown in Fig. 2C) and then calculating the radius of the arc using the following equation:

$$r = \frac{4h^2 + w^2}{8h}$$

The petiole arc angle (A) was then calculated using the following equations:

$$A = 2arcsin\left(\frac{w}{2r}\right) \quad \text{if} \quad r > h$$

$$\text{Or} \quad A = 360° - 2arcsin\left(\frac{w}{2r}\right) \quad \text{if} \quad r < h$$

For the leaflet arc angle, expressing the curvature of the leaflet, the first leaflet of the sixth leaf was cut in half along the main vein, laid flat and unstretched on a surface, and the width (w) and height (h) of the arc formed by the main vein were measured (as shown in Fig. 2D). Then, using the same formulas applied for the petiole arc angle, the leaflet arc angle was calculated.

The contents of four carbohydrates, namely, starch, sucrose, glucose and fructose, were assayed in the first leaflets of the sixth leaves using a method combined and modified from Smith and Zeeman (2006) and Cools and Terry (2012). The leaves were harvested at specific

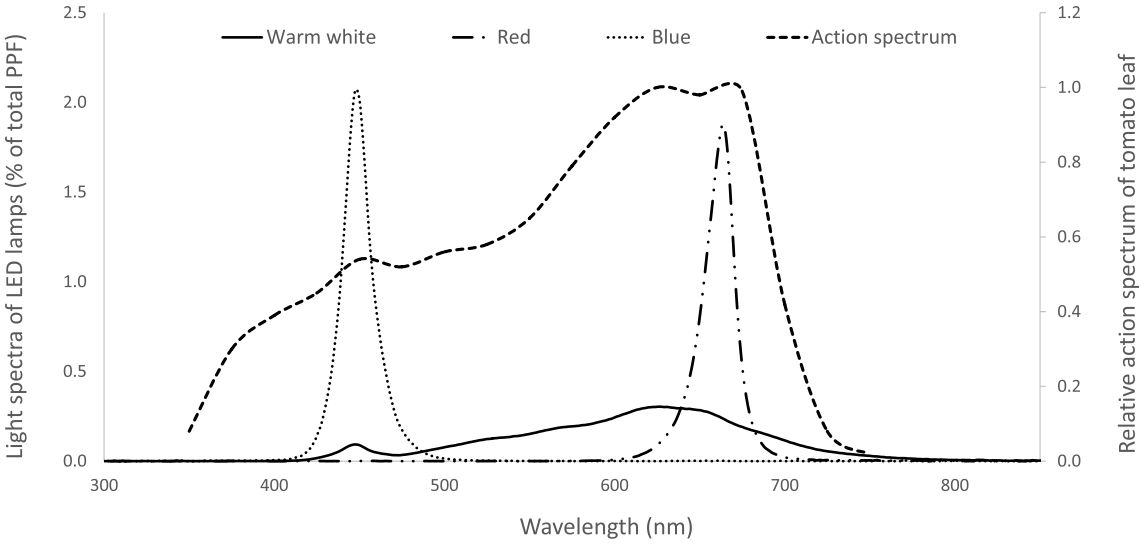

Fig. 1. Light spectra of warm white, red and blue LED lamps used in the experiment plotted against the photosynthetic action spectrum of tomato leaf (McCree, 1972). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

timepoints (0, 1, 7, 14, 15, 19 and 24 h) on day 14. The main vein of the leaflet was removed, and the remaining leaflet blade was snap-frozen in liquid nitrogen and then freeze-dried at – 80 °C for 1 week and stored at – 18 °C until processing. To account for the possibly uneven distributions of carbohydrates between the tips and bases of the leaflets, an entire half of the leaflet blade (20–40 mg) for every sample was used for the assay. The freeze-dried leaflet blade was homogenized and extracted with 5 mL of 80% methanol at 75 °C for 15 min. The pellet was extracted again two more times. The supernatants of three extractions were pooled together, dried at room temperature, and then re-suspended in 5 mL of water, and the solution was used for the sucrose, glucose and fructose assay. The pellet was also left to dry at room temperature before addition of 5 mL of water and vortexing well to yield a fine mixture, of which 200 µL was drawn into a screw cap eppendorf, boiled in a water bath for 30 min and then let to cool down before addition of 200 µL of 200 mM sodium acetate buffer (pH 5.5) containing 0.5 U α-amylase and 6 U amyloglucosidase (Sigma-Aldrich, USA). For each sample, another mixture was prepared using the same procedure but without the addition of enzymes to serve as a control. The mixture was incubated for 12 h at 37 °C, then centrifuged at 10,000 × *g* for 10 min, and the supernatant containing glucose digested from starch was used for the assay. The contents of sugars were assayed with the Megazyme K-SUFRG assay kit (Megazyme U.C., Ireland) using the protocol provided by the manufacturer with modifications for a 96-well plate.

Histochemical staining of hydrogen peroxide (H_2O_2) or superoxide

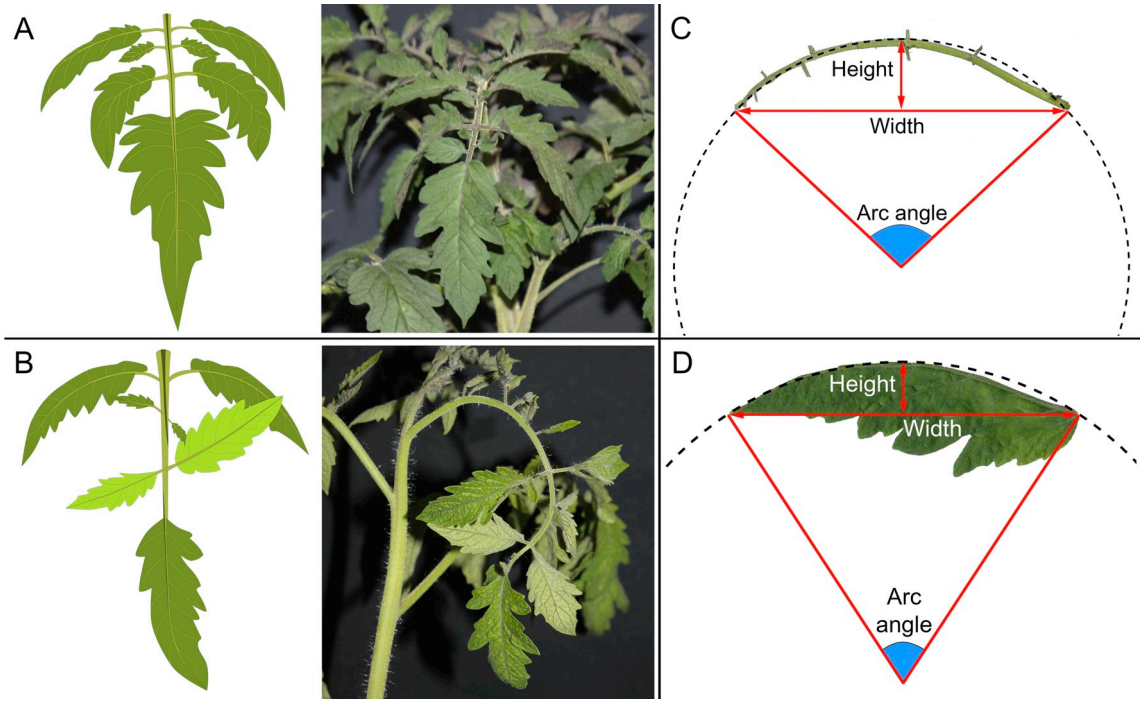

Fig. 2. Evaluation method for leaf epinasty. (A) Illustration and representative image (taken from treatment R50B150) of a normal tomato leaf; (B) Illustration and representative image (taken from treatment WW) of a twisted tomato leaf. Notice the rotation of the leaf along the petiole axis; (C) and (D) Illustration of the method to measure the heights and widths of the arcs formed by the petioles and leaflets, from which the arc angles of the petioles and leaflets can be calculated.

anion (O_2^-) was also performed using the first leaflets of the sixth leaves according to the method reported by Daudi and O'Brien (2012) and Kumar et al. (2014). The entire leaflet was harvested at the end of the subjective photoperiod on day 14 and submerged in 10 mL of 10 mM sodium phosphate buffer (pH 7.0) containing 1 mg·mL^{-1} 3,3′-diaminobenzidine (DAB, Sigma-Aldrich, USA) and 0.05% (v/v) Tween 20, or 10 mL of 50 mM sodium phosphate buffer (pH 7.5) containing 2 mg·mL^{-1} nitro blue tetrazodium chloride (NBT, Thermo Fisher Scientific Inc., USA). It was then placed under a vacuum for 5 min and incubated at 25 °C with shaking at 100 rpm for 7 h (for DAB) or 45 min (for NBT). A set of leaflets from the WW treatment was incubated in a solution of 10 mM sodium phosphate buffer (pH 7.0) and 0.05% v/v Tween 20 without DAB or 50 mM sodium phosphate buffer (pH 7.5) without NBT for use as a negative control. Subsequently, the leaflet was bleached by boiling twice in bleaching solution (ethanol/acetic acid/glycerol, 3/1/1, v/v/v) for 15 min each. The image of the stained leaflet was obtained on a white background.

2.4. Statistical analysis

The experiment was conducted using randomized complete block design. SAS 9.1 software (SAS Institute Inc., USA) was used for statistical analyses. The ANOVA test was performed among the treatments using CL (R200B0, R150B50, R100B100, R50B150, R0B200 and WW). For parameters with significant ANOVA results, the CL treatments were ranked using Duncan's multiple range test, with $p = 0.05$. Each CL treatment was also compared with the control by the Student's T-test. Arcsine square root transformation was performed on the data of the percentage of twisted leaves before statistical analysis to normalize the distribution of the data following the method described by McDonald (2014).

3. Results

3.1. Plant growth and morphology under continuous light of different spectral distributions compared to the normal photoperiod

Among CL treatments with different ratios of red and blue lights, the plant fresh and dry weight tended to increase as the percentage of red light PPF increased (Table 1 and Fig. 3). Treatments with 100%, 75% and 50% red light resulted in a significantly higher plant dry weight than the control with 14/10-h photoperiod. In contrast, continuous monochromatic blue light resulted in the lowest biomass gain among all the treatments, which was significantly lower than the control treatment, indicating that plant growth was strongly inhibited by this treatment. Plants exposed to all CL treatments had a higher percentage of leaf, stem and whole plant dry matter than control treatment plants (Table 2). A higher percentage of red light resulted in a longer shoot and nodal length, although the shoot and nodal length were longer in the presence of 100% blue light than 75% blue light and 25% red light (Table 3). Leaf areas of plants exposed to continuous white light and monochromatic blue light treatments were significantly smaller than the control, indicating that the CL at these spectra had a strong adverse effect in tomato plants.

3.2. Leaf chlorosis and epinasty under continuous light of different spectral distributions compared to the normal photoperiod

Reflecting plant growth, leaf chlorosis result showed the highest percentage of chlorotic areas under continuous white light and monochromatic blue light (Fig. 4). CL at 100% red light resulted in less severe chlorosis than white and 100% blue CL. The combinations of both red and blue light almost completely alleviated leaf chlorosis (the percentages of chlorotic area were not significantly different from the control). In contrast, leaf epinasty, represented by the percentage of twisted leaves, petiole arc angle and leaflet arc angle with high agreement, was more severe at a higher percentage of red light, while almost no symptoms of epinasty were apparent in plants grown under CL with a percentage of blue light equal to or greater than 50% (Table 4).

3.3. Leaf carbohydrate accumulation and H_2O_2 and O_2^- contents under continuous light of different spectral distributions compared to the normal photoperiod

Starch content in the first leaflets of the sixth leaves of tomato plants increased when the light quality shifted from a spectrum with more blue light to a spectrum with more red light (Fig. 5A). Monochromatic red light resulted in the highest starch content in the leaves, comparable to that of continuous white light, while plants had almost the same amount of starch in leaves under continuous monochromatic blue light as under the normal photoperiod (14/10 h). Glucose and fructose contents showed an almost similar pattern among CL treatments, although the contents in the control treatment were the highest among all treatments (Fig. 5B and C). For sucrose, white CL resulted in the highest content in leaves, while monochromatic blue light again resulted in the lowest content, comparable to that of the control treatment (Fig. 5D). Statistical comparisons of the contents of each carbohydrate at 0, 7, 14, 19 and 24 h on day 14 are provided in Table 5.

Histochemical staining of H_2O_2 in the first leaflet of the sixth leaves showed very little H_2O_2 in all treatments (Fig. 6A). Red light ratios from 50% to 100% appeared to be associated with higher H_2O_2 contents in the leaves compared to the other treatments, but the differences were

Table 1
Fresh and dry weights of different parts and whole plant of tomato plants grown under CL with different light qualities compared to the control (14/10-h photoperiod) (n = 3).

Treatment	Fresh weight (g)				Dry weight (g)			
	Leaf	Stem	Root	Whole plant	Leaf	Stem	Root	Whole plant
R200B0	23.3 a^{x*y}	38.6 a**	11.1 abc*	73.0 a**	3.58 ab*	2.24 a**	0.56 bc*	6.39 a**
R150B50	21.4 a^{ns}	26.1 bc*	12.4 ab*	59.9 b^{ns}	3.67 a*	1.84 ab*	0.71 ab*	6.22 a*
R100B100	23.5 a^{ns}	24.5 bc^{ns}	13.1 a*	61.1 b^{ns}	3.44 ab^{ns}	1.75 b^{ns}	0.73 a*	5.91 ab*
R50B150	20.9 a^{ns}	25.3 bc^{ns}	10.7 bc^{ns}	56.8 b^{ns}	2.89 b^{ns}	1.24 c^{ns}	0.59 abc^{ns}	4.72 b^{ns}
R0B200	16.5 b*	19.6 c**	4.9 d*	41.0 c**	2.08 c*	0.98 c**	0.25 d*	3.30 c**
WW	15.8 b*	30.2 b^{ns}	9.0 c^{ns}	55.0 b^{ns}	3.50 ab*	1.84 ab**	0.48 c^{ns}	5.82 ab**
Control	20.2	31.5	7.9	59.7	2.41	1.37	0.41	4.19
ANOVA^z	**	***	***	***	***	***	***	***
CV^t (%)	10.39	13.24	12.34	9.07	11.94	13.57	15.16	12.20

x: Means followed by the same letter within the same column are not significantly different from each other.
y: ns, *, **: not significantly or significantly different at $p = 0.05$ or 0.01, respectively, compared to the control.
z: **, ***: significantly different among CL treatments at $p = 0.01$ or 0.001, respectively.
t: coefficient of variation.

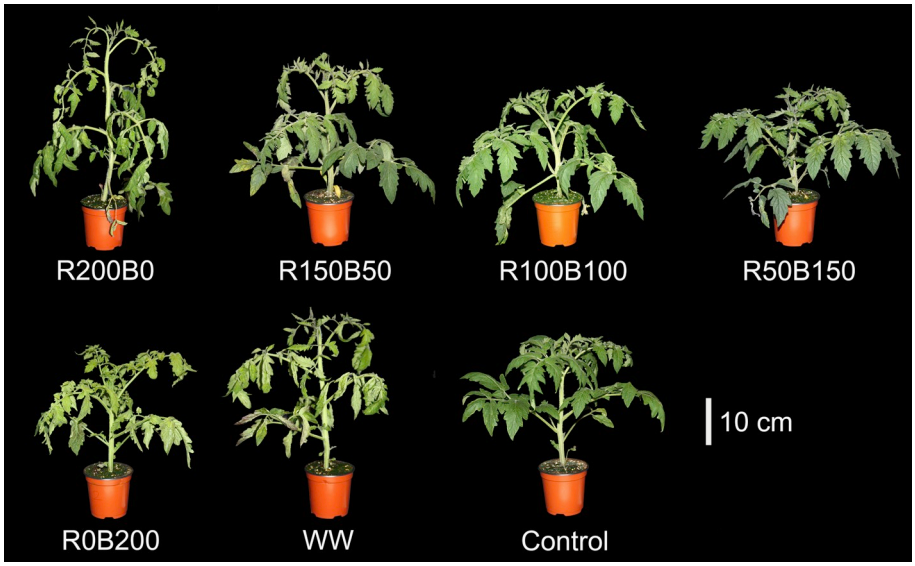

Fig. 3. Tomato plants after 14 days under CL with different light qualities compared to the control (14/10-h photoperiod).

Table 2
Percentage of dry matter in different parts and whole plant of tomato plants grown under CL with different light qualities compared to the control (14/10-h photoperiod) (n = 3).

Treatment	% dry matter			
	Leaf	Stem	Root	Whole plant
R200B0	16 b^{x*y}	5.8 b**	5.1^{ns}	8.8 c**
R150B50	18 b**	7.0 a*	5.7^{ns}	10.4 ab**
R100B100	15 b**	7.2 a**	5.5^{ns}	9.7 b**
R50B150	14 b*	6.0 b*	5.4^{ns}	8.8 c*
R0B200	13 b**	5.0 c**	5.1^{ns}	8.1 c**
WW	25 a**	6.0 b**	5.2^{ns}	10.7 a*
Control	12	4.3	5.2	7.0
ANOVA^{z}	***	***	ns	***
CV^{t} (%)	10.39	13.24	12.34	9.07

x: Means followed by the same letter within the same column are not significantly different from each other.
y: ns, *, **: not significantly or significantly different at $p = 0.05$ or 0.01, respectively, compared to the control.
z: ns, ***: not significantly or significantly different among CL treatments at $p = 0.001$, respectively.
t: coefficient of variation.

not dramatic. Similarly, although with more significant differences, treatments with high red light ratios (50%–100% red light) also resulted in high O_2^- contents in the leaves (Fig. 6B). Interestingly, monochromatic blue light treatment exhibited very low H_2O_2 and O_2^- contents, despite the highest level of chlorosis in this treatment. This finding suggested that oxidative damage by H_2O_2 or O_2^- might not be the direct cause of leaf chlorosis induced by CL.

4. Discussion

4.1. The red:blue light ratio had a significant effect on growth, morphology and leaf chlorosis under CL conditions

The results of this study showed that the growth, morphology and injury level of tomato plants under CL conditions was indeed affected by the light spectral quality. Continuous monochromatic blue light inflicted chlorosis and inhibited growth more severely than continuous monochromatic red light. This result agrees with previous studies (Matsuda et al., 2016; Velez-Ramirez et al., 2017), in which overnight illuminations with blue light, accompanied by day time illumination with either white light or red-blue light combination, resulted in greater damage to the plants than illumination with red light. However, the combination of monochrome red and blue lights, especially at ratios of 1:1 and 1:3 (red to blue), significantly alleviated leaf chlorosis even more than monochromatic red light. In contrast, continuous white light, which contained both the red and blue wavelength, still caused severe leaf chlorosis. Similarly, Velez-Ramirez et al. (2017) also reported that continuous illumination with a red-blue light combination at a ratio of 4 to 1 resulted in a higher leaflet Fv/Fm than overnight illumination

Table 3
Other growth and morphological parameters of tomato plants grown under CL with different light qualities compared to the control (14/10-h photoperiod) (n = 3).

Treatment	Root length (cm)	Shoot length (cm)	Nodal length of 2nd node (cm)	Stem diameter (mm)	No of open leaves	Leaf area (cm^2)
R200B0	53.5 abc^{xnsy}	39.3 a**	3.45 a^{ns}	9.3^{ns}	9.0 a**	970.5 a^{ns}
R150B50	56.6 ab^{ns}	24.1 c^{ns}	1.51 d**	10.2^{ns}	8.1 bc^{ns}	869.9 ab^{ns}
R100B100	60.6 a^{ns}	18.4 d**	1.28 e**	10.4^{ns}	7.7 d^{ns}	912.2 ab^{ns}
R50B150	59.8 a^{ns}	15.5 e**	1.26 e**	9.7^{ns}	7.8 cd*	813.6 abc^{ns}
R0B200	47.3 c^{ns}	18.5 d**	1.99 c*	9.5^{ns}	8.3 b^{ns}	744.3 bc**
WW	50.0 bc^{ns}	33.5 b*	2.29 b*	9.9^{ns}	8.4 b*	663.1 c**
Control	51.6	27.4	2.99	9.5	8.0	1041.6
ANOVA^{z}	*	***	***	ns	***	*
CV^{t} (%)	8.44	5.03	6.06	4.37	2.10	10.79

x: Means followed by the same letter within the same column are not significantly different from each other.
y: ns, *, **: not significantly or significantly different at $p = 0.05$ or 0.01, respectively, compared to the control.
z: ns, *, ***: not significantly or significantly different among CL treatments at $p = 0.05$ or 0.001, respectively.
t: coefficient of variation.

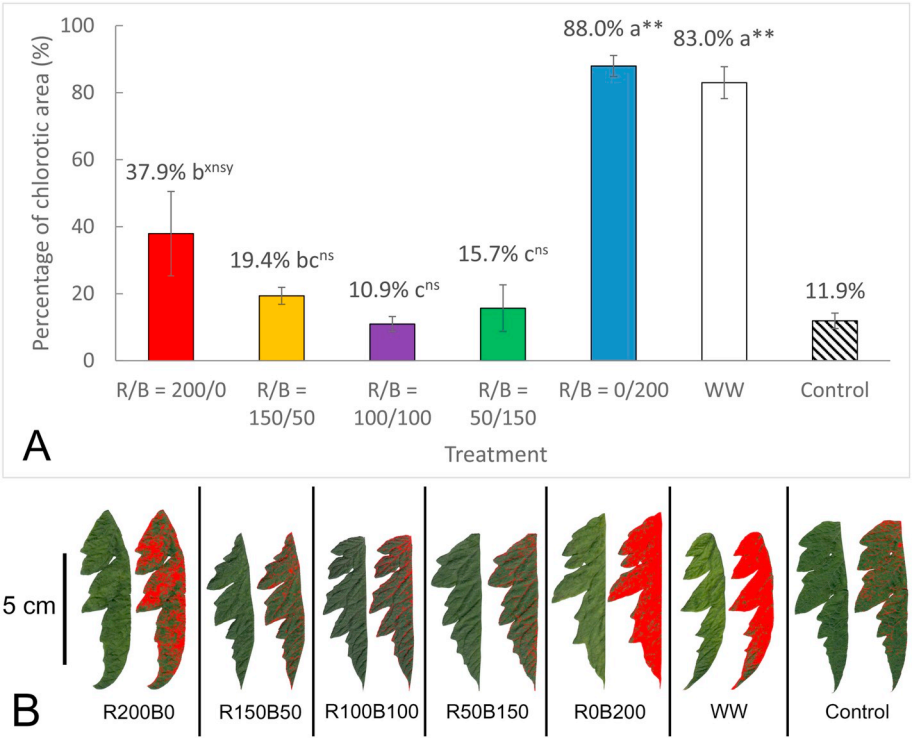

Fig. 4. (A) Percentage of leaf chlorotic area on the first leaflets of the sixth leaves of tomato plants grown under CL with different light qualities compared to 14/10-h photoperiod; (B) Representative images of the leaflets in the treatments before and after processed with ImageJ program. Red areas mark chlorotic spots. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
x: Means followed by the same letter are not significantly different from each other.
y: ns, **: not significantly or significantly different at $p = 0.01$ compared to the control.
Bars represent the standard error.

Table 4
Leaf epinasty parameters of tomato plants grown under CL with different light qualities compared to the control (14/10-h photoperiod) (n = 3).

Treatment	% twisted leaves	Petiole arc angle (degree)	Leaflet arc angle (degree)
R200B0	45.4^{x} a^{y*z}	60 ab^{ns}	57 a*
R150B50	4.2 b^{ns}	44 bc^{ns}	33 b^{ns}
R100B100	0.2 c**	28 cd^{ns}	19 c^{ns}
R50B150	0.0 c**	17 d^{ns}	15 c**
R0B200	1.4 bc**	22 cd^{ns}	19 c**
WW	45.9 a*	75 a^{ns}	54 a*
Control	19.8	48	32
ANOVA^{t}	***	**	***
CV^{u} (%)	56.78	19.65	21.77

x: Arcsine square root transformation was performed on the data of percentage of twisted leaves before statistical analysis to normalize data distribution.
y: Means followed by the same letter within the same column are not significantly different from each other.
z: ns, *, **: not significantly or significantly different at $p = 0.05$ or 0.01, respectively, compared to the control.
t: **, ***: significantly different among CL treatments at $p = 0.01$ or 0.001, respectively.
u: coefficient of variation.

with monochromatic red or blue light or continuous white light. These results support a complicated interaction among different light wavelengths on the induction of leaf chlorosis in tomato under continuous illumination. Both red and blue lights showed the capability to induce chlorosis, but a balanced ratio among these two wavelengths seemed to allow the plants to alleviate the damage. White light, however, might contain other wavelengths that could either nullify this effect or strongly induce chlorosis by themselves. Further studies with other monochromatic lights and their combinations with red or blue light are required to unravel this mystery.

4.2. Leaf chlorosis and leaf epinasty seemed to be two distinct injuries in tomato under CL that might follow different mechanisms

Epinasty is an important leaf injury besides leaf chlorosis in tomato plant grown under CL (Cushman and Tibbitts, 1998). Interestingly, in this experiment the level of leaf epinasty did not correlate with leaf chlorosis. As represented by the percentage of twisted leaves, petiole arc angle and leaflet arc angle with high agreement, leaf epinasty was significantly more severe at high red light ratios (75–100% red light). Under continuous monochromatic red light, leaf epinasty was as severe as with white CL. In contrast, a blue light ratio that was equal to or higher than 50% completely alleviated the leaf epinasty. Similar results were obtained in geranium by Fukuda et al. (2008), who showed that directional irradiation with blue light helped alleviate leaf epinasty caused by red light illumination. This contrast to leaf chlorosis implies that chlorosis and epinasty are two distinct injuries caused by CL that may follow completely independent mechanisms. It should be noted that plants grown under white light with a normal photoperiod (14/10-h light/dark cycle) also demonstrated a considerable level of leaf epinasty, although it was still significantly lower than under continuous white light and continuous monochromatic red light. This result suggests that leaf epinasty may not necessarily be caused by CL but rather intensified under CL conditions.

In the practical aspect, these results, combined with excessive plant height, which is not desirable in tomato transplants, may discourage the use of continuous 100% red light in tomato transplant production despite the highest biomass gain. Red:blue ratios of 3:1 or 1:1, which resulted in much shorter stems as well as reduced levels of both leaf chlorosis and epinasty, might be more suitable choices. These red to blue ratios also provided comparable plant biomass gains to 100% red light and significantly higher plant biomass gains than the control with a 14/10-h photoperiod, revealing the possibility and benefits of using CL with a suitable spectrum in the production of tomato transplants.

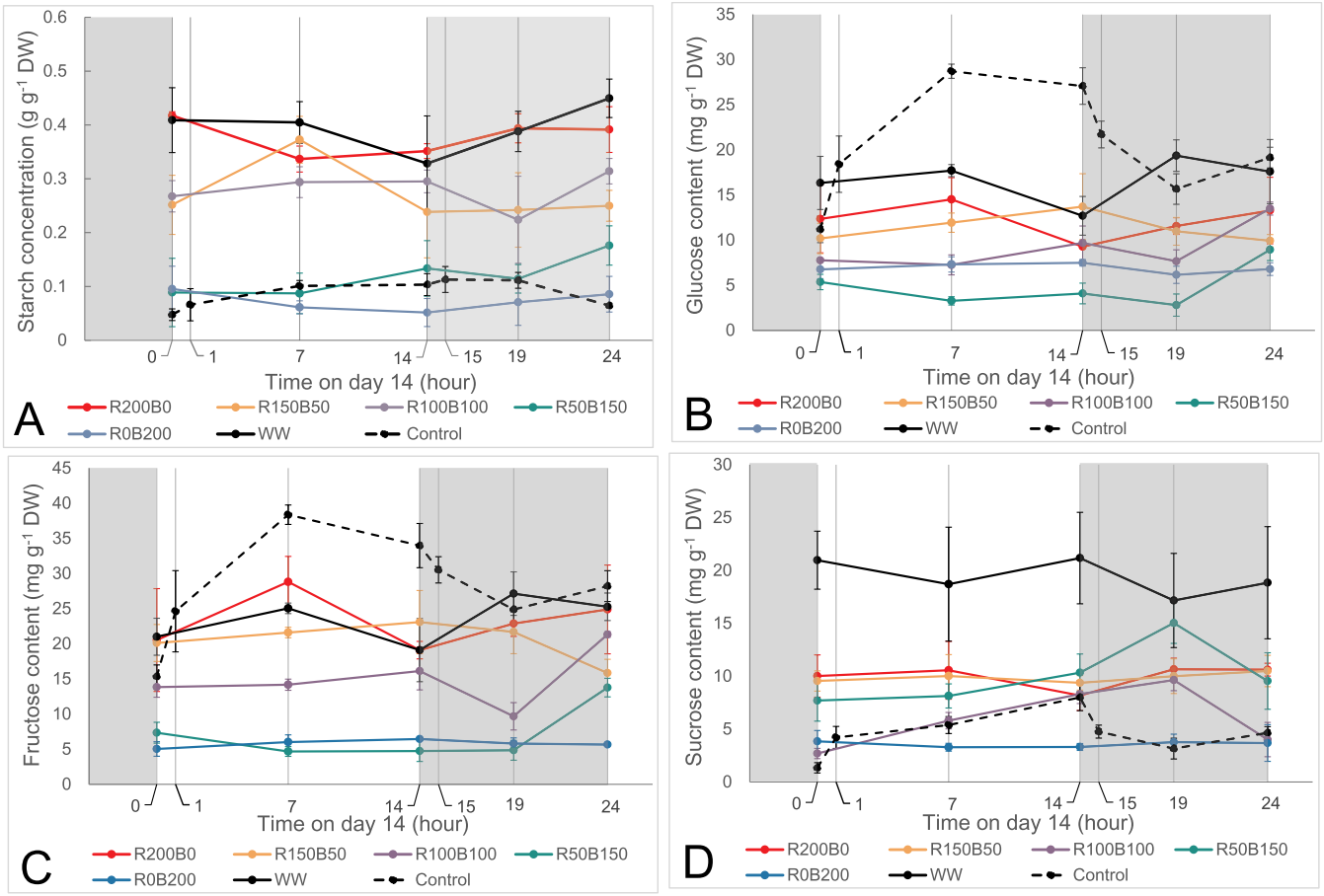

Fig. 5. Carbohydrate contents of the first leaflets of the sixth leaves of tomato plants grown under CL with different light qualities compared to the control (14/10-h photoperiod). (A) Starch content; (B) glucose content; (C) fructose content; (D) sucrose content. Gray areas mark the subjective dark period.
Bars represent the standard error.
Statistical analyses are provided in Table 5.

4.3. Carbohydrate accumulation might not be the main cause of CL-induced leaf chlorosis in tomato

One of the earliest and still the most popular hypotheses about the cause of CL injury in tomato is carbohydrate overaccumulation. This hypothesis was proposed mostly based on the observation that the leaves of tomato plants grown under CL accumulate large amounts of carbohydrates, most notably starch (Arthur et al., 1930; Ho, 1979; Bradley and Janes, 1985; Dorais et al., 1996; Demers et al., 1998). This phenomenon occurs in CL-grown tomato because tomato leaves have low export rates of photoassimilates compared to other species such as pepper (Ho, 1979; Dorais et al., 1996) and require a dark period to export all the photosynthetic products accumulated during the photoperiod. Therefore, under CL conditions, carbohydrates continuously accumulate in tomato leaves without being exported. This accumulation has been speculated to cause various adverse effects including photosynthesis inhibition through feedback effect, induction of photodamage due to such inhibition, induction of ethylene production and leaf senescence by the high concentrations of sugars, among others (Velez-Ramirez et al., 2011), and thus to ultimately result in the chlorosis of tomato leaves.

However, after more than 80 years of research on this topic, little concrete evidence has been acquired to demonstrate that the overaccumulation of carbohydrates in leaves of CL-grown tomato is directly related to leaf chlorosis and not just an irrelevant side effect of CL. In contrast, evidence against this hypothesis has been gradually accumulated. Several early studies (Highkin and Hanson, 1954; Hillman, 1956) have revealed that unnatural photoperiods such as 6 h light/6 h dark or 24 h light/24 h dark, which should result in the same amount of carbon fixation by photosynthesis with the normal 12-h light/12-h dark photoperiod, could also cause injuries similar to CL in tomato. Matsuda et al. (2014) reported that even with the same daily light integral, CL still inflicted distinct chlorosis on tomato plants while a 12/12 h photoperiod gave completely healthy plants, either under a constant or a diurnally varied temperature. The results of that research indeed showed that the soluble sugar contents of leaves were comparable between the CL and normal photoperiod treatments at the end of the photoperiod, while the starch content was even higher under the normal photoperiod than under CL. Haque et al. (2017) also confirmed that a daily temperature variation of 10° largely alleviated chlorosis and improved the Fv/Fm value of tomato leaves under CL compared to a constant temperature, while in contrast to leaf injury, the carbohydrate contents in leaf were comparable or even higher in response to daily temperature variation treatment than constant temperature treatment, both in the early (day 8) and late (day 15) stages of continuous illumination. Altogether, these results cast some doubts on the hypothesis that carbohydrate overaccumulation is the direct cause of CL-induced leaf chlorosis in tomato.

In this research, our results also showed no correlation between leaf carbohydrate contents with the percentage of leaf chlorotic area. Plants exposed to continuous monochromatic blue light, which suffered the most severe chlorosis, actually had the lowest contents of both starch and soluble sugars compared to the other treatments. Starch and sucrose contents in the first leaflet of the sixth leaves of plants in this treatment were equivalent while glucose and fructose contents were significantly lower than in the control. Furthermore, continuous

Table 5
Statistical analysis of carbohydrate contents of the first leaflets of the sixth leaves of tomato plants grown under CL with different light qualities compared to the control at 0, 7, 14, 19 and 24 h on day 14 (n = 3).

Carbohydrate	Time on day 14 (h)	0	7	14	19	24
	Treatment					
Starch	R200B0	$a^{x**\ y}$	ab^{**}	a^{**}	a^{**}	ab^{*}
	R150B50	b^{ns}	ab^{*}	ab^{ns}	ab^{ns}	dc^{*}
	R100B100	ab^{**}	b^{*}	ab^{**}	abc^{ns}	bc^{**}
	R50B150	c^{ns}	c^{ns}	bc^{ns}	bc^{ns}	de^{ns}
	R0B200	c^{ns}	c^{ns}	c^{ns}	c^{ns}	e^{ns}
	WW	a^{*}	a^{*}	a^{ns}	a^{**}	a^{**}
	ANOVA^{z}	**	**	*	**	**
	CV^{t} (%)	32.05	21.72	41.89	37.48	21.13
Glucose	R200B0	ab^{ns}	ab^{*}	ab^{***}	b^{ns}	ab^{ns}
	R150B50	ab^{ns}	b^{***}	a^{*}	b^{ns}	b^{**}
	R100B100	b^{ns}	c^{***}	ab^{**}	bc^{*}	ab^{*}
	R50B150	b^{*}	c^{***}	b^{**}	d^{**}	b^{**}
	R0B200	b^{ns}	c^{***}	ab^{**}	cd^{*}	b^{**}
	WW	a^{ns}	a^{***}	a^{**}	a^{ns}	a^{ns}
	ANOVA	*	***	*	***	ns
	CV (%)	37.8	21.14	35.67	23.26	32.72
Fructose	R200B0	a^{ns}	a^{ns}	a^{*}	a^{ns}	a^{ns}
	R150B50	a^{ns}	b^{**}	a^{ns}	a^{ns}	ab^{*}
	R100B100	ab^{ns}	c^{***}	a^{*}	b^{**}	ab^{*}
	R50B150	b	d^{***}	b^{**}	b^{**}	bc^{**}
	R0B200	b	d^{***}	b^{***}	b^{**}	c^{***}
	WW	a^{ns}	ab^{**}	a^{*}	a^{ns}	a^{ns}
	ANOVA	*	***	***	***	**
	CV (%)	43.47	17.21	28.64	23.13	28.29
Sucrose	R200B0	b^{*}	ab^{ns}	bc^{ns}	abc^{**}	ab^{**}
	R150B50	b^{**}	b^{ns}	bc^{ns}	abc^{*}	ab^{*}
	R100B100	c^{ns}	b^{ns}	bc^{ns}	bc^{**}	b^{ns}
	R50B150	bc^{ns}	b^{ns}	b^{ns}	ab^{*}	b^{ns}
	R0B200	c^{ns}	b^{ns}	c^{ns}	c^{ns}	b^{ns}
	WW	a^{*}	a^{ns}	a^{ns}	a^{ns}	a^{ns}
	ANOVA	***	*	**	*	*
	CV (%)	32.41	49.03	35.09	34.44	48.89

x: Treatments with the same letter within the same column are not significantly different from each other.
y: ns, *, **, ***: not significantly or significantly different at $p = 0.05$, 0.01 or 0.001, respectively, compared to the control.
z: ns, *, **, ***: not significantly or significantly different among CL treatments at $p = 0.05$, 0.01 or 0.001, respectively.
t: coefficient of variation.

monochromatic red light resulted in the highest starch contents, which were equivalent to that of white CL treatment, while the leaf chlorosis level in this treatment was significantly lower than under continuous white light and monochromatic blue light. The two treatment with red:blue ratios of 1:1 and 1:3, which had equivalent and lowest levels of leaf chlorosis among all the CL treatments, had significantly different contents of both starch and soluble sugars from each other. The linear regression analysis between the percentage of leaf chlorotic area and leaf average contents of starch and soluble sugars on day 14 among CL treatments showed almost no correlation (coefficient of determination ranging from 0.0004 to 0.1221, Supplemental Fig. 1), indicating that the level of leaf chlorosis caused by CL could not be explained by the content of any of the above carbohydrates.

Interestingly, however, the average contents of glucose and fructose in the first leaflet of the sixth leaves on day 14 among CL treatments seemed to show relatively high correlations with the arc angle of the petioles (coefficient of determination of 0.9454 and 0.8788, respectively, Supplemental Figs. 2A and B) and the first leaflets (coefficient of determination of 0.7488 and 0.7633, respectively, Supplemental Figs. 2C and D) of the same leaves. Glucose has been reported to increase auxin biosynthesis (Sairanen et al., 2012) and transport (Mishra et al., 2009), while the fructose content is closely related to that of

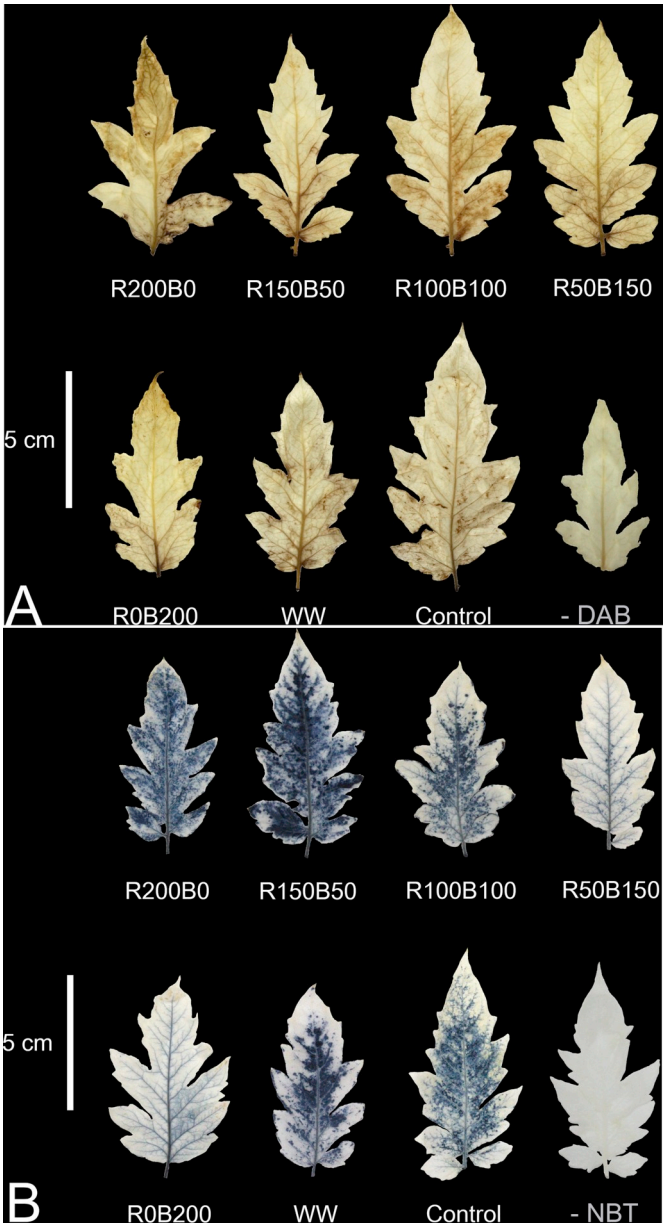

Fig. 6. Histochemical staining of H_2O_2 (A) and O_2^- (B) in the first leaflets of the sixth leaves of tomato plants grown under CL with different light qualities compared to the control (14/10-h photoperiod). Leaflet from WW treatment was used for the negative control (– DAB and – NBT).

glucose according to Zeeman (2015). Given that auxin has also been found to induce leaf curvature and epinasty in tomato (Kazemi and Kefford, 1974) and tobacco (Keller and Volkenburgh, 1997; Kawano et al., 2003), it is conceivable that the increase in petiole and leaflet curvatures under the continuous white light and monochromatic red light treatments was induced, in some parts, by the higher contents of glucose in the leaves. However, it should not be assumed that the glucose concentration was the sole cause of the leaf and leaflet curvatures in tomato, as red light with a very low intensity (1–10 $\mu mol \cdot m^{-2} \cdot s^{-1}$) alone has also been reported to be able to alter auxin polar transport in the tomato hypocotyl (Liu et al., 2011), suggesting other mechanisms other than glucose content through which light wavelength could affect auxin distribution and leaf/leaflet curvature.

4.4. H_2O_2 and O_2^- might not be involved in the induction of leaf chlorosis under continuous light conditions

Reactive oxygen species are common but very destructive by-products of many physiological processes in plants, including photosynthesis, and therefore the equilibrium between reactive oxygen species production and scavenging must always be strictly controlled. When this equilibrium is disrupted, such as under excessive light intensity or abiotic stresses, the consequence is, among many other things, the destruction of photosystem II (Sharma et al., 2012), which can result in leaf chlorosis. Therefore, it has been speculated that leaf chlorosis under CL in tomato can be induced by reactive oxygen species accumulation, most particularly H_2O_2 and O_2^-, which is a consequence of the feedback inhibition of photosynthesis caused by the accumulation of carbohydrates (Velez-Ramirez et al., 2011). As shown by the results of this study, H_2O_2 and O_2^- seemed to be induced by red light, as tomato leaves exposed to treatments with high red light ratios (50%–100%) appeared to have higher H_2O_2 and O_2^- contents, while the leaves under monochromatic blue light treatment were almost devoid of H_2O_2 and O_2^- (Fig. 6A and B). This result was similar to the results reported by Yang et al. (2018). However, similar to carbohydrates, H_2O_2 and O_2^- contents did not show any correlation with the percentage of chlorotic area in tomato leaves, and in the case of H_2O_2 remained relatively low in all treatments. This result agrees with the findings of Haque et al. (2017), who showed that the contents of H_2O_2 and O_2^-, as well as the activities of some common scavenging enzymes, were higher in tomato plants grown under CL with daily temperature variation compared to under CL with constant temperature, even though the later resulted in more severe leaf chlorosis. Taken together, these results suggest that H_2O_2 and O_2^- may not be involved in the induction of leaf chlorosis under CL in tomato.

5. Conclusions

The results of this study demonstrated that the light spectral quality had profound impacts on the effect of CL in tomato regarding growth, morphology, leaf injuries (chlorosis and epinasty) and leaf carbohydrate contents. Contrasting data for leaf chlorosis and epinasty revealed that these might represent two distinctive injuries that can be induced by CL via very different, independent mechanisms. Continuous monochromatic blue light was confirmed to strongly inhibit growth and to cause significant leaf chlorosis, while continuous monochromatic red light induced severe epinasty and excessive stem elongation. The level of leaf chlorosis showed no correlation to carbohydrate contents or H_2O_2 and O_2^- contents, suggesting that these compounds might not be directly responsible for the induction of leaf chlorosis by continuous light. However, the glucose and fructose contents showed relatively high correlations to the petiole and leaflet curvatures of the sixth leaves. A balanced red:blue ratio of 3:1 or 1:1 was effective in alleviating leaf injury as well as providing good biomass gain, and it could be applied for the cultivation of tomato under CL.

Acknowledgements

We sincerely thank Dongsub Kim, Suyeon Lee and Byungkwan Lee for the technical support in this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.06.004.

Funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author contribution

Minh Duy Pham: Conceptualization, Methodology, Investigation, Formal Analysis, Data Curation, Writing – Original Draft.

Hyunseung Hwang: Methodology, Resources, Investigation, Writing – Review and Editing.

Seonwoo Park: Methodology, Resources.

Meiyan Cui: Investigation.

Hyein Lee: Investigation.

Changhoo Chun: Methodology, Supervision, Validation, Writing – Review and Editing, Funding Acquisition.

Declarations of interest

None.

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