Continuous Light from Red, Blue, and Green Light-emitting Diodes Reduces Nitrate Content and Enhances Phytochemical Concentrations and Antioxidant Capacity in Lettuce

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ABSTRACT. Light-emitting diodes (LEDs) have shown great potential for plant growth and development, with higher luminous efficiency and more flexible and feasible spectral control compared with other artificial lighting. The combined effects of red and blue (RB) LED with or without green (G) LED light and white LED light on lettuce (*Lactuca sativa* L.) growth and physiology, including nitrate content, chlorophyll fluorescence, and phytochemical concentration before harvest, were investigated. Continuous light exposure at preharvest can effectively reduce nitrate accumulation and increase phytochemical concentrations in lettuce plants. Nitrate accumulation is dependent on the spectral composition and duration of treatment: lettuce exposed to continuous RB (with or without G) LED light with a photosynthetic photon flux (PPF) of 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ exhibited a remarkable decrease in nitrate content at 24 hour compared with white LED light treatment at the same PPF. In addition, RB LED light (R:B = 4:1) was more effective than white LED light at the same PPF in facilitating lettuce growth. Moreover, continuous LED light for 24 hours significantly enhanced free-radical scavenging activity and increased phenolic compound concentrations. We suggest that 24 hours continuous RB LED with G light exposure can be used to decrease nitrate content and enhance lettuce quality.

Vegetables play an important role in human nutrition and health because they contain a wide variety of beneficial compounds, including phytochemicals, minerals, and vitamins (Connor et al., 2005; Li and Kubota, 2009). Soilless techniques (e.g., hydroponic growth systems) have been recognized as a promising cultivation method for commercial production of vegetables. Hydroponic systems can improve water/nutrient use efficiency and crop yields, while minimizing the use of chemicals for fertilizers, especially nitrogen, and for pest and disease control (Luo et al., 1993). However, vegetables, especially leaf vegetables, grown in hydroponics can accumulate high levels of nitrate and other harmful substances; e.g., oxalic acid (Chen et al., 2014; Zhang et al., 2005). Excess nitrate is harmful because a small percentage of nitrates can convert to nitrite, which forms nitrosamines; these are potential cancer-causing agents (Gangolli et al., 1994; Mensinga et al., 2003). Therefore, keeping nitrate concentrations in vegetables below legal limits is essential for food safety.

Light is one of the most important environmental factors affecting plant growth, the biosynthesis of endogenous substances, and the accumulation of exogenous substances (e.g., nitrate and metal ions; Bian et al., 2015). Among the different wavelengths of light, red and blue light are the most effective in driving photosynthesis (Bouly et al., 2007). In addition, other wavelengths, such as far-red and green light, have effects on plant growth and development (Folta and Maruhnich, 2007; Li and Kubota, 2009; Tsormpatsidis et al., 2008). LEDs provide great advantages over other types of horticultural lighting. Some research has been conducted into the effects of LED light quality on plant growth and development because of the ability to separate and mix various wavelengths of LEDs, their space efficiency, and long life and minimal heating (Morrow, 2008; Samuolienė et al., 2012; Zhang et al., 2011). Kim's group showed that RB light was effective in facilitating biomass accumulation in lettuce and that adding suitable levels of G light to RB light was more effective in driving photosynthesis than the combination of RB light treatment alone (Kim et al., 2004a).

Photosynthetic capacity affects nitrate metabolism and accumulation in plants. Nitrate contents are negatively correlated with the amount of photosynthetic products (Champigny, 1995), e.g., carbohydrates. These products can be increased during continuous light (CL), whereas nitrate is generally stored in the vacuole in the dark (Cheung et al., 2014). The stored nitrate can be released to the cytosol during the day and reduced by nitrate reductase (Martinoia et al., 1981). Thus, our

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hypothesis is that CL and/or the combination of G with RB light could potentially decrease the nitrate contents and increase the nutrient contents in vegetables at preharvest stage. Current research on the effects of CL on photosynthesis and plant growth has mainly focused on the circadian clock and induced injury (Velez-Ramirez et al., 2011). Less is known about the effect of continuous LED light on the response of the photosynthetic system and on changes in the concentrations of endogenous antioxidant substances and phytochemicals. In addition, we are not aware of any reports addressing the effects of adding G LED light to RB LED light during preharvest CL treatment.

Lettuce is a major greenhouse-grown vegetable that is consumed worldwide, and is also a model crop for studying the light quality response (Kim et al., 2004a, 2004b; Li and Kubota, 2009; Samuolienė et al., 2012). However, lettuce has a great capacity for accumulating nitrate in its leaves (Santamaria, 2006). The main objective of this study is to investigate whether LED light and the combination of G with RB LED light have positive effects on reducing nitrate contents and increasing phytochemical levels and antioxidant capacity in lettuce under CL treatment. The results of this research will aid in designing suitable light sources for the production of high-quality vegetables in greenhouses.

Materials and Methods

PLANT MATERIAL AND GROWTH CONDITIONS. Butterhead lettuce (Rijk Zwaan, De Lier, The Netherlands) was seeded in plastic seedling trays (72 × 35 cm) filled with 3 peat : 1 vermiculite (v/v) and grown in a controlled growth chamber. The photoperiod, day/night temperature, relative humidity, and CO₂ levels in the growth chamber were maintained at 12 h, 25/20 ± 1 °C, 70% ± 5%, and 400 μmol·mol⁻¹, respectively. During this period, water was added daily to maintain the moistness of the substrate and replenish evapotranspiration losses. At 14 d after germination, when the seedlings had their second true leaves, similarly sized plants (≈8 mm in height) were transplanted into a recirculating hydroponic cultivation system in another growth chamber. The photoperiod, day/night temperature, relative humidity, and CO₂ levels in the growth chamber were maintained at same conditions as before. The composition of the applied nutrient solution was the following (in millimolars): 5.94 KNO₃, 1.42 MgSO₄,

1.00 NH₄H₂PO₄, 0.44 KH₂PO₄, 2.12 Ca(NO₃)₂, 4.29 ethylenediaminetetraacetic acid (EDTA)-Fe, 4.84 × 10⁻² H₃BO₃, 1.35 × 10⁻² MnSO₄, 1.35 × 10⁻³ ZnSO₄, 5 × 10⁻⁴ CuSO₄, 4 × 10⁻⁴ (NH₄)₆MoO₂₄. The nutrient solution was replaced with fresh solution every 7 d throughout the experiment.

LIGHT CONDITIONS AND TREATMENTS. To investigate whether light quality affects nitrate content and other nutritional qualities, we used white [W (400–700 nm)] LED light and other different LED light conditions: combined R (660 nm) and B (460 nm) LED light, with or without G (530 nm) LED light. Treatments with three replicates (150 plants per treatment in an area of 1.6 m²; 50 plants per replicate) consisted of five light conditions: 1) RB LEDs with a 12-h photoperiod as a control (RB-CK), 2) RB LEDs with preharvest continuous light (RB-CL), 3) RBG LEDs with preharvest continuous light (RBG-CL), 4) W LEDs with a 12-h photoperiod (as the second control; W-CK), and 5) W LEDs with preharvest continuous light (W-CL). The ratio of RB LEDs was 4:1 and the ratio of RBG LEDs was 4:1:1 (Table 1). Plants were grown under RB LEDs with a photoperiod of 12 h and a *PPF* of 200 μmol·m⁻²·s⁻¹ before treatment with RBG-CL, RB-CL, or RB-CK. The lettuce plants in the W-CL and W-CK treatments were first grown with W LEDs with a photoperiod of 12 h and a *PPF* of 200 μmol·m⁻²·s⁻¹ before carrying out the light treatments. These LED light sources (VQ-G200; Vanq Technology, Shenzhen, China) were maintained ≈20 cm above the canopies of the plants. The *PPF* and spectrum of the LED light sources were monitored daily by a quantum sensor (LI-190SA; LI-COR, Lincoln, NE) and a spectrometer (USB 200; Ocean Optics, Dunedin, FL), respectively (Supplemental Fig. 1). The *PPF* was maintained at 200 μmol·m⁻²·s⁻¹ by adjusting the distance between LED light sources and the plant canopies.

At the end of the dark period at 21 d after planting in the hydroponic cultivation system (35 d after germination), the lettuce plants were subjected to the various light treatments. Nonreflective black separators were placed between adjacent treatments to prevent light contamination. When the treatments were initiated, the nutrient solution was replaced with fresh nutrient solution to avoid any possible changes in the nutrient solution.

PLANT SAMPLING. When the treatments were initiated, 12 lettuce plants were sampled from each light treatment (four plants per replicate; three replicates per light treatment) at intervals of 12 h for a total 72 h. The leaves of these plants

Table 1. The spectra data and photoperiod or light duration of white (W) light-emitting diode (LED) light control (W-CK), red (R) and blue (B) LED light control [RB-CK (R:B = 4:1)], continuous light (CL) by W LED (W-CL), CL by RB LED [RB-CL (R:B = 4:1)], and RB-CL plus green (G) LED light [RBG-CL (R:B:G = 4:1:1)] treatments (n = 10). CL treatments were used to investigate the effects of CL on nitrate content, phytochemical concentrations, and antioxidant capacity in lettuce compared with controls.

Parameter	Treatments				
	W-CK	W-CL	RB-CK	RB-CL	RBG-CL
	Photon flux [mean ± SE (μmol·m ⁻² ·s ⁻¹)] ^z				
Blue (400–500 nm)	83.8 ± 1.3	84.0 ± 1.5	40.1 ± 1.9	40.7 ± 1.9	33.6 ± 1.6
Green (500–600 nm)	84.0 ± 1.1	84.5 ± 1.1	0	0	33.4 ± 0.9
Red (600–700 nm)	33.8 ± 1.2	33.6 ± 1.3	161.7 ± 3.2	162.5 ± 4.5	134.8 ± 3.4
<i>PPF</i> (400–700 nm)	201.6 ± 3.1	202.1 ± 2.3	201.8 ± 4.2	203.2 ± 4.2	201.8 ± 5.3
	Spectral ratios				
Red:blue or red:blue:green	0.4:1:1	0.4:1:1	4:1	4:1	4:1:1
	Photoperiod or light duration (h)				
	12	72	12	72	72

PPF = photosynthetic photon flux.

^zData of photon flux in each light wavelength are shown as the mean of 10 replicates, which were recorded and averaged over 10 locations in the plant canopy by a quantum sensor and a spectrometer.

without petioles were immediately treated with liquid nitrogen after collection and then stored at -80°C for phytochemical and antioxidant enzyme measurements. Twelve additional lettuce plants were also harvested from each light treatment at the first (0 h) and last point of the time course (72 h). They were used to calculate the initial and final dry weight (DW) and fresh weight (FW) of the shoots and roots, the leaf area, and the leaf mass per area (LMA).

MEASUREMENT OF NITRATE CONCENTRATIONS. Leaf nitrate concentrations were evaluated using a spectrophotometric method as described by Cataldo et al. (1975) with slight modifications. Leaf tissue (2 g) was pulverized in liquid nitrogen and then suspended in 10 mL of distilled water. The sample was boiled for 30 min at 100°C in a water bath. The extracted samples were cooled with tap water, filtered, and diluted to 25 mL with distilled water. The extract (0.1 mL) was further diluted with 0.4 mL of 5% (w/v) salicylic acid-concentrated sulfuric acid. After 20 min, 9.5 mL of 8% (w/v) NaOH solution was added. The final nitrate concentration was measured using the absorbance monitored at 410 nm with respect to its standard curve.

MEASUREMENT OF PIGMENTS. The method described by Torrecillas et al. (1984) was used to measure the chlorophyll (Chl) content. Leaf tissue (100 mg) was subjected to extraction in 5 mL of 80% (v/v) acetone at 4°C for 72 h. The absorbance of the extraction solution measured at 470, 645, and 663 nm against 80% (v/v) acetone as a blank by an ultraviolet and visible light (UV-VIS) spectrophotometer (UV-180; Shimadzu, Kyoto, Japan) was used to calculate the contents of Chl *a*, Chl *b*, and carotenoids with a microplate reader (Benchmark; Bio-Rad Laboratories, Omaha, NE).

MEASURE OF TOTAL PHENOLIC COMPOUNDS. The total phenolic compound concentrations were determined using the method described by Ragaee and Abdel-Aal (2006). Leaf tissue (1 g) was pulverized in liquid nitrogen and subjected to extraction with 10 mL of 80% (v/v) methanol. The extract was shaken for 30 min before centrifugation at 2012 g_n for 20 min. The extract (1 mL) was then diluted with 1 mL of Folin-Ciocalteu reagent and 2 mL of 7.5% (w/v) Na_2CO_3 solution. After 20 min, the absorbance was monitored using an UV-VIS spectrophotometer (UV-180) at 765 nm against water as a blank. The total phenolic compounds were calculated using a calibration curve with gallic acid as a standard.

MEASUREMENT OF DPPH FREE-RADICAL SCAVENGING ACTIVITY. The free-radical scavenging capacity of the lettuce leaf extract was evaluated spectrophotometrically using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging capacity, as described by Ragaee and Abdel-Aal (2006). The methanol extract (1 mL) was diluted with 4 mL of 80% (v/v) methanol and 1 mL of freshly prepared DPPH. Absorbance was monitored at 16 min against 80% (v/v) methanol as a blank and used to calculate the ability of the leaf tissues to scavenge DPPH free radicals (micromoles/gram).

MEASUREMENT OF NITRATE REDUCTASE ACTIVITY. The nitrate reductase (NR) activity was measured using the method described by Rosales et al. (2012) and Yaneva et al. (2002). Leaf tissue was homogenized with 0.1 M phosphate buffer (pH 7.5) and then centrifuged at $17,000\text{ g}_n$ for 15 min at 4°C . The assay mixture for calculating NR activity contained 200 $\mu\text{mol KNO}_3$, 0.2 μmol nicotinamide adenine dinucleotide, and 100 μL of the extraction. After incubation at 30°C for 20 min, 50 μL 1 M zinc acetate was added to stop the reaction. The mixture was centrifuged at 7600 g_n for 5 min, and the absorbance of the supernatant

was measured using an UV-VIS spectrophotometer (UV-180) at 540 nm. The amount of formed NO_2^- was calculated using a standard curve prepared with NaNO_2 . One unit of NR activity was defined as 1 nmol of NO_2^- formed per gram per minute.

MEASUREMENT OF ANTIOXIDANT ENZYME ACTIVITY AND LIPID PEROXIDATION. The leaf tissue was homogenized with ice-cold 50 mM phosphate buffer (pH 7.0). The extract was centrifuged at $20,000\text{ g}_n$ for 30 min at 4°C . The supernatant, referred to as "crude extract," was used for superoxide dismutase (SOD) and peroxidases (POD) enzyme activity assays and lipid peroxidation content determination.

A spectrophotometric method was used to determine SOD activity (Giannopolitis and Ries, 1977). A 3-mL reaction mixture containing 100 μL enzyme extract, 50 mM phosphate buffer (pH 7.8), 0.1 μM EDTA, 13 mM methionine, 75 μM nitro blue tetrazolium, and 2 μM riboflavin was shaken and then illuminated by fluorescent lamps. The absorbance monitored at 560 nm was used to calculate SOD activity. One unit of the SOD activity was defined as the amount of enzyme causing 50% inhibition of the rate of nitroblue tetrazolium chloride reduction.

The guaiacol oxidation method was used to determine POD activity (Maehly and Chance, 1954), and the formation of malondialdehyde (MDA) was used to estimate lipid peroxidation (Schaefer and Bassham, 1977).

MEASUREMENT OF PLANT GROWTH AND LEAF MORPHOLOGY. The lettuce plants sampled from each light treatment at 0 and 72 h were cut at the hypocotyls to measure the shoot and root FW. Leaf area was measured using an automatic area meter (LI-3100; LI-COR). From each leaf, leaf discs (1.28 cm) without leaf margins and veins were cut randomly over the leaf area. They were then dried in an oven at 70°C for 48 h before weighing and calculating the shoot and root DW and the LMA (Fan et al., 2013; Hogewoning et al., 2010).

MODULATED CHLOROPHYLL FLUORESCENCE ANALYSIS. When the treatments were initiated, 12 lettuce plants were randomly selected from each light treatment, and the Chl fluorescence in the second-youngest, fully expanded leaf was measured using the portable photosynthetic apparatus (LI-6400XT-40; LI-COR) according to the method described by Yan et al. (2012). The interval of Chl fluorescence measurements was 12 h. The minimum (F_o) and maximum (F_m) Chl fluorescence levels were determined after the leaves had been dark adapted for 30 min. The maximum Chl fluorescence of the light-adapted state (F_m') and the steady-state Chl fluorescence (F_s) under each light treatment (monitored at each time interval) were used to calculate the following Chl fluorescence parameters of photosynthetic system II (PSII) (Huner et al., 1998; Lichtenthaler et al., 2005): the maximal quantum yield (F_v/F_m) = $(F_m - F_o)/F_m$; the effective quantum yield (Φ_{PSII}) = $(F_m' - F_s)/F_m'$; nonphotochemical quenching (qN) = $(F_m - F_m')/(F_m - F_o)$; photochemical quenching (qP) = $(F_m' - F_s)/(F_m' - F_o)$; relative deviation energy from a complete balance between PSI and PSII ($1 - \text{qP}$) = $1 - (F_m' - F_s)/(F_m' - F_o)$; relative electron transport rate (ETR) = $\Phi_{\text{PSII}} \times PPF \times f_a \times f_{\text{PSII}}$, where PPF is the photosynthesis photon flux ($\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), f_a is the light absorption rate of leaf (0.85), and f_{PSII} is the proportion of PSII accounting for the photosynthesis system (0.5). The net photosynthesis rate (P_n) at 0 and 72 h was determined with a portable photosynthetic apparatus (LI-6400XT-40), which also was used for the Chl fluorescence determination.

STATISTICAL ANALYSIS. All means were calculated based on the results obtained from 12 plants (4 plants per replication with three replicates per light treatment). The means of the photon flux of light sources were calculated from 10 *PPF* measurement locations over the plant canopy. All of the data were evaluated by analysis of variance using SAS software (version 8.1; SAS Institute, Cary, NC), and significant differences between means were assessed by Duncan's multiple range test at $P \leq 0.05$ or $P \leq 0.01$ (Wang et al., 2009).

Results

PLANT GROWTH AND LEAF MORPHOLOGY. Before harvest, applying 72 h of CL significantly increased lettuce growth, as shown in Table 2 and Fig. 1. DW and shoot-to-root ratios (S/R) were both significantly higher under the CL treatments (W-CL, RB-CL, and RBG-CL) than under the other treatments (Table 2). The initial (0 h) FW and DW of the shoots and roots under the RB-CK, RBG-CL, and RB-CL treatments were almost 2-fold higher than those under the W LED light treatments (W-CK and W-CL), indicating that combined R and B light (R:

B = 4:1) was more effective in facilitating lettuce plant growth than W LED light at the same *PPF* (200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). After 72 h of CL treatment, the final FW of the shoots was the highest (29.39 g) under the RBG-CL treatment among the applied treatments, followed by RB-CL, RB-CK, W-CL, and W-CK, which showed the lowest FW (17.80 g). However, the final root FW under RB-CL was similar to that under RBG-CL, but significantly lower than that under RB-CK. The final shoot DW under RBG-CL and RB-CL did not differ significantly, but the final shoot DW under RB-CL was significantly higher than that under W-CL. No significant differences were observed in the final root DW among RB-CK, RBG-CL, and RB-CL. Interestingly, the S/R value obtained under RBG-CL was significantly higher than that under RB-CL. However, S/R did not differ significantly between RB-CL and W-CL. These results suggest that among the CL treatments, RBG-CL was more effective in enhancing the shoot FW and DW of the lettuce plants.

When the light treatments were initiated (0 h), leaf area for RB-CK, RB-CL, and RBG-CL were significantly higher than those under W-CK and W-CL. However, the levels of P_n and LMA among all the treatments were not different. At 72 h, the

Table 2. Initial (0 h) and final (72 h) fresh weight (FW), dry weight (DW), and shoot-to-root ratio (S/R DW) in lettuce in response to white (W) light-emitting diode (LED) light control (W-CK), red (R) and blue (B) LED light control [RB-CK (R:B = 4:1)], continuous light (CL) by W LED (W-CL), CL by RB LED [RB-CL (R:B = 4:1)], and RB-CL plus green (G) LED light [RBG-CL (R:B:G = 4:1:1)] treatments at 0 and 72 h (n = 12). The photoperiod and light duration for controls and CL treatments was 12 and 72 h, respectively. The photosynthetic photon flux for all treatments was 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

Treatments	FW (g)				DW (g)						S/R (ratio)	
	Shoot		Root		Shoot		Root		Shoot + Root			
	0 h	72 h	0 h	72 h	0 h	72 h	0 h	72 h	0 h	72 h	0 h	72 h
W-CK	8.05b ^z	17.80 d	0.95 b	1.62 d	0.47 b	0.97 d	0.06 b	0.11c	0.54 b	1.08 c	7.82 a	8.76 c
W-CL	8.01 b	21.29 c	0.95 b	2.01 c	0.46 b	1.71 c	0.06 b	0.13 bc	0.53 b	1.83 b	8.08 a	12.71 ab
RB-CK	16.04 a	26.43 b	1.60 a	2.50 a	0.85 a	1.63 c	0.10 a	0.17 ab	0.95 a	1.80 b	7.78 a	9.42 c
RB-CL	16.21 a	28.14ab	1.60 a	2.32 ab	0.83 a	2.13 b	0.12 a	0.19 a	1.04 a	2.32 a	6.79 a	11.12 b
RBG-CL	16.62 a	29.39 a	1.62 a	2.08 b	0.88 a	2.32 a	0.12 a	0.17 ab	1.00 a	2.49 a	7.43 a	13.64 a

^zMeans within the same column followed by different lower case letters are significantly different between treatments at $P \leq 0.05$ according to Duncan's multiple range test.

Fig. 1. The growth of lettuce under white (W) light-emitting diode (LED) light control (W-CK), red (R) and blue (B) LED light control [RB-CK (R:B = 4:1)], continuous light (CL) by W LED (W-CL), CL by RB LED [RB-CL (R:B = 4:1)], and RB-CL plus green (G) LED light [RBG-CL (R:B:G = 4:1:1)] treatments for 72 h. Bar represents 5 cm. The photoperiod and light duration for controls and CL treatments was 12 and 72 h, respectively. The photosynthetic photon flux for all treatments was 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

leaf area and LMA for all CL treatments were significantly increased compared with controls (W-CK and RB-CK) (Table 3). However, levels of P_n for all CL treatments were lower than controls. The leaf area for RBG-CL was the highest, followed by RB-CL and then W-CL. LMA for RBG-CL, RB-CL, and W-CL were not significantly different at 72 h (Table 3).

Furthermore, most leaves of lettuce showed better growth under CL treatment compared with control treatments. The plants produced more leaves of about equal dimensions, and round, tightly formed heads, which possess an attractive appearance which could potentially improve commercial value (Fig. 1).

NITRATE CONTENT. Figure 2 showed the significant change of nitrate content in leaf under W LEDs ($\approx 517 \text{ mg}\cdot\text{kg}^{-1}$) compared with under R and B combination LEDs ($\approx 468 \text{ mg}\cdot\text{kg}^{-1}$) before the light treatments. The nitrate content under W-CK and W-CL increased gradually between 12 and 60 h and between 12 and 48 h, respectively, and declined afterward. However, nitrate content under W-CL was markedly lower than that under W-CK between 24 and 72 h (Fig. 2A). At 12 h, the nitrate content under RBG-CL was significantly reduced to $396.8 \text{ mg}\cdot\text{kg}^{-1}$, compared with RB-CK ($461.5 \text{ mg}\cdot\text{kg}^{-1}$). Remarkably, the leaf nitrate content under RBG-CL and RB-CL at 24 h was dramatically reduced to 222.7 and $296.7 \text{ mg}\cdot\text{kg}^{-1}$, respectively, compared with the other light treatments (Fig. 2B). Nitrate content under RBG-CL and RB-CL were both significantly lower than that under RB-CK between 36 and 72 h. Compared with the other light conditions, the RBG-CL treatment showed the lowest leaf nitrate content throughout the experiment, indicating that RBG-CL was more efficient in reducing the nitrate content in lettuce leaves than the other studied CL treatments.

CHLOROPHYLL FLUORESCENCE. Under different types of CL treatment, the levels of F_v/F_m at 24 h were markedly different from controls. Irradiation with W-CL, RBG-CL, and RB-CL resulted in gradual declines in F_v/F_m , from ≈ 0.83 at 0 h to 0.81 or 0.79 at 24 h (Fig. 3A).

There was no significant change in Φ_{PSII} or qP under RB-CL and W-CL compared with the corresponding control treatments (RB-CK and W-CK) between 0 and 24 h. The values of Φ_{PSII} and qP for RB-CL were comparable to those for RBG-CL, but were higher than those for W-CL between 0 and 24 h (Fig. 3B and C).

Φ_{PSII} and qP were increased under all of the CL treatments between 24 and 48 h, with a concomitant decrease in qN (compared with the levels measured at 24 h). F_v/F_m , Φ_{PSII} , and qP began to decrease after 48 h under RBG-CL and RB-CL. However, the levels of qN recorded under RBG-CL and RB-CL were comparable to that recorded under RB-CK (Fig. 3B). This result suggests that continuous LED light treatment exceeding 48 h might begin to inactivate and damage the photosynthetic apparatus in the lettuce leaves.

The levels of ETR under CL treatments were markedly higher than under RB-CK and W-CK between 24 and 72 h. Furthermore, the ETR under RBG-CL was higher than that under RB-CL between 24 and 72 h (Fig. 3E). The values of $1-qP$ obtained under the CL treatments were similar to or lower than those observed in the controls (RB-CK or W-CK) between 0 and 24 h. However, $1-qP$ under RBG-CL and RB-CL gradually increased after 48 h (Fig. 3F); i.e., the balance of the excitation energy between PSI and PSII was shifted under RBG-CL and RB-CL as the duration of CL exceeded 48 h.

PHYTOCHEMICAL CONCENTRATION AND DPPH FREE-RADICAL SCAVENGING ACTIVITY. Table 4 showed that the phytochemical

Table 3. Initial (0 h) and final (72 h) lettuce leaf area, leaf mass per area (LMA), and the net photosynthesis rate (P_n) under white (W) light-emitting diode (LED) light control (W-CK), red (R) and blue (B) LED light control [RB-CK (R:B = 4:1)], continuous light (CL) by W LED (W-CL), CL by RB LED [RB-CL (R:B = 4:1)], and RB-CL plus green (G) LED light [RBG-CL (R:B:G = 4:1:1)] treatments at 0 and 72 h ($n = 12$). The photoperiod and light duration for controls and CL treatments was 12 and 72 h, respectively. The photosynthetic photon flux for all treatments was $200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

Treatments	Leaf area (cm^2)		LMA ($\text{g}\cdot\text{cm}^{-2}$)		P_n [CO_2 ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)]	
	0 h	72 h	0 h	72 h	0 h	72 h
W-CK	182.1 b ^z	377.6 d	0.003 a	0.002 b	4.54 a	4.46 ab
W-CL	180.5 b	552.3 c	0.003 a	0.003 a	4.35 a	3.38 c
RB-CK	292.8 a	452.3 d	0.003 a	0.002 b	4.97 a	5.38 a
RB-CL	295.3 a	722.5 b	0.003 a	0.003 a	4.67 a	3.91 b
RBG-CL	289.5a	820.5 a	0.003 a	0.003 a	4.74 a	4.08 b

^zMeans within the same column followed by different lower case letters are significantly different between treatments at $P \leq 0.05$ according to Duncan's multiple range test.

Fig. 2. Changes in nitrate content in lettuce leaves under white (W) light-emitting diode (LED) light control (W-CK), red (R) and blue (B) LED light control [RB-CK (R:B = 4:1)], continuous light (CL) by W LED (W-CL), CL by RB LED [RB-CL (R:B = 4:1)], and RB-CL plus green (G) LED light [RBG-CL (R:B:G = 4:1:1)] treatments. (A) Nitrate content under W-CK and W-CL. (B) Nitrate content under RB-CK, RB-CL, and RBG-CL. The photoperiod and light duration for controls and CL treatments was 12 and 72 h, respectively. The photosynthetic photon flux for all treatments was $200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Error bars show $\pm\text{SE}$ ($n = 12$). *, **Indicate significant differences compared with control (W-CK or RB-CK) at each time point at $P \leq 0.05$ and $P \leq 0.01$, respectively, according to Duncan's multiple range test.

concentrations in the lettuce leaves were substantially affected by CL treatment. The total phenolic compounds and DPPH radical scavenging activity under W-CL were higher, but carotenoids showed a marked decrease compared with W-CK

Fig. 3. Changes in the maximum quantum yield of the photosystem II (PSII) in dark-adapted leaves [F_v/F_m (A)], the actual quantum yield of PSII [Φ_{PSII} (B)], photochemical quenching in light-adapted leaves [qP (C)], non-photochemical quenching [qN (D)], the relative electron transport rate of PSII [ETR (E)], and the relative deviation of the excitation from a complete balance between PSI and PSII [$1-qP$ (F)] in lettuce leaves under white (W) light-emitting diode (LED) light control (W-CK), red (R) and blue (B) LED light control [RB-CK (R:B = 4:1)], continuous light (CL) by W LED (W-CL), CL by RB LED [RB-CL (R:B = 4:1)], and RB-CL plus green (G) LED light [RBG-CL (R:B:G = 4:1:1)] treatments. The photoperiod and light duration for controls and CL treatments was 12 and 72 h, respectively. The photosynthetic photon flux for all treatments was $200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Error bars show $\pm\text{SE}$ ($n = 12$).

at 24 h. Compared with RB-CK, the total phenolic compounds, DPPH radical scavenging activity, and carotenoids in lettuce leaves under RBG-CL, were significantly increased at 12 h, but carotenoids was lower than that under RB-CL at 24 h. These results indicate that changes in the light spectrum under CL treatment significantly affected the secondary metabolism in lettuce. The total phenolic compounds and DPPH free radical scavenging activity under RB-CL and RBG-CL both significantly higher, but carotenoids were markedly lower than observed under RB-CK at 36 h. Similarly, the total phenolic compounds and DPPH free radical scavenging activity under all CL treatments were significantly higher, but carotenoids were markedly lower than controls between 36 and 72 h (data not shown; Supplemental Table 1). Furthermore, significant reductions in the Chl content and Chl *a/b* ratio was only observed under RBG-CL at 24 h during the 72 h CL treatment (data not shown; Supplemental Table 2).

ACTIVITY OF NITRATE REDUCTASE. Under CL treatment, NR activity changes were similar to these observed in lettuce under a normal light period (controls) (Fig. 4A and B). NR activity under W-CL gradually increased and markedly higher than W-CK between 36 and 48 h; however, NR activity became lower than that under W-CK after 60 h (Fig. 4A). NR activity under RBG-CL increased markedly by 72.23% at 12 h, 70% at

24 h, and 93.82% at 60 h compared with RB-CK. However, significant increases in NR activity under RB-CL were only obtained at 24 and 60 h compared with RB-CK (Fig. 4B).

ANTIOXIDANT ENZYME ACTIVITY AND LIPID PEROXIDATION. The effects of CL on SOD and POD activity and MDA content in lettuce plants depend on the light duration and the light spectrum of the light sources. SOD activity and MDA content under W-CL were both significantly higher than that under W-CK from 24 h (Fig. 5A and C), but the activity of POD under W-CL was markedly lower than that under W-CK between 24 and 48 h (Fig. 5B).

SOD activity under RBG-CL treatment was higher than that under RB-CL between 12 and 60 h. Compared with RB-CK, a significantly higher SOD activity under RB-CL was only observed from 36 to 72 h (Fig. 5D). This indicates that adding G light could induce SOD activity to reduce light stress. Unlike the response of SOD activity, POD activity under RBG-CL was significantly lower at 12 h and between 48 and 72 h, but markedly higher at 24 and 36 h than the activity under RB-CK. However, the activity of POD under RB-CL was significantly lower than that under RB-CK between 24 and 72 h (Fig. 5E). MDA content under RBG-CL and RB-CL were higher than that under RB-CK between 24

and 60 h (except at 36 h), but MDA content under RBG-CL was lower than that under RB-CL at 12, 24, and 72 h. (Fig. 5F). Taken together, these results indicate that CL for more than 24 h may damage plant leaves.

Discussion

The present investigation was undertaken in an effort to elucidate if continuous LED light and the combination of G with B and R light exposure at preharvest can effectively reduce nitrate accumulation. Our results clearly show that CL by RB and RBG LED dramatically affect the nitrate content at preharvest stage. The change of nitrate in green tissue is closely linked to internal physiological activities, including the balance between the absorption of light energy and the fixation of inorganic carbon. The nitrate content in plants is inversely correlated with the concentration of soluble, nonstructural forms of carbon, such as sugars and organic acids (Champigny, 1995), because nitrate and soluble, nonstructural forms of carbon play complementary roles in maintaining cell turgor (Veen and Kleinendorst, 1985). We observed that nitrate content reduced dramatically under both RB-CL and RBG-CL treatments between 0 and 24 h. This may be explained that CL treatment increased carbohydrate synthesis and led to an

Table 4. Total phenolic compounds, carotenoids, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity in lettuce in response to white (W) light-emitting diode (LED) light control (W-CK), red (R) and blue (B) LED light control [RB-CK (R:B = 4:1)], continuous light (CL) by W LED (W-CL), CL by RB LED [RB-CL (R:B = 4:1)], and RB-CL plus green (G) LED light [RBG-CL (R:B:G = 4:1:1)] treatments at 12, 24, and 36 h (n = 12). The photoperiod and light duration for controls and CL treatments was 12 and 72 h, respectively. The photosynthetic photon flux for all treatments was 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

Time (h)	Treatments	Total phenolic compounds ($\text{mg}\cdot\text{g}^{-1}$ FW)	Carotenoids ($\text{mg}\cdot\text{g}^{-1}$ FW)	DPPH radical scavenging activity ($\mu\text{mol}\cdot\text{g}^{-1}$ FW)
12	W-CK	1.44 b ^z	0.13 b	3.16 a
	W-CL	1.50 b	0.13 b	3.20 a
	RB-CK	1.55 b	0.13 b	2.70 c
	RB-CL	1.51 b	0.12 b	2.82 c
	RBG-CL	1.73 a	0.15 a	2.93 b
24	W-CK	1.43 c	0.12 b	3.25 d
	W-CL	1.69 b	0.10 c	3.90 b
	RB-CK	1.76 a	0.12 b	3.51 c
	RB-CL	1.70 ab	0.13 a	4.26 a
	RBG-CL	1.74 ab	0.095 d	4.18 a
36	W-CK	1.44 b	0.13 c	3.10 d
	W-CL	1.47 b	0.11 d	3.88 b
	RB-CK	1.17 c	0.15 a	3.44 c
	RB-CL	1.55 a	0.13 c	4.26 a
	RBG-CL	1.57 a	0.14 b	4.30 a

^zMeans within the same column followed by different lower case letters are significantly different between treatments at $P \leq 0.05$ according to Duncan's multiple range test.

Fig. 4. Changes in nitrate reductase (NR) activity in lettuce leaves under white (W) light-emitting diode (LED) light control (W-CK), red (R) and blue (B) LED light control [RB-CK (R:B = 4:1)], continuous light (CL) by W LED (W-CL), CL by RB LED [RB-CL (R:B = 4:1)], and RB-CL plus green (G) LED light [RBG-CL (R:B:G = 4:1:1)] treatments. (A) NR activities under W-CK and W-CL. (B) NR activities under RB-CK, RB-CL, and RBG-CL. The photoperiod and light duration for controls and CL treatments was 12 and 72 h, respectively. The photosynthetic photon flux for all treatments was 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Error bars show $\pm\text{SE}$ (n = 12). *, **Indicate significant differences compared with control (W-CK or RB-CK) at each time point at $P \leq 0.05$ and $P \leq 0.01$, respectively, according to Duncan's multiple range test.

increase in the supply of ferredoxin and NADPH, which are used for the reduction of nitrate in leaves (Huner et al., 1998; Manzano et al., 1976; Velez-Ramirez et al., 2011). However, as the duration of CL is increased, de novo synthesis of NR and NAD(P)H can decrease (Lillo, 1994). Pilgrim et al. (1993) has showed circadian and light-regulated expression of NR in *Arabidopsis thaliana* (L.) Heynh. The daily synthesis and destruction of NR leads to the circadian rhythm in NR activity and this circadian rhythm appears even under CL treatment (Ramalho et al., 1995). This kind of circadian rhythm in NR activity was also observed in our study (Fig. 4). This supports the suggestion that changes in NR, NAD(P)H, and circadian-regulated expression of NR may be the reason leading to nitrate content under RB-CL and RBG-CL, first decreasing and then increasing during CL treatment.

In the present study, the similar LMA and Chl content under all CL treatments at 72 h (Table 3 and Supplemental Table 2) indicates a similar capacity of light energy absorption across all of the CL treatments (Evans and Poorter, 2001). However, the lower leaf area and P_n for W-CL (Table 3) would lead to less light interception for photosynthesis and carbohydrate synthesis compared with the other CL treatments (Bugbee and Salisbury, 1988), as apparent from the lower final DW obtained under W-CL than under RB-CL and RBG-CL (Table 2). Compared with other treatments, the higher FW and DW under RB-CK, RB-CL, and RBG-CL at 0 h might likely be due to the capacity for greater light interception per unit ground area, leading to an increased leaf thickness and shoot leaf area (Niinemets, 1999). However, there was no significant difference in P_n and LMA among various light treatments at 0 h. For this study, we focused on the effect of the CL. We believe that the trends we have recorded are likely to represent some of the most prominent differences (e.g., FW and DW in shoots and DW in S/R) between CL-grown and CK-grown. Under low carbohydrate synthesis conditions, more nitrates can accumulate and store in vacuoles as an osmoticum to maintain

Fig. 5. Changes in (A, D) superoxide dismutase (SOD) and (B, E) peroxidases (POD) activities and (C, F) malondialdehyde (MDA) content in lettuce under white (W) light-emitting diode (LED) light control (W-CK), red (R) and blue (B) LED light control [RB-CK (R:B = 4:1)], continuous light (CL) by W LED (W-CL), CL by RB LED [RB-CL (R:B = 4:1)], and RB-CL plus green (G) LED light [RBG-CL (R:B:G = 4:1:1)] treatments. The photoperiod and light duration for controls and CL treatments was 12 and 72 h, respectively. The photosynthetic photon flux for all treatments was $200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Error bars show $\pm\text{SE}$ ($n = 12$). * **Indicate significant differences compared with control (W-CK or RB-CK) at each time point at $P \leq 0.05$ and $P \leq 0.01$, respectively, according to Duncan's multiple range test.

cell turgor (Steingröver et al., 1986). This might explain the higher nitrate content observed under W-CL than under the other CL treatments.

In this study, the FW of lettuce plants was higher under RBG-CL than under the other treatments studied. This result is consistent with those obtained by Sommer and Franke (2006), who found that treatment of radish (*Raphanus sativus* L.) and carrot (*Daucus carota* L.) seeds with G laser light enhanced the FW of the plants at the time of harvest. Similarly, studies have demonstrated that the combination of G light with RB light is more effective than the combined use of RB light alone in facilitating lettuce growth (Kim et al., 2004a, 2004b). G light shows a greater ability to penetrate into plant canopy (Smith, 1994). During preharvest CL treatment, the leaves in the lower canopy under RBG-CL were able to use the transmitted G light to drive photosynthesis (Kim et al., 2004b), and the addition of G light in the RBG-CL treatment reduced the impact of leaf senescence and shading within the canopy on photosynthesis (Preece and Read, 1993). Furthermore, G light transmits deeper within leaves and drives photosynthesis with more efficient than either R or B light in the deeper layers of leaves, whereas R or B light is absorbed in the more superficial layers (Sun et al., 1998). Photosynthesis involves both carbon assimilation and nitrate reduction (Noctor and Foyer, 1998). This may in part

explain why lettuce under RBG-CL had higher FW and DW and the concomitant lower nitrate contents. However, Urbonavičiūtė et al. (2007) found that a combination of 86% R light and 14% B light effectively reduced the nitrate content of harvest stage lettuce.

With light duration increasing, excessive carbohydrate accumulation under CL could lead to a greater use of absorbed energy for generating more reactive oxygen species (ROS) (Cakmak and Kirkby, 2008; Huner et al., 1998). Under environmental stresses, plants often enhance antioxidant enzyme activities and secondary metabolites, such as phenolic compounds and terpenoids, to scavenge ROS to protect plant against oxygen damage (Grassmann et al., 2002). This could explain why total phenolic compounds, DPPH radical scavenging activity, and SOD activity under CL treatments were higher than under controls between 24 and 72 h (Fig. 5).

In the present study, F_v/F_m exhibited a sustained decline under CL treatments compared with the corresponding controls between 0 and 24 h. F_v/F_m under CL was lower than under controls from 24 to 72 h, but F_v/F_m showed similar trend as controls. Given that these measurements include dark periods (and possible circadian clock effects), these results are not surprising.

The decline in F_v/F_m in this study indicated that CL results in photoinhibition. However, the values of Φ_{PSII} , qP , and ETR obtained under the RB-CL, RBG-CL, and W-CL treatments were higher or equal to the values under RB-CK and W-CK from 0 to 24 h, suggesting that the photoinhibition resulting from the RB-CL, RBG-CL, and W-CL treatments between 0 and 24 h did not affect the photosynthetic capacity of the lettuce plants. This was a dynamic photoinhibition that protected the photosynthetic apparatus from damage (Osmond, 1994). However, the decrease in F_v/F_m , Φ_{PSII} , and qP and a concomitant increase in $1-qP$ under RB-CL and RBG-CL after 48 h indicates that the CL began to inactivate and damage the photosynthetic apparatus in the lettuce leaves photosynthetic system (Nixon et al., 2010), as shown by the significant lower P_n under RB-CL and RBG-CL at 72 h (Table 3).

Photosynthesis is a very complex process, which requires the assembly of functional chloroplasts regulated by light and the coordination of the expression of nuclear- and chloroplast-encoded genes (Nelson and Langdale, 1989). Although the light-dependent regulation of photosynthesis by the enzymatic properties of individual enzymes is well studied, a detailed description derived from large-scale gene expression mapping is still lacking. The fluctuation of NR activity and other photochemicals under CL treatment in this study may result

from the circadian clock effects (Deng et al., 1990; Ramalho et al., 1995). The circadian clocks of plants regulate the fluxes of solutes and changes of solute concentrations (Haydon et al., 2011). It is believed that there is circadian regulation of transcripts for nitrate reduction and nutrient transport in plants. The circadian clock based on a transcription–translation feedback loop may be satisfactory to explain the underlying mechanism for the rhythms known in each organism (Lillo et al., 2001). Therefore, recent advances in genomics and transcriptomics might allow us to understand the photosynthetic machinery and regulation, including the photosynthetic control of CL induction and NR-related gene expression, in future research. The findings from this study will also be an important contribution to understanding how light quality and photoperiod affect phytochemical synthesis, which may have biological significance for secondary metabolites.

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Supplemental Fig. 1. Light spectral distributions of white (W) light-emitting diode (LED) light control (W-CK), red (R) and blue (B) LED light control [RB-CK (R:B = 4:1)], continuous light (CL) by W LED (W-CL), CL by RB LED [RB-CL (R:B = 4:1)], and RB-CL plus green (G) LED light [RBG-CL (R:B:G = 4:1:1)] treatments. CL treatments were used to investigate the effects of CL on nitrate content, phytochemical concentrations, and antioxidant capacity in lettuce compared with controls. The photoperiod and light duration for controls and CL treatments was 12 and 72 h, respectively. The photosynthetic photon flux for all treatments was $200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The spectra were recorded at the top of the lettuce canopy. Each line curve represents the average of 10 replicates monitored under each light source.

Supplemental Table 1. Total phenolic compounds, carotenoids, and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity in lettuce in response to white (W) light-emitting diode (LED) light control (W-CK), red (R) and blue (B) LED light control [RB-CK (R:B = 4:1)], continuous light (CL) by W LED (W-CL), CL by RB LED [RB-CL (R:B = 4:1)], and RB-CL plus green (G) LED light [RBG-CL (R:B:G = 4:1:1)] treatments at 0, 48, 60, and 72 h ($n = 12$). The photoperiod and light duration for controls and CL treatments was 12 and 72 h, respectively. The photosynthetic photon flux for all treatments was $200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

Time (h)	Treatments	Total phenolic compounds ($\text{mg}\cdot\text{g}^{-1}$ FW)	Carotenoids ($\text{mg}\cdot\text{g}^{-1}$ FW)	DPPH radical scavenging activity ($\text{mg}\cdot\text{g}^{-1}$ FW)
0	W-CK	1.36 a ^z	0.13 a	2.89 a
	W-CL	1.35 a	0.13 a	2.96 a
	RB-CK	1.32 b	0.13 a	3.03 a
	RB-CL	1.33 b	0.12 a	2.78 a
	RBG-CL	1.33 b	0.12 a	2.79 a
48	W-CK	1.4 e	0.15 a	3.49 c
	W-CL	1.83 b	0.13 b	3.89 b
	RB-CK	1.50 d	0.14 a	3.20 d
	RB-CL	2.03 a	0.13 b	5.78 a
	RBG-CL	1.58 c	0.14 a	4.00 b
60	W-CK	1.40 d	0.14 b	2.92 d
	W-CL	1.90 a	0.13 c	5.65 a
	RB-CK	1.38 d	0.15 a	3.17 c
	RB-CL	1.82 b	0.13 c	4.46 b
	RBG-CL	1.66 c	0.14 b	4.54 b
72	W-CK	1.56 c	0.16 b	3.29 e
	W-CL	1.68 b	0.16 b	4.87 a
	RB-CK	1.78 a	0.17 a	4.06 d
	RB-CL	1.56 c	0.13 d	4.65 b
	RBG-CL	1.70 b	0.14 c	4.57 bc

^zMeans within the same column followed by different lower case letters are significantly different between treatments at $P \leq 0.05$ according to Duncan's multiple range test.

Supplemental Table 2. Chlorophyll (Chl) content and Chl *a* to Chl *b* (Chl *a/b*) ratio in lettuce in response to white (W) light-emitting diode (LED) light control (W-CK), red (R) and blue (B) LED light control [RB-CK (R:B = 4:1)], continuous light (CL) by W LED (W-CL), CL by RB LED [RB-CL (R:B = 4:1)], and RB-CL plus green (G) LED light [RBG-CL (R:B:G = 4:1:1)] treatments for 72 h (n = 12). The photoperiod and light duration for controls and CL treatments was 12 and 72 h, respectively. The photosynthetic photon flux for all treatments was 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

Time (h)	Treatments	Chl <i>a/b</i> (ratio)	Chl content ($\text{mg}\cdot\text{g}^{-1}$ FW)
0	W-CK	3.45 a ^z	0.87 a
	W-CL	3.50 a	0.85 a
	RB-CK	3.36 a	0.80 a
	RB-CL	3.47 a	0.84 a
	RBG-CL	3.60 a	0.85 a
12	W-CK	3.34 a	0.87 a
	W-CL	2.97 a	0.78 a
	RB-CK	3.39 a	0.85 a
	RB-CL	3.61 a	0.83 a
	RBG-CL	3.60 a	0.86 a
24	W-CK	3.02 d	0.84 a
	W-CL	3.42 a	0.70 ab
	RB-CK	3.46 a	0.81 a
	RB-CL	3.74 a	0.80 ab
	RBG-CL	3.17 c	0.61 b
36	W-CK	3.30 b	0.76 b
	W-CL	3.22 b	0.91 a
	RB-CK	3.58 a	0.94 a
	RB-CL	3.42 ab	0.89 ab
	RBG-CL	3.40 ab	0.91 a
48	W-CK	3.28 b	1.00 a
	W-CL	3.26 b	0.94 ab
	RB-CK	3.31 b	0.94 ab
	RB-CL	3.71 a	0.90 b
	RBG-CL	3.41 ab	0.91 b
60	W-CK	3.20 b	1.00 a
	W-CL	3.36 ab	0.94 ab
	RB-CK	3.50 ab	1.02 a
	RB-CL	3.47 ab	0.86 b
	RBG-CL	3.62 a	0.89 b
72	W-CK	3.15 b	1.14 a
	W-CL	3.67 a	1.07 ab
	RB-CK	3.92 a	1.03 ab
	RB-CL	3.74 a	0.93 b
	RBG-CL	3.78 a	0.96 b

^zMeans within the same column followed by different lower case letters are significantly different between treatments at $P \leq 0.05$ according to Duncan's multiple range test.