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Effect of green light on nitrate reduction and edible quality of hydroponically grown lettuce (Lactuca sativa L.) under short-term continuous light from red and blue light-emitting diodes

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Highlights

- Continuous light (CL) increased edible nutrition quality and concomitantly decreased nitrate content.
- Green light supplementation promoted nitrate reductase (NR) and nitrite reductase (NiR) related gene expression.
- Inclusion green light induced the activities of nitrogen assimilation enzymes under short-term CL.

Abstract:

Most leafy vegetables can accumulate large amounts of nitrate, which are often associated with harmful effects on human health. Nitrate assimilation in plants is determined by various growth conditions, especially light conditions including light intensity, light duration and light spectral composition. Red and blue light are the most important since both drive photosynthesis. Increasingly, recent evidence demonstrates a role for green light in the regulation of plant growth and development by regulating the expression of some specific genes. However, the effect of green light on nitrate assimilation has been underestimated. In this study, lettuce (Lactuca sativa L. cv. Butterhead) was treated with continuous light (CL) for 48 h by combined red and blue light-emitting diodes (LEDs) supplemented with or without green LED in an environment-controlled growth chamber. The results showed that nitrate reductase (NR) and nitrite reductase (NiR) related-gene expression and nitrate assimilation enzyme activities were affected by light spectral composition and light duration of CL. Adding green light to red and blue light promoted NR and NiR expressions at 24 h, subsequently, it reduced expression of these genes during CL. Compared with red and blue LEDs, green light supplementation significantly increased NR, NiR, glutamate synthase (GOGAT) and glutamine

synthetase (GS) activities. Green-light supplementation under red and blue light was more efficient in promoting nutritional values by maintaining high net photosynthetic rates (P_n) and maximal photochemical efficiency (F_v/F_m).

Keywords: light spectra; nitrogen metabolism enzymes; gene expression; nitrate; continuous light; Lactuca sativa L.

1. Introduction

Nitrogen (N) plays an important role in plant growth and development (Wang et al., 2002). Nitrate is one of the most abundant N sources in natural and agricultural systems. It is absorbed in the root and mobilized to other organs. When the absorption of nitrate exceeds its assimilation, nitrate will accumulate in plants, particularly in hydroponic growing system. Excessive nitrate accumulation is known to be a common problem in most crops, especially in leaf vegetables (Bóbics et al., 2015; Cárdenas-Navarro et al., 1999).

It has been proved that consuming more vegetables every day can help people keep healthy, since phytochemicals (carotenoids and phenols, etc.) in vegetables are major sources of antioxidants in human diets and play important roles in alleviating age-relative diseases (Connor et al., 2005; Martínez-Sánchez et al., 2008; Mou, 2009). Along with tomatoes, lettuce is another major vegetable grown in greenhouses and is also the most important salad vegetable. Lettuce is most popular consumed as raw leaves due to its taste and high nutritional value, such as ascorbic acid, carotenoids, and other antioxidant substances. However, lettuce is a hyperaccumulator of nitrates and easily accumulates high nitrates in its leaves (Escobar-Gutierrez et al., 2002). High levels of nitrates (usually nitrate level $\geq$ 700 mg kg^{-1}) in edible parts of vegetables have been implicated in increasing the risk of some diseases, such as methaemoglobinemia and gastric cancer (Bruning-Fann and Kaneene, 1993). In order to prevent the risk of these diseases, the legal limit of nitrate to trade lettuce in European countries is 50-140 mg per day (Santamaria, 2006). Therefore, exceeded nitrate intake represents a risk for emergence of diseases which has aroused great concern. (Bian et al., 2015; Lin et al., 2013; Pérez-López et al., 2013; Samuolienė et al., 2012).

Nitrate reductase (NR) is one of the key enzymes in regulating nitrate assimilation, which catalyses the reduction of nitrate to nitrite in plants (Sivasankar et al., 1997). Regulation of NR involves a hierarchy of transcriptional and post-transcriptional controls (Yanagisawa, 2014). Light and carbohydrates influence NR at the transcription and translation levels. NR expression has been found to influence N uptake and reduction. For example, the AtSIZ1, in Arabidopsis, has been shown to control nitrogen assimilation by promoting sumoylation of NRs (Park et al., 2011). After nitrate reduction, nitrite is reduced to ammonium by the second enzyme of the pathway, the nitrite reductase (NiR). Previous studies have provided evidences that the activities of NiR, glutamate synthase

(GOGAT) and glutamine synthetase (GS) can indirectly affect nitrate assimilation in plants (Barneix, 2007; Ruiz et al., 1999; Temple et al., 1998). Furthermore, the co-regulation of NR and NiR expression is not only important for nitrate assimilation but also a vital mechanism for preventing the accumulation of deleterious metabolic intermediates and energy saving for plant growth, especially under a biotic and/or abiotic stress environment (Małolepsza, 2007).

Light is one of the most important environmental factors in regulating plant growth and development (Kim et al., 2004; Li and Kubota, 2009). For plants, light is not only the driving force for photosynthesis but also the transduction signal to regulate gene expression via photoreceptors. Recently, light-emitting diodes (LEDs) have received considerable attention. LEDs now offer cheap, cool, controllable sources of light that can selectively and quantitatively provide different wavelengths. Previous studies have demonstrated that the combination of red (600–700 nm) and blue light (400–500 nm) is an effective lighting source for plant growth (Bian et al., 2015; Hogewoning et al., 2010). However, other light spectra, such as green light and far-red light, also have profound effects on plant procession via phytochromes and/ or cytochromes (Folta and Maruhnich, 2007; Urrestarazu et al., 2016). Green light absorbed by anthocyanins can prevent photo-degradation of light-labile plant defence secondary metabolites, such as thiarubrine A, which is easily degraded after visible light or UV light exposure (Gould et al., 2010). In addition, green light can increase plant defence mechanisms via triggering special gene expression (Nagendran and Lee, 2015; Zhang et al., 2012).

We previously reported that adding green light to red and blue light had a positive effect on reducing nitrate content in lettuce under continuous light treatment. The suitable light spectral ratio for red, blue and green light is 4:1:1 (Bian et al., 2016). However, little is known regarding the NR and NiR gene expression and its enzyme activity in lettuce under different light spectra of short-term CL. Therefore, in this study we present (1) nitrate reduction enzyme activities and related gene expression and (2) the edible quality of lettuce under short-term CL by different LED light spectral composition. It also highlights effect of green light on nitrate reduction, edible quality of lettuce and expression of nitrate assimilation related genes under short-term continuous light with red and blue light. The result of this study could enable a better understanding of the effect of green light on nitrate reduction under short-term continuous light for producing high quality leaf vegetables in greenhouse and environment-controlled facilities.

2. Materials and methods

2.1. Plant materials and growth conditions

Lettuce (Lactuca sativa L. cv. Butterhead) seeds were sown in plastic trays filled with seed-peat mixture (1:1, v/v) substrate and germinated under fluorescent lamps (TL–D 36W, Philips) with 150 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) of 12 h d^{-1} in an environmentally controlled chamber. The day/night temperature, CO_2 level, and relative humidity in the growth chamber were 25

/20 °C, 400 μmol mol^{-1} and 75%, respectively. Water was added daily to maintain the moistness of the substrate and replenish evapotranspiration losses.

When lettuce seedlings had two true leaves, they were transplanted to 40–L containers of Hoagland solution (pH = 6.8 ± 0.2, EC = 1.9 ± 0.1 dS m^{-1}). These plants were randomly grown under a combination of red (R, peak at 660 nm) and blue LEDs (B, peak at 460 nm) (R:B = 4:1) or a combination of red/blue light with the addition of green light (G, peak at 530 nm) LEDs (R:B:G = 1:1:1). No-reflect black separators were placed between different light sources to avoid light contamination. To minimize any effects from uneven light between plants, the containers were systematically moved every other day. The PPFD was monitored daily by a spectroradiometer (Avaspec–2048CL, Avantes, Apeldoorn, The Netherlands) and was maintained at 200 μmol m^{-2} s^{-1} by adjusting the distance between the light sources and plant canopies. Other environmental factors were maintained at similar levels to those at the seedling stage. The nutrition solution was renewed every week.

2.2. Light treatment

At the end of the dark period, 20 d after being transplanted, plants were transferred to environment-controlled growth chamber (temperature 25 °C) under PPFD of 200 μmol m^{-2} s^{-1}. There were five treatments. The details of these treatments are summarized in Table 1. The plants grown under red and blue LEDs were treated with CL (RB-CL) or were treated with supplemental green LEDs (RBG-CL). The light ratios for RB-CL and RBG-CL were 4:1 and 4:1:1, respectively. The plants grown under red and blue LEDs (R:B= 4:1) with a photoperiod of 12 h were used as control (RB-control). Furthermore, plants grown under red, blue and green LEDs were randomly divided into two groups. One group was treated with CL using previous LED light sources (rbg-CL, r:b:g=1:1:1), while the other received CL treatment by previous LED light sources but without green light LEDs (rb-CL, r:b= 1:1). rbg-CL was used to imitate the light spectra of white light–the most common standard illuminants used as target white points for RGB mixing calculations (Boray et al., 1989; Park et al., 2012). rbg-CL was used to further investigate if there is any different effect of green light on regulating nitrate metabolism when compared with rb-CL and RBG-CL. Adding rbg-CL treatment to this study could have an impact and value to the practical application. During the experiment, other environmental conditions were set as similar to those at the seedling stage. There were four replicates per treatment with 48 plants in total.

2.3. Measurements of net photosynthetic rate and chlorophyll fluorescence

The second-youngest and fully expanded leaves were used for monitoring the net photosynthetic rate (P_n) and chlorophyll fluorescence using a portable photosynthetic apparatus with a fluorescent chamber (LI–6400XT, Li–Cor, NE, USA) based on the method described by Weng et al. (2008).

Minimal (F_o) and maximal (F_m) fluorescence of dark-adapted leaves were monitored after being dark-adapted for 30 min. The values of F_o and F_m were used to calculate the maximum potential photochemical efficiency (F_v/F_m). The temperature, light intensity and CO_2 concentration in the leaf

chamber of LI–6400XT were controlled at 25 °C, 200 μmol m^{-2} s^{-1} and 400 μmol mol^{-1}, respectively. The actinic light in the leaf chamber was supplied with red/blue light source. Each treatment consisted of four to six replicates.

2.4. Measurement of pigment, nutritional value and lipid peroxidation

After P_n and chlorophyll fluorescence measurement, leaf samples were collected from the second-youngest and fully expanded leaves. Chlorophyll was extracted in 5 mL of 80% (v/v) acetone buffer at 4 °C for 72 h in a dark condition and its concentration was calculated using the equations described by Torrecillas et al. (1984). Each treatment was replicated four times.

Leaf samples (0.1 g) from the second youngest and fully expanded leaves were homogenized in ice-cold potassium phosphate buffer (50 mM, pH = 7.5). The extracts were filtered using four cheesecloth layers and centrifuged at 15,599 rpm for 15 min at 4 °C. The supernatant was used to determine soluble protein and soluble sugar based on the methods of Bradford (1976) and Yemm and Willis (1954), respectively. Ascorbic acid analysis was performed with HPLC using the method described by Asami et al. (2003). The spectrophotometric methods described by Ragaee and Abdel-Aal (2006) were used to measure total phenolic compounds and the free radical-scavenging capacity of the lettuce leaf extract. The free radical-scavenging capacity in lettuce leaves was represented as the 2,2–diphenyl–1– picrylhydrazyl (DPPH) free radical-scavenging capacity. Each treatment consisted of four replications.

The formation of malondialdehyde (MDA) in leaves was used to estimate lipid peroxidation with the equation described by Schaedle and Bassham (1977). There were four replications for each treatment.

2.5. Nitrate and nitrite content determination

Leaf samples (0.5 g) collected from the third-youngest, fully expanded leaves were used to determine nitrate content. The absorbance monitored at 410 nm was used to calculate nitrate content by the equation of Cataldo et al. (1975). For nitrite content determination, the leaf sample was homogenized using sulphanilamide and N– (1– Naphthyl)-ethylene-diamine dihydrochloride. The extract was used to estimate nitrite content based on the method described by Stevens and Oaks (1973). There were four replications per treatment.

2.6. Nitrate reductase and nitrite reductase activity assay

The activities of nitrogen reduction enzymes were measured using leaf samples collected from the third-youngest, fully expanded leaves. Nitrate reductase (NR; EC 1.6.6.6) activity was determined according to the method described by Rosales et al. (2012). 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 milligram of protein per min.

Nitrite reductase (NiR; EC 1.6.6.4) activity was spectrophotometrically determined (Mendez and Vega, 1981). The absorbance monitored at 540 nm was used to calculate NiR activity. One unit of NiR activity was taken as 1 μmol NO_2^- catalysed per milligram of protein per min.

2.7. Glutamate synthase and glutamine synthetase activity assay

The leaf sample was homogenized with ice-cold 50 mM KH_2PO_4 buffer (pH 7.5), containing 1% (w/v) insoluble polyvinylpyrrolidone, 1.5% (w/v) soluble casein, 2 mM EDTA and 2 mM dithiothreitol. The extract was centrifuged at 3,000 × g for 5 min at 4 °C and then centrifuged again at 12,000 rpm for 20 min at 2 °C. The supernatant, referred as 'crude enzyme', was used for the glutamate synthase enzyme (GOGAT; EC 1.4.1.13) and glutamine synthetase (GS; EC 6.3.1.2) activity assay.
The method described by Cánovas et al. (1991) was used for GS activity measurement. The activity of GS was expressed as μmol γ– glutamylhydroxamate formed per gram per minute. A spectrophotometric method was used to calculate GOGAT activity (Singh and Srivastava, 1986). The GOGAT activity was defined as μmol NADH oxidized per gram per minute.

2.8. Gene expression analysis

The total RNA was isolated from the leaf sample using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted total RNAs were treated with RNase-free DNase I (Invitrogen, Carlsbad, CA, USA) to avoid any genomic DNA contamination before the reverse-transcription reaction as the manufacturer's instructions. The total RNAs were quantified using a NanoDropTM 2000C spectrophotometer before and after DNase I treatment. First-strand cDNA was synthesized using a RevertAid First-stand cDNA synthesis kit (Quanta Biosciences, Gaithersberg, MD, USA). The qRT–PCR was performed by a 7500 Real–Time PCR System (Applied Biosystems, Forest City, CA, USA). An initial denaturing temperature at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 56 °C for 30 s and a melting curve 50–95 °C using PerfeCta SYBR Green FastMix (Quanta Biosciences). Primers of actin were used as a house-keeping gene. Real-time qPCR was repeated four times based on the four separate RNA extracts from four samples. Threshold values (CT) generated from the ABI PRISM 7500 software Tool (Applied Biosystems) were used to quantify relative gene expression by the comparative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Leaf samples were collected before CL treatment (0 h) and after a 6, 12, 24 or 48 h time–course. The second youngest and full expanded leaves were used to investigate related gene expression of NR and NiR. The NR gene sequence (Accession No: KP122207.1) of lettuce (Lactuca sativa L.) was used for assaying NR expression. The NiR gene sequences corresponding to the top BLAST hit were identified within the Compositae Genome Project EST database through sequence homology to known NiR (Lactuca dolichophylla, Accession No: KJ545658.1) from the existing lettuce sequence database in GenBank.

Primers for NR and NiR were designed by Primer Premier 6.0 (Biosoft International, Palo, CA, USA) with the setting of a primer length of 18–24 bp, a melting temperature 58–62 °C, a CG content 30–70% and product size 100–200 bp. The primers of Lactuca sativa L. actin described by Ebisawa et al. (2008) were used as the reference gene primers. The forward and reversed primers for LsActin were (5'–AGGTGTCATGGTTGGCATGGGA–3') and (5'–TGTTCTTCAGGGGCGACACG–3'), respectively. The forward and reverse primers for LsNR were (5'–GGAGGTGGGAAGAAAGTGACA–3') and (5'–TAGCGACCAAAAACACCAACA–3'), respectively; these for LsNiR were (5'–CACCACACGTCAAAACTGGC–3') and (5'–GAGTGGATTGCCAACTGGGT–3'), respectively.

2.9. Statistical analyses

All of the data were subjected to one-way ANOVA analysis of variance using SAS software (Version 8.1; SAS Institute, Cary, NC, USA). Significant differences between means were determined by Duncan's multiple range test at the $P < 0.05$ level.

3. Results

3.1. Net photosynthesis rate and photosynthetic capacity

Compared with the RB-Control, the values of P_n under RB-CL, rb-CL and rbg-CL were all decreased after CL treatment. The P_n for all CL treatments at 48 h was lower than that at 24 h. There was no significant difference in P_n between rbg-CL and RB-CL at 24 and 48 h. The lowest P_n was observed under rb-CL throughout CL treatment. The P_n of RBG-CL treated plants was decreased at 48 h, but the value was higher than that under rbg-CL. However, the value of P_n for RBG-CL was comparable to that of control at 24 h (Fig. 1A). Compared with control, RB-CL and rb-CL led to decreases in F_v/F_m, and the values at 48 h were lower than that at 24 h. A significant decrease in F_v/F_m was observed under rbg-CL at 48 h. However, F_v/F_m for RBG-CL was comparable to that for RB-control at 24 and 48 h (Fig. 1B).

3.2. Edible quality of lettuce

The editable quality of lettuce under CL by different light spectral LEDs is summarized in Table 2. Compared with RB-Control, DPPH free radical-scavenging capacity, ascorbic acid, soluble sugar and soluble protein of lettuce all increased after CL for 24 and 48 h. The values of these studied parameters at 48 h were higher than these at 24 h. This indicates that increasing duration of CL has positive effect on promoting nutrition substance accumulation in lettuce. The DPPH free radical-scavenging capacity and soluble protein content under RB-CL and RBG-CL were higher than these under rb-CL and rbg-CL, but these parameters did not show significance between RB-CL and RBG-CL, and between rb-CL and rbg-CL. Ascorbic acid concentration in plants grown under RBG-CL showed the highest and the second highest ascorbic acid content was observed in plants grown under

rbg-CL, followed by RB-CL, rb-CL and then RB-control. There was no significant difference in ascorbic acid among RB-CL, rb-CL and rbg-CL at 24 and 48 h. These results suggest that green light shows positive on ascorbic acid accumulation. Furthermore, the concentration of soluble sugar in plants grown under RBG-CL were the highest among the studied light treatments.

3.3. Lipid peroxidation

The lipid peroxidation in lettuce plants was represented as the formation of MDA. The MDA content increased with the prolongation of CL duration, as shown by the higher MDA content at 48 h than that at 24 h. Compared with RB-Control, the MDA contents of CL treated lettuce (except RBG-CL at 24 h) were significantly increased at 24 and 48 h. The MDA was the highest under rb-CL, followed by RB-CL, rbg-CL, RBG-CL and then RB-control (Fig. 2), indicating the lipid peroxidation caused by CL depends on light spectral composition and green light alleviates membrane oxidation under CL.

3.4 Contents of nitrate, nitrite and activities of nitrogen assimilation enzymes

Nitrate content and activities of nitrogen assimilation enzymes under different light spectral composition of CL are summarized in Table 3. The nitrate content in CL treated plants was significantly lower than that under the control. Among CL treatments, the lowest nitrate content was obtained under RBG-CL, followed by rbg-CL, RB-CL and then rb-CL at 24 and 48 h. However, there was no significant difference among RB-CL, rb-CL and rbg-CL at 48 h. It is notable that the nitrate content of CL treated plants at 48 h was higher than that at 24 h. Furthermore, there were no significant differences in nitrite content among these CL treatments (Supplementary information Table S1). Compared with the control, NR activity of CL treated plants was significantly increased at 24 h. The NR activity for rb-CL was lower than that for the control at 48 h, but this parameter for other CL treatments was comparable to that for control. Under CL treatment, the activities of NiR, GS and GOGAT were significantly higher than that of the control at 24 h and the highest activities of these enzymes were observed under RBG-CL. At 48 h, the activities of NiR, GS and GOGAT for CL treated plants were lower than or comparable to those of control. However, the activities of NiR and GS under RBG-CL were higher than these under other CL treatments.

3.4. Nitrate reductase and nitrite reductase related gene expressions

The expressions of the NR and NiR gene under CL treated plants were measured with different the light spectral compositions (Fig. 3). Compared with control (RB-control), the transcripts for NR were up-regulated under RBG-CL and rbg-CL between 12 and 36 h, respectively, whereas significant increases in NR expression for RB-CL and rb-CL were only observed at 24 h. However, the transcripts of NR under CL treatment were down-regulated at 48 h (Fig. 3A). Compared with control, the transcripts of NiR for rb-CL were up-regulated at 12 h, and at 24 h for RB-CL, RBG-CL and rbg-CL. At 24 h, the expression levels of NiR for RB-CL, RBG-CL and rbg-CL treated plants were 2.74-, 5.26-

and 2.43-fold greater compared with the expression in control plants. Interestingly, the expression of NiR for CL treated plants was decreased at 48 h (Fig. 3B). There was no significant changes between light treatments at 0 h, 6 h and 36 h time course.

3.5. Correlations between nitrate content and soluble sugar/soluble protein, and between nitrate reductase activity and related gene expression/other nitrogen assimilation enzymes

Under different light spectral CL, nitrate contents were negatively correlated with soluble sugar (Fig. 4A) and soluble protein (Fig. 4B). However, the significant correlation between nitrate contents and soluble protein was only observed at 48 h (R^2_{48}= 0.879, $P < 0.05$; Fig. 4B), indicating a significant increase of soluble protein might be attributed to nitrate assimilation. The NR activity positively corrected with the transcripts of NR and NiR at 24 and 48 h, respectively (Fig. 5 A and B), indicating that the expressions of NR and NiR positive affected NR activity in lettuce under CL treatment. At 24 and 48 h, there was a positive correlation between the activity of NR and NiR (R^2= 0.905, $P < 0.05$ at 24 h; R^2= 0.951, $P < 0.01$ at 48 h) and GS (R^2= 0.863, $P < 0.05$ at 24 h; R^2= 0.880, $P < 0.05$ at 48 h), respectively (Fig. 6 A and B). In contrast, a significant linear relationship between the activity of NR and GOGAT was only observed at 24 h (Fig. 6 C).

4. Discussion

In the present study, our data demonstrated that the effect of CL on reducing nitrate content depends on light spectral composition and light duration. Adding green light to red and blue light shows positive effects on nitrate assimilation by increasing the activity and expression of nitrate assimilation related genes NR and NiR. It is well known that there is a strong relationship between photosynthesis and nitrate assimilation in plants, since nitrate assimilation is an energy-consuming pathway and approximately 25% of the energy generated by photosynthesis can be used for driving nitrate assimilation (Solomonson and Barber, 1990). Terashima et al. (2009) reported that green light drove photosynthesis more efficiently than red light. Apart from energy consumption, nitrate assimilation requires carbon skeletons (2– oxoglutarate) provided by photosynthesis. Adding green light to red and blue LEDs enhances lettuce growth by enhancing plant photosynthesis (Kim et al., 2004). In the present study, there were strong negative correlations between soluble sugar, soluble protein and nitrate content (Fig. 4A and B), indicating the significant decrease in nitrate content might partly be attributed to the constant carbon skeleton supplementation from photosynthesis in lettuce plants. This view is supported by the study of Morcuende et al. (1998). Compared with other CL treatments, the higher P_n and F_v/F_m (Fig. 1), sucrose content (Table 2), and concomitantly lower nitrate content under RBG-CL and rbg-CL (Table 1) indicate adding green light to red and blue light can provide more ferredoxin and carbon skeletons for both photosynthesis and nitrate assimilation under CL. The result is similar as previous discovery of Lillo and Appenroth (2001) and Commichau et al., (2006).

In plants, the first step in nitrate assimilation is catalysed by NR enzymes, which is defined as the most important and limiting step in the acquisition of nitrogen (Campbell, 1999). The NR activity and its related-gene expression is highly modulated by light (Sherameti et al., 2002). There are significant differences in NR activity and NR expression among different light spectral CL treatments. These results demonstrated that the NR activity and NR expression are subjected to light spectra composition of CL (Jonassen et al., 2008; 2009). Nagendran and Lee (2015) reported that green light could promote plant growth and up-regulate special gene expressions under biotic or abiotic stress. Our results first demonstrated that green light has a positive effect on NR and NiR expressions under short-term CL treatment, as shown by qRT-PCR analysis − the higher transcripts of NR and NiR under RBG-CL and rbg-CL compared with RB-CL and rb-CL (Fig. 3A and B). In the present study, the significate positive correlation between NR activity and NR expression (Fig. 5A) indicated that, under short-term CL treatment, the NR activity was subject to NR expression (Jonassen et al., 2009). The significant nitrate reduction in lettuce under CL at 24 h may lie in the fact that the higher NR activity aroused by the increase of NR expression leads to enhanced nitrate reduction (Nawaz et al., 2017). Furthermore, the present work shows the decrease in NR activity was accompanied by a decrease in the transcripts of NR and NiR after 48 h under CL treatment. This finding agrees with a previous data reported by Foyer et al. (1998). Jonassen et al. (2009) also reported that the decrease in NR activity in Arabidopsis leaves resulted from the decrease of NR transcript levels with the prolongation of CL. The re-accumulation of nitrate at 48 h in this study was probably associated with the decrease of NR activity caused by the down-regulation of NR gene expression (Fig. 4 A) (Gojon et al., 1998; Kyaing et al., 2012).

In the second step of the nitrate assimilation pathway, nitrite is catalysed to ammonium by ferredoxin-NiR, and the expression of NiR affects the build-up of nitrate in the nitrate assimilation pathway. In our study, a significant positive correlation between NR activity and the transcript level of NiR was observed, because NiR activity is subject to the regulation of NiR (Kyaing et al., 2012) and the co-regulation of NR activity and NiR activity is required for the deleterious accumulation of nitrite (Faure et al., 1991). In addition, nitrite contents were comparable among different light spectral treatments under CL light, although there was significant difference between NiR expression and NR activity. Our results regarding nitrite content suggested that there is a constant flow between the formation and the reduction of nitrite. Contrary to our finding, Davenport and Maunders (2008) reported that over expression of NiR in tobacco reduced nitrite content. However, Kyaing et al. (2012) reported that over expression of NiR in tobacco showed increased NiR activity but did not show significant effect on reducing nitrite content.

In plants, the reduction of nitrate produces ammonium, which is subsequently incorporated into organic nitrogenous compounds by the enzymatic cycle of GS/GOGAT. Nitrate assimilation is subject to feedback inhibition of its intermediates, such as glutamate or glutamate (Solomonson and Barber, 1990). In our study, the significant positive correlations between activities of NiR, GS and GOGAT and the activity of NR (Fig. 6) suggest that nitrate assimilation under short-term CL is also subject to the

feedback and indirect regulation of the activities of NiR, GS and GOGAT (Barneix, 2007). Most importantly, our data demonstrated a positive effect of green light on inducing nitrogen assimilation enzyme activity under CL treatment, as shown by nitrate assimilation enzyme analysis – the higher NR, NiR and GOGAT for both RBG-CL and rbg-CL (Table 3).

Light is not only driving force for photosynthesis, but also plays a very important role as an external signal for g expression of genes related to nitrate assimilation and activity of its enzymes (Lillo and Appenroth, 2001). Jonassen et al. (2009) reported that continues red light and far-red light regulated the activity and the expression of NR gene via phytochrome A and phytochrome B, respectively, and bZIP transcription factors HY5 and HYH were the positive regulators of this pathway. In the present study, the positive effects of adding green light to red and blue light on the gene expression and enzyme activity of nitrate assimilation suggest that in addition to phytochromes, some other photoreceptors and/or transcription factors may also be involved in the regulation of nitrate assimilation under CL treatment (Lillo, 2008). Therefore, further studies via a functional genomics approach and genome editing techniques on the identification of regulatory genes and their functions for nitrate assimilation and photosynthesis will be valuable.

5. Conclusions

We conclude that short-term continuous light (CL) significantly decreased nitrate content in lettuce leaves. Green light plays an important role in regulating the expression of some specific genes (e.g. NR and NiR) and improving nutritional value that gives us the chance to promote healthy habits and a positive change in diets using naturally grown crops as a base of diets instead of tablets. Further research in crop types based on the corresponding genetic analysis and followed by transcriptomic analysis will provide important information for crop breeding with better food quality.

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Abbreviations

CL, continuous light; LEDs, light-emitting diodes; F_v/F_m, maximal photochemical efficiency of PSII; GS, glutamine synthetase; GOGAT, glutamate synthase; MDA, malondialdehyde; NR, nitrate reductase; NiR, nitrite reductase; NR, nitrate reductase related gene; NiR, nitrite reductase related gene; P_n, net photosynthetic rate.

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Figure Captions

Fig.1. Effects of light spectra on the net photosynthetic rate (P_n; A) and maximum potential photochemical efficiency (F_v/F_m; B) of lettuce leaves under continuous light (CL) for 24 and 48 h, respectively. Error bars through data points show ± SE (n = 4 or 6).

Fig. 2. Effect of light spectra on MDA content in lettuce leaves under continuous light (CL). Error bars through data points show ± SE (n = 4).

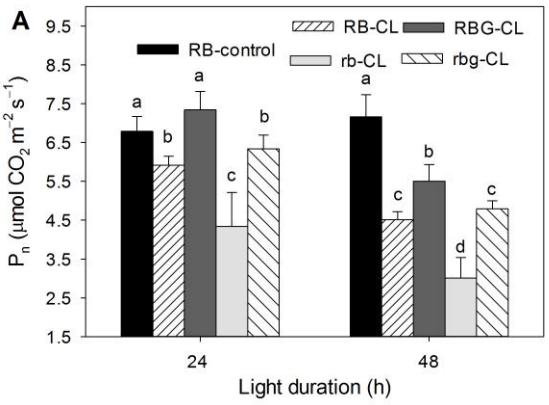
Fig. 3. Related gene expressions of nitrate reductase (NR) and nitrite reductase (NiR) under different wavelengths of continuous light (CL). Error bars through data points show ± SE (n = 4).

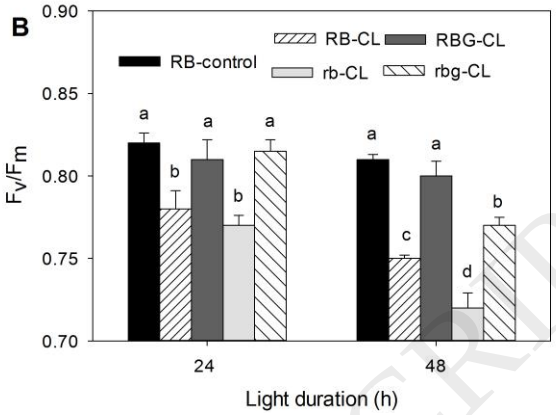
Fig. 4. The relationship between soluble sugar content and nitrate concentration under continuous light (CL) at 24 and 48 h, respectively. Error bars through data points show ± SE (n = 4).

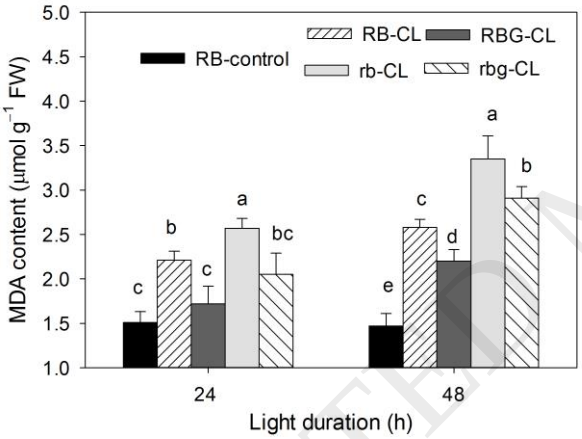
Fig. 5. Correlation analysis reveal links between s of nitrate reductase (NR) related-gene expression (A), nitrite reductase (NiR) related-gene expression (B) and NR activity under different light spectral continuous light (CL). Error bars through data points show ± SE (n = 4).

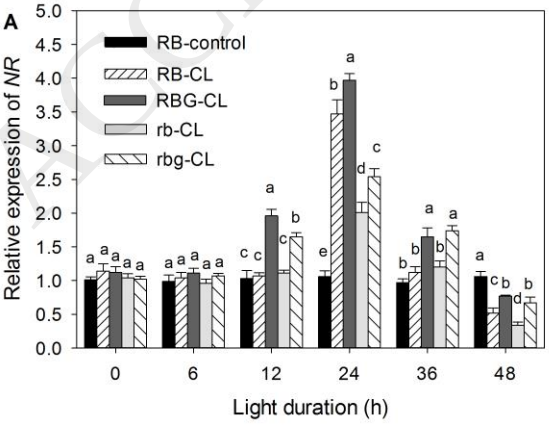
Fig. 6. Correlation analysis revealed links between activities of nitrite reductase (NiR; A), glutamine synthetase (GS; B), glutamate synthase (GOGAT; C) and nitrate reductase activity under different light spectral continuous light (CL). Error bars through data points show ± SE (n = 4).

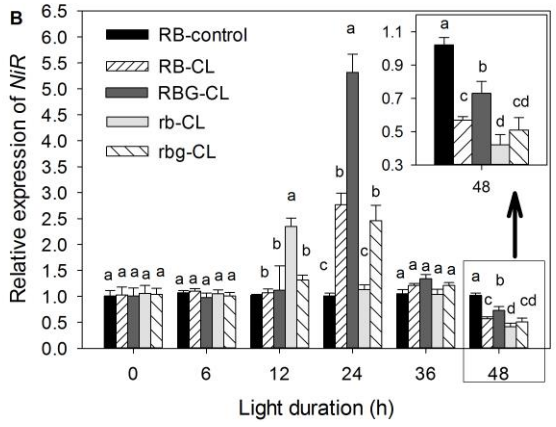
Figures:

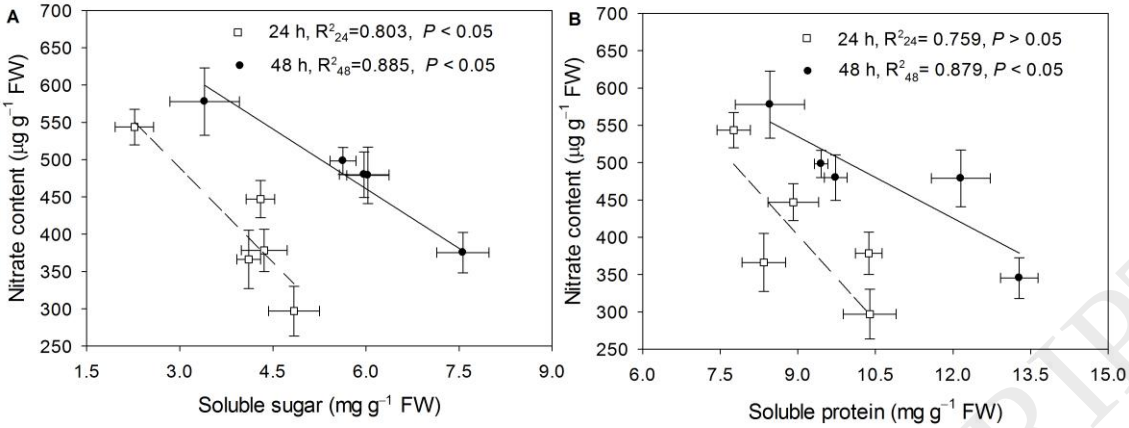


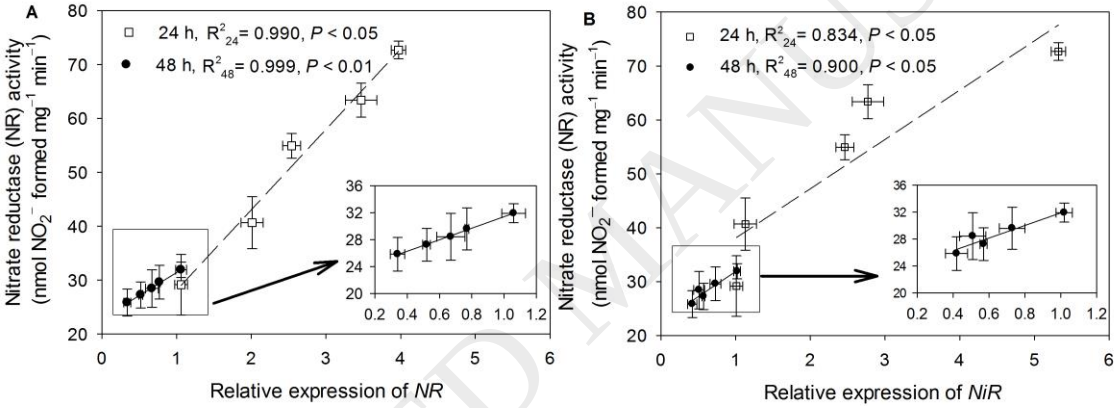


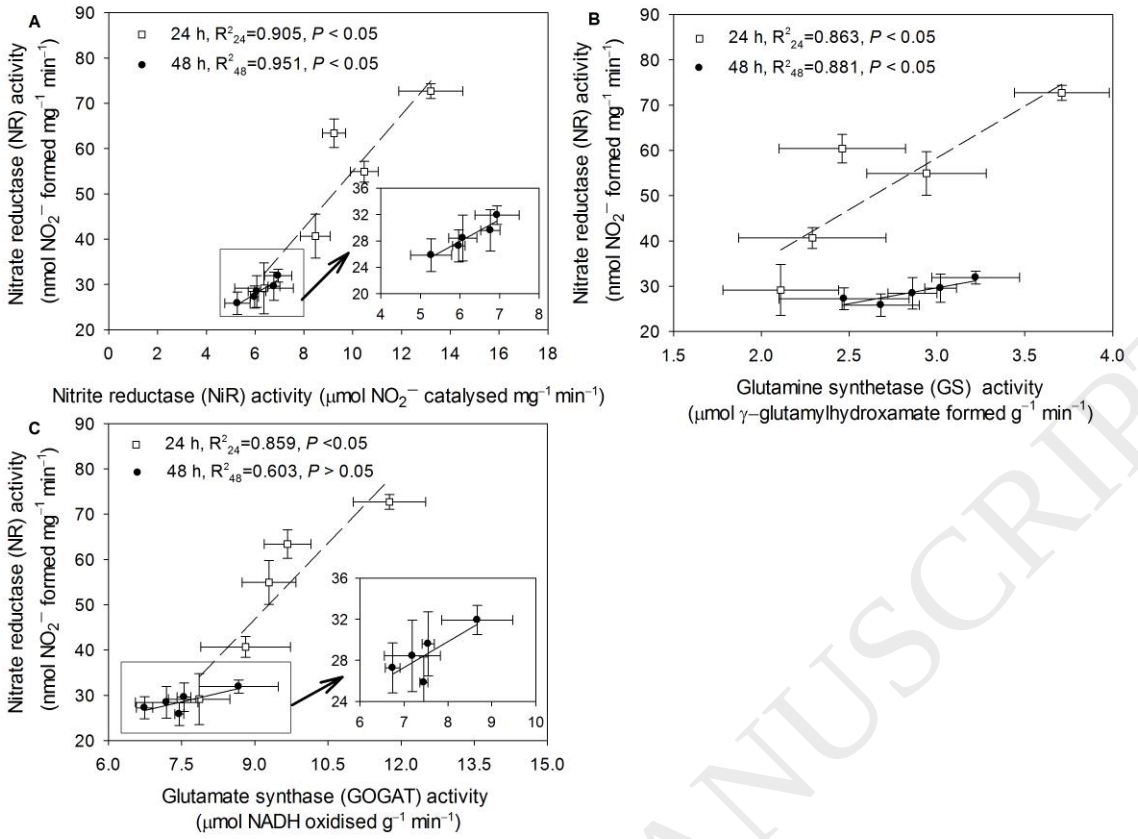












Tables:

Table 1. List of light spectral details and light duration applied for different light treatments.

Treatments	Before light treatment (from transplanting until the light treatment)			Light treatment (from the end of the dark period until 20 d after transplanting)		
	Light sources	Light quality ratio		Light sources	Light quality ratio	Light duration
RB-control	RB LEDs	R:B= 4:1		RB LEDs	R:B= 4:1	12 h light /12 h dark
RB-CL						48 h light /0 h dark
RBG-CL				RBG LEDs	R:B:G= 4:1:1	
rb-CL	RBG LEDs	R:B:G= 1:1:1		RB LEDs	R:B= 1:1	
rbg-CL				RBG LEDs	R:B:G= 1:1:1	

R, red light; B, blue light; G, green light; LEDs, light emitting diodes. RB and rb, combined R and B with a ratio at 4:1 and 1:1, respectively. RBG and rbg, combined R and B plus G with a ratio at 4:1:1 and 1:1:1, respectively. CL, continuous light. The light intensity of all treatments was 200 μmol m^{-2} s^{-1}.

Table 2. Total phenolic compounds, 2, 2⁻ diphenyl⁻1⁻ picrylhydrazyl (DPPH) free radical-scavenging capacity, soluble protein, soluble sugar and ascorbic acid in lettuce leaf under short-term continuous light treatments for 24 and 48 h by different light spectral LEDs (n = 4).

Parameters	RB-control	RB-CL	RBG-CL	rb-CL	rbg-CL
24 h					
Total phenolic compounds (mg g^{-1})	1.76 ± 0.12 a	1.70 ± 0.21 a	1.74 ± 0.11 a	1.81 ± 0.25 a	1.71 ± 0.15 a
DPPH free radical-scavenging capacity (μmol g^{-1})	3.11 ± 0.21c	4.26 ± 0.32 a	4.18 ± 0.16 a	3.87 ± 0.12 b	3.79 ± 0.11 b
Ascorbic acid (mg g^{-1})	0.87 ± 0.05 d	1.64 ± 0.32 bc	2.51 ± 0.19 a	1.23 ± 0.16 c	2.13 ± 0.22 b
Soluble sugar (mg g^{-1})	2.27 ± 0.31 c	4.36 ± 0.37 ab	4.84 ± 0.41 a	4.30 ± 0.23 ab	4.11 ± 0.19 b
Soluble protein (mg g^{-1})	7.76 ± 0.32 c	10.37 ± 0.26 a	10.39 ± 0.51 a	8.91 ± 0.49 b	8.34 ± 0.42 bc
48 h					
Total phenolic compounds (mg g^{-1})	1.50 ± 0.13 c	2.03 ± 0.10 b	2.31 ± 0.14 a	1.89 ± 0.17 b	1.95 ± 0.20 b
DPPH free radical-scavenging capacity (μmol g^{-1})	3.32 ± 0.34 c	5.65 ± 0.71 a*	4.87 ± 0.52 a*	3.89 ± 0.26 b	4.25 ± 0.18 b*
Ascorbic acid (mg g^{-1})	1.02 ± 0.09 c	2.18 ± 0.16 b*	2.67 ± 0.24 a	1.82 ± 0.23 b*	2.44 ± 0.11 a
Soluble sugar (mg g^{-1})	3.10 ± 0.56 c	6.03 ± 0.34 b*	7.65 ± 0.42 a*	5.63 ± 0.21 b*	5.97 ± 0.40 b*
Soluble protein (mg g^{-1})	8.46 ± 0.67 d	12.15 ± 0.51b*	13.28 ± 0.36 a*	9.45 ± 0.13 c*	9.73 ± 0.22 c*
The significant differences (at $P < 0.05$) in each parameter among treatments are indicates by different letters. * indicates significant differences between same parameter determined at 24 and 48 h.					

Table 3. Nitrate content, enzymatic activities of nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), glutamate synthase (GOGAT) in lettuce exposed to continuous light for 24 and 48 h by different light spectral LEDs (n = 4).

Treatments	Nitrate ($\mu g\ g^{-1}$)	NR (nmol NO_2^- formed mg^{-1} min^{-1})	NiR ($\mu mol\ NO_2^-$ mg^{-1} catalysed min^{-1})	GS ($\mu mol\ \gamma$–glutamylhydroxamate formed g^{-1} min^{-1})	GOGAT (μmol NADH oxidised g^{-1} min^{-1})
24 h					
RB-control	543.70 ± 23.81 a	29.16 ± 5.62 e	6.36 ± 1.20 d	2.21 ± 0.33 b	7.86 ± 0.63 c
RB-CL	378.45 ± 28.32 c	63.39 ± 3.14 b	9.23 ± 0.43 c	2.46 ± 0.56 b	9.67 ± 0.48 b
RBG-CL	297.05 ± 33.23 d	72.70 ± 1.64 a	13.20 ± 1.31 a	3.71 ± 0.87 a	11.76 ± 0.74 a
rb-CL	447.12 ± 24.78 b	40.67 ± 4.83 d	8.47 ± 0.61 c	2.29 ± 0.42 b	8.81 ± 0.92 b
rbg-CL	366.23 ±3 8.99 c	54.92 ± 2.29 c	10.47 ± 0.57 b	2.64 ± 0.34 b	9.29 ± 0.55 b
48 h					
RB-control	577.83 ± 45.03 a	31.92 ± 1.14 a	6.94 ± 0.56 a	2.60 ± 0.25 b	8.67 ± 0.81 a
RB-CL	478.92 ± 37.84 b	27.25 ± 2.43 ab	5.97 ± 0.16 b	2.47 ± 0.37 b	6.74 ± 0.17 c
RBG-CL	345.42 ± 27.21 c	29.60 ± 3.12 ab	6.77 ± 0.24 a	3.02 ± 0.09 a	7.55 ± 0.14 b
rb-CL	498.37 ± 18.40 b	25.84 ± 2.49 b	5.27 ± 0.52 b	2.68 ± 0.22 b	7.45 ± 0.09 b
rbg-CL	479.94 ± 33.41 b	28.44 ± 3.48 ab	6.07 ± 0.36 b	2.86 ± 0.14 ab	7.19 ± 0.69 bc

Different letters in the same column indicate significant differences ($P < 0.05$) based on the Duncan's multiple range test.