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Investigating pulsed LED effectiveness as an alternative to continuous LED through morpho-physiological evaluation of baby leaf lettuce (*Lactuca sativa* L. var. *Acephala*)

Awais Ali[a,*], Piero Santoro[b], Antonio Ferrante[a], Giacomo Cocetta[a]

[a] Department of Agricultural and Environmental Sciences, Universita Degli Studi Di Milano, via Celoria, 2, Milano (MI) 20133, Italy
[b] MEG Science., via Aleardo Aleardi 12, Milano (MI) 20154, Italy

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A B S T R A C T

Photoperiod, light intensity, and spectral quantum distribution (SPD) affect plant development and physiology. Light determines morphological signals, influences plant behavior and regulates metabolism in addition to providing energy for photosynthesis. In this experiment, lettuce (*Lactuca sativa*) was grown in an indoor LED-equipped chamber, operated in a pulsed and continuous mode, with an average photosynthetic photon flux density (PPFD) at a seedling level of 150 μmole s^1 m^2, photoperiod of 16 h for growing cycle of 30 days. The primary aim of this study was to observe the effects of varying LED on the growth and quality of the produce. Regardless of the treatments, in both continuous and pulsed LED, an increment in the yield, leaf length and leaf width of lettuce was recorded in comparison to the control, which was managed in a glasshouse under controlled environmental conditions using a winter cropping cycle. *In-vitro* physiological analysis of lettuce revealed the outperformance of the continuous LED treatment over the pulsed LED as well as the control in terms of total sugars, chlorophyll concentration, carotenoids, phenolic index, and sucrose accumulation. Continuous LED treatment has also resulted in a significant reduction in nitrate content, a commercially vital parameter, making it the most advantageous and effective of all the treatments performed. However, the production of anthocyanins, an antioxidant released during stress, was enhanced under pulsed LED which requires further investigation and improvements to achieve an improved metabolite profile of lettuce with a minimal energy usage and cost.

1. Introduction

The inevitable demand for high quality of fruits and vegetables is a precursor for urban horticulture. Numerous supplementary lights have been utilized in various research, medicinal and food industries (Dutta gupta et al., 2019). High Pressure Sodium (HPS), Tubular Fluorescent lamps (TFL), Metal halides (MH) and Light emitting diodes (LED) are some of the available options in the market. However, the high energy and productions costs appear as a hindrance to all other options except LED's, which comes in various monochromatic options of near infra-red (NIR), red, green, blue and UV apart from the recipes of various combinations. LED provides a relatively lower energy cost compared to other artificial light options however, there is still room for further improvement in this regard. Moreover, controlled indoor exposure to LED lights can influence the production of various primary and secondary metabolites in plants marking them a potential subject for indoor cultivations.

Several studies have demonstrated the role of LED lights in promoting leaf pigmentation and variegation which are prime picks for consumers (De keyser et al., 2019). It also has applications in microgreens to enhance primary and secondary metabolism and the accumulation of various bioactive compounds (Frąszczak and Kula-Maximenko, 2022). Edible flowers have preferred these days for their consumption as a whole flower or as part of high-end cuisine and carotenoids intake. LEDs increased carotenoid concentrations in edible flowers opened a broad room for further investigations of their usage (Kopsell et al., 2016). In contrast, aromatic herbs exhibit increased accumulation of phenols, anthocyanins and carotenoids when subjected to various LED recipes (Piovene et al., 2015). Furthermore, an indoor chamber or glasshouse equipped with LED's not only provides the best suited photosynthetic active radiation (PAR) but also ensures better control of temperature, humidity and water availability for the plant to display a better growth and overall production.

* Corresponding author.
E-mail address: awais.ali@unimi.it (A. Ali).

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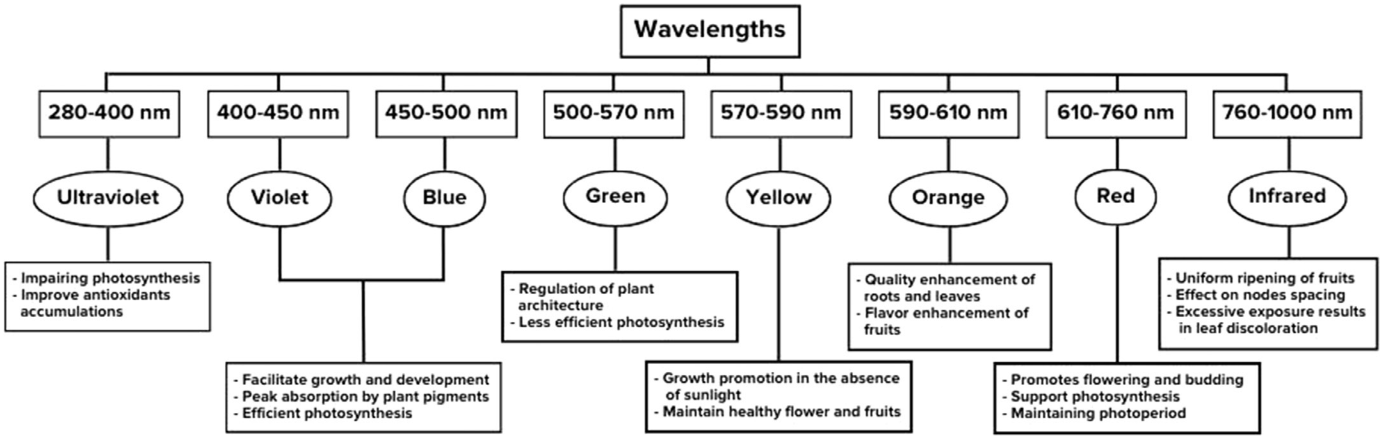

Fig. 1. Commercially available multicolor LED's wavelengths and their effects on plants.

Lettuce (*Lactuca sativa* L.) is an important leafy green vegetable, grown because of its economic relevance, rapid growth and short cropping cycle. It is one of the most widely and occasionally consumed salads and is preferred for its beneficial traits and the valuable content of fiber, vitamins (A, C and K) and antioxidants (Phenolic Acids, Anthocyanins and Flavonoids) (Toscano et al., 2021). It is also valuable source of minerals such as calcium (Ca^{2+}), potassium (K^{+}), iron ($Fe^{2+,3+}$), zinc (Zn^{2+}), phosphorus ($P^{3+,5+}$), magnesium (Mg^{2+}), and manganese ($Mn^{2+,4+}$) (Kim et al., 2016). Natural light has a wide spectrum and is beneficial for plants to manufacture necessary minerals and antioxidants. On the other hand, manipulating light to a spectrum better perceived by plants, favors them to exhibit substantial performance in the growth and accumulation of essential metabolites. A broad body of literature is available on the use of LEDs in protected cultivation and indoor farming and their effect on several growth and quality parameters as shown in Fig. 1 (Yan et al., 2019). Red LEDs are known to enhance biomass accumulation, stem elongation, and leaf expansion whereas the blue LEDs are involved in the production of chlorophyll, assisting leaves in stomatal opening and overall improved photosynthesis (Ouzounis et al., 2015a). Green LED light has little impact on photosynthesis and photomorphogenesis but a greater tendency to penetrate the lower canopy and assist leaves in photosynthesis and carbon assimilation (Smith et al., 2017). In addition to monochromatic wavelengths, red, green and blue in combination are known to be effective in biomass accumulation and increased lettuce growth (Johkan et al., 2012).

In the current study, pulsed LED treatment was tested to maximize the goal of productivity and nutritional profile of lettuce. Continuous LED was used as a reference to determine the growth and quality of the produce. The control was managed in a glasshouse to provide a controlled and uniform environment for all treatments being studied. By testing a number of physiological indices, including total chlorophyll, total sugars, sucrose, nitrate, total carotenoids, anthocyanins and the phenolic index, lettuce quality was assessed using both destructive and non-destructive measurements. Additionally, to support the conclusions on the potential impact of LED, the overall yield, leaf length, leaf width, and dry weight of lettuce were considered.

2. Materials and methods

2.1. Plant material and experimental set up

The experiment was conducted on baby leaf lettuce (*Lactuca sativa* L. var. *Acephala*) which was grown hydroponically in an indoor experimental growing chamber as shown in Fig. 2. Seeds were sown in plastic trays filled with perlite with an expected plant density of 1150 plants/m^2. An optimized Hoagland's solution was used as plant growing media. The concentrations (expressed as mM) of nutrients provided are: 12N-NO_3, 3.8N-NH_4, 2.8 P, 8.4K, 3.5 Ca, 1.4 Mg and Hoagland's concentration for micronutrients.

The LED growing chamber is a bespoke tool designed specifically for this experiment by MEG Science™. It has two shelves which are shielded from each other in order to create two light sealed chambers for independent LED operations. Each shelf is equipped with a planar shape light engine, water cooling system and powered by 8

Fig. 2. Experimental setup of LED chamber with and without LED operation. Continuous LED application was applied at the top shelve of the LED chamber while the pulsed LED at the bottom shelve with an automatic operational setting.

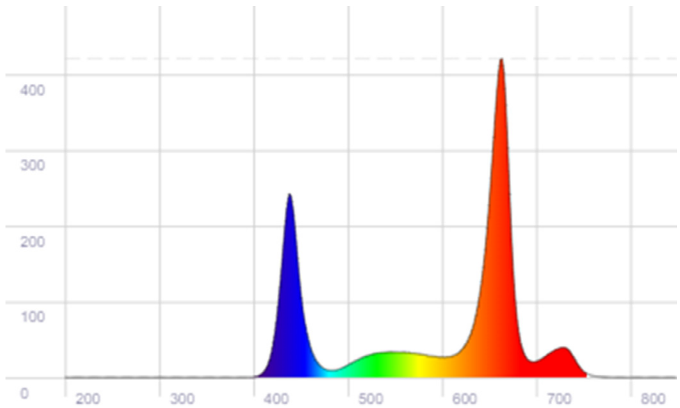

Fig. 3. Spectral quantum distribution used for both the growing chamber experiments; continuous and pulsed light mode (R : 77.1%; G+Y :17.9%; B : 5%).

independent LED's channels with wavelengths peaks ranging from 450 nm to 730 nm. Digital $\lambda^{®}$, a Web App based control software, allows to create dynamic lighting recipes in both operational mode; continuous and pulsed light.

A total of two independently controlled LED chambers were used in the experiment with two shelves each, set up with the same spectral power distribution (SPD) and same average PPFD, but different operational mode. In the first case, the continuous light recipe was chosen based on the previous experiment (Loconsole et al., 2019) having an average PPFD at the seedling level of 150 μmole s^{1} m^{2} with a photoperiod of 16 h for a growing cycle of 30 days. In the second case, the pulsed light recipe had dynamic mode in which the light pulsed with a frequency of 2 Hz and a duty cycle of 0.5 as shown in Fig. 3. The irradiance peak of the pulse was set up at an average PPFD of 150 μmole s^{1} m^{2} and the photoperiod length was the same of the continuous light recipe; 16 h for a growing cycle of 30 days. The average temperature in the growing chamber was 22 $\pm$ 1 °C. Four trays were grown for each condition (continuous and pulsed light), two for each chamber. The energy demand (W) was measured for each LED management condition, using a plug digital wattmeter (Table 1).

An additional set of plants (4 trays) were cultivated in an experimental glasshouse during winter season, under monitored conditions and natural light, with an average PPFD of 65 μmole s^{1} m^{2} (maximum: 110 μmole s^{1} m^{2} and minimum 30 μmole s^{1} m^{2}) and an average temperature of 21 $\pm$ 1 °C.

2.2. Non-destructive *in vivo* estimations by using MPM-100 Multi-Pigment-Meter

A week before harvest and at harvest time, *in vivo* estimation of chlorophyll, flavanols and anthocyanins contents were done using an MPM-100 Multi-Pigment-Meter (ADC BioScientific Ltd, UK) on expanded leaves (Cerovic et al., 2008). However, data taken only at harvesting stage has been presented here to demonstrate the status of the plants under the applied LED exposures and in glasshouse. A week old adaxial leaves surfaces were selected for the measurement and leaves midribs were avoided while placing the fluorescence detector for the readings.

Table 1
Measurement of the energy demand resulting from the different LED management, continuous and pulsed.

LED growing condition	Energy demand (W/h)	Operative hours	Length of the growing cycle (Days)
Continuous	39.8	16	30
Pulsed 2 Hz	24	16	30

2.3. Non-destructive *in vivo* estimation by using Handy PEA+ fluorimeter

This procedure was carried out at harvesting in order to measure the leaves light utilization and health status of photosystem II. The chlorophyll *a* fluorescence was estimated on dark adapted (30 min) week old leaves using a portable fluorimeter (Handy PEA; Hanstech, Kings Lynn, UK). The parameters measured were the maximum quantum efficiency pf photosystem II (Fv/Fm) and the leaf fluorescence performance index (PI), which provided information about the relative leaf functionality. The PI includes three independent parameters: the intensity of active reaction centres (RCs), the efficiency of electron transport and the probability that an absorbed photon will be trapped by the RCs.

2.4. Determination of yield and morphological traits

The yield was measured separately by harvesting the total produce under each treatment. Dry weight was measured by considering the water percentage of the samples after drying the sample at 110 °C for three days. Leaf length and leaf width were measured in centimetres. Leaf length was measured from the pointy tip of the leaves to the point where the leaf was attached to the petiole while the width was measured by considering the longest extension of two points on the leaf blade perpendicular to the leaf length axis.

2.5. Determination of total chlorophylls and carotenoid contents

Total chlorophylls and carotenoids were extracted from the fresh leaf tissues (around 50 mg) in 5 mL of methanol 99.9%. The samples were kept in a dark room at 4 °C for 24 h. Absorbance readings were measured using spectrophotometer at 665.2 nm and 652.4 nm for chlorophyll pigments and 470 nm for total carotenoids. Chlorophyll and carotenoid concentrations were calculated using Lichtenthaler's formula (Lichtenthaler, 1987).

2.6. Determination of nitrate contents

The nitrate content was assessed based on Cataldo's method (Cataldo et al., 1975). Around 1 g of leaves was ground in with 4 mL of distilled water. The extract was centrifuged (ALC centrifuge-model PK130R) at 4000 rpm for 15 min and the supernatant was recovered and used for the colorimetric determination of nitrate and sugars. Twenty μL of the sample was added to 80 μL of 5% salicylic acid in sulphuric acid and to 3 mL of 1.5 N NaOH. The samples were cooled at room temperature and the spectrophotometric readings were done at 410 nm. The nitrate content was estimated based on a KNO_3 standard calibration curve (0–10 mM).

2.7. Determination of sucrose and total sugars contents

The extracts used were the same prepared for the nitrate content (see above). The sucrose assay was performed by mixing 0.2 mL of leaf extract with 0.2 mL 2 M NaOH and incubated in a water bath at 100 °C for 10 min. Then 1.5 mL hot resorcinol buffer (containing 30% hydrochloric acid, 1.2 mM resorcinol, 4.1 mM thiourea 1.5 M acetic acid) was added to samples and incubated in a Dubnoff bath (PID) at 80◦C for another 10 min. After cooling at room temperature, the O.D. was determined at 500 nm and a sucrose standard curve (0–2 mM) was used for calculating the final concentration (Rorem et al., 1960).

The total sugars concentration was assessed spectrophotometrically following the anthrone method (Yemm and Willis, 1954) with slight modifications. The anthrone reagent (10.3 mM) was prepared dissolving anthrone in ice-cold 95% H_2SO_4. Then 0.5 mL of extract was placed on top of 2.5 mL anthrone reagent and kept in ice for 5 min. After that, the mix was vortexed vigorously, heated at 95 °C

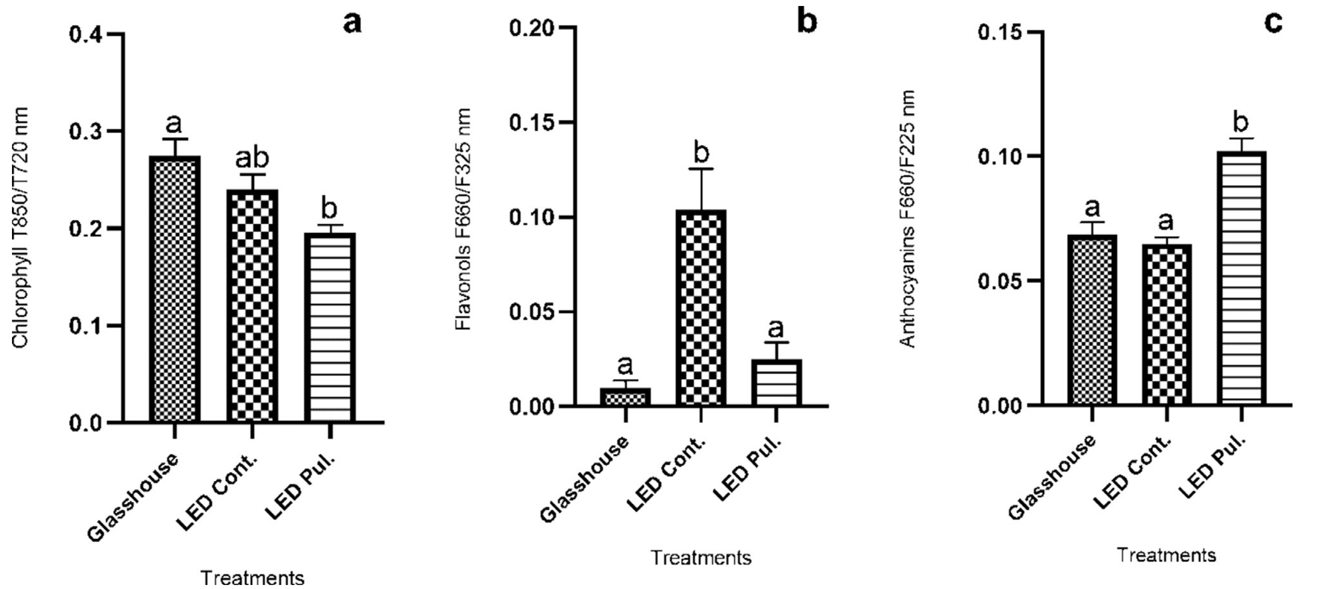

Fig. 4. *In vivo* readings taken through MPM 100 multipigment meter (a) chlorophyll, (b) flavanols and (c) anthocyanins observed in the leaves of lettuce subjected to continuous LED, pulsed LED and control (glasshouse) treatments. Values are means (n = 20 ± S.E). Different letters indicate significant differences among different treatments after one way ANOVA followed by Tukey multiple comparisons test ($p < 0.05$).

for 10 min and left to cool in ice. Readings were performed at 620 nm and total sugars concentration was calculated, based on a glucose calibration (0–4 mM).

2.8. Determination of phenolic index and total anthocyanin contents

For the extraction of the phenolic compounds, around 50 mg of leaves were placed in 5 mL of acidified methanol (1% HCl v/v) and extracted overnight in the dark. The phenolic index was calculated as the absorbance measured at 320 nm. The phenolic index was used as an indication of the total phenolics content. In this method, the total phenols were estimated by measuring absorbance at 320 nm using an UV–Vis spectrophotometer, as previously showed (Ke and Saltveit, 1989).

The total anthocyanins were measured from the same extracts. The concentration of anthocyanins was expressed as cyanidin-3-glucoside equivalents and determined spectrophotometrically at 535 nm using an extinction coefficient εM of 29,600 (Ferrante et al., 2004).

All spectrophotometric determinations have been performed using the Evolution 300 UV–Vis spectrophotometer (Thermo Scientific).

2.9. Statistical analysis

Data were subjected to a one-way analysis of variance (ANOVA) followed by Tukey multiple comparisons test. Analyses were performed using GraphPad Prism version 6 for Windows (GraphPad Software; La Jolla, California, USA, www.graphpad.com).

3. Results

3.1. Non-destructive estimation using MPM-100 Multi-Pigment-Meter

As shown in Fig. 4a, the plants grown in glasshouse (control) had the highest *in vivo* estimation of chlorophyll whereas the pulsed LED exhibited the lowest estimated chlorophyll values among the three treatments. However, in Fig. 4b, the estimation of flavanols showed an entirely different pattern from that of chlorophyll, with considerably higher significant levels of flavanols in the continuous LED treatment compared to non-significant flavanols in the control and pulsed LED treatment. These estimated values clearly explain how LED induced the increase in production of necessary metabolites in treated plants. Moreover, in contrast to the lowest values of anthocyanin concentrations in continuous LED treatment, a much higher anthocyanin has been recorded in pulsed LED treatment which suggested that pulsed LED treated lettuce perceived it as a stress and responded by accumulating higher amount of anthocyanins, as shown in Fig. 4c.

3.2. Non-destructive *in vivo* estimation of using Handy PEA+ fluorimeter

Using a Handy PEA+ fluorimeter, an *in vivo* estimation of the maximum quantum efficiency of the photosystem II as well as the overall leaf fluorescence performance index was evaluated. As shown in Fig 5a, the quantum maximum efficiency of photosystem II decreased

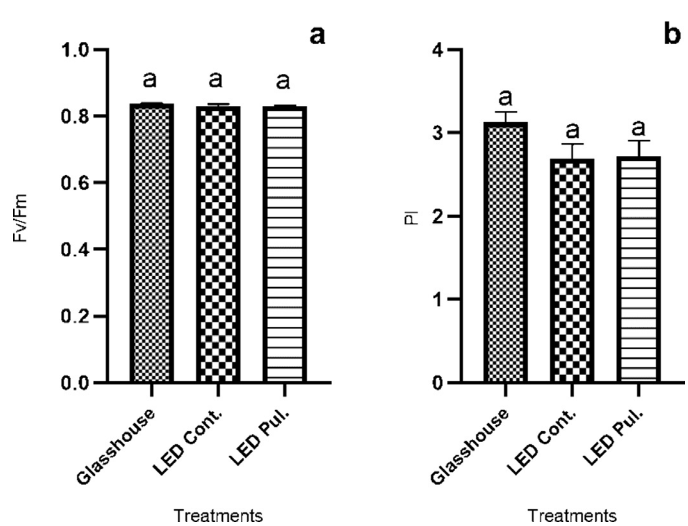

Fig. 5. *In vivo* readings using Handy PEA + fluorimeter (a) maximum quantum efficiency of photosystem II, (b) performance index, of lettuce leaves treated with continuous LED, pulsed LED and control (glasshouse) treatments. Values are means (n = 20 ± S.E). Different letters indicate statistically significant differences among different treatments after one way ANOVA followed by Tukey multiple comparisons test ($p < 0.05$).

slightly in both continuous LED (0.8295) as well as pulsed LED (0.8307) treatments compared to the control (0.8381). Moreover, in Fig. 5b, reduction in the overall leaf fluorescence performance index was also observed in both LED treatments, with LED continuous at the performance index value (2.690) and LED pulsed at (2.714) compared to the control which showed the performance index values at (3.130). However, none of these slight variations were significant.

3.3. Yield, water content, leaf length and leaf width under different LED applications

The yield was calculated as the total amount of biomass produced for each treatment as shown in Fig. 6a. According to these findings, it was much greater in the continuous LED (246.8 g) than in the pulsed LED (115.2 g) and control (73.34 g). Water content was calculated as a percentage, as shown in Fig. 6b, in which the pulsed LED showed significantly higher water contents than the control but non-significantly higher water contents than the continuous LED treatment. For all the treatments under evaluation, leaf length (Fig. 6c) and leaf width (Fig. 6d) were measured in cm. For each of the three distinct treatments, a comparable trend was observed for both the parameters. The leaf length and width were both soaring with the continuous and pulsed LED treatments compared to the control. Although all treatments were statistically significant in terms of leaf width at harvest with continuous LED, which had the widest leaves, a significant difference was observed in leaf length only between the continuous LED and the other two treatments.

3.4. Physiological and quality evaluation of lettuce under different treatments

Lettuce treated with different LEDs as well as the control showed different amounts of chlorophyll, both *a* and *b*. Although the continuous LED treatment had a slightly higher chlorophyll content than the control, it was not statistically significant compared to the control. Both LED treatments had significant chlorophyll concentration, with

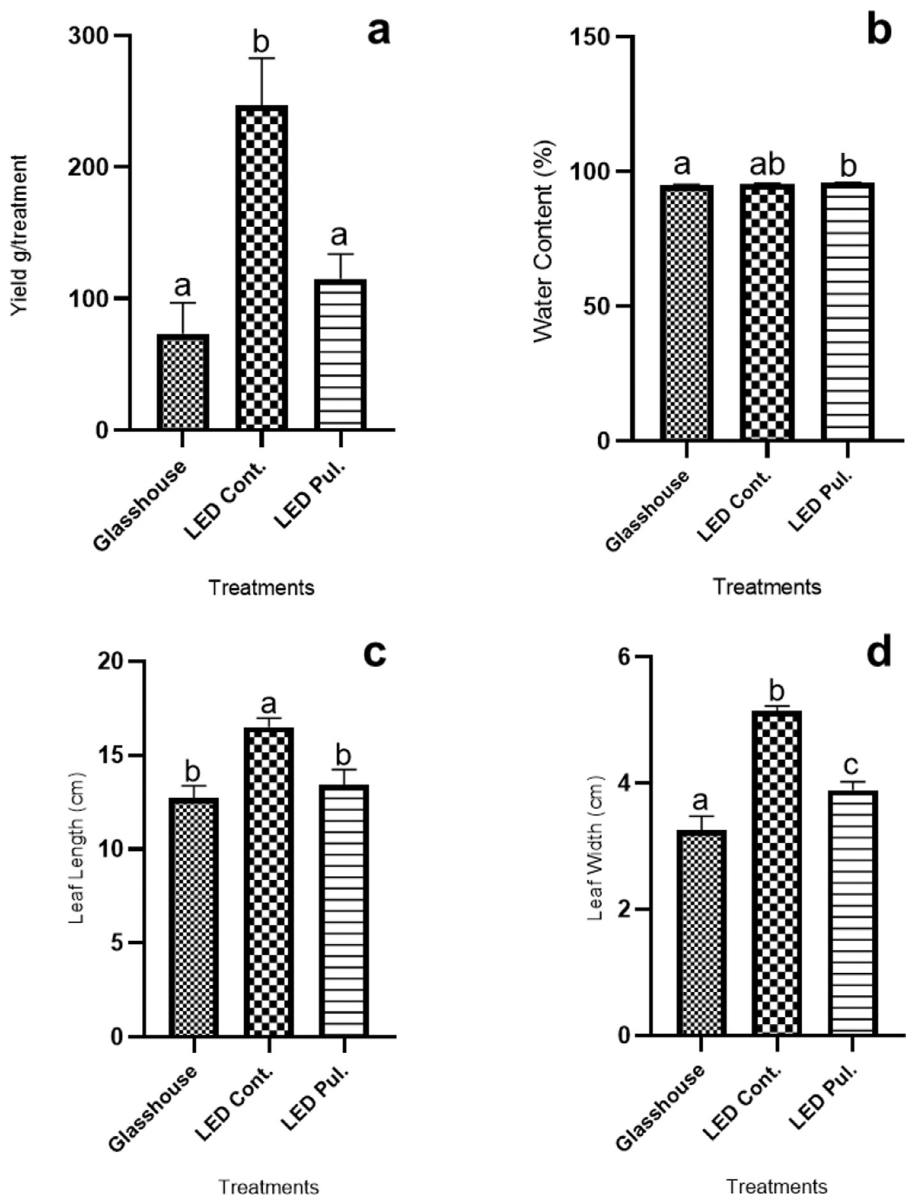

Fig. 6. (a) Yield (g/treatment), (b) water content (%), (c) leaf length (cm) (d) leaf width (cm) of lettuce grown under continuous LED, pulsed LED treatments and control (glasshouse). Values are means (n = 12 ± S.E). Different letters indicate statistically significant differences among different treatments after analyzed by one way ANOVA followed by Tukey multiple comparisons test ($p < 0.05$).

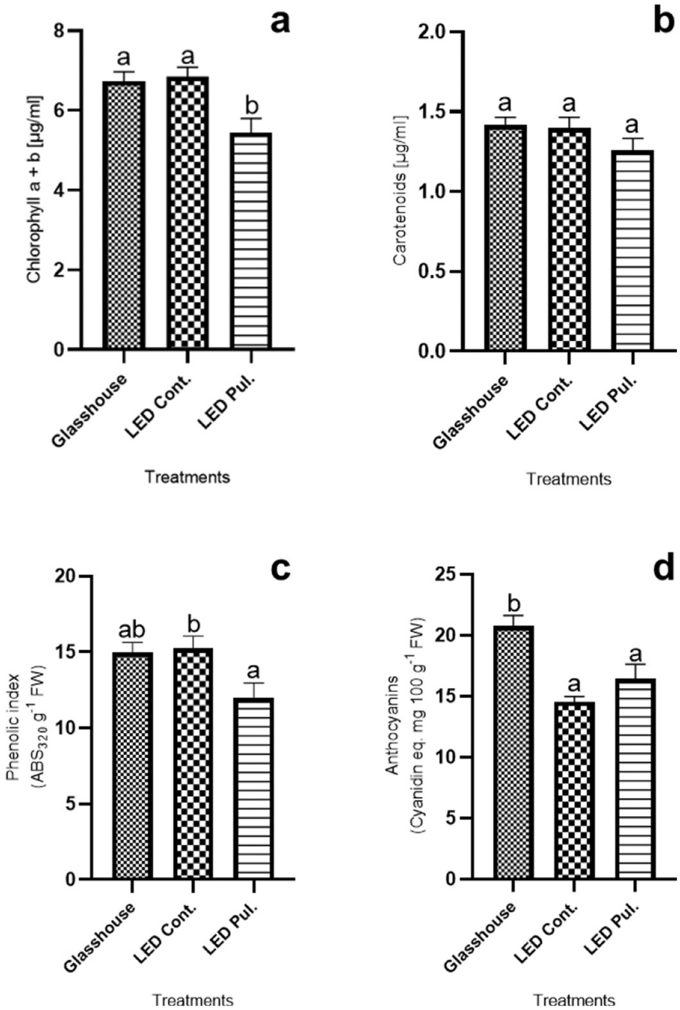

Fig. 7. (a) Chlorophyll (*a* & *b*), (b) carotenoids, (c) phenolic index, (d) anthocyanins, measured under continuous LED, pulsed LED treatments and control (glasshouse). Values are means (n = 12 ± S.E). Different letters indicate statistically significant differences among different treatments after analyzed by one way ANOVA followed by Tukey multiple comparisons test ($p < 0.05$).

pulsed LED showing a marked reduction in chlorophyll compared to continuous, as shown in Fig. 7a. The highest accumulations of total carotenoids were observed in the control compared to both continuous LED and pulsed LED treatments which suffered a subsequent drop in carotenoids accumulation. However, despite of the modest variations in carotenoids accumulations, which varied from 1.258 of pulsed LED to 1.417 g mg^{-1} of control, non-significant differences were observed among all the treatments as shown in Fig. 7b.

Both continuous and pulsed LED showed significant differences in the phenolic index as shown in Fig. 7c however, pulsed LED had significantly lower values of phenolic index compared to both the control and continuous LED treatment. Furthermore, non-significant differences were found between the LED treatments in terms of anthocyanins although there were slightly higher anthocyanins accumulation in the pulsed LED treatment than in the continuous LED treatment. These results are in line with those recorded with MPM-100, where the pulsed LED showed a reasonable increased estimated anthocyanin compared to the continuous LED treatment. In general, plants released anthocyanin during stress which indicates that the lettuce in this particular experiment perceived pulsed LED as a stress and coped up by releasing higher levels of this antioxidant in leaves. However, control showed significantly higher anthocyanins accumulations compared to both LED treatments which might be the reason for the fluctuating PPFD values of the control in the glasshouse, as shown in Fig. 7d.

As reported in Fig. 8a, there was an intriguing significant drop in nitrate levels while using continuous LED (216.2 mg kg^{-1}), compared to the control (455.3 mg kg^{-1}) and the pulsed LED (422.8 mg kg^{-1}) which showed non-significant higher levels of nitrate compared to the continuous LED treatment. Moreover, in contrast to the control and pulsed LED treatment, which showed non-significant lower total sugars contents, a significantly higher total sugar was recorded under the continuous LED treatment as shown in Fig. 8b. The range spans from 0.7240 mg g^{-1} FW of total sugars under the pulsed LED to a maximum of 1.205 mg g^{-1} FW of total sugars in the continuous LED treatment. As for total sugars, continuous LED treatment showed significantly higher sucrose levels than both control and the pulsed LED as shown in Fig. 8c. These findings revealed that the continuous LED treatment favorably influenced lettuce sugar metabolism.

4. Discussion

Plants respond to ultraviolet, infrared, and visible light spectra in a fascinating manner. These wavelengths have been modified and

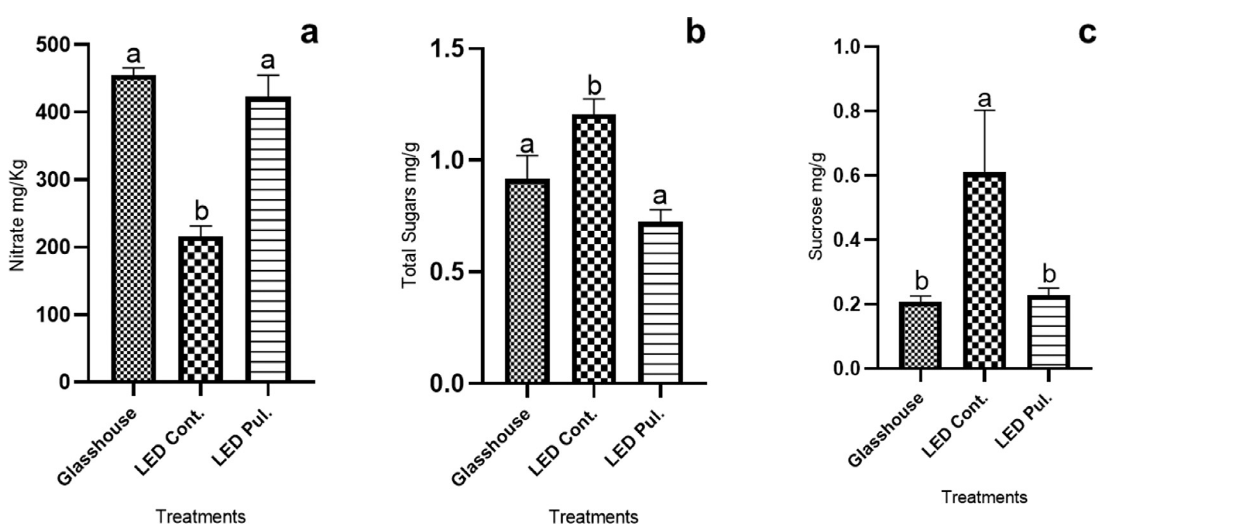

Fig. 8. (a) Nitrate content (b) total sugar content (c) sucrose content, measured under continuous LED, pulsed LED treatments and control (glasshouse). Values are means (n = 12 ± S.E). Different letters indicate statistically significant differences among different treatments after analyzed by one way ANOVA followed by Tukey multiple comparisons test ($p < 0.05$).

modulated in wide range of ways, from long to short, and have been shown to be beneficial for signaling to produce vital physiological reactions as well as an effective fuel to produce fresh food (Pattison et al., 2018). By switching to LED in a regulated setting, rapid generation cycling has been realized, assisting researchers in global food security and crop enhancement initiatives.

4.1. In vivo *estimated parameters using 100 MPM Multi-Pigment-Meter and fluorimeter*

Non-destructive estimation of various pigments using MPM-100 was carried out in this experiment. This portable device is used to measure the amount of chlorophyll in leaves by leaf transmissions in the far red and near infrared regions while it uses ratio fluorescence to estimate the anthocyanin and flavanols content. MPM uses digitally controlled modulated light rather than non-modulated fluorescence detection. A higher estimate of chlorophyll was recorded in continuous LED compared to pulsed LED in this study. However, this photosynthetic pigment was found in excess in control (Fig. 4). The present results are in contrast to those reported by Miliauskienė et al. (2021) in which lettuce subjected to two pulsed treatments had higher levels of chlorophyll *b*oth *a* and *b* as well as carotenoids than lettuce subjected to the continuous treatment which revealed that different cultivation methods, duty cycle and frequency of the pulsed LED, all play an important role in determining the amount and chemistry of photosynthetic pigments in LED controlled experiments. Intriguingly, MPM-100 measurements conducted *in vivo* revealed higher anthocyanin levels in the pulsed LED treatment which differ from the previously studied pulsed LED mode on chilli plants by Olvera-Gonzalez et al. (2021) who conducted an experiment utilizing pulsed mode of LED to investigate the quantum efficiency of Photosystem II. Although there is insufficient evidence to explain the role of pulsed treatment in boosting anthocyanins biosynthesis, this research opens up a room for investigation of antioxidant accumulation in plants under a pulsed LED mode. The increased flavanol contents in recent research can be explained by the already established reasoning that blue light, in particular, has a significant role in the biosynthesis of bioactive chemicals, particularly flavonoids (Matysiak and Kowalski, 2021). Moreover, in another experiment in which lettuce was exposed to blue light in addition to red light, Mickens et al. (2018) recorded a spike in the production of secondary metabolites. The current finding as well as previous studies, all indicated the role of continuous red and blue light in the stimulation of the flavanols accumulation in lettuce, which in turn helps the plant to control overall growth and development, one of the main reasons for the increased yield of LED assisted productions.

The *Chl* fluorescence of green plants is an intricate indicator of photosynthetic potential. Green plants absorb light, using some of it for photosynthesis, some for dissipation of heat and some for *Chl* fluorescence. The Fv/Fm determine the photochemical efficiency of photosystem II. Varied Fv/Fm values among the LED treatments, pulsed LED demonstrated slight increased Fv/Fm values compared to the continuous LED treatments therefore we propose that the pulsed LED was effective in increasing the rate of photosynthesis. However, the noted order of this increased photosynthetic rate would be control> puled>continuous, keeping the quite negligible differences of Fv/Fm values in mind. These results are in line with the findings of Gao et al. (2020) in which high proportion of blue light was found to be responsible for an improved photosynthetic rate but a decreased heat dissipation ability of PS II. In a related study, Ouzounis et al. (2015b) observed that *Phalaenopsis* responded with a decreased Fv/Fm in treatments comprising entirely of red LED compared to those with proportions of blue light. Moreover, no significant differences were observed in the quantum efficiency of PS II by Son et al. (2018) in their research on irradiated lettuce using continuous and pulsed LED treatments of different monochromatic and LED recipes which again depends on the appropriate selection of duty cycle as well as the frequency of the pulsed and species under investigation. Sobczak et al. (2021) described leaf fluorescence performance index (PI), a very sensitive measure that offers quantitative data on the general health and vitality of plants. When tested under stressful circumstances, the PI is a very good biophysical indication, according to Kalaji et al. (2016). The PI in present study followed the same trend as that of Fv/Fm, which indicated that the pulsed LED was the efficient in minimizing the loss of absorbed light energy and hence exhibited a better PI than continuous LED. The higher photosynthetic pigments led to higher light absorption and eradication of reactive oxygen species in the presence of pulsed light resulting in a positive performance index of lettuce.

4.2. *Yield and leaf morphological response of lettuce towards LED*

According to the present study, lettuce cultivated under continuous LED lighting produced noticeably more than lettuce grown under pulsed LED lighting or in a glasshouse (control). This suggested that this LED treatment was the most effective for enhancing the yield. These results are in line with those of Mickens et al. (2018) who observed an increase in yield and dry weight when lentil, basil and mint species were subjected to a red-blue LED exposure. According to previous studies, the makeup of the red and blue spectra fits well with the absorption spectra of both chlorophyll and carotenoids pigments, suggesting that an increase in plant growth, which is related to greater photosynthesis, may have an impact on biomass and yield (Lin et al., 2021). Additionally, red light has been shown to be effective in increasing the fresh shoot weight of lettuce (Borowski et al., 2015), which supports the idea that using LEDs as monochromatic lights or in recipes may have some effect on altering photosynthesis and ultimately increasing the yield and dry weight of produce as previously observed (Rahman et al., 2021). Miliauskienė et al. (2021) recently discovered that pulsed light with red and blue LED, when handled with different frequencies, increases the produce's fresh and dry weight as well as the total leaf area. In the same experiment that pulsed LEDs with a 0.5 kHz frequency tend to boost lettuce's total biomass and leaf area. Moreover, the B/R/G/FR LED in ratio of (1:1:0.07:0.64) imposed stem elongation, which had an impact on the overall quality of the growing plants, while also promoting the growth and yield statistics in both red and green lettuce (Alrajhi et al., 2023). Viršilė et al. (2019) suggested that rather than the photoperiod itself, it is the LED intensity that gives variances in several growth parameters. On the other hand, higher LED intensities lowered the leaf area, the number of leaves generated, and the plant height in red leaf lettuce and moderate LED intensities were found to be more helpful in increasing fresh weight.

Nevertheless, this study supports the findings of Matysiak et al. (2021) in terms of leaf morphology in which an enhanced leaf head circumference, leaf number, and increased fresh weight of Romaine lettuce leaves were recorded under the red, green, and blue LED lighting. However, in contrast to recent research by Watson et al. (2018), we found that leaf length and width increased under both LED treatments compared to the glasshouse. We therefore conclude that this trend is a result of the rapid generation cycling of the LED. Furthermore, taking into account the present results, it is necessary to examine the effects of pulsed LED on yield, leaf length, and width by extending the harvesting period to exclude the rapid generation cycling effects of continuous LED while keeping energy consumption in mind, as well as to observe how pulsed LED with a prolonged LED exposure affects the leaf morphology in the production of lettuce indoors.

4.3. *Quality evaluation and investigating the physiological responses in lettuce*

In general, crop quality is determined by its nutritional contents as well as its appeal to consumers. Leafy vegetables are largely consumed because they contain antioxidants such as phenolic compounds and carotenoids which are beneficial for human health. They also provide useful components such as soluble proteins and carbohydrates. Referring to the previously obtained results of do Nascimento Vieira et al. (2015), it was observed in the present study that continuous LED treatment increases the overall chlorophyll content (*chl a* and *chl b*) in lettuce. For some reason, the pulsed LED treatment deviated from this pattern and showed lower values for the chlorophyll. However, the findings of the current study showed a distinct pattern from those of Camejo et al. (2020), who found that lettuce's chlorophyll did not significantly change when exposed to different light conditions such as W-LED or RB-LED illuminations. According to study by Fu et al. (2017), plants produce less chlorophyll under higher illuminations but still perform photosynthesis effectively, while under low illuminations, there is an increase in chlorophyll content to maintain the effectiveness of photosynthetic functions. Moreover, the chlorophyll content was found to be proportional to the blue LED light by Hogewoning et al. (2010), on the basis of which we can assume that the higher chlorophyll content in continuous LED treatment compared to pulsed LED is due to the lower exposure of lettuce plants to blue LED under the set time period of growth until harvesting. Moreover, 'Elizium', one of the micro cultivars of lettuce demonstrated a tendency to accumulate a comparatively low level of nitrate and a relatively high amount of chlorophyll, carotenoid, polyphenol, and flavonoid in a vertical hydroponic system at two levels 200 μmol m^{-2} s^{-1} and 160 μmol m^{-2} s^{-1} of LED light intensity (Grzegorzewska et al., 2023).

Carotenoids are secondary metabolites that help plants absorb energy for photosynthesis and provide photoprotection on photochemical quenching (Carvalho et al., 2011). This claim is related to current research on the accumulation of carotenoids in lettuce, which has shown a similar pattern to that of chlorophyll concentration confirming its role in protecting leaves against photo-bleaching. The use of LED and its function in manipulating carotenoids has received extensive attention over the years (Carvalho and Folta, 2014). Researchers have concluded that when plants are exposed to red, blue, or RB LED lights instead of white LED lights, there is a noticeable rise in the aforementioned pigments. In Brassica sprouts, three carotenoid biosynthesis genes phytoene synthase (PSY), β-cyclase (β-LCY), and β-carotene hydroxylase (β-OHASE$_1$) underwent an increase in transcript levels when treated with combination of blue and white LEDs. Meanwhile only in pak choi brassica, blue and white LEDs increase the carotenoid levels by 14% when compared to white LEDs alone and by 19% when compared to red and white LEDs (Frede et al., 2023). When lettuce was exposed to 70% *R* + 30% B LED, Amoozgar et al. (2017) found a higher accumulation of carotenoids in leaves. The production of more pigments aids plants in promoting a better light absorption, controlling reactive oxygen species, and improving shoot development. In the current study, improved yield, longer and wider leaves and greater carotenoids accumulation under continuous LED treatment all in favored of higher chlorophyll content.

Sugars assist plants in a wide range of structural, functional and defense processes and help to shape their nutritional profiles. Increased sugar accumulation in the continuous LED treatment compared to that in the pulsed LED and glasshouse treatments (control) was observed. These results are consistent with those of Wojciechowska et al. (2015), who found that "lamb's lettuce" *Valerianella locusta* produced more when exposed to a red-blue LED ratio of 9:1, as measured by yield, dry matter content, and total sugars content. Red-blue LED have also been demonstrated to boost the accumulation of sugars in plants (Khan et al., 2021). The increase in the sugar levels under LED treatments are in direct proportion to the chlorophyll production which was found maximum under continuous LED treatment and hence resulted in an increased and efficient photosynthesis which ultimately yielded higher accumulation of sugars in the leaves of continuous LED treated lettuce. Higher soluble sugar contents have also been recorded by Soufi et al. (2023) in lettuce when subjected to red/blue and monochromatic red LED spectra. Li et al. (2017) exposed tomato plants to monochromatic red and blue LEDs, in addition to the 3R1B LED recipe and observed an increased accumulation of carbohydrates and starch. Hence, supplemental LED lights are beneficial not only to increase the overall productivity of the produce but also to increase the deposition of overall sugar contents in the fruits and crops (Viršilė et al., 2020).

Phenolic compounds, including anthocyanins are the most prevalent secondary metabolites in plants and are widely distributed across the plant kingdom. They are typically used by plants to protect themselves against ultraviolet radiation, diseases, parasites, and predators, as well as to produce distinctive hues (Appolloni et al., 2022). Moreover, some phenolic compounds, are widely recognized as key components of the nutritional value of plant-derived foods, because of their important health-related properties (Jesus et al., 2022). There was a slight increase in the phenolic index observed in the continuous LED treatment compared to the control. However, a decline in the phenolic index was noticed in the pulsed treatment. However, anthocyanins were lower in both LED treatments than in the control which might be due to the fluctuating PPFD of the glasshouse which resulted in excessive release of antioxidants by the lettuce to sustain proper growth and development. Similarly, a gradually increase in light intensity during the growth and developmental stages was found to be potent in plant behavioral responses including an increase in overall biomass productions (Jin et al., 2023). In addition, Mickens et al. (2018) stated that LED support of natural light can increase the synthesis of phenols in romaine baby leaf lettuce, firmly reiterating the findings of this study. The biosynthesis and accumulation of secondary metabolites, particularly phenols, are light dependent. According to a recent study, blue LEDs are the most effective light spectrum for promoting the expression of the genes phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and dihydroflavonol-4-reductase (DFR), which are necessary for the synthesis of anthocyanins and phenols (Giliberto et al., 2005). We can presume that the pulsed LED was not sufficiently effective to enhance the stimulation of specific enzymes to promote the accumulation of these metabolites given the increasing trend of phenols caused by blue LED or BR LED. However, in addition to the quality and intensity of the light, leaf growth phases are also crucial for the buildup of these secondary metabolites which has already been highlighted in terms of rapid generation cycling achieved through continuous LED treatment (Azad et al., 2020). The anthocyanin content, expression of the flavonoid-3-O-glucosyltransferase (UFGT), CHS, and Rubisco small subunit genes, as well as the net photosynthetic rate were all most strongly induced by the red/blue continuous LED light according to Soufi et al. (2023). Moreover, the physiological and developmental activities of plants are halted by changes in source-sink balance and the principal energy source for this coordination is sucrose (Durand et al., 2018). In the present research, it was observed that the higher accumulation of sucrose occurred in the continuous LED while there were no significant differences in pulsed LED and the control. According to the findings from Li et al. (2017), 3R1B LED treatment in tomato plants increased the buildup of sucrose in the leaves. Additionally, increased activity of the enzymes sucrose-phosphate synthase (SPS) and sucrose synthase (SS) results in increased sucrose metabolism which in this study can supposedly be achieved and the reason for enhanced sucrose contents in continuous LED treatment. However, sucrose-phosphate synthase oversees producing sucrose from glucose and fructose (Lombardo et al., 2011). According to past studies, red LED light promotes accumulation of photosynthetic products in plants. However, the buildup of sucrose increases when red LED light is combined with blue LED light (Zheng et al., 2008). Since photosynthesis and growth are facilitated by the spectral quantum, distribution of red and blue LED and the chlorophyll absorption spectrum, the enhanced sucrose contents in continuous LED compared to pulsed LED and control justifies our findings.

Nitrates are found in all plant tissues and are important for healthy growth and development. Leafy vegetables such as spinach and lettuce contain high nitrate content and are consumed worldwide daily (Iammarino et al., 2014). Owing to the harmful effects of nitrates on the human body, foods with less nitrates are preferred in the diet and a European regulation (EU: 1258/2011) exists to regulate the nitrate content of foods. Leafy vegetables such as tatsoi (Simanavičius and Viršilė, 2018), basil (Piovene et al., 2015), spinach (Ohashi-Kaneko et al., 2007), rocket (Signore et al., 2020), and lettuce (Bian et al., 2020) have been shown to contain less nitrate when exposed to LED lights. The continuous LED treatment in this experiment has shown a startling reduction but the pulsed LED and control treatments both had significantly greater nitrate concentrations. It is believed that red light specifically and in combinations with red and blue, is effective in boosting the activity of nitrate reductase (NR). In addition to light quality and intensity Ferrón-Carrillo et al. (2021) hypothesized that the developmental stage of Romaine lettuce is a key factor for nitrate accumulation. The length of vegetation greatly impacted on nitrate reductase activity (NR) to the light treatment itself while 9R:1B LED formula is found to be ideal for reducing nitrate in *Valerianella locusta* (Wojciechowska et al., 2016). Furthermore, basil, fenugreek, and dill microgreens experienced a decline in nitrate level when exposed to continuous blue LED light, and chervil microgreens had a significant decrease in nitrate content when exposed to continuous red LED light (El Haddaji et al., 2023). Although the pulsed LED treatment tested in the present work has determined a reduction in nitrates compared to control, the outcomes were still unsatisfactory compared to the continuous LED treatment. This might be because the spectra requirement vary considerably between different physiological processes. To achieve the intended goal of a lowered nitrate concentration, additional research is planned to adjust the temporal exposure, frequency, duty cycle and intensity of the pulsed LED treatment in future indoor lettuce production.

5. Conclusion

In summary, the yield and nutritional composition of lettuce are considerably affected by the quality and spectral quantum distribution of LED light. Among the studied LED treatments with an average PPFD of 150 μmole s^1 m^2, photoperiod of 16 h and growing cycle of 30 days, the LED with a continuous exposure was found to be the most potent at increasing the overall output of lettuce in terms of yield, leaf length and leaf width. Additionally, a definite increase in chlorophyll content was observed under continuous LED application compared to the pulsed LED and control which suggested the efficiency of the photosynthetic pigments along with yielding higher aggregation of carotenoids, phenolic index, sucrose, and total sugars in the produce. In addition to the secondary metabolites, nitrate contents which is the preferred and healthy end consumers preference, significantly decreased under continuous LED. However, the pulsed LED treatment, outperformed the glasshouse in terms of yield and various aspects of producing antioxidants while being managed to exhibit a better leaf performance index and Fv/Fm values compared to the continuous LED, creating an opportunity to broaden the research work using this special pulsed LED mode to obtain the required outputs, such as minimal energy usage, high yield and quality for commercial agricultural production. As a first step in the pulse LED light characterization, a preliminary estimation of the energy demand showed a significant reduction in the energy consumption due to the application of pulsed LED light. Further studies will allow a deeper investigation (including a cost analysis), aiming to determine the best pulsing condition (in terms of frequency, duty cycle and length of the growing cycle) to improve the economic sustainability of this novel cultivation approach.

Authors contribution

AA, GC, AF: structured the experimental concept. AA: conducted the experiments, analyzed the data and wrote the manuscript. GC: assisted in experiments, data analysis, reviewed and edited the manuscript. PS: manufactured the LED chamber for the experiment and assisted in manuscript editing.

Declaration of Competing Interest

The author Piero Santoro is employed by the company MEG Science. All other authors declare no competing interests.

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