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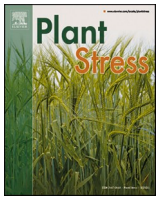

Red light improved photosynthesis and defence-related metabolism in tomato in a leaf level-dependent manner

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ABSTRACT

This study explores the long-term effects of nocturnal red light treatment on tomato plants, focusing on photosynthesis, metabolism, and defence mechanisms. The impact of red light treatment applied at night to both young and old leaves at various times of day was investigated. The results showed that, after three weeks, nocturnal red light treatment improved photosynthetic efficiency in both upper and lower leaves, particularly in the morning and at noon. This enhancement in photosynthesis was associated with higher CO_2 assimilation. Interestingly, older leaves, which usually demonstrate reduced photosynthetic activity, also benefited from red light exposure. Additionally, red light treatment resulted in significant changes to the accumulation of starch and sugars. Higher levels of starch were found in the leaves in the afternoon, alongside increased levels of sucrose in both leaves. Metabolomics analysis revealed that the level of several defence-related metabolites, including 4-guanidinobutyric acid, fucose and quinic acid, increased following exposure to red light. This suggests that red light applied at night could have a priming effect, enhancing defence against pathogens. Conversely, the levels of some metabolites, such as myo-inositol and N-acetylaspartate, decreased, indicating adjustments in plant stress responses. Red light influenced plant height and leaf area and enhanced callose content in older leaves. Moreover, red light can hard plants against fungal pathogens, such as *Botrytis cinerea*. These results suggest that exposure to red light at night is an effective, environmentally friendly approach to enhance the resilience of tomato plants and optimise greenhouse farming practices, particularly through supplemental or interlighting systems.

1. Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most widely grown horticultural crops worldwide, with an estimated 192 million tonnes grown on nearly 5.3 million hectares in 2023 according to Food and Agriculture Organization (FAO, 2023). Although traditionally grown in open fields, greenhouse production is an increasingly important part of the market as consumer demand for sustainable and high quality produce continues to grow (Chanda et al., 2021). This provides to farmers a much more controllable environment where various factors such as lighting, temperature, etc. can be altered to better suit the needs of the crop and reduce the seasonality of the fresh produce (Utasi et al., 2023). Hydroponic systems allow growers to easily control the ratio of each nutrient supplied to plants to optimise vegetative and reproductive stages, as well as fruit size and even flavour (Martínez et al., 2024). However, inside the greenhouse, light is usually not evenly distributed due to the structure and/or plant spacing (Zhang et al., 2020). For example, leaves at the top of the canopy have sufficient light exposure, while leaves in the interior and at the bottom of the plant may suffer from light deficiency, leading to significant reductions in photosynthetic rate (Trouwborst et al., 2010) and even weakened plants that are more susceptible to pathogen attack in the high ratio of far red light (Xiao et al., 2022). The traditional way to combat the infections that occur (apart from prevention) is the use of chemical pesticides (Roca-Couso

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et al., 2021), but due to the increasing use of such chemicals, pathogens often adapt quickly, which temporary changes can lead to acquiring permanent resistance through genetic, epigenetic and physiological changes (Diao et al., 2020; Fisher et al., 2022). The extensive use of pesticides is not only harmful to human health, but also has negative effects on biodiversity therefore the European Commission adopted the EU Action Plan Towards a Zero Pollution for Air, Water and Soil which aimed to reduce nutrient losses and the use of chemical pesticides by 50 % until 2030 (European Commission, 2021). Since 2018, the total amount of active substances has decreased continuously due to removal or not getting approval (Marchand, 2023), so researchers are looking for alternative and environmentally friendly methods to help plants to combat these attacks and limit the use of sprays To overcome these difficulties in large-scale greenhouse crop production, different lighting techniques using light emitting diodes (LEDs) at different times and methods are attracting considerable scientific interest in the hope of improving plant health and even yield (Camejo et al., 2020; Wu, 2021; Darko et al., 2023; Zhao et al., 2024; Paponov and Paponov, 2025).

As in most plants, light plays a central role in the optimal growth and development of tomato, and also has a significant influence on other processes related to defence responses, photosynthesis or circadian rhythm (Kong et al., 2021; Venkat and Muneer, 2022). With the help of their numerous light-sensing photoreceptors, plants are able to detect the changes that occur in the quality, quantity and duration of light (Teixeira, 2020; Qi et al., 2022), allowing them to make various adaptations in response to changes in the aforementioned parameters (Paik and Huq, 2019; Pierik and Ballaré, 2021). These receptors are categorized according to the wavelength of light they detect: Red light receptors are called phytochromes, five of which are found in tomato (phyA, phyB1, phyB2, phyE, phyF), blue light and UV-A sensing is performed by phototropins (phot1,2), cryptochromes (cry1–3), F-box proteins, and recently a UV-B receptor called UVR-8 has also been discovered (Paik and Huq, 2019; Sanchez et al., 2020). Among photoreceptors, the most abundant is phyB, which is responsible for sensing red light and inducing downstream responses through phytochrome interacting factors (PIF1–8) (Hoang et al., 2019). Red light is known to have significant effects on plant defence mechanisms (Gallé et al., 2021), and phytochromes are known to regulate a wide range of processes such as seed germination, flowering time (Franklin and Quail, 2010), and even fruit ripening and quality (Gong et al., 2021). In addition, red light can delay chlorophyll degradation and the expression of senescence-associated genes (Jiang et al., 2019; Song et al., 2020), which can be critical under high far-red light conditions in the lower leaf levels of plants. Thus the application of red light supplementation/interlighting can be important because the majority of tomato plants in greenhouse cultivation grow under low red/far red light conditions which can enhance the susceptibility of plants to various pathogens such as *Botrytis cinerea* (Ingle et al., 2015). This necrotrophic fungus can infect around 1000 plant species, including tomato, causing significant crop yield losses worldwide by damaging leaves, stems, flowers and fruits of tomato (Elad et al., 2016; Diao et al., 2020). Red light directly affects *Botrytis cinerea* by influencing its growth, development, and virulence. Research suggests that red light exposure can also regulate fungal morphogenesis, stress responses, and even photoreceptor activity (Schumacher, 2017). It was found that, red light significantly reduced *B. cinerea*-induced lesions in leaves of tomato (Hui et al., 2017) and enhanced plant defence in broad bean (Khanam et al., 2005) and strawberry (Meng et al., 2019; 2020; 2023; Lauria et al., 2023). However, despite the beneficial effects of interlighting on CO_2 assimilation and biomass production as well as defence, it can have significant costs in terms of electricity consumption (Maeda et al., 2022; Lanoue et al., 2022). Therefore, shortening the timing and duration of illumination has become a focus of recent research (Zhao et al., 2024). Thus, the application of short periods of red light during the dark period can be an effective way to enhance plant defence responses (Kukri et al., 2024). Namely, nocturnal red light application positively affects plant defence responses during the dark period, when fungal infection occurs (Yu and Fischer, 2019), and enhances defence-related phytohomone-mediated signalling (Yang et al., 2015), antioxidant- and detoxification mechanisms (Pelsőczi et al., 2023). In addition, well-timed red light exposure at night is beneficial for carbohydrate metabolism (Meng et al., 2020) and improves growth and yield under greenhouse conditions (Medelo et al., 2023). Therefore, the use of supplemental red light could provide growers a versatile and efficient tool to optimise plant growth, improve crop quality and maximise productivity while minimising resource use and environmental impact in the future, but some fundamental biological questions remained unanswered.

One of the key questions is how nocturnal light treatment, which disrupts the dark period but enhances plant defence responses, affects normal plant growth, photosynthesis and metabolism. Plants are known to have intrinsic oscillators that track and predict changes in light and dark cycles (Greenham and McClung, 2015), but to be considered a circadian oscillator they must also persist under continuous conditions. Light and dark oscillations can affect a wide range of cellular and organism-wide processes, one of which is carbohydrate metabolism (Venkat and Muneer, 2022). Many scientific articles describe how the circadian rhythm plays a crucial role in synchronising starch and sugar metabolism pathways (Kim et al., 2017). Most plants accumulate starch in their leaves during the photoperiod, which is then mobilised at night to provide the energy and carbon needed for growth (de Barros Dantas et al., 2023). Starch accumulation and degradation show a period of about 24 h that may be under the control of the circadian clock, and indeed plants that are somehow disrupted in their circadian rhythm show differences in biomass production and growth (Chew et al., 2014). There are several points at which the circadian oscillator could intervene in carbohydrate metabolism, for example in the case of SNF1-related kinase 1, after its activation by the β subunit of SnRK1 (AKINβ1), which inhibits sucrose 1,6-bisphosphatase and sucrose phosphate synthase, thus blocking sucrose synthesis (Halford et al., 2003; Kulma et al., 2004). This time, sucrose is exported and consumed by sink tissues (young leaves). The level of AKINβ1 is increased under darkness and by LATE ELONGATED HYPOCOTYL (LHY) (Pokhilko et al., 2014). Another component in this model is the osmo-sensitive kinase OsmK, which is activated during the day and creates a positive feedback loop in the afternoon, activating source supply with trehalose 6-phosphate by sink demand (Pokhilko and Ebenhöh, 2015). At night, OsmK accelerates starch degradation, thereby upregulating sucrose production (Kim et al., 2017). PIF4 and PIF5 also regulate sugar consumption through the circadian clock after being activated by the morning clock proteins LHY/CCA1 or inhibited by the evening complex (EC) (Niwa et al., 2009). Since the circadian clock regulates starch utilisation and photosynthesis, it is necessary to analyse these changes in the different leaf levels during long-term nocturnal red light exposure.

In this paper, we assessed the impact of nocturnal red light treatment on the metabolic changes of young and old leaves at various times of day. We aimed to analyse photosynthetic activity and carbohydrate metabolism, the key parameters for leaf and tomato growth, as well as defence responses against *Botrytis cinerea* infection. We focused and compared the upper and lower leaves of tomato plants with and without red light application. We hypothesise that nocturnal red light exposure enhances the defence of tomatoes against *Botrytis cinerea* by modulating circadian-regulated photosynthesis and carbohydrate metabolism, particularly increasing early-day CO_2 assimilation and sucrose accumulation. These metabolic adjustments, along with red light-induced alterations in defence-related metabolites and cell wall composition, improve pathogen resistance without compromising overall photosynthetic efficiency or the key leaf parameters. This study will contribute to our understanding of the physiological and biochemical effects of red light and could serve sustainable, environmentally friendly approaches to enhancing the resilience of tomato plants and optimising greenhouse farming practices using supplemental or interlighting systems.

2. Materials and methods

2.1. Plant growing and experimental conditions

The experiment was carried out using the wild-type tomato (*Solanum lycopersicum* L.) variety Moneymaker. After germination of the seeds in a thermostat set at 27 °C, the seedlings were placed in perlite in a greenhouse under controlled growth conditions (200 μmol m^{-2} s^{-1} photosynthetic flux density with LED lights, (PPFD; white LED (5700 K) supplemented with FAR LEDs; PSI, Drasov, Czech Republic) 12/12 h light/dark period (6:00–18:00 light period; 18:00–6:00 dark period), 24/22 °C day/night temperature and 55 %−60 % relative humidity (Czékus et al., 2021). After two weeks of growth, the plants were transferred to 500 mL pots for hydroponic culture (Poór et al., 2011), the nutrient solution was changed three times a week and the plants were grown for a further four weeks.

2.2. Red light treatments

Five-week-old plants were treated with red light starting from midnight for half an hour using LEDs (V-TAC; Plovdiv; Bulgaria; 50 mW m^{-2}; 590–660 nm, maximum: 630 nm). Samples were taken at 3:00 a.m., 9:00 a.m., 12:00 pm. and 3:00 pm. after 1 or 3 weeks of red light exposure.

2.3. Measurements of photosynthetic activity

Photosynthetic activity was measured using a Dual-PAM-100 instrument (Heinz-Walz, Effeltrich, Germany) (Klughammer and Schreiber, 1994; Schreiber, 2008). Prior to measurements, leaves were kept in the dark for 15 min to determine the minimum fluorescence yield in the dark-adapted state (F0), after which a saturation pulse (800 ms, 12,000 μmol photons m^{-2} s^{-1}; 635 nm) was used to determine the maximum fluorescence in the dark-adapted state (Fm). In the presence of actinic light (220 μmol(photon) m^{-2} s^{-1}), the light-adapted steady--state fluorescence (F_s) was measured and the maximum fluorescence level (F_m') in the light-adapted state was recorded with saturation pulses. The actinic light was then switched off and the minimum light-adapted fluorescence level (F_0') was measured by illuminating the leaf with far-red light (5 μmol(photon) m^{-2} s^{-1}) for 3 s (Borbély et al., 2025). Using these parameters, we determined the following factors indicating the efficiency of the photosynthetic apparatus: the maximum quantum yield of PSII (variable fluorescence (F_v)/maximum fluorescence (F_m)), the quantum yields of PSI (Y(I)) and PSII (Y(II)), the non-photochemical quenching (NPQ) (Zhang et al., 2014). Three samples were taken from different plants per treatment and three biological replicates were measured. Means ± SE were calculated based on all data from the biological replicates.

Net photosynthetic rate was determined using a portable photosynthesis system (LI-6400, LI-COR, Inc.; Lincoln, NE) as described by Poór et al. (2011). Leaves were illuminated (200 μmol m^{-2} s^{-1} PPFD) in the chamber and data were recorded after 10 min. Conditions were constant throughout the measurements (25 °C, 65±10 % relative humidity and controlled CO_2 supply of 400 μmol mol^{-1}).

2.4. Measurement of starch and sugar contents

100 mg of leaf tissue was homogenised in liquid nitrogen, then 1 mL of 80 % (v/v %) ethanol was added to the powder and boiled at 80 °C for 30 min. The solution was then centrifuged at 2600 × *g* for 10 min and the supernatant was used to determine the starch and sugar content of the samples. Spectrophotometer (Kontron, Milan, Italy) at 630 nm was used for the determination of absorbance of samples (Hansen and Møller, 1975). Three samples were taken from different plants per treatment and three technical replicates were also measured from these samples. All experiments were performed in triplicate. Means ± SE were calculated based on all data from the biological replicates.

2.5. Sample collection and processing for metabolic analysis

After three weeks of red light irradiation, leaf samples for metabolome analysis were collected at 3 a.m. and 3 pm. Among the investigated leaves the older ones were referred as "Lower leaf" and more recently developed leaves as "Upper leaf". We aimed to collect one leaf per plant in the 150–200 mg weight range, if the leaves did not reach the required weight, two leaves were combined. After collection, the samples were placed into 15 mL vials containing zirconia beads and 7 mL of solvent mixture containing 40 % acetonitrile, 40 % methanol and 20 % water (v/v %), then were immediately frozen in liquid nitrogen. The samples were homogenized in 10 cycles for 30 s followed by 30 s of cooling using an automated homogenizer (Precellys Evolution Touch, Bertin Technologies SAS, Montigny-le-Bretonneux, FRANCE). After homogenization, the lysate was centrifuged at 12,000× *g* for 15 min at −5 °C.

For metabolomic analysis, an ultrahigh-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) system was used, consisting of a Thermo Q-Exactive Focus mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Dionex Unimate 3000 UHPLC system. Chromatographic separation was done using an XBridge Premier BEH Amide column (2.1 mm × 150 mm, 2.5 μm; Waters Corporation, Milford, MA, USA). From each samples 5 μL of supernatant was injected into the system. The gradient elution was performed at a flow rate of 400 μL/min, starting with 98 % of solvent A (97.5 % acetonitrile/2.5 % water (v/v %)) gradually changing to 80 % of solvent B (20 % acetonitrile/80 % aqueous (v/v %) phase containing 20 mM ammonium acetate and 20 mM ammonia) over 9 min. Semi-quantitative metabolomic data were collected in MS1 scan mode under negative ionization. Instrument settings included: sheath gas flow at 55 L/min, auxiliary gas at 14 L/min, sweep gas at 4 L/min, a probe heater temperature of 440 °C, a capillary temperature of 280 °C, and a capillary voltage of 2500 V. Scanning was performed in the *m/z* range of 100–800 with an AGC target of 3×10^6 and a maximum injection time of 250 ms. To ensure data quality, pooled quality control (QC) samples were injected periodically throughout the batch in accordance with the recommendations of Broadhurst and co-workers (Broadhurst et al., 2018). For MS2 acquisition, data-dependent MS mode was used with normalized collision energies of 10, 30, and 50 in both positive and negative ionization modes for metabolite confirmation. Five samples were taken from different plants per treatment and three technical replicates were also measured from these samples. Means ± SE were calculated based on all data from the biological replicates.

2.6. Metabolomic data analysis

For metabolite identification and signal extraction, exact masses (considering only deprotonated ions) and retention times from the Mass Spectrometry Metabolite Library were utilized. Peak intensity values were acquired by processing the raw data using TraceFinder™ Software version 5.1 (Thermo Fisher Scientific, Waltham, MA, USA). Metabolite signal intensities were extracted based on a retention time window of ±0.25 min and a mass accuracy tolerance of ±2.5 ppm. The resulting metabolite data were normalized using Probabilistic Quotient Normalization (and Quality Control-based Robust LOESS Signal Correction following the method described by Dunn and coworkers (Dunn et al., 2011).

Metabolites that are normally present in leaf samples were selected for MS2-level confirmation and are listed in the supplement table. Putative identification of these molecules at the MS2 level was carried out using Compound Discoverer 3.2 (Thermo Fisher Scientific, Waltham, MA, USA), with spectral matching performed against the Endogenous Metabolites class of the mzCloud database and our in-house MS2 library based on MSMLS metabolite library. As a result, the metabolites were

identified at the highest achievable confidence level (Alseekh et al., 2021).

2.7. Physiological measurements

For physiological measurements fully developed leaves with 5 leaflets were collected, in case of plants treated for 1 week this meant the 4th or 3rd leaf level, whereas in the case of 3-week-long-treated plants older (1st leaf with 5 leaflets) and younger leaves (2nd leaf from the top) were collected as well. For the determination of fresh and dry weight leaves without any damage were measured immediately after sampling, then dried at 60 °C for 2 days. Photographs of the detached leaves were also taken for the leaf area measurements using a documentation system (Dark hood DH-50 equipped with Canon camera, Biotec-Fischer GmbH; Reiskirchen; Germany) and ImageJ software (https://imagej.net/ij/). The internodes were measured with a ruler above and below of the leaves from which sample collection was done. Nine samples were taken from different plants per treatment. All experiments were performed in triplicate. Means ± SE were calculated based on all data from the biological replicates.

2.8. Preparation of leaf cross sections

Fresh tomato leaf segments were cut from the middle part of the lamina then fixed in 4 % (w/v) paraformaldehyde (PFA) solution according to the protocol of Barroso et al. (2006). After fixation, samples were thoroughly rinsed with distilled water to remove the extra fixative solution and prepare the leaf samples for embedding. Subsequently, the tissues were embedded in 5 % (w/v) agarose following the method of Zelko et al. (2012). The embedded leaf segments were sectioned using a vibratome (Zeiss-Microm, HM650V) into uniform 100 μm thick slices. The leaf cross sections of each treatment were put into PBS (phosphate-buffered saline) buffer then transferred on microscopic slides and covered for the measurements of leaf thickness (Zhu et al., 2021). All microscopic photos were prepared using Zeiss Axiovert 200 M inverted microscope (Carl Zeiss, Jena, Germany) equipped with a digital camera (AxiocamHR, HQ CCD, Carl Zeiss, Jena, Germany) and all measurements were executed by Axiovision Rel. 4.8 software (Carl Zeiss, Jena, Germany). Nine samples were taken from different plants per treatment. All experiments were performed in triplicate. Means ± SE were calculated based on all data from the biological replicates.

2.9. Callose staining

At the selected time points, nine leaf discs were collected from three different plants for callose staining. The leaf discs were cleared by soaking them in 96 % ethanol overnight at room temperature, after which they were washed three times with distilled water. The cleared leaves were then stained with 0.05 % aniline blue in a 0.07 M phosphate buffer solution (pH 8) overnight (Po-Wen et al., 2013). Fluorescence of the aniline blue stain was detected in the samples using a Zeiss Axiowert 200 M fluorescence microscope applying DAPI filter set (exc.: 365 nm, em.: 445/50 nm) (Carl Zeiss AG, Jena, Germany), which was equipped with a high-resolution digital camera (Axiocam HR, Carl Zeiss AG, Jena, Germany). Data were evaluated using AXIOVISION REL. 4.8 (Carl Zeiss, Munich, Germany).

2.10. Tomato-botrytis cinerea pathosystem

For the infection *B. cinerea* SZMC 21,472 hyphal suspension (10^4 colony-forming unit/mL) grown on potato dextrose broth (0.1 × PDB; Sigma–Aldrich) was used. The abaxial side of detached lower and upper leaves were inoculated with 10 μL suspension, by dropping at three points, then kept on dank filter paper under normal day/light conditions at 24/22 °C for 3 days, then stained using Evans blue dye according to Tóth et al. (2020). After staining photographs were taken using documentation system and the stained areas (indicating cell death in the case of Evans blue dye) were analysed using ImageJ software. Three samples were taken from different plants per treatment. All experiments were performed in triplicate. Means ± SE were calculated based on all data from the biological replicates.

2.11. Statistical analysis

The experiments were conducted from different sowings at least three times, from each sampling we collected three replicates. Results are expressed as mean ± *S*.E. The effects of red light treatments and differences between older and younger leaves were analysed by one-way ANOVA, supplemented by post hoc pairwise comparisons using Duncan's multiple range test. All statistical analyses were performed using SigmaPlot 11 software (Systat Software Inc., San Jose, California, USA). Means marked with different letters are significantly different at $p \leq 0.05$.

3. Results

First, the short- and long-term effects of 30 min-long of red light application at night on the photosynthetic activity of tomato plants were investigated. As photosynthesis is highly dependent on the time of day, three time points were chosen for analysis: 9:00 in the morning, 12:00 in the middle of the light period and 15:00 in the afternoon. After one week of red light exposure at night, no significant changes were found in the chlorophyll fluorescence parameters of the developed leaves (Table 1).

After three weeks of red light exposure at midnight, lower, old and upper developed leaves were examined and it was found that red light increased the yield of both photosystems [Y(I) and Y(II)] in upper leaves at 9:00 a.m. and in old lower leaves at noon (Table 2). Interestingly, no significant changes in yield parameters were observed in either leaf level in the afternoon (Table 2). At the same time, Y(I) and Y(II) were basically lower in the lower leaves and the non-photochemical quenching (NPQ) was lower in the upper leaves as compared to each other from 12:00 (Table 2). Neither the maximum quantum yield of PSII (F_v/F_m) nor NPQ changed with the treatments at any time points (Table 2).

Based on measurements of net photosynthetic rate at different times of the day, red light application at night significantly increased CO_2 assimilation at 9:00 and 12:00 after one week (Fig. 1A). A similar trend was found after three weeks of red light treatment at night in the upper young leaves, where the net photosynthetic rate was higher at all-time points compared to untreated controls and old leaves (Fig. 1B). At the same time, three weeks of red light at night also increased by 41 % the generally lower CO_2 assimilation in the older lower leaves at 9:00 in the morning (Fig. 1B).

Based on the analysis of the photosynthetic products, the starch content was lower (24 %) in the red light-treated leaves at 3:00 compared to the control (Fig. 2A). At the same time, increased starch levels were measured at 12:00 (64 %) and 15:00 (48 %) after one week of nocturnal red light treatment (Fig. 2A). Similarly, starch levels were decreased by red light exposure at 3:00 after three weeks in both investigated leaf levels of tomato (Fig. 2B). A similar trend was found after three weeks in the case of later measurements, nocturnal red light treatments increased starch content in both leaf levels at 12:00 and 15:00, with the highest values measured in the upper leaves (Fig. 2B).

In contrast to starch, sucrose levels were increased by night time red light application at all times recorded, including at dawn (Fig. 3A). Similarly, sucrose content was higher at 3:00 a.m. in both leaf levels examined after the three weeks of nocturnal red light treatments (Fig. 3B). Sucrose content was generally higher in upper leaves compared to lower leaves, and red light application also increased sucrose levels in lower leaves at 12:00 (15 %) and 15:00 (20 %) and in upper leaves at 9:00 (9 %) and 15:00 (14 %) (Fig. 3B).

Based on the time-dependent changes in photosynthesis and photosynthetic primer products in different leaf levels of tomato plants, a

Table 1
Effects of red light exposure on chlorophyll fluorescence parameters of tomato plants leaves after one week.

1. week	Treatments					
Parameters	9:00 Control	9:00 Red light	12:00 Control	12:00 Red light	15:00 Control	15:00 Red light
F_v/F_m	0.81+0.00[a]	0.80+0.00[a]	0.80+0.00[a]	0.81+0.00[a]	0.80+0.00[a]	0.80+0.00[a]
Y(I)	0.78+0.02[a]	0.79+0.01[a]	0.80+0.01[a]	0.76+0.02[a]	0.76+0.02[a]	0.75+0.01[a]
Y(II)	0.65+0.01[a]	0.63+0.01[a]	0.62+0.01[a]	0.60+0.02[a]	0.55+0.02[a]	0.55+0.01[a]
NPQ	0.19+0.04[a]	0.18+0.02[a]	0.20+0.02[a]	0.25+0.03[a]	0.42+0.09[a]	0.42+0.04[a]

Changes in chlorophyll fluorescence parameters of tomato plant leaves at 9:00, 12:00 and 15:00 after 30-min-long nocturnal red light treatments applied for one week (mean + SE, $n = 9$). Data with different letters indicate significant differences at the $p \leq 0.05$ level (Duncan's multiple comparisons) at each time point.

metabolomic analysis was performed after three weeks of nocturnal red light exposure to identify the major metabolites regulated by red light in a time-dependent manner in lower and upper leaves of tomato plants (Fig. 4). The levels of four metabolites, 4-guanidinobutanate, fucose, N-methylglutamate and quinate were elevated by the nocturnal red light treatment in the upper leaves both at 3:00 a.m. and at 3:00 pm. (Table 3). In contrast, the levels of four metabolites were significantly decreased: myo-inositol, N-acetylaspartate, nicotinate, trigonelline (Table 3). Interestingly, the levels of alanine and dihydroxyacetone phosphate changed inversely, both levels were increased by red light at 3:00 a.m., but both decreased in the afternoon (Table 3). At the same time, the level of other important metabolites changed in a diurnal manner after exposure to red light, such as mevalonate and phenylalanine contents increased in the upper leaves, but 1-aminocyclopropane-1-carboxylate, proline, and sorbitol decreased at 3:00 a.m. (Table 3). In the afternoon, serotonine level increased whereas methionine and trigonelline decreased (Table 3). In the lower leaves, only quinate and dihydroorotate increased significantly whereas guanosine and lysine decreased independently of the time of day (Table 3). Methionine levels changed inversely, decreasing at 3:00 a.m. but increasing in the afternoon in the lower leaves (Table 3). At 3:00 a.m., 3-dehydroshikimate, alanine, aspartate and mevalonate levels increased, but proline decreased in the lower leaves (Table 3). In the afternoon, methionine, fructose and glucose-6-phosphate levels, among others, increased significantly, but trigonelline, lysine decreased in the lower leaves following red light at night for three weeks (Table 3).

Metabolic profiling was carried out after nocturnal red light treatment, and the leaf fresh and dry mass and growth of the tomato plants were analysed (Table 4). After one week, red light application at midnight resulted in a significant increase in internode length but a decrease in leaf thickness (Table 4). At the same time, nocturnal red light treatment significantly decreased the fresh mass of both the upper and lower leaves, but did not affect their dry mass (Table 4). Additionally, leaf area decreased and leaf thickness reduced in the lower leaves, but increased in the upper leaves (Table 4). The internode length increased, but the stem diameter remained unchanged following nocturnal red light exposure (Table 4).

Not only nocturnal red light pre-treatment influenced the level of certain metabolites and growth/mass of leaves, it also affected cell wall composition, such as callose content (Fig. 6). Even after just one week, nocturnal red light treatment significantly increased callose content (15 %) in the leaves compared to the control (Table 5). In addition, three weeks of nocturnal red light treatment also increased (10 %) callose in the lower leaves, but decreased it in the upper leaves (Table 5).

Fig. 5

The effects of red light exposure at night on plant defence responses were investigated following infection of lower and upper tomato plant leaves with *B. cinerea* (Fig. 6). It was found that one week of nocturnal red light application significantly inhibited the infection in tomato plant leaves (Table 6). This significant effect of was also evident after three weeks of nocturnal red light application (Fig. 6) and it was more pronounced in the lower leaves than in the upper leaves (Table 6).

4. Discussion

Light intensity and quality are key importance in the regulation of many physiological and biochemical processes in plants such as photosynthesis and metabolism (Borbély et al., 2022). The importance of light quality and specific light conditions is receiving increasing attention, with a growing number of scientific studies addressing the physiological and molecular biological effects of specific wavelengths of light (Vitale et al., 2020; Gallé et al., 2021; Pashkovskiy et al., 2022; Vitale et al., 2022; Vereshchagin et al., 2024). However, many questions remain unanswered, such as the effects of specific wavelengths of light on different levels of leaves (Paponov et al., 2020). Exploring these effects offers a more comprehensive understanding of how to optimize cultivation practices such as interlighting for tomato farming (Appolloni et al., 2022), emphasizing their implications for growth, biomass production, photosynthetic efficiency, metabolism and defence mechanism of leaves. Therefore, the aim of this study was also to monitor metabolic changes in lower older and upper young leaves of tomato to help develop new greenhouse procedures. Among the various agricultural practices, it is well known that night break with light can enhance the photosynthetic capacities and yield of tomato (Tewolde et al., 2016), but many fundamental biological changes remained unclear in this process. In this work firstly was investigated the changes in photosynthetic activity and carbohydrate metabolism after short-term red light illumination at night. In addition, changes in the different leaf levels (upper, young and lower, old) following red light treatments were also monitored, which may also contribute to more successful carbohydrate metabolism and defence against fungal infection, especially in the old, lower leaves. This can be crucial, because photosynthetic activity and metabolism of lower leaves are significantly reduced as compared to younger, upper leaves of the intact plants (Tari et al., 2014). In addition, higher far-red light exposition and dark period can promote the signal of competition, as well as help the fungal pathogens e.g. due to alleviation of jasmonic acid (JA)- and SA-dependent signalling pathways (Ballaré and Pierik, 2017; Gallé et al., 2021). Therefore, long-term physiological and biochemical changes induced by red light at 3:00 a.m. and in the afternoon were compared using metabolomics data to elucidate the diurnal red light-induced defence mechanisms in tomato. It can be crucial, because some beneficial effects of nocturnal red light application can be dependent on the day/night time as carbohydrate metabolism is highly dependent on the circadian rhythm of plants (Graf et al., 2010; Venkat and Muneer, 2022).

First, three time points of the daytime were chosen for analysis of photosynthetic efficiency: at 9:00 in the morning, at 12:00 in the middle of the light period and at 15:00 in the afternoon. Based on the time curve analysis, after one week of nocturnal red light treatments for 30 min resulted in no significant changes in the chlorophyll fluorescence parameters of mature leaves of tomato plants. At the same time, after the three-weeks-long nocturnal red light application, Y(I) and Y(II) was enhanced in upper leaves at 9:00 a.m. and in old lower leaves at noon. Others also found that red light application enhanced Y(II) in spinach leaves (Vitale et al., 2020). At the same time, this is the first time, when not only the beneficial effects of red light on PSII were reported (Yang et al., 2022) in both leaf levels (Papanov et al., 2020), but also its

Table 2
Effects of red light treatment on chlorophyll fluorescence parameters in leaves of tomato plants after three weeks.

3. week	Treatments											
Parameters	Lower leaves		Upper leaves		Lower leaves		Upper leaves		Lower leaves		Upper leaves	
	9:00 Control	9:00 Red light	9:00 Control	9:00 Red light	12:00 Control	12:00 Red light	12:00 Control	12:00 Red light	15:00 Control	15:00 Red light	15:00 Control	15:00 Red light
F_v/F_m	$0.81+0.00^{a}$	$0.80+0.00^{a}$	$0.81+0.00^{a}$	$0.81+0.00^{a}$	$0.80+0.00^{a}$	$0.81+0.01^{a}$	$0.81+0.00^{a}$	$0.80+0.00^{a}$	$0.80+0.00^{a}$	$0.80+0.00^{a}$	$0.81+0.00^{a}$	$0.81+0.00^{a}$
Y(I)	$0.74+0.00^{c}$	$0.73+0.02^{c}$	$0.81+0.02^{b}$	$0.84+0.00^{a}$	$0.65+0.01^{c}$	$0.73+0.03^{b}$	$0.82+0.00^{a}$	$0.80+0.01^{a}$	$0.71+0.01^{b}$	$0.70+0.02^{b}$	$0.82+0.00^{a}$	$0.80+0.01^{a}$
Y(II)	$0.56+0.01^{c}$	$0.56+0.03^{c}$	$0.62+0.03^{b}$	$0.69+0.00^{a}$	$0.49+0.01^{c}$	$0.55+0.04^{b}$	$0.66+0.01^{a}$	$0.63+0.01^{a}$	$0.54+0.01^{b}$	$0.53+0.03^{b}$	$0.65+0.01^{a}$	$0.64+0.01^{a}$
NPQ	$0.31+0.06^{a}$	$0.21+0.06^{a}$	$0.26+0.10^{ab}$	$0.17+0.01^{b}$	$0.51+0.10^{a}$	$0.49+0.13^{a}$	$0.17+0.01^{b}$	$0.18+0.02^{b}$	$0.38+0.04^{a}$	$0.55+0.11^{a}$	$0.25+0.04^{b}$	$0.28+0.03^{b}$

Changes in chlorophyll fluorescence parameters of tomato plant leaves at 9:00, 12:00 and 15:00 after 30-min-long nocturnal red light treatments applied for three weeks (mean + SE, $n = 9$). Data with different letters indicate significant differences at the $p \leq 0.05$ level (Duncan's multiple comparisons) at each time point.

contribution to improve PSI activity which changes are highly dependent on the daytime. Therefore, it can be concluded that applying red light at night has a beneficial effect on both photosystems and both leaf levels of tomato plants.

At the same time, not only the light reactions of both photosystems are improved by daytime-dependent manner and independently from leaf levels by nocturnal red light exposure, but photosynthetic CO_2 assimilation was also increased already after one-week-long red light exposure at night in tomato leaves, similarly to the changes in Y(I) and Y (II). Moreover, the net photosynthetic rate was also higher at all investigated time points upon nocturnal red light treatments compared to untreated controls. In addition, the basically lower CO_2 assimilation in the older leaves as compared to the upper leaves was improved by the light treatments in the morning. However, CO_2 assimilation induced by red light was higher in the upper leaves in the morning than in the old leaves. These results confirmed the beneficial effects of using supplemental red light in boosting the photosynthetic rate of greenhouse plants (Vitale et al., 2020; Maeda et al., 2022; Lanoue et al., 2022; Utasi et al., 2023) but also serve the first evidence related to daytime- and leaf-level dependent effects of a special light spectra application for a short time.

Besides the analysis of photosynthetic activity, the products of this process was analysed. It is well known that carbohydrate metabolism and starch/sugar accumulation during the day and decomposition at dawn are highly circadian-regulated processes (Graf et al., 2010; Venkat and Muneer, 2022). In addition, it is also well known that, there are differences in the carbohydrate metabolism between young and old leaves because young leaves are actively photosynthesizing and are considered source organs, producing more carbohydrates (Baxter et al., 2003; Zhu et al., 2018; Zhao et al., 2025) but the potential beneficial effects of red light application comparing two different leaf levels remained unknown. Thus we added a new sampling time, at 3:00 a.m. at dawn to our measurements to check the leaf level-dependent changes (Kukri et al., 2024). Based on the results, starch content was significantly lower in the red light-treated leaves at this time point compared to the control suggesting the inhibition of starch deposition, while sugar content was higher in both leaf levels examined after three weeks of nocturnal red light treatments. At the same time, significantly higher starch accumulation was found in both leaf levels at 12:00 pm. and 15:00 pm. in the afternoon after one and three weeks of the nocturnal red light treatments, with the highest values measured in the upper leaves. However, sucrose content was also higher in these time points in both leaf levels, especially in the upper leaves after exposure to red light in both sampling time. These results are consistent with further findings indicating increased carbohydrate metabolism in the upper leaves (Baxter et al., 2003) as well as the enhanced photosynthetic activity in this leaf level under red light illumination. At the same time, this carbohydrate plays not only key role in the growth and development of plants, as well as in the determination of biomass and yield production (Tang et al., 2022), but is also crucial in the effective defence reactions of plants regulated by defence-related phytohormones such as JA (Machado et al., 2017).

Production of the primary products of photosynthesis are dependent on the time of day and leaf level, which suggests important changes in different leaf metabolites after nocturnal red light application. Thus, metabolomics profiling was performed to reveal the effects of nocturnal red light treatment on tomato leaves. At the same time, the daytime- and leaf-level-dependent effects of red light were analysed. Thus, samples were taken from the lower and upper leaves at dawn (at 3:00 a.m.), three hours after red light exposure, as well as in the afternoon (at 15:00 pm.), 12 h later. Based on the results of the metabolic profile, metabolic changes of young tomato leaves were more sensitive to nocturnal red light treatment at dawn compared to lower leaves (27 vs. 14 metabolites changed), but in the afternoon, the metabolic profile of old leaves changed more compared to upper leaves (26 vs. 24 metabolites) confirming the significant leaf-level and time dependent effects of red light application. Interestingly, a similar number of metabolites increased and

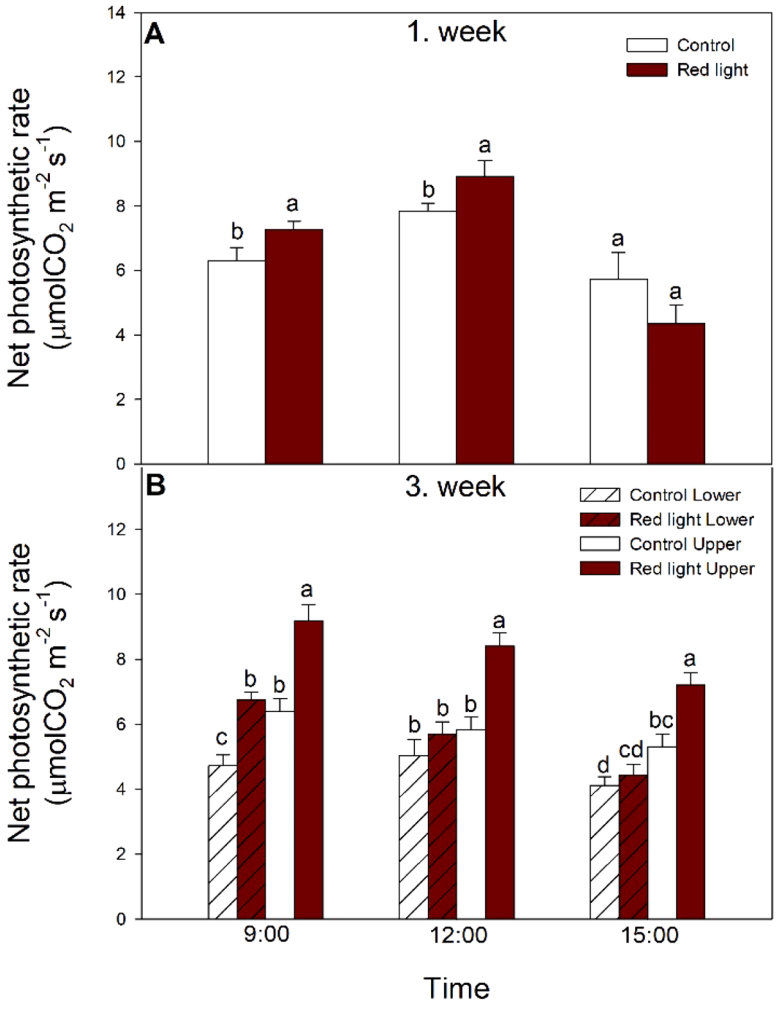

Fig. 1. Changes in the net photosynthetic rate of tomato plant leaves at 9:00, 12:00 and 15:00 after 30-min-long nocturnal red light treatments applied for one (A) and three weeks (B) (mean + SE, $n = 9$). Data with different letters indicate significant differences at the $p \leq 0.05$ level (Duncan's multiple comparisons) at each time point.

decreased in both leaf levels at each time point. Based on the analysis of these molecules, 4-guanidinobutanate, fucose, N-methylglutamate and quinate levels were elevated by the nocturnal red light treatment in the upper leaves both at dawn and in the afternoon. Based on a recent finding, the organic acid 4-guanidinobutanate plays crucial role in the flooding stress tolerance in watermelon (He et al., 2023). Fucose also plays a role in the abiotic defence responses of plants, as its levels increased in the presence of arbuscular mycorrhizal fungi, which boosts the drought tolerance of trifoliate orange (Zheng et al., 2024). It also contributes to drought tolerance in *Ilex paraguariensis* (Acevedo et al., 2019) and *Vigna unguiculata* (Gomes et al., 2020), as well as salt tolerance in *Solanum melongena* (Harsha et al., 2024). The accumulation of the glutamic acid derivative N-methyl-L-glutamic acid also plays a role in defence responses upon various stress stimuli (Zhang et al., 2018; Mu et al., 2022), as well as in the nitrogen metabolism of plants (Tian et al., 2020). Quinate is involved in the shikimate pathway, which is responsible for the biosynthesis of aromatic amino acids such as tryptophan, tyrosine and phenylalanine. It is also a ubiquitous building block in plants for the production of antimicrobial phytoalexins such as brassinin in *Brassica* species whose antifungal activity has been well documented (N'guyen et al., 2021; Shelden et al., 2016). It is known that quinate plays a role in salt stress tolerance (Chang et al., 2019). In addition, quinate hydroxycinnamoyl transferase which uses quinate as a substrate, is considered an essential enzyme for regulating lignin biosynthesis and composition as part of defence, which depends on light intensity and quality (Clé et al., 2008; Alimohammadi et al., 2012). However, the levels of four metabolites decreased significantly when red light was applied at night: myo-inositol, N-acetylaspartate, nicotinate and trigonelline. Consumption of myo-inositol can contribute to changes in membrane structure, as well as acting as a signal for sugar metabolism and reducing oxidative stress in tomato (Valluru and Van den Ende, 2011). N-acetylaspartate, a derivative of aspartic acid, can form a conjugate with indole-3-acetic acid to influence plant growth (Sitbon et al., 1993). A decrease in nicotinate can indicate increased nicotinamide adenine dinucleotide synthesis as part of the salvage pathway, or the formation of nicotinic acid N-glucoside or trigonelline (N-methylnicotinic acid) (Ashihara et al., 2012). At the same time, the level of trigonelline also decreased during the day in tomato leaves. Trigonelline is a well-known alkaloid in tomato (Tyihak et al., 1988), and can act as an antifungal agent against *Alternaria alternata*, for example (Muñoz Hoyos et al., 2024). Based on the results, the level of other metabolites changed differently with red light treatment in a time-of-day-dependend manner: alanine and dihydroxyacetone phosphate levels increased at 3:00 a.m., but both decreased in the afternoon. These compounds play a role in stress tolerance (López-Gresa et al., 2010; Li et al., 2013) and the systemic resistance of plants (Nandi et al., 2004). Furthermore, levels of mevalonate and phenylalanine increased in the upper leaves, while levels of 1-aminocyclopropane-1-carboxylate, proline and sorbitol decreased at 3:00 a.m. The accumulation of mevalonate suggests the synthesis of defence-related volatile terpenoid compounds (Vickers

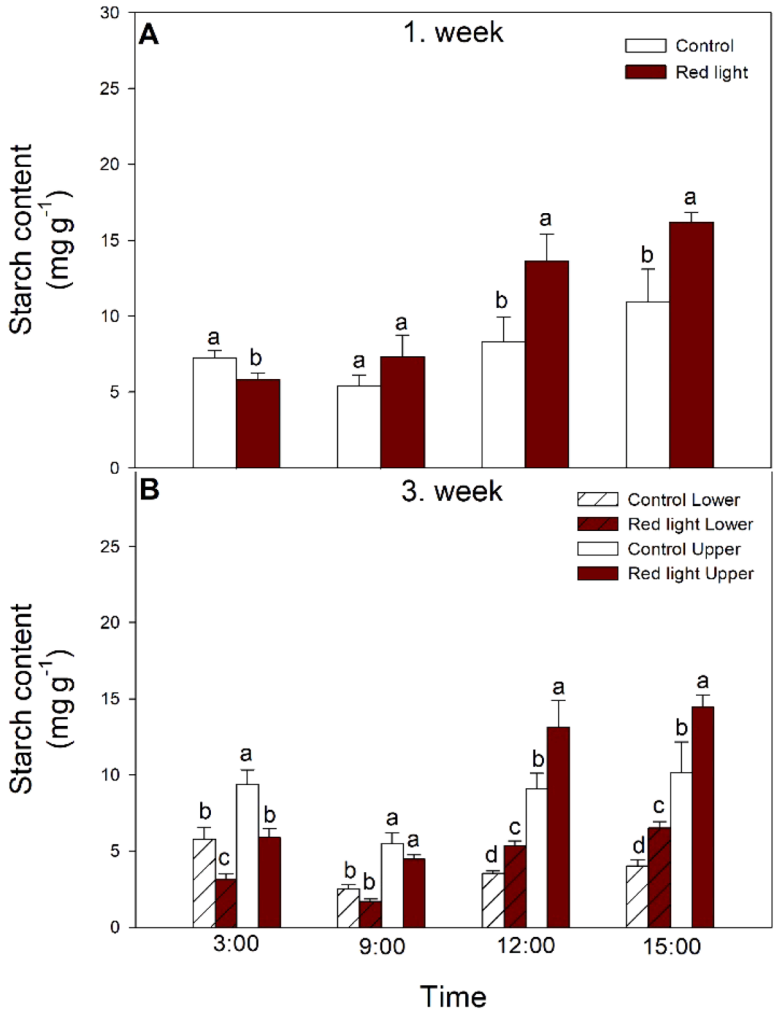

Fig. 2. Changes in the starch content of tomato plant leaves at 3:00, 9:00, 12:00 and 15:00 after 30-min-long nocturnal red light treatments applied for one (A) and three weeks (B) (mean + SE, $n = 9$). Data with different letters indicate significant differences at the $p \leq 0.05$ level (Duncan's multiple comparisons) at each time point.

et al., 2014), whereas the consumption of precursors of the defence-related phytohormone ethylene can enhance its biosynthesis (Kendrick and Chang, 2008). Conversely, the accumulation of phenylalanine may indicate the activation of another defence-related phytohormone, the salicylic acid (Shine et al., 2016), which is regulated by red light (Yang et al., 2023). Interestingly, the serotonin content of the upper leaves increased in the afternoon following nocturnal red light exposure. Both serotonin and salicylic acid are derived from chorismate (Maeda and Dudareva, 2012). It has recently been shown that serotonin plays a role in removing reactive oxygen species and protecting plant tissues from fungal infections (Sun et al., 2025). In contrast to the upper leaves, only quinate and dihydroorotate increased significantly in the lower leaves, while guanosine and lysine decreased independently of the time of day. As the substrate of dihydroorotate dehydrogenase, dihydroorotate can play a role in basal defence against pathogen infection in plants (Rolly et al., 2020). Additionally, lysine degradation can influence plant immunity (Ding et al., 2016). At 3:00 a.m., the levels of 3-dehydroshikimate, alanine, aspartate, and mevalonate increased, while methionine and proline decreased in the young leaves, demonstrating significant red light-induced changes in amino acid metabolism in old leaves. In the afternoon, methionine, fructose, glucose-6-phosphate and others levels increased significantly, while trigonelline and lysine decreased. Changes in carbohydrates acting as priming agents in old leaves can contribute to a more active defence reaction against fungal pathogens (Morkunas and Ratajczak, 2014). It can therefore be concluded that these significant time-of-day- and leaf-level-dependent changes in these tomato metabolites can contribute to the priming effects of red light applied at night. At the same time, further research using mutants and transgenic plants is needed to prove their red light-dependent role in the defence reaction of tomato.

Significant changes in defence-related metabolites can influence plant growth and development (Cipollini et al., 2018). Only one week of nocturnal red light application resulted in a significant increase in internode length, a change that was also observed after three weeks, despite no changes to the stem diameter. Red light was found to positively influence the height of tomatoes in a temperature-dependent manner, as opposed to other light intensities, which are associated with enhanced photosynthetic activity (Kong et al., 2021) and sugar accumulation (Thwe et al., 2020). A decrease in leaf area thickness, especially in lower leaves, can indicate changes in energy allocation through shoot growth, photosynthesis, and the synthesis of defence-related metabolites (Kim et al., 2019; Vitale et al., 2020). Moreover, the callose content in these leaves increased significantly, which can be the reason of the energy allocation and the enhanced carbohydrate metabolism upon nocturnal red light exposure (Li et al., 2023). These processes are regulated by the interaction between three phytohormones: gibberellins, auxin, and ethylene, which are affected by red light (Kurepin and Pharis, 2014). These significant changes at different leaf levels also highlight the potential for the timed and planned application of red light as an interlighting system in future.

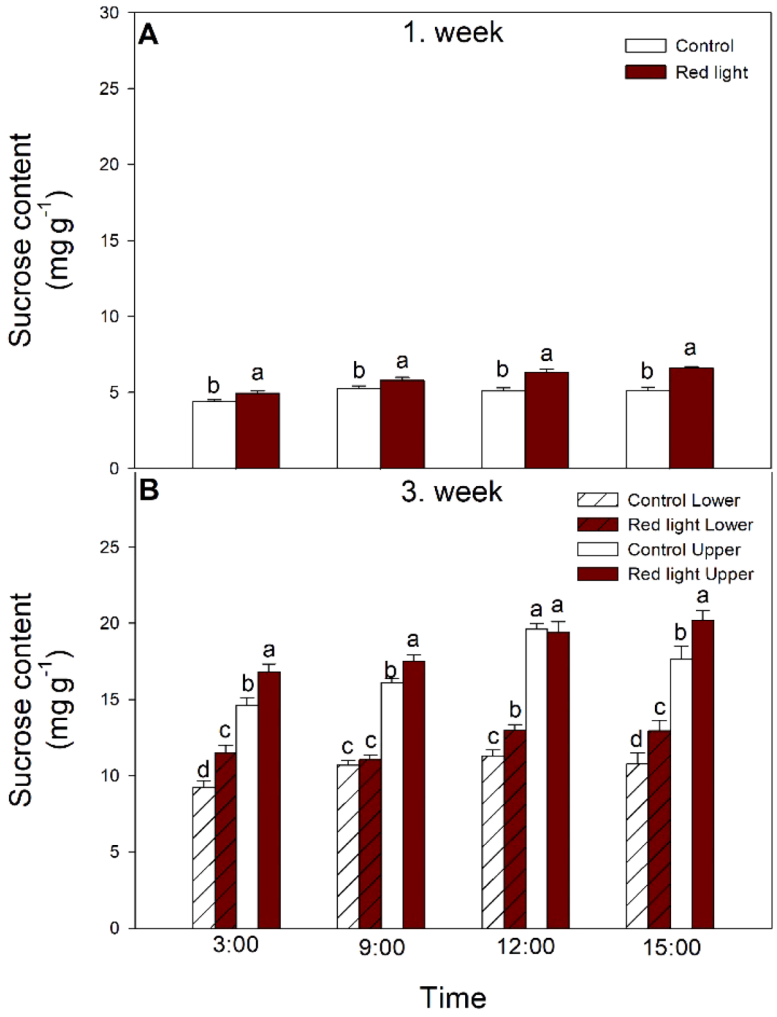

Fig. 3. Changes in the starch content of tomato plant leaves at 3:00, 9:00, 12:00 and 15:00 after 30-min-long nocturnal red light treatments applied for one (A) and three weeks (B) (mean + SE, $n = 9$). Data with different letters indicate significant differences at the $p \leq 0.05$ level (Duncan's multiple comparisons) at each time point.

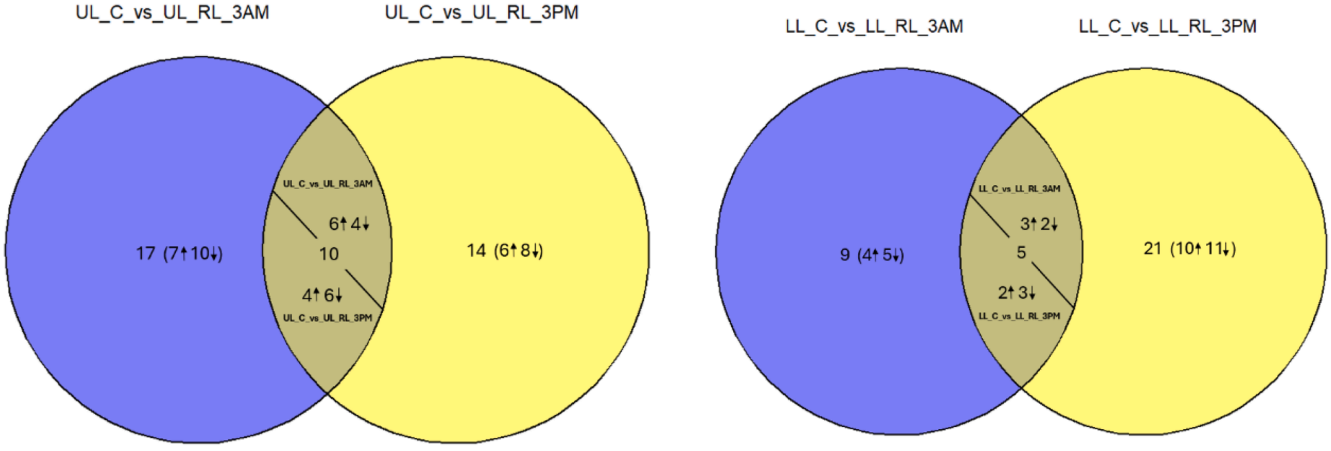

Fig. 4. The results of the metabolome analysis revealed significant changes following 30-min-long nocturnal red light treatments at midnight on the upper and lower leaves of tomato plants, as measured at dawn (3:00 a.m.) and in the afternoon (3:00 pm.). The Venn diagram shows the overlap of the number of metabolites in the five comparisons: C (Control); LL (Lower leaves); RL (Red light treatment); and UL (Upper leaves); up arrow (increased metabolites); down arrow (decreased metabolites).

Table 3
The effects of nocturnal red light treatment on the changes in certain metabolites' level of upper and lower tomato plant leaves at dawn (3:00 a.m.) and in the afternoon (3:00 pm.).

	Upper leaves					Lower leaves			
Daytime	Metabolites	*P*-value	Comparison	Changes	Daytime	Metabolites	*P*-value	Comparison	Changes
At dawn 3:00 a.m.	4-GUANIDINOBUTANOATE	0.039334	UL_RL_vs_UL_C_3AM	Increased	At dawn 3:00 a.m.	3-DEHYDROSHIKIMATE	0.004086	LL_RL_vs_LL_C_3AM	Increased
	ALANINE	0.016091	UL_RL_vs_UL_C_3AM	Increased		ALANINE	0.004872	LL_RL_vs_LL_C_3AM	Increased
	CITRULLINE	0.039789	UL_RL_vs_UL_C_3AM	Increased		ASPARTATE	0.010165	LL_RL_vs_LL_C_3AM	Increased
	D-RIBOSE_5-PHOSPHATE	0.012229	UL_RL_vs_UL_C_3AM	Increased		DIHYDROOROTATE	0.009641	LL_RL_vs_LL_C_3AM	Increased
	DIHYDROXYACETONE_PHOSPHATE	0.009288	UL_RL_vs_UL_C_3AM	Increased		MEVALONATE	0.035445	LL_RL_vs_LL_C_3AM	Increased
	FUCOSE	0.00101	UL_RL_vs_UL_C_3AM	Increased		QUINATE	0.043571	LL_RL_vs_LL_C_3AM	Increased
	GLYCEROL_3-PHOSPHATE	0.013488	UL_RL_vs_UL_C_3AM	Increased		GUANOSINE	0.003745	LL_RL_vs_LL_C_3AM	Decreased
	METHYL_GALACTOSIDE	0.01031	UL_RL_vs_UL_C_3AM	Increased		KYNURENINE	0.008919	LL_RL_vs_LL_C_3AM	Decreased
	MEVALONATE	0.041612	UL_RL_vs_UL_C_3AM	Increased		LYSINE	0.007495	LL_RL_vs_LL_C_3AM	Decreased
	N-FORMYL-L-METHIONINE	0.027414	UL_RL_vs_UL_C_3AM	Increased		METHIONINE	0.030819	LL_RL_vs_LL_C_3AM	Decreased
	N-METHYLGLUTAMATE	0.014973	UL_RL_vs_UL_C_3AM	Increased		MYOINOSITOL	0.049592	LL_RL_vs_LL_C_3AM	Decreased
	PHENYLALANINE	0.006668	UL_RL_vs_UL_C_3AM	Increased		NICOTINATE	0.01616	LL_RL_vs_LL_C_3AM	Decreased
	QUINATE	0.009529	UL_RL_vs_UL_C_3AM	Increased		PHOSPHOENOLPYRUVATE	0.025688	LL_RL_vs_LL_C_3AM	Decreased
	1-AMINOCYCLOPROPANECARBOXYLATE	0.010058	UL_RL_vs_UL_C_3AM	Decreased		PROLINE	0.004516	LL_RL_vs_LL_C_3AM	Decreased
	ERYTHRITOL	0.006579	UL_RL_vs_UL_C_3AM	Decreased					
	GLUTAMATE	0.022593	UL_RL_vs_UL_C_3AM	Decreased					
	INOSINE_5′-DIPHOSPHATE	0.012785	UL_RL_vs_UL_C_3AM	Decreased					
	MYOINOSITOL	0.027322	UL_RL_vs_UL_C_3AM	Decreased					
	N-ACETYLASPARTATE	0.020305	UL_RL_vs_UL_C_3AM	Decreased					
	NICOTINATE	0.007496	UL_RL_vs_UL_C_3AM	Decreased					
	PHOSPHOENOLPYRUVATE	0.015629	UL_RL_vs_UL_C_3AM	Decreased					
	PROLINE	0.027041	UL_RL_vs_UL_C_3AM	Decreased					
	RHAMNOSE	0.012312	UL_RL_vs_UL_C_3AM	Decreased					
	SORBITOL/MANNITOL	0.001435	UL_RL_vs_UL_C_3AM	Decreased					
	TRIGONELLINE	0.000713	UL_RL_vs_UL_C_3AM	Decreased					
	XANTHINE	0.020579	UL_RL_vs_UL_C_3AM	Decreased					
	XANTHURENATE	0.034721	UL_RL_vs_UL_C_3AM	Decreased					
Afternoon 3:00 pm.	4-GUANIDINOBUTANOATE	0.031652	UL_RL_vs_UL_C_3PM	Increased	Afternoon 3:00 pm.	2-AMINOISOBUTYRATE/AMINOISOBUTAN	0.006033	LL_RL_vs_LL_C_3PM	Increased
	ADENOSINE-MONOPHOSPHATE	0.034758	UL_RL_vs_UL_C_3PM	Increased		ADENOSINE-MONOPHOSPHATE	0.0004	LL_RL_vs_LL_C_3PM	Increased
	CYTIDINE_MONOPHOSPHATE	0.002389	UL_RL_vs_UL_C_3PM	Increased		BETA-NICOTINAMIDE_ADENINE_DINUCLE	0.04987	LL_RL_vs_LL_C_3PM	Increased
	DIHYDROOROTATE	0.046341	UL_RL_vs_UL_C_3PM	Increased		CYTIDINE_MONOPHOSPHATE	0.000102	LL_RL_vs_LL_C_3PM	Increased
	FUCOSE	0.031452	UL_RL_vs_UL_C_3PM	Increased		D-RIBOSE_5-PHOSPHATE	0.003934	LL_RL_vs_LL_C_3PM	Increased
	KYNURENINE	0.044814	UL_RL_vs_UL_C_3PM	Increased		DIHYDROOROTATE	0.023542	LL_RL_vs_LL_C_3PM	Increased
	N-METHYLGLUTAMATE	0.003549	UL_RL_vs_UL_C_3PM	Increased		FRUCTOSE	0.047969	LL_RL_vs_LL_C_3PM	Increased
	QUINATE	0.006303	UL_RL_vs_UL_C_3PM	Increased		GLUCOSE_6-PHOSPHATE	0.044993	LL_RL_vs_LL_C_3PM	Increased
	SEROTONIN	0.032341	UL_RL_vs_UL_C_3PM	Increased		INOSINE_5′-DIPHOSPHATE	6.22E-05	LL_RL_vs_LL_C_3PM	Increased
	TYRAMINE	0.024411	UL_RL_vs_UL_C_3PM	Increased		METHIONINE	0.041458	LL_RL_vs_LL_C_3PM	Increased
	ALANINE	0.028149	UL_RL_vs_UL_C_3PM	Decreased		N-ACETYLALANINE	0.03407	LL_RL_vs_LL_C_3PM	Increased
	CITRATE	0.008642	UL_RL_vs_UL_C_3PM	Decreased		QUINATE	0.020798	LL_RL_vs_LL_C_3PM	Increased
	DIHYDROXYACETONE_PHOSPHATE	0.012708	UL_RL_vs_UL_C_3PM	Decreased		STACHYOSE	0.030897	LL_RL_vs_LL_C_3PM	Increased
	GLYCERALDEHYDE_3-PHOSPHATE	0.042884	UL_RL_vs_UL_C_3PM	Decreased		ALLANTOIN	0.0023	LL_RL_vs_LL_C_3PM	Decreased
	MALATE	0.023786	UL_RL_vs_UL_C_3PM	Decreased		CITRATE	0.002262	LL_RL_vs_LL_C_3PM	Decreased
	METHIONINE	0.020596	UL_RL_vs_UL_C_3PM	Decreased		CYTIDINE	2.14E-06	LL_RL_vs_LL_C_3PM	Decreased
	MYOINOSITOL	0.02153	UL_RL_vs_UL_C_3PM	Decreased		GUANOSINE	2.39E-05	LL_RL_vs_LL_C_3PM	Decreased
	N-ACETYLASPARTATE	0.004622	UL_RL_vs_UL_C_3PM	Decreased		LYSINE	0.014188	LL_RL_vs_LL_C_3PM	Decreased
	N-ACETYLLEUCINE	0.002719	UL_RL_vs_UL_C_3PM	Decreased		LYXOSE	0.027923	LL_RL_vs_LL_C_3PM	Decreased
	N-ACETYLMETHIONINE	0.013183	UL_RL_vs_UL_C_3PM	Decreased		METHYL_GALACTOSIDE	0.04246	LL_RL_vs_LL_C_3PM	Decreased
	NICOTINATE	0.002621	UL_RL_vs_UL_C_3PM	Decreased		SUBERATE	0.033284	LL_RL_vs_LL_C_3PM	Decreased
	PIPECOLATE	0.049733	UL_RL_vs_UL_C_3PM	Decreased		TRANS-ACONITATE	0.004362	LL_RL_vs_LL_C_3PM	Decreased
	TRANS-ACONITATE	0.032888	UL_RL_vs_UL_C_3PM	Decreased		TRIGONELLINE	0.003938	LL_RL_vs_LL_C_3PM	Decreased
	TRIGONELLINE	0.002502	UL_RL_vs_UL_C_3PM	Decreased		URATE	0.0363	LL_RL_vs_LL_C_3PM	Decreased
						URIDINE	0.000242	LL_RL_vs_LL_C_3PM	Decreased
						XANTHINE	0.007643	LL_RL_vs_LL_C_3PM	Decreased

Changes in the level of certain metabolites in the upper and lower leaves of tomato plants measured at dawn (3:00 a.m.) and in the afternoon (3:00 pm.) following 30-min-long nocturnal red light treatment applied for 3 weeks ($n = 5$). There are significant differences in the data at the $p \leq 0.05$ level (Duncan's multiple comparisons) at each time point.

Table 4
Effects of nocturnal red light treatment on biomass production and growth of tomato plants.

	Treatments					
	1. week		3. week			
			Lower leaves		Upper leaves	
Parameters	Control	Red light	Control	Red light	Control	Red light
Fresh mass (mg)	749.51+27.81^{a}	738.74+3674^{a}	964.34+45.18^{a}	782.32+54.57^{bc}	833.27+49.13^{b}	667.11+26.56^{c}
Dry mass (mg)	80.60+5.66^{a}	70.17+5.32^{a}	109.50+5.04^{a}	110.10+7.07^{a}	114.63+8.69^{a}	113.40+6.34^{a}
Leaf area (cm^{2})	70.76+2.36^{a}	67.51+3.02^{a}	87.08+4.12^{a}	69.46+3.88^{bc}	76.21+4.77^{ab}	64.23+2.20^{c}
Leaf thickness (μm)	147.65+2.78^{a}	137.04+3.38^{b}	148.42+2.96^{a}	133.40+2.27^{b}	118.71+1.62^{d}	124.05+1.91^{c}
Length of internode (cm)	5.76+0.17^{b}	6.85+0.17^{a}	7.60+0.25^{b}	8.75+0.36^{a}	6.50+0.23^{c}	7.47+0.33^{b}
Stem diameter (mm)	2.52+0.10^{a}	2.57+0.11^{a}	4.87+0.90^{a}	4.80+0.09^{a}	4.27+0.12^{b}	4.22+0.13^{b}

Changes in the key parameters of leaves and growth of tomato plants after 30-min-long nocturnal red light treatments applied for one and three weeks (mean+SE, $n = 18$). Data with different letters indicate significant differences at the $p \leq 0.05$ level (Duncan's multiple comparisons) at each time point.

Table 5
Effects of nocturnal red light treatment on callose content in tomato plants leaves.

	Treatments					
	First week		Third week			
			Lower leaves		Upper leaves	
Callose content	Control	Red light	Control	Red light	Control	Red light
A.U.	1693+252^{b}	1952+265^{a}	5549+42^{b}	6062+42^{a}	5076+44^{c}	4774+49^{d}

Changes in the callose content (A.U.; Arbitrary Units) after 30-min-long nocturnal red light treatments applied for one and three weeks (mean+SE, $n = 9$). Data with different letters indicate significant differences at the $p \leq 0.05$ level (Duncan's multiple comparisons) at each time point.

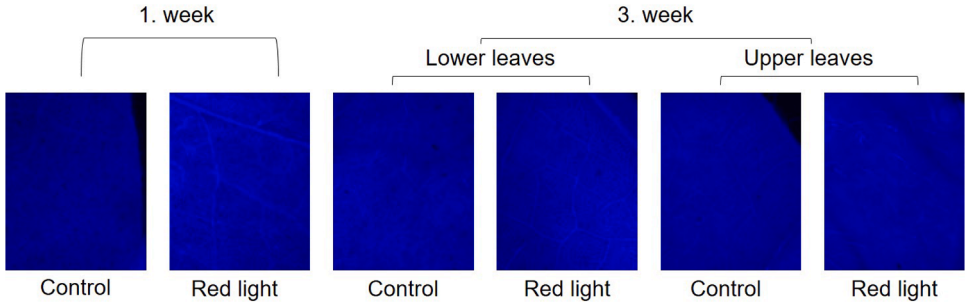

Fig. 5. Representative images showing callose content in the lower and upper leaves of tomato plants, visualised using aniline blue staining. The plants were pre-treated with 30 min of nocturnal red light for one and three weeks.

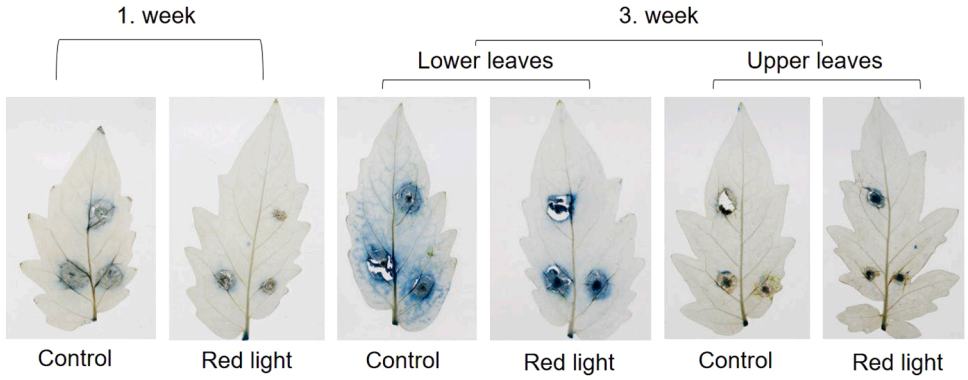

Fig. 6. Representative images showing necrotic zones caused by *Botrytis cinerea* infection on the upper and lower tomato plant leaves, as visualised by Evans Blue staining. The plants were pre-treated with 30 min of nocturnal red light for one and three weeks.

The potential priming effects of nocturnal red light application against fungal pathogens were investigated using the tomato-*B. cinerea* pathosystem (Tóth et al., 2019). Due to significant accumulation of defence-related metabolites and energy allocation for enhanced photosynthesis and carbohydrate metabolism, as well as cell wall reinforcement by callose, nocturnal red light treatments were able to promote priming effects against *B. cinerea* infection in tomato plant leaves, which were detected even after three weeks of red light application. At

Table 6
Effects of nocturnal red light treatment on the *Botrytis cinerea* infection in leaves of tomato plants.

	Treatments					
	First week		Third week			
			Lower leaves		Upper leaves	
Parameter	Control	Red light	Control	Red light	Control	Red light
Lesion diameter (cm^2)	0.23+0.02[a]	0.17+0.01[b]	0.65+0.06[a]	0.57+0.03[a]	0.27+0.02[b]	0.19+0.02[c]

Changes in the area of necrotic zones caused by *B. cinerea* after 30-min-long nocturnal red light treatments applied for one and three weeks (mean+SE, $n = 9$). Data with different letters indicate significant differences at the $p \leq 0.05$ level (Duncan's multiple comparisons) at each time point.

the same time, the red light was more effective at inhibiting *B. cinerea* infection in younger leaves than in older ones. Similarly, the beneficial effects of long-term red light treatment have been reported against *Pseudomonas syringae* (Yang et al., 2015) and *B. cinerea* (Hui et al., 2017; Kukri et al., 2024). At the same time, the beneficial effects of nocturnal red light treatment against pathogenic fungi were also confirmed in the lower leaves, where the level of callose content was higher.

Thus, our work demonstrated, that nocturnal red light application can elicit time-of-day–dependent physiological and metabolic changes in tomato plants, such as enhanced photosynthetic activity even in the older leaves, moreover priming of defence responses against fungal pathogens. Therefore, using a specific light spectrum for a short time during the dark period can serve as an eco-friendly tool not only to optimize sustainable greenhouse crop production, but also for limiting diseases in greenhouse tomato farming and improving plants' resilience against biotic stresses. This can be achieved through supplemental- or interlighting systems that influence the defence responses of plants in a light-level-dependent manner, however, the enhanced defence responses in plants affect growth parameters (Cao et al., 2016). Consequently, these findings provide an important knowledge for future studies on light-mediated plant responses and nocturnal light priming, particularly in relation to optimizing lighting strategies for sustainable crop production.

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CRediT authorship contribution statement

Péter Koprivanacz: Writing – original draft, Visualization, Investigation, Data curation. **András Kukri:** Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Dávid Milodanovic:** Investigation. **Atina Martics:** Investigation. **Zalán Czékus:** Writing – review & editing, Investigation, Funding acquisition, Data curation. **Ágnes Gallé:** Writing – review & editing, Visualization, Investigation, Funding acquisition. **Réka Szőllősi:** Visualization, Methodology, Investigation. **László Galgóczy:** Writing – review & editing, Methodology, Investigation. **Roland Tengölics:** Validation, Software, Methodology, Investigation. **Nadeem Iqbal:** Investigation. **Attila Ördög:** Writing – review & editing, Investigation. **Péter Poór:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2025.101104.

Data availability

Data will be made available on request.

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