

Differential Physiological and Growth Responses of Lettuce Seedlings to Extreme Thermal Stress Analyzed through Chlorophyll Fluorescence

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Author Contributions

Yu Kyeong Shin (Y.K.S.) and Jun Gu Lee (J.G.L.) designed the study. Yu Kyeong Shin (Y.K.S.) performed the experiments and analyzed the data. Y. K. S. and J. G. L. drafted and revised the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Abstract

This study sought to monitor changes in growth parameters, chlorophyll fluorescence (CF) levels, and proline and chlorophyll contents in lettuce seedlings grown under different temperature conditions. Ten-day-old lettuce seedlings grown under a fluorescent lamp in a closed plant production system having a constant temperature (24/18°C), humidity of 60 ± 3%, and a light period of 14/10 h (day/night) were kept at 24/18°C for three days in a closed chamber under white warm LED lighting at 220 ± 10 μmol·m⁻²·s⁻¹. The seedlings were then grown under different temperature conditions [low: 8/4°C (day/night), control: 24/20°C, and high: 40/36°C] in a closed and controlled plant production system for eight days. The CF parameters, proline amount, and chlorophyll content were measured at 0, 2, 4, 6, and 8 d after the initiation of the treatment, while the growth parameters (fresh weight, dry weight, leaf length, and hypocotyl length) were measured at the end of the experiment. The quantum yield of nonregulated energy dissipation in PSII [Y(NO)] increased on the second day and then decreased at low temperatures. Non-photochemical quenching (NPQ) and the fluorescence decrease ratio (Rfd) significantly decreased under both high- and low-temperature stress compared to those in the control. Growth parameters were retarded at low temperatures, whereas leaves became dry at high temperatures. The chlorophyll content decreased significantly, whereas the proline content increased only at low temperatures. These results indicate that Y(NO), NPQ, and Rfd can be used to detect temperature stress in lettuce seedlings.

Additional key words: abiotic stress, leafy vegetables, photosynthesis, phytochemical, rapid detection

Introduction

Lettuce is a representative leafy vegetable commonly grown in open fields and controlled environments, such as plant factories. It is primarily distributed and consumed fresh, making it easily accessible to people of all ages (Khan et al. 2008; Cheowtirakul and Linh 2010). The optimal growth

temperature for lettuce generally ranges from 15 to 25°C, and temperatures outside this range are known to have direct and indirect negative effects on the photosynthetic process (Anfoka et al. 2016; Banks 2018). Maintaining an appropriate temperature is crucial for cultivating high-quality crops because temperature is one of the most significant factors affecting the marketability of lettuce (Chen et al. 2021; Yan et al. 2021).

Lettuce tends to bolt when exposed to temperatures above 30°C for extended periods, which results in reductions in the leaf number, leaf length, leaf color, chlorophyll content, photosynthesis, and soluble sugar content (Hawrylak-Nowak et al. 2018; Liu et al. 2021). These temperature-induced changes in photosynthetic parameters are particularly evident in lettuces grown in open fields and greenhouses, where environmental conditions fluctuate naturally, unlike in closed plant factories with precise climate control systems that can maintain optimal growing conditions. High temperatures accelerate lettuce bolting, causing the leaves to become bitter. This is associated with an increase in the respiration rate and the accumulation of cellular oxidants, leading to the production of proline and soluble sugars as an adaptive response to heat stress (Choudhury et al. 2017; Argosubekti 2020; Giordano et al. 2021). Conversely, when exposed to temperatures below 15°C, lettuce exhibits reduced photosynthesis, respiration rates, and water absorption levels, as well as a decrease in cell turgor within mesophyll cells, reduced synthesis of secondary metabolites, chlorosis, and yellowing of the leaf margins (Streb et al. 2008; Mattila et al. 2020). Cold stress plays a critical role in the osmotic regulation mechanism, increasing plant resistance by generating proline to maintain the cellular water balance and alleviating osmotic pressure under abiotic stress conditions (Fahad et al. 2017; Guan et al. 2024; Yaseen et al. 2025). Additionally, the accumulation of soluble sugars is an essential regulator that is positively correlated with plant cold tolerance, enhanced water retention capacity, and improved cold resistance (Wei et al. 2023; Li et al. 2024).

Various nondestructive assessment tools have been developed to quantify and monitor temperature stress levels, all of which can be applied as part of a cultivation management strategy (Baker and Rosenqvist 2004; Tsai et al. 2019). Nondestructive assessment tools offer significant advantages over traditional destructive methods, including real-time monitoring capabilities, preservation of plant integrity throughout the growth cycle, reductions in sampling variability, the ability to track the same plants over time, and minimized labor requirements. These benefits make nondestructive techniques particularly valuable for both research applications and in commercial cultivation settings. Temperature stress is known to have direct and indirect effects on photosynthetic processes, leading to increased interest in chlorophyll fluorescence measurement techniques that can effectively assess photosynthetic activity (Pastenes and Horton 1996; Qiu et al. 2003; Song et al. 2014; Yan et al. 2021). Chlorophyll fluorescence measurement techniques allow for sensitive observations of changes in the photosynthetic process and are used to diagnose abiotic and biotic stresses across different plant species and growth stages (Na et al. 2014; Kalaji et al. 2016; Guidi et al. 2019). Exposure to high temperatures (35 – 42°C) during crop cultivation increases photorespiration and destabilization through Photosystem II (PSII), and damage to the electron transport chain can impair the photosynthetic machinery (Sharma et al. 2015; Hou et al. 2016; Chen et al. 2018; Yan et al. 2021). Chlorophyll fluorescence, which can effectively monitor PSII, is a useful indicator of heat stress along with key parameters such as F_v/F_m , $Y(PSII)$, and NPQ (Yamane et al. 1997; Hou et al. 2016; Bhandari et al. 2018). Additionally, heat damage in cold-tolerant and temperate crops such as paprika, Chinese cabbage, and chrysanthemum under high-temperature stress has been analyzed (Oh and Koh 2014; Janka et al. 2015; Bhandari et al. 2018).

Cold stress is closely related to the temperature of the thylakoid membrane, where photosynthesis occurs, leading to the emission of chlorophyll fluorescence, a sensitive signal generated during photosynthesis (Hou et al. 2018; van Buer et al.

2019). As the leaf temperature decreases rapidly, many plant species exhibit a strong correlation between F_v/F_m , an indicator of the maximum quantum efficiency of photochemistry, and freezing or supercooling effects (Oliveira and Peñuelas 2005; Xu et al. 2022). Numerous studies of wheat, *Arabidopsis*, and tomato have shown that photochemical and non-photochemical types of fluorescence quenching are closely related to cold stress (Demmig-Adams et al. 2014; Ruban 2016; Lu et al. 2022; Mishra et al. 2023; Jan et al. 2024). Research on various high- and low-temperature forms of stress has discussed their negative effects on PSII efficiency and light energy utilization outside the optimal temperature range (Oh and Koh 2014; Weng et al. 2021; Mesa et al. 2024). High- and low-temperature stress effects vary depending on the developmental stage, species, and plant cultivar. Previous studies of lettuce used chlorophyll fluorescence to investigate the effects of differing amounts of light intensity, salinity, and drought (Woo et al. 2008; Fu et al. 2012; Yao et al. 2018; Shin et al. 2020a, 2020b; Chen et al. 2021). Many of these studies focused on the mid-stage cultivation period, when lettuce is exposed to high or low temperatures, primarily emphasizing the transient effects of temperature fluctuations (Koseki and Isoke 2005; Becker 2014; Janka et al. 2015; Carotti et al. 2020; Yan et al. 2021). As is well known, plants exhibit dynamic morphological and physiological changes in response to both transient temperature shocks and continuous temperature fluctuations (May et al. 2013). Therefore, the main objective of the present study was to monitor the dynamic changes in lettuce under varying temperature conditions by means of the nondestructive tracking of chlorophyll fluorescence imaging, the chlorophyll content, and proline and sugar levels. This approach will be valuable for those seeking a better understanding of the effects of extremely high- and low-temperature stress outside the optimal temperature range for lettuce growth.

Materials and Methods

Plant materials and cultivation settings

The lettuce cultivar *Cheong Chi Ma* (Asia Seed Co., Seoul, Korea), known for its widespread consumption in Korea, was used. Seeds were germinated in 50-hole plug trays (Bumnong Co., Korea) filled with a commercial potting medium (Nongwoobio Co., Korea). Sub-irrigation was applied daily for 20 minutes over twelve days. At ten days after sprouting, seedlings were maintained for three days in a growth chamber set at 24°C/20°C (day/night), with a 14/10 h photoperiod, $60 \pm 3\%$ relative humidity, and an LED light intensity near $220 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. After this acclimatization period, the plants were subjected to the following ten-day temperature treatments: low (8/4°C), control (24/20°C), and high (40/36°C) in a fully enclosed environment. The temperature treatments were designed to induce significant low- and high-temperature stress while maintaining plant viability. The low temperature of 8°C was selected because it is below the optimal growth range for lettuce and is known to impair photosynthetic efficiency and water uptake without causing immediate tissue damage. Conversely, a high temperature of 40°C was chosen to simulate extreme heat conditions often experienced in greenhouse environments, where photorespiration is accelerated, Photosystem II (PSII) destabilization occurs, and oxidative stress responses are promoted. These temperature settings enabled a systematic evaluation of physiological responses in lettuce under cold and heat stress.

Chlorophyll fluorescence imaging

Photosynthetic efficiency and related chlorophyll fluorescence parameters were assessed using a FluorCam 7 instrument (Photon Systems Instruments, Drásov, Czech Republic). Prior to the measurements, leaves were dark-adapted for 20 minutes. Evaluations were conducted on days 0, 2, 4, 6, and 8 following temperature exposure. For each treatment group, all ten randomly selected seedlings (from a pool of 100 uniform individuals) were measured per time point. During imaging, the distance between the camera and the plants was maintained at approximately 17–20 cm. Fluorescence imaging was conducted on the entire set of leaves of each seedling, as opposed to selecting a specific leaf position. The parameters F_0 , F_m , F_v/F_m , $Y(PSII)$, NPQ, qP , qL , qN , F_v'/F_m' , $Y(NO)$, $Y(NPQ)$, and Rfd were recorded and analyzed using the system's default software protocols.

Growth characterization

Growth traits were examined in ten selected seedlings per treatment, at five time points spaced two days apart. Measurements were taken of the leaf size (length, width), number of leaves, and biomass. Shoot and root fresh weights were recorded using an electronic balance, and dry weights were obtained post-drying at 70°C for 72 hours. Leaf tissues used for chlorophyll fluorescence were retained, freeze-dried, ground to a powder, and stored at -80°C for biochemical assays.

Proline determination

To quantify proline, 50 mg of lyophilized tissue powder was extracted using 3% sulfosalicylic acid. The homogenate was shaken, centrifuged, filtered, and subjected to a colorimetric analysis following a modified protocol based on Bates et al. (1973). Acid ninhydrin and acetic acid were added to the extract, followed by heating, cooling, and toluene separation. Absorbance at 520 nm was recorded using a multi-well plate reader. Proline levels were calculated against a standard curve generated with L-proline solutions.

Chlorophyll estimation

Chlorophyll a and b were extracted with methanol from 20 mg of freeze-dried plant material, following the procedure of Warren (2008). After shaking and centrifugation, absorbance readings at 652 and 665 nm were obtained, and concentrations were calculated by means of optical correction formulas.

Sugar profiling and sweetness estimation

Soluble sugars were extracted by incubating 50 mg of dried sample in distilled water at 80°C, followed by centrifugation and filtration. The supernatant was analyzed using a 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a carbohydrate-specific column and a refractive index detector. Separation conditions included a 75:25 acetonitrile-water mobile phase. Glucose, fructose, and sucrose (Sigma-Aldrich, St. Louis, MO, USA) were identified based on retention time matching with standards, and concentrations were used to compute the total sweetness index (TSI) according to the method of Magwaza and Opara (2015), incorporating individual sugar sweetness coefficients.

Data analysis

All results were expressed as the means $\pm$ standard errors (SE). Growth traits were measured with five biological replicates per treatment, and chlorophyll fluorescence parameters were assessed using ten biological replicates. Biochemical analyses were performed using samples from five individual plants, with each measurement conducted in three technical replicates. One-way and two-way ANOVA, along with a correlation analysis, were carried out using R software (version 4.0.2), with significance determined at $p < 0.05$.

Results

Effects of high and low temperatures on growth parameters and the phenotype of lettuce

Morphological changes in lettuce seedlings subjected to high- and low-temperature stress are shown in Fig. 1. Under low-temperature stress, lettuce seedlings exhibited abnormal growth compared to the control and high-temperature stress conditions. Low-temperature stress had a detrimental effect on the growth parameters of lettuce seedlings compared to the control and high-temperature stress conditions. Most growth parameters, including the shoot fresh weight, shoot dry weight, number of true leaves, leaf length, and leaf width, were significantly lower under both high- and low-temperature stress treatments than in the control on day 8 (Table 1). Among the three temperature conditions, low-temperature stress led to greater differences between the maximum and minimum values of the shoot fresh weight (5.4-fold), shoot dry weight (3-fold), leaf length, and leaf width. Although high-temperature stress showed significant differences from the control, growth development under high-temperature stress was better than that under low-temperature stress.

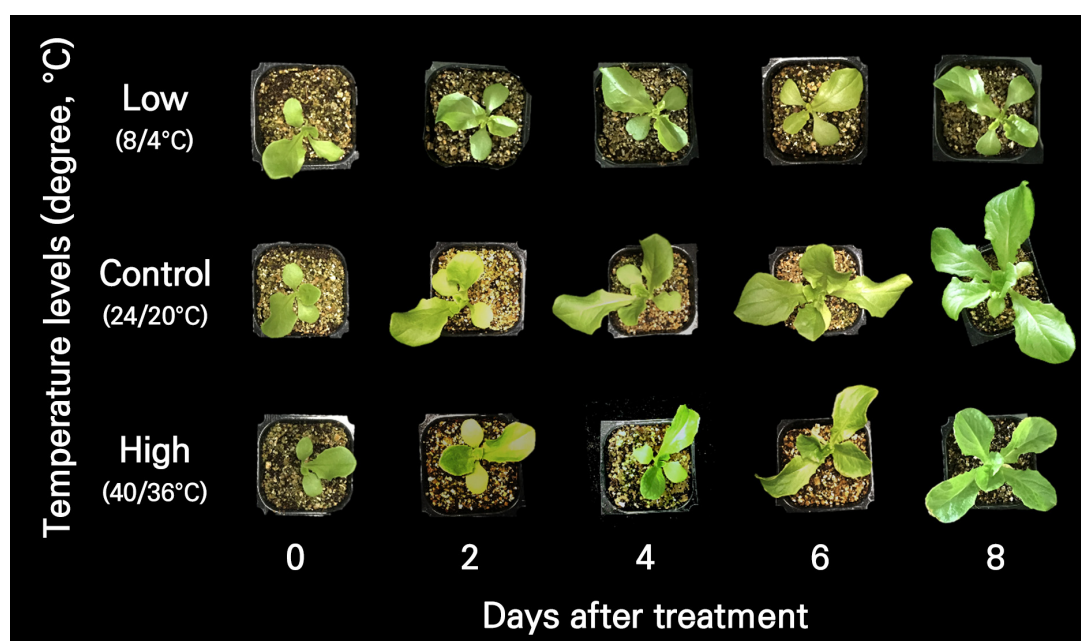


Fig. 1. Changes in the visual appearance of lettuce seedlings grown under control (24/20°C), high- (40/36°C), and low- (8/4°C) temperature conditions during the progressive treatment times.

Table 1. Effects of temperature levels (control (24/20°C), high (40/36°C), and low (8/4°C)) on the growth parameters of the lettuce seedlings after eight days of treatment

Days after treatment (DAT)	Temperature level (TL)	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (mg)	Number of leaves (ea)	Leaf length (cm)	Leaf width (cm)	Ratio of L/W
0	Control	0.14 ± 0.02 ^z	0.01 ± 0.00	0.06 ± 0.01	0.03 ± 0.00	1.00 ± 0.00	3.67 ± 0.40	1.90 ± 0.17	1.93 ± 0.09
2	Control	0.95 ± 0.09 a ^y	0.70 ± 0.03 b	0.80 ± 0.03 a	0.05 ± 0.03 a	2.00 ± 0.00 a	4.73 ± 0.35 a	2.53 ± 0.15 a	1.88 ± 0.22 a
	High	0.91 ± 0.06 a	0.73 ± 0.02 b	0.81 ± 0.01 a	0.05 ± 0.00 a	1.67 ± 0.58 a	3.90 ± 0.44 a	2.40 ± 0.35 a	1.66 ± 0.39 a
	Low	0.79 ± 0.02 b	0.69 ± 0.01 b	0.79 ± 0.06 b	0.08 ± 0.03 b	1.00 ± 0.00 b	3.53 ± 0.55 b	1.77 ± 0.06 b	2.00 ± 0.31 b
4	Control	1.10 ± 0.10 a	0.77 ± 0.01 b	0.89 ± 0.03 a	0.77 ± 0.02 b	3.00 ± 0.00 a	4.97 ± 0.76 a	2.50 ± 0.30 a	1.99 ± 0.24 a
	High	0.97 ± 0.07 ab	0.75 ± 0.04 b	0.82 ± 0.09 ab	0.71 ± 0.06 b	3.00 ± 0.00 a	4.30 ± 0.36 a	2.20 ± 0.17 a	1.96 ± 0.20 a
	Low	0.91 ± 0.05 b	0.76 ± 0.01 b	0.84 ± 0.01 b	0.87 ± 0.18 b	1.33 ± 0.58 b	3.47 ± 1.03 b	1.90 ± 0.36 b	1.90 ± 0.19 b
6	Control	1.63 ± 0.19 a	1.26 ± 0.06 a	1.26 ± 0.06 a	1.07 ± 0.01 a	4.33 ± 0.58 a	5.07 ± 0.49 a	2.77 ± 0.32 a	1.83 ± 0.04 a
	High	1.45 ± 0.07 ab	1.23 ± 0.03 a	1.23 ± 0.03 ab	1.09 ± 0.01 a	3.67 ± 0.58 ab	4.40 ± 0.20 ab	1.97 ± 0.59 ab	2.38 ± 0.74 ab
	Low	1.24 ± 0.01 c	1.16 ± 0.00 b	1.16 ± 0.00 b	1.06 ± 0.00 a	1.67 ± 0.58 c	3.87 ± 0.21 b	2.07 ± 0.06 b	1.87 ± 0.15 b
8	Control	2.10 ± 0.11 a	1.13 ± 0.00 a	1.09 ± 0.07 a	0.78 ± 0.02 a	5.67 ± 0.58 a	5.93 ± 0.55 a	2.97 ± 0.31 a	2.00 ± 0.03 a
	High	1.76 ± 0.11 b	1.13 ± 0.02 a	1.00 ± 0.06 b	0.78 ± 0.02 a	5.67 ± 0.58 a	4.77 ± 0.57 b	2.37 ± 0.15 ab	2.01 ± 0.13 a
	Low	1.25 ± 0.05 c	1.06 ± 0.01 a	0.88 ± 0.02 c	0.77 ± 0.01 a	2.00 ± 0.00 b	3.97 ± 0.25 b	2.00 ± 0.10 b	1.99 ± 0.13 a
DAT		**	NS	**	NS	**	NS	NS	NS
TL		**	**	**	**	**	**	**	NS
DAT × TL		NS ^x	NS	NS	NS	NS	NS	NS	NS

^zData are presented as the mean values from five replicates.^yDifferent letters within the same column indicate significant differences (Duncan's test, $p < 0.05$).^xNS, non-significant; **, significance at $p < 0.01$.

Identification of early responses to temperature stress using chlorophyll fluorescence kinetics

To assess the photosynthetic performance of the plants under the control, high, and low temperature stress conditions, chlorophyll fluorescence quenching curves were measured every two days from day 0 to day 8 of temperature stress using the chlorophyll fluorescence quenching protocol Quenching Act 2 (Shin et al. 2020b). Typical chlorophyll fluorescence curves of lettuce seedlings subjected to temperature stress, measured over five sessions, are shown in Fig. 2. Day 2 was the earliest point at which pattern differences in the chlorophyll fluorescence curves between the temperature stress treatments and the control could be visually observed, with these differences becoming more pronounced from day 4 onwards compared to day 0 (Fig. 2). In seedlings subjected to low-temperature stress, a decline in most parameters began on day 2, and they continued to decrease as the treatment duration increased, especially in comparison with the control (Fig. 2B). In seedlings subjected to high-temperature stress, the parameters measured with the actinic light turned on were higher, whereas those in the dark tended to decrease relative to the control (Fig. 2C). Additionally, on day 8, plants exposed to high-temperature stress exhibited high fluorescence values relative to those of the control, whereas those under low-temperature stress showed a significant decrease (Fig. 2D). These findings suggest that both high- and low-temperature stress induces anomalies in the mechanisms involved in the photosynthetic processes of these plants.

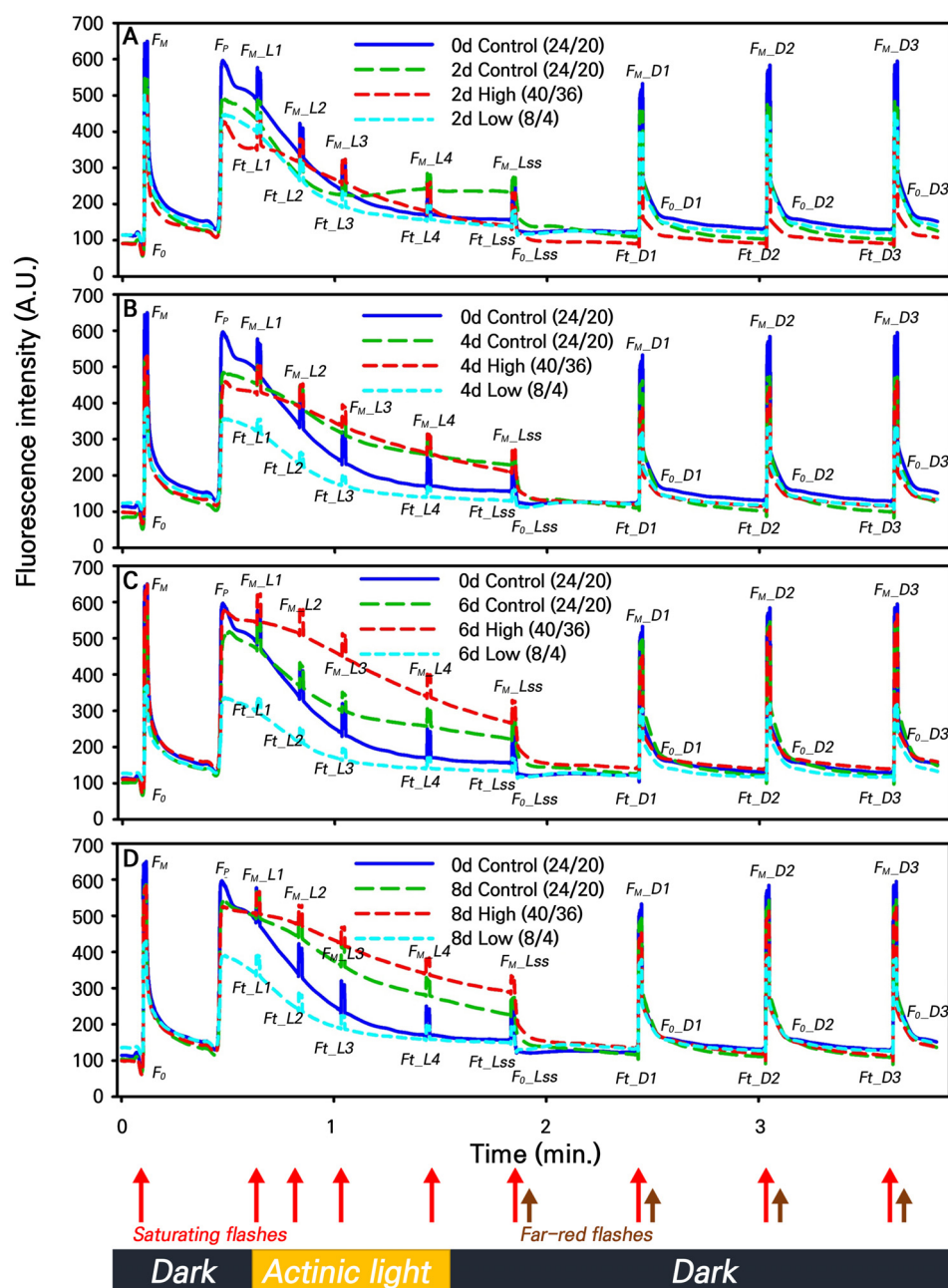


Fig. 2. Representative kinetic chlorophyll fluorescence intensity of lettuce (*Lactuca sativa* L.) in the control (24/20°C), high (40/36°C), and low (8/4°C) treatments at day 2 (A), day 4 (B), day 6 (C), and day 8 (D) after the introduction of temperature stress. Arrows indicate the timings of the saturating flashes (red) and far-red flashes (brown), and values represent the average of ten replicates per genotype and treatment. Readers can refer to “Abbreviations” for all fluorescence parameters.

Effects of temperature stress on the CF parameters

To examine the efficiency of photochemical energy utilization, the parameters F_0 , F_m , F_v/F_m , F_v'/F_m' , $Y(\text{PSII})$, qP , and qL were analyzed, along with NPQ and qN , which represent non-photochemical quenching, and Rfd , which indicates the chlorophyll reduction ratio (Fig. 3). Overall, the low-temperature treatment showed a tendency to decrease compared to

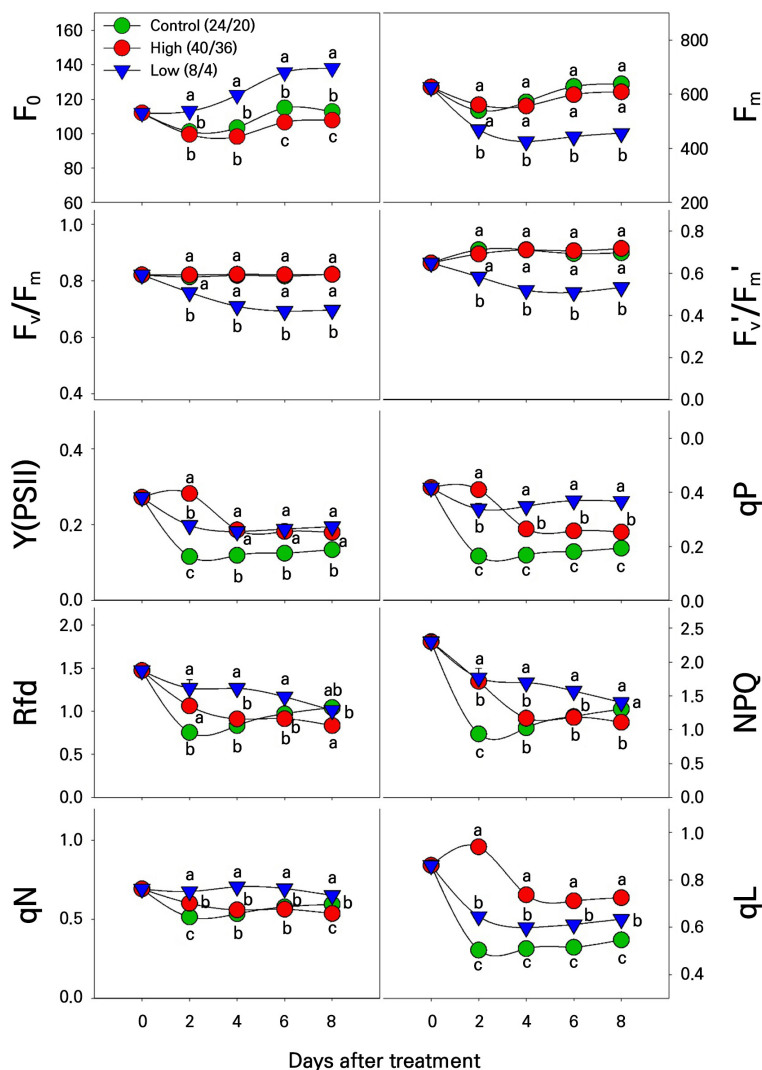


Fig. 3. Changes in the CF parameters (F_0 , F_m , F_v/F_m , F_v'/F_m' , $Y(PSII)$, qP , Rfd , NPQ , qN , and qL) of lettuce seedlings grown under control (24/20°C), high (40/36°C), and low (8/4°C) temperature conditions for different time periods. Each plot point represents the mean $\pm$ SE of ten biological replicates.

the control and high-temperature treatments throughout the experimental period, with significant differences observed (Table 2). Under low-temperature conditions, F_0 consistently increased, whereas F_m decreased. During the experiment, the average F_v/F_m value for the control group remained stable at 0.8322 ± 0.002 . This result was similar to that observed under high-temperature conditions, whereas a significant decrease was observed in plants exposed to low temperatures as early as day 2 ($p < 0.001$). The parameter F_v'/F_m' , indicative of photochemical energy efficiency, decreased under low-temperature conditions. Meanwhile, $Y(PSII)$ and qP showed relatively high values in both the high- and low-temperature treatments compared to the control throughout the experimental period, with significant trends observed. Rfd displayed differences beginning on day 2, but after day 4, no significant changes were observed compared to the control and high-temperature treatments. In addition, NPQ and qN exhibited trends similar to those of Rfd , although it was challenging to discern significant differences despite the similarity in the value ranges of these parameters.

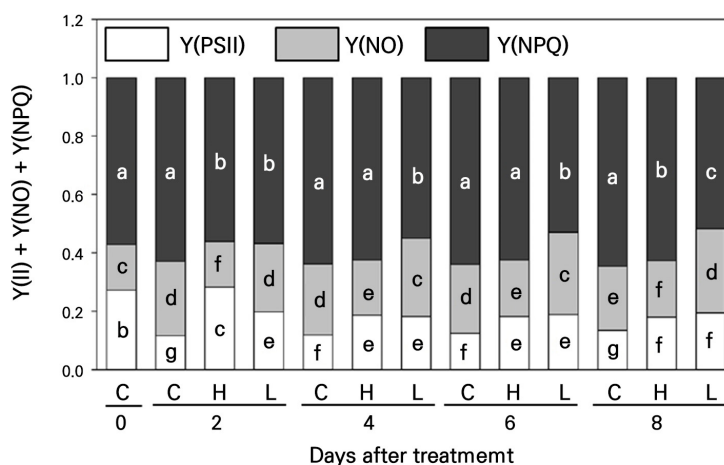
Table 2. Summary of the analysis of the chlorophyll fluorescence (CF) parameter of lettuce seedlings at the temperature and multiple treatment times

Parameters	Temperature levels (TL) ^z		Treatment Time (TT) ^y		TL × TT	
	F-Value	Significance	F-Value	Significance	F-Value	Significance
F ₀	207.97	*** ^x	43.19	***	5.13	***
F _m	281.62	***	14.77	***	7.56	***
F _v /F _m	1271.24	***	17.03	***	28.96	***
F _v '/F _m '	717.12	***	17.30	***	9.63	***
Y (PSII)	45.82	***	62.26	***	10.62	***
NPQ	30.03	***	30.06	***	6.83	***
qN	94.94	***	22.43	***	5.90	***
qP	96.22	***	58.18	***	11.91	***
Rfd	24.97	***	59.56	***	9.32	***
Y (NO)	214.93	***	47.81	***	10.84	***

^zTemperature levels: control (24/20°C), high (40/36°C), and low (8/4°C).^yTreatment times: 0, 2, 4, 6, and 8 days.^x*, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. NS: non-significant.

Effects of temperature stress on energy partitioning

The utilization of all light energy absorbed by the plants at different temperature levels was examined using energy-partitioning parameters (Fig. 4). Starting from an initial Y(PSII) value of 0.27 ± 0.01 on day 0, all treatments showed a significant reduction of approximately 50%. On day 8, Y(NO) increased significantly, with the control group showing an increase to 28.92%, the high-temperature treatment to 19.67%, and the low-temperature treatment showing the highest increase at 45.93%. Y(NPQ) also increased on day 8, with the control group at 11.52% and the high-temperature treatment at 8.69%, whereas the low-temperature treatment showed a relative decrease of -10.70%. Throughout the experiment, Y(NPQ) values were highest in the control group on days 2, 4, 6, and 8, whereas Y(PSII) values were consistently lower in the control group across all measurement days.

**Fig. 4.** Changes in the CF parameters (Y(II), Y(NO), and Y(NPQ)) of lettuce seedlings grown under control (C, 24/20°C), high (H, 40/36°C), and low (L, 8/4°C) temperature conditions for different time periods. Each plot point represents the mean $\pm$ SD of ten biological replicates.

Changes in Chl, proline, and sugar contents under high- and low-temperature stress conditions

During the high- and low-temperature stress treatments, the Chl a, Chl b, and total chlorophyll contents significantly decreased (Fig. 5A, 5B, 5C, and Table 3). In the low-temperature stress group, a continuous decline in Chl a (3.91 ± 0.01), Chl b (1.08 ± 0.06), and the total chlorophyll content (4.99 ± 0.05) was observed starting on day 2 of the treatment. In the high-temperature treatment, a significant difference in the Chl a content began to emerge on day 4, whereas the Chl b content decreased by day 6. The control group showed a continuous increase in the chlorophyll content after day 4, ultimately resulting in a 22.00 ± 0.1 % increase compared to day 0. Under low-temperature conditions, the proline content began to increase on day 2 and showed a sharp increase of 5.67-fold by day 4 (Fig. 5D). In the high-temperature stress

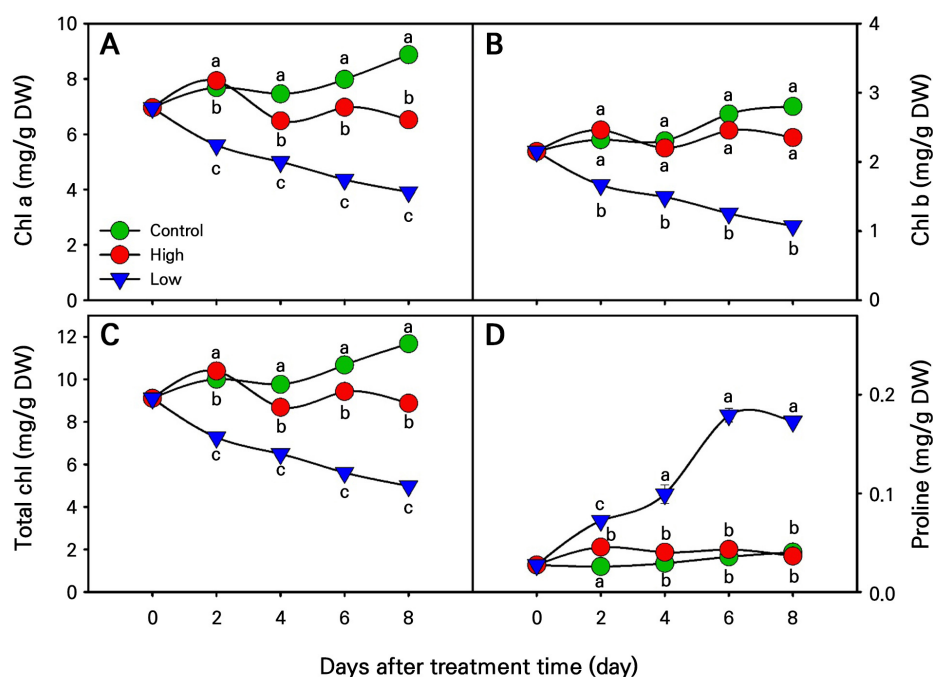


Fig. 5. Changes of chlorophyll a (A), chlorophyll b (B), and total chlorophyll (C), and proline (D) contents in lettuce seedlings at 0, 2, 4, 6, and 8 days after control (24/20°C), high (40/36°C), and low (8/4°C) treatment times. Multiple line plots represent the mean $\pm$ SE of three replicates. Chl: Chlorophyll, Total chl: Total chlorophyll.

Table 3. Summary of the analysis of the chlorophyll fluorescence (CF) parameters of lettuce seedlings at the temperature and multiple treatment times

Parameters	Temperature levels (TL) ^z		Treatment time (TT) ^y		TL $\times$ TT	
	F-Value	Significance	F-Value	Significance	F-Value	Significance
Chl a	10639.8	*** ^x	468.2	***	504.2	***
Chl b	486.7	***	12.4	***	21.3	***
Total Chl	4797.5	***	176.6	***	218.0	***
Proline	1266.7	***	84.0	***	92.8	***

^zTemperature levels: control (24/20°C), high (40/36°C), and low (8/4°C).

^yTreatment time: 0, 2, 4, 6, and 8 days.

^x*, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. NS: non-significant.

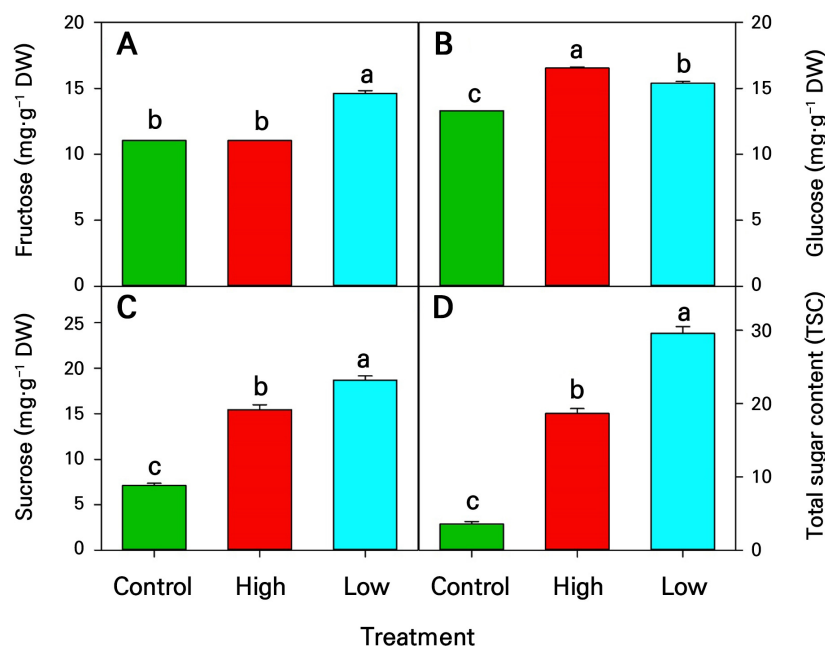


Fig. 6. Changes in fructose (A), glucose (B), sucrose (C), and total sugar contents (TSC) (D) in lettuce seedlings grown under control (24/20°C), high (40/36°C), and low (8/4°C) temperature conditions. Each bar represents the mean $\pm$ SE of three replicates.

group, the proline content showed some significant differences compared to the control on day 2 but then decreased by day 4, resulting in no significant difference compared to the control. On the final day of the temperature treatment, the fructose content under high-temperature conditions was similar to that under normal conditions but was 1.3 times higher under the low-temperature conditions (Fig. 6A). The glucose content was 1.15 times higher under high-temperature conditions and 1.16 times higher under low-temperature conditions than that in the control (Fig. 6B). The sucrose content was 2.17 times higher under high-temperature conditions and 2.62 times higher under low-temperature conditions (Fig. 6C). The total sugar content was elevated under both the high- (1.36 times) and low- (1.54 times) temperature conditions (Fig. 6D).

Correlation analysis

A correlation analysis was conducted to determine the relationships among the chlorophyll fluorescence parameters, growth parameters, chlorophyll content, and proline content (Fig. 7). Pearson's correlation coefficients (r) were calculated and correlations with $p < 0.05$ were considered statistically significant. The analysis revealed that F_0 exhibited a negative correlation with F_v and F_v/F_m ($r = -0.78$, $p < 0.01$ and $r = -0.82$, $p < 0.01$, respectively), but showed no significant relationship with the other chlorophyll fluorescence parameters examined in this study, in this case qN , qP , Rfd , and $Y(NO)$ ($p > 0.05$). All of the other chlorophyll fluorescence parameters showed significant positive or negative correlations with correlation coefficients ranging from $r = 0.65$ to $r = 0.89$ ($p < 0.01$). The chlorophyll content was found to have a strong positive correlation with F_v/F_m and $Y(PSII)$ ($r = 0.76$, $p < 0.01$ and $r = 0.72$, $p < 0.01$, respectively), consistent with results observed in *Arabidopsis* and pepper. However, the magnitudes of these correlations differed, likely

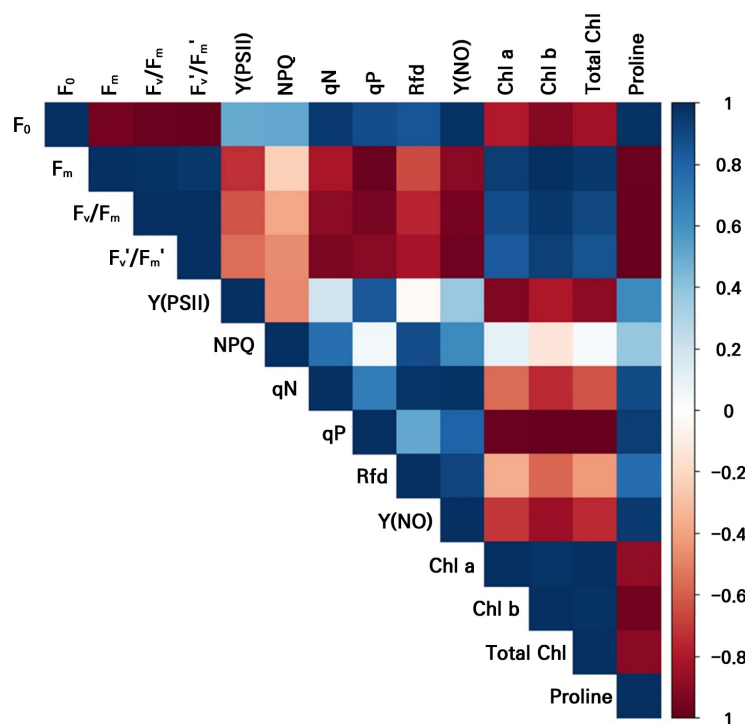


Fig. 7. Correlation analysis for CF parameters, chlorophyll, and proline contents in lettuce seedlings regardless of the treatment and temperature level at eight days. Blue circles represent positive correlations, whereas red circles represent negative correlations. Color intensity is proportional to the correlation coefficients, as shown in the legend to the right. Readers can refer to Table 1 for detailed information pertaining to the CF parameters.

due to the varying levels of heat and cold stress tolerance across the different lettuce varieties and due to genetic resources. In contrast, qP and Rfd were not significantly correlated with NPQ or qN ($p > 0.05$). These findings are consistent with those of related studies. The proline content exhibited a strong positive correlation with F_0 ($r = 0.68$, $p < 0.01$) and a negative correlation with F_m , F_v/F_m , and F_v'/F_m' ($r = -0.71$, $p < 0.01$; $r = -0.75$, $p < 0.01$; $r = -0.77$, $p < 0.01$, respectively). Additionally, the proline content was strongly and negatively correlated with $Chl\ a$, $Chl\ b$, and the total chlorophyll content ($r = -0.69$, -0.65 , and -0.72 respectively, all $p < 0.01$). These results are similar to those observed in studies of salt and drought stress treatments in lettuce.

Discussion

Plants undergo various external and internal changes in response to temperature stress, ultimately triggering appropriate defense mechanisms. According to certain morphological changes (Fig. 1), slow leaf development and leaf discoloration can be considered early physiological responses to temperature stress. Similar findings have been reported in tomatoes and Arabidopsis, where growth reductions due to cold stress were reported (Koseki and Isobe 2005; Sakamoto and Suzuki 2015; Heidari et al. 2021). In lettuce, growth parameters under high-temperature stress also differed from those in the control group (Jenni et al. 2013). The reduction in most growth parameters under cold stress can be attributed to decreased photosynthetic activity, insufficient transpiration, and an imbalance in nutrient uptake (Pérez-Torres et al. 2006; Mattila

et al. 2020). Specifically, cold stress induces photoinhibition under light conditions, which adversely affects photosynthetic levels (Hetherington et al. 1989; Huner et al. 1993). Under high-temperature stress, although growth and development occurred, visual changes in the leaf color, reductions in the leaf length and width, and leaf distortion were observed (Table 1). The decrease in growth was likely due to a reduction in photosynthetic activity and failure to maintain normal transpiration during this period. These results are consistent with those reported by Hawrylak-Nowak et al. (2018) and Yan et al. (2021). However, as shown in Table 1, there were no significant interactions between temperature levels and treatment duration ($\text{DAT} \times \text{TL}$) for most growth parameters, indicating that temperature stress responses manifested relatively early and remained relatively stable over the 8-day treatment period. The lack of cumulative effects over time suggests that either the imposed temperature stress was quickly absorbed by the plants' physiological adjustment mechanisms or that the duration of stress exposure was insufficient to cause progressive physiological deterioration. These findings imply that the primary effects of temperature stress on lettuce seedlings were immediate rather than time-dependent.

Temperature stress is one of the most significant factors limiting photosynthesis, as it restricts stomatal function and other factors involved in photosynthesis. Chlorophyll fluorescence imaging is a valuable tool for monitoring photochemical and non-photochemical energy utilization. The chlorophyll fluorescence curves demonstrated distinct patterns under both high- and low-temperature conditions compared to the control, indicating a close association between temperature and energy utilization processes during photosynthesis. Under cold conditions, a reduction in energy use efficiency was observed in lettuce (Fig. 2), consistent with the findings of Hou et al. (2016), who reported similar variations in chlorophyll fluorescence under both high- and low-temperature stress. In addition to the chlorophyll fluorescence parameters, the curves obtained using the Quenching Act 2 protocol provided more intuitive and rapid identification of chlorophyll fluorescence levels. This suggests that the Quenching Act 2 protocol could serve as an effective tool for the diagnosis of abiotic and biotic forms of stress. The temporal dynamics of these chlorophyll fluorescence patterns revealed crucial information about the progression of stress responses over the 8-day treatment period. During the initial exposure (0–2 days), plants stressed under both high and low temperatures showed moderate deviations in fluorescence curves compared to control plants, but maintained similar curve shapes. However, as the exposure time increased, these patterns diverged more dramatically, with cold-stressed plants exhibiting progressively flattened curve profiles by days 4–6, indicating an increasing inability to utilize absorbed light energy effectively. This time-dependent deterioration of fluorescence characteristics suggests cumulative damage to the photosynthetic apparatus rather than a simple temporary inhibition. The underlying mechanism likely involves time-dependent disruption of thylakoid membrane organization under prolonged temperature stress, as reported by Yamamoto (2016) in spinach chloroplasts. Interestingly, the Quenching Act 2 protocol revealed different levels of temporal sensitivity between the fast and slow relaxation components of non-photochemical quenching. The fast-relaxing component, associated with the xanthophyll cycle, showed significant alterations within two days of temperature stress, whereas the slow-relaxing component, linked to photoinhibition and D1 protein damage, exhibited a gradual increase that became pronounced only after four to six days of continuous stress. This differential time course suggests a sequential activation of photoprotective mechanisms, with rapid adjustments to energy dissipation systems preceding longer-term protective responses. Similar time-dependent progression of photosynthetic adjustments under temperature stress has been observed in various crop species, including rice (Li et al. 2018) and cucumber (Wang et al. 2019), suggesting a conserved temporal response pattern across different

plant species. The gradual changes in chlorophyll fluorescence parameters over the experimental period also provide insight into the plants' acclimation capacity. The initial decline in energy use efficiency under cold stress was partially stabilized between days 4–6, suggesting the activation of compensatory mechanisms, before deteriorating further by day 8. This non-linear temporal pattern indicates a complex stress response with distinct phases of shock, attempted acclimation, and eventual failure of protective systems under continued stress exposure. The practical implication of these findings is that the timing of stress detection through chlorophyll fluorescence analysis is critical when devising effective intervention strategies in agricultural settings.

The F_v/F_m' ratio, obtained under illuminated conditions, reflects the maximum potential efficiency of PSII under light conditions and is commonly used to assess the impact of light-induced stress (Baker 2008). A continuous decline in F_v/F_m' under low-temperature conditions indicates inefficient energy utilization under these conditions (Aazami et al. 2021). The parameters $Y(II)$, qL , and qP , which represent photochemical energy efficiency, showed lower utilization efficiency rates under low temperatures than under the control and high-temperature conditions (Bhandari et al. 2018; Aazami et al. 2021). In our study, this indicates a decline in the utilization of photosynthetic energy under low-temperature stress in the lettuce cultivars examined, consistent with findings by Hou et al. (2016), who reported similar responses in watermelon seedlings. Additionally, Lu et al. (2022) observed reduced photosynthetic efficiency in tomato seedlings under cold stress conditions. Although all treatments showed a declining trend from day 0 onwards, the control group exhibited a gradual increase over time. In particular, the reduction in $Y(PSII)$, which is a key parameter related to photochemical efficiency measurements, under both low- and high-temperature conditions can be attributed to excessive photorespiration at high temperatures (Argosubekti 2020).

Non-photochemical quenching (NPQ) can be used to assess the photoprotective processes that plants use to adapt to stressful environments (Murchie and Lawson 2013). On day 2 of the treatment here, NPQ decreased but showed a gradual increase in the control group from day 4 onwards, continuing to decline, however, under both high- and low-temperature conditions. This indicates that lettuce grown under these types of temperature stress is unable to activate protective mechanisms due to the progression of senescence (Gorbe and Calatayud 2012). Furthermore, an increase in qN suggests heightened thermal dissipation, with a significant difference observed under the low-temperature conditions compared to the other treatments. This implies that photoprotective mechanisms are activated to safeguard Photosystem II from cold stress (Oliveira and Peñuelas 2005). Finally, Rfd , which reflects the activity of PSII and the electron transport process when the plant is exposed to light, continuously declines at both high and low temperatures, indicating reduced photosynthetic efficiency (Bron et al. 2004; Bhandari et al. 2018). A more rapid decline under high-temperature stress suggests that photorespiration caused by high temperatures leads to a swift reduction in fluorescence, signifying the inefficient use of light energy.

Chlorophyll a and b, the primary photosynthetic pigments in plant leaves, are known to increase or decrease upon exposure to abiotic and biotic stress factors (Barbagallo et al. 2003; Kalaji et al. 2016; Taïbi et al. 2016). The continuous decline observed under cold stress suggests that the negative impact of low temperatures in the presence of light leads to decreased photosynthetic activity, damage to chloroplast membranes and structures and the inhibition of chlorophyll biosynthesis (Li et al. 2010; van Buer et al. 2019). Similar findings have been reported in various plant genetic resources and growth stages under cold stress, where the chlorophyll content was reduced (Koç et al. 2010; Hussain et al. 2023). Conversely, although the high-temperature stress treatment visually exhibited lighter leaf coloration than the control

plants, the actual analysis showed no significant difference in the chlorophyll content. This result is consistent with observations involving other plant species.

Proline, a multifunctional amino acid, is synthesized and accumulates in plants in response to various abiotic stresses, serving as a key osmoprotectant and stabilizer of cellular structures (Verslues and Sharma 2010). The extent of proline accumulation is known to vary depending on the type and severity of the stress, including salinity, drought, heat, and cold (Trovato et al. 2008; Toscano et al. 2019). In the present study, a progressive increase in the proline content was observed in lettuce seedlings subjected to low-temperature conditions, indicating an osmotic adjustment response to cold stress. In contrast, seedlings exposed to high-temperature stress exhibited minimal changes in proline levels. This phenomenon is attributable to the nature of heat stress, which predominantly induces oxidative stress through the overproduction of reactive oxygen species (ROS) rather than imposing significant osmotic stress (Amini et al. 2015). As a result, plants experiencing high temperatures may preferentially activate antioxidant defense mechanisms over osmolyte accumulation pathways. These findings are consistent with previous reports demonstrating limited proline accumulation under heat stress conditions in lettuce.

The temperature conditions in the lettuce seedlings significantly influenced fructose, glucose, sucrose, and total sugar content findings. Both high- and low-temperature conditions lead to a substantial increase in specific sugar contents, with particularly pronounced increases in all sugars under cold conditions (Fahad et al. 2017; Wang et al. 2018; Yu et al. 2022). These findings suggest that temperature changes play a critical role in plant carbohydrate metabolism and sugar accumulation.

This study analyzed the effects of temperature stress on the correlation among chlorophyll fluorescence (CF) parameters, photosynthetic efficiency indicators, chlorophyll content levels, and proline levels. Major CF parameters such as F_v/F_m showed strong positive correlations with other parameters, indicating a close relationship with the PSII efficiency. In contrast, the proline content was negatively correlated with CF parameters, suggesting a potential decrease in photosynthetic efficiency with increased stress. The chlorophyll content (Chl a, Chl b, and total chlorophyll) also showed strong positive correlations, highlighting its crucial role in photosynthetic capacity. These results enhance our understanding of the physiological responses and energy utilization efficiency of plants under temperature stress conditions.

Conclusions

This study presented changes in the chlorophyll fluorescence, growth, and secondary metabolite levels in lettuce under three temperature treatments, providing insights into the detection of temperature stress responses. The chlorophyll fluorescence curves exhibited the potential for detecting responses to high- and low-temperature stress in lettuce. Existing fluorescence parameters have identified highly sensitive chlorophyll fluorescence indicators at the canopy level that respond to temperature stress. Changes in energy-partitioning parameters revealed photoprotective mechanisms under cold conditions. Additionally, chlorophyll fluorescence parameters, the chlorophyll content, and proline and sugar levels were strongly correlated with the physiological characteristics. The findings of this study suggest that a correlation analysis of the chlorophyll fluorescence curves and a secondary metabolite analysis can provide comprehensive information pertaining to the effects of temperature stress on photosynthesis and secondary metabolism processes.

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