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Long-term high temperature stress decreases the photosynthetic capacity and induces irreversible damage in chrysanthemum seedlings

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Abstract: To study the effects of long-term and short-term high temperature stress and recovery on the physiological functions and appearance quality of chrysanthemums, a controlled experiment with chrysanthemums was conducted. The treatments were 25 °C for 3 days (T_1D_3), 25 °C for 9 days (T_1D_9), 41 °C for 3 days (T_2D_3) and 41°C for 9 days (T_2D_9). The results indicated that there is no significant difference between the T_1D_3 and T_1D_9 groups. Conversely, the total chlorophyll content (Chl), net photosynthetic rate (P_N), and maximum quantum yield of Photosystem II (PSII) (F_V/F_M) under T_2D_3 and T_2D_9 decreased by 27.07%, 43.30%, 5.62%, and 44.85%, 68.22%, 8.29%, respectively. The JIP-test results showed that the T_2D_9-stressed plants had a lower efficiency and functional antenna size, and a higher activity of the reaction centre than T_2D_3. The contents of malondialdehyde, soluble protein and proline increased by 3.67 nmol/g FM, 298.75 μg/g, and 192.99 μg/g, and the antioxidant enzymes activities were inhibited significantly under T_2D_9. After the stress was relieved, Chl, P_N, and F_V/F_M under T_2D_3 recovered to the same level as T_1D_3, while T_2D_9 did not. Furthermore, the diameter of the flowers in T_2D_3 showed no significant difference with the chrysanthemums under T_1D_3. However, the plants in T_2D_9 recovered poorly. Both the diameter of the flowers and the anthocyanin under T_2D_9 reduced significantly comparing with T_1D_9, indicating that the damage in the chrysanthemum seedlings caused by long-term high temperature was irreversible.

Keywords: antioxidant enzymes; chlorophyll fluorescence; heat stress; photosynthesis; reactive oxygen species

Chrysanthemum morifolium is one of the most popular ornamental cut or potted plants in the world (Zhang et al. 2020; Chumber, Jhanji 2022). The potted autumn chrysanthemum, which is basically in full bloom from October to November, generally needs to be planted in the summer in most areas of China, such as the Jiangsu, Henan, Anhui, and Shandong Provinces (Zhang et al. 2021). However, most parts of China are hot in the summer, especially in the middle and lower reaches of the Yangtze River, which is mainly affected by the East Asian subtropical anticyclone in summer, belonging to a subtropical monsoon humid climate. The extreme temperature is as high as 42 °C that lasts for 3–15 days, which has become the main factor limiting the growth and development of the chry-

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santhemum (Zhang et al. 2005; Zhou et al. 2018; Luo et al. 2022). The suitable temperature of chrysanthemum seedlings for growth ranges from 16 to 25 °C. Previous studies demonstrated that high temperatures could cause various limits to the growth and development of plants (Nakano et al. 2020; Xu et al. 2020a; Yang et al. 2022). For example, Xue et al. (2018) found that the growth indicators, such as the leaf width, petiole length, and per unit area yield, decreased with increased high-temperature stress of 25–38 °C. Zhang et al. (2021) demonstrated that temperatures above 35 °C can severely limit the photosynthetic characteristics and photosynthetic pigment content of the chrysanthemum leaves of plants. Yang et al. (2019) indicated that short-term heat shock at 40 °C helped improve the heat tolerance of tomatoes and the damage was reversible.

The frequencies and durations of the occurrence of extreme temperature events in the summer have significantly increased since the 1990 s (Kim, Choi 2017), which has become a major abiotic stress leading to delayed flowering and abnormal production of chrysanthemums (Huh et al. 2004; Nozaki, Fukai 2008).

Photosynthesis is the basic source of energy and organic matter, such as carbohydrates, amino acids, and proteins, that is required for the plants' survival (Jurczyk et al. 2019; Kadir et al. 2006; Yang et al. 2020). High temperature stress has proven to be one of the most important environmental factors affecting the physiological traits related to the photosynthesis of plants (Singh, Singh 2015; Zhang et al. 2022). It has been demonstrated that the main components of plant photosynthesis, including carbon reduction cycles, thylakoid electron transport, and the control of the stomatal conductance, can be destroyed by high temperatures (Allen, Ort 2001; Singh, Singh 201). Whereas, if the stress is detected in time, damage can be avoided and the plants can regain their full photosynthetic capacity (Crafts-Brandner, Law 2000; Crafts-Brandner, Salvucci 2000; Salvucci et al. 2001).

High temperatures might severely affect the photosynthetic electron transport system of plants and even cause Photosystem II (PSII) inactivation and thylakoid disorganisation, which might be irreversible (Havaux 1993; Berry, Bjorkman 2003; Xu et al. 2021b). Chlorophyll fluorescence, which can be used to measure changes in the PSII photochemistry, CO_2 assimilation, and linear electron flux *in vivo*, is generally considered to be an effective probe to learn the photosynthesis and fluorescence parameters of plants under high temperature stress (Larcher 1995; Maxwell, Johnson 2000; Baker 2008; Shi et al. 2022). Chlorophyll fluorescence is closely related to most photosynthesis reactions and provides various information about the effects of plants under adverse stress. Previous studies demonstrated that the maximum quantum yield of PSII (F_V/F_M) of dark-adapted leaves is an excellent indicator to detect thermal stress (Andrews et al. 1995; Fracheboud et al. 1999; Lu et al. 2020). Besides, the JIP-test has been extensively used as another indicator for abiotic stress by analysing the polyphasic rise of the chlorophyll (Strasser et al. 2000; Mathur et al. 2011; Su et al. 2022).

Previous research manifested that the photosynthesis intensity of plants decreased sharply under high temperature stress (Lu et al. 2017; Li et al. 2020). The cell structure, gene expression and metabolism of the cells in the leaf suffered severe damage during this process (Gill, Tuteja 2010; Li, Yang 2021). Meanwhile, the contents of the lipid peroxidation and reactive oxygen species (ROS) of the leaves increased, causing photoinhibition for crops due to the great toxicity to the proteins, lipids and nucleic acids (Takahashi, Murata 2008; Gill, Tuteja 2010).

In biological systems, however, antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), play a vital role in fighting against oxidative stress and helping maintain normal cellular components and metabolic function (Begara-Morales et al. 2017). A high concentration of ROS may destroy antioxidant defence system, ultimately resulting in irreversible damage to the photosynthetic apparatus (Munné-Bosch, Pintó-Marijuan 2017; Zheng et al. 2021). Besides, another common indicator for the measurement of plant oxidative stress, malondialdehyde (MDA), is widely used to determine the non-enzymatic forms of lipid peroxidation (Tsikas 2017). Previous studies demonstrated that the MDA content of plants significantly increased under extreme temperature stress, and the longer the stress duration lasted, the more obvious the increase was (Xu et al. 2020c, 2021b; Zhang et al. 2022). Cheng et al. also concluded that the MDA of chrysanthemums under extreme low temperatures significantly increased (Cheng et al. 2018). However, research on the variety of the MDA for chrysanthemums at long-term high temperatures is lacking.

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The reproductive growth of chrysanthemums was affected by the high temperature stress in the summer, including delayed flowering and decreased anthocyanin content (Shaked-Sachray et al. 2002). The senescence rate of small inflorescences for summer chrysanthemums was significantly accelerated under temperatures above 30 °C (Sun et al. 2013). High temperature stress had a more serious impact on the growth of chrysanthemum plants. The new leaves could not normally spread under a short-term treatment with 40 °C, and the middle and underneath leaves prolapsed and dried under the long-term treatment (Sun et al. 2013; Cho, Kim 2021).

Previous studies on the effects of heat stress on plants basically concentrated on short-term treatments (Seliem et al. 2020; Xu et al. 2021a), while ignoring the harm of long-term high temperature stress on the physiological traits related to the photosynthesis and growth of plants. Moreover, whether the effects caused by the short-term or long-term high temperatures were both reversible was still unclear. In addition, previous studies on the impacts of plants under high temperatures mainly concentrated on single aspect of either the physiological mechanism or appearance quality (Long et al. 2022). Investigations into changes to the internal physiological indicators (including photosynthetic characteristics, antioxidant enzyme activity, proline, protein), as well as the appearance quality indicators (including the diameter and anthocyanin content of the flowers) of chrysanthemums under long-term and short-term heat stress is lacking. Therefore, the study was conducted to explore whether different effects existed on the chrysanthemum's physiological characteristics as well as its appearance quality under long-term and short-term high temperature stress.

MATERIAL AND METHODS

Plant material and growth conditions. The experiments were carried out from April to July 2021 in Venlo-type glasshouse of the Agricultural Meteorological Experimental Station at Nanjing University of Information Science and Technology (32°14'N, 118°42'E). Chrysanthemum seedlings (*Chrysanthemum morifolium* cv. 'Hongmian') were grown in 22 × 15 × 15.5 cm pots filled with a soil substrate:vermiculite:perlite mixture of 1:1:1 (v:v:v) and cultivated in an artificial climate chamber (BDW40, Conviron, Canada). The conditions were set as: a 25/15 °C (day/night) temperature, 60 ± 5% relative humidity, a 12/12 h light/dark photoperiod, and a photosynthetic photon flux density (*PPFD*) of 1 000 ± 25 μmol/m^2/s. The plants were watered once every two days with tap water and once a week with water containing N fertiliser.

Experimental materials and treatments. Healthy and uniform chrysanthemum seedlings, 15-cm in height, were selected and transferred into other artificial climate chambers for the experimental treatments. As shown in Table 1, the experiment was designed for four treatments, which were 25/15 °C (day/night) for 3 days (T_1D_3), 25/15 °C (day/night) for 9 days (T_1D_9), 41/31 °C for 3 days (T_2D_3) and 41/31 °C for 9 days (T_2D_9). The potted plants in the 41/31 °C room were then transferred into the 25/15 °C room after the treatments. All the plants were permitted to recover for 5, 10, and 15 days. The environmental conditions of each artificial climate chamber during the experiment were as follows: a photoperiod of 10/14 h (day/night), relative humidity of 60 ± 5%, and an illumination intensity of 1 000 ± 25 μmol/m^2/s. Three healthy plants per treatment were used for the measurements and three biological repetitions were measured on the 5th to 8th fully expanded healthy leaves from the top on each plant in the same way in all the groups.

Photosynthetic pigment content. The Chl *a* and Chl *b* contents were assayed by the method of Arnon (1949). A crushed leaf sample was placed in a glass tube with a 4.5:4.5:1 acetone:ethanol:water ratio by volume for 48 h in darkness until the photosynthetic pigments in the leaves were completely extracted. Then, the absorbance values were measured at 663 and 646 nm by using an ultraviolet spectrophotometer (Cary 50 Conc UV-VIS, Varian, Victoria, Australia). The Chl *a* and

Table 1. Experimental design table

Treatment	Temperature (°C)	Duration (days)
T_1D_3	25	3
T_1D_9	25	9
T_2D_3	41	3
T_2D_9	41	9

T_1D_3 – 25/15 °C (day/night) for 3 days; T_1D_9 – 25/15 °C (day/night) for 9 days; T_2D_3 – 41/31 °C for 3 days; T_2D_9 – 41/31 °C for 9 days

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Chl *b* contents were determined according to the following formulas:

$$\text{Chl } a = 13.95 \times D_{663} - 6.68 \times D_{646}$$

$$\text{Chl } b = 24.96 \times D_{646} - 7.23 \times D_{663}$$

$$\text{Chl} = \text{Chl } a + \text{Chl } b$$

where: Chl *a*, Chl *b* – the Chl *a* and Chl *b* contents in the chrysanthemum leaves (mg/g FM); Chl – the sum of Chl *a* and Chl *b*; D_{663}, D_{646} – the absorbance measured at 663 and 646 nm.

Gas-exchange parameters. The measurements of the gas-exchange parameters were conducted on the 5th to 8th fully expanded healthy leaves from the top between 9:00–11:00 am with an LI-6400 (LI-COR Bioscience Inc., USA) portable photosynthesis system. The stomatal conductance (g_s), intercellular CO_2 concentration (C_i), net photosynthetic rate (P_N), transpiration rate (E), vapour pressure deficit (*VPD*) were obtained automatically by the LI-6400 under a photosynthetic photon flux density (*PPFD*) of 1 000 μmol/m^2/s. The CO_2 concentration and relative humidity in the leaf chamber was set as 400 ± 10 ppm and 60 ± 5%, respectively. The stomatal limitation value (L_s) was then determined by the following formula according to Xu et al. (2019):

$$L_s = (C_a - C_i)/C_a \times 100\%$$

where: C_a, C_i – atmospheric and intercellular CO_2 concentration, respectively.

Chl fluorescence parameters. The 5th to 8th fully expanded functional leaves from the top were selected to measure the Chl fluorescence parameters between 9:00–11:00 am by using a plant efficiency analyser (Handy PEA, Hansatech Instrument, UK). Before each fluorescence measurement, a leaf clip was attached to the sample leaf of the experimental plants for 30 min to achieve dark adaption. The minimal fluorescence (F_0), maximal fluorescence (F_M), maximal variable fluorescence (F_V), maximum quantum yield of the PSII (F_V/F_M) were measured automatically. The following fluorescence parameters reflecting the photosynthetic activity of PSII based on the JIP-test were calculated according to Yusuf et al. (2010):

absorption flux (of antenna Chls) (ABS) per reaction centre (RC):

$$ABS/RC = M_o\,(1/V_J) \times (1/\phi_{Po})$$

trapping flux (leading to Q_A reduction) (TR_0) per RC:

$$TR_0/RC = M_o\,(1/V_J)$$

electron transport flux (further than Q_A^-) (ET_0) per RC:

$$ET_0/RC = M_o\,(1/V_J) \times \Psi_o$$

electron flux reducing end electron acceptors at the PSI acceptor side (RE_0) per RC:

$$RE_0/RC = M_o\,(1/V_J)\,\Psi_o\,\delta_{Ro}$$

quantum yield for reduction of end electron acceptors at the PSI acceptor side (ϕ_{Ro}):

$$\phi_{Ro} = RE_0/ABS = TR_o/ABS\,(1 - V_I)$$

performance index (potential) for energy conservation from exciton to the reduction of intersystem electron acceptors (PI_{ABS}):

$$PI_{ABS} = (RC/ABS)[\phi_{Po}/(1 - \phi_{Po})][\,\Psi_o/(1 - \Psi_o)]$$

performance index (potential) for energy conservation from exciton to the reduction of PSI end acceptors (PI_{total}):

$$PI_{total} = PI_{ABS}(\delta_{Ro}/1 - \delta_{Ro})$$

where: M_o – approximated initial slope (m/s) of the fluorescence transient normalized on the maximal variable fluorescence F_V; V_J – relative variable fluorescence at the J-step; Ψo – efficiency that an electron moves further than Q_A^-; δ_{Ro} – efficiency with which an electron from the intersystem electron carriers moves to reduce end electron acceptors at the PSI acceptor side (RE).

Antioxidant enzymes activities. The crushed leaf samples (0.5 g) were ground with 5 mL of a phosphate buffer (pH 7.8) in a mortar. The homogenate was then centrifuged at 4 000 rpm at 4 °C for 20 min. The supernatant was detected to determine the activities of the antioxidant enzymes. The superoxide dismutase (SOD; EC 1.15.1.1) activity, expressed as U/mg (protein), was measured by the method of using nitro blue tetrazolium (NBT)

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described by Dhindsa et al. (1981). The catalase (CAT; EC 1.11.1.6) activity, expressed as μmol (H_2O_2 oxidised)/min/mg (protein), was determined by the potassium permanganate titration method according to Aebi (1984). The peroxidase (POD, EC 1.11.1.7) activity, expressed as μmol (H_2O_2 reduced)/min/mg (protein), was measured based on the guaiacol chromogenic method according to Kwak et al. (1995).

Lipid peroxidation. The estimation of the malondialdehyde (MDA) content is considered an effective method for the determination of lipid peroxidation according to Camejo et al. (2005). The fresh leaf samples (0.2 g) were ground with 5 mL of a phosphate buffer (pH 6.5) in a mortar and then centrifuged at 4 000 rpm at 4 °C for 20 min. Then, 2 mL of the supernatant was reacted with 2 mL of 0.6% thiobarbituric acid (TBA) and incubated at 100 °C for 30 min to produce the chromogen thiobarbituric acid-malondialdehyde (TBA-MDA). The mixture was then cooled rapidly and centrifuged at 4 000 rpm at 4 °C for 10 min. The supernatant was measured at 600, 532 and 400 nm by using the ultraviolet spectrophotometer (Cary 50 Conc UV-VIS, Varian, Victoria, Australia). The MDA content, expressed as nmol/g FM, was calculated based on the following formulas:

$$MDA = 6.45(A_{532} - A_{600}) - 0.56A_{450}$$

where: A_{532}, A_{600} and A_{450} – the absorbance measured at 532, 600 and 450 nm, respectively.

Measurement of soluble protein and proline contents. The fresh leaf samples were ground with 3 mL of the phosphate buffer (pH 7.8) placed on an ice pack and centrifuged at 6 000 rpm at 4 °C for 15 min. The supernatant was then used to assay the soluble protein content by the method of Coomassie brilliant blue G-250, as described by Whitham et al. (1994). The soluble protein content was expressed as μg/g.

The leaf tissue (0.2 g) was set into a glass tube, 5 mL of 3% sulfosalicylic acid was added and then boiled for 10 min. Two millilitres (2 mL) of extraction was placed into another glass tube and 2 mL of glacial acetic acid was added along with 4 mL of 2.5% ninhydrin solution to each tube. Then, the tubes were boiled with shaking for 60 min. Four millilitres (4 mL) of toluene was added into each tube when they cooled down to room temperature and then they were centrifuged at 3 000 rpm for 5 min. The supernatant was determined at 520 nm by using the ultraviolet spectrophotometer (Cary 50 Conc UV-VIS, Varian, Victoria, Australia). The proline content was calculated by using commercial standard L-proline.

Diameter of the flowers and anthocyanin of the chrysanthemums. The diameter of the flowers and the anthocyanin of the chrysanthemums after the treatments were measured and recorded at the peak flowering stage. The anthocyanin was extracted and quantified by the water bath shaking extraction from 1 g of the flower petals as described by Oren-Shamir and Nissim-Levi (1999). Three biological repetitions were measured on the fully blooming healthy flowers on each plant in the same way in all the groups.

Data analysis. The data reported in all the figures and tables were expressed as the mean of three replicates ± standard deviation (SD). The statistical analysis of the data was conducted by using SPSS 21.0 (SPSS, Chicago, USA). The statistical differences between all the treatments were evaluated by using the one-way analysis of variance (ANOVA) method ($P < 0.05$). All the figures were drawn by Origin Pro 8.0 (OriginLab, Northampton, MA).

RESULTS

Photosynthetic pigment content. The total Chl contents of the chrysanthemum plants for the T_1D_3 and T_1D_9 groups were both basically unchanged during the treatment and recovery (Table 2). Moreover, the Chl contents of the plants under the T_1D_3 and T_1D_9 treatments were even 0.04 and 0.12 mg/g FM, respectively, higher than before. After 15 days of recovery, the difference between the T_1D_3 and T_1D_9 groups was only 0.03 mg/g FM, which was much higher than that between the T_2D_3 and T_2D_9 groups (0.71 mg/g FM). However, the Chl content of chrysanthemum leaves decreased by 27.07% and 44.85% after the T_2D_3 and T_2D_9 treatments compared to the T_1D_3 and T_1D_9 ones, respectively. After the stress was relieved, the Chl content of the plants under the T_2D_3 treatment increased gradually, and there was no significant difference to the values for the T_1D_3 group after 15 days of recovery. However, the Chl content under the T_2D_9 stress decreased by 9.33% after 15 days of recovery, which was significantly lower than that of the T_1D_9 group.

Gas-exchange parameters. The gas-exchange parameters of the chrysanthemum plants showed

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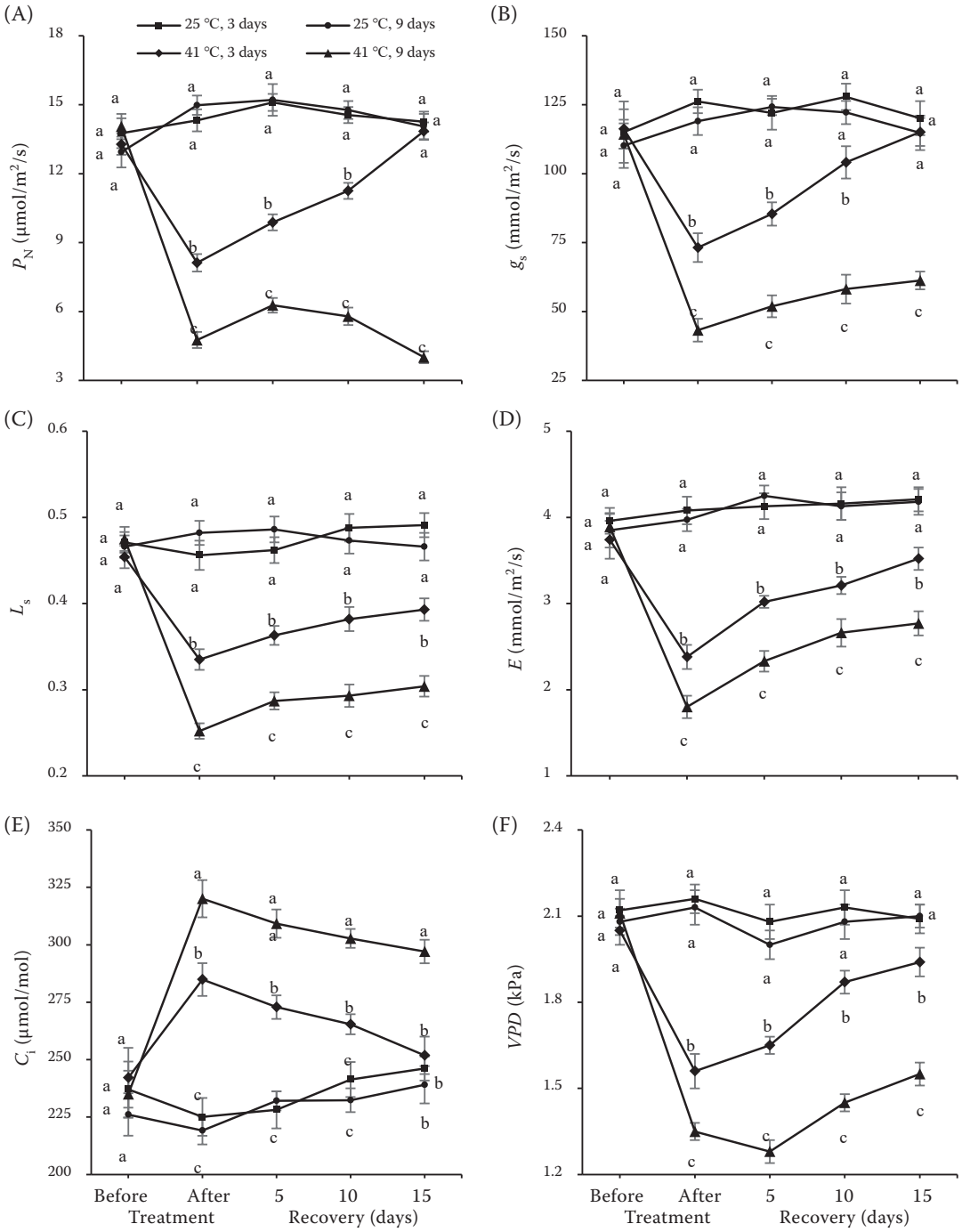

Figure 1. Effects of the treatment and recovery on the photosynthetic parameters in the chrysanthemum leaves after exposure to high temperature stress for a long time: net photosynthetic rate (P_N) (A), stomatal conductance (g_s) (B), stomatal limitation value (L_s) (C), transpiration rate (E) (D), intercellular CO_2 concentration (C_i) (E), vapour pressure deficit (VPD) (F)

Different lowercase letters in the same column represent significant differences at a level of 0.05 by Duncan's test

no significant difference between the T_1D_3 and T_1D_9 groups during the treatment and recovery. The P_N under the T_1D_9 group was 4.61% higher than that under the T_1D_3 group. After treatment, however, the P_N under the T_2D_3 and T_2D_9 treatments decreased significantly by 43.30% and 68.22%, respectively, compared to the T_1D_3 and T_1D_9 (Figure 1A) treatments. Meanwhile, the g_s and L_s significantly

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decreased after exposure to high temperature stress for 3 and 9 days, but the C_i increased significantly (Figure 1B, C, E). With the extension of the recovery period, the P_N, g_s, and L_s of the chrysanthemum plants increased gradually under the T_2D_3 and T_2D_9 treatments except for the P_N under the T_2D_9 treatment, which presented an increase at first and then a decrease, while the C_i of the chrysanthemum plants showed a downward trend. After 15 d of recovery, the P_N, g_s, and C_i under T_2D_3 showed no significant difference from that of the T_1D_3 group, while the P_N, g_s, and C_i under T_2D_9 were still 71.46%, 46.63% lower and 24.26% higher than T_1D_9, respectively. In addition, the high temperature stress inhibited not only the photosynthetic capacity, but also the transpiration of the chrysanthemum plants. It caused a decrease in the *VPD* by 27.78% and 36.62% after the T_2D_3 and T_2D_9 treatments compared to the T_1D_3 and T_1D_9 treatments, respectively.

Chl fluorescence parameters. Figure 2 shows the changes in the F_0, F_V, F_M and F_V/F_M during the treatment and recovery in four groups. After the T_2D_3 and T_2D_9 treatments, the F_0 values increased significantly by 25.04% and 46.34% compared to the T_1D_3 and T_1D_9 groups, and then began to decline when the plants were transferred to the suitable environment. The F_V, F_M and F_V/F_M of T_2D_3 and T_2D_9 group showed a decrease after the treatments and then showed an increasing trend during recovery. After 15 days of recovery, the F_0, F_V, F_M and F_V/F_M values under the T_2D_3 treatment basically recovered to the T_1D_3 level. However, the F_V, F_M and F_V/F_M values under the T_2D_9 treatment were still 24.10%, 27.01% and 3.84% lower than those of the T_1D_9 group, respectively. As for the plants under the T_1D_3 and T_1D_9 treatments, the F_0, F_V, F_M and F_V/F_M showed no significant difference between those two groups.

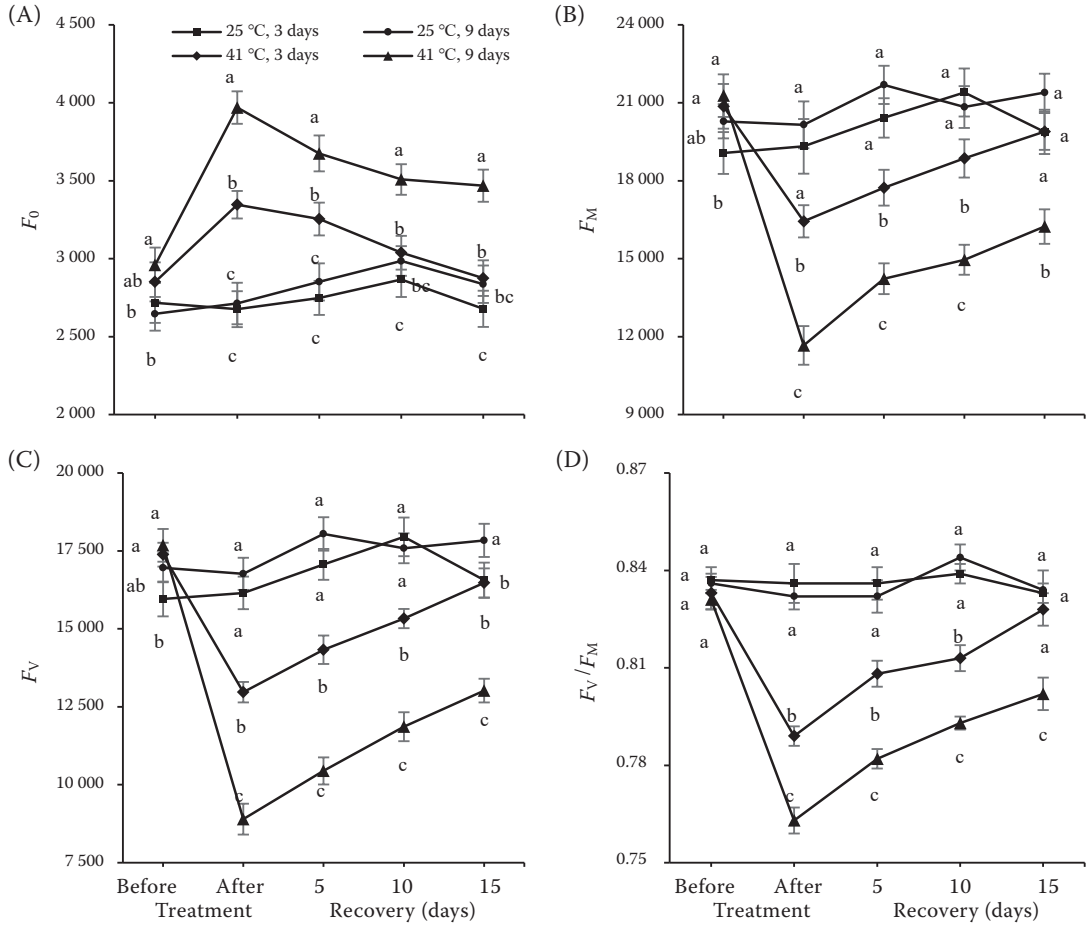

Figure 2. Effects of the treatment and recovery on the F_0, F_V, F_M and F_V/F_M of the chrysanthemum leaves after exposure to high temperature stress for a long time: minimal fluorescence (F_0) (A), maximal fluorescence (F_M) (B), maximal variable fluorescence (F_V) (C) and maximum quantum yield of the PSII (F_V/F_M) (D)

Different lowercase letters in the same column represent significant differences at a level of 0.05 by Duncan's test

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After exposure to high temperature stress for 9 days, the ABS/RC, TR_0/RC, ET_0/RC and RE_0/RC increased by 42.86%, 40.24%, 37.50% and 52.94%, respectively, compared to the T_1D_9 group (Table 3). The T_2D_9 stress also negatively affected either the PI_{ABS} or PI_{total} and the values significantly decreased by 50.96% and 48.90%, respectively, compared to the plants grown at 25 °C for 9 days. After 15 days of recovery, the values of the plants under the T_2D_9 stress could not return to a similar level to that of the T_1D_9 group. Similarly, the trends of all the fluorescence parameters above the plants subjected to high temperature stress for 3 days were similar to that under the T_2D_9 treatment. However, the ET_0/RC, RE_0/RC and PI_{total} values under T_2D_3 recovered to the T_1D_3 level after the subsequent recovery. In addition, there was no significant difference between the fluorescence parameters above the plants for the T_1D_3 and T_1D_9 groups during the entire experience.

Antioxidant enzymes activities. The SOD, CAT and POD activities increased in different degrees after the T_2D_3 and T_2D_9 treatments. Plants grown at 41 °C for 9 days showed the highest SOD, CAT and POD activities. With the prolongation of the recovery period, the activities of the antioxidant enzymes of the plants under the T_2D_3 treatment rapidly decreased and the POD activity even recovered to the same level as the T_1D_3 group after 15 days of recovery. However, the high temperature stress for 9 days severely affected the plants and the SOD, CAT and POD activities of T_2D_9 were still significantly different from that of the T_1D_9 group after recovery for 15 days.

Lipid peroxidation. The MDA contents showed no significant difference between the chrysanthemum plants in the T_1D_3 and T_1D_9 groups during the experiments (Table 4). The difference in the MDA contents between the T_1D_3 and T_1D_9 groups was less than 0.30 nmol/g FM. However, the MDA contents of the plants which was exposed to high temperature stress for 3 and 9 days increased by 63.13% and 93.94%, respectively, compared to the T_1D_3 and T_1D_9 groups, suggesting that the longer the high temperature stress duration is, the more significant the increment. After the stress was relieved, the MDA contents of the plants under the T_2D_3 and T_2D_9 treatments gradually decreased in different degrees. However, it was still 17.01% higher than the T_1D_3 group at 15 days of recovery under the T_2D_3 stress, which was in contrast to 52.36% for the T_2D_9 group compared to the T_1D_9 group.

Soluble protein and proline contents. The plant samples showed similar soluble protein and proline contents under the T_1D_3 and T_1D_9 groups while they showed an increase at first and then a downward trend under the T_2D_3 and T_2D_9 treatments throughout the experiment (Figure 4). The peaks of the protein and proline contents appeared in the T_2D_9 group after treatment, which was 2.2 and 3.3 times higher than T_1D_9, respectively. At the beginning of the recovery, the soluble protein and proline contents of the heat-treated leaves under the T_2D_9 group reduced more slowly compared with the T_2D_3 group. After 15 days of recovery, the protein and proline contents of T_2D_3 and T_2D_9 showed recovery rates of 21–37% and 9–25%, respectively, but could not recover to the T_1D_3 and T_1D_9 levels. The soluble protein and proline contents under the T_1D_3 and T_1D_9 treatments during the entire experiment were lower than 260 and 100 μg/g, respectively.

Diameter of the flowers and anthocyanin of the chrysanthemums. As an ornamental plant, both the size and colour of the flowers are vital morphological indicators for assessing the appearance quality of chrysanthemums. The diameter and anthocyanin content of flowers could exactly reflect the size and colour of the chrysanthemums, respectively (Xu et al. 2020b). According to Table 5, there was no significant differences in the two indices of the chrysanthemums between the T_1D_3 and T_1D_9 groups. In addition, the diameter of the flowers under the T_2D_3 treatment also showed no significant difference with the chrysanthemums under 25 °C for 3 or 9 days. Although the anthocyanin of the plants of the T_2D_3 group was lower than that of T_1D_3, the mean difference was just 6.09 μg/g compared to T_1D_3. However, the diameter of the flowers and the anthocyanin content under the T_2D_9 treatment significantly decreased by 40.41% and 69.04% compared to the T_1D_9 group, respectively.

DISCUSSION

Photosynthesis is one of the main determinators to supply the necessary energy for a plant's growth and development (Lu et al. 2017). In this study, long-time high temperature stress caused a significant decrease in the P_N (Figure 1A), which led to the severe inhibition of the photosynthesis. The reasons for the decline in the photosynthesis include stomatal factors and non-stomatal factors

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Table 2. Effects of the treatment and recovery on the total chlorophyll content in the chrysanthemum leaves after exposure to high temperatures for a long time (mg/g FM)

Treatment	Before treatment	After treatment	Days of recovery		
			5	10	15
T_1D_3	1.29 ± 0.06[a]	1.33 ± 0.06[a]	1.44 ± 0.05[a]	1.41 ± 0.03[a]	1.43 ± 0.04[a]
T_1D_9	1.24 ± 0.04[a]	1.36 ± 0.03[a]	1.42 ± 0.02[a]	1.38 ± 0.06[ab]	1.46 ± 0.07[a]
T_2D_3	1.33 ± 0.05[a]	0.97 ± 0.05[b]	1.08 ± 0.03[b]	1.26 ± 0.07[b]	1.39 ± 0.03[a]
T_2D_9	1.31 ± 0.08[a]	0.75 ± 0.02[c]	0.71 ± 0.06[c]	0.75 ± 0.04[c]	0.68 ± 0.02[b]

T_1D_3 – 25/15 °C (day/night) for 3 days; T_1D_9 – 25/15 °C (day/night) for 9 days; T_2D_3 – 41/31 °C for 3 days; T_2D_9 – 41/31 °C for 9 days; different lowercase letters in the same column represent significant differences at a level of 0.05 by Duncan's test

(Farquhar, Sharkey 1982). The results showed that the T_2D_3 and T_2D_9 stresses caused a decrease in the P_N of the chrysanthemum leaves, while the C_i increased, suggesting that the decline in the photosynthesis was not caused by a reduction in the supply of CO_2 due to a decrease in the g_s, but was caused by the non-stomatal limitations that hindered the utilisation of CO_2, leading to the accumulation of intercellular CO_2 (Gerganova et al. 2017; Liu et al. 2019; Allen, Ort 2001). In addition, the total Chl contents of the chrysanthemum leaves also decreased due to the T_2D_3 and T_2D_9 stresses. After 15 days of recovery, the total Chl contents under the T_2D_3 stress restored to a similar level as the T_1D_3 group, while this did not happen under the T_2D_9 stress (Table 2). The reason was mainly that the long-term heat stress aggravated the oxidative stress of the cells and inactivated the membrane system, resulting in irreversible damage.

A large decline in the P_N accompanied by a significant and reversible decline in the F_V/F_M, as the major result of the T_2D_3 stress, which eliminated the PSII inhibition, occurred especially in the process of recovery (Figure 3A). In other words, the photoinhibition under the T_2D_3 stress did not cause the PSII damage, but it was the photoprotective mech-

Table 3. Effects of the treatment and recovery on the JIP-test analysis in the chrysanthemum leaves after exposure to high temperatures for a long time

Treatment		ABS/RC	TR_0/RC	ET_0/RC	RE_0/RC	ϕ_{Ro}	PI_{ABS}	PI_{total}
Before treatment	T_1D_3	0.94 ± 0.05[a]	0.77 ± 0.05[a]	0.52 ± 0.03[a]	0.17 ± 0.03[a]	0.142 ± 0.005[a]	11.36 ± 1.15[a]	2.49 ± 0.23[a]
	T_1D_9	0.98 ± 0.04[a]	0.76 ± 0.03[a]	0.51 ± 0.02[a]	0.15 ± 0.02[a]	0.131 ± 0.006[a]	10.61 ± 1.32[a]	2.20 ± 0.32[a]
	T_2D_3	1.02 ± 0.05[a]	0.72 ± 0.05[a]	0.53 ± 0.03[a]	0.14 ± 0.03[a]	0.136 ± 0.006[a]	13.14 ± 2.04[a]	2.55 ± 0.19[a]
	T_2D_9	0.93 ± 0.08[a]	0.78 ± 0.04[a]	0.49 ± 0.04[a]	0.17 ± 0.02[a]	0.139 ± 0.004[a]	10.17 ± 1.52[a]	2.30 ± 0.25[a]
After treatment	T_1D_3	0.99 ± 0.04[c]	0.80 ± 0.04[c]	0.51 ± 0.02[c]	0.16 ± 0.03[c]	0.138 ± 0.003[c]	10.28 ± 1.08[a]	2.21 ± 0.33[a]
	T_1D_9	1.05 ± 0.05[c]	0.82 ± 0.05[c]	0.48 ± 0.04[c]	0.17 ± 0.02[c]	0.134 ± 0.004[c]	9.95 ± 1.76[a]	2.27 ± 0.18[a]
	T_2D_3	1.32 ± 0.03[b]	1.02 ± 0.04[b]	0.59 ± 0.03[b]	0.21 ± 0.01[b]	0.203 ± 0.002[b]	6.65 ± 1.65[b]	1.52 ± 0.15[b]
	T_2D_9	1.50 ± 0.04[a]	1.15 ± 0.03[a]	0.66 ± 0.02[a]	0.26 ± 0.01[a]	0.341 ± 0.003[a]	4.88 ± 1.23[c]	1.16 ± 0.12[c]
15 days of recovery	T_1D_3	1.03 ± 0.04[c]	0.75 ± 0.04[c]	0.51 ± 0.03[b]	0.15 ± 0.02[b]	0.134 ± 0.003[c]	10.29 ± 1.86[a]	2.13 ± 0.21[a]
	T_1D_9	0.95 ± 0.05[c]	0.73 ± 0.02[c]	0.53 ± 0.02[b]	0.18 ± 0.02[ab]	0.132 ± 0.004[c]	11.01 ± 1.69[a]	2.46 ± 0.37[a]
	T_2D_3	1.12 ± 0.04[b]	0.85 ± 0.05[b]	0.52 ± 0.04[b]	0.18 ± 0.03[ab]	0.144 ± 0.002[b]	9.03 ± 1.32[b]	2.04 ± 0.12[a]
	T_2D_9	1.21 ± 0.03[a]	0.93 ± 0.04[a]	0.59 ± 0.02[a]	0.21 ± 0.02[a]	0.213 ± 0.002[a]	6.77 ± 1.05[c]	1.65 ± 0.16[b]

ABS/RC – apparent antenna size of the active PSII per reaction centre; TR_0/RC – trapped energy flux per reaction centre; ET_0/RC – electron transport flux per reaction centre; RE_0/RC – electron flux reducing the end electron acceptors at the PSI acceptor side per reaction centre; ϕ_{Ro} – quantum yield for the reduction of the end electron acceptors at the PSI acceptor side; PI_{ABS} – performance index (potential) for energy conservation from the exciton to the reduction of intersystem electron acceptors; PI_{total} – performance index (potential) for energy conservation from the exciton to the reduction of PSI end acceptors; T_1D_3 – 25/15 °C (day/night) for 3 days; T_1D_9 – 25/15 °C (day/night) for 9 days; T_2D_3 – 41/31 °C for 3 days; T_2D_9 – 41/31 °C for 9 days; different lowercase letters in the same column represent significant differences at a level of 0.05 by Duncan's test

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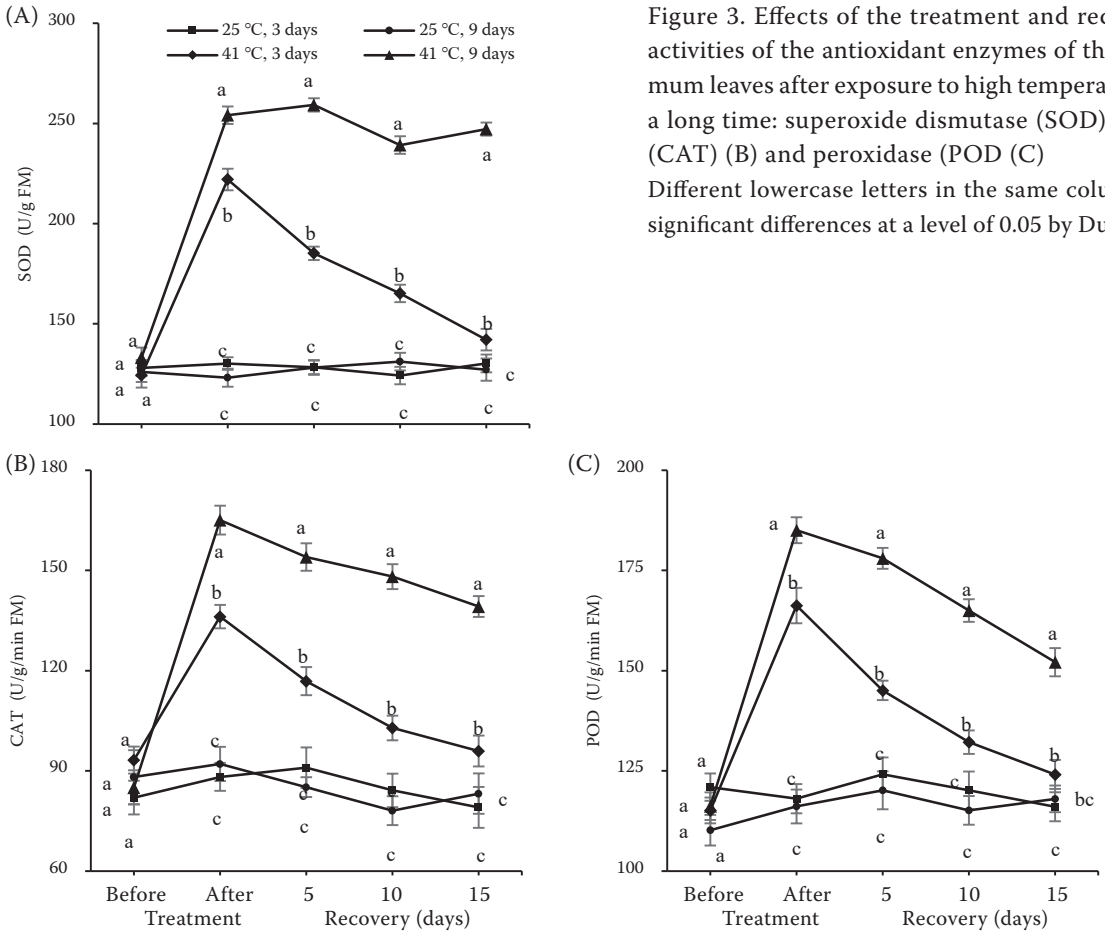

Figure 3. Effects of the treatment and recovery on the activities of the antioxidant enzymes of the chrysanthemum leaves after exposure to high temperature stress for a long time: superoxide dismutase (SOD) (A), catalase (CAT) (B) and peroxidase (POD (C)

Different lowercase letters in the same column represent significant differences at a level of 0.05 by Duncan's test

anism of the plants grown under adverse stress (Xu et al. 2020c). Similar results were reported in previous research studies that concentrated on temperature stress (Xu et al. 2019, 2020a, b). However, the ratio of F_V/F_M under 41 °C for 9 days did not recover to the group levels of 25 °C for 9 days after recovery for 15 days, suggesting that the photoinhibition of the chrysanthemum seedlings induced by the T_2D_3 stress was irreversible. Besides, the decline in the photosynthesis usually resulted in the accumulation of superfluous photon energy and PSII photoinhibition. According to Yamamoto et al. (2014), the inactivation of the PSII reaction centre was divided into two types, namely, reversible inactivation and the destruction of the reaction centre. In this study, after 15 days of recovery, the F_0 value under the T_2D_3 stress could recover to a similar level compared to the T_1D_3 group (Figure 2A), showing that short-term high temperature stress caused the reversible inactivation of the PSII reaction centre. However, the sharp decrease in the F_M and F_V, which were unrecoverable, indicated that the T_2D_9 stress

Table 4. Effects of the treatment and recovery on the lipid peroxidation (expressed as the malondialdehyde content) in the chrysanthemum leaves (nmol/g FM)

Treatment	Before treatment	After treatment	Days of recovery		
			5	10	15
T_1D_3	3.83 ± 0.11^a	3.77 ± 0.13^c	3.92 ± 0.08^c	4.05 ± 0.12^c	3.88 ± 0.09^c
T_1D_9	3.75 ± 0.09^a	3.96 ± 0.12^c	3.85 ± 0.13^c	3.92 ± 0.12^c	3.82 ± 0.08^c
T_2D_3	3.90 ± 0.08^a	6.15 ± 0.11^b	5.33 ± 0.12^b	4.87 ± 0.14^b	4.54 ± 0.11^b
T_2D_9	4.01 ± 0.10^a	7.68 ± 0.13^a	7.12 ± 0.12^a	6.38 ± 0.11^a	5.82 ± 0.09^a

T_1D_3 – 25/15 °C (day/night) for 3 days; T_1D_9 – 25/15 °C (day/night) for 9 days; T_2D_3 – 41/31 °C for 3 days; T_2D_9 – 41/31 °C for 9 days; different lowercase letters in the same column represent significant differences at a level of 0.05 by Duncan's tes

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Table 5. Effects of the treatment on the diameter of the flowers and the anthocyanin content of the chrysanthemums in the full-bloom stage

Treatment	T_1D_3	T_1D_9	T_2D_3	T_2D_9
Diameter of flowers (cm)	9.52 ± 0.71[a]	10.27 ± 0.56[a]	8.72 ± 0.48[a]	6.12 ± 0.35[b]
Anthocyanin (μg/g)	74.78 ± 2.12[a]	76.35 ± 3.33[a]	68.69 ± 2.93[b]	23.64 ± 1.77[c]

T_1D_3 – 25/15 °C (day/night) for 3 days; T_1D_9 – 25/15 °C (day/night) for 9 days; T_2D_3 – 41/31 °C for 3 days; T_2D_9 – 41/31 °C for 9 days; different lowercase letters in the same column represent significant differences at a level of 0.05 by Duncan's test

resulted in the severe destruction of the PSII reaction centre (Figure 2B, C).

A sequence of parameters, including the absorption flux (of antenna Chls) per RC (ABS/RC), trapping flux (leading to Q_A reduction) per RC (TP_0/RC), electron transport flux (further than Q_A^-) per RC (ET_0/RC), and electron flux reducing end electron acceptors at the PSI acceptor side per RC (RE_0/RC) can describe the energy conversion effectively (Yusuf et al. 2010). This study suggested that the energy for absorption, transportation, and dissipation was more severely affected under the long-term high temperature stress than that under the short-term heat shock. The chrysanthemum plants which were exposed to the T_2D_3 stress expressed higher stability in the energy flux system and utilised the excitation energy better than that under the T_2D_9 stress. Besides, the quantum yield for the reduction of end electron acceptors at the PSI acceptor side (ϕ_{Ro}) decreased under adverse stress, showing the efficiency of the intermediate energy transduction. The performance index (PI_{total}) is the most sensitive parameter of the JIP-test, including partial 'potentials' for energy conservation (Kalaji et al. 2017, 2018). It was observed that the PI_{total} of the T_1D_9 and T_2D_3 groups presented an increase indicating 'gain for energy conservation' during the recovery process, while an insignificant increase was observed in the T_2D_9 treatment manifesting in the plants exposed to the T_2D_9 stress having a poor ability to conserve energy.

Plants, exposed to different adverse stresses, might produce ROS continuously, which can lead to oxidative damage by causing disruption of the membrane lipids or DNA chain reactions, presenting a great challenge to the growth and development of plants (Cao et al. 2019; Pinto-Marijuan, Munne-Bosch 2014). However, plants can exploit the ROS-scavenging enzymes, including SOD, POD and CAT, to reduce the ROS and keep an adequate reduction or oxidation balance (Xu et al. 2019). The final product of membrane liposome peroxidation is malondialdehyde, which is used to measure the degree of peroxidation of the membrane-bound liposome, where the higher the malondialdehyde content, the more severe the membrane damage (Jiang, Huang 2001). In this study, both the high temperature treatments increased the activities of the above enzymes (Figure 3), suggesting that chrysanthemum seedlings started to utilise self-protection mechanisms to adapt to the hostile environment and reconstruct a balance between the production and removal of ROS by adjusting the activities of the ROS-scavenging enzymes (Kaushik, Aryadeep 2014; Mittler 2017). Whereas, plants under the T_2D_3 stress produced smaller changes in the biochemical responses than the plants under the T_2D_9 stress in the antioxidant enzymes activities and malondialdehyde content, indicating that the plants under short-term heat stress maintained cell integrity better than those under long-term heat stress. After 15 days of recovery, the SOD, POD and CAT activities under the T_2D_3 stress recovered to a similar level as the plants grown at a normal temperature, but not under the T_2D_9 stress, suggesting that the T_2D_9 stress broke the balance between the production and removal of ROS, which was irreversible.

The soluble protein accumulation is considered an adaptive response of plants to adverse stress. Proline is produced by plants to adjust the osmotic water potential, and proline may play a certain role in alleviating the damage caused by dehydration (Kishor et al. 1995; Sánchez et al. 1998). In the present research, we noticed that the soluble protein and proline contents were enhanced under the high temperature stress, while they did not change significantly in the T_1D_3 and T_1D_9 groups. After 15 days of recovery, the soluble protein and proline contents of the plants under the T_2D_9 stress did not recover to a normal level as T_1D_9, indicating that it was the long-term heat stress rather than the long accumulation process that caused damage to the plants.

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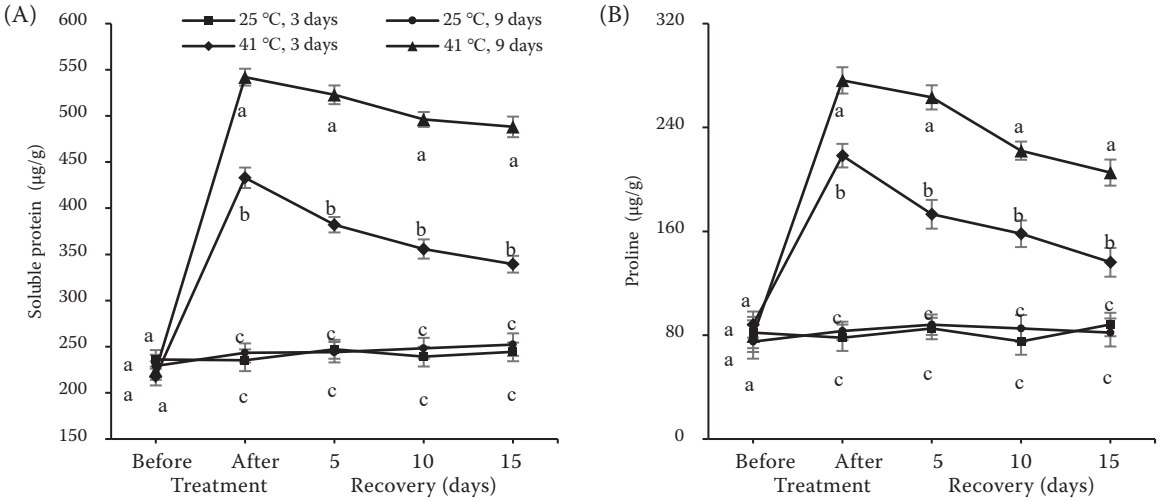

Figure 4. Effects of the treatment and recovery on the soluble protein (A) and proline contents (B) of the chrysanthemum leaves after exposure to high temperature stress for a long time

Different lowercase letters in the same column represent significant differences at a level of 0.05 by Duncan's test

The diameter and anthocyanin content of the flowers are two effective indices used to reflect the appearance and internal quality of the chrysanthemum. There was also no significant difference in either the flower diameter or anthocyanin content between the T_1D_3 and T_1D_9 groups, the same as other indices above, which strongly proved that the long-term treatment at 25 °C during the seedling stage would not cause damage to the chrysanthemum, compared with the short-term treatment. In addition, the diameter of the flowers under T_2D_3 showed no significant difference with the T_1D_3 and T_1D_9 groups, indicating that the damage caused by the short-term high temperature at 41 °C in the seedling stage of the chrysanthemum was reversible. Conversely, the diameter of the flowers and the anthocyanin content under the T_2D_9 treatment reduced sharply comparing to T_1D_9, suggesting that long-term stress at 41 °C would cause irreversible damage in both the appearance and internal quality of the chrysanthemum.

CONCLUSION

The chlorophyll content, P_N, g_s, L_s, F_V/F_M, PI_{total} presented a downward trend after high temperature stress at 41 °C for both 3 and 9 days, which indicated that heat stress caused damage to the photosynthetic capacity in the chrysanthemum seedlings. After the stress was relieved, the indicators above, as well as antioxidant enzymes activities, the malondialdehyde, soluble protein and proline contents of the plants under 41 °C for 3 days recovered to the same level as the group under 25 °C for 3 days. In addition, the diameter of the flowers and anthocyanin content of the plants under 41 °C for 3 days presented no significant difference with those under 25 °C for 3 and 9 days, while the group under 41 °C for 9 days did not, which suggests that the damage to the chrysanthemum seedlings caused by the long-term high temperature stress was irreversible. Conversely, the harm induced by the short-term heat stress could recover to the normal level after a period of at least 15 days.

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