Exogenous spermidine enhances the photosynthetic and antioxidant capacity of citrus seedlings under high temperature

Xu Chao\textsuperscript{a,b}, Tang Yuqing\textsuperscript{a}, Liu Xincheng\textsuperscript{a}, Yang Huidong\textsuperscript{a}, Wang Yuting\textsuperscript{a}, Hu Zhongdong\textsuperscript{a}, Hu Xinlong\textsuperscript{a}, Liu Buchun\textsuperscript{b}, and Su Jing\textsuperscript{c}

\textsuperscript{a}Key Laboratory of Horticultural Plant Genetics and Physiology, Institute of Horticulture, Jiangxi Academy of Agricultural Sciences, Nanchang, P. R. China; \textsuperscript{b}Institute of Environment and Sustainable Development in Agriculture, CAAS/National Engineering Laboratory of Efficient Crop Water Use and Disaster Reduction/Key Laboratory of Agricultural Environment, Ministry of Agriculture and Rural Affairs, Beijing, P. R. China; \textsuperscript{c}Nanjing Institute of Environmental Sciences, MEE, Nanjing, P. R. China

**ABSTRACT**

Studies have not fully explained the underlying mechanism of spermidine-mediated heat tolerance. This study investigated the possible role of spermidine (Spd) in regulating citrus heat tolerance. The results showed that exogenous Spd effectively alleviated the limitation of high temperature (HT) on photosynthesis. Exogenous Spd increased the chlorophyll content, net photosynthetic rate, intercellular carbon dioxide concentration, stomatal conductance, maximum and effective quantum yield of PSII photochemistry, nonphotochemical quenching coefficient, and electron transport rate in citrus seedlings under HT stress, but declined the stomatal limitation value. In addition, Spd treatment promoted the dynamic balance of the citrus enzymatic and non-enzymatic antioxidants system. Spd application significantly increased the activity of superoxide dismutase, peroxidase, catalase, ascorbic acid, and glutathione and the expression level of corresponding genes at high temperature, while reducing the content of H$_2$O$_2$ and malondialdehyde. Therefore, our findings suggested exogenous Spd significantly ameliorated citrus physiological and photosynthetic adaptation under HT stress, thereby providing helpful guidance for citrus cultivation in HT events.

**Introduction**

Because of global warming, frequent high temperature (HT) has become one of the major environmental threats that seriously limits plant growth, metabolism, and productivity.\textsuperscript{1} According to the latest IPCC synthesis report (IPCC 2021), the global surface temperature is supposed to increase by 1.5°C until the middle of the century and that would result in crop yield reductions of 2.5–16%. The most vital physiological and biochemical processes in plants are extremely susceptible to HT stress.\textsuperscript{2} The HT stress substantially decreases the photosynthesis capability and photosynthetic pigments content in plant leaves by Zhang et al.,\textsuperscript{3} reducing the photosynthetic activity in plants. The stress is associated with stomatal limitations, such as stomatal closure, and/or non-stomatal limitations. In the photosynthetic apparatus, these limitations include chlorophyll degradation, chloroplast ultrastructure damage, degradation of membrane, and the enzymatic proteins.\textsuperscript{4} The HT stress also causes oxidative damage in plant cells due to the excess production of reactive oxygen species (ROS), such as hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH$^\cdot$), superoxide (O$_2^\cdot$), among others etc.\textsuperscript{5} These damages result in cell membrane lipid peroxidation, discoloration of pigments, proteins inactivation, RNA and DNA molecules damage, which will eventually lead to plant death.\textsuperscript{6} Plants have natural defense mechanisms to resist oxidative stress to some extent by regulating underlying enzymatic and non-enzymatic antioxidant defense systems. Enzymatic antioxidants include superoxide dismutase (SOD), peroxidase (POD) and ascorbate peroxidase (APX), etc., which can maintain the balance between the production and scavenging of ROS by removing excess ROS in plants and protect plants from ROS-induced damage.\textsuperscript{7,8} Therefore, the photosynthetic capacity and antioxidant capacity are highly important for normal growth and development of plants under HT stress. Non-enzymatic antioxidants include ascorbic acid (AsA), glutathione (GSH), phenolic compounds, and tocopherols, which can regulate the functions of main cells and participate in the removal of ROS produced by plants during stress.\textsuperscript{9,10}

Polyamines (PAs) are the ubiquitous low-molecular-weight aliphatic amines that are involved in the regulation of plant growth and development.\textsuperscript{11} In higher plants, the major PAs are the diamine putrescine (Put), spermidine (Spd), and the two

**CONTACT** Hu Zhongdong \textsuperscript{a} hzdt204028@163.com \ Key Laboratory of Horticultural Plant Genetics and Physiology, Institute of Horticulture, Jiangxi Academy of Agricultural Sciences, Nanchang, P. R. China; Hu Xinlong \textsuperscript{a} 1373572417@qq.com \ Key Laboratory of Horticultural Plant Genetics and Physiology, Institute of Horticulture, Jiangxi Academy of Agricultural Sciences, Nanchang, P. R. China; Liu Buchun \textsuperscript{b} liubuchun@caas.cn \ Institute of Environment and Sustainable Development in Agriculture, CAAS/National Engineering Laboratory of Efficient Crop Water Use and Disaster Reduction/Key Laboratory of Agricultural Environment, Ministry of Agriculture and Rural Affairs, Beijing, P. R. China; Su Jing \textsuperscript{c} sujing@nies.org \ Nanjing Institute of Environmental Sciences, MEE, Nanjing, P. R. China

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tetraamines, spermine (Spm) and thermospermine (T-Spm).12 Research in recent years showed that PAs are also positive regulators implicated in defense responses to various types of environmental stresses such as salinity, chilling, drought, heat stress, and heavy metals.13,14 The PAs also have the roles in stimulating seed germination, modulating root morphology, promoting flower bud differentiation, inhibiting shoot branching, and increasing fruit set rate.15,16 In large, plants experiencing abiotic stress tend to increase PA levels, which provides tolerance by changing hormone balance, inducing antioxidant enzymes, and stabilizing membrane integrity and function.17 Spd, a common PA in plants, is involved in alleviating the detrimental effects on plants of various abiotic stresses. The accumulation of Spd in plants under heat stress is results from the improvement of high-temperature resistance. The Spd-mediated protection has a close association with the maintained chloroplast and mitochondria ultra-structures. It enhances photosynthesis and HT tolerance in wheat.18 Moreover, the Spd pretreatment alleviates the negative effects of HT on the photochemical efficiency of the photosystem II (PSII) of lettuce seedlings.19,20 Furthermore, exogenous Spd regulates antioxidant enzyme activity, reducing superoxide anion rate and malondialdehyde (MDA) content to maintain plant cell membrane stability.21 Even so, the information regarding the effects of exogenous Spd on photosynthesis and the antioxidant machinery of heat-stressed plants is limited. Therefore, it is essential to obtain a better understanding of how Spd regulates the photosynthetic performance of plants under HT stress conditions.

Citrus is the most extensively produced tree fruit crop in southern China.22 The suitable temperature for citrus growth is 15–28°C, and thus the continuous high temperature in summer inhibits citrus growth and development.23 Nonetheless, to the best of our knowledge, few studies have been focused on the potential of Spd in improving the heat tolerance in citrus. This study explored the possible role of Spd in improving heat tolerance in citrus, based on changes in photosynthetic characteristics, gas exchange traits, chlorophyll fluorescence parameters, and stability of the antioxidant system.

Materials and methods

Plant material and growth condition

The conducted experiments were from July to September 2021 in the Venlo-type glasshouse of the institute of Horticulture in Jiangxi Academy of Agricultural Sciences, Nanchang, China. The experimental ground lies between latitude 28°51′N, longitude 115°95′E, and altitude of 53 m above sea level. Individual healthy and uniform three-year-old citrus seedlings (Newhall navel orange (Citrus × sinensis cv. Osbeck)), a lowbush citrus cultivar, which is popular in southern China, were planted in pots with a top diameter of 40 cm, a bottom diameter of 50 cm, and a height of 70 cm. The pots filled with sandy loam containing organic matter of 17.75 g kg⁻¹, total nitrogen of 0.97 g kg⁻¹, total phosphorus 1.82 g kg⁻¹, total potassium 10.33 g kg⁻¹, readily available phosphorus 17.92 mg kg⁻¹, readily available potassium 136.47 mg kg⁻¹, and pH 6.63. These all elements were the most suitable soil physical and chemical properties for citrus seedlings growth. Before the experiment, all plants were raised for 7 d in an artificial climate chamber (A1000, Conviron, Canada) with day/night temperature of 28/18°C (day/night), light intensity of 800 μmol m⁻² s⁻¹, photoperiod of 12/12 h (light/dark), and relative humidity of approximately 65%.

After pre-treatment, put all pots in a completely randomized design in a factorial arrangement and seedlings then divided into the following four groups. Two groups were subjected to a temperature of 28/18°C (day/night) and sprayed with distilled water and 0.5 mmol L⁻¹ Spd (0.5 mmol L⁻¹ Spd is the empirical value based on the previous experiments of the research group), respectively [28/18°C + H₂O (CK, normal control) and 28/18°C + 0.5 mmol L⁻¹ Spd (Spd)]. The other two groups were subjected to HT treatment at 38/28°C and sprayed with distilled water and 0.5 mmol L⁻¹ Spd, respectively [38/28°C + H₂O (HT) and 38/28°C + 0.5 mmol L⁻¹ Spd (HT + Spd)]. They uniformly spray-leaves on both sides until the solution on the leaves formed fine mist-like droplets. Each treatment consisted of five (5) pots and three replications of each experimental unit, with 60 pots in total. The environmental conditions of each artificial climate chamber were as follows: photoperiod of 12/12 (day/night), the illumination intensity of 800 μmol m⁻² s⁻¹ and relative humidity of approximately 65%. Citrus seedlings were foliar sprayed with Spd purchased from Sigma-Aldrich every two days and this treatment continued for six days before the HT treatments in which Spd sprayed evenly on the leaf surface and the back of the leaf until the front, the back leaf surfaces were wet, and no liquid dripped. The collection of leaf samples was on the 3rd day of HT treatment and the relevant physiological indicators were determined.

Measurement of chlorophyll content

Chlorophyll a (Chla), chlorophyll b (Chlb), total chlorophyll, and carotenoid (Car) contents were determined according to the method reported by Arnon24 with minor modifications. A leaf sample of 0.2 g was ground in a mortar and placed into a 25 ml centrifuge tube containing 20 ml 95% acetone. The tube was sealed and placed in a dark place until the pigment in the leaves was completely extracted. Chla, Chlb, and total Chl concentrations were measured using ultraviolet/visible absorption spectrophotometry (UV-9100, LabTech, China) and absorbance was measured at 663 nm, 645 nm, and 470 nm. The following formulae were used for calculations: Chla = 12.7A₆₆₃ – 2.59 A₆₄₅; Chlb = 22.9A₆₄₅ – 4.67A₆₆₃; total Chl = 34.5 × A₆₆₃×1000, Car = (1000A₁₉₇₀ – 3.27 Chla – 104 Chlb) /229. The content of Chl and Car are expressed as mg g⁻¹ (FM).

Measurement of gas exchange parameters and light response curves

Gas-exchange parameters were measured by using the portable photosynthesis system (Li-6400XT, Li-COR Inc., USA), as described previously by Yang et al.25 Measurements were conducted between 9:00 and 11:00 hours. Before the measurement, each leaf was induced for 10 minutes in the leaf chamber (2 × 3 cm) with constant light intensity (800 μmol m⁻² s⁻¹).
During the measurement, the light irradiation, leaf chamber temperature, and CO₂ concentration were maintained at 800 μmol m⁻² s⁻¹, 25°C, and 400 μmol mol⁻¹ respectively. The LI-6400 program recorded the Intercellular carbon dioxide concentration (Cᵢ), stomatal conductance (gₛ), and net photosynthetic rate (Pₛ). The stomatal limit value (Lₛ) was calculated by the equation Lₛ = 1 − Cᵢ/Cₑ, where Cₑ represents the atmospheric CO₂ concentration.³

The light response curves under different treatments were measured based on Hermann et al.²⁶ The light intensity gradient of PAR was set as 0, 20, 50, 80, 100, 300, 500, 700, 900, 1100, 1200, and 1500 μmol m⁻² s⁻¹ for 120 s each. The environmental parameters in the leaf chamber were the same as when measuring the gas exchange parameters.

It was necessary to fit the light response curve to accurately obtain the parameters on the light response curve such as the light compensation point (LCP), the light saturation point (LSP), maximum net photosynthetic rate (Pₘₐₓₑₙ), and the apparent quantum yield (AQₑ). Light response curves were simulated by a non-orthogonal hyperbolic model (Equation 1) using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) as described previously by Farquhar et al.²⁷

\[ Pₛ(I) = \frac{al + Pₘₐₓ - [(al + Pₘₐₓ)² - 4θaIPₘₐₓ]^{0.5}}{2θ} - R_d \tag{1} \]

where, \( Pₛ(I) \) is the net photosynthetic rate, \( I \) is the light intensity, \( θ \) is the curvature of the curve, and \( a \) is the slope of the plant photosynthesis versus light response curve at \( I = 0 \), also called initial quantum efficiency, \( Pₘₐₓ \) is the maximum net photosynthetic rate and \( R_d \) is the dark respiration rate.

**Measurement of chlorophyll fluorescence parameters**

Chl fluorescence was measured using a pulse amplitude modulation fluorometer (PAM-2500, Walz, Germany) after leaves were dark-adapted for 20 min. The maximum quantum yield of PSII photochemistry (Fᵥ/Fₘ), nonphotochemical quenching (NPQ), the effective quantum yield of PSII photochemistry (ΦPSII), and electron transport rate (ETR) were measured. Chl fluorescence parameters were calculated following the method reported by Jan et al.²⁸

**Measurement of malondialdehyde (MDA) and hydrogen peroxide (H₂O₂)**

MDA content was measured by the thiobarbituric acid (TBA) method described in a study conducted by Hodges et al.²⁹ Firstly, 0.5 g of fresh leaves were ground with 5 mL of 10% trichloroacetic acid (TCA) in a mortar, and the extract was centrifuged for 20 min at 4,000 × g at 4°C. Next, 2 mL of the supernatant taken in a 5-mL centrifuge tube and 2 mL of 0.67% thiobarbituric acid (TBA) was added. The tube containing a well-mixed sample immersed in a boiling-water bath for 30 min, and re-centrifuged after cooling. The absorbance of the supernatant was recorded at 532, 450, and 600 nm using ultraviolet/visible absorption spectrophotometry (UV-9100, LabTech, China). The MDA content of expressed as μmol g⁻¹.

H₂O₂ was determined by using the method reported by Wang et al.³⁰ with minor modification. 0.5 g of fresh leaves homogenized with 5 mL of 0.1% (w/v) TCA in an ice bath. After centrifugation at 12,000 × g for 20 min, 0.5 mL of the supernatant was then added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide. The ultraviolet/visible absorption spectrophotometry (UV-9100, LabTech, China) used again to measure absorbance of the supernatant at 390 nm, with the content of H₂O₂ expressed as μmol g⁻¹.

**Measurement of enzymatic and non-enzymatic antioxidants and related gene expression**

The specific enzyme activity detection kit purchased by Nanjing Jiancheng Bioengineering Institute facilitated to calculate the activities of SOD, CAT, and POD, using 0.1 g of samples individually. The activity (1 U) of SOD was defined as the number of enzymes required to reduce nitrotetrazolium blue chloride (NBT) to half of that of the control group. The activity (1 U) of CAT was defined as a 0.1 absorbance reduction at 240 nm, POD activity (1 U) was defined as a 0.01 absorbance reduction at 470 nm. While the SOD, CAT, and POD activities were expressed as U g⁻¹. The application of The method of Li determined the contents of ASA and GSH. The contents of ASA and GSH were μmol g⁻¹.³¹ The relative expression levels of sod1, cat1, and cevi16 genes were completely determined by Shanghai Sheng Gong Biological Engineering Co., Ltd with separate 10 g samples.

**Statistical analysis**

The experimental data were statistically analyzed using one-factor analysis of variance (ANOVA). Analysis of the significance of differences between control and each treatment was performed by the Duncan multiple-range test at the 0.05 level of confidence using SPSS statistical software (SPSS, Chicago, IL, USA). The data of each index in the figure were presented as the mean ± standard deviation (SD) of three biological replications, with three samples of each replication. Calculations were performed using Microsoft Excel (Microsoft, USA). Graphs were plotted using GraphPad Prism 7.05 (GraphPad Software, San Diego, CA, USA).

**Results**

**Photosynthetic pigments**

High-temperature stress caused a sharp decrease in the pigment contents (Figure 1). The Chlα, Chlβ, total Chl and Car contents in only the heat-stressed seedlings decreased by 25%, 25%, 19%, and 27%, respectively, in contrast with their corresponding controls. Conversely, the application of exogenous melatonin to heat-stressed seedlings led to the restoration of the Chlα, Chlβ, total Chl, and Car contents compared to citrus seedlings subjected to HT stress, which amounted to 21%, 23%, 9%, and 18%, respectively.
Light response curves

Light response curves (LRCs) ranging from 0 to 1500 μmol m\(^{-2}\) s\(^{-1}\) were used to investigate the photosynthetic capacity of the citrus plants (Figure 2). With the increase in light intensity, LRCs in citrus leaves increased rapidly. However, the LRCs became steady after the light intensity reached 800 μmol m\(^{-2}\) s\(^{-1}\). The CK and Spd group showed the higher LRC, while LRC under HT was only 43% of the control when the light intensity reached 1500 μmol m\(^{-2}\) s\(^{-1}\). Application of Spd effectively improved the LRC under HT treatment, as exhibited by the middle curve.

As shown in Table 1, HT stress led to a decline in \(P_{\text{Nmax}}\), AQE, and LSP, but an increase in LCP. Compared with the control, the \(P_{\text{Nmax}}\), AQE, and LSP in citrus leaves under HT treatment decreased by 64%, 44%, and 32%, respectively, and the LCP increased by 40%. However, exogenous application of Spd significantly increased \(P_{\text{Nmax}}\), AQE, and LSP under HT conditions in citrus by 44%, 54%, and 23%, respectively, but reduced LCP by 9%.

**Gas exchange parameters**

The values of \(P_{\text{N}}\), \(C_{i}\), and \(g_{s}\) significantly decreased in both heat-stressed citrus seedlings with and those without Spd treatment compared to non-stressed plants (Figure 3). However, the reductions were more conspicuous in the seedlings exposed only to heat stress. The Spd-treated seedlings reversed the
Figure 3. Effects of exogenous spermidine treatment on gas exchange parameters of citrus plants exposed to high temperature stress. Note: Different letters indicate statistically significant by Duncan multiple-range test at $P < .05$. Data were expressed as the mean ± standard error of three independent biological replicates. Key: PN – net photosynthetic rate, gs – stomatal conductance, Ci – intercellular carbon dioxide concentration, Ls – stomatal limitation value.

Figure 4. Effects of exogenous spermidine treatment on Chl fluorescence parameters of citrus plants exposed to high temperature stress. Note: Different letters indicate statistically significant by Duncan multiple-range test at $P < .05$. Data were expressed as the mean ± standard error of three independent biological replicates. Key: $Fv/Fm$ – maximal quantum yield of PSII photochemistry, NPQ – non-photochemical quenching, $\Phi_{PSII}$ – effective quantum yield of PSII photochemistry, ETR – electron transport rate.
inhibitory effects of high temperatures on those parameters, with \( P_N \), \( C_i \) and \( g_s \) levels substantially increased by 68\%, 43\%, and 95\%, respectively, compared with untreated stressed seedlings. Conversely, the \( L_s \) in the Spd-treated plants under heat stress sharply elevated by 29\%, compared with those of the plants subjected to heat stress alone. In addition, no statistically significant differences observed between the control and Spd-only seedlings regarding these gas exchange parameters.

**Chl fluorescence parameters**

As shown in Figure 4, under normal growth conditions, Spd had no significant effects on the \( F_v/F_m \), \( \Phi_{PSII} \), NPQ, and ETR values. However, heat stress significantly inhibited the \( F_v/F_m \), \( \Phi_{PSII} \), and ETR values by 23\%, 250\%, and 61\%, respectively, and increased the NPQ value 3 times. In addition, the values of NPQ decreased while the values of \( F_v/F_m \), \( \Phi_{PSII} \), and ETR increased with Spd supplementation in citrus seedlings exposed to heat stress.

**Table 2. Effects of exogenous spermidine (Spd) on MDA and \( \text{H}_2\text{O}_2 \) contents in citrus seedling leaves under high-temperature stress.**

| Treatment | MDA content [nmol g\(^{-1}\)] | \( \text{H}_2\text{O}_2 \) content [μmol g\(^{-1}\)] |
|-----------|-------------------------------|---------------------------------|
| CK        | 28.74 ± 2.13\(^a\)           | 51.89 ± 4.25\(^c\)            |
| Spd       | 28.38 ± 2.22\(^c\)           | 52.33 ± 2.10\(^c\)            |
| HT        | 98.03 ± 3.71\(^a\)           | 102.32 ± 6.44\(^a\)           |
| Spd + HT  | 42.05 ± 2.34\(^b\)           | 67.66 ± 3.19\(^b\)            |

Note: For each variable, different lowercase letters indicate statistically significant by Duncan multiple- range test at \( P < 0.05 \). Data were expressed as the mean ± standard error of three independent biological replicates. Key: MDA – malondialdehyde, \( \text{H}_2\text{O}_2 \) – hydrogen peroxide.

**\( \text{H}_2\text{O}_2 \) contents and lipid peroxidation**

As shown in Table 2, under normal growth conditions, the addition of Spd had no significant effects on \( \text{H}_2\text{O}_2 \) and MDA contents. HT stress progressively increased the contents of \( \text{H}_2\text{O}_2 \) and MDA in citrus leaves. Compared to CK, \( \text{H}_2\text{O}_2 \) and MDA contents increased significantly by 2 times and 2.44 times, respectively. On the contrary, spraying exogenous Spd can effectively alleviate the damage to citrus seedlings under high-temperature stress. Spd-treated plants under heat stress resulted in a significant decrease in \( \text{H}_2\text{O}_2 \) and MDA contents (52.4\% and 72.4\%, respectively) compared to stress treatment with HT alone.

**Antioxidant enzyme activities and related gene expression**

HT stress led to significant regulation of antioxidant defense in citrus leaves. Activities of antioxidant enzymes (SOD, POD, and CAT) increased in citrus plants due to the imposition of HT than those in non-stressed plants (Figure 5). However, exogenously applied Spd caused a further increase in antioxidant enzyme activity of stressed plants. Furthermore, spraying the seedlings with exogenous Spd enhanced the activities of antioxidant enzymes in non-stressed plants as well. In addition, with the application of exogenous Spd under HT stress, the gene expression of \( sod1 \), \( cve16 \), and \( cat1 \) was significantly promoted by 19.6\%, 27.6\%, and 48.19\% compared with the control group, respectively (Table 3).

![Figure 5. Effects of exogenous spermidine treatment on the activity of SOD, POD and CAT in citrus plants exposed to high temperature stress. Note: Different letters indicate statistically significant by Duncan multiple- range test at \( P < 0.05 \). Data were expressed as the mean ± standard error of three independent biological replicates. Key: SOD – superoxide dismutase, POD – peroxidase, CAT – catalase.](image-url)
Table 3. Effects of exogenous spermidine (Spd) on the transcript level of sod1, celt16, and cat1 in citrus seedling leaves under high-temperature stress.

| Treatment | sod1 relative expression | celt16 relative expression | cat1 relative expression |
|-----------|--------------------------|-----------------------------|--------------------------|
| CK        | 5.34 ± 0.03^3            | 1.46 ± 0.02^2              | 1.84 ± 0.02^2           |
| Spd       | 5.88 ± 0.02^3            | 1.73 ± 0.02^2              | 2.03 ± 0.01^1           |
| HT        | 6.73 ± 0.04^6            | 2.32 ± 0.01^1             | 3.32 ± 0.04^6           |
| Spd + HT  | 8.05 ± 0.03^3            | 2.96 ± 0.03^3             | 4.92 ± 0.01^1           |

Note: For each variable, different lowercase letters indicate statistically significant results by Duncan multiple-range test at P < 0.05. Data were expressed as the mean ± standard error of three independent biological replicates. Key: sod1 – superoxide dismutase 1 gene, celt16 – peroxidase gene, cat1 – catalase 1 gene.

Ascorbic acid and glutathione contents

As shown in Figure 5, spraying Spd under normal temperature increased the ASA content, albeit with little change, and there was no significant difference in ASA content compared with that in the control treatment. Under HT stress conditions, the ASA content tended to increase, which was 0.75 times that of the normal temperature controls. Exogenously applied Spd under HT stress increased the ASA content and there was no significant difference in ASA content compared with that in the HT treatment. The variation trend of GSH content under HT stress and in response to Spd spraying was consistent with the variation in ASA content.

Discussion

Chlorophyll is the basic unit of plant energy systems during the process of photosynthesis. However, Chl is very fragile and easily destroyed by HT stress, which has been confirmed by many other authors, and also has been presented in our experiments (Figure 1), probably due to, under HT stress, the structure and function of Chl were severely destroyed, and the activity of Chl degrading enzyme was significantly increased. However, the addition of exogenous Spd could enhance photosynthetic pigment-synthesis or slow its degradation rate. The results were consistent with these reports. Photosynthesis is highly sensitive to high-temperature stress and is often inhibited before other cell functions are impaired. Previous research demonstrated that the stomatal limiting factors cause a decrease in photosynthetic rate. The suggested mechanism is due to the reduction of leaf gs by hindering the supply of CO2 in chloroplasts. However, some studies believed that the inhibition of photosynthesis was caused by non-stomatal factors, which was reflected by the higher CO2 concentration in mesophyll cells, but the lower affinity of Rubisco for CO2. In our study, PN decreased, accompanied by different degrees of gs and Ci increased and Lns decreased significantly under heat stress (Figure 3), which indicated that the decrease of PN might be due to the effect of non-stomatal factors. However, with the application of Spd, the PN increased and the values of Lns reduced under HT conditions. The underlying mechanism might be due to Spd treatment promoting the transport of CO2, the protein and gene expression of Ribulose-1,5-Bisphosphate Carboxylase (Rubisco) and phenol pyruvate carboxylase. In addition, HT also significantly influenced the photosynthetic curve characteristic parameters, such as reduced PNmax, AQE, and LCP, and significantly increasing the LSP (Table 1), which was consistent with the research results by Zhou et al. (2015), but the application of Spd could effectively alleviate the influence of HT stress on the characteristic parameters of the light response curve.

Chlorophyll fluorescence technique is a practical method to study the plant photosynthetic apparatus, especially the function of PSII. Chl fluorescence signal and its measured parameters have been successfully used to probe and elucidate injury to the PSII from various stresses. Fv/Fm represents the maximal quantum yield of PSII photochemistry, and its value is very stable, between 0.78 and 0.84. In our study, the Fv/Fm decreased in the HT-stressed leaves, which implied the quantum of absorption into the heart and fluorescence increased, and the absorption quantum converted into chemical fixation energy decreased. The treatment with exogenous Spd before the HT stress, significantly alleviated the decrease of Fv/Fm and ETR, which indicated that Spd might alleviate the effect of HT on the photosynthetic process by improving the photosynthetic electron transport. Moreover, the ΦPSII decreased, whereas NPQ increased under HT stress. This indicates that the electron transfer of photosynthetic electrons was blocked, and the efficiency of photosynthetic pigments converting light energy into chemical energy decreased. Pretreatment with exogenous Spd under HT stress could improve ΦPSII, but reduce NPQ, which might be that exogenous Spd could protect PSII from excessive energy damage, and enhance the stability of the reaction center of the photosystem, which is conducive to the conversion of light energy into chemical energy, thereby providing enough energy for carbon assimilation.

The effect of HT stress on chlorophyll and photosynthetic apparatus suggested to relating to the production of ROS. Under abiotic stress conditions, the production of ROS can exceed the scavenging capability of the cell, which has the potential of damaging the biomolecules leading to cell death. MDA is one of the most known secondary products of lipid peroxidation, and it can be used as a marker of cell membrane injury. Exogenous Spd treatment effectively inhibits the accumulation of H2O2 and MDA in plants under HT stress, which has been confirmed in wheat, rice, and other plants. In this study, we observed that H2O2 and MDA contents significantly increased under HT stress, suggesting cell membrane damage; however, H2O2 and MDA contents decreased significantly when exogenous Spd was spayed under HT stress. A reduction of H2O2 and MDA suggested that exogenous Spd showed a protective effect against membrane damage under HT stress.

To reduce oxidative stress, plants have developed a series of both enzymatic and non-enzymatic detoxification systems to deal with ROS, controlling the formation and removal rates, and thereby protecting cells from oxidative damage. The antioxidant enzyme system, comprising SOD, POD, and CAT, is an important system that protects cells from the negative effects of ROS. Our experimental results showed that under HT treatment, SOD, POD, and CAT activities in leaves showed varying degrees of increase (Figure 4). These results suggested that to protect themselves from HT stress, plants increase the activity of their antioxidant enzymes, thereby reducing adversity stress, which is consistent with previous research.
results.\textsuperscript{58,59} Our research confirmed that Spd was involved in ROS scavenging under HT conditions. Therefore, the reversal of HT stress by Spd is due to its ability in improving antioxidant capacity and increasing the scavenging of ROS.

The AsA-GSH cycle is an important non-enzyme system in plants that maintains the oxidative environment in cells by regulating the content of both AsA and GSH and plays a protective role in plants.\textsuperscript{60} The AsA is an effective active oxygen scavenger\textsuperscript{61} and GSH is widely distributed in plant tissues. It can participate directly or indirectly in the detoxification of ROS.\textsuperscript{62} The GSH is meanwhile involved in methylglyoxal (MG) detoxification.\textsuperscript{62} In this study, the AsA and GSH contents in citrus leaves tended to increase under HT stress (Figure 6), which was consistent with the results of the study on the effect of heat stress on lettuce,\textsuperscript{19} and the levels of these two antioxidants further increased after the application of exogenous Spd. Our results showed that exogenous Spd could increase the heat resistance of citrus seedlings under HT stress by increasing AsA and GSH levels.

**Conclusion**

HT stress negatively influences photosynthesis and the chlorophyll fluorescence parameters in citrus seedlings. The application of exogenous Spd improved photosynthesis, maintained the balance in ROS metabolism and alleviated the damage caused by HT stress. In addition, the results also indicated that exogenous Spd could alleviate the damage of citrus seedlings under HT stress by improving photosynthetic efficiency, enhancing the activities of enzymatic and non-enzymatic antioxidants, and reducing oxidative stress in leaves. Therefore, exogenous Spd treatment might be a suitable approach to improve the tolerance of citrus seedlings under HT stress.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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**References**

1. Raza A, Razzaq A, Mehmood SS, Zou X, Zhang X, Lv Y, Xu J. Impact of climate change on crops adaptation and strategies to tackle its outcome: a review. Plants. 2019;8:34. doi:10.3390/plants8020034.
2. Mittler R, Fink A, Goloubinoff P. How do plants feel the heat? Trends Biochem Sci. 2012;37:118–125. doi:10.1016/j.tibs.2011.11.007.
3. Zhang JH, HUANG WD, LIU YP, PAN QH. Effects of temperature acclimation pretreatment on the ultrastructure of mesophyll cells in young grape plants (Vitis vinifera L. cv. Jingshiu) under cross-temperature stresses. J Integr Plant Biol. 2005;47:959–970. doi:10.1111/j.1744-7909.2005.00109.x.
4. Song Y, Chen Q, Ci D, Shao X, Zhang D. Effects of high temperature on photosynthesis and related gene expression in poplar. Bmc Plant Biol. 2014;14:1–20. doi:10.1186/1471-2229-14-111.
5. Gerganova MT, Faik AK, Velitchkova MY. Acquired tolerance of the photosynthetic apparatus to photo-inhibition as a result of growing Solanum lycopersicum at moderately higher temperature and light intensity. Funct Plant Biol. 2019;46:555–566. doi:10.1071/FP18264.
6. Muhlemann JK, Younts TL, Muday GK. Flavonols control pollen tube growth and integrity by regulating ROS homeostasis during high-temperature stress. PNAS. 2018;115:E11188–E11197. doi:10.1073/pnas.1811492115.
7. Chung IM, Kim JJ, Lim JD, Yu CY, Kim SH, Hahn SJ. Comparison of resveratrol, SOD activity, phenolic compounds and free amino acids in rehmannia glutinosa under temperature and water stress. Environ Exp Bot. 2006;56:44–53. doi:10.1016/j.envexpbot.2005.01.001.
8. Xu C, Yang ZQ, Yang SQ, Wang L, Wang MT. High humidity alleviates photosynthetic inhibition and oxidative damage of tomato seedlings under heat stress. Photosynthetica. 2020;58:146–155. doi:10.36215/ps.2019.168.
9. Li L, Gu W, Li C, Li W, Li C, Li J, Wei S. Exogenous spermidine improves drought tolerance in maize by enhancing the antioxidant defence system and regulating endogenous polyamine metabolism. Crop Pasture Sci. 2018;69:1076–1091. doi:10.1071/CP18271.
planets.

wavelength-sensitive

Exogenous

Zhou

2020
doi:

temperature

through

maize

photolysis:

physiological

drought

Acta

Plant.

in

engineered

and

spermidine

Zandalinas

Umutoni

the

Velarde-Buendía

SY,

2019

doi:

curves-linking

system

with

Pea

enhances

non-stomatal

and

non-stomatal

limitations

to

high

heat

stress

Plant

Cell

Environment.

2000;

23:

235–250.

doi:10.1046/j.

I.

A,

Calatayud

A,

P,

K,

DeLong

Huang

Brestic

A,

Q,

B,

Hodges

Planets.

1949;

24:1.

doi:10.1104/

pp.24.1.1.

Yang

ZQ,

Xu

C,

Wang

MT,

Zhao

HL,

Zheng

YJ,

Huang

HJ,

Vuyuziga

F,

Umutoni

MA.

Enhancing

the

thermolterence

of

tomato

seedlings

by

heat

shock

treatment.

Photosynthetica.

2019;

57:

1184–1192.

doi:10.1139/fs.2019.127.

Herrmann

HA,

Schwartz

J,

Johnson

GN.

From

empirical
to

theoretical

models

of

light

response

curves-linking

photosynthetic

and

metabolic

acclimation.

Photosynth Res.

2020;

145:5–14.

doi:10.1007/s11210-019-00681-2.

Farquhar

J,

Savirano

J,

Airieau

S,

Thiemens

MH.

Observation

of

wavelength-sensitive

mass-independent

sulfur

isotope

effects

during

SO2

photolysis:

implications

for

the

early

atmosphere.

J

Geophys Res.

Planets.

2001;

106:32829–32839.

doi:10.2920/2000JE001437.

Jan

S,

Alyemeni

MN,

Wijaya

L,

Alam

P,

Siddique

KH,

Ahmad

P.

Interactive

effect

of

24-epibrassinolide

and

silicon

ameliorates

cadmium

stress

via

the

modulation

of

antioxidant
defense

and

glyoxalase

systems

and

macronutrient

content

in

Pisum

sativum

L.

seedlings.

Bmc

Plant

Biol.

2018;

18:1–18.

doi:10.1186/s12870-018-1359-5.

Hodges

DM,

DeLong

JM,

Forney

CF,

Prange

RK.

Improving

the

thioarbituric

acid-reactive

substances

assay

for

estimating

lipid

photodestruction

in

plant

tissues

containing

anthocyanin

and

other

interfering

compounds.

Plant.

1999;

207:604–611.

doi:10.1007/s11120-017-0355-0.

Maxwell

K,

Johnson

GN.

Chlorophyll

fluorescence—a

practical

guide.

J

Exp

Bot.

2000;

51:659–668.

doi:10.1093/jexbot/51.345.659.
46. Poudyal D, Rosenqvist E, Ottosen C. Phenotyping from lab to field–tomato lines screened for heat stress using Fv/Fm maintain high fruit yield during thermal stress in the field. Funct Plant Biol. 2018;45:44–55. doi:10.1071/FP17317.

47. Jahan MS, Guo S, Sun J, Shu S, Wang Y, Abou El-Yazied A, Alabdallah NM, Hikal M, Mohamed MH, Ibrahim MF. Melatonin-mediated photosynthetic performance of tomato seedlings under high-temperature stress. Plant Physiol Bioch. 2021;167:309–320. doi:10.1016/j.plaphy.2021.08.002.

48. Yuan L, Yuan Y, Liu S, Wang J, Zhu S, Chen G, Hou J, Wang C. Influence of high temperature on photosynthesis, antioxidative capacity of chloroplast, and carbon assimilation among heat-tolerant and heat-susceptible genotypes of nonheading Chinese cabbage. Hortscience. 2017;52:1464–1470. doi:10.21273/HORTSCI12259-17.

49. Camejo D, Jiménez A, Alarcón JJ, Torres W, Gómez JM, Sevilla F. Changes in photosynthetic parameters and antioxidant activities following heat-shock treatment in tomato plants. Funct Plant Biol. 2006;33:177–187. doi:10.1071/FP05067.

50. Guo Y, Zhou H, Zhang L. Photosynthetic characteristics and protective mechanisms against photooxidation during high temperature stress in two citrus species. Sci Hortic-Amsterdam. 2006;108:260–267. doi:10.1016/j.scienta.2006.01.029.

51. Gill SS, Tuteja N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Bioch. 2010;48:909–930.

52. Rahman M, Rahman K, Sathi KS, Alam M, Nahar K, Fujita M, Hasanuzzaman M. Supplemental selenium and boron mitigate salt-induced oxidative damages in glycine max L. Plants. 2021;10:2224. doi:10.3390/plants1012224.

53. Jing J, Guo S, Li Y, Li W. The alleviating effect of exogenous polyamines on heat stress susceptibility of different heat resistant wheat (Triticum aestivum L.) varieties. Sci Rep-Uk. 2020;10:1–12.

54. Tang S, Zhang H, Li L, Liu X, Chen L, Chen W, Ding Y. Exogenous spermidine enhances the photosynthetic and antioxidant capacity of rice under heat stress during early grain-filling period. Funct Plant Biol. 2018;45:911–921. doi:10.1071/FP17149.

55. Foyer CH, Noctor G. Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. Physiol Plant. 2003;119:355–364. doi:10.1046/j.1399-3054.2003.00223.x.

56. Mittler R. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 2002;7:405–410. doi:10.1016/S1360-1385(02)02312-9.

57. Noctor G, Foyer CH. Ascorbate and glutathione: keeping active oxygen under control. Annu Rev Plant Biol. 1998;49:249–279. doi:10.1146/annurev.arplant.49.1.249.

58. Almeselmani M, Deshmukh PS, Sairam RK, Kushwaha SR, Singh TP. Protective role of antioxidant enzymes under high temperature stress. Plant Sci. 2006;171:382–388. doi:10.1016/j.plantsci.2006.04.009.

59. Zhang X, Ervin EH, Schmidt RE. Plant growth regulators can enhance the recovery of Kentucky bluegrass sod from heat injury. Crop Sci. 2003;43:952–956. doi:10.2135/cropsci2003.9520.

60. Bartoli CG, Buet A, Gergoff Grozelf G, Galatro A, Simontacchi M. Ascorbate-glutathione cycle and abiotic stress tolerance in plants.In: Hossain M, Munné-Bosch S, Burritt D, Diaz-Vivancos P, Fujita M, Lorence A, editors. Ascorbic acid in plant growth, development and stress tolerance. Cham: Springer International Publishing: 2017. p. 177–200.

61. Foyer CH, Noctor G. Ascorbate and glutathione: the heart of the redox hub. Plant Physiol. 2011;155:2–18. doi:10.1104/pp.110.167569.

62. Hasanuzzaman M, Nahar K, Hossain M, Mahmud J, Rahman A, Inafuku M, Oku H, Fujita M. Coordinated actions of glyoxalase and antioxidant defense systems in conferring abiotic stress tolerance in plants. Int J Mol Sci. 2017;18:200. doi:10.3390/ijms18010200.