Exogenous Melatonin and Salicylic Acid Enhance the Drought Resistance of Hibiscus (Hibiscus syriacus L.) by Regulating Photosynthesis and Antioxidant System

Ruiyang Yan
Shandong Agricultural University
Jinying Liu
Shandong Agricultural University
Shuyong Zhang
Shandong Agricultural University
jing Guo (jingguo@sdau.edu.cn)
Shandong Agricultural University

Research Article

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Abstract

Drought is among the environmental stress factors that causes a decrease in plant productivity. A hot topic in abiotic stress physiology research is how to alleviate drought stress during plant growth. Exogenous substances have been observed to play a positive role in regulating plant responses to drought. Hibiscus (*Hibiscus syriacus* L.) has high ornamental and medicinal value. However, there is a shortage of reports focused on exogenous substances that can alleviate stress caused by environmental factors in hibiscus. This study used 1-year-old *H. syriacus* var. 'elegantissimus' seedlings as experimental materials. We investigated the roles and physiological mechanisms of melatonin (MT) and salicylic acid (SA) on hibiscus during drought stress by observing plant growth status and photosynthetic physiological parameters. The results showed that compared with the treatment of only drought stress, exogenous MT and SA increased the chlorophyll content of plants, enhanced photosynthesis, alleviated photoinhibition, and protected the photosystem. On the other hand, exogenous MT and SA increased the expression of antioxidant enzyme genes (*Cu/Zn-SOD*, *POD-20*, *CAT*, and *APX-1*) and improved their enzymatic activity. This reduced the ROS content and alleviated the drought-induced oxidative damage. In addition, MT and SA induced the expression of drought-responsive genes *HsNCED* and *HsDBF1*. In summary, Exogenous melatonin and salicylic acid can enhance drought resistance of hibiscus by regulating its photosynthetic system, antioxidant enzyme activity, and drought-related genes. The study results provide a crucial scientific basis and theoretical support for the cultivation management and subsequent care of hibiscus.

1. Introduction

Various adverse environmental factors, such as soil drought, heavy metal pollution, and high temperature, often influence the growth and development of most plants under natural conditions (Zhu et al. 2021). Of them, soil drought is one of the most severe environmental stressors leading to a decrease in plant productivity. This decrease slows down plant growth or accelerates plant aging by affecting various plant physiological and biochemical processes (Luo et al. 2022; Talbi et al. 2020). Drought can cause plant chloroplast degradation, resulting in damage to the photosynthetic system, inhibition of photosynthetic electron transfer, an increase in excess excitation energy, and ultimately accumulation of reactive oxygen species (ROS) in the chloroplast. This excessive ROS accumulation can damage the cell structure, exacerbate PS photoinhibition, reduce the light energy utilization efficiency, and cause oxidative damage in plants (Che et al. 2018; Wang et al. 2022). Drought stress can decrease the relative water content of plant leaves, leading to wilted leaves and curved stem tips and affecting the normal plant growth. It can also cause an increase in MDA content and electrical conductivity in plant leaves, thereby damaging the cytomembrane (Zeng et al. 2022). In addition to the plant's own response mechanisms, various horticultural measures can be used to alleviate drought stress-induced damage. Among these measures, the use of plant growth regulators for enhancing plant drought resistance has become a research hotspot (Altaf et al. 2022).

Melatonin (N-acetyl-5-methoxytryptamine, MT) is a natural indoleamine compound. It was first found to be widely present in plants in 1995 (Dubbels et al. 1995). MT plays a crucial role in resistance to abiotic stress. For example, MT treatment alleviated chlorophyll degradation and increased photosynthetic rate in drought stress-exposed *Malus hupehensis* seedlings (Li et al. 2012). Luo et al. (2022) reported that previous MT treatment can reduce the leaf MDA content in drought stress-exposed rice (*Oryza sativa* L.) seedlings. MT can also improve the photosynthetic capacity of apple (*Malus domestica* Borkh.) exposed to drought stress (Wang et al. 2013). Additionally, MT is a powerful antioxidant that can clear ROS by increasing antioxidant enzyme activity and reducing drought stress-induced oxidative damage in plants. Under drought stress, MT promoted the activity of antioxidant enzymes in rice (*O. sativa* L.) seedlings and cleared excess ROS (Luo et al. 2022). Previous MT treatment in two *Malus* species increased their own antioxidant enzyme activity and inhibited H_2O_2 production under drought stress (Li et al. 2015).

Salicylic acid (SA) is a naturally occurring phenolic compound belonging to a class of plant growth regulators (Hayat et al. 2010). SA is a crucial player in improving plant tolerance to drought stress when sprayed on plants. Shemi et al. (2021) reported that SA can increase the photosynthetic rate of drought stress-exposed maize (*Zea mays* L.) by increasing its chlorophyll content and reducing light inhibition. SA-pretreated barley (*Hordeum vulgare* L. *cv* Nosrat) exhibited Fa better photosynthetic ability and less damage to the photosystem under drought stress (Habibi 2012). Under drought stress, spraying SA could remove excessive ROS by increasing the antioxidant enzyme activity in *Vicia faba* L., decreasing MDA content and electrical conductivity, and thus reducing oxidative damage (Dawood et al. 2022). Kang et al. (2012) found that wheat (*Triticum aestivum* L.) seedlings treated with 0.5 mM SA exhibited alleviation of drought stress-induced leaf wilting.

Hibiscus (*Hibiscus syriacus* L.) (Family: Malvaceae) is widely distributed in East Asia and South Asia (Lee et al. 1999). Current research on hibiscus mainly focuses on its cultivation techniques, and ornamental and medicinal properties. Hibiscus boasts various flower colors and a beautiful tree shape. Its flowers, fruits, roots, stems, and bark can be used to treat dysentery, cancer, and ulcers, which makes it highly valuable for both garden viewing and medicinal purposes (Eo et al. 2020; Zhang et al. 2022). However, research on the physiological and ecological characteristics of hibiscus and their relationship with environmental factors is limited. Only a few studies have investigated the effects of salt stress on the photosynthetic capacity and reactive oxygen metabolism of hibiscus seedlings (Lu et al. 2022). Reports on exogenous substances that alleviate hibiscus drought stress resistance are lacking.

To clarify the drought resistance of hibiscus seedlings and explore ways to improve their drought resistance, this study analyzed the growth morphology, physiological and biochemical characteristics, chlorophyll fluorescence characteristics, and related gene expression levels of seedlings under drought stress after they were sprayed with SA and MT. The response patterns of photosynthesis, chlorophyll fluorescence, and antioxidant enzyme activity of hibiscus to soil drought and exogenous substances were elucidated. This study offers a vital scientific basis and theoretical foundation hibiscus cultivation and introduction.

2. Materials and Methods

2.1 Plant Materials and Growth Conditions

Cutting branches of *H. syriacus* var. 'elegantissimus' were purchased from Jiashan County Ecological Technology Company, China. The cuttings were propagated in March 2022 and cultivated in a greenhouse located at the Experimental Station of Shandong Agricultural University (36°09' N, 117°09' E). Approximately 600 hibiscus branches, each with one bud on the top and bottom and measuring approximately 5 cm, were selected and planted in planting trays measuring 6 cm × 5 cm × 8 cm by using a nursery substrate. The trays were maintained with water at the bottom to ensure the soil had adequate moisture in the hole plates. After rooting, the branches were transplanted into nutrient bowls measuring 23 cm × 18 cm. The bowls contained a cultivation substrate of garden soil and nutrient soil at a 1:1 ratio. In total, 180 hibiscus seedlings (height: 38 ± 2 cm; ground diameter: 3.4 ± 0.5 cm), were selected for the experiment.

2.2 Experimental Design and Exogenous Substances Treatment

The experiment consisted of four treatment groups: W (normal watering and soil relative moisture content controlled at 65% ± 4%), D (drought stress), D + MT (drought stress with 100 μmol L^{-1} MA), and D + SA (drought stress with 500 μmol L^{-1} SA). MT and SA were applied using a leaf spraying method. Considering full irrigation and beginning to stop water supply as the 0th day, MT and SA and deionized water were sprayed at 6 p.m. every day for the first 3 days (− 3 days, − 2 days, − 1 day), respectively, on the front and back of the leaves. From day 0, hibiscus of each treatment group was fully irrigated and the test was started. The water supply was stopped for 21 days, and exogenous substances were no longer sprayed during the whole test. Sampling or photosynthetic indices were measured at five time points: T1 (0 day, soil relative moisture content 70% ± 5%), T2 (5 days, soil relative moisture content 35% ± 0.8%), T3 (10 days, soil relative moisture content 19% ± 2.6%), T4 (15 days, soil relative moisture content 11% ± 3.4%), and T5 (21 days, soil relative moisture content 4.2% ± 1.2%). Each treatment group included 45 hibiscus seedlings, and the middle and upper mature leaves of 9 seedlings were sampled at each time point, of which every 3 seedlings were a biological replicate. We maintained a total of three biological replicates.

2.3 Determination of Gas Exchange Parameters

Leaf gas exchange parameters of the seedlings in each treatment group were measured at the five time points of the experiment, with measurement taken from 8 to 11 a.m. To reduce differences in photosynthetic physiological parameters caused by dynamic changes in time, an alternate measurement method was used between different treatment groups and different leaf replicates during specific measurements. This ensured comparability and accuracy of the observed data among different treatments. In each treatment group, three healthy mature third or fourth leaves of the upper part of the hibiscus seedling were randomly selected for in vivo determination, and each leaf was recorded 3–5

times. For each treatment group, three hibiscus seedlings were randomly selected, and the third or fourth mature healthy leaf located at the middle to upper part of the plant and facing the sun was measured. Three to five recordings were taken for each hibiscus leaf by using an LI-COR 6800 photosynthesis measurement system (LI-COR, Inc., USA) to determine the net CO_2 assimilation rate (P_n), transpiration rate (T_r), stomatal conductance (G_s), and intercellular carbon dioxide concentration (C_i). Water use efficiency (WUE) was calculated using the formula P_n/T_r. During the gas exchange parameter measurement, photosynthetically active radiation (PAR) was set to 1200 μmol m^{-2} s^{-1} by using a controllable artificial light source in the photosynthesis system, and the temperature was set to be consistent with the atmospheric temperature. The CO_2 concentration was set at 400 μmol mol^{-1} by using a CO_2 controller.

2.4 Determination of Light Response Curve

At each of the five time points during the experimental treatments, three seedlings from each treatment group were randomly selected, and the third or fourth mature healthy leaf located at the middle to upper part of the plant were used to measure the light response process of hibiscus leaves under different treatments by using the LI-COR 6800 photosynthesis measurement system. To minimize the effects of external light fluctuations, measurements were taken at approximately 8–11 a.m. on completely sunny days. Each leaf measurement was repeated three times, and the average was taken. During the measurement, the PAR was set from high to low at 1800, 1600, 1400, 1200, 1000, 800, 600, 400, 200, 150, 100, 50, and 0 μmol m^{-2} s^{-1}, with each gradient controlled for 180 s. The CO_2 concentration was set at 400 μmol mol^{-1} by using a CO_2 controller. The relative humidity was 55%, and the atmospheric temperature was 28°C. The instrument automatically recorded the net CO_2 assimilation rate (Pn). Linear regression was performed on the initial part (PAR < 200 μmol m^{-2} s^{-1}) of the Pn curve to obtain the light compensation point (LCP), dark respiration rate (R_d), and apparent quantum yield (AQY).

2.5 Chlorophyll Fluorescence Determination

Chlorophyll fluorescence parameters of hibiscus seedlings were measured at the same time as the gas exchange parameter measurements. Six seedlings were randomly selected from each treatment group, and the third or fourth mature healthy leaf located at the middle to upper part of the plant was selected for each measurement. A total of 6 replicates were maintained. The maximum fluorescence (Fm') was measured after a steady state was attained through photochemical action under 600 μmol m^{-2} s^{-1} light. The initial fluorescence (Fo), maximum fluorescence (Fm), and maximum photochemical efficiency (*Fv/Fm*) were measured after the 30-min dark treatment. The non-photochemical quenching coefficient (*NPQ*) coefficient was calculated using the formula provided by Baker (2008):

$$Fv/Fm = (Fm - Fo) / Fm$$

$$NPQ = (Fm - Fm) Fm'$$

2.6 Determination of Leaf Relative Water Content

At each of the five time points, three hibiscus seedlings were randomly selected from each treatment group. One completely expanded and similarly sized leaf from the middle of each seedling was selected and weighed for fresh weight. Then, the leaves were immersed in deionized water in a storage box and brought back to the laboratory to avoid light. After 24 h, the leaves were removed from the box and weighed for saturated weight. The leaves were then placed in an oven at 80°C until a constant weight was obtained, and the dry weight was recorded. The following equation was used for calculation (Ghoulam et al. 2002):

$$RWC\ (\%) = [(FW - DW)/(TW - DW)] \times 100$$

FW: fresh weight of the leaf, DW: dry weight of the leaf, and TW: saturated weight of the leaf.

2.7 Chlorophyll Content Measurement

The photosynthetic pigment content was determined using the mixed solvent extraction method (Lichtenthaler and Wellburn 1983). Fresh samples (0.5 g) were cut into strips and soaked in 80% acetone solution at room temperature in the dark until the leaves turned completely white. The supernatant was collected, and the absorbance was measured at 663, 645, and 670 nm.

2.8 ROS Determination and Staining

Hydrogen peroxide (H_2O_2) and superoxide radical (O_2^-) contents were determined according to Beijing Solarbio Science & Technology Co., Ltd. (www.solarbio.com) by strictly adhering to the instructions for the determination.

The experimental method of H_2O_2 staining was slightly modified by Shi et al. (2010). A 3,3'-diaminobenzidine (DAB) solution (1 mg mL^{-1}, pH 3.8) was prepared using 10 mM phosphate buffer (pH 7.8). Fresh intact leaves were immersed in the DAB solution at room temperature in the dark until brown spots produced by the reaction between DAB and H_2O_2 were visible. The leaves were then bleached with 95% ethanol until the brown spots became clearly visible and stored in 70% ethanol. O_2^- staining was performed according to the methods of Kumar et al. (2014) and Fryer et al. (2002). NBT powder was dissolved in 50 mM phosphate buffer (pH 7.5) to prepare 2 mg ml^{-1} NBT staining solution; the solution was freshly prepared before use. Fresh intact leaves were immersed in the prepared solution at room temperature in the dark overnight. The lacto-glycerol-ethanol (1:1:4 by volume) mixture was used to remove chlorophyll from the leaves for observing the blue spots.

2.9 Determination of Antioxidant Enzymes Activities

The 0.1 g of plant samples subjected to different treatments were collected and ground under ice-cold conditions after adding 1 mL of 50 mM phosphate-buffered saline (PBS, pH 7.8, and 1% polyvinylpyrrolidone). The homogenate was centrifuged at 12,000 rpm and 4°C for 20 min, and the supernatant was used for determining antioxidant enzyme activity. Superoxide dismutase (SOD),

peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) activities were determined using the methods described by Wu et al. (2003), Maehly (1954), and Gakmak (1994).

2.10 Quantitative Real-Time PCR Analysis

Total RNA was extracted using the TransZol Up Plus RNA Kit (TransGen Biotech, Beijing, China). Then, cDNA was synthesized using TransScript Uni All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (TransGen Biotech, Beijing, China). Real-time fluorescence quantitative PCR was performed using PerfectStart Green qPCR SuperMix (TransGen Biotech, Beijing, China), with hibiscus 18S rRNA acting as the reference gene. All primers used are listed in Table S1. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.11 Statistical Analysis

Three biological replicates were maintained for each treatment group. Independent sample t-test were performed using SPSS Statistics 26.0 software (SPSS Inc., Chicago, IL, USA). Data processing and analysis were conducted using Origin 2021 (Lab, USA).

3. Results

3.1 Phenotypes and Leaf Relative Water Content

To investigate the effects of MT and SA on alleviation of drought stress-induced changes in the growth characteristics of hibiscus seedlings, the seedlings were photographed from the onset of the stress phenotype. At T4, compared with the W treatment group, hibiscus seedlings in the D, D + MT, and D + SA treatment groups started exhibiting slight leaf wilting (Fig. 1A). At this stage, the leaf relative water content of the seedlings treated with D + MT and D + SA increased by 19.5% and 21.4%, respectively, compared with that of the seedlings treated with D (Fig. 1C). At T5, the leaves in the D treatment group exhibited severe water loss, wilting, stem bending, and drooping; these indicated significant damage. However, the leaves in the D + MT and D + SA treatment groups only exhibited slight wilting and drooping, but the stem tips exhibited no bend (Fig. 1B). Additionally, the leaf relative water content in the D + MT and D + SA groups increased significantly by 11.8% and 17.8%, respectively, compared with that in the D treatment group (Fig. 1C). These results suggest that spraying MT and SA can alleviate drought stress-induced harm to the growth of hibiscus seedlings, and the mitigation effect of spraying SA on water loss of the seedlings was stronger than that of MT.

3.2 Light Response Curve of P_n

At T1 and T2, the net CO_2 assimilation (P_n) of seedlings in each treatment group sharply increased at < 400 μmol m^{-2} s^{-1} PAR (Fig. 2A-B). At T3, P_n of the D, D + MT, and D + SA treatment groups exhibited the light saturation phenomenon (Fig. 2C). However, compared with the D treatment group, at 1600 μmol m^{-2} s^{-1} PAR, P_n of the D + MT and D + SA treatment groups increased by 31.62% and 39.45%, respectively. As

the drought intensified, at T4, the light saturation points of the D and D + SA treatment groups appeared at 1200 and 1600 μmol m^{-2} s^{-1} PAR, respectively, whereas no light saturation phenomenon was observed in the D + MT treatment group (Fig. 2D). At T5, all drought stressed-exposed groups exhibited varying degrees of light inhibition, with the light saturation points of the D, D + MT, and D + SA treatment groups appearing at 400, 800, and 1000 μmol m^{-2} s^{-1} PAR, respectively (Fig. 2E). These results suggest that MT and SA can increase the light saturation point of hibiscus seedlings and alleviate drought stress-induced photoinhibition. The SA-treated plants exhibited a stronger ability to utilize strong light under drought stress than the MT-treated plants.

3.3 Light Response Characteristics

As the experiment continued, the LCP and Rd of the W, D, and D + SA treatment groups displayed a trend of bimodal variation, while the D + MT treatment group exhibited an upward trend, followed by a downward trend and then an upward trend again (Table 1). At T4 and T5, the LCP was significantly lower in the W, D + MT, and D + SA treatment groups than in the D treatment group, especially at T5, where the LCP was reduced by 86.21%, 92.44%, and 73.36%, respectively. As the drought stress continued, the AQY of the W treatment group remained stable, whereas the D treatment group exhibited a continuous decrease and the D + MT and D + SA treatment groups exhibited an upward trend, followed by a downward trend. At T4 and T5, the AQY was significantly higher in the W, D + MT, and D + SA treatment groups than in the D treatment group, especially at T5, where the AQY increased by 335.23%, 152.27%, and 153.41%, respectively. The aforementioned results showed that the MAT-treated seedlings had a stronger ability to use weak light than the SA-treated seedlings under drought stress.

Table 1
Effect of melatonin (MT) and salicylic acid (SA) on the light response characteristics of hibiscus.

Time	Treatment	LCP (μmol m^{-2} m^{-1})	R_d (mmol m^{-2} m^{-1})	AQY (mol mol^{-1})
T1	W	44.23 ± 2.75	1.83 ± 0.14	0.041 ± 0.0007
	D	43.94 ± 10.12	1.88 ± 0.43	0.043 ± 0.0002
	D + MT	19.20 ± 5.06	0.78 ± 0.23	0.039 ± 0.0022
	D + SA	30.44 ± 5.30	1.12 ± 0.19	0.037 ± 0.0005***
T2	W	8.93 ± 2.41*	0.34 ± 0.08*	0.036 ± 0.0010
	D	18.78 ± 1.82	0.75 ± 0.09	0.040 ± 0.0013
	D + MT	20.59 ± 13.68	0.83 ± 0.54	0.041 ± 0.0010
	D + SA	16.13 ± 8.23	0.65 ± 0.32	0.041 ± 0.0013
T3	W	16.24 ± 7.32	0.59 ± 0.30	0.035 ± 0.0032
	D	36.38 ± 4.32	1.40 ± 0.16	0.039 ± 0.0008
	D + MT	22.84 ± 8.68	0.85 ± 0.33	0.037 ± 0.0012
	D + SA	42.88 ± 4.97	1.68 ± 0.16	0.040 ± 0.0017
T4	W	14.70 ± 3.40*	0.54 ± 0.12	0.036 ± 0.0011***
	D	32.73 ± 2.20	0.73 ± 0.05	0.022 ± 0.0006
	D + MT	8.67 ± 1.99**	0.25 ± 0.06**	0.028 ± 0.0012*
	D + SA	1.59 ± 0.87***	0.04 ± 0.02***	0.025 ± 0.0006*
T5	W	26.83 ± 1.87***	1.02 ± 0.04	0.038 ± 0.0019***
	D	194.50 ± 5.30	1.73 ± 0.29	0.009 ± 0.0013
	D + MT	14.70 ± 1.02**	0.33 ± 0.01*	0.022 ± 0.0008**
	D + SA	51.82 ± 12.67***	1.13 ± 0.22	0.022 ± 0.0015**

Values represent means ± SE of three replicates. Asterisks indicate statistical significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; independent samples t-test) of the W, D + MT and D + SA treatment groups compared to D treatment group in the same treatment time.

Note

LCP, light saturation point; Rd, dark respiration rate; AQY, apparent quantum yield.

3.4 Gas Exchange Parameters

At T1 and T2, the P_n of seedlings in all treatment groups was relatively high (Fig. 3A). At T3, the P_n value of the D treatment group decreased sharply, whereas those of the W, D + MT, and D + SA treatment groups remained at a higher level, which were significantly higher than that of the D treatment group by 1.25, 1.33, and 1.39 times ($p < 0.05$), respectively. As drought stress intensified, at T5, the P_n values of the D, D + MT, and D + SA treatment groups reached the lowest point, decreasing by 4.67, 2.71, and 1.70 times compared with those at T1, respectively. The P_n value of the W treatment group only decreased by 1.16 times compared with that at T1. Changes in the T_r and G_s of hibiscus in each treatment group exhibited a consistent trend, and no significant difference was observed among the treatment groups at T1 and T2 (Fig. 3B, 3C). At T3, the T_r and G_s of the D + MT and D + SA treatment groups decreased significantly and were significantly lower than those of the D treatment group ($p < 0.05$). As the stress time continued, the T_r and G_s of each drought treatment group continued to decrease and reached the lowest point at T5, whereas the T_r and G_s of the D + MT and D + SA treatment groups were significantly higher than those of the D treatment group ($p < 0.05$). Compared with the D treatment group, the overall decline in the W treatment group was relatively small. From T1 to T4, the C_i of the D, D + MT, and D + SA treatment groups displayed a continuous downward trend, whereas at T5, it showed a sharp upward trend (Fig. 3D). The C_i of the W treatment group remained relatively stable. In the early stages of the experiment, no significant difference in WUE was observed among the treatment groups (Fig. 3E). At T3, the D + MT and D + SA treatment groups exhibited a significant increase in WUE compared with the D treatment group by 2.72 and 4.45 times, respectively ($p < 0.05$). As drought stress intensified, the WUE of each drought treatment group increased significantly and reached the highest point at T5. Compared with those at T1, the WUE of the D, D + MT, and D + SA treatment groups increased by 10.46, 12.38, and 13.19 times, respectively. The WUE of the W treatment group only increased by 2.04 times compared with that at T1, indicating a relatively small overall change.

3.5 Chlorophyll Content

Chl a and Chl b contents increased to varying degrees among the treatment groups as the treatment time was extended, but the degree of increase varied (Fig. 4). At T1, Chl a in the D + MT and D + SA treatment groups was significantly higher than that in the D and W treatment groups ($p < 0.05$), while no significant difference in Chl b was observed among the groups ($p > 0.05$). At T2, Chl a and Chl b contents were significantly higher in the D + MT treatment group than in the D treatment group ($p < 0.05$), whereas Chl a in the W treatment group was significantly lower than that in the D treatment group ($p < 0.05$). As drought stress intensified, the Chl a and Chl b contents of each drought treatment group increased sharply at T4, but no significant difference was observed among them. By contrast, the Chl a and Chl b contents of the W treatment group were significantly lower than those of the D treatment group ($p < 0.05$). At T5, the Chl a and Chl b contents of each drought treatment group further increased and reached their peak values. The Chl a and Chl b contents of the W treatment group remained significantly lower than those in the D treatment group. By contrast, the Chl a and Chl b contents of the D + MT and D + SA treatment groups

were significantly higher than those of the D treatment group ($p < 0.05$), and no significant difference was observed between the D + MT and D + SA treatment groups. This indicates that MT and SA application can significantly increase the chlorophyll content of hibiscus leaves in the late stage of drought stress.

3.6 Chlorophyll Fluorescence Parameters

Fv/Fm values remained at a relatively high level among the treatment groups from T1 to T3, and no significant difference was observed among them (Fig. 5A). With the extension of treatment time to T4, the *Fv/Fm* values decreased, but the degree of decrease varied. This indicated that severe water stress had significantly reduced the photosynthetic efficiency of Photosystem II (PSII). Compared with the W treatment group, *Fv/Fm* values in the D treatment group significantly decreased by 6.09%. By contrast, the D + MT and D + SA treatment groups exhibited higher *Fv/Fm* values than the D treatment group, but no significant difference was observed ($p > 0.05$). At T5, compared with those at T4, the *Fv/Fm* values rebounded slightly, with the values in the D, D + MT, and D + SA treatment groups increasing by 3.67%, 2.98%, and 3.26%, respectively. However, the *Fv/Fm* values in the D treatment group still significantly decreased by 2.71% compared with those in the W treatment group ($p < 0.05$). By contrast, the *Fv/Fm* values in the D + MT and D + SA treatment groups were higher than those in the D treatment group, but no significant difference was observed ($p > 0.05$).

The *NPQ* reflects the plant's ability to dissipate excess light energy through heat. *NPQ* values remained at a relatively high level among the treatment groups at T1 and T2, but no significant difference was observed (Fig. 5B). As the experiment continued, the *NPQ* value of each treatment group decreased significantly at T3. With continuous drought stress, the value rebounded at T4 and T5. Furthermore, compared with the D treatment group, the *NPQ* value of the D + MT and D + SA treatment groups significantly increased by 24.02% and 21.49% ($p < 0.05$), respectively, at T5.

3.7 ROS Metabolism

At T1, O_2^- and H_2O_2 contents in the W and D treatment groups were higher, possibly because of the high soil moisture content in the early stage of the experiment that was unsuitable for the normal growth of hibiscus. However, spraying MT and SA alleviated water stress to a certain extent (Fig. 6A-B). At T3, the O_2^- content in the W and D + MT treatment groups was significantly lower than that in the D treatment group. After T3, with an increase in drought stress, the O_2^- content in the D treatment group increased significantly, while that in the D + MT and D + SA treatment groups increased slowly, except for the D + SA treatment group in the T3 period, which was significantly lower than that in the D treatment group. This indicated that MT and SA could effectively remove O_2^-, and the time of MT scavenging O_2^- was earlier than that of SA scavenging O_2^-. At T4 and T5, drought stress increased H_2O_2 content in the hibiscus leaves (Fig. 6B), with the D treatment group exhibiting significantly higher levels than the W treatment group ($p < 0.05$). However, the H_2O_2 content was significantly lower in the D + SA treatment group than in the D treatment group at both T4 and T5 ($p < 0.05$). By contrast, the H_2O_2 content was significantly lower

in the D + MT treatment group than in the D treatment group only at T5 ($p < 0.05$). This indicates that MT and SA application can effectively remove H_2O_2, with SA clearing H_2O_2 earlier than MT. The tissue staining results for O_2^- and H_2O_2 are shown in Fig. 6C-D. The area of blue and brown spots on the leaves increased significantly at T5, and the area of spots on the leaves treated with exogenous substances was smaller than that on the hibiscus leaves subjected to drought stress alone, which is consistent with the results of O_2^- and H_2O_2 contents.

3.8 Antioxidant System

During the entire experiment, SOD activity exhibited a gradual decrease with an increase in drought stress duration (Fig. 7A). At T5, SOD activity in the W and D + SA treatment groups was significantly higher than that in the D treatment group ($p < 0.05$). Changes in POD, CAT, and APX activities were relatively consistent, demonstrating a significant increase as the drought stress severity increased (Fig. 7B-D). Compared with those at T3, POD, CAT, and APX activities in the D treatment group increased by 69.6%, 13.3%, and 28.6% at T4, and by 101.01%, 26.67%, and 40% at T5, respectively. Moreover, POD, CAT, and APX activities in the D + MT and D + SA treatment groups were higher than those in the D treatment group at T4 and T5, with a significant difference observed at T5 ($p < 0.05$). Moreover, the expression levels of antioxidant enzyme-related genes (*Cu/Zn-SOD*, *POD-20*, *CAT*, and *APX-1*) were upregulated in the D + MT and D + SA treatment groups compared with the D treatment group at T4 and T5 (Fig. 7E–H). These results indicate that exogenous MT and SA can improve antioxidant enzyme activity to a certain extent by inducing the expression of antioxidant enzyme-related genes.

3.9 Drought-Related Gene Expression

To further analyze the molecular mechanism underlying exogenous MT- and SA-mediated improvement in the drought resistance of hibiscus, we analyzed the expression levels of homologous genes of two representative drought-responsive genes reported in other studies (Hwang et al. 2018; Saleh et al. 2006), named *HsNCED*, and *HsDBF1*. qRT-PCR was used to study and analyze the expression levels of these two genes in exogenous substance-treated hibiscus seedlings under drought stress. As the water deprivation time was extended, the *HsNCED* expression level gradually increased from T3 and reached its highest value at T5 (Fig. 8A), while the *HsDBF1* expression level significantly increased at T3 (Fig. 8B). This indicated that these two genes are responsive to drought stress in hibiscus. Compared with the D treatment group, MT and SA application induced *HsNCED* and *HsDBF1* upregulation at T4 (Fig. 8C-D), and SA induced high *HsNCED* and *HsDBF1* expression at T5 (Fig. 8E-F). This suggests that exogenous MT and SA may regulate the drought resistance of hibiscus by further regulating *HsNCED* and *HsDBF1.*

4. Discussion

As a limiting environmental factor for plant growth, drought can inhibit respiration and photosynthesis to some extent and affect the physiological and ecological characteristics of plants (Yang et al. 2021; Zeng

et al. 2022). MT and SA alleviate the adverse effects of drought on plants (Campos et al. 2019; Kang et al. 2012). Hibiscus is an excellent ornamental and medicinal plant. However, research on MT and SA application (spraying) for improving the drought resistance of hibiscus is currently lacking. In this study, on analyzing the physiological indices of hibiscus treated with MT and SA for improving its drought resistance, we found that they could effectively alleviate drought-induced photosynthetic inhibition and reduce ROS accumulation.

Chlorophyll is a green pigment present in plant chloroplasts, and its content can, to some, extent, reflect the strength of photosynthetic ability and growth status (Li et al. 2018). Drought stress is known to cause chlorophyll degradation in apple (*M.* × *domestica*) (Jia et al. 2021) and grapevine (*Vitis vinifera* L.) (Zeng et al. 2022), thereby affecting their normal growth. MT and SA application has been reported to effectively increase the chlorophyll content of apple (Wang et al. 2013) and purslane (*Portulaca oleracea* L.) (Saheri et al. 2020), and reduce chlorophyll degradation. In the present study, spraying exogenous MT and SA significantly increased the chlorophyll content in the late drought period, indicating that these exogenous substances may induce chlorophyll synthesis in hibiscus. This result is similar to the research results of apple (Wang et al. 2013) and spinach (*Spinacia oleracea* L. cv.) (Akpinar and Cansev 2022) under drought stress. Our study found that spraying exogenous MT and SA can increase the relative leaf water content of plants and alleviate drought stress-induced leaf wilt and stem tip bending. Some studies have shown that *DBF1* overexpression in *Arabidopsis thaliana* can effectively alleviate drought stress-induced leaf wilting (Saleh et al. 2006). Other studies have suggested that *NCED* overexpression in *A. thaliana* can enhance its water retention capacity under drought stress, mitigating leaf wilt (Hwang et al. 2018). Here, MT and SA induced upregulation of *HsNCED* and *HsDBF1* expression in the late stage of drought stress. Therefore, we speculated that MT and SA can further enhance the water retention capacity of hibiscus leaves and reduce leaf damage by upregulating *HsNCED* and *HsDBF1* expression.

In plants, drought stress can cause photoinhibition, reducing the photosynthetic efficiency of PS and PS (Wang et al. 2021). Experiments have shown that exogenous MT and SA can alleviate the photoinhibition of drought stress-exposed apple (Wang et al. 2013) and maize (Shemi et al. 2021) plants by increasing the chlorophyll content. By measuring the photosynthetic capacity of the plants, we found that exogenous MT and SA alleviate drought stress-induced photoinhibition of hibiscus (Fig. 3A). In the late stage of drought stress, the T_r value in the D + MT and D + SA treatment groups was higher than that in the D treatment group, which indicated that exogenous MT and SA may maintain a certain transpiration level under severe drought stress to preserve plant growth and alleviate drought stress. From T1 to T4, the P_n and C_i values of hibiscus leaves decreased with a decrease in G_s. However, at T5, the P_n and G_s values continued to decline, whereas C_i sharply increased. This indicated a shift from stomatal limitation to non-stomatal limitation at this time (Medrano et al. 2002). In addition, the study results showed that the WUE of plants increased under drought stress. The WUE of MT- and SA-treated hibiscus was significantly higher than that of plants under drought stress alone ($p < 0.05$). Therefore, MT and SA are believed to enhance the drought resistance of hibiscus by increasing its water use efficiency in the late stage of drought stress. This is consistent with the results in apple (Wang et al. 2013) and barley (Habibi 2012).

The AQY reflects the efficiency of light energy conversion of plants, especially the efficiency of using weak light. The height of the LCP reflects the plant's ability to use weak light (Craine and Reich 2005). The smaller the LCP value, the stronger the plant's ability to use weak light. In this study, drought stress decreased hibiscus AQY, but exogenous MT and SA reduced the decrease in AQY. The AQY of MT- and SA-treated plants was were significantly higher than that of hibiscus without exogenous substance treatment. As drought stress intensified, the LCP value of each drought treatment group exhibited an upward trend at T5, and the LCP value of MT-and SA-sprayed hibiscus was lower than that of plants under drought stress alone. These results indicate that drought stress inhibits the plant's ability to use weak light, while spraying exogenous MT and SA can help improve this ability and enhance the tolerance of hibiscus plants to drought stress.

Chlorophyll fluorescence technology has the characteristics of rapid, sensitive, and non-destructive detection. It is widely used to detect drought stress-induced damage to the photosynthetic system (Giglou et al. 2022; Lichtenthaler and Rinderle 1988). *Fv/Fm* represents the potential quantum efficiency of PS and can reflect the photosynthetic inhibition of plants. The optimal *Fv/Fm* value for most plants is approximately 0.83 (Maxwell and Johnson 2000). The *Fv/Fm* value of all treatment groups was > 0.8, and the *Fv/Fm* value of the D + MT and D + SA treatment groups were higher than that of the D treatment group at both T4 and T5. The *Fv/Fm* value did not decrease significantly with drought stress aggravation, which may be due to the alleviation of plant photoinhibition by increased chlorophyll content, which avoids serious damage to the photosynthetic system. *NPQ* is a photoprotective regulatory mechanism where plants convert excess light energy into heat to alleviate photodamage. *NPQ* is considered a necessary mechanism for protecting cells from light damage (Sperdouli and Moustakas 2012). Drought stress leads to an increase in *NPQ* in *Jatropha curcas* (Silva et al. 2015) and *Medicago sativa* (Huihui et al. 2020). In this study, drought stress increased *NPQ* in hibiscus seedlings, and exogenous MT and SA further increased the *NPQ* of plants, indicating that spraying exogenous substances on hibiscus seedlings can increase the heat dissipation capacity, convert excess light energy into heat, reduce light absorption by drought stress-exposed plants, and alleviate drought stress-induced photoinhibition damage to plants.

ROS in plant cells is primarily generated within organelles such as chloroplasts, mitochondria, and peroxisomes that have a high oxidative metabolic activity or a strong electron flow rate (Gill and Tuteja 2010). Drought stress can increase ROS accumulation in plants and cause harm to plant cells (Wang et al. 2021). The reaction centers of PS and PS in chloroplasts are crucial sites for ROS production, and photosynthetic inhibition in plants can lead to excessive ROS production (Asada 2006; Che et al. 2018). In this study, with the aggravation of drought stress, the hibiscus photosynthetic rate exhibited a decreasing trend and varying degrees of photoinhibition appeared. To verify whether ROS would be generated in hibiscus seedlings under photoinhibition, the H_2O_2 and O_2^- contents of leaves at different drought stress levels were measured, and tissue staining for H_2O_2 and O_2^- was performed using DAB and NBT. According to the results, drought stress increased ROS content in hibiscus plants, whereas exogenous MT and SA significantly alleviated the ROS increase ($p < 0.05$), thus reducing damage to hibiscus leaf cells.

This result is consistent with the results of studies on *V. faba* L. (Dawood et al. 2022)d *sativa* L. (Luo et al. 2022).

To prevent excessive ROS accumulation, plants activate enzymatic and non-enzymatic antioxidant systems to scavenge ROS (Nadarajah 2020). Antioxidant enzymes, including SOD, POD, CAT, APX, and GR, are a major player in the defense system of plants against biotic and abiotic stressors (Altaf et al. 2022). SOD can convert O_2^- into H_2O_2 and is considered the first step in resisting oxidative damage (Zeng et al. 2022). POD, CAT, and APX convert the produced H_2O_2 into H_2O and O_2 to prevent ROS-induced oxidative damage (Hasanuzzaman et al. 2020). Under drought stress, MT not only increased the antioxidant enzyme activity of tomato (*Solanum lycopersicum* L.) but also upregulated the expression of antioxidant enzyme-related genes (Altaf et al. 2022). Saheri et al. (2020) found that SA can increase the antioxidant enzyme activity of purslane under drought stress. Consistent with these findings, we here observed that hibiscus plants treated with MT and SA after the later stages of drought stress exhibited higher antioxidant enzyme activity and significantly upregulated *Cu/Zn-SOD*, *POD-20*, *CAT*, and *APX-1* expression than plants subjected to drought stress alone. This suggests that exogenous MT and SA can enhance the antioxidant enzyme activity of hibiscus under drought stress, reduce ROS accumulation, and prevent oxidative damage to the plants.

5. Conclusions

We propose a simplified model to describe the role of exogenous MT and SA in drought tolerance (Fig. 9). The pretreatment with 100 μM MT and 500 μM SA could alleviate drought-induced leaf wilting and shoot tip bending in hibiscus. MT and SA increased the photosynthetic pigment content, photosynthesis, and heat dissipation capacity of leaves. As the expression and enzyme activity of antioxidant enzyme (SOD, POD, CAT, APX)-encoding genes increased, the content of oxidative stress markers (O_2^-, H_2O_2) reduced and drought-induced photoinhibition and oxidative damage alleviated. In addition, in the late drought stress stage, exogenous MT and SA may increase the drought resistance of hibiscus by inducing the expression of drought response genes *HsNCED* and *HsDBF1*. The study results can provide a theoretical basis for hibiscus cultivation and management in arid and semi-arid areas.

Declarations

Supplementary Materials:

The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Primer sequences for quantitative real-time PCR.

Author Contributions:

Conceptualization, R.Y. and J.G.; methodology, R.Y. and J.L; software, R.Y.; validation, R.Y. and J.L.; formal analysis, R.Y.; investigation, R.Y. and J.L.; resources, S.Z.; data curation, R.Y. and J.L.; writing—original

draft preparation, R.Y.; writing—review and editing, J.G. and S.Z.; visualization, R.Y. and J.L.; supervision, J.L.; project administration, S.Z.; funding acquisition, J.G. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement:

All data relevant to the main findings of this study are included within the article.

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Conflicts of Interest:

The authors declare no conflict of interest.

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Figures

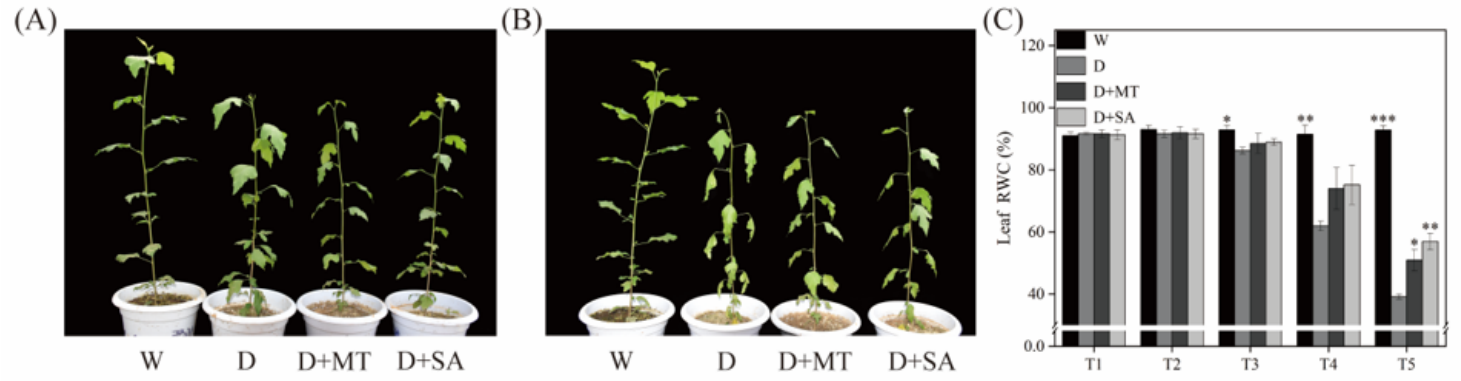

Figure 1

Effects of melatonin (MT) and salicylic acid (SA) treatments on the growth characteristics and reactive water content of hibiscus. (A) Phenotypes in T4. (B) Phenotypes in T5. RWC, leaf relative water content. Values represent means ± SE of three replicates. Asterisks indicate statistical significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; independent samples t-test) of the W, D + MT and D + SA treatment groups compared to D treatment group in the same treatment time.

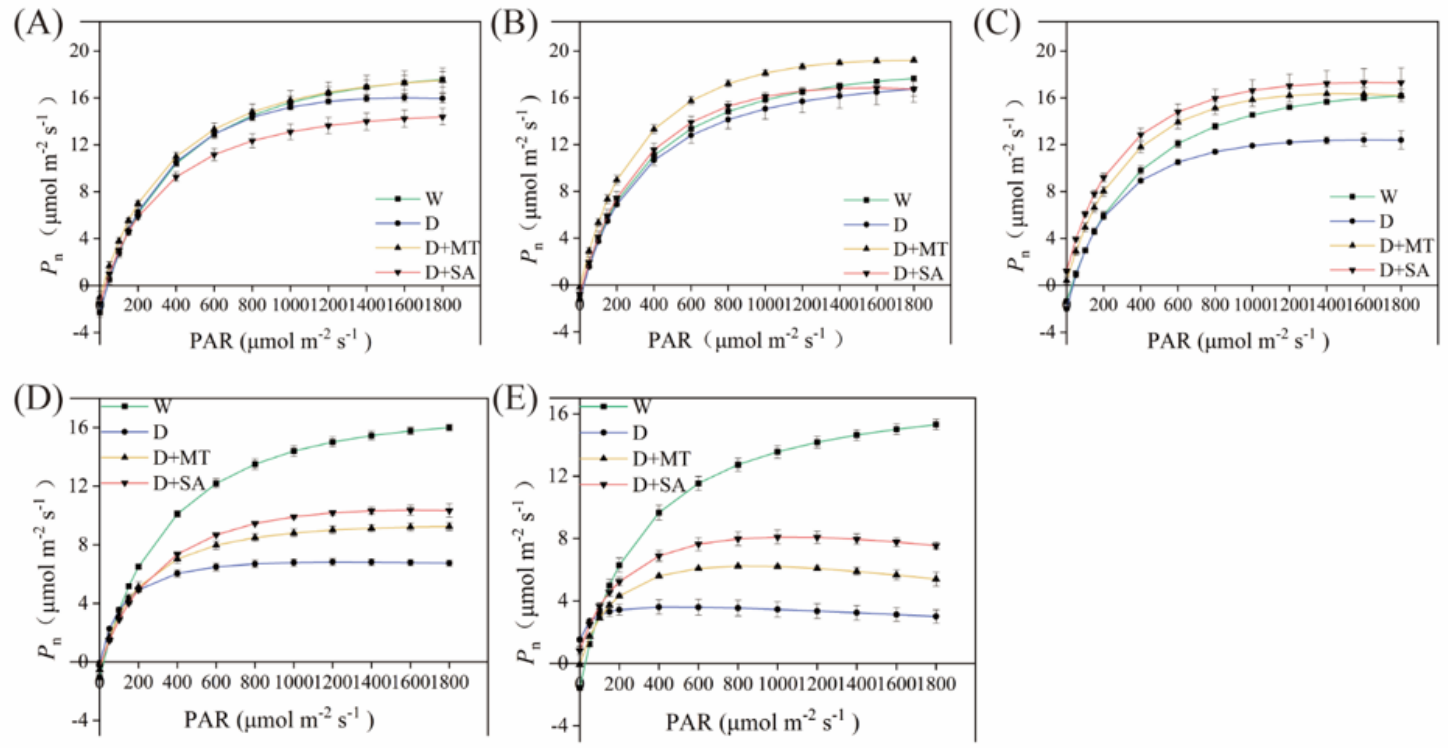

Figure 2

Effect of melatonin (MT) and salicylic acid (SA) on the light response of net photosynthetic rate (P_n) in hibiscus. (A-E) Light response curves of P_n in T1-T5. Values represent means ± SE of three replicates.

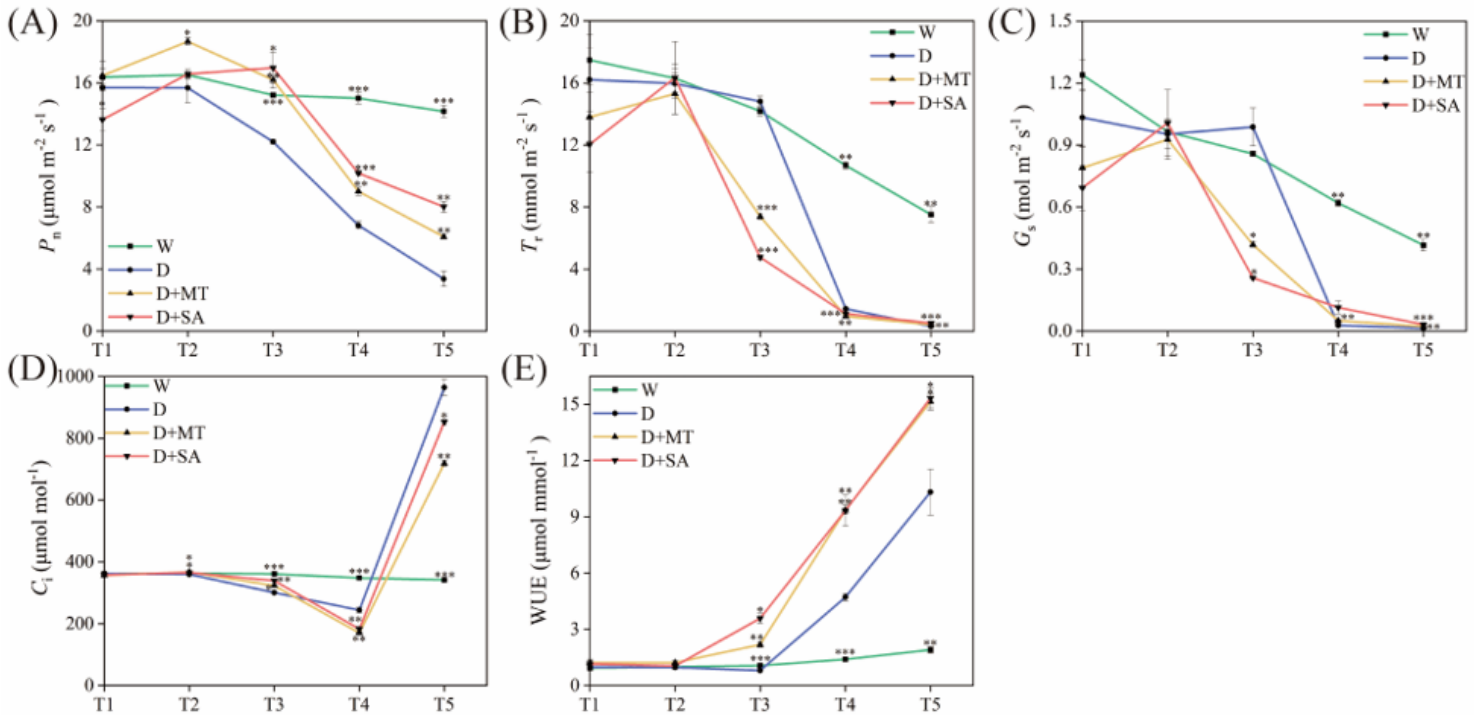

Figure 3

Effect of melatonin (MT) and salicylic acid (SA) on gas exchange parameters in hibiscus. (A) Net CO_2 assimilation (P_n). (B) Transpiration (T_r). (C) Stomatal conductance (G_s). (D) Intercellular carbon dioxide concentration (C_i). (E) Water use efficiency (WUE). Values represent means ± SE of three replicates. Asterisks indicate statistical significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; independent samples t-test) of the W, D + MT and D + SA treatment groups compared to D treatment group in the same treatment time.

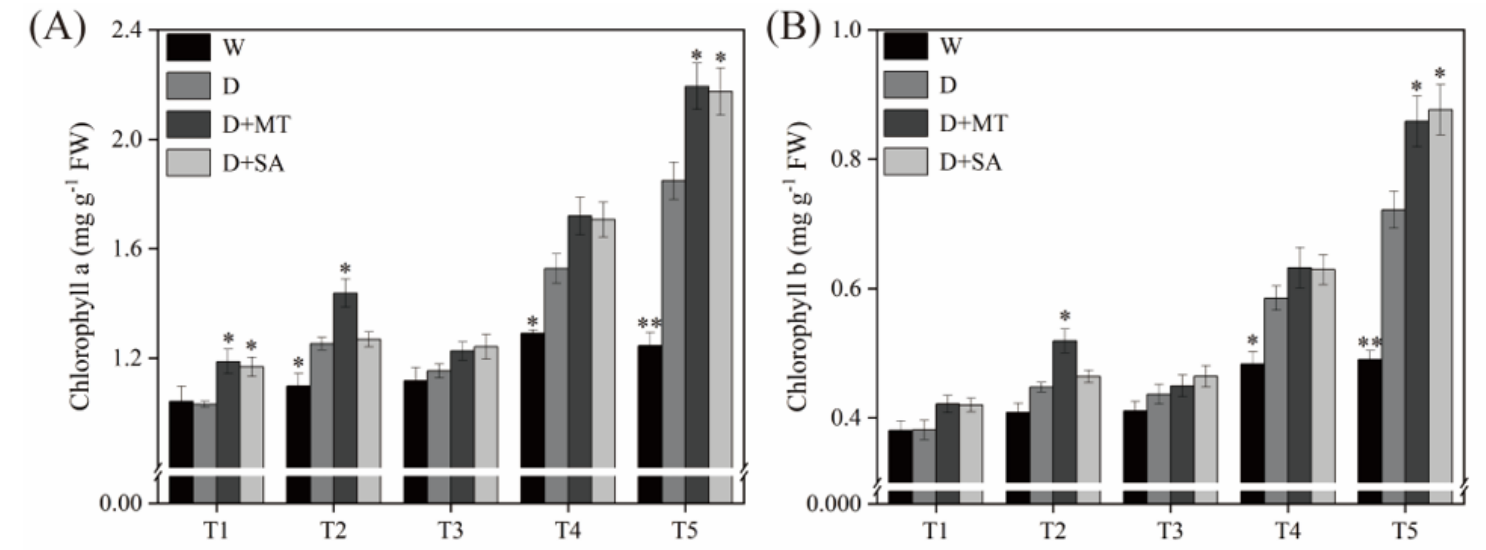

Figure 4

Effect of melatonin (MT) and salicylic acid (SA) on chlorophyll content in hibiscus. Values represent means ± SE of three replicates. Asterisks indicate statistical significance (*, $p < 0.05$; **, $p < 0.01$; independent samples t-test) of the W, D + MT and D + SA treatment groups compared to D treatment group in the same treatment time.

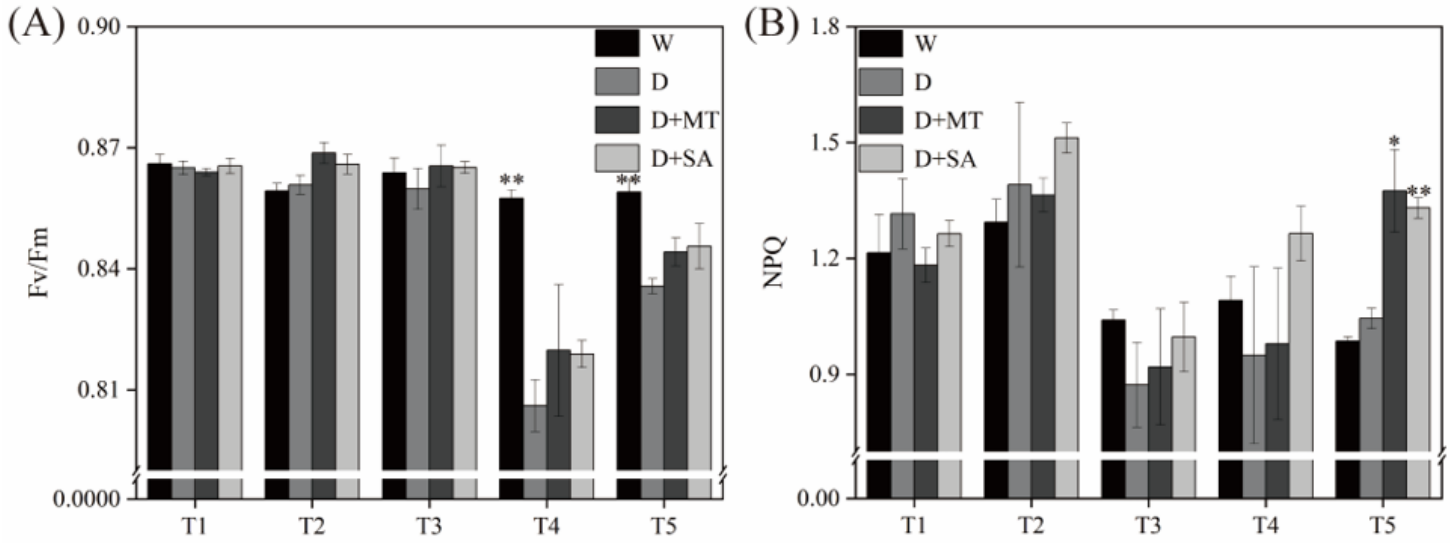

Figure 5

Effect of melatonin (MT) and salicylic acid (SA) on chlorophyll fluorescence parameters in hibiscus. (A) Maximum optical efficiency (*Fv/Fm*). (B) Non-photochemical quenching (*NPQ*). Values represent means ± SE of three replicates. Asterisks indicate statistical significance (*, $p < 0.05$; **, $p < 0.01$; independent samples t-test) of the W, D + MT and D + SA treatment groups compared to D treatment group in the same treatment time.

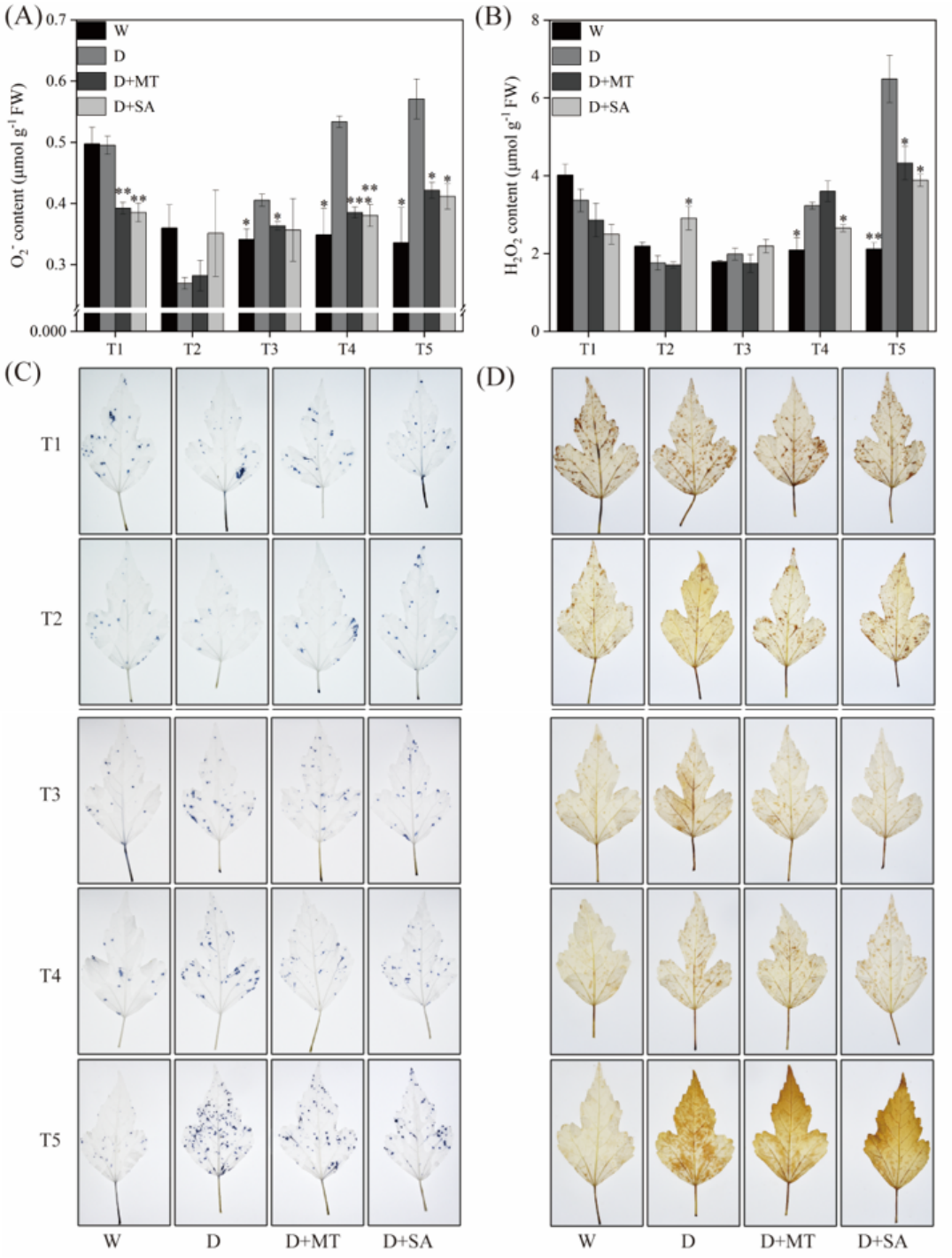

Figure 6

Effect of melatonin (MT) and salicylic acid (SA) on reactive oxygen species content in hibiscus. (A) Superoxide anion (O_2^-) content. (B) Hydrogen peroxide (H_2O_2) content. (C) Histochemical detection of O_2^- by NBT staining. (D) Histochemical detection of H_2O_2 by DAB staining. Values represent means ± SE of three replicates. Asterisks indicate statistical significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; independent samples t-test) of the W, D + MT and D + SA treatment groups compared to D treatment group in the same treatment time.

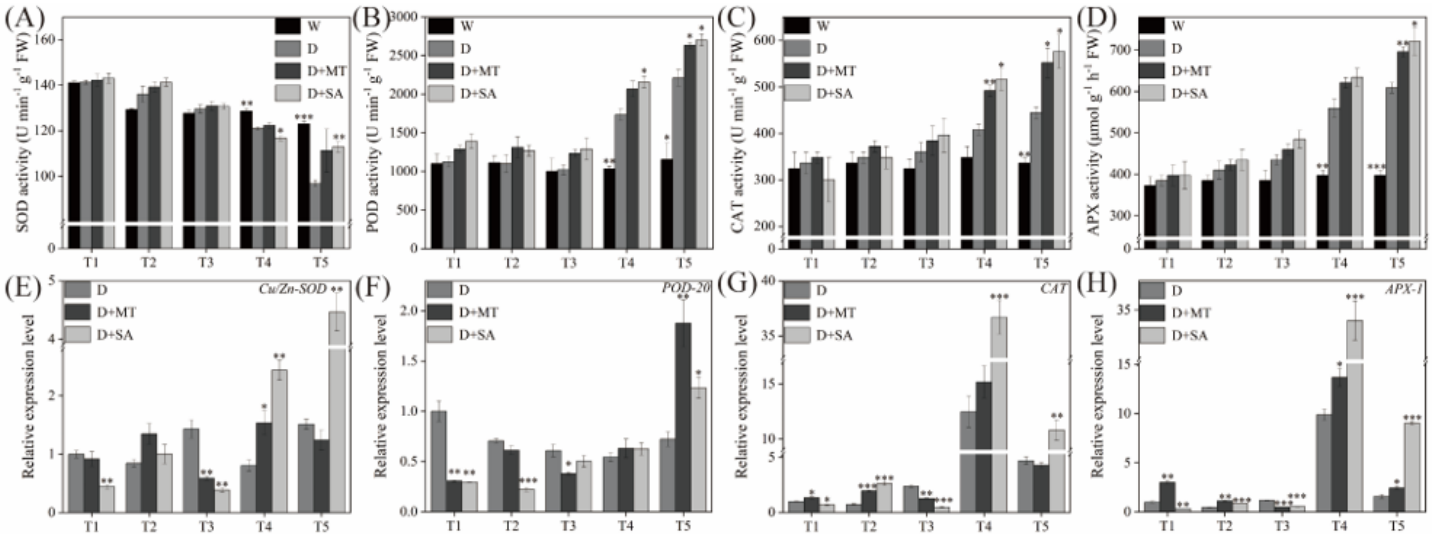

Figure 7

Effect of melatonin (MT) and salicylic acid (SA) on antioxidant system in hibiscus. Values represent means ± SE of three replicates. Asterisks indicate statistical significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; independent samples t-test) of the W, D + MT and D + SA treatment groups compared to D treatment group in the same treatment time.

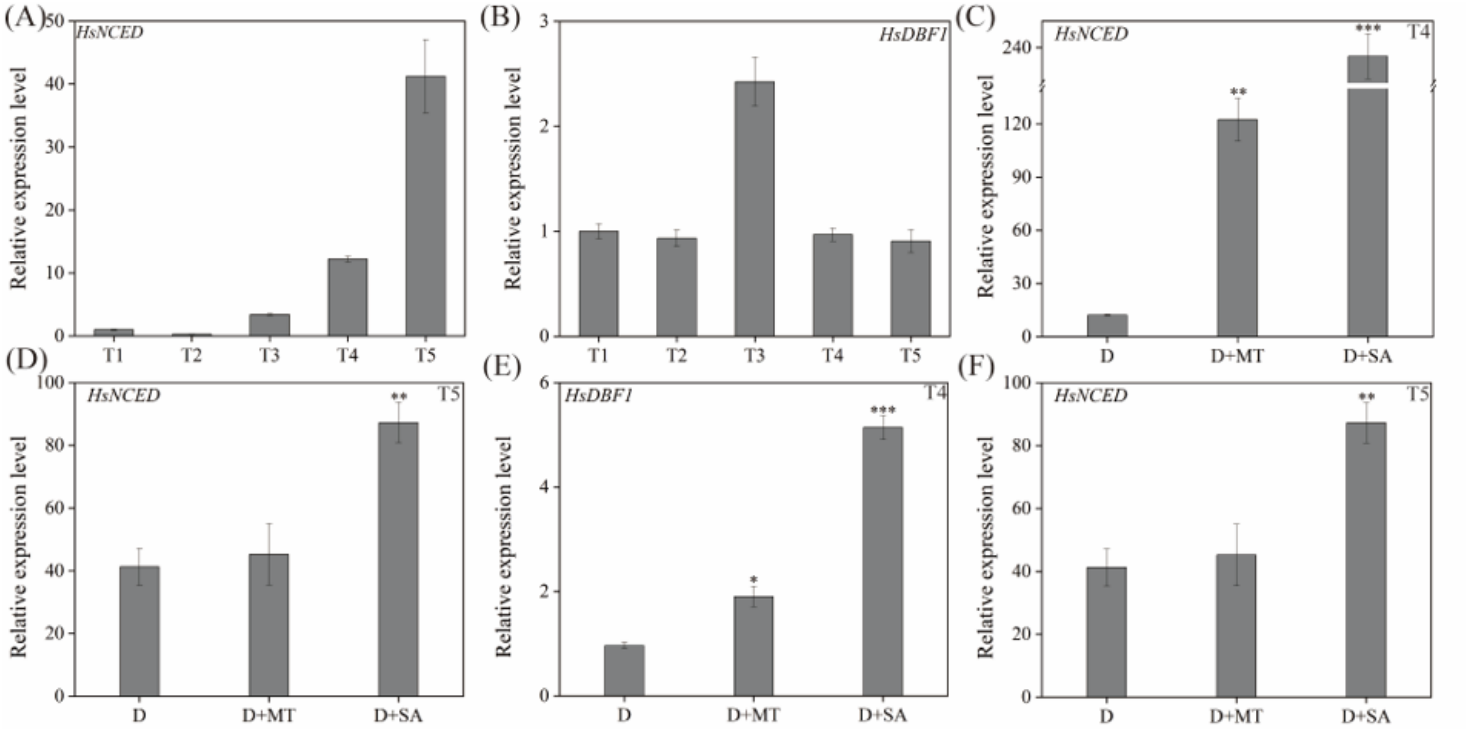

Figure 8

Effect of melatonin (MT) and salicylic acid (SA) on the expression of *HsNCED* and *HsDBF1*in hibiscus. (A) and (B) Expression level of *HsNCED* and *HsDBF1* in hibiscus of D treatment group. Values represent means ± SE of three replicates. Asterisks indicate statistical significance (*, $p < 0.05$; **, $p< 0.01$; ***, $p < 0.001$; independent samples t-test) of the W, D + MT and D + SA treatment groups compared to D treatment group in the same treatment time.

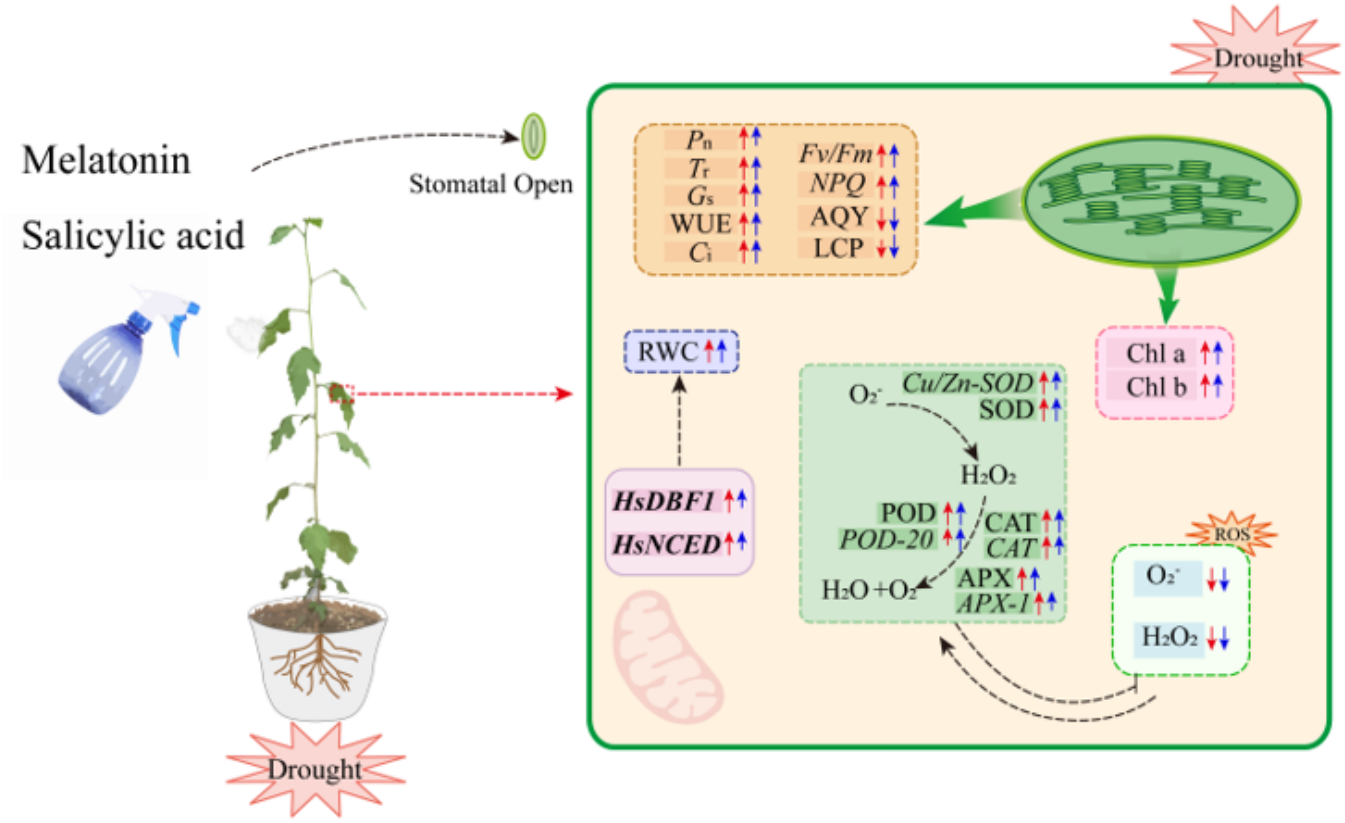

Figure 9

Schematic diagram of the physiological mechanisms by which exogenous melatonin and salicylic acid enhance drought resistance in hibiscus. The red line represents salicylic acid, the blue line represents melatonin, and the length of the line indicates the strength of regulation.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable.docx