

# Exogenous melatonin alleviated growth inhibition and oxidative stress induced by drought stress in apple rootstock

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**Abstract:** Drought stress is one of the major environmental obstacles that limit the production and development of apples (*Malus domestica* Borkh.). The role of melatonin is well known in the protection of plants under environmental stresses. In this study, we investigated the effect of melatonin on apple rootstock *M. hupehensis* Rehd under drought stress. The results showed that drought inhibited the growth of *M. hupehensis* and dramatically reduced root surface area, root volume, the number of tips and forks, and root diameter. Drought-induced growth inhibition was significantly decreased by adding melatonin. Net photosynthetic rate (Pn), intercellular CO<sub>2</sub> concentration (Ci), stomatal conductance (Gs), transpiration rate (Tr), were markedly reduced under drought stress. However, the application of melatonin could mitigate the damage to the photosynthetic apparatus and increase the quantum yield of PSII photochemistry. Additionally, generation of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and superoxide radicals (O<sub>2</sub><sup>•-</sup>) sharply increased in apple leaves after 4 days under drought stress, and the accumulation of electrolyte leakage (EL) represented oxidative stress, while by applying melatonin under drought stress, the generation of O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub> were significantly reduced and protected the membrane from drought damages. These results suggest that the adverse effects of drought can be minimized by applying melatonin to apples.

## Introduction

Drought is one of the most important environmental stresses that affect plant growth and development worldwide (Chaves *et al.*, 2003). Drought stress inhibits plant growth via many physiological processes, including chlorophyll biosynthesis, photosynthesis, energy consumption, and reactive oxygen species (ROS) metabolism (Barchet *et al.*, 2014; Liang *et al.*, 2019; Qiang *et al.*, 2019). Photosynthesis is one of the physiological processes which is sensitive to environmental stresses (Johnson *et al.*, 2015). It has been reported that drought inhibits photosynthesis through stomatal limitation and non-stomatal limitation (Barchet *et al.*, 2014; Bai *et al.*, 2019). Stomatal closure is one of the earliest responses of plants to drought stress, reducing transpiration and photosynthetic rate (Chaves *et al.*, 2003). Notably, drought stress induced over-reduction of the electron transport chain, which in turn leads to photooxidation and excessive accumulation of ROS, such as superoxide radicals (O<sub>2</sub><sup>•-</sup>)

and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Bano *et al.*, 2012; Liu *et al.*, 2012). These ROS are very reactive and damage the photosynthetic apparatus, reduce the photosynthetic efficiency, and ultimately result in cell death (Yin *et al.*, 2005; Gupta *et al.*, 2017; Gururani *et al.*, 2015).

To overcome drought, plants have developed a variety of adaptation mechanisms, such as movement of leaves or chloroplasts, photorespiration, and ROS scavenging system (Yin *et al.*, 2005; Liu *et al.*, 2012; Basu *et al.*, 2016). Drought resistance and adaptation in plants developed via morphological structure and physiological changes in leaves. Plants can induce stomatal closure, accumulation of compatible solutes, and biosynthesis of wax to avoid drought (Galmés *et al.*, 2007). Additionally, the ROS scavenging system is also important for the physiological mechanisms which are supportive of drought resistance in plants. Enhanced ROS scavenging system alleviates oxidative damage and induces some molecular chaperones to protect the plant from damage (Bai *et al.*, 2009; Basu *et al.*, 2016). Drought stress was reported that stimulate the ROS scavenging system in apple leaves under mild to moderate stress, but that was lower under severe stress because of antioxidant enzyme damage (Liu *et al.*, 2012). Furthermore, plants are regulated

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by the absorption of immobile nutrients *via* root systems under drought stress (Hanslin *et al.*, 2019).

The application of biostimulators is one of the promising and practical strategies to enhance stress tolerance (Wei *et al.*, 2015; Debnath *et al.*, 2019). Melatonin is known as a biopromoter and plays important roles in regulating plant growth, development, and stress tolerance (Wang *et al.*, 2012; Arnao and Hernández-Ruiz, 2014; Fan *et al.*, 2018). In recent years, an increasing number of studies have reported the physiological functions of melatonin in plants to serve as the first line of defense against environmental stresses. Previous studies suggested that drought tolerance has increased by applying melatonin under environmental stress, such as drought, salinity, ultraviolet radiation, chilling, and heat (Posmyk *et al.*, 2009; Galano *et al.*, 2011; Li *et al.*, 2012; Zhang *et al.*, 2014; Wei *et al.*, 2015). Stress alleviation effect of melatonin relies on various factors, including inhibiting chlorophyll degradation, improving the photosynthesis, enhancing the antioxidant system, and directly scavenging free radicals (Wang *et al.*, 2012; Liu *et al.*, 2015; Ke *et al.*, 2018; Debnath *et al.*, 2019; Asif *et al.*, 2020).

Apple (*Malus × domestica* Borkh.) is one of the most economically important fruits worldwide, with the largest area of cultivation in China (Liu *et al.*, 2012). Most apple trees are grown in arid or semi-arid regions, and drought is considered as the main environmental factor restricting the development and production of apples (Bai *et al.*, 2019). The development of drought resistance is a priority goal in apples. Rootstocks play a vital role in productive apple orchards because of their contributions to water and nutrient uptake and abiotic stress resistance (Valverdi *et al.*, 2019). *M. hupehensis*, which originates in China, is an excellent rootstock because of its resistance to abiotic stress. In addition, *M. hupehensis* is apomictic, resulting in uniform phenotypes and genotypes. So, it is widely used as rootstock for apple production (Huang *et al.*, 2018). Evidence suggested melatonin is known as a biopromoter and plays important roles in regulating plant drought tolerance (Fleta-Soriano *et al.*, 2017; Liang *et al.*, 2019). Therefore, the objective of this work was to examine the influence of melatonin on the seedling growth, root architecture, photosynthesis, chlorophyll fluorescence of *M. hupehensis* exposed to drought.

## Materials and Methods

### Plant materials and experimental design

Seeds of *M. hupehensis* were laminated by sand at 4°C for 35 to 40 days (d). Then, one germinated seed was planted in one plastic pot (6 cm × 6 cm) filled with soil/organic substrate (1:5, v:v), the plastic pots were then placed in a greenhouse at 20–25/15–20°C (day/night), with a 16-hour photoperiod (photosynthetically active radiation, 180–200 μmol m<sup>-2</sup> s<sup>-1</sup>), and the relative humidity was approximately 70%–80%. After growing for 70 d, the seedlings were assigned to a pretreatment group that received irrigation water containing 0.2 mM melatonin, whereas others continued to receive irrigation water containing 0 mM melatonin. After the 10-d pretreatment was completed, the seedlings were assigned to

one of three treatments: (1) Control (CK), plants normally watered; (2) Drought treatment (D), plants exposed to progressive drought by withholding irrigation; and (3) Drought + melatonin treatment (DM), plants receiving melatonin pretreatment for 10 d and then exposed to drought stress. Each treatment contained three replications with 30 plants in each replicate, resulting in a total of 90 plants per treatment. At 0, 1, 2, 3, and 4 d after treatment, photosynthetic measurements were recorded, and leaves were sampled from plants of each treatment.

### Root growth

After the treatments, the seedlings were taken out, and the roots were then washed with water. The root system was scanned with a scanner (SNAPSCAN 310, Agfa, Beijing, China), and root length, root surface area, root volume, number of root tips, and branching angle of each root were measured by root image analysis software WinRHIZO (Canada, Regent Instruments).

### Photosynthetic and chlorophyll fluorescence

Photosynthetic measurements were determined with a Li-Cor 6400 portable photosynthesis system (Li-6400XT, LICOR, Lincoln, Nebraska, USA). The net photosynthetic rate (Pn), stomatal conductance (Gs), intercellular CO<sub>2</sub> concentration (Ci), and transpiration rate (Tr) were recorded from mature leaves between 09:00 AM and 11:00 AM. Chlorophyll fluorescence parameters in leaves were measured with a portable photosynthetic efficiency analyzer (DUAL-PAM-100, WALZ, Germany). Leaves were dark-adapted for 30 min in leaf clips before measurements. Measurements were taken between 10:00 AM and 02:00 PM from six leaves per treatment. Chlorophyll fluorescence parameters were calculated according to Genty *et al.* (1989).  $Fv/Fm = (Fm - F0)/Fm$ ,  $qP = (Fm' - Fs)/(Fm' - F0)$ ,  $NPQ = (Fm - Fm')/Fm'$ . That,  $Fv/Fm$  is the maximal potential quantum efficiency of photosystem II;  $Fm$  is the maximum fluorescence (in the dark-adapted leaves);  $F0$  is the minimum chlorophyll fluorescence; the photochemical quenching value was evaluated using  $qP = (Fm' - Fs)/(Fm' - F0)$  formula representing the photochemical energy conversion of the PS II reaction centers when the primary acceptor QA has been oxidized;  $Fv$  is the variable fluorescence (dark) ( $Fm - F0$ ); and  $NPQ$  is non-photochemical quenching.

### Determination of chlorophyll content and electrolyte leakage

Chlorophyll was extracted from 0.1 g of leaf tissue in 80% (v/v) acetone under darkness at 25°C. After centrifugation for 15 min at 13000×g, values were measured at A663, A645, and A470 with a spectrophotometer (UV-9000S, Shanghai, China). Total chlorophyll (Chl t), chlorophyll a (Chl a), and chlorophyll b (Chl b) contents were calculated according to the equations (Porra *et al.*, 1989). Electrolyte leakage (EL) was determined according to the method described by Dionisio-Sese and Tobita (1998).

### Histochemical detections of H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>•-</sup>

H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>•-</sup> were detected by histochemical staining with 3,3'-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT), respectively, according to the method described by

Rodríguez and Taleisnik (2012). For  $H_2O_2$  measurement, leaves were stained in 1 mg/mL DAB solution and incubated for 12 h at room temperature in darkness, then photographed. For  $O_2^{\cdot-}$  measurement, leaves were stained in 0.1 mg/mL NBT in 25 mM K-HEPES buffer (pH 7.9) for 1 h. Then, the samples were kept at 25°C for an additional 4 h. After 12 h of incubation, leaves were decolorized by immersing them in boiling 70% ethanol to detect the deep brown polymerization product. After cooling, photographs were taken after placing the leaves on the bench.

#### Statistical analysis

Data were statistically analyzed with IBM SPSS Statistics 17.0 (IBM China Company, Ltd., Beijing, China). Results were represented as the mean values  $\pm$  standard deviation. The statistical significance between treatments was assessed by one-way analysis of variance at  $P < 0.05$ .

## Results

#### Exogenous melatonin improved the growth parameters

*M. hupehensis* responded to drought stress with morphological changes of the seedling showing leaf wilting due to dehydration (Fig. 1). However, melatonin-pretreated plants under drought stress exhibited a significant improvement compared to control plants. To evaluate the effect of melatonin on root traits under drought stress, we investigated the total root length, root diameter, root surface area, root volume, and the number of forks. Compared to control, root surface area, number of tips, forks, and root diameter significantly decreased under drought stress (Table 1). However, after treatment with irrigation water containing melatonin, the root surface area, root volume, and root diameter significantly increased, and were 42.71%, 133.33%, and 36.36% greater than those in the drought stress treatment, respectively.

Additionally, the effects of exogenous melatonin on the root length proportion of *M. hupehensis* were detected under drought stress (Fig. 2). There was no obvious change

in the proportion of short root lengths among control, drought stress, and melatonin-treated plants. However, drought reduced the proportion of medium and long root lengths, especially for the length of 3.0–3.5 cm. In contrast, melatonin significantly increased the proportion of medium and long root lengths.

#### Exogenous melatonin enhanced photosynthesis and fluorescence parameters

Drought stress caused a continuous decline in gas exchange parameters over time. With the extension of drought treatment time, all gas exchange parameters decreased sharply, except  $C_i$ , which exhibited a smooth decline (Fig. 3). However, the application of melatonin resulted in higher  $G_s$ ,  $C_i$ , and  $T_r$  under drought stress. At the beginning of the second day, the  $P_n$  increased then decreased until the end of the second day. Additionally, the application of melatonin effectively improved  $P_n$ .

Chlorophyll fluorescence measurement technology was used to explore the effects of melatonin priming on the photosynthetic capacity of apple leaves under drought stress (Fig. 4).  $F_v/F_m$  and  $qP$  of apple leaves decreased sharply, over time, under drought treatment compared to control ones. However, drought stress remarkably increased the  $F_o$  value. In contrast, melatonin application improved the photochemical efficiency of *PSII*, which was expected to be sensitive to some environmental stresses, suggesting that melatonin priming could reduce damage to *PSII* of apple leaves under drought stress.

#### Exogenous melatonin enhanced chlorophyll content

To illustrate the role of melatonin on chlorophyll content under drought stress, we detected the content of Chl a and Chl b. After the first 2 d, all chlorophyll a and b content and the ratio of Chl a/b smoothly declined (Fig. 5). Then, a sharp decrease was observed during the last days under drought stress. However, the reduction of Chl caused by drought stress was significantly alleviated by melatonin treatment.

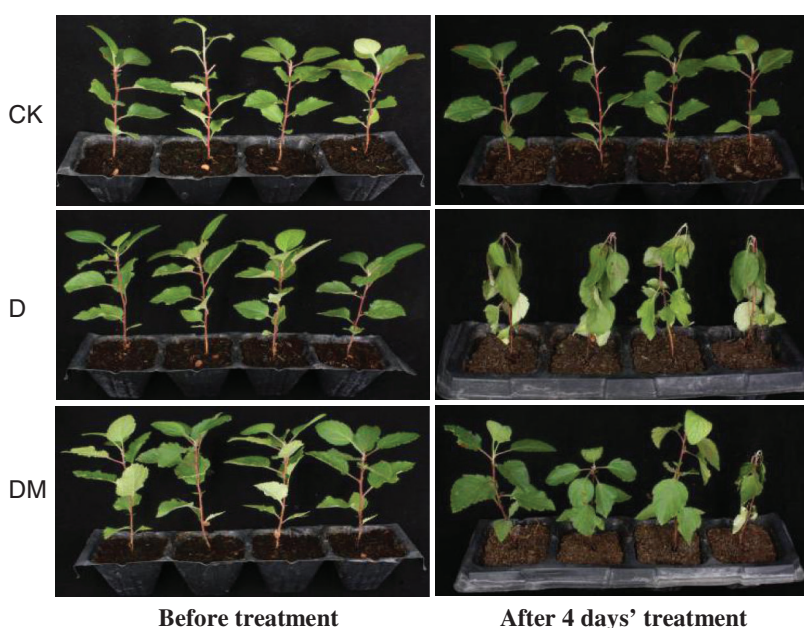


FIGURE 1. Growth status of *M. hupehensis* under drought conditions for 4 d. CK: plants not pretreated with drought and melatonin; D: plants under drought stress; DM: melatonin-pretreated plants under drought stress.

TABLE 1

Effects of exogenous melatonin on the total root length, root surface area, root volume, root forks, the number of root tips, and root diameter of *M. hupehensis* under drought stress

Root architecture parameters	Treatment		
	CK	D	DM
Total root length (cm)	314.1 ± 37.5 b	313.9 ± 60.7 b	391.3 ± 42.8 a
Root surface area (cm <sup>2</sup> )	68.5 ± 9.2 b	59 ± 12.3 c	84.2 ± 13.9 a
Root volume (cm <sup>3</sup> )	1.2 ± 0.3 b	0.9 ± 0.3 b	2.1 ± 0.7 a
Number of tips	467.9 ± 200.4 a	395.1 ± 121.5 b	444.3 ± 123.7 a
Forks	1896.2 ± 257.0 a	1645.2 ± 372.4 b	1737.6 ± 204.6 a
Root diameter (mm)	1.2 ± 0.2 ab	1.1 ± 0.2 b	1.5 ± 0.2 a

Note: Data are mean values ± SD (N = 15). Different letters in the same row indicate a significant difference among treatments according to LSD' test ( $P < 0.05$ ).

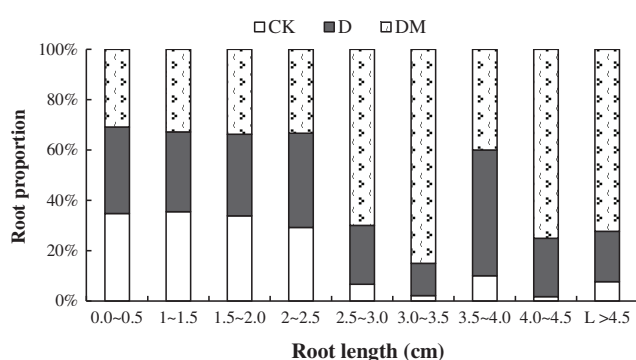


FIGURE 2. Root proportions of *M. hupehensis* under drought conditions with and without melatonin application. Data are mean values of 15 measurements ± standard deviation. CK: plants not pretreated with drought and melatonin; D: plants under drought stress; DM: melatonin-pretreated plants under drought stress.

#### Exogenous melatonin delayed membrane lipid peroxidation

The data showed that EL increased significantly with an increase in the duration of drought stress with maximum EL recorded at 4 d (Fig. 6). Compared to the control plants, the melatonin exhibited a significant effect on the drought response, which significantly decreased EL of the melatonin-treated plants.

#### Exogenous melatonin declined $O_2^{\bullet-}$ and $H_2O_2$ contents

As shown in Fig. 7, photographs of the stained leaves against a contrasting background were documented for oxidation conditions.  $H_2O_2$  was visualized as a reddish-brown stain formed by the reaction of DAB with the endogenous  $H_2O_2$ .  $O_2^{\bullet-}$  was detected as a dark blue stain of the formazan compound formed as a result of NBT reacting with the endogenous  $O_2^{\bullet-}$ . When the time of drought treatment reached 2 d, the accumulation of  $O_2^{\bullet-}$  and  $H_2O_2$  was not significantly different from that of the melatonin treatment. However,  $H_2O_2$  and  $O_2^{\bullet-}$  were sharply increased in apple leaves under drought stress after 4 d compared to the control ones. Exogenous application of melatonin significantly reduced the generation of  $O_2^{\bullet-}$  and  $H_2O_2$ , and the highest efficiency of  $O_2^{\bullet-}$  and  $H_2O_2$  removal was observed in the drought stress treatment after 4 d.

#### Discussion

Apple is known to be a large water consumer and drought stress is the major limitation for high quality and yield of apple (Atkinson et al., 1999; Liu et al., 2012). Identifying ways to produce more apples with less water consumption is essential for the high quality and yield of apples. Melatonin

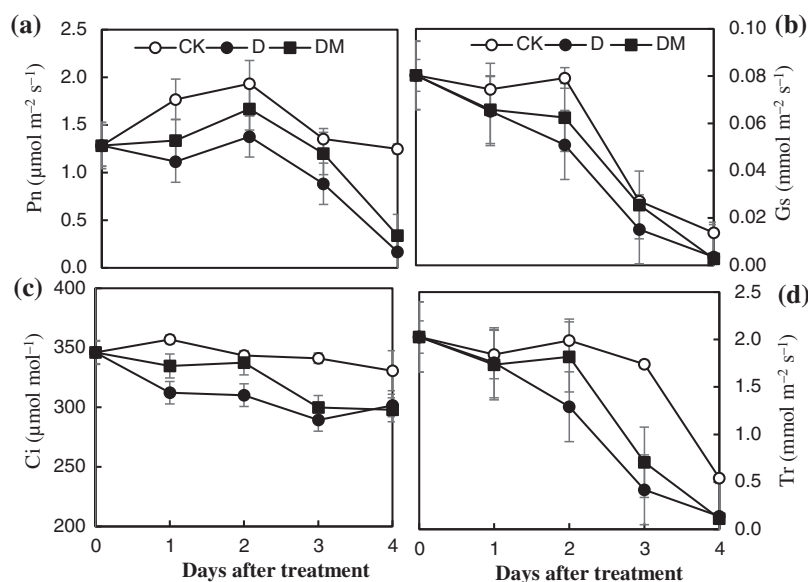
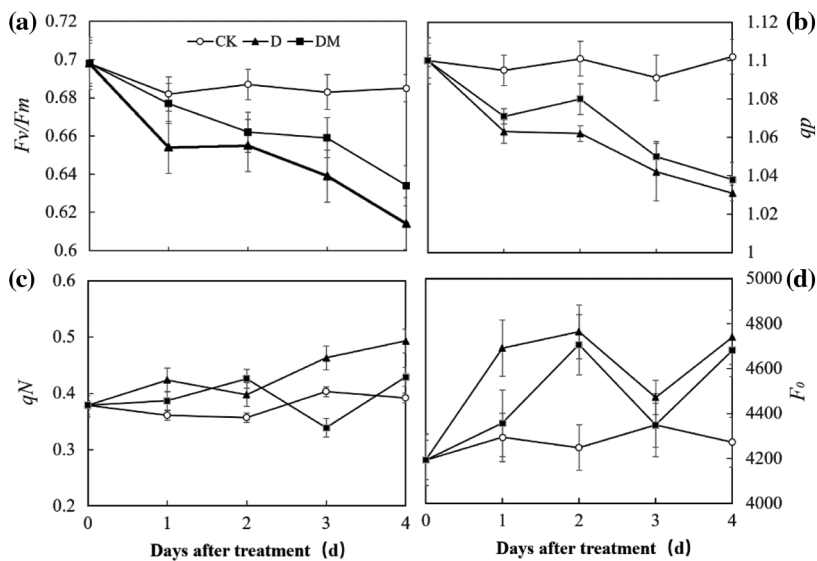
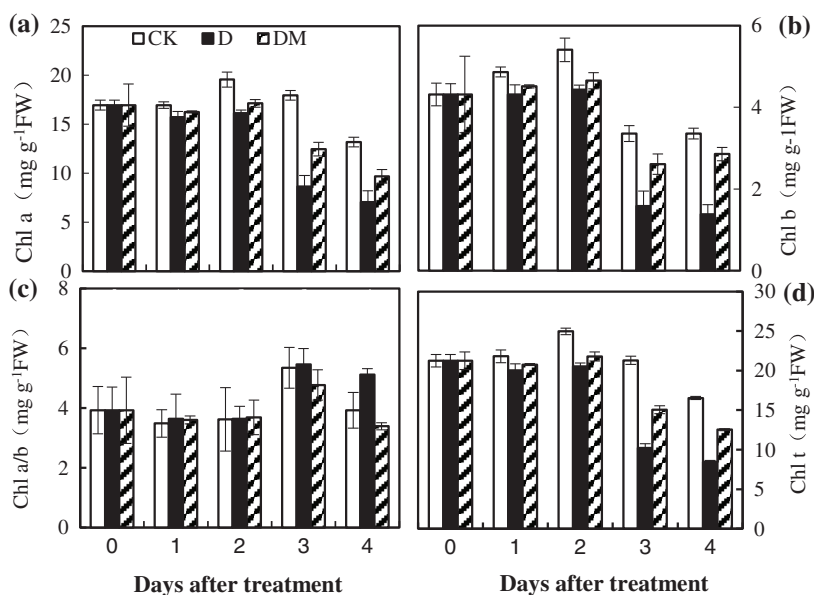


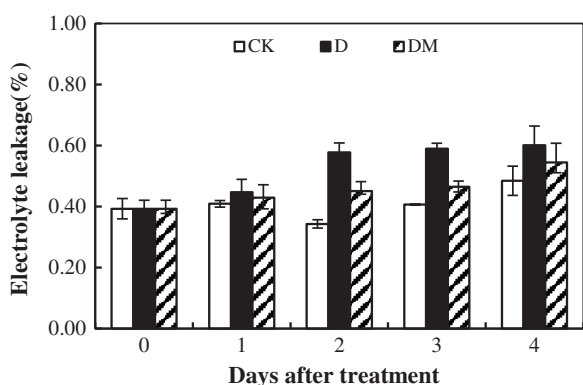
FIGURE 3. Effects of exogenous melatonin on the net photosynthetic rate ( $P_n$ ), stomatal conductance ( $G_s$ ), internal  $CO_2$  concentration ( $C_i$ ), and transpiration ( $Tr$ ) of *M. hupehensis* under drought stress. Each point represents the mean value from 6 replicates and the vertical bars indicate the standard deviations.



**FIGURE 4.** Effects of exogenous melatonin on *Fv/Fm*, *qP*, *qN*, and *F0* of *M. hupehensis* under drought stress. Each point represents the mean value from 6 replicates and the vertical bars indicate the standard deviations.



**FIGURE 5.** Effects of exogenous melatonin on Chl a, Chl b, Chl a/b, and the Chl t ratio of *M. hupehensis* under drought stress. Each point represents the mean value from 4 replicates and the vertical bars indicate the standard deviations.



**FIGURE 6.** Effects of exogenous melatonin on electrolyte leakage of *M. hupehensis* under drought stress. Each point represents the mean value from 4 replicates and the vertical bars indicate the standard deviations.

has been reported to play key roles in regulating environmental stresses tolerance (Galano *et al.*, 2011; Zhang *et al.*, 2014; Arnao and Hernández-Ruiz, 2015). In the

present study, the protective effects of melatonin in drought response were investigated in *M. hupehensis*. The results showed that plants were wilting and that the leaves were seriously dehydrated, and almost all the measured root architecture parameters were significantly reduced under drought conditions. However, melatonin-treated plants under drought stress exhibited an effectively improved state. This agrees with the findings that melatonin-treated maize had a higher dry mass compared with the untreated plants under drought (Ye *et al.*, 2016; Fleta-Soriano *et al.*, 2017).

It has been reported that growth inhibition was related to photosynthesis (Galmés *et al.*, 2007; Gururani *et al.*, 2015; Fleta-Soriano *et al.*, 2017). In the present study, drought stress caused a decline in all gas exchange parameters, especially for Pn on the last day of the treatment. Drought can result in photooxidation and reductions in photosynthetic efficiency (Skirycz and Inzé, 2010; Ma *et al.*, 2015). The current results proved that exogenously applied melatonin could effectively ameliorate photosynthetic inhibition caused by drought. Additionally, exogenous

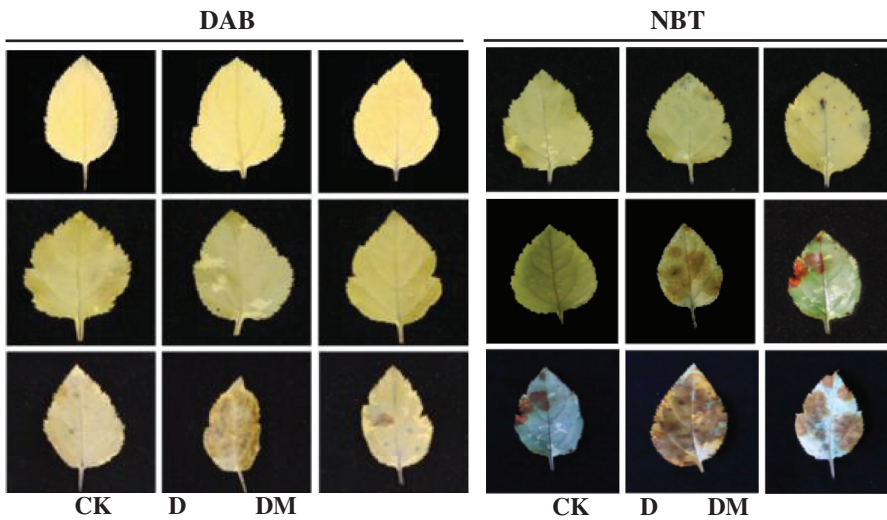


FIGURE 7. Effects of exogenous melatonin on H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>•-</sup> accumulation of *M. hupehensis* under drought stress. H<sub>2</sub>O<sub>2</sub> production in *M. hupehensis* leaves was detected using DAB after 4 d of drought stress. O<sub>2</sub><sup>•-</sup> production in *M. hupehensis* leaves was detected using NBT after 4 d of drought stress.

application of melatonin significantly increased the proportion of medium and long root lengths. Roots play an effective role in soil water use by conveying it to the aerial parts of plants more effectively during drought (Palta and Turner, 2019). Previous research has also provided evidence for the potential role of melatonin in the rooting of plants (Li et al., 2012).

Stress-induced photosynthetic apparatus damage in the plant causes a decrease in photosynthesis, which is one of the main reasons for excessive accumulation of ROS (Bai et al., 2009; Liu et al., 2015; Gupta et al., 2017). Therefore, keeping the photosynthetic organ in good condition is important for the enhancement of plant tolerance to stress. Our study showed that drought significantly induced accumulation of H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>•-</sup>, which resulted in higher EL. Similar results were obtained in maize (Liu et al., 2012). Also, Goharrizi et al. (2020a; 2020b; 2020c) showed that drought and osmotic stresses can increase the oxidative stress parameters, thereby elevating the content of EL. Oxidative stress caused by drought was removed via adding

melatonin, which effectively protected apple plants from damage. To some extent, this could be attributed to the function of melatonin as a direct free radical scavenger (Galano et al., 2011; Arnao and Hernández-Ruiz, 2015). Substantial evidence supports that melatonin could play a vital role as an antioxidant molecule, especially under abiotic stress conditions (Li et al., 2012; Liu et al., 2015; Ye et al., 2016; Fleta-Soriano et al., 2017).

Chlorophyll plays an extremely critical role in plants, which absorb light energy and transport electrons to the reaction center during photosynthesis (Gururani et al., 2015). In plants under various environmental stresses, chlorophyll synthesis can be disrupted (Ahammed et al., 2018). Previous studies showed that exogenous melatonin helped in maintaining normal levels of chlorophyll in salt-stressed apples (Li et al., 2012). In our study, exogenous melatonin enhanced the level of chlorophyll under drought stress, suggesting that melatonin was linked to enhance the synthesis of chlorophyll. In addition, melatonin significantly decreased Chl a/Chl b at 4 d. This indicated that melatonin was able to inactivate the inhibitory effect of drought

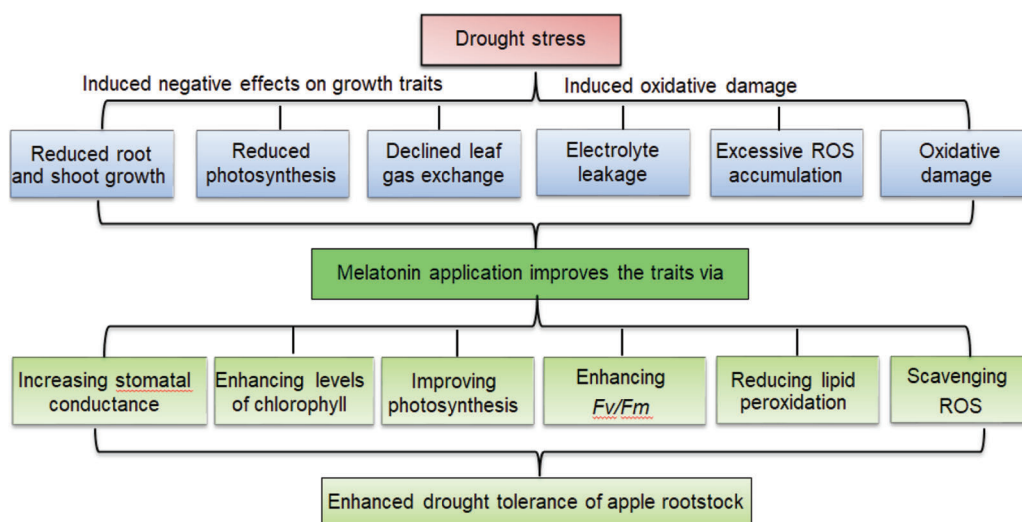


FIGURE 8. Proposed model depicting melatonin-mediated drought tolerance in apple rootstock. Drought caused oxidative damage and inhibited plant growth of apple rootstock seedlings. Melatonin alleviated the adverse effects of drought stress on the growth by enhancing capacity of photosynthesis, improving chlorophyll fluorescence, and inhibiting the degradation of chlorophyll, as well as directly scavenging H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>•-</sup> to reduce oxidative damage.

stress on Chl b. The function of the photosynthetic apparatus was studied using chlorophyll fluorescence (Tang *et al.*, 2016; Oikonomou *et al.*, 2019). The current results showed that F0 increased under drought stress, implying that the PSII reaction center was damaged, which led to the decline of Fv/Fm. However, melatonin had a favorable effect and relieved drought-induced changes to F0 and Fv/Fm. These results suggested that 0.2 mM melatonin is an effective method to prevent damage to the PSII and to enhance the drought tolerance of apple rootstock.

## Conclusions

Application of melatonin mitigated the growth inhibition, delayed membrane lipid peroxidation, and reduced oxidative damage in *M. hupehensis* under drought stress. Melatonin alleviated the adverse effects of drought stress on the growth of apple seedlings by enhancing the capacity of photosynthesis, improving chlorophyll fluorescence, and inhibiting the degradation of chlorophyll, as well as directly scavenging H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>•-</sup> to reduce oxidative damage induced by drought stress. Thus, melatonin has been proved to improve drought stress tolerance in apple plants. Based upon the results of the current study, we present a model depicting melatonin-mediated drought tolerance of apple rootstock (Fig. 8).

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**Author Contributions:** TB and MW conceived and designed research; JG, CS, and ZW conducted experiments; XZ, SS, JJ, and MW reviewed and edited the manuscript. All the authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare that they have no conflicts of interest to report regarding the present study.

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