

Article

# Melatonin Stimulates Activities and Expression Level of Antioxidant Enzymes and Preserves Functionality of Photosynthetic Apparatus in Hickory Plants (*Carya cathayensis* Sarg.) under PEG-Promoted Drought

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**Abstract:** Nowadays, drought is one of the major abiotic factors which negatively affects growth and development of several fruit tree species, including Chinese hickory plants (*Carya cathayensis* Sarg.). The present investigation was conducted to study the possible positive effects of melatonin in drought resistance of *C. cathayensis* plants along with associated mechanisms. It was observed that melatonin pre-treatment applied before limited water availability significantly contrasted drought-promoted negative effects in terms of plant growth and physiological responses. Significant improvement was observed in key biological parameters like relative water content, net photosynthetic rate, stomatal conductance, transpiration rate, maximum photosynthetic efficiency of photosystem II (PSII), and PSII electron transport rate. Antioxidant apparatus was also stimulated by melatonin and enhanced activities of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) were noticed along with higher accumulation of proline. Gene expression studies herein revealed that melatonin promoted the up-regulation of the expression of SOD (70.7%), CAT (32.7%), and APX (66.5%) genes. As a consequence, accumulation of malondialdehyde by-products and leaf symptoms were reduced in melatonin-treated plants. All these observations offer the clear evidence that pre-treatment with melatonin ameliorate the performance of Chinese hickory plants against drought stress.

**Keywords:** antioxidant system; hickory plant; reactive oxygen species; water stress

## 1. Introduction

*Carya cathayensis* Sarg. (Chinese hickory) is a nut tree, native to eastern China and mainly distributed around Tianmu Mountains in southern Anhui province and northeast Zhejiang province. The oil content of Chinese hickory kernel is up to 70%, and the oil is rich in unsaturated fatty acids with high nutritional value, making it one of the most economically important trees of China [1,2].

Drought is one of the major abiotic stresses which limits crop yield and plant development [3,4]. Drought stress typically reduces the relative water content (RWC) and total chlorophyll content in leaves [5]. The decline in RWC of plant tissue limits directly the overall plant growth and causes toxicity to plant cells [6]. Drought also negatively affects the photosynthetic process of plants [7] by limiting stomatal function and disrupting normal functioning of photosystems, particularly photosystem II (PSII) [8]. Additionally, drought triggers non-stomatal limiting factors and redox imbalance in leaf cells, which also contribute in altering the photosynthetic process in plants under limited water availability [9]. Redox imbalance due to drought causes oxidative damage in plant cells [10]. The main cause of oxidative stress is the over production and accumulation of reactive oxygen species (ROS) in plants cells under abiotic stresses, including drought [10–14]. However, plants try to overcome the excessive accumulation of ROS by activation of their antioxidative system, which is composed by both enzymatic and non-enzymatic components [15–19]. Drought stimulates the activities of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and peroxidase (POD) [20–22]. Additionally, non-enzymatic antioxidants are also stimulated under drought conditions to preserve plant functionality [23].

Polyethylene glycol (PEG) is used to simulate a condition of drought in experimental studies and, at the same time, PEG molecules with molecular weight greater than 3000 are not absorbed by plant cells [24] thereby avoiding toxic side effects [25]. Melatonin is a multifunctional biomolecule and exerts multiple physiological and biochemical functions in plants [26–28]. Moreover, melatonin application has been shown to promote the plant resistance against a plethora of abiotic stress conditions such as salinity [29,30], drought [31,32], low temperature [33], boron [34,35], and heavy metal toxicity [36]. Melatonin can also improve the net photosynthetic rate of plants under various abiotic stresses, principally by maintaining the function of PSII and protecting chlorophyll molecules from degradation [26]. This reduction is attributable to the decline in oxidative stress accompanied by a lower level of cell damage and cell wall peroxidation [37,38]. Additionally, melatonin promotes the scavenging of ROS in plants under stress conditions by modulating the activities of antioxidants [39,40] via up-regulating the expression of related genes [29].

In view of the ameliorative roles of melatonin in plants under stress, in the present study, leaves of Chinese hickory seedlings were pretreated with melatonin solutions before being subjected to PEG-induced drought stress. To explore the possible role exerted by melatonin, physiological (gas exchange and chlorophyll fluorescence analyses), biochemical (pigment, malondialdehyde (MDA) and proline (Pro) concentration, activity of SOD, CAT, APX), and molecular aspects related to antioxidant enzymes (expression of *SOD*, *CAT*, and *APX* genes) were studied under drought stress.

## 2. Materials and Methods

### 2.1. Plant Material

The current study was conducted in the greenhouse of Zhejiang Agriculture and Forestry University (N 30°23', E 119°72'), Hangzhou, China, from June to November of 2018. The growth conditions were: 25–35 °C, 70% (relative humidity), and 12:12 h (light:dark photoperiod). Seedlings of *C. cathayensis* Sarg. (one-year-old) were transplanted in plastic pots with an inner diameter of 13 cm and height of 17 cm. Pots were filled with mixture of soil (clay) vermiculite and perlite (4:3:1), and the field water holding capacity of 80% was maintained.

### 2.2. Experimental Setup

Before the beginning of the experiment, Chinese hickory plants were sprayed with five different concentrations of melatonin: 0, 25, 50, 100, and 200 µM. Melatonin spraying on leaves of Chinese hickory was done (once) every day for five consecutive days. The net photosynthetic rate of plants was measured on the second day after the last spray. On the basis of preliminary experiments, 100 µM concentration of melatonin was selected for final experimentation. Experimental plants were divided

into two groups, which were sprayed with distilled water and 100  $\mu\text{M}$  melatonin (once) every day for five consecutive days. Each treatment included three set of seedlings (each set was comprised of three pots with one seedling in each pot). Subsequently, the plants were subjected to drought stress the day after the last spray with distilled water or 100  $\mu\text{M}$  melatonin. Drought stress was induced by using a PEG-6000 solution supplied with a half strength (1/2) Hoagland's nutrient solution [41]. In total, there were eight treatment groups in this experiment: (1) Adequate moisture (CK), (2) mild drought stress (P1) 1/2, i.e., Hoagland nutrient solution with 15% PEG concentration, (3) moderate drought stress (P2) 1/2, Hoagland nutrient solution with 25% PEG concentration, (4) severe drought stress (P3) 1/2, Hoagland nutrient solution with 35% PEG concentration, (5) adequate moisture + melatonin pretreatment (M), 100  $\mu\text{M}$  melatonin, (6) 15 % PEG + 100  $\mu\text{M}$  melatonin (M-P1), (7) 25 % PEG + 100  $\mu\text{M}$  melatonin (M-P2), (8) 35 % PEG + 100  $\mu\text{M}$  melatonin (M-P3).

### 2.3. Determination of Leaf Gas Exchange Parameters

The net photosynthetic rate ( $Pn$ ), transpiration rate ( $Tr$ ), stomatal conductance ( $Cond$ ), and intercellular carbon dioxide concentration ( $Ci$ ) of first, second, and third fully-expanded leaves (from the top) were measured by a Li-6400 portable photosynthesis measurement system (LICOR, Lincoln, NE, USA). Light intensity was 1260  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ,  $\text{CO}_2$  concentration was 400  $\mu\text{mol} (\text{CO}_2) \text{ mol}^{-1}$ , leaf chamber temperature was 25 °C, relative humidity was 60%, and each measurement was repeated three times. Some other parameters like water use efficiency (WUE) and stomatal limiting factor ( $Ls$ ) were calculated as  $\text{WUE} = Pn/Tr \text{ mmol}$ ,  $Ls = 1 - (Ci/Ca)$  ( $Ca$  = ambient  $\text{CO}_2$ ).

### 2.4. Determination of Chlorophyll Fluorescence

Portable pulse-modulated chlorophyll fluorescence fluorometer (pam-2500, Walz, Germany) was used to measure chlorophyll fluorescence parameters. Chlorophyll fluorescence parameters were measured in homogeneous top fully-expanded leaves used for gas exchange determinations. First, a dark adaptation clip was placed on the leaf, and the values of  $Fo$  and  $Fm$  were measured in dark-adapted leaves (30 min) before and after a saturating pulse (8000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for 1 s), whilst the maximal PSII photochemical efficiency [ $Fv/Fm = (Fm - Fo)/Fm$ ] and the operating PSII efficiency [ $Y(II) = (Fm' - Fs)/Fm'$ ] were calculated according to [9]. ( $Fm'$  = maximum Chl fluorescence in the light-adapted state,  $Fs$  = steady state Chl fluorescence).

### 2.5. Relative Water Content of Leaves

The fresh leaves (three leaves from the top for each replicate, except top three leaves which were not fully expanded) were immersed in distilled water, and their weight was measured every 12 h until their weight no longer increased, and their saturated water concentration weight ( $Tw$ ) was measured.

### 2.6. Total Chlorophyll Content

Chlorophyll concentration was estimated according to the method of Arnon et al. [42]. One g of fresh leaves were homogenized in 80% acetone (1 mL) and centrifuged for 20 min (1500 g). After that supernatant was separated in a new tube and absorbance was recorded at wavelengths of 645 and 663 nm.

### 2.7. Antioxidant Enzyme Activity

An aliquot of 500 mg of fresh leaves was homogenized in 4.5 mL of phosphate buffer (50 mM, pH 7.0) followed by centrifugation for 20 min (1500 g, 4 °C). Supernatant was transferred to a separate tube and it was used as plant extract to determine the enzyme activities. The method Zhu et al. [43] was used to estimate SOD activity. The activity of ascorbate peroxidase (APX) was determined by the method of Jia et al. [44]. To determine activities of SOD and POD, we used two enzyme assay kits,

following the instructions provided by the manufacturer. SOD assay kit number A001-2 and APX assay kit number A123 were provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Catalase activity was determined according to Azevedo et al. [45]. One mL of phosphate buffer (50 mM, pH 7.0) was mixed with 2.5  $\mu$ L of hydrogen peroxide solution (30%) followed by the addition of 15  $\mu$ L of supernatant (plant extract). Change in absorbance in the reaction mixture was recorded at 240 nm.

#### 2.8. Malondialdehyde (MDA) and Proline (Pro) Concentration

The concentration of MDA by-products was determined by the thiobarbituric acid method of Zhao et al. [46]. An aliquot of 500 mg of fresh leaves was homogenized in 2.5 mL of trichloroacetic acid (TCA, 0.1%). Crushed samples were centrifuged for 15 min (10,000 g, 4 °C). The supernatant was separated and 0.5 mL of extract was mixed with 2 mL TCA (20%), followed by incubation of reaction mixture at 95 °C (30 min). Then, the mixture was kept in ice followed by centrifugation for 15 min at 10,000 g, 4 °C. Absorbance of supernatant was taken immediately at 532 and 600 nm.

The concentration of Pro was estimated by the ninhydrin method by Bates et al. [47]. An aliquot of 500 mg of fresh leaves was homogenized in 5 mL of aqueous sulfosalicylic acid (3%) followed by filtration (Whatman N°1 filter paper) of mixture. One mL of filtered extract was mixed with 1 mL each of acid-ninhydrin and glacial acetic acid followed by 1 h incubation in boiling water. After that, the reaction mixture was immediately placed in an ice bath, followed by the addition of 2 mL of toluene. The reaction mixture was used to record absorbance at 520 nm.

#### 2.9. Quantitative Real-Time PCR

Total RNA was extracted from 100 mg of fresh leaves and 1  $\mu$ g of total RNA was used to synthesize cDNA using PrimeScript RT Master Mix (Takara). Expression of genes was estimated in the RT-PCR system C1000 Touch Thermal Cycler (Bio-Rad) using ChamQ SYBR qPCR Master Mix (Vazyme). *Actin* was used as a housekeeping and primer information is mentioned in Supplementary Table S1. The reaction consisted of total 45 cycles (95 °C for 10 s followed by 57 °C for 10 s, and finally 72 °C for 20 s). Expression change was determined by using formula  $2^{-\Delta\Delta Ct}$  [48]. All the experiments were done using three biological and technical replicates.

#### 2.10. Statistical Analysis

The experiment was set up following a completely-randomized design. All data were subjected to Bartlett's test to assess homoscedasticity of data across populations. Data were subjected to one-way ANOVA and the means were then separated by the minimum significant difference (LSD) method ( $p < 0.05$ ) using the SPSS-16 software. Graphs were plotted using the SIGMAPLOT 12.5. All the data presented are the mean  $\pm$  standard deviation of the three independent replicates.

### 3. Results

Drought stress resulted in severe toxic effects on the phenotype of Chinese hickory leaves at 30 days after treatment (DAT). However, melatonin pretreatment alleviated the drought-induced leaf damage (Figure 1).

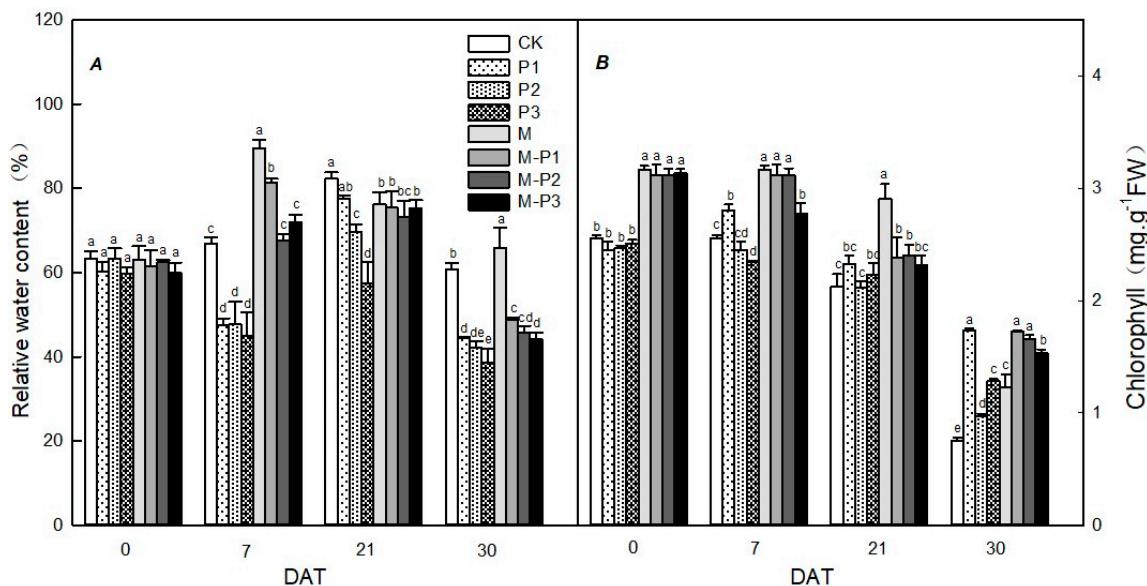
Compared with control conditions, drought stress significantly reduced the RWC of leaves. Melatonin pre-treatment effectively alleviated the drought induced negative impact on RWC. (Figure 2A). At 30 DAT, RWC of leaves of drought stressed plants was significantly lower than that of the control group. Only slight (but significant) differences were recorded at 30 DAT between plants pre-treated and non-pre-treated with melatonin. RWC in the leaves of the M, M-P1, and M-P3 treatment groups increased by 8.14%, 10.29%, and 14.68%, compared with the CK, P1, and P3 treatment groups, respectively (30 DAT). Conversely, melatonin improved strongly the RWC of pre-treated plants (compared to the non-pre-treated) at seven and 21 DAT.



**Figure 1.** Effect of melatonin on Chinese hickory leaf phenotype and visible symptoms. Control (CK), 15% polyethylene glycol (PEG) (P1), 25% PEG (P2), 35% PEG (P3), 100  $\mu\text{M}$  melatonin (M), 15% PEG + 100  $\mu\text{M}$  melatonin (M-P1), 25% PEG + 100  $\mu\text{M}$  melatonin (M-P2), 35% PEG + 100  $\mu\text{M}$  melatonin (M-P3).

In the absence of drought stress, the application of melatonin to the leaves of Chinese hickory improved the total chlorophyll concentration (Figure 2B). At 30 DAT, the total chlorophyll concentration in the M, M-P2, and M-P3 treatment groups was increased by 70.23%, 41.85%, and 19.87%, compared with the CK, P2, and P3 treatment groups, respectively.

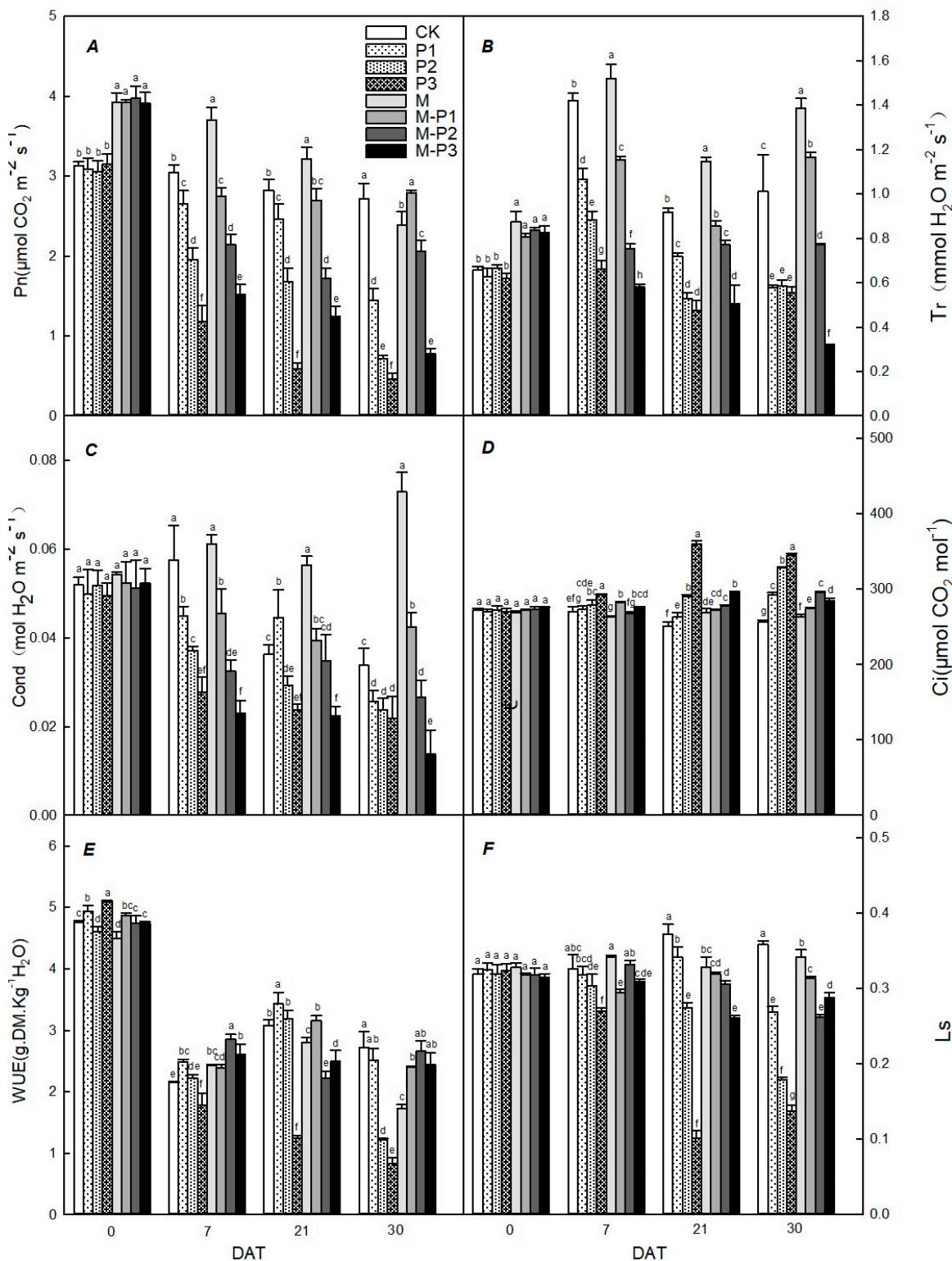
Foliar application of melatonin effectively improved the values of  $P_n$  in Chinese hickory plants under drought stress, as well as in control plants (Figure 3A). At 30 DAT, the net photosynthetic rate of M-P1, M-P2, and M-P3 treatment groups was increased by 93.10%, 188.60%, and 66.52%, compared with P1, P2, and P3 treatment group, respectively. Melatonin spraying also increased the transpiration rate of leaves (Figure 3B). More effectiveness of melatonin application was observed at 21 DAT, where transpiration rate of M, M-P1, M-P2, and M-P3 treatment group was improved by 24.95%, 19.11%, 46.13%, and 6.32%, as compared to CK, P1, P2, and P3 treatment groups, respectively. The stomatal conductance of leaves was reduced under drought stress. However, application of melatonin recovered the stomatal conductance in plants subjected to drought stress (Figure 3C). At 30 DAT, stomatal conductance of M, M-P1, and M-P2 treatment group was increased by 115.70%, 65.63%, and 12.18%, in comparison to that of CK, P1, and P2 treatment groups, respectively, while in M-P3 it was reduced. At seven and 21 DAT, M-P1 and MP-2 showed a significant increase in stomatal conductance, whereas M-P3 showed no significant difference (Figure 3C). Drought stress lead to an increase in the concentration of intercellular  $\text{CO}_2$  in leaves, which was further reduced after melatonin application (Figure 3D). At 30 DAT, the intercellular  $\text{CO}_2$  concentration of M, M-P1, M-P2, and M-P3 groups was reduced by 6.07%, 10.04%, and 17.33%, in comparison to that of P1, P2, and P3 groups, respectively.



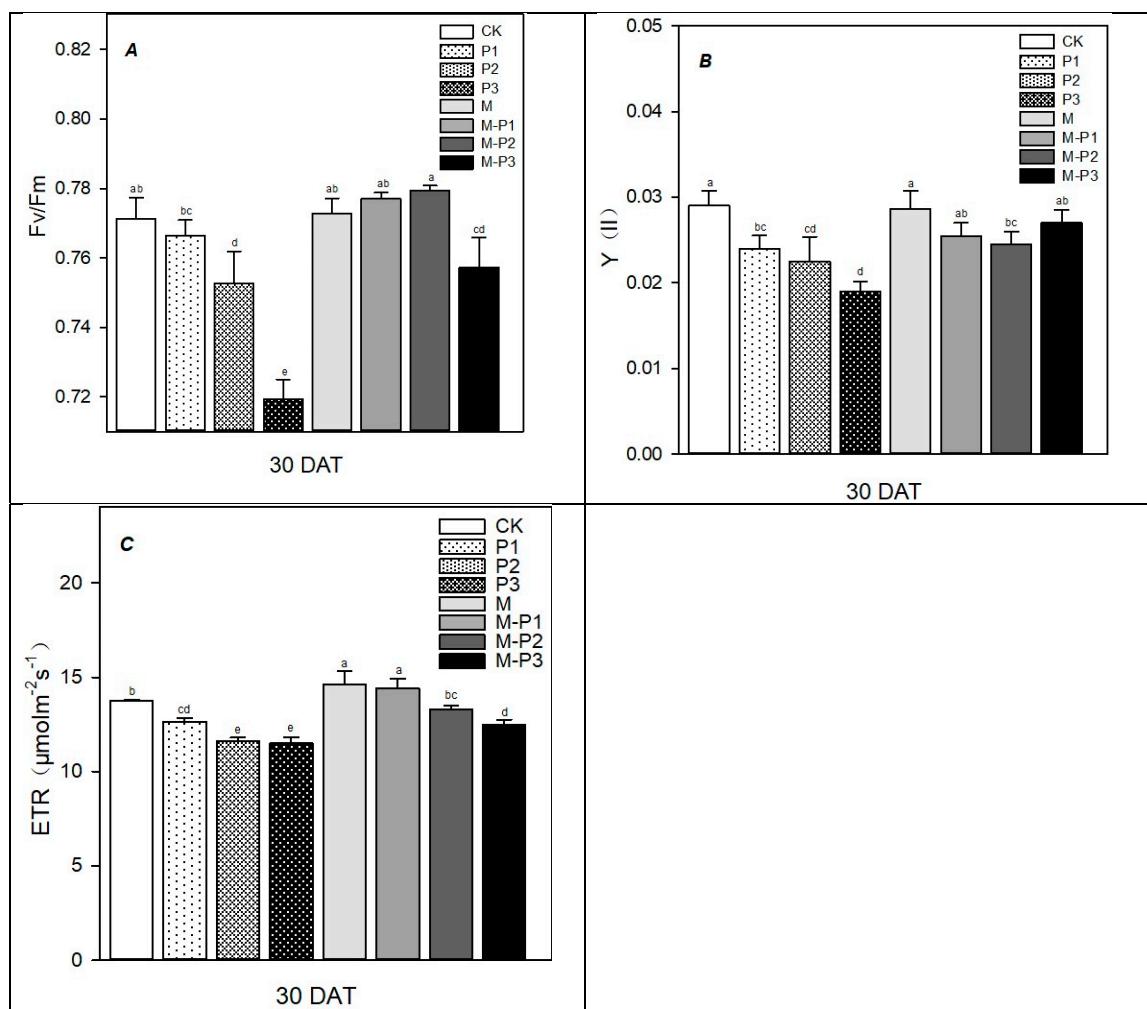
**Figure 2.** Effects of melatonin on relative water content (RWC) (A) and total chlorophyll (B) concentration. Each data point represents the mean  $\pm$  SD of three independent replicates. According to least significance difference (LSD) test, the mean values represented by different letters are significantly different ( $p < 0.05$ ). Control (CK), 15% PEG (P1), 25% PEG (P2), 35% PEG (P3), 100  $\mu$ M melatonin (M), 15% PEG + 100  $\mu$ M melatonin (M-P1), 25% PEG + 100  $\mu$ M melatonin (M-P2), 35% PEG + 100  $\mu$ M melatonin (M-P3).

Values of WUE were improved after application of melatonin in drought-stressed plants (Figure 3E). The maximum recovery of WUE was observed in M-P2 (118.08%) and M-P3 (192.91%) groups when compared to the corresponding drought-stressed groups which were non-pretreated with melatonin, i.e., P2 and P3 (30 DAT). Additionally, melatonin application also improved Ls value in plants under drought conditions. It was observed that at 30 DAT, values of Ls were recovered by 16.53% (M-P1), 46.02% (M-P2), and 108.50% (M-P3) as compared to P1, P2, and P3 treatment groups, respectively (Figure 3F).

Short-term drought stress has little effect on values of Fv/Fm, but increasing the duration of drought, the damage to PSII was gradually increased as it was evident from the significant reduction of Fv/Fm values. Melatonin application resulted in a recovery of Fv/Fm values in drought-stressed plants (Figure 4A). Similarly, Y (II) also recovered in melatonin-treated drought-stressed plants. Compared with P1, P2, and P3, the Y (II) of M-P1, M-P2, and M-P3 groups was increased by 6.25%, 8.89%, and 42.11%, respectively (Figure 4B). Values of the ETR also increased with the application of melatonin. In M-P1, M-P2, and M-P3 groups, ETR was increased by 13.83%, 14.66%, and 8.70%, as compared with values of P1, P2, and P3 groups, respectively (Figure 4C).

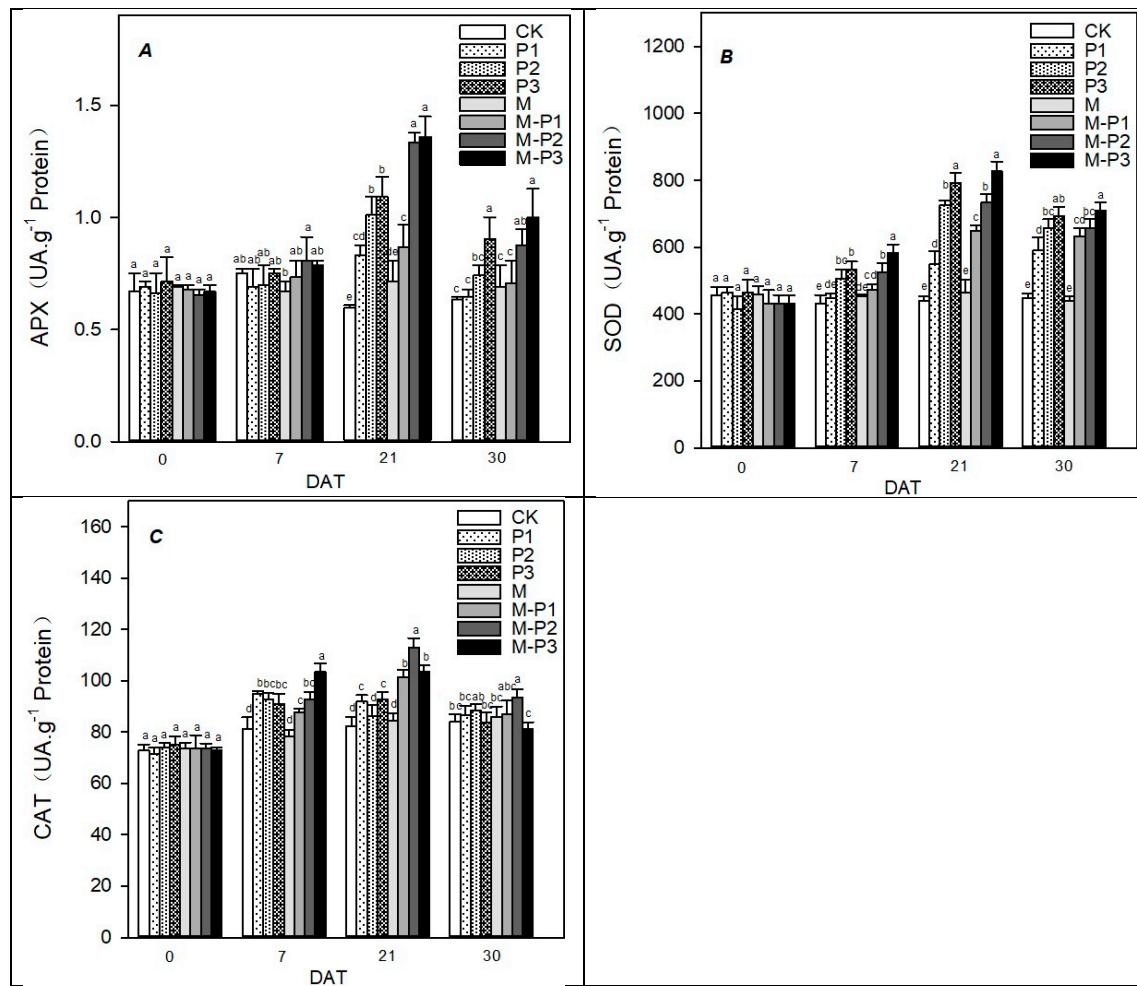


**Figure 3.** Effects of melatonin on gas exchange parameters in Chinese hickory leaves: Net photosynthetic rate ( $Pn$ ) (A), transpiration rate ( $Tr$ ) (B), stomatal conductance ( $Cond$ ) (C), intercellular  $\text{CO}_2$  concentration ( $Ci$ ) (D), water use efficiency (WUE) (E), and stomatal restriction factors ( $L_s$ ) (F). Each data point represents the mean  $\pm$  SD of three independent replicates. According to LSD test, the mean values represented by different letters are significantly different ( $p < 0.05$ ). Control (CK), 15% PEG (P1), 25% PEG (P2), 35% PEG (P3), 100  $\mu\text{M}$  melatonin (M), 15% PEG + 100  $\mu\text{M}$  melatonin (M-P1), 25% PEG + 100  $\mu\text{M}$  melatonin (M-P2), 35% PEG + 100  $\mu\text{M}$  melatonin (M-P3).



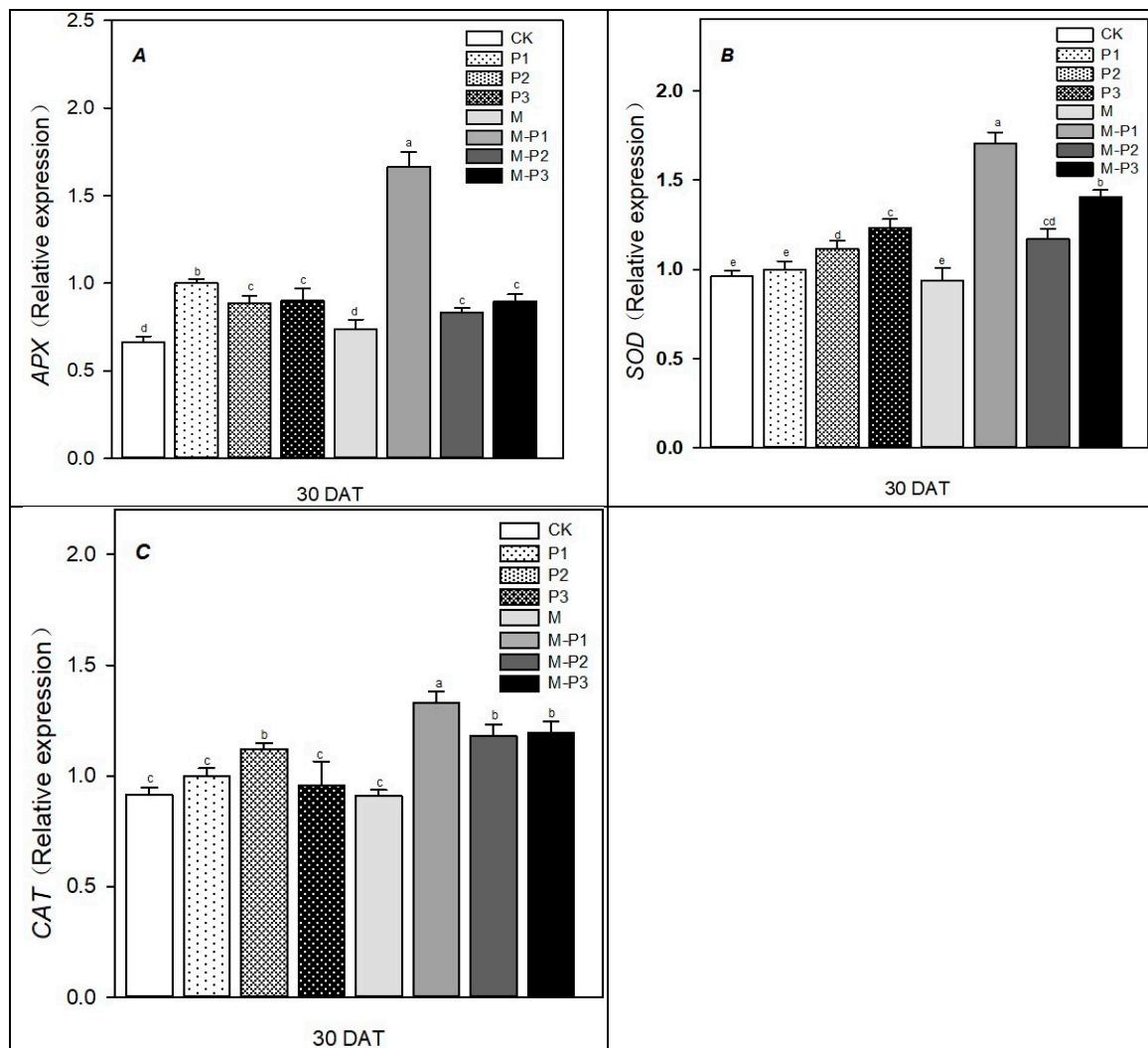
**Figure 4.** Effects of melatonin on photosystem II (PSII) photochemical efficiency (Fv/Fm; A), maximal photochemical efficiency (Y II; B) and electron transport rate (ETR; C) of leaves. Each data point represents the mean  $\pm$  SD of three independent replicates. According to LSD test, the mean values represented by different letters are significantly different ( $p < 0.05$ ). Control (CK), 15% PEG (P1), 25% PEG (P2), 35% PEG (P3), 100  $\mu\text{M}$  melatonin (M), 15% PEG + 100  $\mu\text{M}$  melatonin (M-P1), 25% PEG + 100  $\mu\text{M}$  melatonin (M-P2), 35% PEG + 100  $\mu\text{M}$  melatonin (M-P3).

Compared with well-watered plants, the antioxidant enzyme activities (SOD, CAT, APX) in leaves were enhanced under drought stress, and the application of melatonin further enhanced the activities of the antioxidant enzymes measured in our experiment (Figure 5). At 21 DAT, change in SOD activity was maximum in M-P1 group (18.38%) as compared to P1 group. The highest activity of POD was measured in melatonin-treated plants after seven DAT, where its activity was increased by 13.06% in M-P2 group as compared to P2 group. Similarly, the APX activity was also maximally increased in M-P2 group (31.86%, 21 DAT) in comparison to that of P2 group.



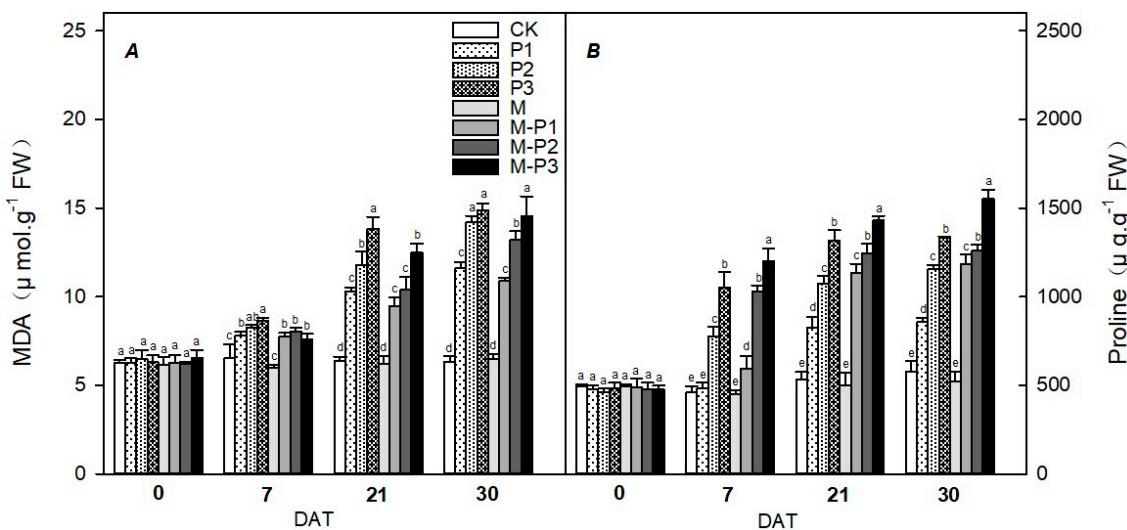
**Figure 5.** Effects of melatonin on the activity ascorbate peroxidase (APX); (A), superoxide dismutase (SOD; B) and catalase (CAT; C) in leaves. Each data point represents the mean  $\pm$  SD of three independent replicates. According to LSD test, the mean values represented by different letters are significantly different ( $p < 0.05$ ). Control (CK), 15% PEG (P1), 25% PEG (P2), 35% PEG (P3), 100  $\mu$ M melatonin (M), 15% PEG + 100  $\mu$ M melatonin (M-P1), 25% PEG + 100  $\mu$ M melatonin (M-P2), 35% PEG + 100  $\mu$ M melatonin (M-P3).

The relative expression of *APX*, *SOD*, and *CAT* genes was observed to be up-regulated in melatonin pre-treated Chinese hickory plants growing under drought stress (30 DAT). The maximum up-regulation in the expression levels was noticed in M-P1 group as compared to corresponding P1 treatment group (Figure 6). The gene expression was up-regulated by 66.50%, 70.74%, and 32.7% for *APX* (Figure 6A), *SOD* (Figure 6B), and *CAT* (Figure 6C), respectively, when P1 treatment group was compared with M-P1 treatment group.



**Figure 6.** Impact of melatonin on the relative expression of genes encoding ascorbate peroxidase (APX); (A), superoxide dismutase (SOD; B) and catalase (CAT; C) in Chinese hickory leaves. Data represents the mean  $\pm$  SD of three independent replicates. Mean values with different letters are significantly different from each other ( $p < 0.05$ ). Control (CK), 15% PEG (P1), 25% PEG (P2), 35% PEG (P3), 100  $\mu$ M melatonin (M), 15% PEG + 100  $\mu$ M melatonin (M-P1), 25% PEG + 100  $\mu$ M melatonin (M-P2), 35% PEG + 100  $\mu$ M melatonin (M-P3).

It was observed that drought stress increased MDA and proline concentration in the leaves. Of note, melatonin pre-treatment reduced the levels of MDA by-products accumulation and increased the proline concentration (Figure 7). The concentration of MDA at 21 DAT was maximum reduced by 11.91% in M-P2 group as compared to P2 group (Figure 7A). Proline concentration was also noticed to increase maximum in M-P2 group (32.63%, at seven DAT) as compared to moderate drought stress group (P2) (Figure 7B).



**Figure 7.** Effects of melatonin on malondialdehyde (MDA) (A) and proline (B) in Chinese hickory leaves. Each data point represents the mean  $\pm$  SD of three independent samples. According to LSD test, the mean values represented by different letters are significantly different ( $p < 0.05$ ). Control (CK), 15% PEG (P1), 25% PEG (P2), 35% PEG (P3), 100  $\mu\text{M}$  melatonin (M), 15% PEG + 100  $\mu\text{M}$  melatonin (M-P1), 25% PEG + 100  $\mu\text{M}$  melatonin (M-P2), 35% PEG + 100  $\mu\text{M}$  melatonin (M-P3).

#### 4. Discussion

Drought is one of the most common abiotic stresses, which affects seed germination, plant growth, metabolism, and overall the plant yield. In the present study, drought stress seriously altered the leaf status by reduced RWC and total chlorophyll concentration of Chinese hickory leaves. Melatonin pretreatment ameliorated the effect of drought as evident by lower impairment of RWC and chlorophyll concentration, which might be due to the regulation of water balance and less degradation of chlorophylls [31,49].

In the present study, melatonin pre-treatment efficiently improved  $P_n$  with respect to drought stressed plants which were not treated with melatonin. Principally, melatonin limits stomata limitations and preserve the transpiration rate of Chinese hickory leaves. Additionally, melatonin is also observed to preserve from non-stomata limitation, thereby protecting PSII from the adverse effects of drought, according to other researches [31,49]. Moreover, under drought stress, plants improve their WUE in the attempt to limit their water loss [50,51], which is consistent with the results of present study. We found indeed that the levels of WUE in melatonin pre-treated Chinese hickory leaves were improved under drought conditions.

The process of photosynthesis is affected by stomatal opening and intracellular environment, and drought stress caused both stomatal and non-stomatal limitations [52]. Stomatal limitations can reduce the intercellular carbon dioxide concentration of the leaves by reducing the stomatal opening and, in turn, reducing the photosynthetic yield of plants. Among other biochemical limitations, photosystem II is the most vulnerable part of photosynthetic apparatus when the plants are subjected to environmental cues, such as drought conditions [53,54]. In the present study, reduction of  $F_v/F_m$ , Y II, and ETR were observed under drought stress. Of note, melatonin pre-treatment resulted in a lower impact of drought to those PSII parameters. This improvement after melatonin treatment might be due to the protection of chloroplast structure from drought-induced injuries [55]. Drought stress causes imbalance in ROS metabolism [56], and excessive accumulation of ROS leads to membrane peroxidation, chlorophyll degradation, and reduced photosynthetic performance of plants [49]. Antioxidant defense systems can counteract excessive ROS production in plants to protect them from oxidative damage [57]. Melatonin can directly scavenge  $\text{H}_2\text{O}_2$  in plant cells [58] by up-regulating the gene expression of key antioxidant enzymes under stress [59] along with stimulating the activities of antioxidant

enzymes [60]. In the current investigation, the activity of SOD, POD, and APX increased under drought stress, and their activities were further triggered in melatonin-pretreated plants. This was effectively supporting for a stimulating activity by melatonin to antioxidant enzymes. The enhancement of antioxidant enzymes was also associated with the melatonin-mediated up-regulation of transcript levels of genes encoding for APX, SOD, and CAT, which is in agreement with previous findings [61].

As a common osmotic regulator and antioxidant in plants, the increase of proline content under stress is considered to be the main way to improve the resistance of plants [23,62], and the content of proline under stress is often an indicator of the tolerance of plants to stress [63]. In the present study, MDA and proline levels in plants were increased under drought stress, indicating that the membrane lipid peroxidation of plant cells was increased under drought stress, and the stress resistance of plants to drought was gradually improved by accumulation of proline. After treatment with melatonin, MDA by-products levels decreased, while proline accumulation further accumulated. Melatonin reduces membrane lipid peroxidation in plants by increasing antioxidant capacity [27]. Moreover, enhanced proline accumulation may be due to a direct regulation of proline biosynthesis triggered by melatonin at biochemical and molecular level [32,64].

## 5. Conclusions

This study showed that drought stress significantly reduced photosynthetic capacity of Chinese hickory plants, and long-term drought can damage the PSII. Melatonin spraying promotes the enhancement of the antioxidative enzymatic system in Chinese hickory plants under drought stress, thus ameliorating the redox balance and preserving the leaf physiological status. Results of the present experiment highlight the possibility of using a melatonin-based pre-treatment in Chinese hickory to enhance the performances of this widely cultivated and economically-important tree species in China when subjected to limited water availability.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2073-4395/9/11/702/s1>, Table S1: Primers used for gene expression analysis.

**Author Contributions:** Conceptualization, B.Z., H.Y., and D.Y.; Methodology, investigation, formal analysis, J.W., J.C., A.S., and S.T.; Resources, B.Z., H.Y., and D.Y.; Writing original draft, J.W. and A.S.; Writing—review and editing, A.S., B.Z., H.Y., M.L., and D.Y.

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