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Exogenous Melatonin Improves Waterlogging Tolerance in Wheat through Promoting Antioxidant Enzymatic Activity and Carbon Assimilation

Shangyu Ma [1,2], Panpan Gai [1], Bingjie Geng [1], Yanyan Wang [3], Najeeb Ullah [4], Wenjing Zhang [1], Haipeng Zhang [1], Yonghui Fan [1] and Zhenglai Huang [1,2,*]

1 Key Laboratory of Wheat Biology and Genetic Improvement on Southern Yellow & Huai River Valley, College of Agronomy, Anhui Agricultural University, Ministry of Agriculture and Rural Affairs, Hefei 230036, China
2 Jiangsu Collaborative Innovation Center for Modern Crop Production, Nanjing 210095, China
3 Agricultural Park Management Center, Anhui Agricultural University, Hefei 230036, China
4 Faculty of Science, Universiti Brunei Darussalam, Jalan Tungku Link, Gadong BE1410, Brunei
* Correspondence: huangzhenglai@ahau.edu.cn

Abstract: In a pot experiment, we explored the regulatory pathways through which melatonin (MT) protects wheat growth and grain yield loss from waterlogging injury. Two wheat cultivars, Yangmai 18 and Yannong 19, were exposed to seven days of soil waterlogging at flowering. Melatonin (100 μmol·L^{-1}) was sprayed before and after waterlogging to explore its regulation on root growth, photosynthetic characteristics, dry matter accumulation, and grain yield. Soil waterlogging intensified malondialdehyde (MDA) and O_2^- production rates in wheat tissues, impairing leaf photosynthesis, biomass accumulation, and final grain yield formation. In this study, the roots waterlogged at 7 days after anthesis (DAA) accumulated 20.9%, 76.2%, 17.6%, 28.5%, and 5.6% higher MDA content, O_2^- production rate, pyruvate decarboxylase (PDC), lactate dehydrogenase (LDH), and alcohol dehydrogenase (ADH) activities, respectively, in Yangmai 18, and 25.7%, 74.8%, 35.8%, 70.8%, and 30.7% higher in Yannong 19, respectively, compared with their respective non-waterlogged controls. Further, Yangmai 18 achieved a maximum net photosynthetic rate (Pn) reduction of 22.1% at 7 DAA, while the maximum Pn reduction of Yannong 19 was 27.4% at 14 DAA, respectively, compared with their respective non-waterlogged plants. Thus, waterlogging decreased total dry matter accumulation, 1000-grain weight (TGW), and total grain yield by 14.0%, 13.8%, and 16.2%, respectively, in Yangmai 18, and 16.0%, 8.1%, and 25.1%, respectively, in Yannong 19. Our study also suggests that exogenously applied melatonin can protect wheat root tissues from waterlogging-induced oxidative injury by upregulating antioxidant enzymes and sustaining leaf photosynthesis. The plants treated with melatonin showed better water status and less oxidative damage, which was conducive to maintaining a higher photosynthetic capacity, thereby improving the waterlogging tolerance of wheat. For example, compared with waterlogged plants, melatonin treatments significantly reduced MDA content, O_2^- production rate, PDC, LDH, and ADH activities by 7.7%, 25.4%, 2.6%, 32.1%, and 3.2%, respectively, in Yangmai 18, and 6.7%, 17.9%, 4.1%, 22.0%, and 15.3%, respectively, in Yannong 19. MT treatments significantly increased total dry matter accumulation, TGW, and yield by 5.9%, 8.7%, and 14.9%, respectively, in Yangmai 18, and 3.2%, 7.3%, and 26.0%, respectively, in Yannong 19.

Keywords: wheat; waterlogging; melatonin; anaerobic respiration; photosynthetic characteristics; yield

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1. Introduction

Waterlogging is a major agricultural constraint that limits the growth and productivity of many crops worldwide [1,2]. For example, long-term soil waterlogging can reduce the yield of major crops such as wheat and cotton by 25% and 60%, respectively [3]. In waterlogged soil, limited oxygen supply causes hypoxic conditions, and the plant roots shift their metabolism from aerobic to anaerobic to sustain energy synthesis under

O_2^--limited environments [4,5]. When oxygen-dependent energy production pathways (such as aerobic respiration) are suspended, glycolysis and fermentation processes are enhanced to sustain ATP synthesis for plant growth [6]. During anaerobic respiration, lactate dehydrogenase (LDH) and alcohol dehydrogenase (ADH) catalyze ethanol and lactic acid fermentation in plants to sustain energy metabolism. In contrast, pyruvate decarboxylase (PDC) is involved in anaerobic and aerobic metabolisms [7]. During this transitional energy-deficient phase, reactive oxygen species (ROS) and malondialdehyde (MDA) are induced in waterlogged plants. Excess ROS damage cell membranes, nucleic acids, and proteins directly or indirectly, and inhibit plant growth [8,9]. Studies have shown that most crops cannot tolerate sustained waterlogging [10]. For example, tomato leaves wilted after only 4–6 h of flooding [11]. Three days of waterlogging causes leaf wilting and yellowing of tobacco plants [12], although cotton plants may sustain growth up to 5 days of soil waterlogging [13,14].

Our earlier studies suggest that wheat plants are moderately waterlogging-tolerant, sustaining biomass assimilation in response to 3 days of waterlogging. However, extended waterlogging (6–9 days) significantly reduces wheat crop growth and yield formation [15]. Some crop species have developed complex regulatory strategies to sustain growth under waterlogged environments, including enzymatic and non-enzymatic systems [16]. In addition, various plant growth regulators can also induce tolerance to waterlogging in plants [17]. More recently, melatonin (MT), a novel bioregulator, has received extensive attention in plant research to regulate plant stress tolerance [18]. MT scavenges superoxide by capturing the free radicals and protects cells from oxidative injury [19]. Previous studies show that foliar spraying 100 μM of MT can eliminate ROS, alleviate oxidative damages, resist waterlogging, and consequently reduce yield loss [20]. MT also acts as a signaling molecule at the cellular level, and it upregulates many antioxidant enzymes, thereby increasing their efficiency [21]. Positive effects of MT application have been recorded on root growth [22] and phytohormone synthesis [23], nitrogen uptake and assimilation [24], carbon assimilation [23], and the final yield of different crops [25].

Exogenously applied MT has been found effective in increasing the tolerance to salinity in cucumber [26], cold stress in wheat seedlings [27], and heat stress in rice [28]. The MT-indued abiotic stress tolerance in plants is associated with its capacity to modulate several antioxidants and 54 secondary metabolites, including amino acids, organic acids, sugars, and sugar alcohols [29]. In this study, we explored how waterlogging and melatonin regulate carbon assimilation and translocation in different plant organs and how these changes translate into grain yield formation, which has not been performed for the wheat crop before. Post-waterlogging stress modifications in growth, physiology, and anaerobic metabolism of wheat plants were studied to understand the mechanism of MT-induced regulation of waterlogging tolerance in wheat. We also explored the regulation of grain development under waterlogging and melatonin application.

2. Materials and Methods

2.1. Planting and Cultivation Management of Wheat

Experiments were carried out at the Wanzhong Experimental Station of Anhui Agricultural University in Guohe Town, Lujiang County, Hefei City, Anhui Province (117°01′ E, 30°57′ N), in 2020 and 2021. The soil samples from the field's 0–30 cm plough layer in the experimental area before sowing were analyzed. It contained 23.4 $g\cdot kg^{-1}$ of organic matter, 1.0 $g\cdot kg^{-1}$ of total nitrogen, 121.0 $mg\cdot kg^{-1}$ of alkaline hydrolyzed nitrogen, 33.6 $g\cdot kg^{-1}$ of available phosphorus, 356.0 $mg\cdot kg^{-1}$ of available potassium, and 6.3 pH. Then, 2.5 kg of sieved dry soil containing 1.0 g of compound fertilizer ($N + P_2O_5 + K_2O \geq 51\%$), 0.3 g of urea (total nitrogen $\geq$ 46.4%), and 25.0 g of organic fertilizer was mixed evenly for each pot (28 cm high, 13 cm in diameter).

Five wheat seeds were sown on 10 November 2020, and three seedlings with similar plant sizes were retained at the three-leaf stage (Zadoks decimal growth stage [30], Z13). Each pot was top-dressed with 0.2 g of urea at the stem elongation stage (Z31).

2.2. Experimental Design

Two commercial wheat cultivars, i.e., Yangmai 18 (waterlogging-tolerant) and Yannong 19 (waterlogging-sensitive), were used in this study [15]. The experiment was conducted in a completely randomized design with four treatments (Table 1).

Table 1. Experimental design of soil condition and leaf spraying.

Treatment	Soil Condition	Leaf Spraying
CK + QS	Non-waterlogged	Distilled water
CK + MT	Non-waterlogged	100 μmol·L^{-1} melatonin
WL + QS	Waterlogged	Distilled water
WL + MT	Waterlogged	100 μmol·L^{-1} melatonin

The treatment pots were artificially waterlogged when 50% of the plants were flowering (Z65). All pots were divided into four groups with sixty pots each, of which two groups were waterlogged for seven days. The pots used for waterlogging treatment were moved into a pool (3.0 × 1.0 × 0.3 m) and then watered by hand with tap water, ensuring that a 2 cm layer of water was maintained above the soil surface for 7 days (Figure 1).


(**A**) (**B**)

Figure 1. The field under the soil water treatment: the pool (**A**) and the pots under waterlogging (**B**).

Following waterlogging treatment, the pots were removed from the water and allowed to drain freely, and the plants were irrigated normally until maturity. Melatonin or distilled water was evenly sprayed one day before and after each waterlogging event. A hand sprayer was used to supply 100 μM of melatonin on foliage to both waterlogged and non-waterlogged plants for melatonin treatments. The other two groups of plants were sprayed with distilled water. Tween-20 (0.01%) was added with both melatonin and distilled water. Daily weather data for precipitation and temperature were obtained from a nearby automated weather station located in Lujiang County. The weather data and the treatment time are shown in Figure 2.

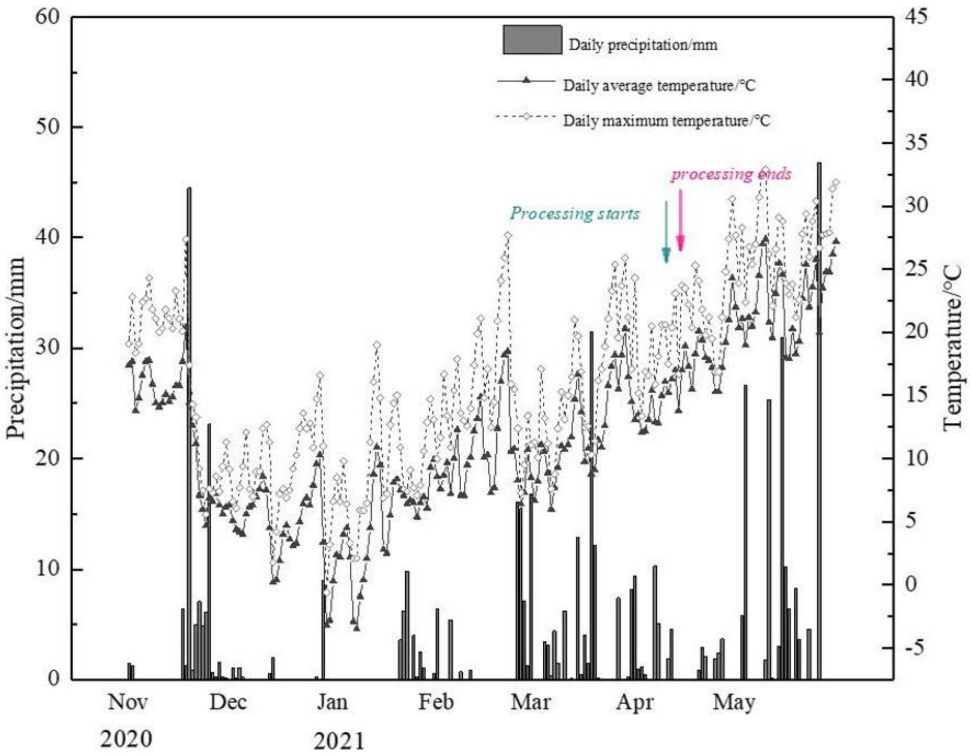

Figure 2. Daily precipitation and temperature during the wheat growing season.

2.3. Measurement Items and Methods

Approximately 120 stems of each group were tagged at flowering (Z65). The greenness and gas exchange of flag leaf and spikes were measured from the tagged stems of each group.

2.3.1. Root Dry Weight, Antioxidant Enzymes, Malondialdehyde (MDA), and O_2^- Production Rate

Six pots with the same number of tagged stems from each group were harvested at 7, 14, 21, and 28 days after flowering. The harvested plants were partitioned into spike, stem, leaf, and root tissues. Three pots of root samples were dried to a constant weight in a forced-draft oven at 70 °C for dry weights.

The fresh root samples (1 cm of root tips) from the remaining three pots were stored in liquid nitrogen until analysis. Root samples were analyzed for antioxidant enzymes, such as superoxide dismutase (SOD) and peroxidase (POD), and MDA contents using the previously described methods [31]. The production rate of reactive oxygen species, i.e., superoxide anion (O_2^-), was determined with the hydroxylamine method [32].

2.3.2. Anaerobic Respiration-Related Enzyme Activities in Roots

Three pots of fresh root samples were stored in liquid nitrogen seven days after flowering until analysis. The anaerobic metabolism was determined based on lactate dehydrogenase (LDH), alcohol dehydrogenase (ADH), and pyruvate decarboxylase (PDC) concentrations following procedures described in the manufacturer guidelines of their assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.3.3. Photosynthetic Characteristics of Flag Leaves

Five tagged stems from each group were used to measure the relative chlorophyll content (SPAD units), net photosynthetic rate (Pn), and actual photochemical efficiency (ΦPSII) at 0, 7, 14, and 21 days after flowering. Five flag leaves were measured for each treatment and averaged.

The SPAD value was measured using a handheld chlorophyll meter (SPAD-502, Soil Plant Analysis Development, Minolta, Japan).

Pn was measured using a CIRAS-3 photosynthesis analyzer (PP Systems, Amesbury, MA, USA). Measurements were taken from 09:00 to 11:00 am under constant CO_2 concentration (400 μmol·mol^{-1}), leaf temperature (25 °C), and light (1200 mol·m^{-2}·s^{-1}) conditions [33].

The ΦPSII values were measured using a portable fluorometer (PAM-2500, Walz, Germany) when the weather was clear and dark-adapted for 30 min before the measurement from the middle part of the leaves [34].

2.3.4. Grain-Filling Characteristics

Spike samples were collected from the tagged stems from the flowering to the crop maturity every week, and ten spikes (about three pots) were taken from each group each time. The harvested spikes were dried to a constant weight in a forced-draft oven at 70 °C to obtain the 1000-grain weight.

Days after flowering (t) and 1000-grain weight (Y) were measured each time as independent and dependent variables, respectively. The logistic equation $Y = K/(1 + e^{A + Bt})$ was used to fit the grain growth process, where K is the maximum 1000-grain weight that can be achieved at the end of grouting, A and B are the parameters of the equation, and the coefficient of determination, R^2, is used to represent the goodness of fit. According to the logistic equation and the first- and second-order derivatives of the equation, a series of grouting parameters were derived [35]. The calculation methods of grain-filling parameters are shown in Table 2.

Table 2. Various grouting parameter expressions and calculation methods.

Parameter Description	Calculation Method
Beginning of peak grouting period (d)	$t_1 = [A - \mathrm{Ln}(2 + 1.732)]/(-B)$
End date of peak grouting period (d)	$t_2 = [A + \mathrm{Ln}(2 + 1.732)]/(-B)$
Grouting end date (Y up to 99% K) (d)	$t_3 = (4.59512 + A)/(-B)$
Time to maximum filling rate (d)	$T_m = -A/B$
Maximum filling rate (mg·grain^{-1}·d^{-1})	$V_m = -BK/4$
The duration of grain-filling main (d)	$T = t_3$
Average filling rate (mg·grain^{-1}·d^{-1})	$V_a = K/t_3$
Duration of the slight-increase period (d)	$T_1 = t_1$
Duration of the fast-increase period (d)	$T_2 = t_2 - t_1$
Duration of the slow-increase period (d)	$T_3 = t_3 - t_2$
Accumulation of the slight-increase period (g·1000 grain^{-1})	$W_1 = K/(1 + e^{A + Bt1})$
Accumulation of the fast-increase period (g·1000 grain^{-1})	$W_2 = K/(1 + e^{A + Bt2}) - W_1$
Accumulation of the slow-increase period (g·1000 grain^{-1})	$W_3 = K/(1 + e^{A + Bt3}) - W_2 - W_1$
Grain-filling rate of the slight-increase period (mg·grain^{-1}·d^{-1})	$V_1 = W_1/T_1$
Grain-filling rate of the fast-increase period (mg·grain^{-1}·d^{-1})	$V_2 = W_2/T_2$
Grain-filling rate of the slow-increase period (mg·grain^{-1}·d^{-1})	$V_3 = W_3/T_3$

2.3.5. Grain Yield and Its Components

The total number of spikes per plant and grains per spike of six pots were counted at the crop maturity, and each pot was separately threshed, weighed, and converted into yield when the water content was 13%.

2.4. Statistical Analysis

Data were statistically analyzed using SSPS software for Windows (version 22.0). The treatment means were compared at $\alpha = 0.05$ to identify significant differences. Multiple comparisons were performed using the least significant difference test with $\alpha = 0.05$ to determine significant differences among treatments. GraphPad Prism 9 was used to show differences in the studied traits, e.g., root dry weight, SOD and POD activities, MDA content, anaerobic respiration enzymes, and leaf SPAD, Pn, ΦPSII, accumulation, and distribution of dry matter.

3. Results

3.1. Root Growth under Waterlogging and Exogenous MT

3.1.1. Root Dry Weight

The wheat cultivars (Yannong 19 and Yongmai 18) produced a similar amount of dry root biomass under non-waterlogged conditions at flowering. Root dry biomass of Yangmai 18 progressively declined for each subsequent measurement under all the treatments, i.e., 14, 21, and 28 days after anthesis (DAA) (Figure 3A). In contrast, Yannong 19 accumulated maximum root biomass at 14 DAA, which was significantly reduced at 21 and 28 DAA (Figure 3B).

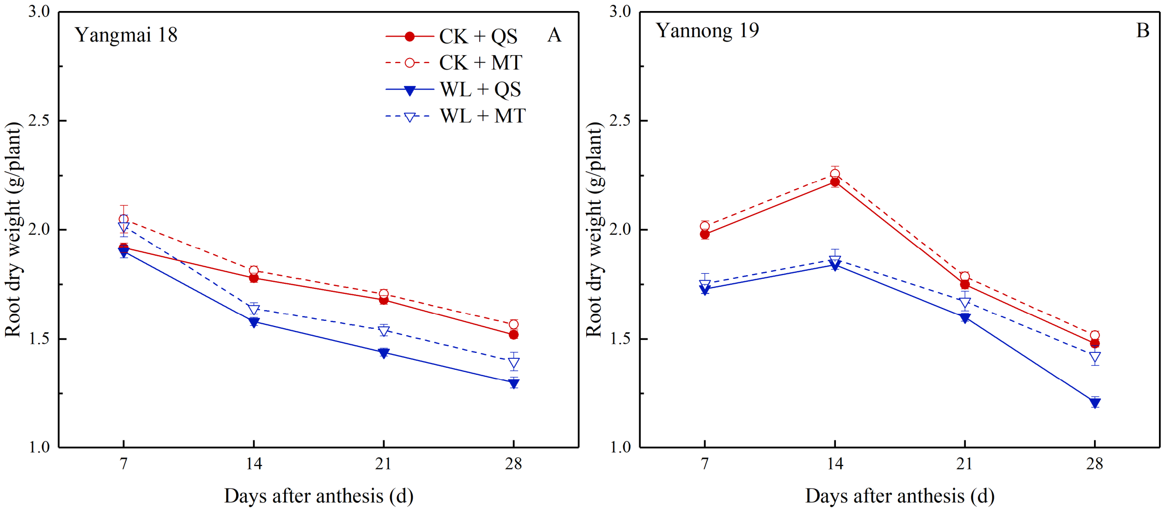

Figure 3. Post-flowering changes in root dry weight (**A**,**B**) under different treatments, i.e., CK + QS: leaf spraying with distilled water under non-waterlogged conditions; CK + MT: leaf spraying with 100 µmol·L^{-1} of melatonin under non-waterlogged conditions; WL + QS: leaf spraying with distilled water under waterlogged conditions; WL + MT: leaf spraying with 100 µmol·L^{-1} of melatonin under waterlogged conditions.

Waterlogging had no significant effect on the root dry biomass of Yangmai 18 at 7 DAA, but at each subsequent measurement, waterlogged roots were significantly lighter than non-waterlogged roots. Waterlogging significantly reduced the root biomass of Yannong 19 at each measurement, with significantly more reduction at 7 and 14 DAA, when waterlogged roots had 12.6% and 17.1% lower root dry weight, respectively, compared with their respective non-waterlogged roots (Figure 3B).

Spraying MT increased the root dry weight of the two wheat cultivars under waterlogging conditions. This difference between the dry weight of waterlogged and non-waterlogged roots increased as the grain filling progressed. At 28 DAA, exogenous MT increased the root dry biomass of Yangmai 18 and Yannong 19 by 7.5% and 17.7%, respectively, compared with their respective waterlogged roots.

3.1.2. Antioxidant Enzyme Activity in Root Tissues

Under non-waterlogged conditions, both studied wheat cultivars showed a similar change trend in the activity of root antioxidant enzymes (i.e., SOD and POD). In general, the SOD activity of both cultivars gradually decreased with each subsequent measurement under all treatments, namely 7, 14, 21, and 28 DAA (Figure 4A,B). In contrast, POD activity in roots showed an upward trend as the plant growth progressed (Figure 4C,D).

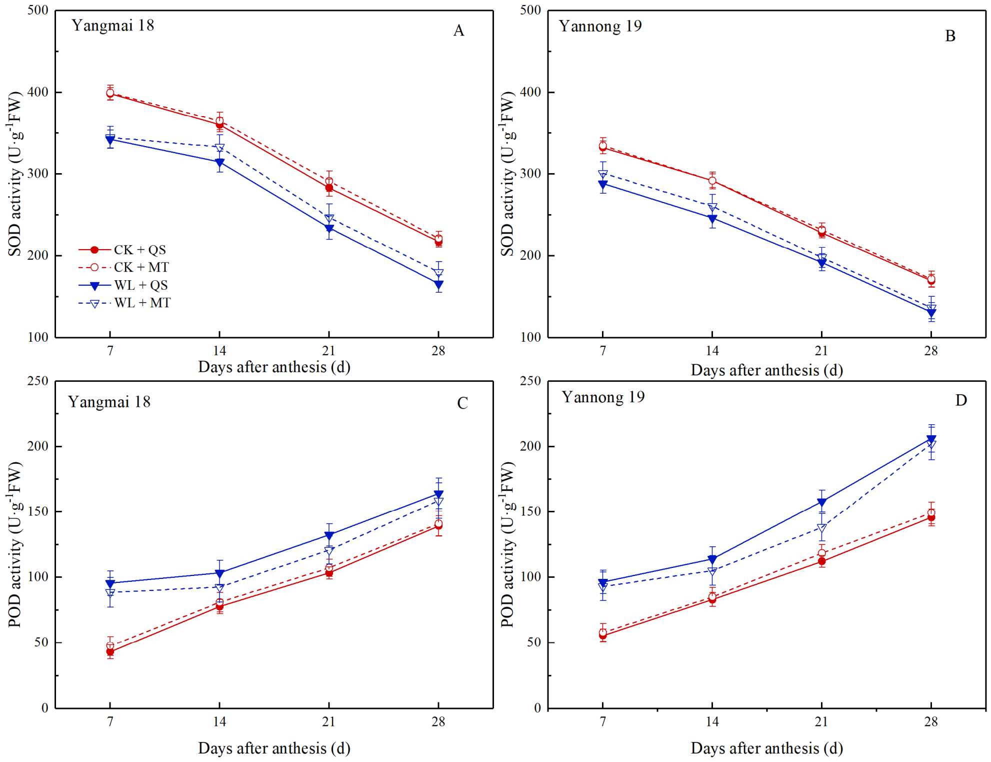

Figure 4. Post-flowering changes in root superoxide dismutase (SOD) and peroxidase (POD) activities (**A**–**D**) under different treatments, i.e., CK + QS: leaf spraying with distilled water under non-waterlogged conditions; CK + MT: leaf spraying with 100 μmol·L^{-1} of melatonin under non-waterlogged conditions; WL + QS: leaf spraying with distilled water under waterlogged conditions; WL + MT: leaf spraying with 100 μmol·L^{-1} of melatonin under waterlogged conditions.

At each measurement, the SOD activity of the waterlogged roots of the two wheat cultivars was significantly lower than the non-waterlogged roots, and the POD activity was significantly higher in waterlogged than non-waterlogged roots. Compared with non-waterlogged roots, waterlogged roots experienced a 122% increase in the POD activity of Yangmai 18 at 7 DAA (Figure 4C) and a 73.2% increase in the POD activity of Yannong 19 at 28 DAA (Figure 4D).

Spraying MT had no significant effect on SOD and POD activities in Yangmai 18 roots at 7 DAA. However, MT-treated roots had significantly higher SOD and significantly lower POD activity in each subsequent measurement than their non-MT-treated roots under waterlogging conditions. In 19 Yannong roots, MT significantly reduced SOD activity by 4.4% and 5.8% at 7 and 14 DAA, respectively, compared with their non-MT-treated controls under waterlogging conditions (Figure 4B). At 7 and 28 DAA, exogenous MT had no significant effect on POD activity in Yannong 19, but it significantly decreased POD activity by 7.6% and 12.6% at 14 and 21 DAA, respectively, compared with their respective waterlogged roots.

The two wheat cultivars (Yangmai 18 and Yannong 19) had a similar MDA content and O_2^- production rate change trend in root tissues under non-waterlogging conditions. MDA content and the O_2^- production rate in Yangmai 18 and Yannong 19 gradually increased in each subsequent measurement under all treatments.

Waterlogging significantly increased MDA content and the O_2^- production rate in both cultivars at each measurement. Compared with non-waterlogged roots at 7 DAA, waterlogged roots have 20.9% and 76.2% higher MDA content and O_2^- production rate, respectively, in Yangmai 18 (Figure 5A,C), and 25.7% and 74.8% in Yannong 19, respectively. However, the difference between waterlogged and non-waterlogged roots declined to 9.9% and 27.4% in Yangmai 18, and 8.0% and 26.4% in Yannong 19, respectively, at 28 DAA (Figure 5B,D).

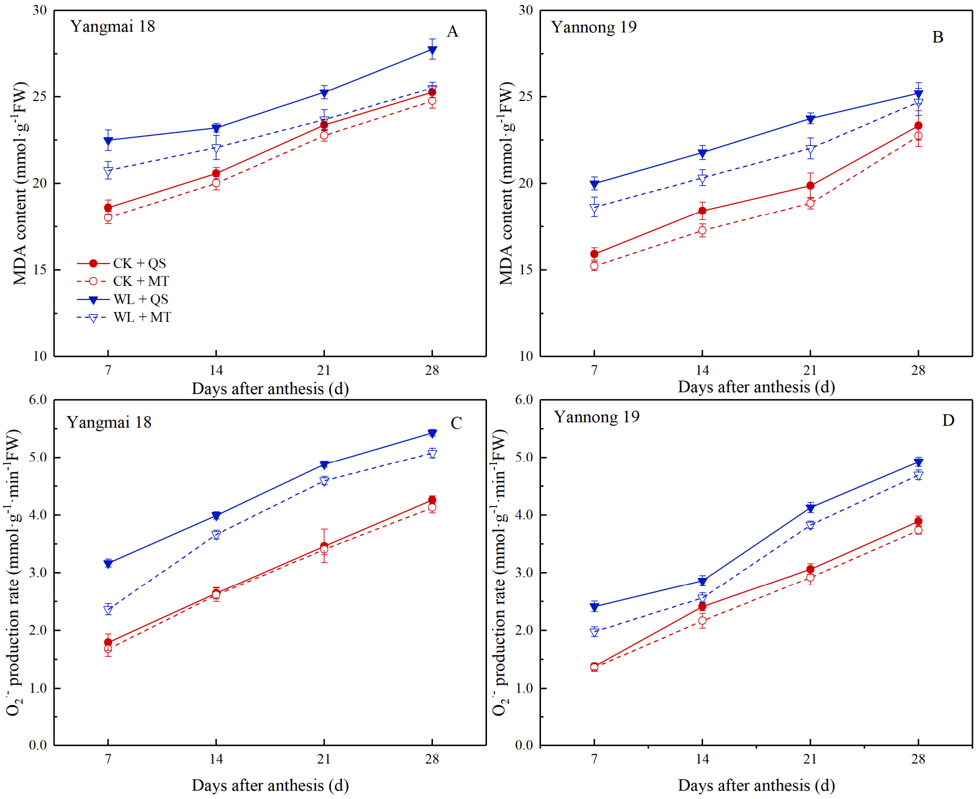

Figure 5. Post-flowering changes in root malondialdehyde (MDA) content and O_2^- production rate (**A**–**D**) under different treatments, i.e., CK + QS: leaf spraying with distilled water under non-waterlogged conditions; CK + MT: leaf spraying with 100 µmol·L^{-1} of melatonin under non-waterlogged conditions; WL + QS: leaf spraying with distilled water under waterlogged conditions; WL + MT: leaf spraying with 100 µmol·L^{-1} of melatonin under waterlogged conditions.

Spraying MT significantly reduced the MDA content and O_2^- production rate in the roots of two wheat cultivars under waterlogging conditions. Compared with waterlogged plants, MT treatments significantly reduced MDA content and the O_2^- production rate by 7.7% and 25.4% in Yangmai 18 and 6.7% and 17.9% in Yannong 19, respectively, at 7 DAA.

3.1.3. Root Anaerobic Respiration

Compared with non-waterlogging treatments, waterlogging significantly increased PDC activity in root tissues in both wheat cultivars ($p < 0.0001$). Spraying MT had no significant effect on the PDC activity ($p > 0.05$) of Yangmai 18 both under waterlogged and non-waterlogged conditions (Figure 6A). However, it significantly reduced the PDC activity in non-waterlogged Yannong 19 roots ($p < 0.0001$) (Figure 6D).

Waterlogging also significantly ($p < 0.0001$) increased the LDH activity in the roots of both wheat cultivars. Under non-waterlogged conditions, MT had no significant effect on the LDH activity of Yangmai 18 roots (Figure 6B), but it significantly reduced the LDH activity of Yannong 19 (Figure 6E). Under waterlogged conditions, MT significantly reduced the LDH activity in both cultivars.

Waterlogging significantly increased the activity of ADH in roots in Yangmai 18 ($p < 0.01$) (Figure 6C) and Yannong 19 ($p < 0.0001$) (Figure 6F). Compared with waterlogging treatments, MT significantly reduced ADH activity in both cultivars.

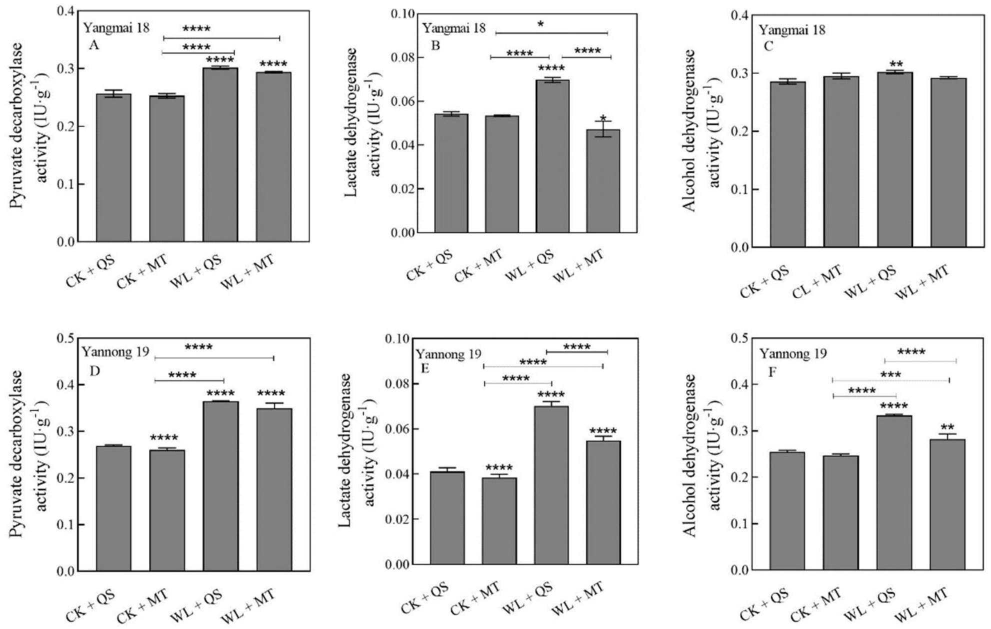

Figure 6. Post-flowering changes in root enzymes' activities related to anaerobic respiration (**A**–**F**) under different treatments, i.e., CK + QS: leaf spraying with distilled water under non-waterlogged conditions; CK + MT: leaf spraying with 100 µmol·L^{-1} of melatonin under non-waterlogged conditions; WL + QS: leaf spraying with distilled water under waterlogged conditions; WL + MT: leaf spraying with 100 µmol·L^{-1} of melatonin under waterlogged conditions. * Significance at the 0.05 level, ** significance at the 0.01 level, *** significance at the 0.001 level, **** significance at the 0.0001 level.

3.2. Photosynthetic Characteristics of Flag Leaves

3.2.1. Relative Chlorophyll Content (SPAD Units)

Both cultivars contained maximum flag leaf SPAD values at 7 DAA, which were progressively reduced as the plant growth proceeded (14, 21, and 28 DAA). Waterlogging also significantly reduced the SPAD values of both cultivars, with an accelerated reduction in Yangmai 18 later during grain filling. For example, compared with their respective non-waterlogged controls, waterlogging reduced the relative chlorophyll content by 2.9 and 14.3 SPAD units, respectively, at 7 and 28 DAA in Yangmai 18 (Figure 7A), whereas this reduction was 2.7 and 4.25 SPAD units, respectively, in Yannong 19 (Figure 7B).

Spraying MT significantly increased the relative chlorophyll content in both cultivars under waterlogging conditions. Further, this positive effect of MT on relative chlorophyll content increased in Yangmai 18 as the plant growth progressed with each subsequent measurement. For example, MT increased the relative chlorophyll content of waterlogged plants of Yangmai 18 by 1.6 SPAD units at 7 DAA, but this increment was 3.1 SPAD units at 28 DAA. In contrast, in waterlogged Yannong 19, MT caused a maximum SPAD increment of 2.2 SPAD units at 14 DAA.

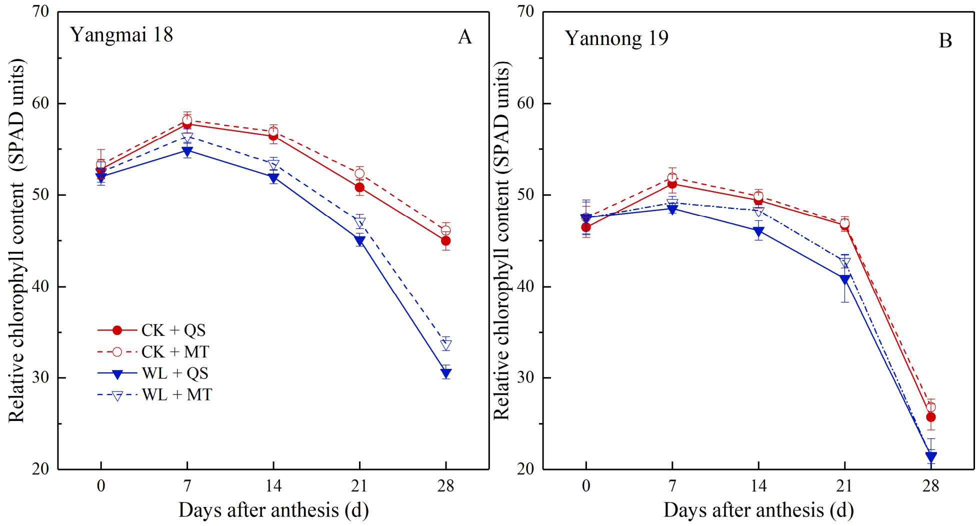

Figure 7. Post-flowering changes in flag leaf relative chlorophyll content (SPAD units) (**A**,**B**) under different treatments, i.e., CK + QS: leaf spraying with distilled water under non-waterlogged conditions; CK + MT: leaf spraying with 100 μmol·L^{-1} of melatonin under non-waterlogged conditions; WL + QS: leaf spraying with distilled water under waterlogged conditions; WL + MT: leaf spraying with 100 μmol·L^{-1} of melatonin under waterlogged conditions.

3.2.2. Net Photosynthetic Rate of Flag Leaves

Both non-waterlogged cultivars showed no significant differences in their flag leaf Pn and Ci at 7 DAA. Further, these plants had a maximum Pn value at 7 DAA, which was gradually reduced as plant growth progressed (Figure 8A,B).

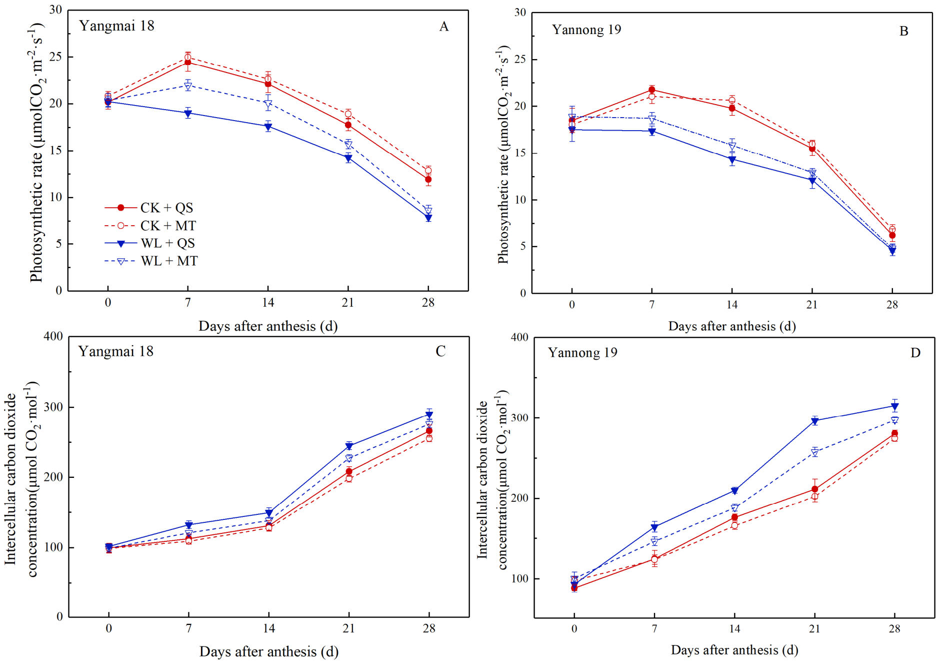

Figure 8. Post-flowering changes in the flag leaf net photosynthetic rate and intercellular CO$_2$ concentration (**A**–**D**) under different treatments, i.e., CK + QS: leaf spraying with distilled water under non-waterlogged conditions; CK + MT: leaf spraying with 100 μmol·L^{-1} of melatonin under non-waterlogged conditions; WL + QS: leaf spraying with distilled water under waterlogged conditions; WL + MT: leaf spraying with 100 μmol·L^{-1} of melatonin under waterlogged conditions.

In each measurement, the Pn of the two wheat cultivars under waterlogged conditions was significantly lower than that of the non-waterlogged flag leaves. At the same time, the Ci was significantly higher than that of the non-waterlogged flag leaves. Further, Yangmai 18 and Yannong 19 plants experienced a maximum Pn reduction of 22.1% at 7 DAA (Figure 7A) and 27.4% at 14 DAA, respectively (Figure 8B). With the advancement of the grain-filling process, the difference in Ci of Yangmai 18 gradually increased compared with the non-waterlogged treatment (Figure 8C), and Yannong 19 had a maximum increment at 21 DAA. It decreased at 28 DAA (Figure 8D).

Spraying MT increased the Pn and decreased the Ci of the two wheat cultivars under waterlogging conditions. Further, the gap between leaf Pn of MT-treated and untreated leaves first increased and then decreased, with both genotypes achieving a maximum Pn increment at different developmental stages. For example, Yangmai 18 had a maximum Pn increment of 15.4% at 7 DAA (Figure 8A), whereas Yannong 19 had a maximum Pn increment of 10.5% at 14 DAA (Figure 8B).

3.2.3. Actual Photochemical Efficiency (ΦPSII)

Under non-waterlogging conditions, both wheat cultivars had a maximum ΦPSII at 7 DAA, which declined during later measurements (14 and 21 DAA). In contrast, ΦPSII values of the waterlogged leaves started declining just after flowering. Waterlogging significantly reduced ΦPSII in both wheat cultivars at each measurement time. Further, wheat cultivars experienced a maximum reduction of ΦPSII at 7 DAA, as the plants showed a degree of recovery in the following measurement. For example, waterlogging decreased ΦPSII by 26.0% in Yangmai 18 and 20.0% in Yannong 19 at 7 DAA, respectively.

Spraying MT increased the ΦPSII in both wheat cultivars under waterlogging conditions. Compared with non-MT-treated plants, Yangmai 18 had a maximum ΦPSII increment of 4.5% at 28 DAA (Figure 9A), whereas Yannong 19 had a maximum ΦPSII increment of 4.9% at 7 DAA (Figure 9B).

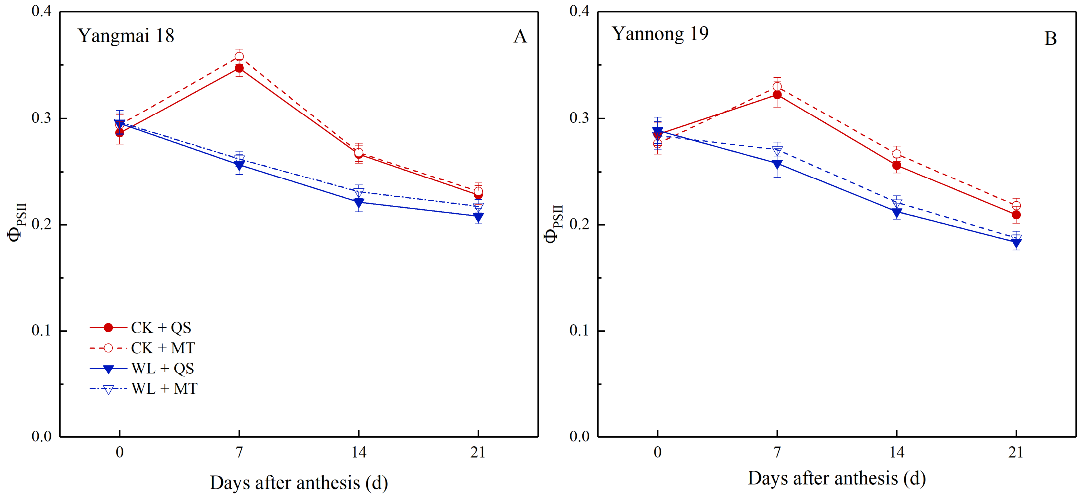

Figure 9. Post-flowering changes in flag leaf actual photochemical efficiency (**A**,**B**) under different treatments, i.e., CK + QS: leaf spraying with distilled water under non-waterlogged conditions; CK + MT: leaf spraying with 100 μmol·L^{-1} of melatonin under non-waterlogged conditions; WL + QS: leaf spraying with distilled water under waterlogged conditions; WL + MT: leaf spraying with 100 μmol·L^{-1} of melatonin under waterlogged conditions.

3.3. Grain-Filling Characteristics

A logistic equation was used to fit the dynamics of dry matter accumulation (Table 3). The coefficient of determination (R^2) of each fitting equation was highly significant ($p < 0.01$). Waterlogging shortened the total grain-filling days (T) of the two wheat cultivars, advanced the appearance date of the maximum grain-filling rate (T_{m}), and shortened the duration of the slight-increase period (T_1), fast-increase period (T_2), and slow-increase period (T_3). Waterlogging increased the maximum grain-filling rate (V_{m}) and average grain-filling rate (V_{a}) of the two wheat cultivars. Spraying MT increased the T in both wheat cultivars, delayed T_{m}, prolonged the duration of T_1, T_2, and T_3, and decreased V_{m} and V_{a} in the two wheat cultivars.

Table 3. Grain-filling parameters under different treatments, i.e., CK + QS: leaf spraying with distilled water under non-waterlogged conditions; CK + MT: leaf spraying with 100 µmol·L^{-1} of melatonin under non-waterlogged conditions; WL + QS: leaf spraying with distilled water under waterlogged conditions; WL + MT: leaf spraying with 100 µmol·L^{-1} of melatonin under waterlogged conditions.

Cultivar	Treatment	Model	Decision Coefficient (R^2)	T_{m} (d)	V_{m} (mg·Grain^{-1}·d^{-1})	T (d)	V_{a} (mg·Grain^{-1}·d^{-1})	T_1 (d)	T_2 (d)	T_3 (d)
Yangmai 18	CK + QS	$Y = 49.3892/(1 + e^{(3.2907 - 0.164413t)})$	0.9993 **	20.0	2.0	48.0	1.0	12.0	16.0	19.9
	CK + MT	$Y = 48.8008/(1 + e^{(3.2846 - 0.179028t)})$	0.9990 **	18.4	2.2	44.0	1.1	11.0	14.7	18.3
	WL + QS	$Y = 41.8093/(1 + e^{(3.6082 - 0.205044t)})$	0.9990 **	17.6	2.1	40.0	1.1	11.2	12.9	16.0
	WL + MT	$Y = 43.4346/(1 + e^{(3.4910 - 0.187974t)})$	0.9981 **	18.6	2.0	43.0	1.0	11.6	14.0	17.4
Yannong 19	CK + QS	$Y = 46.3428/(1 + e^{(3.4303 - 0.175447t)})$	0.9989 **	19.6	2.0	45.7	1.0	12.1	15.0	18.7
	CK + MT	$Y = 46.9292/(1 + e^{(3.3835 - 0.170038t)})$	0.9988 **	19.9	2.0	46.9	1.0	12.2	15.5	19.3
	WL + QS	$Y = 40.0660/(1 + e^{(3.7892 - 0.215242t)})$	0.9992 **	17.6	2.2	39.0	1.0	11.5	12.2	15.2
	WL + MT	$Y = 41.1069/(1 + e^{(3.7016 - 0.204595t)})$	0.9991 **	18.1	2.1	40.6	1.0	11.7	12.9	16.0

Note: Y, 1000-grain weight; t, days after flowering; R^2, determination coefficient; T_{m}, time to maximum filling rate; V_{m}, maximum filling rate; T, the grain-filling duration; V_{a}, average filling rate; T_1, duration of the slight-increase period (d); T_2, duration of the fast-increase period (d); T_3, duration of the slow-increase period (d). ** indicate significant differences at the 0.01 level.

3.4. Dry Matter Accumulation and Distribution

Waterlogging significantly reduced dry matter accumulation in stem + sheath + leaf and grain ($p < 0.05$), although it had no significant effect on dry matter accumulation in the spike axis + glume in both cultivars (Figure 10A,B). Compared with their respective non-waterlogged controls, waterlogged Yangmai 18 and Yannong 19 accumulated 14.0% and 16.0% lower total dry matter, respectively. Compared with waterlogging treatments, MT treatment significantly increased the dry matter accumulation in grain in both cultivars and increased total dry matter accumulation by 5.9% and 3.2% in Yangmai 18 and Yannong 19, respectively.

Waterlogging increased the dry matter distribution in the stem + sheath + leaf and spike + axis + glume, but it decreased the dry matter distribution to the grains of both cultivars. MT application decreased the dry matter distribution in the stem + sheath + leaf and spike + axis + glume and increased the distribution in grain in both cultivars compared with their respective waterlogged plants (Figure 10C,D).

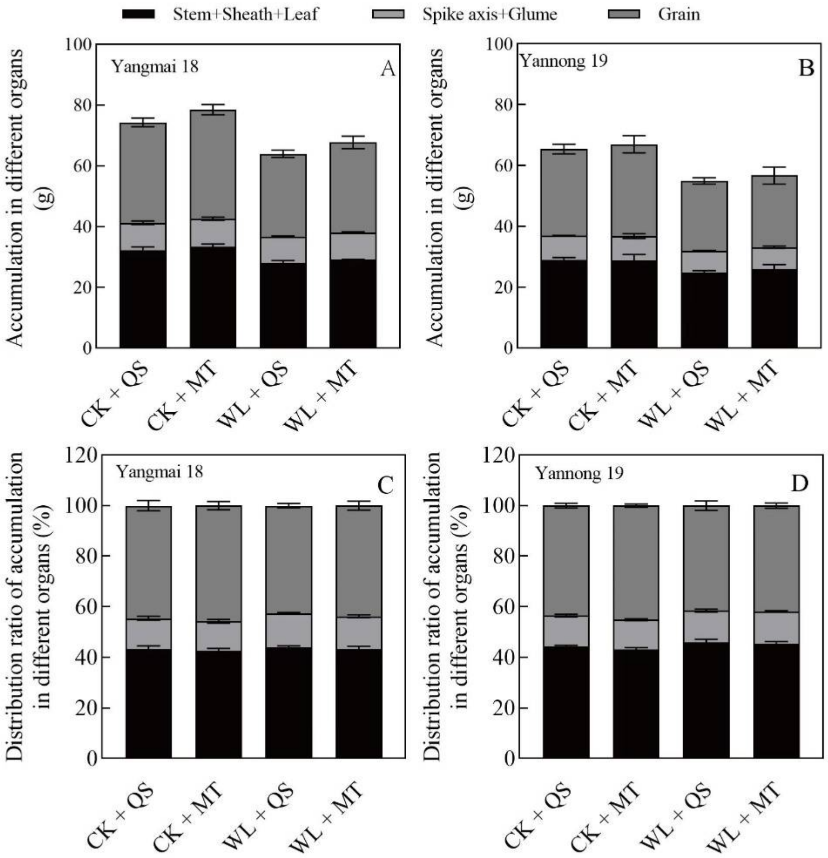

Figure 10. Dry matter accumulation and distribution (**A**–**D**) under different treatments, i.e., CK + QS: leaf spraying with distilled water under non-waterlogged conditions; CK + MT: leaf spraying with 100 μmol·L^{-1} of melatonin under non-waterlogged conditions; WL + QS: leaf spraying with distilled water under waterlogged conditions; WL + MT: leaf spraying with 100 μmol·L^{-1} of melatonin under waterlogged conditions.

3.5. Grain Yield and Its Components

The total number of spikes per plant remained unaffected by any treatments ($p > 0.05$), although soil waterlogging significantly reduced the number of grains per spike, 1000-grain weight (TGW), and yield in both wheat cultivars (Table 4). In contrast, MT significantly increased the final grain yield and TGW of both wheat cultivars. Compared with non-waterlogged treatments, soil waterlogging decreased the number of grains per spike, TGW, and yield by 8.6%, 13.8%, and 16.2%, respectively, in Yangmai 18, and 14.7%, 8.1%, and 25.1%, respectively, in Yannong 19. Spraying MT increased the number of grains per spike, TGW, and yield by 3.8%, 8.7%, and 14.9%, respectively, in Yangmai 18, and 2.8%, 7.3%, and 26.0%, respectively, in Yannong 19. From the analysis of the variance of the F value, the effect of the cultivar on the yield and its components was highly significant ($p < 0.01$), and the effects of waterlogging on yield, grain numbers per spike, and TGW were highly significant ($p < 0.01$). The effects of MT on grain numbers per spike and TGW were significant ($p < 0.05$).

There was a positive correlation between the number of grains per spike, TGW, and yield (Figure 11). The grain number per spike and TGW in Yangmai 18 were generally higher than those in Yannong 19. Further, grains per spike, TGW, and total grain yield under the CK + MT treatments were significantly higher than those under WL + QS treatments.

Table 4. Grain yield and its components under different treatments, i.e., CK + QS: leaf spraying with distilled water under non-waterlogged conditions; CK + MT: leaf spraying with 100 μmol·L^{-1} of melatonin under non-waterlogged conditions; WL + QS: leaf spraying with distilled water under waterlogged conditions; WL + MT: leaf spraying with 100 μmol·L^{-1} of melatonin under waterlogged conditions.

Cultivar	Treatment	Spikes (Pot^{-1})	Kernels (Spike^{-1})	1000-Grain Weight (g)	Grain Yield (g·Pot^{-1})
Yangmai 18	CK + QS	14.7a	49.6a	40.6b	30.1b
	CK + MT	15.3a	51.0a	43.9a	33.2a
	WL + QS	14.7a	45.3b	35.0c	25.2c
	WL + MT	15.0a	47.0b	38.0b	29.0b
Yannong 19	CK + QS	13.7a	47.6a	40.8b	27.5b
	CK + MT	14.0a	49.1a	44.0a	31.2a
	WL + QS	14.7a	40.6b	37.5c	20.6c
	WL + MT	13.7a	41.7b	40.2b	25.9b
F-$_{\text{Cultivar (C)}}$		10.080 **	27.516 **	49.608 **	45.646 **
F-$_{\text{Waterlogging (W)}}$		0.083	73.177 **	271.136 **	26.831 **
F-$_{\text{Melatonin (M)}}$		0.083	4.748 *	5.019 *	1.227
F-$_{\text{C×W}}$		0.750	5.360 *	0.179	0.875 **
F-$_{\text{C×M}}$		2.083	0.024	0.253	8.523
F-$_{\text{W×M}}$		2.083	0.000	1.593	0.019
F-$_{\text{C×W×M}}$		0.750	0.064	0.019	0.000

Note: Different letters in the same column indicate the differences between treatments at $p < 0.05$. F-Cultivar (C), F-Waterlogging (W), and F-Melatonin (M) represent the F values of the cultivar, waterlogging, and melatonin, respectively. F-$_{\text{C×W}}$, F-$_{\text{C×M}}$, F-$_{\text{W×M}}$, and F-$_{\text{C×W×M}}$ represent the F values between each other. * and ** indicate significant differences at 0.05 and 0.01 level.

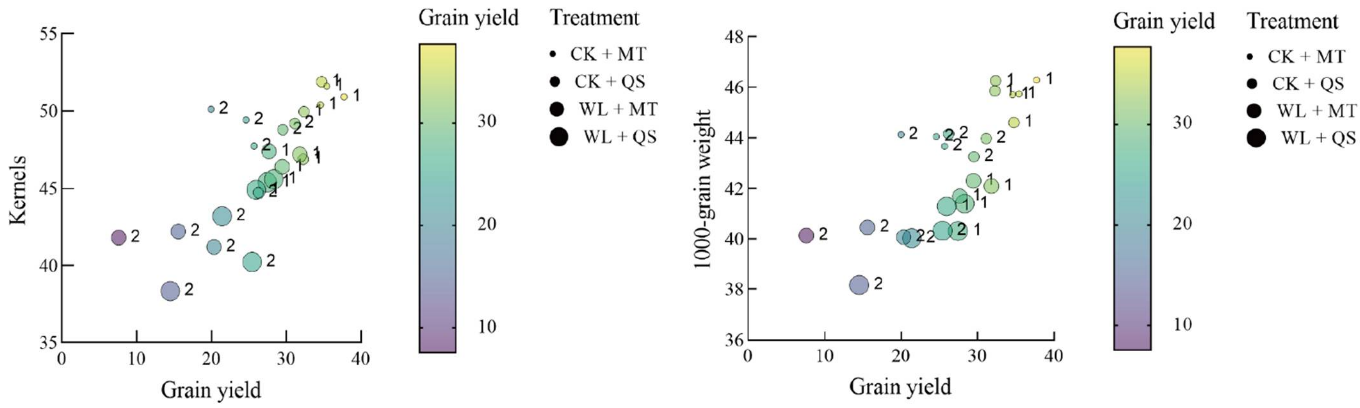

Figure 11. Using a bubble chart to analyze the effect of grain number and thousand-grain weight on yield by cultivar and treatment, i.e., CK + QS: leaf spraying with distilled water under non-waterlogged conditions; CK + MT: leaf spraying with 100 μmol·L^{-1} of melatonin under non-waterlogged conditions; WL + QS: leaf spraying with distilled water under waterlogged conditions; WL + MT: leaf spraying with 100 μmol·L^{-1} of melatonin under waterlogged conditions. 1 represents Yangmai 18, and 2 represents Yannong 19.

3.6. Correlation Analysis between Each Measured Index and Yield in the Root System

In Yangmai 18 at 7 DAA, the grain yield was negatively correlated with the RDW, MDA content, POD activity, O_2^- production rate, PDC activity, LDH activity, and ADH activity, and positively correlated with SOD activity in root tissues. In Yannong 19 at 7 DAA, the final grain yield was negatively correlated with MDA content, POD activity, O_2^-production rate, PDC activity, LDH activity, and ADH activity, and positively correlated with RDW and SOD activity in the roots (Figure 12).

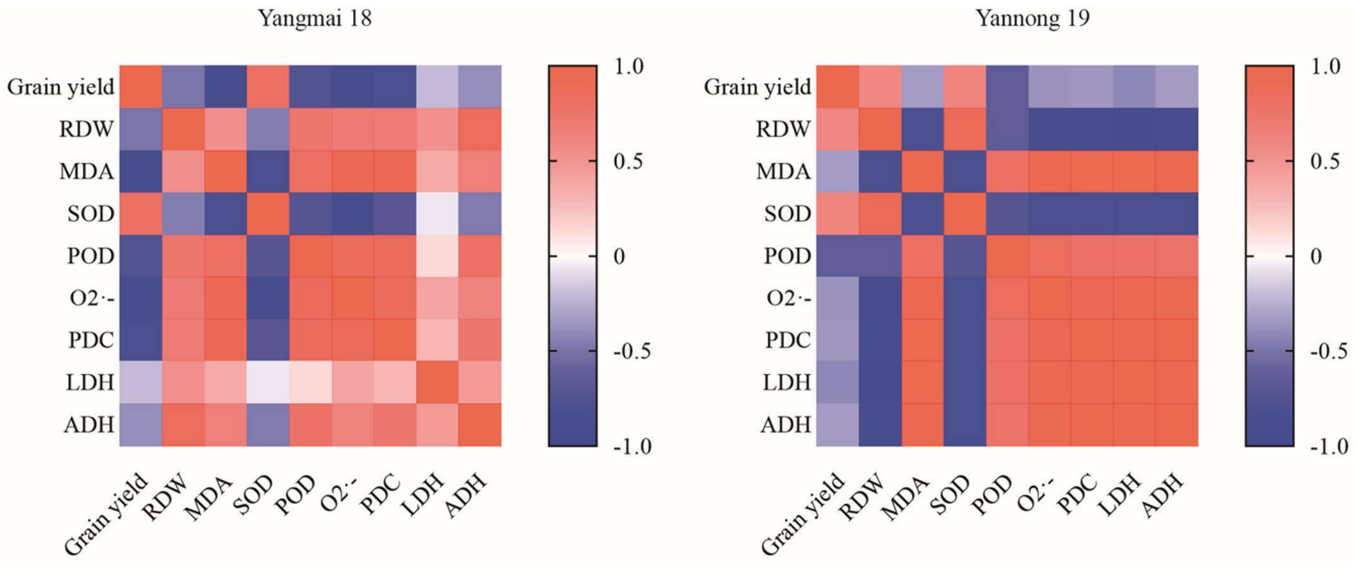

Figure 12. Correlation between wheat root measurement indexes at 7 DAA and yield under different treatments. Abbreviations: RDW, root dry weight; MDA, malondialdehyde content; SOD, superoxide dismutase activity; POD, peroxidase activity; O_2^-, superoxide anion production rate; PDC, pyruvate decarboxylase activity; LDH, lactate dehydrogenase activity; ADH, alcohol dehydrogenase activity.

4. Discussion

Under normal conditions, the production and scavenging of reactive oxygen species in plants are in a dynamic balance, but environmental stress breaks this balance and the content of ROS increases [36]. Excessive water at the early stage of flooding saturates the rhizosphere, and plant roots and soil microorganisms quickly consume the remaining oxygen, causing hypoxia [37]. The cause of oxidative stress is singlet oxygen ($^1O_2$) and superoxide. The accumulation of a large number of compounds, such as anion radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH) [38], caused the leakage of root cytosol, loss of root pressure, and disruption of membrane integrity further exacerbated by waterlogging, which lead to irreversible root cell death [39]. MDA, an end-product of lipid peroxidation of cellular membranes, is often used to quantify the degree of cellular peroxidation in plants [40]. Increased MDA levels have already been reported in waterlogged plants. For example, 10 days of waterlogging caused a 250% increase in MDA contents in alfalfa leaves [41]. In this study, soil waterlogging (7 days) significantly increased MDA contents and O_2^- levels in the root tissues of the two wheat cultivars.

Furthermore, our study showed that MDA and O_2^- levels in waterlogged plants were significantly reduced after spraying MT, suggesting its capacity to mitigate waterlogging-induced oxidative injury. However, under normal growth conditions, spraying MT had little effect on MDA content and the O_2^- production rate in roots. This suggests that MT can inhibit the accumulation of reactive oxygen species in roots to a certain extent, reduce the degree of membrane lipid peroxidation, and reduce damage to cell membranes, slowing the root senescence rate [42].

Plants protect cells from ROS injury by activating an antioxidant enzyme system [43], which can be used as physiological indices to assess plant stress tolerance [44]. Foliar spray MT can increase SOD and POD activity and thus enhance the tolerance of plants to abiotic stress [45]. Our study suggests that melatonin induces waterlogging tolerance in wheat by sustaining aerobic respiration in root tissues and protects leaf photosynthesis from oxidative damage through efficient repression of the ROS burst and consequent mitochondria degradation [46]. This study found that WL + QS treatment significantly reduced SOD activity and increased POD activity in the roots of the two wheat cultivars. Compared with CK + NW, spraying MT increased SOD activity and decreased POD activity in the roots of the two wheat cultivars, consistent with CK + NW. POD has a dual role:

it protects cells by eliminating the accumulated cellular H_2O_2 and catalyzes NADH or NADPH to produce $O_2{}^-$, participates in the degradation of chlorophyll, and accelerates the senescence of the root system [47]. In our experiment, POD activity was elevated under conditions that accelerated root senescence.

Pyruvate fermentation produces energy in two pathways: lactic acid, via either LDH or PDC, turning pyruvate into acetaldehyde, which is then reduced to ethanol by ADH. ADH and PDC play key roles in the ethanol fermentation pathway, and their activity is usually considered one of the critical indexes reflecting the tolerance of plants to waterlogging [37]. Waterlogging-tolerant plants can improve the ethanol fermentation rate by regulating the expression of ADH, PDC, and other related enzyme genes, temporarily providing energy for plant growth under waterlogging [48]. We found that waterlogging activated the anaerobic respiration metabolism of wheat roots. For example, under 7 days of waterlogging, PDC, LDH, and ADH activities of Yangmai 18 roots were increased by 17.6%, 28.5%, and 5.6%, respectively, and of Yannong 19 by 35.8%, 70.8%, and 30.7%, respectively. Compared with waterlogging treatments, spraying MT decreased the PDC, LDH, and ADH activities in Yangmai 18 roots by 2.6%, 32.1%, and 3.2%, respectively, and in Yannong 19 roots by 4.1%, 22.0%, and 15.3%, respectively. The results showed that the anaerobic respiration of Yannong 19 was stronger than Yangmai 18, and lactate fermentation was the primary pathway of NAD+ production in the two studied wheat cultivars. MT improved waterlogging tolerance in both cultivars by enhancing plant aerenchyma formation and inhibiting metabolic enzymes ADH, PDC, and LDH [5].

Literature suggests that leaf photosynthetic inhibition in waterlogged plants could result from non-stomatal limitations [49], poor ΦPSII activity, or inhibited electron transfer [50]. The chloroplast, the main site of melatonin production, is also one of the organelles most affected by ROS. Thus, it requires large amounts of melatonin to maintain its structure and function [41]. Earlier studies suggest that 100 $\mu mol \cdot L^{-1}$ of melatonin applied to the heat-stressed wheat for 15 days significantly increased the photosynthesis and carbohydrate metabolism to provide energy and carbon skeleton to the developing plant under stress [51]. This study found that waterlogging decreased key leaf physiological traits such as SPAD, Pn, and ΦPSII, and increased Ci of both wheat cultivars, which were restored by MT application. This indicates that soil waterlogging inhibits biomass assimilation in wheat plants by impairing multiple biochemical pathways.

Under waterlogged conditions, the wheat root system cannot quickly restore its function and growth, thus reducing above-ground productivity [52]. Studies have shown that sucrose transportation from the above-ground to the underground is reduced by 79–97% under anoxic conditions. The ability of roots to absorb water and transport it to the above-ground parts is reduced, resulting in an 18–60% reduction in leaf water potential and stomata closure, inhibiting photosynthesis and leading to plant wilting and thus to a decrease in above-ground biomass [53]. Significantly positive effects of exogenously applied MT assimilates' transportation to wheat grains have already been reported [54]. Previous studies show that MT can promote nitrogen metabolism by upregulating the activities of N uptake and metabolism-related enzymes and enhancing the nitrogen, nitrate, and protein content in stressed plants [54]. We found that waterlogging decreased the dry matter accumulation in grain and increased the dry matter distribution to vegetative organs in the two wheat cultivars, and spraying MT alleviated the waterlogging-induced damage and yield reduction. We suggest that MT protects the photosynthetic machinery in wheat leaves from waterlogging injury either directly by regulating oxidative stress or indirectly by strengthening the chloroplast capacity to sustain biomass assimilation [24].

The effect of grain-filling parameters on the final crop yield is regulated by genotype [55] and environments, such as soil moisture and temperature [56]. Post-flowering waterlogging reduces the grain-filling period by 1–5 days; consequently, the final grain size is smaller due to poor grain filling [57]. Waterlogging-induced grain yield loss in this study primarily resulted from the reduced thousand-grain weight, but not from the reduced number of spikes or grains per spike. Our study also showed that a seven-day waterlogging event significantly shortened the total grain-filling days of the two wheat cultivars. Although the waterlogged plants compensated for this loss by increasing the maximum grain-filling rate and the average grain-filling rate, they suffered a significant grain yield loss. The reason why waterlogging reduces TGW is mainly due to the total number of days of grain filling, the slight-increase period, the fast-increase period, and the slow-increase period. Spraying MT alleviated the damage resulting from shortening the grain-filling days. In this experiment, the final grain yields of Yangmai 18 and Yannong 19 were decreased by 14.7% and 17.2%, respectively, and spraying MT increased the number of grains per spike, TGW, and yield. Compared with their non-MT-treated plants under waterlogging conditions, MT increased the yield of Yangmai 18 and Yannong 19 by 8.8% and 2.7%, respectively. The results also showed that the damage of waterlogging treatment to Yannong 19 was greater than that of Yangmai 18, and MT showed a better effect in Yangmai 18 than Yannong 19.

5. Conclusions

In general, soil waterlogging significantly increased MDA and $O_2{}^-$ production rates and inhibited leaf photosynthesis traits, leading to poor biomass accumulation and grain yield formation. MT protects wheat roots from waterlogging-induced oxidative injury by upregulating antioxidant enzymes and accelerating anaerobic respiration. We proposed that MT-induced water and nutrient supplies assisted waterlogged plants in sustaining leaf photosynthesis. The plants treated with melatonin showed better water status and less oxidative damage, which was conducive to maintaining a higher photosynthetic capacity, thereby improving the waterlogging tolerance of wheat. Compared with waterlogged plants, melatonin treatments significantly reduced the MDA content, $O_2{}^-$ production rate, PDC, LDH, and ADH activities of the tested wheat genotypes. Melatonin also assisted waterlogged plants in sustaining leaf photosynthesis, dry matter accumulation, and final grain yield. Our study highlights the critical role of carbohydrate synthesis and re-distribution in post-flowering waterlogged wheat crops. The findings are likely to contribute to managing wheat crops in high-rainfall regions.

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