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# Role of Glutathione-Ascorbate Cycle and Photosynthetic Electronic Transfer in Alternative Oxidase-Manipulated Waterlogging Tolerance in Watermelon Seedlings

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**Abstract:** Alternative oxidase (AOX) has been documented to mitigate the oxidative stress caused by abiotic stresses. However, it remains unknown how AOX regulates the antioxidant system and photosynthesis under waterlogging. To address this issue, we used two watermelon (*Citrullus lanatus* L.) cultivars (waterlogging tolerant cultivar 'YL' and sensitive cultivar 'Zaojia8424') as materials and the AOX inhibitor salicylhydroxamic acid (SHAM) to investigate the effects of AOX on photosynthesis and reactive oxygen species metabolism under waterlogging. We found that waterlogging decreased leaf photosynthesis and quantum yield of photosynthesis in watermelon, and the waterlogging tolerant cultivar 'YL' showed higher expression level of *ClaAOX* than the sensitive cultivar 'Zaojia8424'. Net photosynthesis rate was higher in 'YL' than 'Zaojia8424'. Moreover, waterlogging induced photoinhibition in 'Zaojia8424' but not in 'YL'. Meanwhile, waterlogging promoted the accumulation of superoxide and peroxide hydrogen, and triggered oxidative damage. 'YL' suffered from less severe oxidative damage due to increased contents of ascorbate, a higher ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG), a higher activity of ascorbate peroxidase (APX) and catalase (CAT), and enhanced levels of *CAT* and *APX* expression, relative to 'Zaojia8424'. However, the alleviation of photosynthesis and oxidative damage, increased content of ascorbate and higher GSH/GSSG ratio were abolished by SHAM. Our results suggested that photosynthetic electronic transfer and glutathione-ascorbate cycle are involved in waterlogging tolerance mediated by the AOX pathway in watermelon.

**Keywords:** alternative oxidase; photosynthesis; reactive oxygen species; antioxidant system



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## 1. Introduction

Waterlogging is an emerging environmental factor restricting plant productivity due to climate change and influences almost 16% of global cultivated areas [1]. It is estimated that two-thirds of the total crop yield reduction worldwide has been ascribed to waterlogging during 2006–2016 [2]. Waterlogging initiates $O_2$ deprivation within the roots, and triggers molecular, biochemical and physiological changes in both shoots and roots [1,3–6]. Leaf photosynthesis is susceptible to waterlogging. The reduction of net photosynthesis rate (Pn) by waterlogging has been observed in many plants, including cucumber (*Cucumis sativus*) [7], maize (*Zea mays*) [8], peanut (*Arachis hypogaea*) [9], sorghum (*Sorghum bicolor*) [10] and tomato (*Solanum lycopersicum*) [11]. Moreover, both Photosystem II (PSII) and Photosystem I (PSI) are impaired by waterlogging, represented by a decrease in the maximal photochemical efficiency of PSII and PSI [12]. Furthermore, the photosynthetic

electron transport chain was interrupted by waterlogging, indicated by the reduction of effective quantum yield of PSII ($Y_{II}$) [13].

The chloroplast is a primary generating site of reactive oxygen species (ROS), including singlet oxygen ($^1O_2$), superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$), and the hydroxyl radical (·OH) [14,15]. The over-reduction of the photosynthetic system due to environmental stress such as waterlogging causes electron leakage from plastoquinone QA and QB to $O_2$, and consequently results in the production of ROS [16]. It has been reported that $O_2^-$ and $H_2O_2$ can be excessively produced by waterlogging in plant leaves [8,17,18], and the over-accumulation of ROS could lead to oxidative damage to lipids, proteins and nucleic acids [14].

To keep the ROS concentrations under tight control, plants have evolved a sophisticated antioxidant system, including non-enzymatic and enzymatic ROS scavenging mechanisms [19]. Enzymatic ROS scavenging mechanisms comprise superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (GPOD). SODs dismutate $O_2^-$ into $H_2O_2$, which can be detoxified into $H_2O$ by CAT, GPOD and the glutathione-ascorbate cycle. Ascorbate and glutathione scavenge $H_2O_2$ via ascorbate peroxidase (APX) and glutathione peroxidase (GPX) [20,21]. The antioxidant system responds rapidly to waterlogging. For instance, leaf ascorbate and glutathione pools were reduced after only 3 h of anoxia in barley (*Hordeum vulgare*) [18].

Alternative oxidase (AOX), located in the inner membrane of mitochondria, serves as a terminal electron acceptor besides classical cytochrome c oxidase. AOX can lower ROS production in plant cells and has a positive effect on plant performance under abiotic stresses [22,23]. For instance, overexpression of AOX reduced the accumulation of $O_2^-$ and prevented the oxidative damage caused by hypoxia in tobacco (*Nicotiana tabacum*) [24]. However, whether the glutathione-ascorbate cycle is involved in the alleviation of oxidative damage mediated by AOX under waterlogging remains unknown. Moreover, it has been reported that AOX is essential in maintaining of photosynthesis under drought stress [25,26]. However, the role of AOX in photosynthesis under waterlogging remains unclear.

Watermelon (*Citrullus lanatus*) is an important crop worldwide and is often threatened by waterlogging. Recently, we discovered a waterlogging tolerant cultivar of watermelon, 'YL', and found its tolerance to waterlogging was associated with enhanced activity of AOX via maintaining the root respiratory [27].

In this study, we examined the role of the glutathione-ascorbate cycle and photosynthesis in AOX mediated waterlogging tolerance by using the tolerant cultivar 'YL' and a sensitive cultivar 'Zaojia8424'. Salicylhydroxamic acid (SHAM), an AOX inhibitor, was also employed to manipulate AOX activity. Biochemical and physiological parameters associated with the glutathione-ascorbate cycle and photosynthesis were examined.

## 2. Materials and Methods

### *2.1. Plant Genotypes and Waterlogging Treatment*

Two cultivars of watermelon (*Citrullus lanatus*), the waterlogging tolerant cultivar 'YL' and a waterlogging sensitive cultivar 'Zaojia8424', were used in the present study. Seeds of both cultivars were surface sterilized and germinated for 3 days at 30 °C in darkness. Seedlings were transferred into 1 L plastic pots containing commercial substrate (Hangzhou Jinhai Agricultural Technology Co. LTD, China) with one seedling in each pot. All pots were placed in a growth chamber with day/night temperatures of 28 °C/20 °C, light intensity of 600 μmol $m^{-2}$ $s^{-1}$ and 12 h photoperiod.

After reaching the four-leaf stage, the pots were transferred to a tank filled with water to impose waterlogging treatment for 9 days. In the AOX inhibition treatment, 3 mM SHAM was sprayed on leaves every 3 days during waterlogging treatment. The second and third fully expanded leaves were harvested from the start to the end of waterlogging at an interval of 3 d (0, 3, 6 and 9 d after waterlogging stress (DAS)) to measure the contents of glutathione and ascorbate acid, and the activity of antioxidant enzymes as well. These leaves were sampled from a subset of plants, and an individual plant was sampled

once. The oxidative damage and contents of superoxide and hydrogen peroxide were investigated at 9 DAS. The harvested tissues were put into the liquid nitrogen immediately and then stored at −80 °C until analyses. Twenty plants were included in each treatment and in the controls. The experiment was repeated 3 times from May to November 2020.

The shoots were harvested and weighed at the end of waterlogging.

### 2.2. Measurement of Photosynthesis and Chlorophyll Fluorescence

Leaf photosynthesis was measured in four plants randomly selected from each treatment with an open gas exchange system (Li-6800, Li-Cor, USA) at 9 DAS. Net photosynthetic rate (Pn), stomatal conductance (Gs), intercellular $CO_2$ concentration (Ci) and transpiration rate (E) were determined between 9:00 and 12:00 at 800 μmol $m^{-2}$ $s^{-1}$ photosynthetic photon flux density. During measurement, relative humidity was maintained at 70% and leaf temperature was set at 28 ± 0.5 °C in the leaf chamber.

Chlorophyll fluorescence was measured on the same leaf for photosynthesis measurements using a pulse amplitude modulated chlorophyll fluorometer (PAM2500, Walz, Germany) at 9 DAS. Prior to Fv/Fm measurements, the leaf area assayed was dark-adapted for 20 min. The initial fluorescence (Fo) was obtained by application of a low-intensity red measuring light source, and Fm was measured after applying a saturating light pulse of 8000 μmol $m^{-2}$ $s^{-1}$. Minimum (F'o) and maximum (F'm) values of fluorescence in the light-adapted state at 800 μmol $m^{-2}$ $s^{-1}$ were also obtained in this manner. The following parameters were collected from the machine: Fv/Fm (maximum quantum efficiency of PSII photochemistry), F'v/F'm (effective quantum use efficiency of PSII in the light-adapted state), qP (proportion of open PSII), $Y_{II}$ (the effective quantum yield of PSII), $Y_{NPQ}$ (the quantum yield of regulated non-photochemical energy loss in PSII) and $Y_{NO}$ (the quantum yield of non-regulated energy loss in PSII).

### 2.3. Assay of Electrolyte Leakage and Lipid Peroxidation in Leaves

Electrolyte leakage in leaves was assessed by an electrical conductivity meter (DDS-307, Shanghai INESA Scientific Instrument Co., Ltd., Shanghai, China) following the method of Dionisio-Sese and Tobita (1998) [28].

Lipid peroxidation was estimated in terms of thiobarbituric acid reactive substances (TBARS) according to the method of Cakmak and Marschner (1992) [29]. Briefly, 0.3 g leaves were homogenized in 2 mL 0.1% (*v*/*v*) trichloroacetic acid solution on ice. After centrifugation at 10,000× *g* for 5 min, the supernatant was collected and measured spectrophotometrically at 532 and 600 nm. After subtracting the non-specific absorbance at 600 nm, the TBARS concentration was determined by its extinction coefficient of 155 $mM^{-1}$ $cm^{-1}$.

### 2.4. Quantification of $O_2^-$ and $H_2O_2$

For the extraction of $O_2^-$, 0.5 g watermelon leaves were ground in 2 mL potassium phosphate buffer (pH 7.8, 65 mM), and the homogenates were centrifuged at 10,000× *g* for 15 min at 4 °C. Then, 0.5 mL supernatants were mixed with 1.5 mL of reaction buffer (containing 3 mM hydroxylamine hydrochloride, 20 mM sulfanilamide and 2 mM α-naphthylamine in 4 M acetic acid) and incubated for 20 min at 25 °C. The absorbance of the reaction mixture was recorded at 530 nm [30].

The $H_2O_2$ content was assayed according to the method of Cheeseman (2006) [31]. Leaf tissue (0.5 g) was homogenized in 2 mL $HClO_4$ (1.0 M) with polyvinylpyrrolidone. The homogenates were centrifuged at 10,000× *g* for 10 min at 4 °C. Then, 1 mL supernatant was added to 3 mL eFOX reagents, incubated for 20 min, and then the difference in absorbance between 550 and 800 nm was recorded. The $H_2O_2$ content was calculated through a standard curve.

### 2.5. Determination of Antioxidant Enzyme Activity

The extraction and activity determination of antioxidant enzymes were carried out as described by He et al. (2009) [32]. A 0.3 g leaf was ground with 2 mL ice-cold potassium

phosphate buffer (pH 7.8, 50 mM) containing 0.2 mM EDTA and 2% polyvinylpyrrolidone, and the homogenates were centrifuged at 4 °C for 20 min at 12,000× *g*. Then the supernatants obtained were used for enzyme analysis.

The activity of superoxide dismutase (SOD, EC; 1.15.1.1) was assayed by its ability to inhibit the photochemical reduction of nitro blue tetrazolium [33]. The activity of catalase (CAT, EC 1.11.1.6) was quantified by monitoring the reduction of absorbance at 240 nm as the disintegration of $H_2O_2$ using the extinction coefficient 39.4 $mM^{-1}$ $cm^{-1}$ [29]. The activity of guaiacol peroxidase (GPOD, EC 1.11.1.7) was calculated by the increase of absorbance at 470 nm using the extinction coefficient 26.6 $mM^{-1}$ $cm^{-1}$ [34]. The activity of ascorbate peroxidase (APX, EC 1.11.1.11) was measured by the decrease in absorbance at 290 nm with extinction coefficient 2.8 $mM^{-1}$ $cm^{-1}$ [35]. Dehydroascorbate reductase (DHAR, EC; 1.8.5.1) activity was measured by the increase in absorbance at 265 nm using the extinction coefficient 14 $mM^{-1}$ $cm^{-1}$ [35]. Glutathione reductase (GR, EC; 1.6.4.2) activity was calculated by the decrease in absorbance at 340 nm due to NADPH oxidation using the extinction coefficient 6.2 $mM^{-1}$ $cm^{-1}$ [36].

### *2.6. Analysis of Glutathione and Ascorbate*

The extraction of glutathione and ascorbate were carried out as described by Hodges et al. (1996) [37]. Briefly, 0.3 g watermelon leaf was ground in 3 mL of 5% trichloroacetic acid and the homogenate was centrifuged at 4 °C for 10 min at 12,000× *g*. This supernatant then was used for reduced glutathione (GSH), oxidized glutathione (GSSG) and ascorbate measurement.

For the measurement of glutathione, 0.5 mL supernatant was added to the 2 mL reaction solution containing 50 mM potassium phosphate, 0.2 mM 5,5′-dithiobis-(2-nitrobenzoic acid), 0.2 mM NADPH, and 3 units of glutathione reductase [38]. The total glutathione content was calculated by monitoring the absorbance at 412 nm for 1 min. To measure the content of GSSG, 4 µL 2-vinylpyridine was added to 200 µL extract and then the same protocol for total glutathione was used. The content of GSH was attained by deducting GSSG from total glutathione.

For the measurement of ascorbate, 0.2 mL supernatant was added to the 2 mL reaction solution containing 4% trichloroacetic acid (*v*/*v*), 22% o-phosphoric acid (*v*/*v*), 5 mM α,α′-dipyridyl and 10 mM $FeCl_3$ and incubated for 30 min. The absorbance at 520 nm was recorded.

### *2.7. Identification of AOX Amino Acid Sequences from Watermelon Genome*

The conserved amino acid sequence of DBD (Pfam: PF01786 for AOX) was used as a BLAST query against the watermelon genome database (http://cucurbitgenomics.org/organism/21, 1 November 2019), and the full-length amino acid sequences of the AOX proteins from arabidopsis (*Arabidopsis thaliana*) were obtained from National Center for Biotechnology Information (NCBI). The acquired protein sequences were confirmed by Pfam (http://pfam.xfam.org/search, 1 March 2021) and then phylogenetic analyzed with MEGA 5 and ClustalW software.

### *2.8. RNA Isolation and Real-Time Quantitative PCR*

The second fully expanded leaves were harvested at 0, 24, 48 and 72 h after stress (HAS) to measure the expression of genes involved in antioxidant responses. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA 92008, USA) following the manufacturer's protocol. Then, the total RNA was reverse transcribed using the THUNDERBIRD SYBR® qPCR Mix (Toyobo, Osaka, Japan). Quantitative real-time PCR was performed using SYBR Green PCR Master Mix in real-time PCR System (qTower3, Analytik, Jena, Germany). Each reaction (20 µL) contained 4 µL SYBR qPCR Mix, 0.4 µL ROX reference dye, 3 µL cDNA, 0.8 µL each of forward and reverse primers and 11 µL distilled water. PCR was run for 35 cycles of 20 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. *UBCP* was used as reference gene. The specific primers employed for *AOX*, *CSD*, *CAT*, *APX*, *GPOD*,

*DHAR*, *GR*, *RBoh* and *UBCP* can be found in Table S1. The relative gene expression was calculated according to Livak and Schmittgen (2001) [39] and was normalized to the results from the expression of 'Zaojia8424' at 0 HAS.

### *2.9. Statistical Analysis*

Statistical assays were carried out through the analysis of variance (ANOVA) test with SAS software (SAS Institute, Cary, NC, USA) and means were compared by the least significant difference (LSD) test or Tukey's test dependent on the number of groups. Comparisons with $p < 0.05$ were considered significantly different.

## 3. Results

### *3.1. Waterlogging Tolerant Seedlings Exhibited Higher AOX Expression and Better Shoot Growth under Waterlogging*

Here we identified an *AOX* gene (*Cla97C10G192430*) from the watermelon genome and named it as *ClaAOX*. Results from gene expression analysis showed that waterlogging upregulated the expression of *ClaAOX* in both cultivars, with the tolerant cultivar 'YL' showing significantly higher ($p < 0.05$) *ClaAOX* transcript level than the sensitive cultivar 'Zaojia8424' during 72 h of waterlogging stress (Figure 1A). For instance, *ClaAOX* expression at 24 h after stress (HAS) had increased by 201.1% and 510.7% in 'Zaojia8424' and 'YL', respectively, in comparison with the original level.

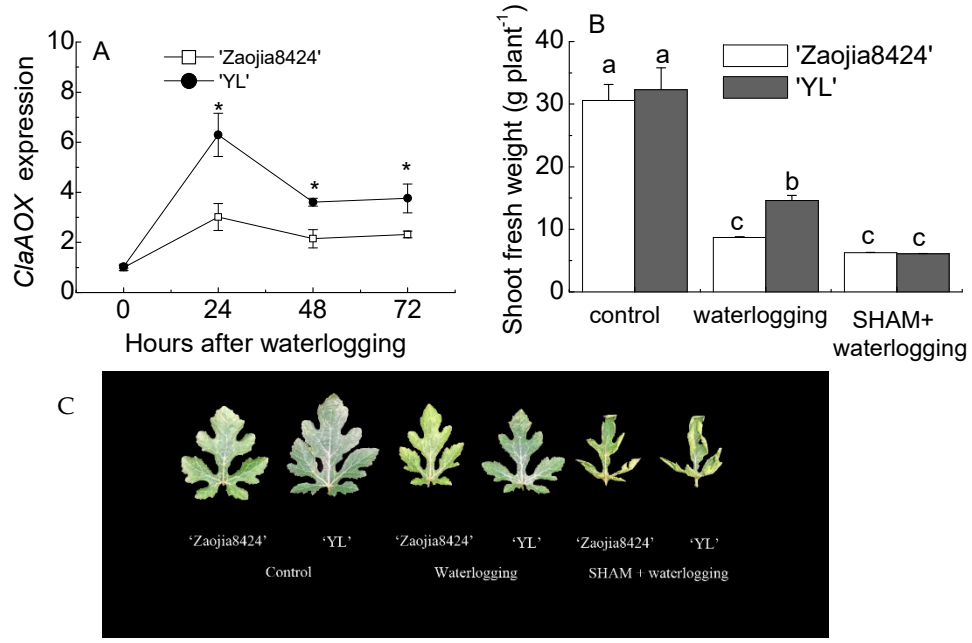


**Figure 1.** Changes of *ClaAOX* expression in watermelon leaves under waterlogging (**A**) and effects of SHAM on plant growth in two cultivars of watermelon ('Zaojia8424' and 'YL') under waterlogging ((**B**) shoot fresh weight. (**C**) Typical leaf phenotype under waterlogging and SHAM). Each histogram represents a mean ± SE of four replicates ($n = 4$). Different letters indicate significant differences between treatments ($p < 0.05$) according to Tukey's test and * indicates a significant difference for 'YL' compared to 'Zaojia8424' at $p < 0.05$ according to the least significant difference (LSD) test.

Shoot fresh weight was reduced by waterlogging, with a more significant ($p < 0.05$) decrease observed in 'Zaojia8424' (Figure 1B). A foliar spray of SHAM, an inhibitor of AOX, exacerbated the reduction of shoot fresh weight in both cultivars. However, there were no significant differences between 'Zaojia8424' and 'YL' in terms of shoot fresh weight under combined treatment of waterlogging and SHAM. Meanwhile, yellowing was observed in 'Zaojia8424' leaves but not in 'YL' leaves, when plants were exposed to waterlogging, and exogenous SHAM accelerated the wilting of both cultivars under waterlogging (Figure 1C).

### *3.2. Waterlogging and AOX Inhibition Reduced the Photosynthesis Performance in Both Cultivars*

Photosynthesis is the main function of a leaf and is easily affected by environmental stresses such as waterlogging. Data in Figure 2 showed that the net photosynthesis rate (Pn), stomatal conductance (Gs) and transpiration rate (E) decreased with the prolonging of waterlogging in both cultivars. However, 'YL' was less affected than 'Zaojia8424' by waterlogging. For instance, Pn of 'YL' was approximately twofold that of 'Zaojia8424' after 6 days of waterlogging. Again, no significant differences were detected in Pn, Gs and E between cultivars in the combination treatment of waterlogging and SHAM (Figure 2A,B,D).

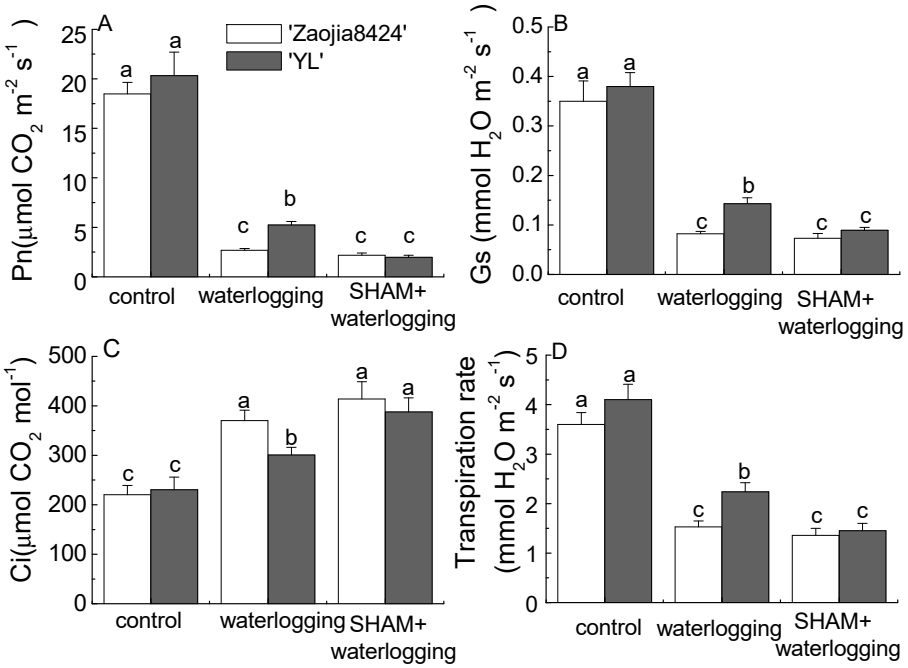


**Figure 2.** Effects of exogenous SHAM on photosynthesis in watermelon leaves under waterlogging treatment. (**A**) Net photosynthesis rate (Pn). (**B**) Stomatal conductance (Gs). (**C**) Intercellular $CO_2$ concentration (Ci). (**D**) Transpiration rate (E). Different letters indicate significant differences according to Tukey's test ($p < 0.05$).

The intercellular $CO_2$ concentration (Ci) was increased by waterlogging in both cultivars (Supplementary Figure S1). A more significant increase was observed in 'Zaojia-8424', compared with that in 'YL'. However, no significant differences in Ci were detected between 'Zaojia8424' and 'YL' in waterlogging plus SHAM treatment (Figure 2C).

We found that Fv/Fm (maximum quantum efficiency of PSII photochemistry) and F'v/F'm (effective quantum use efficiency of PSII in the light-adapted state) was decreased significantly ($p < 0.05$) during waterlogging in 'Zaojia8424', whereas was not affected in 'YL' (Figure 3A,C). Exogenous SHAM decreased Fv/Fm and F'v/F'm in both cultivars under waterlogging (Figure 3A,C).

qP (proportion of open PSII) and $Y_{II}$ (the effective quantum yield of PSII) was decreased by waterlogging in both cultivars, with a more profound reduction detected in 'Zaojia8424' (Figure 3B,D). $Y_{NPQ}$ (the quantum yield of regulated non-photochemical energy loss in PSII) was enhanced by waterlogging in both cultivars, with more significant increase observed in 'Zaojia8424' (Figure 3E). $Y_{NO}$ (the quantum yield of non-regulated energy loss in PSII) rose during waterlogging in 'Zaojia8424' rather than 'YL' (Figure 3F).

Interestingly, there were no significant differences in qP, F'v/F'm, $Y_{II}$, $Y_{NPQ}$, and $Y_{NO}$ between 'Zaojia8424' and 'YL' in the combined treatment of waterlogging and SHAM.

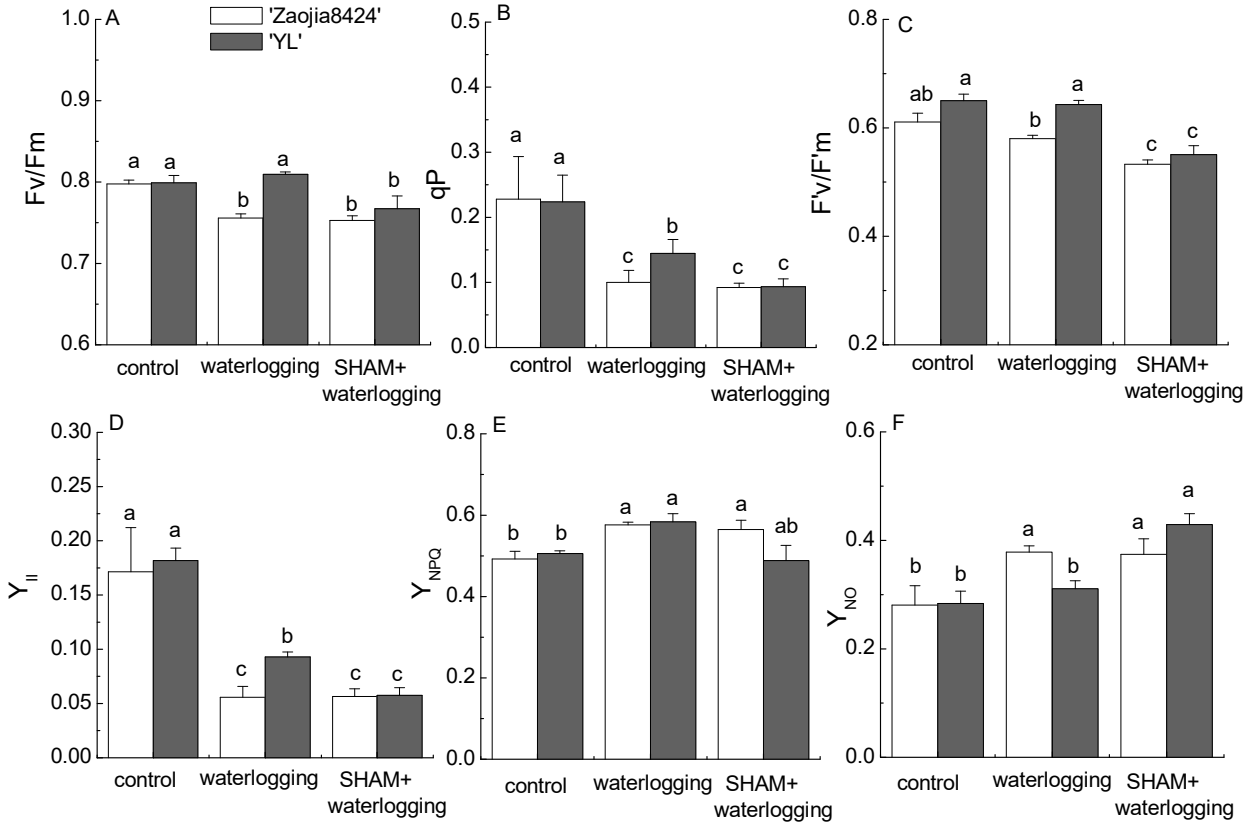


**Figure 3.** Effects of exogenous SHAM on chlorophyll fluorescence parameters in watermelon leaves under waterlogging treatment. (**A**) Fv/Fm (maximum quantum efficiency of PSII photochemistry). (**B**) qP (proportion of open PSII). (**C**) F'v/F'm (effective quantum use efficiency of PSII in the light-adapted state). (**D**) $Y_{II}$ (the effective quantum yield of PSII). (**E**) $Y_{NPQ}$ (the quantum yield of regulated non-photochemical energy loss in PSII). (**F**) $Y_{NO}$ (the quantum yield of non-regulated energy loss in PSII). Different letters indicate significant differences according to Tukey's test ($p < 0.05$).

### *3.3. Inhibition of AOX Activity Exacerbated the Oxidative Damage Induced by Waterlogging*

Membrane permeability and lipid peroxidation are two typical markers of oxidative damage. Results in Figure 4 showed that electrolyte leakage and TBARS content increased significantly ($p < 0.05$) in both cultivars by waterlogging, with a more profound increase in the sensitive cultivar 'Zaojia8424' than the tolerant cultivar 'YL'. In addition, the inhibition of AOX activity by SHAM aggravated the membrane damage, with no significant differences detected between two cultivars.

### *3.4. AOX Inhibition Promoted the ROS Accumulation in Watermelon Leaves during Waterlogging*

The occurrence of oxidative damage is due to the excessive accumulation of ROS. Here, we investigated the effects of waterlogging and AOX inhibition on contents of $O_2^-$ and $H_2O_2$ in watermelon seedlings. Results in Figure 5 showed that $O_2^-$ and $H_2O_2$ contents increased during waterlogging in both cultivars, with less profound increases in $O_2^-$ and $H_2O_2$ content observed in the tolerant cultivar 'YL'. For instance, $O_2^-$ content was increased by about 3 fold and 1 fold in 'Zaojia8424' and 'YL', respectively, after 9 days of waterlogging treatment. In addition, the inhibition of AOX activity by SHAM promoted further increases in $O_2^-$ and $H_2O_2$ content in both cultivars, with no significant differences between cultivars (Figure 5A,B).

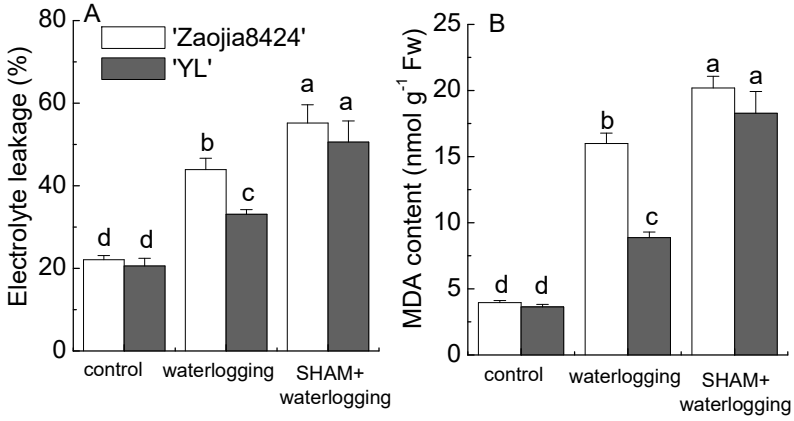


**Figure 4.** Effects of exogenous SHAM on oxidative damage in watermelon leaves under waterlogging treatment. (**A**) Electrolyte leakage. (**B**) TBARS content. Different letters indicate significant differences according to Tukey's test ($p < 0.05$).

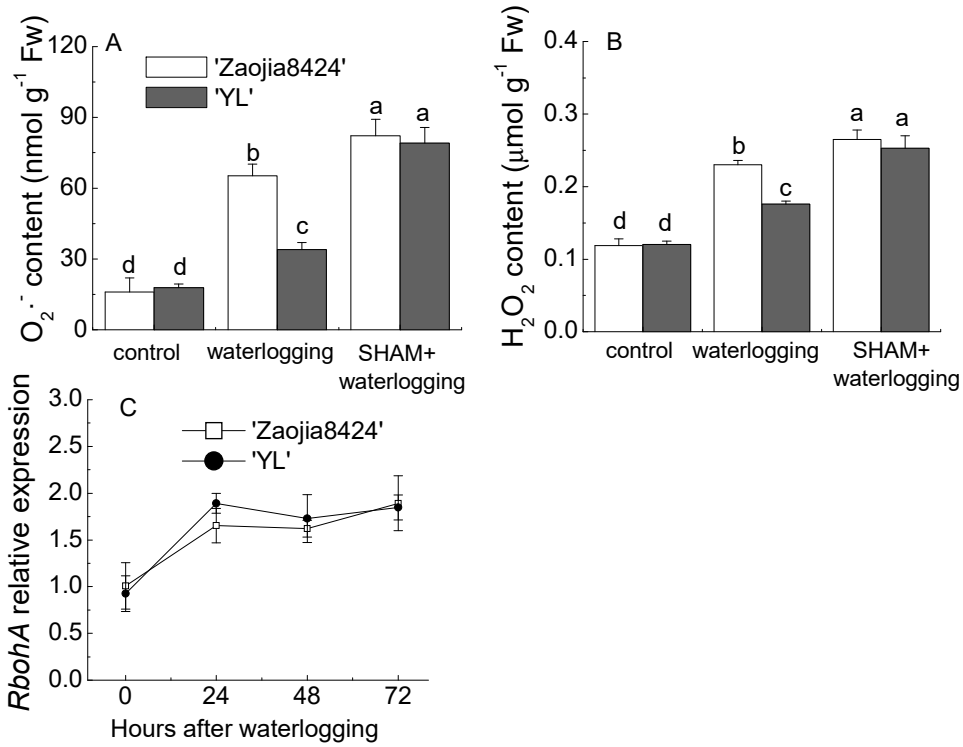


**Figure 5.** Effects of exogenous SHAM on ROS content in watermelon leaves under waterlogging treatment ((**A**), $O_2^-$ content. (**B**), $H_2O_2$ content) and changes of *RbohA* expression in watermelon leaves during waterlogging (**C**). Different letters indicate significant differences according to Tukey's test and * indicates a significant difference for 'YL' compared to 'Zaojia8424' at $p < 0.05$ ($n = 4$) according to LSD test.

Superoxide are generated by the reduction of $O_2$ by NADPH oxidase which is coded by the *Rboh* gene. Results from qPCR analysis showed that waterlogging upregulated the transcript levels of *RbohA* in both cultivars. However, no significant differences of *RbohA* expression were detected between 'Zaojia8424' and 'YL' (Figure 5C).

### *3.5. Responses of Antioxidant Enzymes to Waterlogging in Leaves*

Antioxidant enzymes play crucial roles in the balance of ROS generation and scavenging. Here, we examined the activities of antioxidant enzymes under waterlogging in seedlings of both cultivars. Generally, the activity of SOD, CAT, APX, GPOD, DHAR

and GR showed upward trends under waterlogging (Figure 6A–F). There were no significant differences in the activities of SOD, DHAR, and GR between 'Zaojia8424' and 'YL' (Figure 6A,E,F). However, the tolerant cultivar 'YL' exhibited a significantly ($p < 0.05$) higher CAT activity at 3 DAS and a significantly ($p < 0.05$) higher APX activity at 0 and 3 DAS (Figure 6B,D). Meanwhile, the increase of GPOD activity by waterlogging was more evident in 'Zaojia8424' than in 'YL' (Figure 6C).

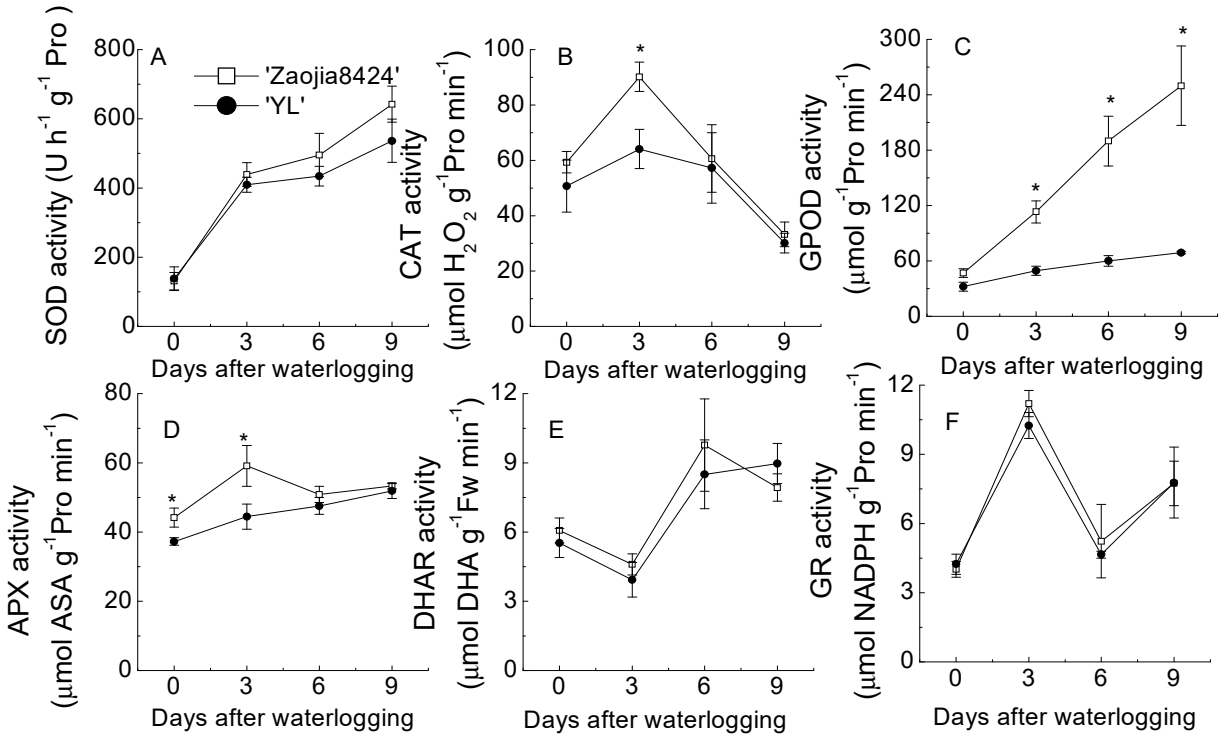


**Figure 6.** Effects of waterlogging on activities of antioxidant enzymes in watermelon. (**A**) SOD. (**B**) CAT. (**C**) GPOD. (**D**) APX. (**E**) DHAR. (**F**) GR. * indicates a significant difference for 'YL' compared to 'Zaojia8424' at $p < 0.05$ according to LSD test.

Furthermore, we investigated the expressions of antioxidant genes. Generally, waterlogging induced the expression of *CSD*, *CAT*, *APX*, *GPOD*, *DHAR*, and *GR* in watermelon leaves (Figure 7A–F). There were no significant differences detected in *CSD*, *DHAR* and *GR* transcript levels between cultivars during 72 h of waterlogging (Figure 7A,E,F). The expressions of *CAT* and *APX* were significantly ($p < 0.05$) higher in the tolerant cultivar 'YL' than the sensitive cultivar 'Zaojia8424' (Figure 6B,D). The relative expression of *GPOD* was significantly ($p < 0.05$) higher in 'Zaojia8424' than in 'YL' during waterlogging (Figure 6C).

### *3.6. AOX Inhibition Hampered the Homeostasis of Glutathione and Ascorbate in Watermelon Seedlings under Waterlogging*

Antioxidant substances are considered to be an important player during ROS scavenging. In the present study, we found that waterlogging decreased the contents of glutathione (GSH), oxidized glutathione (GSSG) and total glutathione in the seedlings of both cultivars (Figure 8A–C). Interestingly, the GSH to GSSG ratio increased significantly ($p < 0.05$) in the tolerant cultivar 'YL' during the second half of the 9 days of waterlogging, which showed a significantly higher level than the sensitive cultivar, 'Zaojia8424' (Figure 8D). The inhibition of AOX dramatically reduced the GSH/GSSG ratio with no significant differences detected between cultivars (Figure 8E).

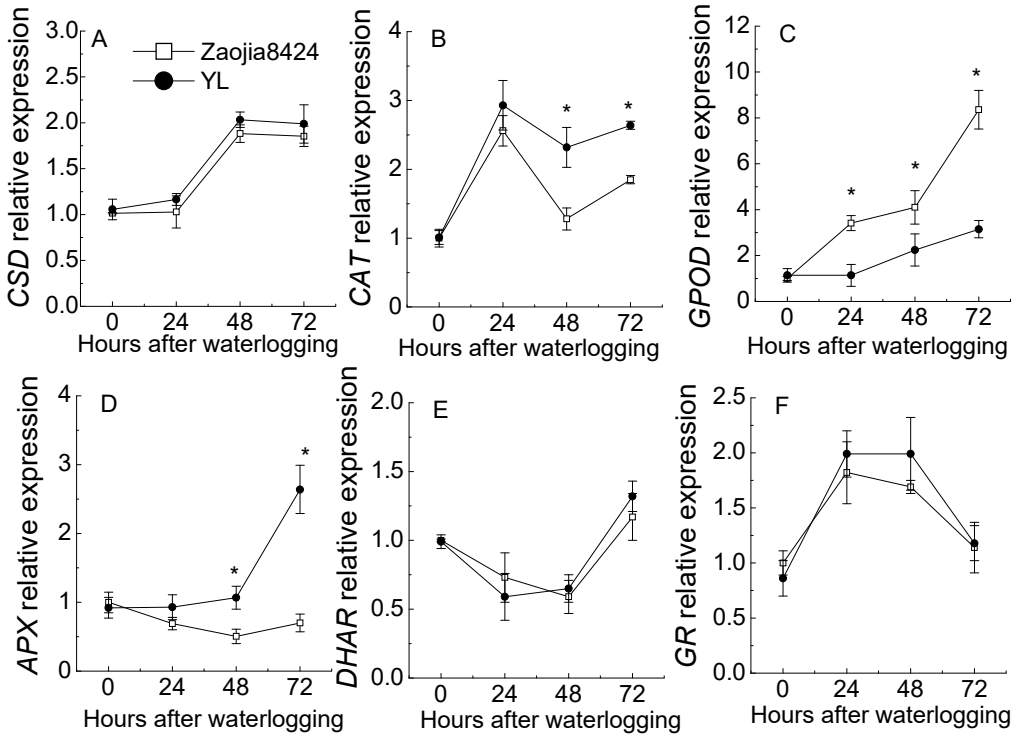


**Figure 7.** Effects of waterlogging on expressions of antioxidant genes in watermelon. (**A**) *CSD*. (**B**) *CAT*. (**C**) *GPOD*. (**D**) *APX*. (**E**) *DHAR*. (**F**) *GR*. * indicates a significant difference for 'YL' compared to 'Zaojia8424' at $p < 0.05$ according to LSD test.

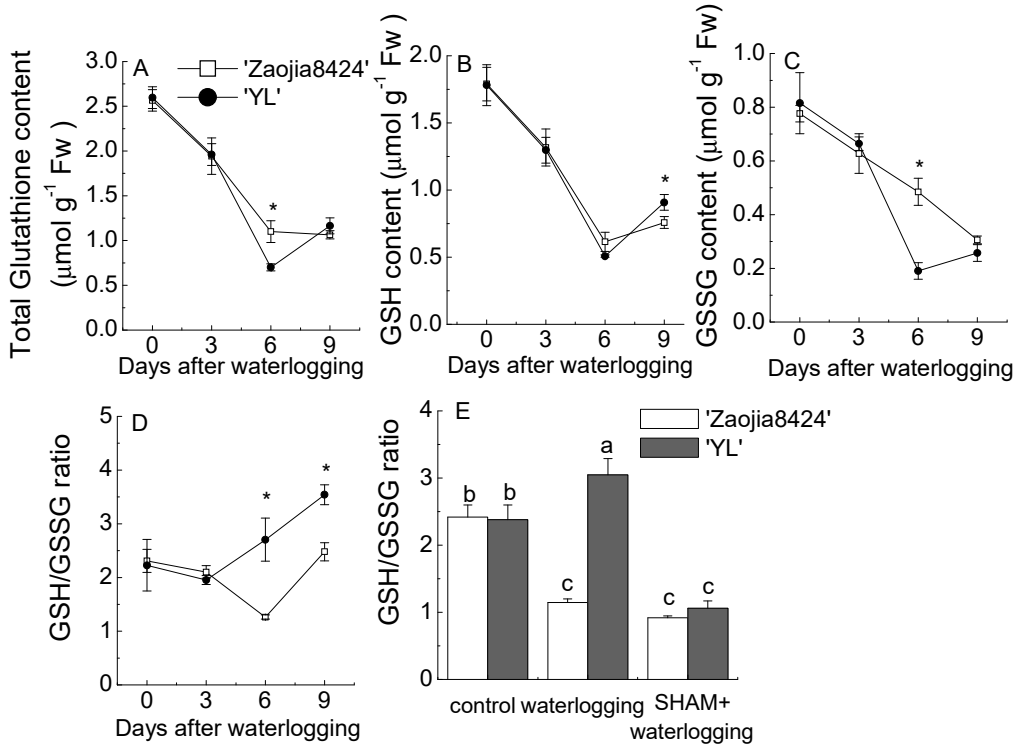


**Figure 8.** Changes of glutathione contents in watermelon leaves during waterlogging and effects of SHAM on GSH/GSSG ratio under waterlogging. (**A**) Total glutathione content under waterlogging. (**B**) GSH content under waterlogging. (**C**) GSSG content under waterlogging. (**D**) GSH/GSSG ratio during waterlogging. (**E**) Effects of SHAM on GSH/GSSG ratio under waterlogging. Different letters indicate significant differences according to Tukey's test and * indicates a significant difference for 'YL' compared to 'Zaojia8424' at $p < 0.05$ ($n = 4$) according to LSD test.

The ascorbate acid (ASC) content in watermelon leaves during waterlogging was also investigated. Results showed that the ASC content increased initially, peaked at 6 DAS, and then declined (Figure 9A). The ASC content was significant higher ($p < 0.05$) in 'YL' than 'Zaojia8424' during 9 days of waterlogging treatment. For instance, ASC content was increased by 84.5% and 184.1% in 'Zaojia8424' and 'YL', respectively, compared with the original level. Additionally, the differences in ASC content between 'Zaojia8424' and 'YL' were diminished by AOX inhibition (Figure 9B).

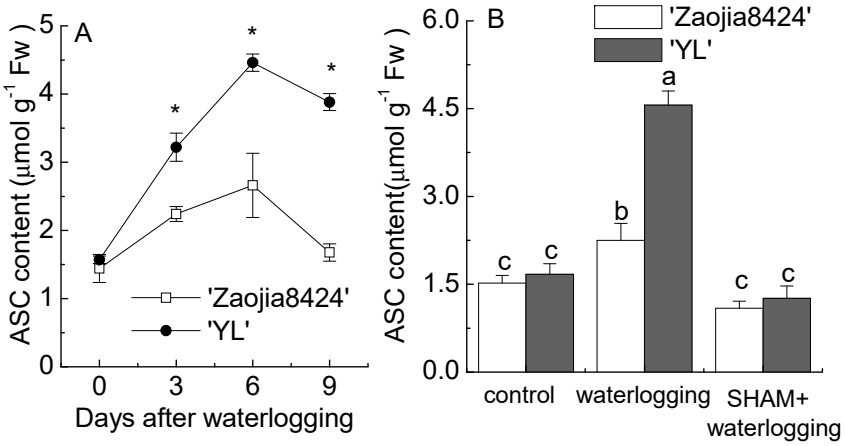


**Figure 9.** Changes of ASC contents in watermelon leaves during waterlogging (**A**) and effects of SHAM on ASC contents in watermelon leaves under waterlogging (**B**). Different letters indicate significant differences according to Tukey's test and * indicates a significant difference for 'YL' compared to 'Zaojia8424' at $p < 0.05$ ($n = 4$) according to LSD test.

## 4. Discussion

Alternative oxidase (AOX) has two subfamilies, AOX1 and AOX2. In general, AOX1 is induced by stresses, whereas AOX2 is thought to be developmentally expressed [40]. In the present study, we found that expression of *ClaAOX* was upregulated sharply in watermelon leaves of both cultivars. In our previous study, we have shown that activity of AOX was induced by waterlogging in watermelon roots [27]. These results indicated that *ClaAOX* might be waterlogging induced. Phylogenetic analysis suggested that *ClaAOX* was grouped in the *AOX2* subfamily (Figure S2). Recently, some isoforms of AOX2 were experimentally validated to be responsive to abiotic stresses. For instance, *CaAOX2A* and *CaAOX2D* were upregulated by drought in legumes [41]. Moreover, tolerant cultivar "YL" displayed the higher level of *ClaAOX* than sensitive cultivar 'Zaojia8424' in leaves during waterlogging stress (Figure 1A).

Photosynthesis inhibition is a typical phenomenon caused by waterlogging. In the present study, the waterlogging tolerant cultivar 'YL' exhibited higher Pn than the sensitive cultivar 'Zaojia8424' during waterlogging treatment (Figure 1A), which is consistent with previous results in sorghum [10], avocado (*Persea americana*) [42] and *Actinidia valvata* [43]. The tolerant cultivar of watermelon 'YL' displayed higher expression of *ClaAOX* compared with the sensitive cultivar 'Zaojia8424' under waterlogging (Figure 1A), and the inhibition of AOX activity diminished with the better performance of Pn in 'YL' under waterlogging (Figure 2A). Thus, it is suggested that the maintenance of photosynthesis under waterlogging in 'YL' was likely due, at least in part, to the AOX pathway. To the best of our knowledge, this is the first report about the involvement of AOX in optimizing photosynthesis under waterlogging. Similarly, it has been reported that inhibition of AOX activity by an exogenous inhibitor or AOX gene silencing reduced Pn when plants were exposed to drought [25], low light [44], low temperature and osmotic stress [45], and heat and high light [46] as well. However, overexpression of AOX increased Pn under drought [26]. Moreover, a single SHAM treatment did not affect the Pn in *Pisum sativum*

under control conditions [45]. Thus, AOX might play a vital role in the maintenance of plant photosynthesis under abiotic stresses.

Reduced Pn by waterlogging is considered to be associated with the damage of the photosynthetic apparatus [47]. Fv/Fm, an indicator of the photoinhibition of PSII [48], decreased to about 0.75 by waterlogging in 'Zaojia8424' (Figure 4A), suggesting the occurrence of photoinhibition. Similar phenomena were observed in *Arabidopsis* [49], sorghum [10], tomato [11] and Jerusalem artichoke (*Helianthus tuberosus*) [12], However, while Fv/Fm was hardly affected in 'YL' by waterlogging, it was decreased sharply by AOX inhibition, suggesting that AOX is likely involved in the maintenance of PSII function under waterlogging. Similarly, Fv/Fm was decreased by severe drought in AOX knockdown plants [26].

The energy absorbed by PSII can be divided into three partitions, the quantum yield of photosynthesis ($Y_{II}$), the quantum yield of regulated non-photochemical energy loss ($Y_{NPQ}$) and the quantum yield of non-regulated energy loss ($Y_{NO}$), according Kramer et al. [50]. Here we found that $Y_{II}$ was reduced by waterlogging in both cultivars (Figure 4B), which is consistent with previous studies [10,49]. Moreover, the decrease of $Y_{II}$ resulted from the decline of qP and Fv'/Fm', since $Y_{II}$ is a product of qP and Fv'/Fm' [51]. Besides, 'YL' exhibited significantly ($p < 0.05$) higher $Y_{II}$ than 'Zaojia8424', but this effect was reduced by AOX inhibition (Figure 4B). Decreased $Y_{II}$ by SHAM implies lower electron transport to carbon fixation [21], which was correlated to the reduced Pn (Figure 2A).

The increase of $Y_{NO}$ indicates the insufficiency of photochemical energy conversion and photo-protective regulatory mechanism, and the excess electron can be used to form singlet oxygen ($^1O_2$) in the chloroplast [52]. $Y_{NO}$ was increased by SHAM in 'YL', suggesting inhibition of AOX could result in more ROS generation in the chloroplast. Since AOX may act as an additional electron sink for photo-generated reductant [25], the inhibition of AOX activity would weaken the sink, and thus impair the photosynthetic capacity.

Oxidative damage occurred when plants are exposed to long term waterlogging [53]. Here, watermelon seedlings suffered from oxidative stress, since increased $O_2^-$ and $H_2O_2$ content and consequently higher TBARS content and electrolyte leakage were detected in both cultivars after waterlogging treatment (Figures 4A,B and 5A,B). The tolerant cultivar 'YL', with higher transcript levels of *ClaAOX*, displayed less severe oxidative damage, compared with the sensitive cultivar 'Zaojia8424'. However, this alleviation of oxidative damage in 'YL' was reduced by SHAM. Similar phenomena were observed in the roots of these two cultivars in our previous study [27]. Other studies have reported that overexpression of *AOX* decreased TBARS contents of tobacco under hypoxia, and knockdown of AOX increased TBARS contents [24]. In addition, *AOX* knockdown plants or mutants had increased contents of $H_2O_2$ when plants were exposed to drought stress [54], arsenic stress [55], nitrogen deficiency and iron deficiency [46]. Overexpression of *AOX* decreased the $H_2O_2$ content under salt stress [56] and drought stress [54]. Thus, AOX might be essential in maintaining plant ROS balance under abiotic stresses.

AOX may ameliorate oxidative stress via two pathways, modulating the production of ROS and inducing the antioxidant capacity. For instance, Maxwell et al. [22] reported that *AOX* antisense tobacco cells exhibited higher levels of ROS, whereas *AOX* overexpression cells displayed lower ROS abundance compared with wild-type cells. They further found that *AOX* overexpression cells had lower expression of genes encoding antioxidant enzymes, such as *SODA*, *SODB*, *CAT* and *GPX* [22]. Thus, AOX has been considered as a key a player in reducing mitochondrial ROS formation in plant cells.

However, the cells they used lacked developed chloroplasts [22]. The responses of the antioxidant system might be different from tissues containing developed chloroplasts. For instance, we found that expression of *CSD*, *CAT*, *APX*, *DHAR* and *GR* were upregulated by waterlogging in watermelon leaves (Figure 7), whereas the expressions of the corresponding genes were downregulated sharply in watermelon roots in our previous study using the same cultivars [27]. These different antioxidant responses to hypoxia between shoot and roots were observed in barley [18] and gray poplar (*Populus* × *canescens*) [3]. Skutnik

and Rychter [18] further suggested that AOX plays a major role in ROS scavenging in roots, whereas antioxidant substances including glutathione and ascorbate are of great importance in the ROS detoxification in leaves.

Thus, the responses of the glutathione ascorbate cycle in watermelon leaves under waterlogging were further examined, where we found that GSH, GSSG and total glutathione contents were decreased by waterlogging (Figure 8A–C). As GSH regenerates ascorbate by reducing DHA through the glutathione-ascorbate cycle [57], the reduced GSH could be attributed to the increase of ASC content under waterlogging (Figure 9A). In addition, the tolerant cultivar 'YL', with a higher expression level of *ClaAOX*, exhibited higher ASC contents compared with the sensitive cultivar 'Zaojia8424'. However, this effect was abolished by AOX inhibition, implying the regulation of the glutathione-ascorbate cycle by AOX. To the best of our knowledge, this is the first report of the involvement of the glutathione-ascorbate cycle in antioxidant responses mediated by AOX under waterlogging. Since ASC is the substrate of APX, a higher content of ASC in 'YL' resulted in enhanced activity of APX and transcript level of *APX* (Figures 6D and 7D), which is beneficial for ROS scavenging.

Interestingly, the GSH/GSSG ratio was increased in the tolerant cultivar 'YL', but not in the sensitive cultivar 'Zaojia8424' (Figure 8D). However, the increased GSH/GSSG ratio in 'YL' was hampered by AOX inhibition (Figure 8E). Similarly, it has been reported that inhibition of AOX by SHAM or *AOX* gene knockdown decreased the GSH/GSSG ratio when plants were exposed to As stress [55] and low temperature [48]. The GSH/GSSG ratio, presenting the redox state of cells, has long been recognized as a signaling cascade [58]. Since CAT is sensitive to glutathione redox [59], the higher GSH/GSSG ratio in 'YL' resulted in the higher activity of CAT and increased expression of *CAT* compared with 'Zaojia8424' (Figures 6B and 7B). Conversely, GPOD is considered to be a biomarker of oxidative stress [60], and thus the lower GSH/GSSG ratio in 'Zaojia8424' would lead to higher activity of GPOD and increased expression of GPOD compared with 'YL' (Figures 6C and 7C).

## 5. Conclusions

Waterlogging decreased leaf Pn and $Y_{II}$ in watermelon. The photosynthesis was less affected in the waterlogging tolerant cultivar 'YL' than the sensitive cultivar 'Zaojia8424'. Moreover, waterlogging induced photoinhibition in 'Zaojia8424' but not in 'YL'. Meanwhile, waterlogging promoted the accumulation of ROS, and triggered oxidative damage. The waterlogging tolerant cultivar 'YL' suffered from less severe oxidative damage due to increased contents of ascorbate, a higher ratio of GSH/GSSG, a higher activity of APX and CAT, and enhanced transcript levels of *CAT* and *APX* compared to the sensitive cultivar 'Zaojia8424'. However, no significant differences were detected in photosynthesis and antioxidant performances between cultivars when AOX activity was inhibited by SHAM application. Our results address the involvement of photosynthetic electronic transfer and glutathione-ascorbate cycle in AOX manipulated waterlogging tolerance in watermelon seedlings.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/horticulturae7060130/s1, Figure S1: Phylogenetic analysis of the alternative oxidase (AOX) amino acid sequences from watermelon genome; Table S1: Primer sequences used in the qRT-PCR experiments.

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