agronomy

MDPI

Article

Drought Stress and High Temperature Affect the Antioxidant Metabolism of Cotton (*Gossypium hirsutum* L.) Anthers and Reduce Pollen Fertility

Jipeng Zhang [1,2], Mengdie Cheng [1], Nan Cao [3], Yongjun Li [4], Shanshan Wang [1], Zhiguo Zhou [1] and Wei Hu [1,*]

[1] College of Agriculture, Nanjing Agricultural University, Nanjing 210095, China; jpzhang03@163.com (J.Z.); chengmengdie@stu.njau.edu.cn (M.C.); yjshan@njau.edu.cn (S.W.); giscott@njau.edu.cn (Z.Z.)
[2] Economic Crop Research Institute, Xinjiang Academy of Agricultural Sciences, Urumqi 830091, China
[3] College of Agriculture, Tarim University, Alar 843300, China; caonan0209@163.com
[4] Soil Improvement Test Station of the Eighth Artillery Station of the Corps, Shihezi 832066, China; pttrglsyz@sina.com
* Correspondence: weihu@njau.edu.cn

Abstract: Both drought and high temperature can influence the antioxidant metabolism of crop reproductive organs in different ways, affecting the fertility of reproductive organs and yield formation. However, the combined effects of drought stress and high temperature on the crop reproductive physiology have not yet been widely considered. In order to broaden our understanding of this mechanism of influence, a pond experiment was conducted using a cotton variety Yuzaomian 9110 divided into four treatment groups: control (CK), drought stress (DS), high temperature (HT), and drought stress coupled with high temperature (DS+HT). Results showed a significant negative correlation between pollen viability and superoxide anion (O_2^-) content, as well as hydrogen peroxide (H_2O_2). Compared with CK, DS did not alter O_2^- content in anthers, but HT treatment resulted in higher anther O_2^-. Compared with single-stress groups, DS+HT further promoted the formation of O_2^- in anthers, leading to more malondialdehyde in anthers. Moreover, a higher H_2O_2 content in anthers was found in DS and HT than in CK. DS+HT did not show altered H_2O_2 content relative to HT treatment, although its H_2O_2 was higher than in DS. Further analyses of the antioxidant enzyme system showed that DS had no significant effect on superoxide dismutase gene (*GhCu/ZnSOD*) expression, but HT and DS+HT significantly downregulated its expression. The expression of *GhCu/ZnSOD* was lower under DS+HT than HT, which might be why O_2^- content was not altered under DS treatment compared with CK and was higher in DS+HT than HT. DS and HT significantly downregulated the expression of the peroxidase gene (*GhPOD*) and catalase gene (*GhCAT*), which should be the main reason for the larger accumulation of H_2O_2 under drought stress and high-temperature conditions. Compared with single-stress groups, DS+HT had lower expression of *GhCAT*, resulting in a larger H_2O_2 content. Regarding the ascorbic acid–glutathione (AsA–GSH) cycle, DS and HT significantly downregulated the expression of monodehydroascorbate reductase gene (*GhMDHAR*) to hinder the production of AsA and upregulated the expression of ascorbate oxidase gene (*GhAAO*) to promote the oxidation of AsA, which was theoretically detrimental to AsA accumulation. However, HT downregulated the expression of the ascorbate peroxidase gene (*GhAPX*), hindering the reduction of H_2O_2 by AsA, which was the reason for AsA and H_2O_2 accumulation. Moreover, DS also significantly upregulated the expression of the dehydroascorbate reductase gene (*GhDHAR2*) to enhance the reduction of dehydroascorbate to form AsA, leading to a higher content of AsA under DS than HT. The combined stress significantly downregulated the expression of *GhAAO* to inhibit the oxidation of AsA but significantly upregulated the expression of *GhMDHAR* and *GhDHAR2*, promoting the AsA production, and downregulated the expression of *GhAPX*, hindering the reduction of H_2O_2 by AsA. All these resulted in increased AsA content under combined stresses. In addition, HT significantly downregulated the glutathione reductase gene (*GhGR*) expression, hindering the reduction of oxidized glutathione (GSSG), which led to the reduction of GSH. However, DS and DS+HT significantly downregulated the glutathione peroxidase gene (*GhGPX*) expression, resulting in the accumulation of GSH. Overall, compared with single-stress treatments, the effects of DS+HT on cotton pollen fertility

Citation: Zhang, J.; Cheng, M.; Cao, N.; Li, Y.; Wang, S.; Zhou, Z.; Hu, W. Drought Stress and High Temperature Affect the Antioxidant Metabolism of Cotton (*Gossypium hirsutum* L.) Anthers and Reduce Pollen Fertility. *Agronomy* **2023**, *13*, 2550. https://doi.org/10.3390/agronomy13102550

Academic Editor: Carla Gentile

Received: 4 September 2023
Revised: 22 September 2023
Accepted: 30 September 2023
Published: 3 October 2023

and peroxide accumulation were more significant. The effects of DS+HT on the antioxidant enzyme system were mainly caused by high temperature, while the mechanism of abnormal accumulation of AsA and GSH caused by DS+HT was different from those of single-stress groups.

Keywords: cotton (*Gossypium hirsutum* L.); drought stress; high temperature; combined stress; pollen viability; antioxidant metabolism

1. Introduction

Global climate change leads to frequent extreme weather, especially drought stress and high temperature, which seriously affect the growth and development of crops and bring new challenges to agricultural production. It was estimated that by the end of this century, the global average temperature will increase by another 1.8–4.0 °C [1,2]. The high temperature seriously affects the physiological and metabolic activities of crops, especially reproductive development, and thus affects the normal fertilization of plants, as one of the main factors for crop yield loss [3,4]. At the same time, the deterioration of the global climate has caused more than one-third of the world's cultivated land to face the problem of insufficient water supply [5]. Drought is also the main abiotic stress factor that limits crop growth, especially drought occurring at the flowering and boll-forming stage, leading to a significant decrease in pollen fertility, which is not conducive to fertilization and fruiting [6], therefore reducing the crop yield [7,8]. Moreover, heat and drought stresses usually occur simultaneously under field conditions, and some studies have explored the effects of combined stress of drought and heat temperature on the physiological and metabolic activities in cotton (*Gossypium hirsutum* L.) leaves, fibers, and seeds [9,10]. However, there are few studies on the effects of the combined stress of drought and high temperature on the physiological and metabolic activities of cotton male organs and their relationship with pollen fertility.

Abiotic stresses, including drought, high temperature, salinization, etc., can destroy the metabolic balance of reactive oxygen species (ROS) and result in ROS accumulation in plant cells [11]. Excessive ROS are among the main causes of male sterility in wheat (*Triticum aestivum* L.) [12], maize (*Zea mays* L.) [13], and rice (*Oryza sativa* L.) [14]. The antioxidant systems responsible for scavenging ROS in plants include enzymatic and nonenzymatic systems. Superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) are three classical active oxygen scavenging enzymes. SOD can disproportionate O_2^- into H_2O_2 and O_2, while CAT and POD are responsible for removing the H_2O_2 toxicity. The former directly converts H_2O_2 into nontoxic H_2O and O_2, while the latter promotes the interaction of H_2O_2 and the substrate to form H_2O [15,16]. The nonenzymatic system for scavenging ROS consists of a class of small molecular antioxidants, including ascorbic acid (AsA), glutathione (GSH), proline, carotenoids, flavonoids, tocopherols, and others. AsA can effectively eliminate O_2^- and ·OH and is also the quenching agent of $^1O_2$ [17]. GSH can scavenge ·OH and $^1O_2$ directly and protect the mercaptan groups of other enzymes [18]. AsA–GSH cycle is an important nonenzymatic antioxidant defense system in plants. The redox process of AsA and GSH involves a variety of enzymes. Ascorbate peroxidase (APX) catalyzes the reaction of AsA and H_2O_2, which is highly specific to AsA. Monodehydroascorbate reductase (MDHAR) catalyzes the reduction of monodehydroascorbic acid (MDHA) to form AsA to complete the redox cycle of AsA. The transformation between GSH and oxidized glutathione (GSSG) mainly depends on glutathione peroxidase (GPX) and glutathione reductase (GR). In addition, dehydroascorbate reductase (DHAR) can catalyze the reduction of dehydroascorbic acid (DHA) and oxidize GSH to GSSG [19].

Drought stress can cause the imbalance of antioxidant metabolism of crops to increase ROS such as O_2^-, ·OH, and H_2O_2 contents [20–22], and the effects of drought on antioxidant metabolism depend on the crop variety and the severity of drought. For example, Rong et al. [23] have confirmed that mild-drought-stressed tobacco (*Nicotiana tabacum* L.)

plants have higher antioxidant enzyme activities, but Fu et al. [24] found that severe drought stress decreased the activities of SOD and CAT in anthers of two rice cultivars. Selote et al. [25] reported that drought significantly influenced the activities of SOD, POD, and CAT in spikelets of the two wheat varieties. The variation in amplitude of these enzyme activities was higher for drought-resistant varieties than drought-sensitive varieties. Regarding the AsA–GSH cycle, it was found that under mild drought conditions, the activities of enzymes (APX, DHAR, MDHAR, and GR) related to the AsA–GSH cycle in apple (*Malus pumila* Mill.) leaves increased to improve the antioxidant capacity; however, under severe conditions, the activities of these enzymes of AsA–GSH circulation system decreased [26]. Under drought stress, the contents of AsA and GSH in spikelets of drought-resistant rice cultivars were significantly increased, while the contents of AsA and GSH in spikelets of drought-sensitive rice cultivars were significantly lower than that in well-watered plants [25]. High temperatures also disturb the antioxidant metabolism in plants [27]. Under high-temperature stress, ROS production rate and content increased during pollen development [28,29], leading to cell death in anthers and pollen abortion [30,31]. It was found that high temperatures eventually lead to a decrease in the activities of antioxidant enzymes (SOD, POD, and CAT) related to antioxidant metabolism in reproductive organs [32]. The contents of AsA and GSH in leaves of tall fescue and perennial ryegrass decreased under high-temperature stress [33]. The activities of enzymes (APX, DHAR, MDHAR, and GR) related to the AsA–GSH cycle were also affected by high temperatures. Zou et al. [34] found that high temperature increased the activities of MDHAR, DHAR, and GR in Chinese cabbage (*Brassica campestris* L.) seedlings and significantly increased the content of AsA and GSH, which was beneficial to the AsA–GSH cycle. A similar study found that the activities of APX, MDHAR, and GR were significantly increased in pears (*Pyrus communis* L.) exposed to short-term high-temperature treatment but decreased significantly with the extension of treatment time [35]. The effects of the combined stresses depend on the intensity and condition of every single stress and cannot be deduced from the plants' responses to individual stresses [36]. Although the effects of drought stress and high temperature on the antioxidant metabolism in crop male reproductive organs and the relationship with pollen fertility have been studied individually, their effects, when combined, have received less attention.

Therefore, exploring the effects of the combined stress of drought and high temperature on the antioxidant metabolism in cotton male reproductive organs and its relationship with pollen fertility can not only improve the mechanism of abiotic stress in cotton reproductive growth but also provide a theoretical basis and technical support for the stress-resistant cultivation of cotton production conducive to coping with the frequent stress caused by climate change.

2. Materials and Methods

2.1. Experimental Design

This experiment was carried out in the Pailou Experimental Station of Nanjing Agricultural University from 2019 to 2020. A total of 6 ponds (4 m length × 4 m width × 1.5 m height and filled with yellow-brown loam soil) were placed under a half-open warming greenhouse to effectively eliminate the effect of precipitation in this experiment. The upland cotton variety Yuzaomian 9110 was planted in nutrition bowls on May 6 and May 12 in 2019 and 2020, respectively. At the three-true-leaves stage, the cotton seedlings were transplanted into the ponds, with line spacing of 75 cm and interplant spacing of 15 cm. In this experiment, the split zone design with temperature treatment as the main plot and water treatment as the subplot was performed in three replications. Before performing the different treatments, all ponds were kept at soil-relative water content (SRWC) of (75 ± 5)% and ambient temperature. Different soil-moisture treatments began at the early stage of flowering. When the soil moisture reached the predetermined level, the temperature treatments were established (Figure S1) according to our previous report [10], using a Temperature Control System (OTC, Southeast Co., Ltd., Ningbo, China) to raise the

ambient temperature 2–3 °C. The mean daily temperatures in the ambient and the elevated temperature areas in 2019 and 2020 are shown in Table S1. The mean daily temperature in the ambient temperature area in 2019 and 2020 were 31.7 °C and 31.2 °C, respectively, lower than the upper limit of 32 °C of the optimum temperature range for cotton development [37]. The average daily temperature in the elevated temperature area in 2019 and 2020 were 34.2 °C and 33.8 °C, respectively, which were higher than the upper limit of the optimum temperature range for cotton development. Hence, the plants in the elevated temperature area were under high temperature stress. On the seventh day after the high-temperature treatment was established, the white flowers blooming were collected as the study objects. Finally, the four treatments were as follows: (1) control (CK), SRWC (75 ± 5)% + ambient temperature, (2) drought stress (DS), SRWC (45 ± 5)% + ambient temperature, (3) high temperature (HT), SRWC (75 ± 5)% + ambient temperature plus 2–3 °C, and (4) drought stress coupled with high temperature (DS+HT), SRWC (45 ± 5)% + ambient temperature plus 2–3 °C.

2.2. Data Collection

2.2.1. Midday Leaf Water Potential (Ψ_{MD})

The midday leaf water potential of cotton was measured from 18 July to 2 August and from 23 July to 7 August in 2019 and 2020, respectively. The Ψ_{MD} of functional leaves of cotton main stem was measured every 3 days. Three cotton leaves were taken from each treatment from 12:00 to 13:00, and the leaf water potential was measured immediately with a 3005 series pressure chamber from Soilmoisture Equipment Company (Burlington, ON, Canada).

2.2.2. Pollen Viability

In 2019, pollen viability was determined by I_2-KI staining [38]. The pollen was shaken onto a glass slide, and 1–2 drops of I_2-KI were then added. The glass slide was incubated at 37 °C for 5 min, and blue-black pollen grains were considered viable. In 2020, pollen viability was determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining [39]. The pollen was shaken into a Petri dish, and 0.5% TTC solution was added. The pollen grains were observed and photographed after 20 min. Red or pink pollen grains were considered viable, and white pollen grains were abortive. The results of pollen viability measurements in 2019 and 2020 are shown in Figure S2 and Table S2.

2.2.3. Anther Peroxide Content

The O_2^- content test kit (No. A052-1-1) provided by Nanjing Jiancheng Bioengineering Institute was used for anther O_2^- extraction and determination. Fresh anther tissue (0.2 g) was ground in a chilled grinder, and then 3.0 mL of 0.1 M PBS extract solution was added to make a homogenate. The mixture was then centrifuged for 10 min at 10,000× *g* at 4 °C, and the absorbance of the supernatant was determined at 550 nm using an ultraviolet spectrophotometer.

The H_2O_2 content test kit (No. A064-1-1) provided by Nanjing Jiancheng Bioengineering Institute was used for anther H_2O_2 extraction and determination. The preparation method of anther H_2O_2 extract was the same as that of the O_2^- extract. Then, the absorbance of the supernatant was determined at 405 nm using an ultraviolet spectrophotometer.

Malondialdehyde (MDA) content was assayed according to Hu et al. [40] with slight modifications. Fresh anther tissue (0.2 g) was ground in a chilled grinder with 5 mL Tris-HCl buffer and centrifuged at 4 °C 12,000× *g* for 10 min. The supernatant was collected, and 0.5 mL supernatant was heated at 100 °C with 2 mL TCA–TBA mixture [containing 10% trichloroacetic acid (TCA) and 0.6% thiobarbituric acid (TBA)] for 20 min. Then, the mixture was centrifuged for 10 min at 10,000× *g*. The absorbance values of the supernatant were determined at the wavelengths of 450, 532, and 600 nm, respectively. The concentration of MDA was calculated as $6.45 \times (OD_{532} - OD_{600}) - 0.56 \times OD_{450}$.

2.2.4. Peroxides Labeling

The male reproductive organs (anthers and filament) were placed in a 7 mL centrifuge tube. DAB staining kit (Wuhan Servicebio Technology Co., Ltd., Wuhan, China) was

selected to label the peroxide. The plant hydrogen peroxide staining solution (DAB) (item: G1022-100ML) should be protected from light during the operation. For the DAB staining solution, 100 mg of DAB (3,3′-Diaminobenzidine tetrahydrochloride) was dissolved in 100 mL pH = 3.8 phosphate buffer. The samples were completely immersed in the dye solution. After dyeing at 25 °C for 6 h, the samples were observed by DVM6 ultra-depth stereoscope (LEICA company, Wetzlar, Germany).

2.2.5. Anther AsA and GSH Contents

The content of AsA was assayed according to Kampfenkel et al. [41] with slight modifications. Fresh anther tissue (0.2 g) was ground in a chilled grinder with 2 mL of 0.2 M PBS extract solution before centrifuged at 4 °C at 10,000× *g* for 10 min. The supernatant was collected. The reaction system includes 400 µL sample extract, 100 µL H_2O_2, 500 µL 10% (*w*/*v*) TCA, 400 µL 42% (*w*/*v*) H_3PO_4, 400 µL 4% (*w*/*v*) bipyridine, and 200 µL 3% (*w*/*v*) $FeCl_3$. All the constituents were thoroughly mixed and placed in a water bath at 42 °C for 40 min. The absorbance was detected at 525 nm.

The test kit (No. A006-2-1) of GSH content provided by Nanjing Jiancheng Bioengineering Institute was used for anther GSH extraction and determination. Fresh anther tissue (0.2 g) was ground in a chilled grinder, and then 1.8 mL of 0.1 M PBS extract solution was added to make a homogenate. The mixture was then centrifuged for 10 min at 10,000× *g* at 4 °C, and the absorbance of the supernatant was determined at 405 nm.

2.2.6. Gene Expression

The samples collected in 2020 were used for gene expression analysis. A FastPure Plant Total RNA lsolation Kit (Vazyme, Nanjing, China) and a Goldenstar^{TM} RT6 cDNA Synthesis Kit Ver. 2 (TsingKe, Beijing, China) were utilized to extract total RNA and generate the cDNAs, respectively. The PCR conditions were initial denaturation at 95 °C for 1 min, 40 cycles at 95 °C for 10 s, and annealing and extension at 60 °C for 15 s. The ubiquitin was used as an internal control. The primers for all genes are presented in Table S3. The relative expression level of tested genes was analyzed using the $2^{-\triangle\triangle Ct}$ method. Three biological replicates were measured per treatment.

2.2.7. Data Analysis

The data were the mean of three replications. The statistical analysis software SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was used to perform a one-way analysis of variance, correlation analysis, and principal component analysis. The LSD method was used to test the significant difference between means set at 0.05. Graphs were created by Origin 2021.

3. Results

3.1. Midday Leaf Water Potential (Ψ_{MD})

In Figure 1, The Ψ_{MD} of CK and HT treatments was similar and relatively stable, about −1.8~−1.6 Mpa and −2.2~−2.0 Mpa in 2019 and 2020, respectively. The Ψ_{MD} of DS and DS+HT treatments was significantly lower than that of CK.

3.2. Pollen Viability and Pollen Germination Rate

Compared with CK, both DS and HT significantly decreased cotton pollen viability (Figure S2 and Table S2), but the decrease was much greater under DS than HT conditions. The pollen viability of DS treatment was only 49.94–55.20% of that of HT treatment. The pollen viability under DS+HT treatment decreased by 73.43–75.37%, compared with CK, which was markedly lower than DS and HT treatments.

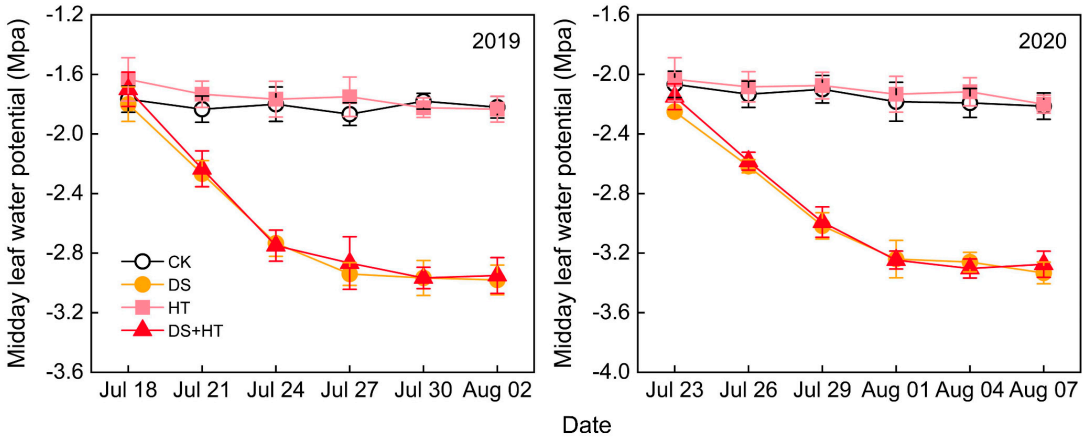

Figure 1. Effects of drought stress, high temperature, and combined stress on cotton's midday leaf water potential in 2019 and 2020. CK—control; DS—drought; HT—high temperature; DS+HT—drought stress coupled with high temperature.

3.3. Peroxides Labeling

The results of DAB staining showed that the color of the male reproductive organ differed among different treatments (Figure 2). Under individual DS and HT treatments, the anther color became darker than that in the CK group. Under DS+HT treatment, the color of the anther was the deepest, indicating that the accumulation of peroxide in anthers under DS+HT treatment was the highest.

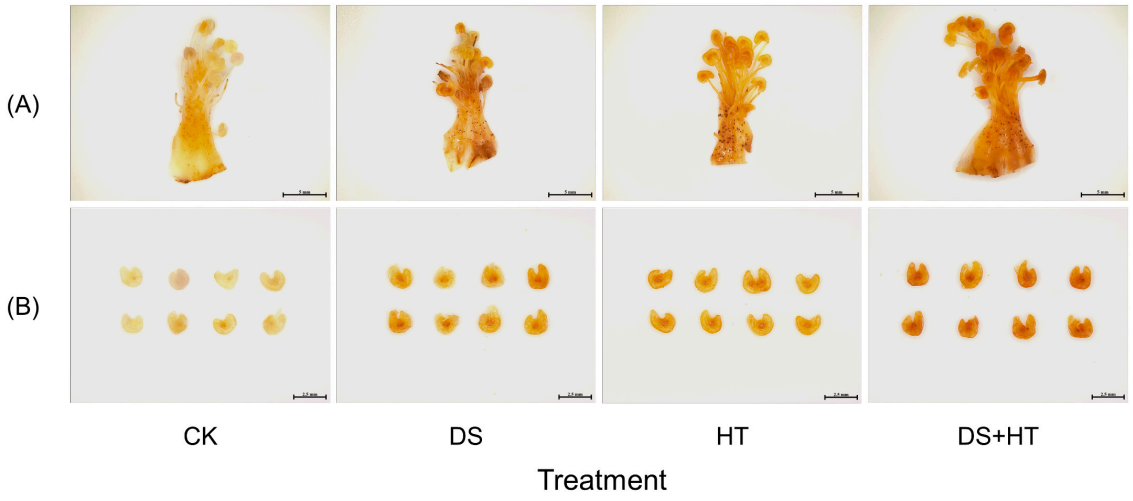

Figure 2. The labeling of peroxides in cotton anthers: (**A**) male reproductive organ and (**B**) single anther under different treatments in 2020. CK—control; DS—drought stress; HT—high temperature; DS+HT—drought stress coupled with high temperature.

3.4. Anther ROS Content

There was no significant difference in O_2^- content between CK and DS treatments in 2019 and 2020 (Figure 3). In 2019, the O_2^- content under HT and DS+HT treatments was significantly higher than that of CK, and there was no significant difference between HT and DS+HT treatments, while in 2020, the O_2^- content under HT and DS+HT treatments was significantly higher than that of CK, and DS+HT treatment had a higher value than HT treatment. Compared with CK, the H_2O_2 content under DS, HT, and DS+HT treatments increased by 7.17–9.30%, 19.49–24.30%, and 24.81–28.38%, respectively (Figure 3), and there was no significant difference in H_2O_2 content between HT and DS+HT treatments in 2019 and 2020.

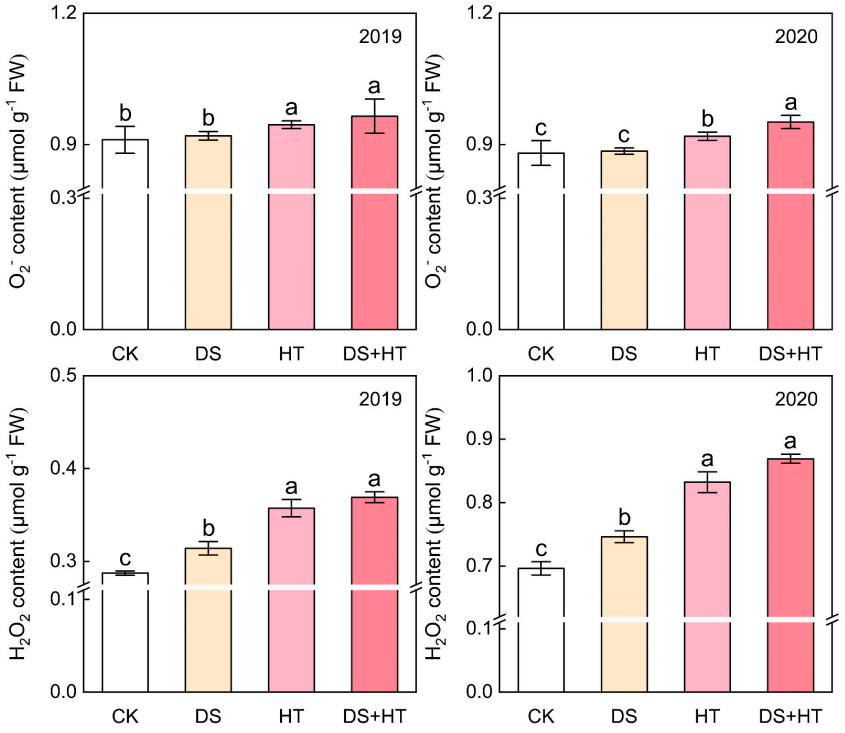

Figure 3. Effects of drought stress, high temperature, and combined stress on O_2^- and H_2O_2 contents of cotton anthers in 2019 and 2020. CK—control; DS—drought stress; HT—high temperature; DS+HT—drought stress coupled with high temperature. Columns labeled with different letters are significantly different at the $p = 0.05$ probability level.

3.5. Anther MDA Content

Compared with CK, the MDA content under DS and HT treatments increased by 24.00–40.22% and 15.63–16.02%, respectively (Figure 4). Compared with CK, the MDA content under the combined stress increased by 47.19–50.34%. Moreover, the MDA content under the combined stress was significantly higher than that under CK and single stress.

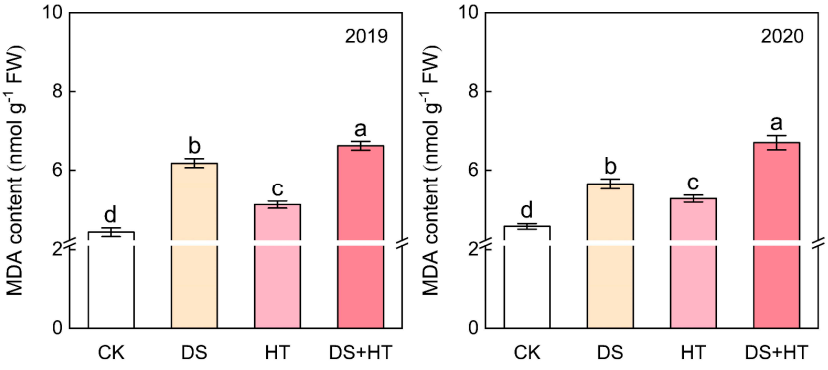

Figure 4. Effects of drought stress, high temperature, and combined stress on MDA content of cotton anthers in 2019 and 2020. CK—control; DS—drought stress; HT—high temperature; DS+HT—drought stress coupled with high temperature. Columns labeled with different letters are significantly different at the $p = 0.05$ probability level.

3.6. Expression of Genes Associated with Antioxidant Enzymes in Cotton Anthers

In Figure 5, DS treatment had no significant effect on the expression of *GhCu/ZnSOD* but significantly downregulated the expressions of *GhPOD* and *GhCAT* to 0.31- and 0.81-fold, respectively, compared with CK. HT treatment significantly downregulated the expressions of *GhCu/ZnSOD*, *GhPOD*, and *GhCAT* to 0.41-, 0.22-, and 0.33-fold, respectively, com-

pared with CK. DS+HT treatment significantly downregulated the expressions of *GhCu/ZnSOD*, *GhPOD*, and *GhCAT* to 0.17-, 0.38-, and 0.28-fold, respectively, compared with CK.

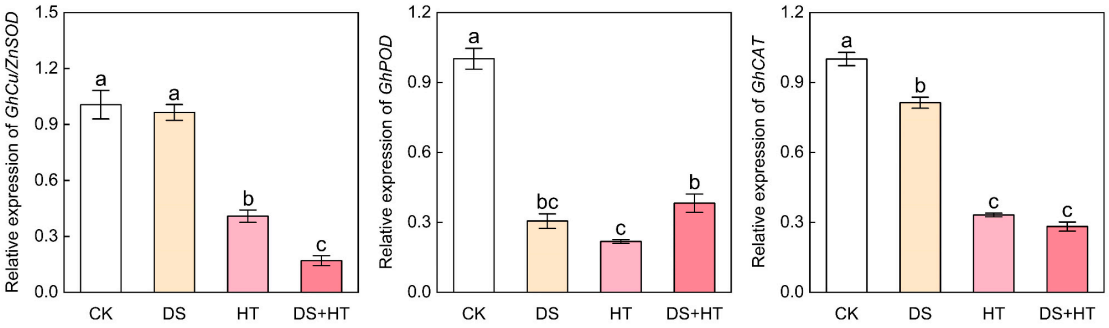

Figure 5. Effects of drought stress, high temperature, and combined stress on the relative expression level of Cu, Zn-superoxide dismutase gene (*Cu/ZnSOD*), peroxidase gene (*POD*), and catalase gene (*CAT*) in cotton anthers in 2020. CK—control; DS—drought stress; HT—high temperature; DS+HT—drought stress coupled with high temperature. Columns labeled with different letters are significantly different at the $p = 0.05$ probability level.

3.7. Anther AsA and GSH Contents

Compared with CK, the AsA content under DS, HT, and DS+HT treatments increased by 29.87–67.58%, 6.54–22.51%, and 21.88–34.89%, respectively (Figure 6). The AsA content under DS treatment was the highest, followed by DS+HT treatment and HT treatment. Compared with CK, the GSH content under DS and DS+HT treatments increased by 26.34–49.34% and 11.12–24.25%, respectively (Figure 6), while the GSH content was significantly decreased by HT treatment by 13.24–15.55%.

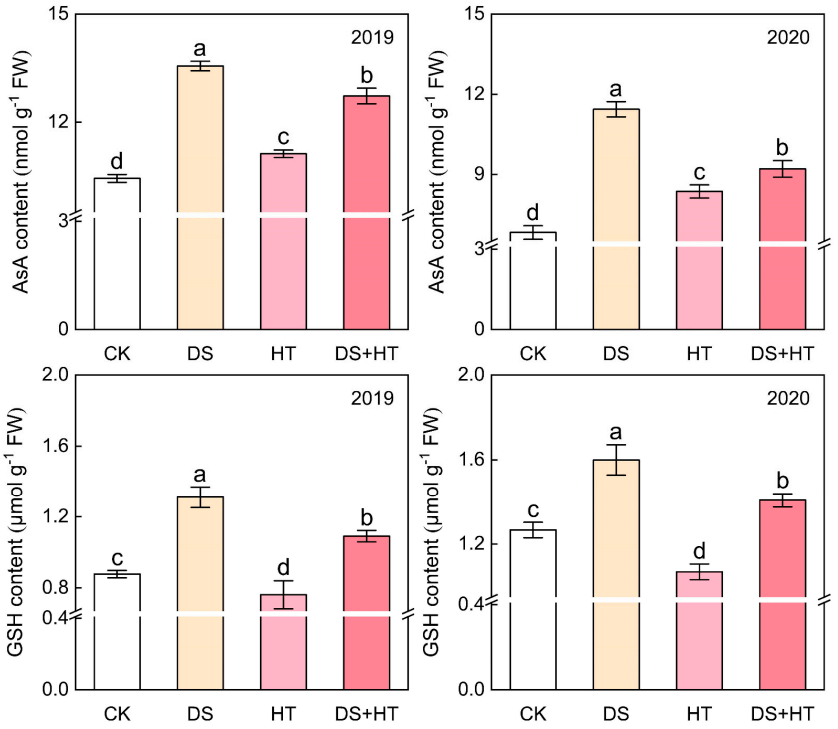

Figure 6. Effects of drought stress, high temperature, and combined stress on AsA and GSH contents of cotton anthers in 2019 and 2020. CK—control; DS—drought stress; HT—high temperature; DS+HT—drought stress coupled with high temperature. Columns labeled with different letters are significantly different at the $p = 0.05$ probability level.

3.8. Expression of Genes Associated with AsA–GSH Cycle in Cotton Anthers

The expression patterns of genes associated with AsA–GSH cycle in cotton anthers were different under single and combined stresses (Figure 7). Compared with CK, DS, HT, and DS+HT treatments significantly downregulated the expression of *GhAPX* in cotton anthers to 0.40-, 0.09-, and 0.34-fold, respectively (Figure 7). Compared with CK, DS and HT treatments significantly downregulated the expression of *GhMDHAR* in cotton anthers to 0.14- and 0.19-fold, respectively, while DS+HT treatment significantly upregulated the expression of *GhMDHAR* in cotton anthers by a 5.93-fold (Figure 7). Compared with CK, DS and DS+HT significantly upregulated the expression of *GhDHAR2* in cotton anthers with 1.58- and 4.38-fold, respectively, while HT treatment had no significant effect on the expression of *GhDHAR2* in cotton anthers (Figure 7). The expression of *GhGR* was markedly decreased by all stress treatments compared with CK, with the decreases being largest under DS+HT conditions, followed by DS, and then HT (Figure 7). Compared with CK, DS and HT significantly upregulated the expression of *GhAAO* in cotton anthers with 1.44- and 1.51-fold, respectively, while DS+HT treatment significantly downregulated the expression of *GhAAO* in cotton anthers to 0.19-fold (Figure 7). Compared with CK, DS and DS+HT significantly downregulated the expression of *GhGPX* in cotton anthers to 0.58- and 0.48-fold, respectively, while HT treatment had no significant effect on the expression of *GhGPX* in cotton anthers (Figure 7).

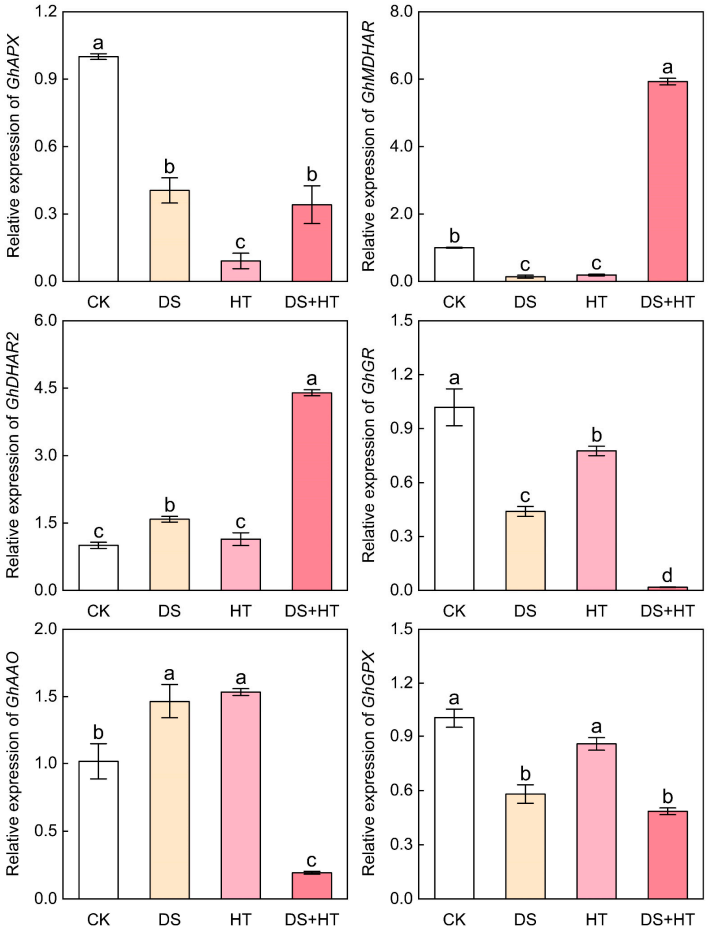

Figure 7. Effects of drought stress, high temperature, and combined stress on the relative expression levels of ascorbate peroxidase gene (*APX*), monodehydroascorbate reductase gene (*MDHAR*), dehydroascorbate reductase gene (*DHAR2*), glutathione reductase gene (*GR*), ascorbate oxidase gene (*AAO*), and glutathione peroxidase gene (*GPX*) in cotton anthers in 2020. CK—control; DS—drought stress; HT—high temperature; DS+HT—drought stress coupled with high temperature. Columns labeled with different letters are significantly different at the $p = 0.05$ probability level.

3.9. Correlation between Pollen Viability and ROS Content

According to correlation analysis, there was a significant negative correlation ($p < 0.01$) between pollen viability, O_2^- content, and H_2O_2 content in 2019 and 2020, respectively (Table S4).

3.10. Principal Component Analysis of Antioxidant Metabolism in Cotton Anthers

In order to visualize the changes in antioxidant metabolism under different treatments, a principal component (PC) analysis was conducted. The results showed that the first two PCs could explain 60.88% and 20.73% of the total variance, respectively (Figure 8). The scores plot showed that PC1 could well separate CK and DS+HT treatment, and the combination of PC1 and PC2 could well separate CK and DS treatment as well as HT treatment. According to the principal component load diagram (Figure 8), the indexes with higher load (the absolute value > 0.4) in the direction of PC1 were MDA, *GhPOD*, *GhAPX*, *GhMDHAR*, *GhDHAR2*, *GhGR*, and *GhAAO*, while those in the direction of PC2 were AsA and GSH. However, the loads of O_2^-, H_2O_2, *GhCu/ZnSOD*, *GhCAT*, and *GhGPX* were approximately equal in the direction of PC1 and PC2.

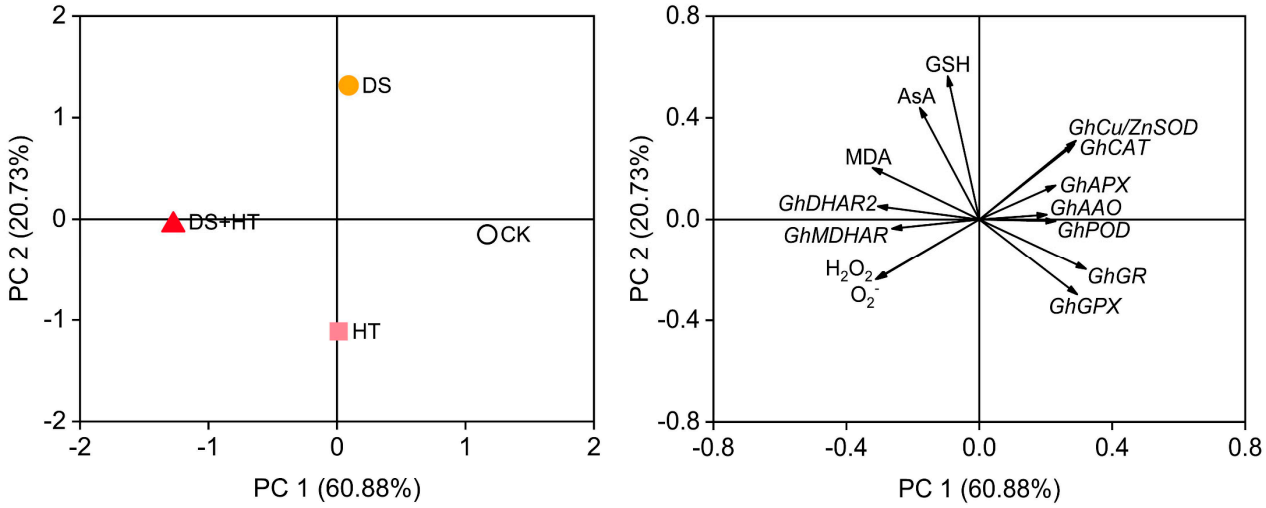

Figure 8. Multivariate analysis based on the antioxidant metabolism in cotton anthers.

4. Discussion

4.1. Effects of Drought Stress and High Temperature on ROS Content and Its Relationship with Pollen Fertility

In this study, two common methods, I_2-KI staining and TTC staining [42], were used to determine the cotton pollen viability in 2019 and 2020, and the results indicated it had good repeatability between years. Compared with CK, both DS and HT significantly decreased cotton pollen viability. Pollen viability under DS+HT was markedly lower than under DS and HT treatments, indicating that drought stress coupled with high temperature led to greater damage to pollen fertility than any single stress. The embodiment of pollen fertility includes a variety of complex metabolic processes. The balancing process of production and scavenging of reactive oxygen species (ROS) is also closely related to pollen fertility [43,44]. In line with their reports, there was a significant negative correlation between pollen viability and O_2^- content, as well as H_2O_2 content in the present study. Previous studies have found that drought stress [45] and high temperature [32] could lead to a significant accumulation of ROS in crop anthers, which was a significant cause of reduced pollen fertility. In this study, the results also showed that, compared with CK, there was a higher content of O_2^- in anthers under HT treatment and a higher H_2O_2 content in anthers under DS and HT treatments. The content of O_2^- in anthers under DS+HT treatment was higher than that under individual stress treatments (except for O_2^- in 2019), meaning that compared with single stress, the combined stress of drought and

high temperature further promoted the formation of O_2^- in anthers. Moreover, DS+HT treatment had more accumulation of malondialdehyde (MDA) in anthers than any single stress, indicating that, compared with single drought or high-temperature stress, higher O_2^- content under combined stress led to greater damage to cell structure. In support of our results, Yan et al. [46] found that O_2^- content together with MDA content in a male tassel of maize under combined stress of heat and drought was significantly higher than that under individual stress, and the difference was the most significant 6–8 days after stress. However, DS+HT treatment did not alter H_2O_2 content relative to HT treatment, although DS+HT had a higher H_2O_2 content than DS treatment, suggesting that under combined stress, the influence of high temperature on H_2O_2 content might play a dominant role.

4.2. Effects of Drought Stress and High Temperature on Antioxidant Enzyme System in Anthers

The antioxidant enzyme system in anthers mainly includes superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT). SOD is the first line of defense for scavenging ROS, and it converts O_2^- to H_2O_2 and O_2 by catalytic disproportionation [15,16]. Fu et al. [24] have found that pollen fertility under drought stress was closely related to the activities of SOD, and Zhao et al. [44] found that the increase in O_2^- content in rice anthers under heat stress was closely related to the decrease in SOD activity. In this study, DS treatment had no significant effect on the expression of *GhCu/ZnSOD*, indicating that the O_2^- disproportionation reaction was not altered, which might be why O_2^- content did not increase significantly under DS treatment compared with CK. The HT and DS+HT treatments significantly downregulated the expression of *GhCu/ZnSOD* in relation to CK, and the expression of *GhCu/ZnSOD* was substantially decreased under the DS+HT treatment compared with HT treatment, indicating that combined stress might further damage the ability to convert O_2^- to H_2O_2 and O_2 by catalytic disproportionation more than single high-temperature stress. Hence, it was also why the content of O_2^- under DS+HT treatment was higher than that under HT treatment. The two enzymes, POD and CAT, are responsible for catalyzing the scavenging of H_2O_2 [15,16]. In this study, DS and HT treatments significantly downregulated the expression of *GhPOD* and *GhCAT*, which was consistent with previous studies where drought stress significantly decreased the activities or gene expression of POD and CAT [47], and the increase in ROS content in rice anthers under high-temperature stress was closely related to the decrease in POD and CAT activities [44]. This also led us to speculate whether the downregulation of *GhPOD* and *GhCAT* could be the main reason for the larger accumulation of H_2O_2 under drought stress and high-temperature conditions. Compared with single stress, the downregulation range of *GhPOD* under DS+HT treatment was the smallest. However, the expression of *GhCAT* decreased to the lowest level under DS+HT treatment, letting us hypothesize that the downregulation of *GhCAT* expression weakened the ability of anthers to scavenge H_2O_2 directly under combined stress, resulting in a large H_2O_2 accumulation.

4.3. Effects of Drought Stress and High Temperature on AsA–GSH Cycle in Anthers

The ascorbic acid–glutathione (AsA–GSH) cycle is an important nonenzymatic pathway for scavenging ROS in plants [48,49]. In this study, all stress treatments significantly increased the content of AsA in cotton anthers, which was consistent with previous studies [50]. Compared with CK, the GSH content under DS increased, while the GSH content significantly decreased under HT treatment, indicating that drought and high temperature had adverse effects on anther GSH content. Consistent with our results, related studies have found the adverse effects of drought stress [25] and high temperature [51] on the content of GSH. Hence, drought and high-temperature effects on GSH content resulted in a higher GSH under DS+HT treatment than under HT but lower than under DS treatment. However, similar to DS treatment, under DS+HT treatment, the GSH content was still higher than under CK, suggesting that drought plays a major role in the effects of combined stress on anther GSH content.

AsA–GSH cycle involves multiple enzymatic reactions. Ascorbate peroxidase (APX) uses AsA as a reductant in the first step of the AsA–GSH cycle and is one of the most important enzymes for scavenging H_2O_2 [52]. Compared with CK, DS, HT, and DS+HT treatments significantly downregulated the expression of *GhAPX*, severely hindering the AsA's reduction of H_2O_2. This can explain why the AsA content was high under drought stress, high temperature, and their combined conditions, as opposed to leading to the increased H_2O_2 content. Monodehydroascorbate reductase (MDHAR) is responsible for reducing monodehydroascorbic acid (MDHA) [53]. Single DS or HT treatment led to a significant downregulation of *GhMDHAR*, which was not conducive to forming AsA. Under the combined stress, *GhMDHAR* was significantly downregulated, suggesting that the effect of combined stress on *GhMDHAR* expression was different from that of any single stress and thus conducive to forming AsA.

Dehydroascorbate reductase (DHAR) can catalyze two reactions: one is to recycle AsA from DHA [54], and the other is to catalyze the oxidation of GSH to GSSG. Ascorbate oxidase (AAO) is responsible for catalyzing the oxidation of AsA to MDHA, but MDHA is unstable and can be easily converted into DHA [55]. Then, the DHA is restored to AsA by DHAR. In this study, the results showed that DS and HT significantly upregulated the expression of *GhAAO*, and there was no significant difference between the two treatments. This indicated that drought stress and high temperature promoted the oxidation of AsA. Drought stress also upregulated the expression of *GhDHAR2*, but the high temperature had no significant effect on *GhDHAR2* expression. It can be seen that drought stress enhanced the process of reducing DHA to AsA compared with high temperature, which was also why the content of AsA under drought stress was significantly higher than that under high temperature. Under the combined stress, *GhAAO* was significantly downregulated, and *GhDHAR2* was significantly upregulated, which hindered the oxidation of AsA and promoted the reduction of oxidized AsA, resulting in the accumulation of AsA content.

Glutathione peroxidase (GPX) can catalyze the reaction of GSH and H_2O_2 to produce GSSG, and glutathione reductase (GR) is an enzyme that reduces GSSG and catalyzes GSH regeneration [49]. The reaction catalyzed by GPX and GR plays a momentous role in maintaining GSH/GSSG balance. In this study, compared with CK, all stress significantly downregulated the expression of *GhGR*, and the amplitude of decline in the DS+HT treatment was the largest, indicating that drought stress and high temperature, especially when combined, were not conducive to GSH regeneration in cotton anthers. In addition, HT had no significant effect on the expression of *GhGPX*, which means that high temperature had no effects on the oxidation of GSH, so the hindrance of GSH regeneration may be the primary reason for the decrease in GSH content under high temperature. However, the DS and DS+HT treatment significantly downregulated the expression of *GhGPX*, indicating that the oxidation of GSH was blocked by drought stress and drought stress coupled with high temperature, which was the main reason for the final higher GSH accumulation than in CK.

5. Conclusions

The variation of pollen viability was closely related to the peroxide content in anthers. DS had no significant effect on the expression of *GhCu/ZnSOD*, so the content of O_2^- did not increase significantly, while the HT and DS+HT significantly downregulated the expression of *GhCu/ZnSOD*, resulting in a significant increase in the content of O_2^-. Although AsA content was higher under DS, HT, and DS+HT, the expression of *GhPOD* decreased under DS and HT, and the expression of *GhCAT* decreased under DS+HT, which led to the decrease in the ability to scavenge H_2O_2, resulting in the increase in H_2O_2 and MDA contents. In addition, DS and HT significantly downregulated *GhMDHAR* to seriously hinder the production of AsA. They upregulated the expression of *GhAAO* to promote the oxidation of AsA but downregulated the expression of *GhAPX* to hinder the reduction of H_2O_2 by AsA, which was the reason for the accumulation of AsA and the H_2O_2 increase. DS also upregulated the expression of *GhDHAR2*, which was beneficial in reducing DHA to AsA. This might be the reason why the content of AsA under DS was significantly higher

than under HT; the DS+HT downregulated the expression of *GhAAO* and inhibited the oxidation of AsA but upregulated the expression of *GhMDHAR* and *GhDHAR2*, which was beneficial to the production of AsA. Downregulated *GhAPX* inhibited the reduction of H_2O_2 by AsA, resulting in an increase in AsA content. In addition, HT significantly downregulated the expression of *GhGR*, blocking the reduction of GSSG and decreasing the content of GSH. In contrast, DS and DS+HT significantly downregulated the expression of *GhGPX*, blocking the oxidation of GSH and leading to the accumulation of GSH. Combined stress had a unique effect on the AsA–GSH cycle. Compared with single stress, combined stress had a major influence on the related genes, including *GhMDHAR*, *GhDHAR2*, *GhGR*, and *GhAAO*, involving the AsA–GSH cycle. Therefore, the combined stress had a more comprehensive impact on the AsA–GSH cycle to influence ROS content. Timely inspection is recommended to avoid severe antioxidant metabolism disorders caused by multiple stresses.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy13102550/s1, Figure S1: Temporal trend in soil relative water content (2019–2020). CK, control; DS, drought stress; HT, high temperature; DS+HT, drought stress coupling with high temperature; Figure S2: Pollen viability ((A), in 2019 and (B), in 2020) under drought stress, high temperature and combined stress. CK, control; DS, drought stress; HT, high temperature; DS+HT, drought stress coupling with high temperature; Table S1: Mean daily temperature in the high temperature area and the ambient temperature area (2019–2020); Table S2: Effects of drought stress, high temperature and coupled stress on cotton pollen fertility in 2019 and 2020. CK, control; DS, drought stress; HT, high temperature; DS+HT, drought stress coupling with high temperature; Table S3: Gene primer sequences used for the quantitative real-time PCR analysis; Table S4: Correlation coefficient among pollen viability and O_2^- as well as H_2O_2 content in 2019 and 2020.

Author Contributions: Conceptualization, W.H.; data curation, J.Z., M.C., N.C. and Y.L.; formal analysis, J.Z. and W.H.; funding acquisition, W.H.; investigation, J.Z., M.C., N.C. and Y.L.; methodology, J.Z. and M.C.; project administration, W.H., Z.Z. and S.W.; supervision, Z.Z. and S.W.; validation, J.Z. and W.H.; writing—original draft, J.Z., M.C. and W.H.; writing—review and editing, J.Z., M.C., N.C. and W.H.; and co-corresponding author W.H. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Natural Science Foundation of China (31630051, 31901463, 32272223), Fundamental Research Funds for the Central Universities (XUEKEN 2022008), China Agriculture Research System of MOF and MARA (CARS-15-14), Collaborative Innovation Center for Modern Crop Production co-sponsored by Province and Ministry (CIC-MCP) and High-Level Talent Introduction Program of Nanjing Agricultural University.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Akasofu, S.I. On the Present Halting of Global Warming. *Climate* **2013**, *1*, 4–11. [CrossRef]
2. Solomon, S.; Qin, D.; Manning, M.; Chen, Z.; Marquis, M.; Averyt, K.B.; Tignor, M.; Miller, H. The Physical Science Basis: Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. *Comput. Geom.* **2007**, *996*, 95–123.
3. Peng, S.; Huang, J.; Sheehy, J.E.; Laza, R.C.; Visperas, R.M.; Zhong, X.; Centeno, G.S.; Khush, G.S.; Cassman, K.G. Rice Yields Decline with Higher Night Temperature from Global Warming. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 9971–9975. [CrossRef] [PubMed]
4. Zinn, K.E.; Tunc-Ozdemir, M.; Harper, J.F. Temperature Stress and Plant Sexual Reproduction: Uncovering the Weakest Links. *J. Exp. Bot.* **2010**, *61*, 1959–1968. [CrossRef]
5. Giorgi, F.; Lionello, P. Climate Change Projections for the Mediterranean Region. *Glob. Planet. Chang.* **2008**, *63*, 90–104. [CrossRef]
6. Guo, C.; Ge, X.; Ma, H. The Rice *OsDIL* Gene Plays a Role in Drought Tolerance at Vegetative and Reproductive Stages. *Plant Mol. Biol.* **2013**, *82*, 239–253. [CrossRef] [PubMed]
7. Pettigrew, W.T. Moisture Deficit Effects on Cotton Lint Yield, Yield Components, and Boll Distribution. *Agron. J.* **2004**, *96*, 377–383. [CrossRef]
8. Wang, R.; Ji, S.; Zhang, P.; Meng, Y.; Zhou, Z. Drought Effects on Cotton Yield and Fiber Quality on Different Fruiting Branches. *Crop Sci.* **2016**, *56*, 1265–1276. [CrossRef]

9. Gao, M.; Xu, B.; Wang, Y.; Zhou, Z.; Hu, W. Quantifying Individual and Interactive Effects of Elevated Temperature and Drought Stress on Cotton Yield and Fibre Quality. *J. Agron. Crop Sci.* **2021**, *207*, 422–436. [CrossRef]
10. Xu, B.; Hu, W.; Gao, M.; Zhao, W.; Wang, Y.; Zhou, Z. Effects of Elevated Air Temperature Coupling with Soil Drought on Carbohydrate Metabolism and Oil Synthesis during Cottonseed Development. *Physiol. Plant.* **2022**, *174*, e13643. [CrossRef]
11. Liu, Z.; Zhu, Y.; Hu, C.; Wei, G.; Yang, L.; Zhang, G. Effects of NaCl Stress on the Growth, Antioxidant Enzyme Activities and Reactive Oxygen Metabolism of Grafted Eggplant. *J. Appl. Ecol.* **2007**, *18*, 537–541.
12. Liu, Z.; Shi, X.; Yan, P.; Duan, Y.; Geng, X.; Ye, J.; Li, S.; Yang, X.; Zhang, G.; Jia, Y.; et al. Tapetal Programmed Cell Death, Antioxidant Response and Oxidative Stress in Wheat Anthers Associated with D^2-type Cytoplasmic Male-Sterility. *Sci. Agric. Sin.* **2017**, *50*, 4071–4086.
13. Kelliher, T.; Walbot, V. Hypoxia Triggers Meiotic Fate Acquisition in Maize. *Science* **2012**, *337*, 345–348. [CrossRef] [PubMed]
14. Liu, L.; Jiang, Y.; Ma, J.; Gao, Z.; Chen, G. Metabolism of Reactive Oxygen Species in Anthers and Leaves of Cytoplasmic Male-sterile Rice. *Chin. Agric. Sci. Bull.* **2016**, *32*, 6–12.
15. Mittler, R. Oxidative Stress, Antioxidants and Stress Tolerance. *Trends Plant Sci.* **2002**, *7*, 405–410. [CrossRef] [PubMed]
16. García-Limones, C.; Hervás, A.; Navas-Cortés, J.A.; Jiménez-Díaz, R.M.; Tena, M.J.P.; Pathology, M.P. Induction of an Antioxidant Enzyme System and Other Oxidative Stress Markers Associated with Compatible and Incompatible Interactions between Chickpea (*Cicer arietinum* L.) and *Fusarium oxysporum* f. sp. *ciceris*. *Physiol. Mol. Plant Pathol.* **2002**, *61*, 325–337. [CrossRef]
17. Li, Y.; Liu, Y.; Zhang, J. Advances in the Research on the AsA–GSH Cycle in Horticultural Crops. *Front. Agric. China* **2010**, *4*, 84–90. [CrossRef]
18. Foyer, C.H.; Noctor, G. Oxidant and Antioxidant Signalling in Plants: A Re-evaluation of the Concept of Oxidative Stress in a Physiological Context. *Plant Cell Environ.* **2005**, *28*, 1056–1071. [CrossRef]
19. Eltayeb, A.E.; Kawano, N.; Badawi, G.H.; Kaminaka, H.; Sanekata, T.; Morishima, I.; Shibahara, T.; Inanaga, S.; Tanaka, K. Enhanced Tolerance to Ozone and Drought Stresses in Transgenic Tobacco Overexpressing Dehydroascorbate Reductase in Cytosol. *Physiol. Plant.* **2006**, *127*, 57–65. [CrossRef]
20. Navari-Izzo, F.; Pinzino, C.; Quartacci, M.F.; Sgherri, C.L.M.; Izzo, R. Intracellular Membranes: Kinetics of Superoxide Production and Changes in Thylakoids of Resurrection Plants upon Dehydration and Rehydration. *Proc. R. Soc. Edinb. Sect. B Biol.* **1994**, *102*, 187–191. [CrossRef]
21. Tambussi, E.A.; Bartoli, C.G.; Beltrano, J.; Guiamet, J.J.; Araus, J.L. Oxidative Damage to Thylakoid Proteins in Water-stressed Leaves of Wheat (*Triticum aestivum*). *Physiol. Plant.* **2000**, *108*, 398–404. [CrossRef]
22. Moller, I.M.; Jensen, P.E.; Hansson, A. Oxidative Modifications to Cellular Components in Plants. *Annu. Rev. Plant Biol.* **2007**, *58*, 459–481. [CrossRef]
23. Rong, Z.; Zhang, X.; Yang, S.; Xu, Z.; Li, J.; Huang, G.; Zhao, J.; Gong, M. Involvement of Antioxidant Defense System in Enhancement of Drought Resistance in Tobacco (*Nicotiana tabacum* L.) Plants Through Circular Drought-hardening. *Plant Physiol. J.* **2012**, *48*, 705–713.
24. Fu, G.; Song, J.; Xiong, J.; Li, Y.; Chen, H.; Le, M.; Tao, L. Changes of Oxidative Stress and Soluble Sugar in Anthers Involve in Rice Pollen Abortion under Drought Stress. *Agric. Sci. China* **2011**, *10*, 1016–1025. [CrossRef]
25. Selote, D.S.; Khanna-Chopra, R. Drought-induced Spikelet Sterility is Associated with an Inefficient Antioxidant Defence in Rice Panicles. *Physiol. Plant.* **2004**, *121*, 462–471. [CrossRef]
26. Ma, Y.; Ma, F.; Ma, X.; Li, M.; Wang, Y.; Han, M.; Shu, H. Effects of Drought Stress on Ascorbic Acid Contents and Activities of Related Metabolic Enzymes in Apple Leaves. *J. Northwest A F Univ. (Nat. Sci. Ed.)* **2008**, *36*, 150–154+160.
27. Liu, X.; Huang, B. Heat Stress Injury in Relation to Membrane Lipid Peroxidation in Creeping Bentgrass. *Crop Sci.* **2000**, *40*, 503–510. [CrossRef]
28. Zhang, G.; Zhang, S.; Xiao, L.; Tang, W.; Xiao, Y.; Chen, L. Effect of High Temperature Stress on Physiological Characteristics of Anther, Pollen and Stigma of Rice During Heading-flowering Stage. *Chin. J. Rice Sci.* **2014**, *28*, 155–166.
29. Ahammed, G.J.; Xu, W.; Liu, A.; Chen, S. Endogenous Melatonin Deficiency Aggravates High Temperature-induced Oxidative Stress in *Solanum lycopersicum* L. *Environ. Exp. Bot.* **2019**, *161*, 303–311. [CrossRef]
30. Nico, D.S.; Danny, G. The Impact of Environmental Stress on Male Reproductive Development in Plants: Biological Processes and Molecular Mechanisms. *Plant Cell Environ.* **2014**, *37*, 1–18.
31. Djanaguiraman, M.; Prasad, P.V.V.; Al-Khatib, K. Ethylene Perception Inhibitor 1-MCP Decreases Oxidative Damage of Leaves Through Enhanced Antioxidant Defense Mechanisms in Soybean Plants Grown under High Temperature Stress. *Environ. Exp. Bot.* **2011**, *71*, 215–223. [CrossRef]
32. Zhao, Q.; Zhou, L.; Liu, J.; Du, X.; Asad, M.; Huang, F.; Pan, G.; Cheng, F. Relationship of ROS Accumulation and Superoxide Dismutase Isozymes in Developing Anther with Floret Fertility of Rice under Heat Stress. *Plant Physiol. Biochem.* **2017**, *122*, 90–101. [CrossRef] [PubMed]
33. Xu, S.; He, X.; Chen, W.; Li, J.; Zhang, J. Effects of Heat Acclimation on High-temperature Stress Resistance and Heattolerance Mechanism of *Festuca arundinacea* and *Lolium perenne*. *Acta Ecol. Sin.* **2008**, *28*, 162–171.
34. Zou, M.; Yuan, L.; Zhu, S.; Liu, S.; Ge, J.; Wang, C. Response of Osmotic Adjustment and Ascorbate-glutathione Cycle to Heat Stress in a Heat-sensitive and a Heat-tolerant Genotype of wucai (*Brassica campestris* L.). *Sci. Hortic.* **2016**, *211*, 87–94. [CrossRef]
35. Li, Y.; Zhang, J. Effect of High-temperature Stress on the Activity of Key Enzymes in the AsA–GSH Cycle in 'Yali' Pears. *Front. Agric. China* **2010**, *4*, 463–467. [CrossRef]

36. Loka, D.A.; Oosterhuis, D.M.; Baxevanos, D.; Noulas, C.; Hu, W. Single and Combined Effects of Heat and Water Stress and Recovery on Cotton (*Gossypium hirsutum* L.) Leaf Physiology and Sucrose Metabolism. *Plant Physiol. Biochem.* **2020**, *148*, 166–179. [CrossRef]
37. Burke, J.J.; Mahan, J.R.; Hatfield, J.L. Crop-specific Thermal Kinetic Windows in Relation to Wheat and Cotton Biomass Production. *Agron. J.* **1988**, *80*, 553–556. [CrossRef]
38. Zuo, D.; Ming, J.; Liu, C.; Wang, L. Advance in Technique of Plant Pollen Viability. *J. Anhui Agric. Sci.* **2007**, *35*, 4742–4745.
39. Liu, X.; Wang, X.; Wang, X.; Gao, J.; Luo, N.; Meng, Q.; Wang, P. Dissecting the Critical Stage in the Response of Maize Kernel Set to Individual and Combined Drought and Heat Stress around Flowering. *Environ. Exp. Bot.* **2020**, *179*, 104213. [CrossRef]
40. Hu, W.; Lv, X.; Yang, J.; Chen, B.; Zhao, W.; Meng, Y.; Wang, Y.; Zhou, Z.; Oosterhuis, D.M. Effects of Potassium Deficiency on Antioxidant Metabolism Related to Leaf Senescence in Cotton (*Gossypium hirsutum* L.). *Field Crops Res.* **2016**, *191*, 139–149. [CrossRef]
41. Kampfenkel, K.; Vanmontagu, M.; Inzé, D. Extraction and Determination of Ascorbate and Dehydroascorbate from Plant Tissue. *Anal. Biochem.* **1995**, *225*, 165–167. [CrossRef]
42. Barrow, J.R. Comparisons among Pollen Viability Measurement Methods in Cotton. *Crop Sci.* **1983**, *23*, 734–736. [CrossRef]
43. Jiang, P.; Zhang, X.; Zhu, Y.; Zhu, W.; Xie, H.; Wang, X. Metabolism of Reactive Oxygen Species in Cotton Cytoplasmic Male Sterility and Its Restoration. *Plant Cell Rep.* **2007**, *26*, 1627–1634. [CrossRef]
44. Zhao, Q.; Zhou, L.; Liu, J.; Cao, Z.; Du, X.; Huang, F.; Pan, G.; Cheng, F. Involvement of CAT in the Detoxification of HT-induced ROS Burst in Rice Anther and Its Relation to Pollen Fertility. *Plant Cell Rep.* **2018**, *37*, 741–757. [CrossRef]
45. Nguyen, G.N.; Hailstones, D.L.; Wilkes, M.; Sutton, B.G. Drought-induced Oxidative Conditions in Rice Anthers Leading to a Programmed Cell Death and Pollen Abortion. *J. Agron. Crop Sci.* **2010**, *195*, 157–164. [CrossRef]
46. Yan, Z.; Liu, D.; Jia, X.; Yang, Q.; Chen, Y.; Dong, P.; Wang, Q. Maize Tassel Development, Physiological Traits and Yield under Heat and Drought Stress during Flowering Stage. *Sci. Agric. Sin.* **2021**, *54*, 3592–3608.
47. Liu, M.; Guo, L.; Yue, Y.; Wu, J.; Fan, X.; Xiao, G.; Teng, K. Physiological and Antioxidant Enzyme Gene Expression Differences Between Female and Male *Buchloe dactyloides* Plants under Drought Stress. *Acta Prataculturae Sin.* **2023**, *32*, 93–103.
48. Gupta, M.; Cuypers, A.; Vangronsveld, J.; Clijsters, H. Copper Affects the Enzymes of the Ascorbate-glutathione Cycle and Its Related Metabolites in the Roots of *Phaseolus vulgaris*. *Physiol. Plant.* **1999**, *106*, 262–267. [CrossRef]
49. Gill, S.S.; Anjum, N.A.; Hasanuzzaman, M.; Gill, R.; Trivedi, D.K.; Ahmad, I.; Pereira, E.; Tuteja, N. Glutathione and Glutathione Reductase: A Boon in Disguise for Plant Abiotic Stress Defense Operations. *Plant Physiol. Biochem.* **2013**, *70*, 204–212. [CrossRef]
50. Xu, S.; Li, J.; Zhang, X.; Wei, H.; Cui, L. Effects of Heat Acclimation Pretreatment on Changes of Membrane Lipid Peroxidation, Antioxidant Metabolites, and Ultrastructure of Chloroplasts in Two Cool-season Turfgrass Species under Heat Stress. *Environ. Exp. Bot.* **2006**, *56*, 274–285. [CrossRef]
51. Li, Y.; Zhang, J.; Zhang, J. Effect of Exogenous Substance Pretreatment on Antioxidative Ability of Pear Fruits under High Temperature and Excessive Sunlight Stress. *Acta Hortic.* **2015**, *1094*, 389–394. [CrossRef]
52. Noctor, G.; Foyer, C.H. Ascorbate and Glutathione: Keeping Active Oxygen under Control. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1998**, *49*, 249–279. [CrossRef] [PubMed]
53. Shila, S.K.; Murali, A.V.M.; Sibasis, S.; Malireddy, K.R.; Arulandu, A. Biochemical and Structural Characterization of a Robust and Thermostable Ascorbate Recycling Monodehydroascorbate Reductase (MDHAR) from Stress Adapted Pearl Millet. *Biochem. Biophys. Res. Commun.* **2023**, *662*, 135–141.
54. Krishna, D.B.; Ali, K.W.; Nellootil, S.S.; Kannapiran, P.; Murali, A.V.M.; Seetharami, R.E.; Balasubramaniam, D.; Anmol, C.; Malireddy, K.R.; Arulandu, A. Plant Dehydroascorbate Reductase Moonlights as Membrane Integrated Ion Channel. *Arch. Biochem. Biophys.* **2023**, *741*, 109603.
55. Gaspard, S.; Monzani, E.; Casella, L.; Gullotti, M.; Maritano, S.; Marchesini, A. Inhibition of Ascorbate Oxidase by Phenolic Compounds. Enzymatic and Spectroscopic Studies. *Biochemistry* **1997**, *36*, 4852–4859. [CrossRef] [PubMed]