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Droughts and Thermo-Priming Enhance Acclimation to Later Drought and Heat Stress in Maize Seedlings by Improving Leaf Physiological Activity

Chen Ru, Xiaotao Hu *, Dianyu Chen and Wene Wang

Key Laboratory of Agricultural Soil and Water Engineering in Arid and Semiarid Areas, Ministry of Education, Northwest A&F University, Xianyang 712100, China

* Correspondence: huxiaotao11@nwsuaf.edu.cn; Tel.: +86-1389-2816-133; Fax: +86-29-87082117

Abstract: Early heat and drought priming may increase the plant's ability to resist later drought and heat stress. However, it remains unclear whether combined heat and drought priming can enhance the acclimation of plants to later combined stress by improving physiological activities. In this study, maize seedlings were first pre-exposed twice to heat, drought, and a combination of stresses followed by recovery, and then subjected to six days of more severe stresses. A considerable reduction in photosynthetic pigment content, stomatal size, and photosynthesis was observed under heat and drought conditions, and the changes in the above indicators were amplified under combined stress conditions. Stress priming improves antioxidant defense and cellular osmoregulation, as indicated by improved superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), glutathione reductase (GR), and ascorbate peroxidase activities, as well as elevated soluble sugar (SS) and proline (Pro) contents. Lower superoxide anion and malondialdehyde contents and injury index in the primed seedlings demonstrated the mitigation of oxidative stress. ROC analysis revealed that SOD and POD had considerable reliability in determining that maize seedlings were experiencing heat stress (AUC = 0.941–0.971); GR and SS were capable of accurately monitoring drought stress that was being experienced by plants (AUC = 0.919–0.958); and SOD, GR, and Pro had more capability for detecting the combination of heat and drought stress (AUC = 0.907–0.958). Collectively, the primed seedlings exhibited better performance than the non-primed seedlings, exhibiting stronger stress acclimation supported by an effective antioxidant defense system and osmoregulatory function.

Keywords: heat stress; drought stress; priming; antioxidant activity; photosynthesis; osmoregulation

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1. Introduction

Heat and drought stress are the two major factors limiting crop growth and yield worldwide [1]. According to the climate prediction model, extreme heat and drought stress are expected to appear increasingly in the future [2]. Maize is one of the most popularly cultivated crops in the world. However, it is mainly planted in semiarid areas and often suffers from high temperatures and drought events [3,4], and this sensitivity may arise in most of the maize-growing areas of northwestern China under future climatic conditions. High temperatures commonly occur in conjunction with drought and coincide with the seedling stage of summer maize, which has a significant negative impact on seedling growth and subsequent yield [5,6].

High temperature and drought are significantly detrimental to the photosynthesis mechanism. When plants are exposed to heat stress, the inactivation of Rubisco, as well as a reduction in chlorophyll content and PSII efficiency, results in a severe reduction in photosynthesis [7–9]. Research has demonstrated that drought stress decreases photosynthesis by reducing the rate of CO_2 diffusion caused by stomatal and nonstomatal restrictions [10,11]. Under stress conditions, the inhibition of photosynthesis limits aboveground plant growth,

resulting in a significant decrease in plant height, leaf area, and biomass accumulation. Furthermore, heat and drought stress lead to excessive accumulation of superoxide radicals in plants, resulting in oxidative damage to proteins, nucleic acids, and cell membranes [1,12]. Higher plants possess ROS scavenging systems in which numerous antioxidant enzymes, such as superoxide dismutase (SOD), peroxidases (PODs), catalase (CAT), and glutathione reductase (GR), play an important role in the antioxidant defense system of plants [13]. Nevertheless, the antioxidant defense mechanism of plants may not be effective in scavenging the overproduced ROS in the presence of severe stresses, resulting in lipid peroxidation and protein degeneration of the cell membrane, which impair the membrane structures and cellular functions [1,12]. Cellular osmoregulation is considered a vital physiological characteristic related to heat and drought tolerance. As osmoregulatory substances, proline, sugar, and soluble protein play crucial roles in resisting stresses [14].

Improving stress tolerance and reducing the negative impacts of abiotic stress are well-known goals in crops. Unfortunately, it is very difficult and complicated to achieve. To date, various exogenous chemical priming methods have been applied to improve crop tolerance to later drought and heat stress, as exemplified by urea and KNO used in drought priming of hybrid maize [15], exogenous salicylic acid used in heat priming of maize [16], and nitrate and sulfate used in drought priming of wheat [17]. However, some are species- and dose-dependent, and more comprehensive knowledge of the underlying potential mechanisms is needed [18]. Heat priming has been shown to reduce leaf temperature via stomatal-regulated evaporation, thus inducing heat stress signals and improving plant acclimation to high temperatures [19,20]. Compared with non-primed plants, the drought-primed plants showed better photosynthesis, photoprotection, antioxidant activity, and biomass accumulation to resist later drought stress [12,21,22]. Furthermore, heat and drought priming could reduce the production of ROS and maintain cellular membrane stability by improving antioxidant capacity [21,23].

In recent years, the majority of studies concerning priming have primarily focused on one-off or individual stress priming [12,20,21,24]. However, it remains largely unknown whether combined heat and drought priming can alleviate the negative impacts of subsequent adverse environmental factors on maize plants. Little attention has been paid to the ability of physiological traits to diagnose heat, drought, and their combined stresses. The hypothesis is that maize seedlings pre-subjected to high temperatures and drought conditions (stress-primed seedlings) are better equipped to cope with subsequent more severe stress events than the non-primed seedlings. The purposes of the present study were: (i) to investigate the difference in pigment content, stomatal morphology, photosynthesis, antioxidant defense, cellular osmoregulation, and growth between the primed and non-primed plants; and (ii) to evaluate the capability of key physiological traits to diagnose individual and combined stresses.

2. Materials and Methods

2.1. Plant Culture and Treatments

A summer maize pot experiment was carried out in two growth chambers at the irrigation test station of Northwest A&F University (108.04° E, 34.20° N). A high-yielding maize variety (*Zea mays* L. cv. Zhengdan 958) from China was used. The pots used in the experiment were 30 cm in diameter. The experimental soil contained 13.10 g/kg organic matter content, 1.08 g/kg total N content, 67.98 mg/kg available N, 136.55 mg/kg available K, and 40.21 mg/kg Olsen-P. To provide the plants' required nutrients, 1.5 g N, 0.9 g P_2O_5, and 1.6 g K_2O per pot were homogenously mixed into the soil before sowing. After emergence, there were four seedlings for each pot.

After sowing, all maize plants were transferred to a suitable temperature (25 °C/17 °C, daily maximum/minimum temperature) and maintained at 70–80% of soil water holding capacity (SWHC). Before priming, the soil moisture of pots destined to drought and combined stress priming was gradually adjusted to 50–60% SWHC. The detailed process of stress priming is shown in Figure 1A. The first step was the transfer of heat- and

combined-stress-primed seedlings to the high-temperature growth chamber (33 °C/25 °C), and the exposure to drought stress of the drought-stress-primed seedlings. Following the first priming, heat- and combined-stress-primed seedlings were moved to the control growth chamber, and then heat-, drought-, and combined-stressed seedlings were subjected to temperature recovery (25 °C/17 °C), moisture recovery (70–80% SWHC), and temperature + moisture recovery for seven days, respectively. Meteorological data from 1990 to 2019 (http://data.cma.cn (accessed on 15 August 2019)) for the major maize-producing areas in the Guanzhong Plain of China showed that the average value of extreme high temperatures during the maize seedling stage was maintained at approximately 36 °C. Therefore, the maximum temperature in high-temperature growth during the subsequent stress was set to 36 °C to simulate the potential trend of increased temperatures in northwest China occurring during the maize seedling period. A second stress priming was performed once the first recovery process was completed (30 June 2020). The priming and recovery processes were kept consistent with the first processes. When the second priming was completed, both the primed and non-primed seedlings (except the control plants) were subjected to more severe stresses for six days, including heat stress (36 °C/28 °C), drought stress (45–55% SWHC), and their combined stress. During the subsequent stress, seven treatments were established with fifteen replicates (pots) per treatment (Figure 1A). Both growth chambers maintained consistent environmental conditions, with a light duration of 14 h, a relative humidity of 60–65%, and a photosynthetically active radiation (PAR) of 1100 μmol m^{-2} s^{-1}. The soil water content of potted plants was determined by weighing, and irrigation was performed from 17:00 to 18:30 (Figure 1B).

2.2. Sampling and Measurement

2.2.1. Sampling Method

Maize seedlings were sampled to measure the aboveground growth traits after the second recovery (ASR) and at the 6th day after stress (6 DAS). At ASR, the 3rd day after stress (3 DAS), and 6 DAS, a portion of the collected leaf samples was immediately analyzed for stomatal morphology and membrane damage index. The remaining leaf samples were cryopreserved (−80 °C) to determine chlorophyll and carotenoid contents and enzyme activities, as well as malondialdehyde, superoxide anion, and osmoregulation substance contents. Five independent replications were performed for the measurements of the above physiological traits. Leaf samples were selected from the last fully expanded leaf.

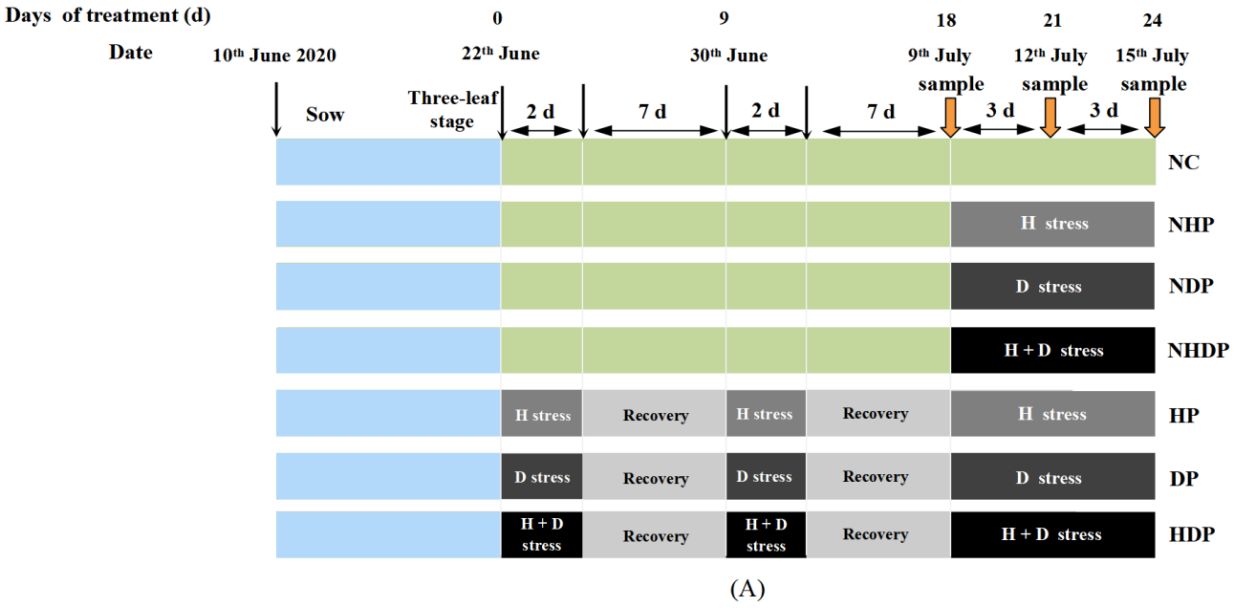


Figure 1. *Cont.*

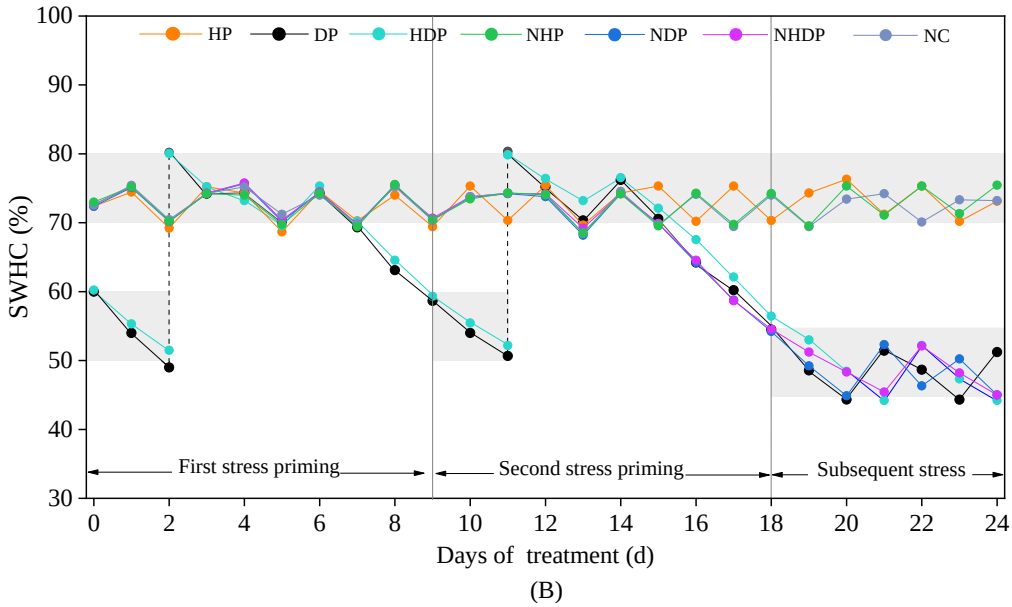


Figure 1. (**A**) Detailed representation of experimental design and treatments. HP, heat priming + later heat stress; DP, drought priming + later heat stress; HDP, combined stress priming + later combined stress; NHP, no heat priming + no later heat stress; NDP, no drought priming + no later drought stress; NHDP, no combined stress priming + no later combined stress; NC, no priming + no later stress. (**B**) Changes in the soil water content.

2.2.2. Growth Traits

The injury degree of maize seedlings was recorded at ASR and 6 DAS. The 5 levels of injury were as follows: (level 0) no injury symptoms; (level 1) yellowing on 1 or 2 leaves; (level 2) yellowing on all leaves; (level 3) 1 or 2 leaves wilting and falling off; and (level 4) all leaves withered and the whole seedling died. For each treatment, six plants from different pots were measured. The injury index was obtained following the method of He [25].

At ASR and 6 DAS, 6 plants were chosen per treatment from distinct pots. A measuring ruler was used to determine the plant height. All leaves were separated from individual plants, and the leaves were scanned using ImageJ software to calculate leaf area. The aboveground dry mass was determined by drying samples of aboveground parts to a constant mass at 80 °C. Between the two sampling stages, the relative growth rate was calculated as the rate of accumulation per unit of existing dry mass [26].

2.2.3. Imprinted Sampling and Microscopic Observation

Three leaves per treatment were selected for stomatal morphological analysis. Colorless and transparent nail polish was applied to the adaxial surface of the median portion of leaves to collect imprint samples of stoma (5 mm × 5 mm). The slides with samples were observed with a Leica optical microscope (Leica Microsystems, Germany) under ×200 (observation of number of stomata) and ×1000 (observation of stomatal length and width) magnification. The slides with samples were observed with a Leica optical microscope. Three visual fields were chosen per leaf, and three photos were taken for each visual field. Five photos were randomly selected to measure the length and width of stomata using Motic Images Plus 2.0 software. Stomatal density was calculated as the total number of stomata per area using Image J software. The formula for calculating stomatal density was as follows [27]:

$$\text{Stomatal density (number/mm}^2\text{)} = \frac{\text{stomatal number in visual field}}{\text{Visual field area}}$$

2.2.4. Leaf Pigment Content and Gas Exchange

A fresh leaf sample (0.5 g) was harvested and chopped with a razor blade. The leaf tissue was extracted in 15 mL of 96% ethanol with gentle agitation for 12 h at room temperature in aluminum-foil-wrapped bottles. Ethanol extracts were separated from tissue debris with filter paper. The tissue was rinsed with 96% ethanol to completely extract residual pigment. The extract and rinse were combined and brought to 25 mL final volume in 96% ethanol. Chlorophyll a (Chla), chlorophyll b (Chlb), and carotenoid (Car) absorbance was measured at 665, 649, and 470 nm using a spectrophotometer. The contents of Chla, Chlb, and Car were assessed with the methods proposed by Lichtenthaler and Wellburn [28]. At ASR, 3 DAS, and 6 DAS, photosynthetic rate (P_n), stomatal conductance (g_s), and transpiration rate (E) were determined using a portable photosynthesis system (LI-6800, LI-COR, NE, USA). All measurements were carried out at a relative humidity of 60%, a CO_2 concentration of about 400 μmol mol^{-1}, and a PAR of 1100 μmol m^{-2} s^{-1}. Instantaneous water use efficiency (WUE_i) is the ratio of P_n and E.

2.2.5. The Membrane Injury Index and MDA and O_2^- Contents

Membrane injury index (MII) was measured using the method of Deshmukh [29]. Leaf samples (0.3 g) were cut into uniformly sized squares and placed in test tubes containing 10 mL of deionized water in two sets. One set was kept at 30 °C for 30 min and another set at 100 °C in a boiling water bath for 20 min, and their electric conductivities, E1 and E2, respectively, were measured. MII was calculated as the ratio of E1/E2. The malondialdehyde (MDA) content was assessed following the protocol of Du and Bramlage [30]. Leaf samples (0.5 g) were homogenized in 10 mL of 10% TCA. The homogenate was centrifuged at 4000 rpm at 4 °C for 15 min. A quantity of 2 mL of 0.6% thiobarbituric acid (TBA) was added to the supernatant, and the mixture was incubated at 100 °C for 20 min. After the tube was centrifuged at 4000 rpm at 4 °C for 10 min, the absorbance values of the supernatant at 532, 600, and 450 nm were recorded. The determination of superoxide anion content was conducted following Wu [31]. A quantity of 1.0 mL of supernatant was mixed with 1.0 mL of phosphate buffer and 0.2 mL of hydroxylamine hydrochloride. The mixture was incubated at 25 °C for 30 min. Then 1 mL each of naphthylamine and aminobenzenesulfonic acid was supplemented and incubated for 15 min at 30 °C. The absorbance at 530 nm was measured.

2.2.6. Antioxidant Enzyme Activity

A fresh leaf sample (0.5 g) was frozen in liquid nitrogen and then homogenized using 7 mL of 50 $mmol^{-1}$ potassium phosphate buffer (pH 7.0, containing 0.1 $mmol^{-1}$ EDTA) with a prechilled mortar and pestle. The homogenate was centrifuged at 8000 rpm for 20 min at 4 °C, and the supernatant was used for the assay of SOD, POD, and CAT activities. Superoxide dismutase (SOD, EC 1.15.1.1) activity was evaluated by determining the reductive inhibition of nitro blue tetrazolium (NBT) [32]. A quantity of 3 mL of reaction mixture contained 50 $mmmol^{-1}$ potassium phosphate buffer (pH 7.0), 13 $mmmol^{-1}$ methionine, 75 $\mu mmol^{-1}$ NBT, 2 $\mu mmol^{-1}$ riboflavin, and 0.2 mL of enzyme extract. One unit of SOD activity was estimated as a 50% reduction in NBT at 560 nm. Peroxidase (POD, EC 1.11.1.7) was assessed in accordance with Kochba [33]. For POD measurement, the enzyme extract was allowed to react with the mixture containing guaiacol acid, phosphate buffer (50 mmol/L phosphate buffer and 30% hydrogen peroxide), and POD activity was measured at 470 nm and expressed as an increase of 0.01 OD value per minute as one enzyme activity unit. Catalase (CAT, EC 1.11.1.6) activity was evaluated by a process suggested by Dhindsa [34]. The enzyme extract was allowed to react with a mixture containing 50 mmol/L phosphate buffer and 15 mmol/L hydrogen peroxide. The absorbance values were measured at 240 nm, and the enzyme activity was determined by using a decrease of 0.01 OD value per minute as a unit.

Glutathione reductase (GR, EC 1.6.4.2) activity was measured according to the methods of Schaedle and Bassham [35] with some modifications. GR was determined by measuring

NADPH oxidation at 340 nm for 3 min in a 1 mL analytical mixture containing potassium phosphate buffer (50 mmol/L), 2 mmol/L Na_2EDTA, 0.15 mmol/L NADPH, 0.5 mmol/L GSSG (glutathione oxidized), and 225 µL enzyme extract. The reaction was initiated by adding NADPH. Background correction was performed using the absorbance of the analytical mixture without NADPH at 340 nm.

A method proposed by Nakano and Asada [36] was used to determine the activity of ascorbate peroxidase (APX, EC 1.11.1.11). The leaf samples (0.5 g) were ground and extracted by adding precooled 50 mmol/L K_2HPO_4-KH_2PO_4 buffer at 1:3 (w/v), and the filtrate was centrifuged at 8000 rpm at 4 °C for 20 min. The supernatant was used as the enzyme extract for determination. The reaction mixture contained 650 µL of potassium phosphate buffer (80 mmol/L), 100 µL of 5 mmol/L ascorbate, 100 µL of 1 mol/L EDTA, 100 µL of 1 mmol/L hydrogen peroxide, and 50 µL of the enzyme extract. APX activity was determined by monitoring the ascorbate oxidation rate at 290 nm at 30 °C for 60 s with a spectrophotometer.

2.2.7. Total Protein and Soluble Protein Content

For protein extraction, plant tissue was extracted with 0.1 $mmol^{-1}$ Na-phosphate pH 6.4 extraction buffer containing 1 $mmol^{-1}$ PMSF (phenylmethyl sulphonyl fluoride) and 0.2% TWEEN. The homogenate was then centrifuged for 15 min at 8000 rpm, and the supernatant was used to determine the total protein content. Total protein content was determined by the method of Lowry [37], using bovine serum albumin (BSA) as a standard. The content of soluble protein was measured as described in Bradford [38] with some modifications. Leaf samples (0.5 g) were homogenized in 5 mL of phosphate buffer (pH 7.8, 50 mmol L^{-1}). The protein extract was obtained from the supernatant after centrifuging the homogenate at 4 °C for 15 min at 8000 rpm. After that, a solution of Coomassie brilliant blue G-250 was mixed with 0.1 mL of protein extract. After standing for 2 min, the absorbance of the mixture was measured at 595 nm.

2.2.8. Soluble Sugar and Proline Contents

The content of soluble sugar (SS) was assessed as described in Li and He [39]. The soluble sugar (SS) content was assessed by the anthrone method. Leaf samples (0.5 g) were boiled with 10 mL of 80% (v/v) ethanol in a water bath (100 °C) for 1 h. After cooling, the supernatant solution was filtered through Whatman No. 10 filter paper. The residue was re-extracted with 10 mL ethanol as described above for 1 h, and the supernatant was filtered again. Finally, the residue was boiled with 10 mL double-distilled water, and a clear solution was collected. All supernatants were mixed, and the volume was increased to 50 mL. A quantity of 1 mL of the sugar sample was added to 4 mL of anthrone reagent. The mixture was heated in a boiling water bath (100 °C) for 8 min, followed by cooling. The optical density of the cooled solution at 630 nm was recorded against a reagent blank. The content of proline (Pro) was assessed as described in Bate [40]. A quantity of 0.5 g leaf material was immersed in 7 mL of 3% sulfosalicylic acid, then extracted in a boiling water bath for 15 min. A quantity of 1.0 mL of supernatant was mixed with 1.0 mL glacial acetic acid and 2.0 mL acidic ninhydrin, then heated in a boiling water bath for 30 min, and the absorbance at 520 nm was determined.

2.3. Data Analysis

SPSS (21.0, SPSS Inc., Chicago, IL, USA) was used for the analysis of variance (ANOVA), correlation analysis, and receiver operating characteristic (ROC) curve analysis [41]. The varimax rotation method was used to rotate the principal component for principal component analysis. The differences among treatments were analyzed following Duncan's method. Pearson correlations were used to evaluate the correlations. MetaboAnalyst 5.0 software was used to draw the heatmap. Pearson correlation analysis was used to assess the correlations between growth and physiological traits using Origin 2021 (Origin

Lab Corporation, Northampton, MA, USA). Principal component analysis and clustering heatmap analysis were performed in MetaboAnalyst 5.0 software [42].

3. Results

3.1. Injury Index and Aboveground Growth

The HDP seedlings had significantly lower plant heights than the control (NC) seedlings at ASR, and the HDP seedlings exhibited a more pronounced reduction in leaf area and aboveground dry mass than the DP seedlings (Table 1). Subsequent stresses significantly increased the injury index of maize seedlings. Compared with the NHP, NDP, and NHDP seedlings, the injury index in the HDP plants decreased by 25.64% at 6 DAS. Later single and combined stresses significantly decreased aboveground growth traits and the relative growth rate in maize seedlings, especially under combined stresses. Nonetheless, combined stress priming could alleviate the growth inhibition induced by stress. At 6 DAS, plant height and leaf area in the HDP seedlings increased by 0.32% and 7.95%, respectively, compared with the HDP seedlings. The beneficial effects of stress priming were also observed in the relative growth rate. The relative growth rate of the HDP seedlings was 1.86-fold higher than that of the NHDP seedlings at 6 DAS.

Table 1. Effects of stress priming on the injury index and aboveground growth traits of seedlings under stress conditions.

Periods	Treatments	Injury Index (%)	Plant Height (cm)	Leaf Area (cm^2/Plant)	Aboveground Dry Mass (g/Plant)	Relative Growth Rate (mg g^{-1} day^{-1})
ASR	HP	0.0 ± 0.00 a	64.53 ± 2.16 a	394.18 ± 9.12 a	1.22 ± 0.06a	-
	DP	0.0 ± 0.00 a	63.01 ± 1.34 b	378.67 ± 8.32 b	1.17 ± 0.11 b	-
	HDP	0.0 ± 0.00 a	61.15 ± 1.55 c	374.21 ± 15.22 b	1.13 ± 0.08 c	-
	NC	0.0 ± 0.00 a	63.21 ± 0.56 ab	401.11 ± 12.42 a	1.25 ± 0.03 a	-
6 DAS	HP	26.5 ± 0.50 d	74.63 ± 2.13 b	618.19 ± 6.80 b	1.96 ± 0.13 b	123.33 ± 4.32 b
	DP	25.4 ± 0.34 d	72.54 ± 1.22 c	590.76 ± 14.20 c	1.88 ± 0.08 c	118.33 ± 3.78 b
	HDP	32.2 ± 1.15 c	68.89 ± 3.21 f	553.21 ± 16.21 d	1.65 ± 0.15 e	86.67 ± 1.88 c
	NC	0.0 ± 0.00 e	78.12 ± 1.28 a	701.15 ± 5.40 a	2.35 ± 0.11 a	183.33 ± 4.21 a
	NHP	31.0 ± 1.00 c	71.56 ± 1.77 d	576.17 ± 21.30 c	1.80 ± 0.09 c	91.67 ± 1.43 c
	NDP	34.8 ± 1.32 b	69.77 ± 0.99 e	555.32 ± 16.30 d	1.71 ± 0.08 d	76.67 ± 3.21 d
	NHDP	43.3 ± 2.48 a	68.67 ± 1.23 f	512.45 ± 7.80 e	1.53 ± 0.04 f	46.67 ± 1.98 e

HP, heat priming + later heat stress; DP, drought priming + later drought stress; HDP, combined stress priming + later combined stress; NHP, no heat priming + later heat stress; NDP, no drought priming + later drought stress; NHDP, no combined stress priming + later combined stress; NC, no stress priming + no later stress. Data are means ± SD (n = 6), where different letters designate significant differences at the 0.05 level.

3.2. Leaf Pigment Contents

Compared with the NC seedlings, the HP, DP, and HDP seedlings displayed a considerable reduction in leaf pigment contents at ASR (Figure 2). Subsequent stresses decreased Chla, Chlb, and Car contents but increased Chla/b. At 3 DAS, the HP, DP, and HDP seedlings had 6.80%, 8.98%, and 6.81% greater Chlb contents than the NHP, NDP, and NHDP seedlings, respectively. Combined stress priming had greater positive effects on Chla, Chlb, and Car contents at 6 DAS than at 3 DAS, and Chla, Chlb, and Car contents in the HDP seedlings increased by 31.38%, 37.61%, and 38.75%, respectively, compared with those in the NHDP seedlings. A significant reduction in Chla/b was observed in the HDP seedlings compared with the NHDP seedlings at 3 DAS. At 6 DAS, the Chla, Chlb, and Car contents of primed seedlings were significantly higher than those of the NP seedlings, respectively; lower Chla/b was found in the HP and HDP seedlings than in the NHP and NHDP seedlings, respectively.

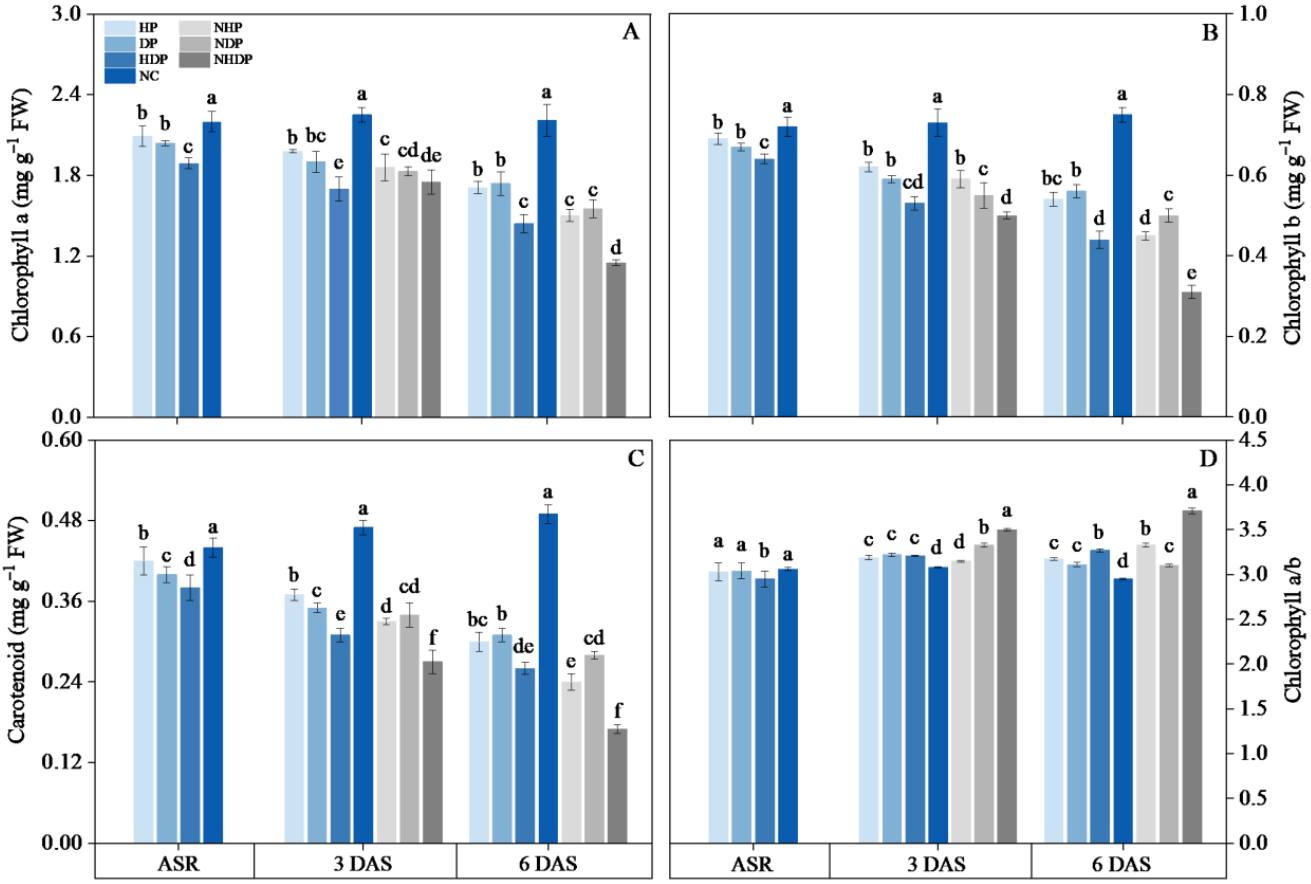


Figure 2. Effects of stress priming on chlorophyll a (Chla, **A**), chlorophyll b (Chlb, **B**), carotenoid (Car, **C**) contents, and chlorophyll a/b (Chla/b, **D**) of seedlings under stress conditions. HP, heat priming + later heat stress; DP, drought priming + later drought stress; HDP, combined stress priming + later combined stress; NHP, no heat priming + later heat stress; NDP, no drought priming + later drought stress; NHDP, no combined stress priming + later combined stress; NC, no stress priming + no later stress. The bars represent the means ± SD (n = 5), where different letters designate significant differences at the 0.05 level.

3.3. Stomatal Morphology and Gas Exchange

Compared with the NC seedlings, stomatal length and stomatal width were markedly reduced by later heat, drought, and combined stress, while stomatal density increased (Figure 3). Compared with no priming, individual and combined stress priming can mitigate the decrease in stomatal length and stomatal width. In comparison with the NHDP seedlings, the stomatal length and stomatal width in the HDP seedlings increased by 11.90% and 32.93% at 3 DAS, respectively. The effect of combined stress priming on stomatal morphology was more pronounced at 6 DAS than at 3 DAS. The HDP seedlings had 23.05% greater stomatal length and 78.57% greater stomatal width at 6 DAS than the NHDP seedlings. A significant reduction in stomatal density was found in the P seedlings compared with the NP seedlings. At 6 DAS, the stomatal density of the HDP seedlings decreased by 10.71% compared with the NHDP seedlings.

The DP and HDP seedlings exhibited lower P_n and g_s than the NC seedlings at ASR. Subsequent single and combined stresses caused significant decreases in P_n, g_s, and E in the primed (P) and non-primed (NP) seedlings (Figure 4). Nonetheless, stress priming mitigated the stress-induced decreases in P_n, g_s, and E. The HP, DP, and HDP seedlings had significantly greater g_s and E than the NHP, NDP, and NHDP seedlings at 3 DAS, respectively. The beneficial effects of stress priming became increasingly pronounced with increased stress duration. The HDP seedlings showed a 41.91% increase in P_n and a 29.29%

increase in g_s at 6 DAS, compared with the NHDP seedlings. A similar regulation of priming was found in *E* (Figure 4A–C). The WUE_i increased significantly under later heat, drought, and combined stress conditions. At 3 DAS, the WUE_i in the P seedlings was lower than that in the NP seedlings, while the WUE_i was 1.94%, 3.89%, and 8.17% higher in the HP, DP, and HDP seedlings at 6 DAS than that in the NHP, NDP, and NHDP seedlings, respectively (Figure 4D).

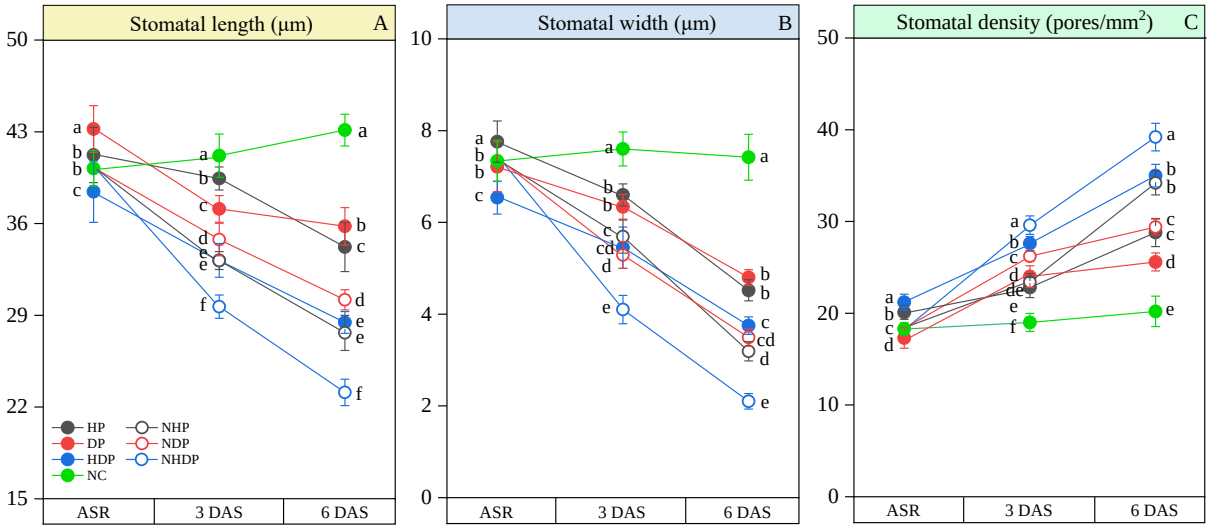


Figure 3. Effects of stress priming on the stomatal length (**A**), stomatal width (**B**), and stomatal density (**C**) of seedlings under stress conditions. HP, heat priming + later heat stress; DP, drought priming + later drought stress; HDP, combined stress priming + later combined stress; NHP, no heat priming + later heat stress; NDP, no drought priming + later drought stress; NHDP, no combined stress priming + later combined stress; NC, no stress priming + no later stress. Data are means ± SD (n = 15), where different letters designate significant differences at the 0.05 level.

3.4. Superoxide Anion and Malondialdehyde Contents

At ASR, MDA and O_2^- contents were significantly higher in the HDP seedlings than in the HP and DP seedlings (Figure 5). Subsequent stresses resulted in a sustained increase in MDA and O_2^- contents. At 3 DAS, O_2^- content in the HP, DP, and HDP seedlings decreased by 22.98%, 20.59%, and 17.51%, respectively, compared with that in the NHP, NDP, and NHDP seedlings, and MDA contents were markedly reduced in the HDP seedlings in comparison with the NHDP at 3 DAS (Figure 5A). At 6 DAS, O_2^- content of the HP, DP, and HDP seedlings was reduced by 27.23%, 19.23%, and 27.70%, respectively, compared with that of the NHP, NDP, and NHDP seedlings. Notably, compared with the NHDP seedlings, the HDP seedlings had a 36.77% increase in MDA content at 6 DAS (Figure 5B).

3.5. Antioxidant Enzyme Activity

The DP and HDP seedlings had significantly higher SOD, CAT, GR, and APX activities at ASR, compared with the NC seedlings (Figure 6). Interestingly, the P seedlings exhibited greater antioxidant enzyme activities than the NP seedlings under the subsequent stress. At 6 DAS, the HP, DP, and HDP seedlings showed increases of 15.84%, 21.78%, and 24.10% in SOD activity, respectively, compared with the NHP, NDP, and NHDP seedlings. Compared with the NP seedlings, the HDP seedlings had more significant increases in POD, CAT, and GR activities at 6 DAS than the HP and DP seedlings. POD, GR, and APX activities of the HDP seedlings increased by 29.58%, 21.33%, and 22.11%, respectively, when compared with the NHDP seedlings. Combined stress priming had a more significant effect on CAT activity at 6 DAS, resulting in the HDP seedlings having 54.28% higher CAT activity than the NHDP seedlings.

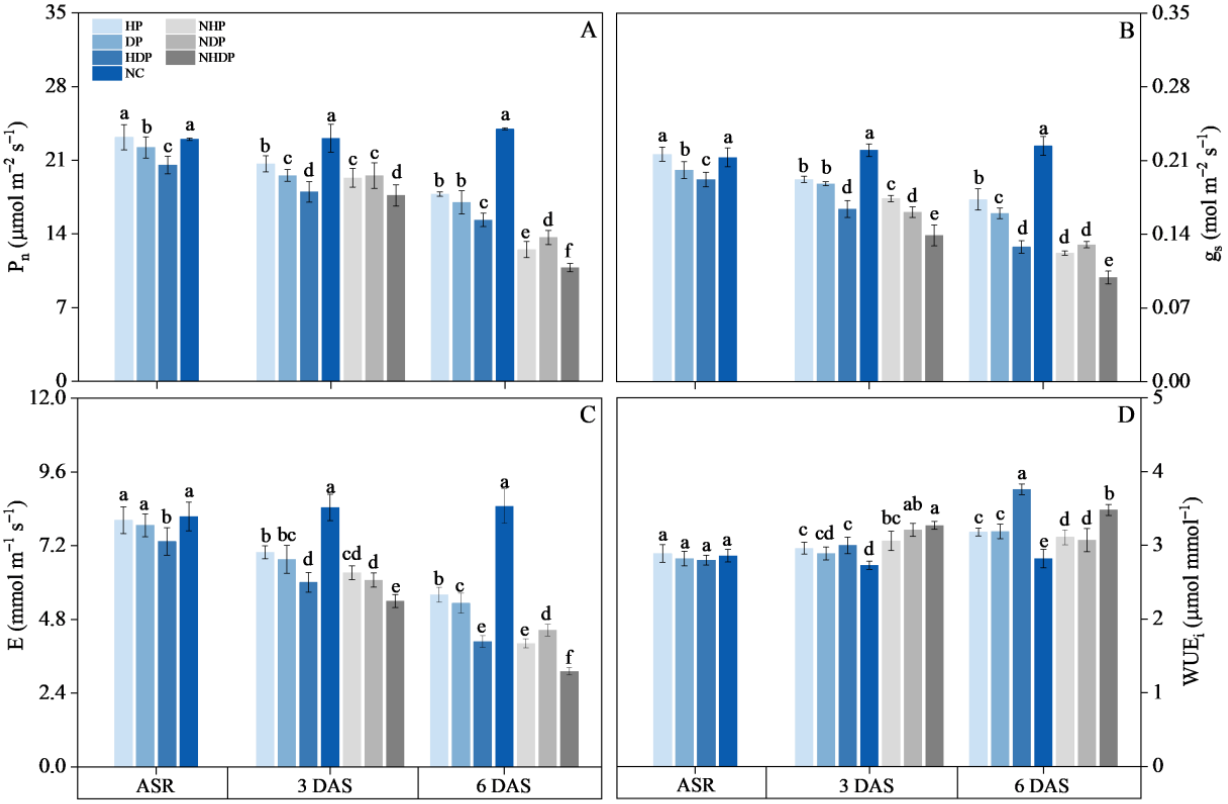


Figure 4. Effects of stress priming on the photosynthesis rate (P_n, **A**), stomatal conductance (g_s, **B**), transpiration rate (E, **C**), and instantaneous water use efficiency (WUE_i, **D**) of seedlings under stress conditions. HP, heat priming + later heat stress; DP, drought priming + later drought stress; HDP, combined stress priming + later combined stress; NHP, no heat priming + later heat stress; NDP, no drought priming + later drought stress; NHDP, no combined stress priming + later combined stress; NC, no stress priming + no later stress. The bars represent means ± SD (n = 10), where different letters designate significant differences at the 0.05 level.

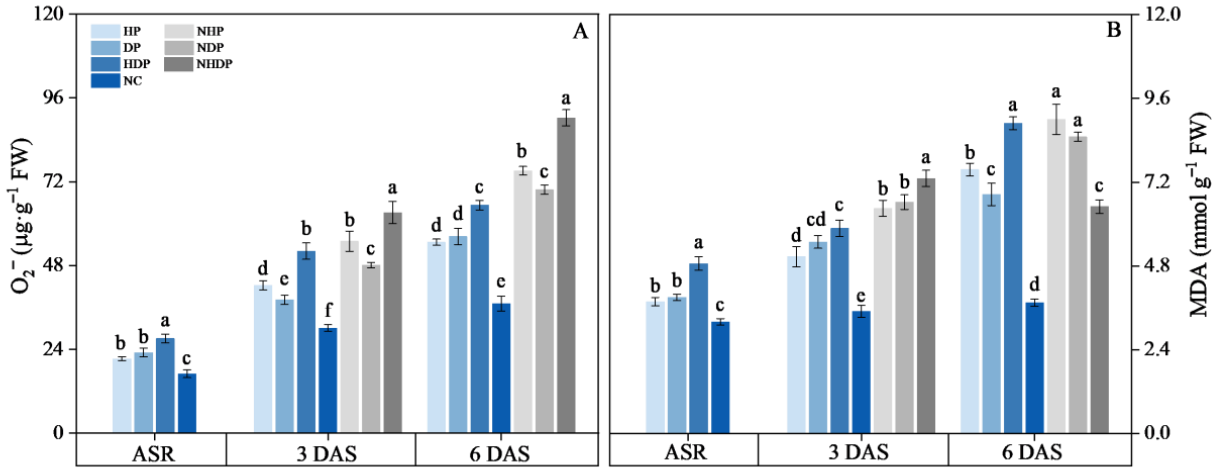


Figure 5. Effects of stress priming on superoxide anion (O_2^-, **A**) and malondialdehyde (MDA, **B**) contents of seedlings under stress conditions. HP, heat priming + later heat stress; DP, drought priming + later drought stress; HDP, combined stress priming + later combined stress; NHP, no heat priming + later heat stress; NDP, no drought priming + later drought stress; NHDP, no combined stress priming + later combined stress; NC, no stress priming + no later stress. The bars represent the ±SDs (n = 5), where different letters designate significant differences at the 0.05 level.

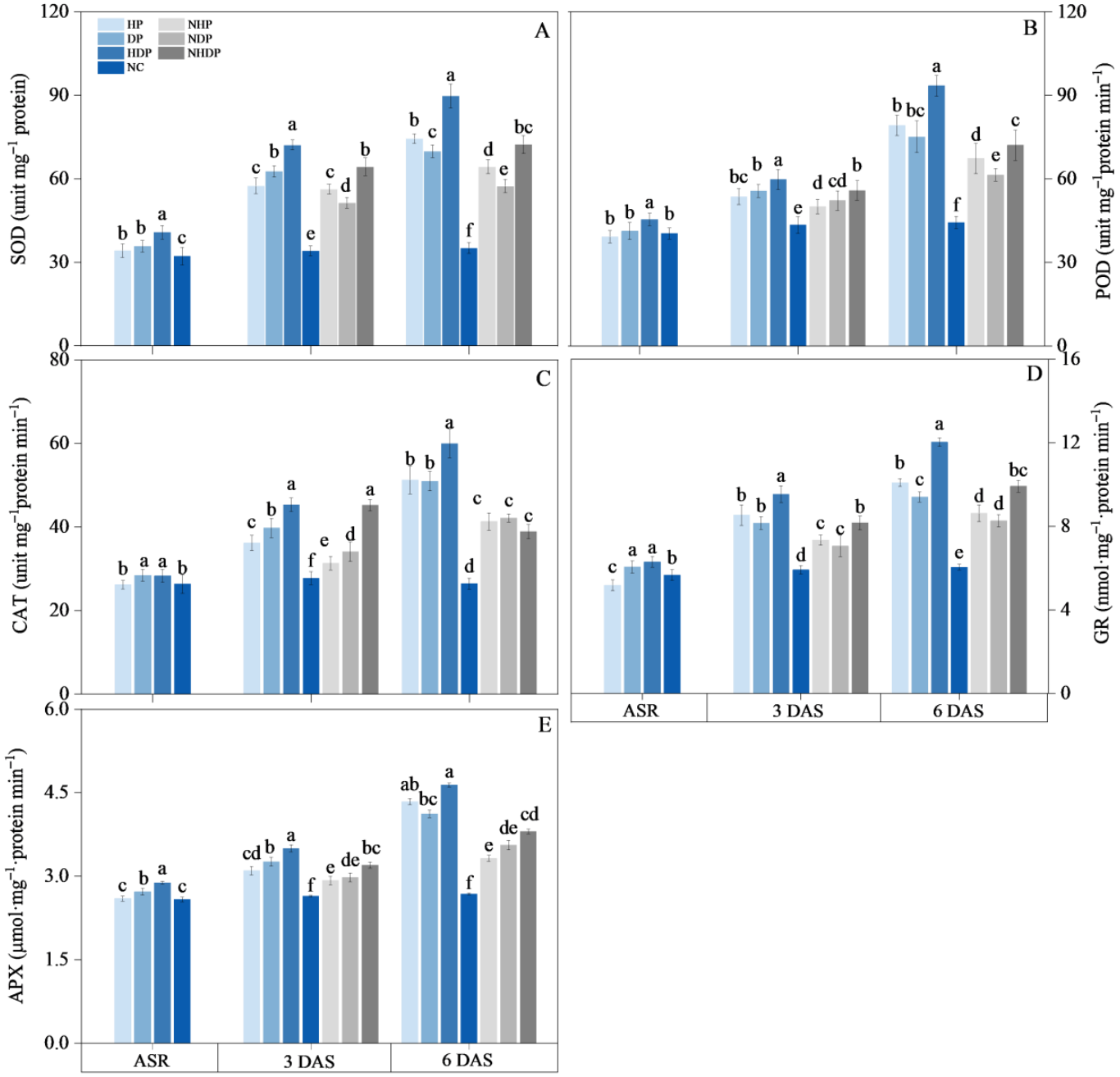


Figure 6. Effects of stress priming on superoxide dismutase (SOD, **A**), peroxidase (POD, **B**), catalase (CAT, **C**), glutathione reductase (GR, **D**), and ascorbate peroxidase (APX, **E**) activities of seedlings under stress conditions. HP, heat priming + later heat stress; DP, drought priming + later drought stress; HDP, combined stress priming + later combined stress; NHP, no heat priming + later heat stress; NDP, no drought priming + later drought stress; NHDP, no combined stress priming + later combined stress; NC, no stress priming + no later stress. The bars represent means ± SD (n = 5), where different letters designate significant differences at the 0.05 level.

3.6. The Contents of Osmoregulatory Substance and Membrane Injury Index

Subsequent single and combined stresses caused a remarkable rise in SP, SS, and Pro contents, especially under combined stress. Meanwhile, stress priming strengthened this increase mechanism (Figure 7). The differences in osmoregulatory substance contents between the P and NP seedlings gradually increased with increasing stress time. At 6 DAS, the HP, DP, and HDP plants had 17.92%, 42.29%, and 42.61% higher SP contents, 14.12%,

20.27%, and 11.90% higher SS contents, and 22.30%, 15.35%, and 19.76% higher Pro contents than the NHP, NDP, and NHDP seedlings, respectively. Later heat stress and drought stress increased the membrane injury index of maize seedlings, with heat stress increasing the index more than drought stress. Nevertheless, the P seedlings had a lower membrane injury index than the NP seedlings. The index in the HDP seedlings decreased by 13.60% at 3 DAS and by 10.16% at 6 DAS, compared with the NHDP seedlings.

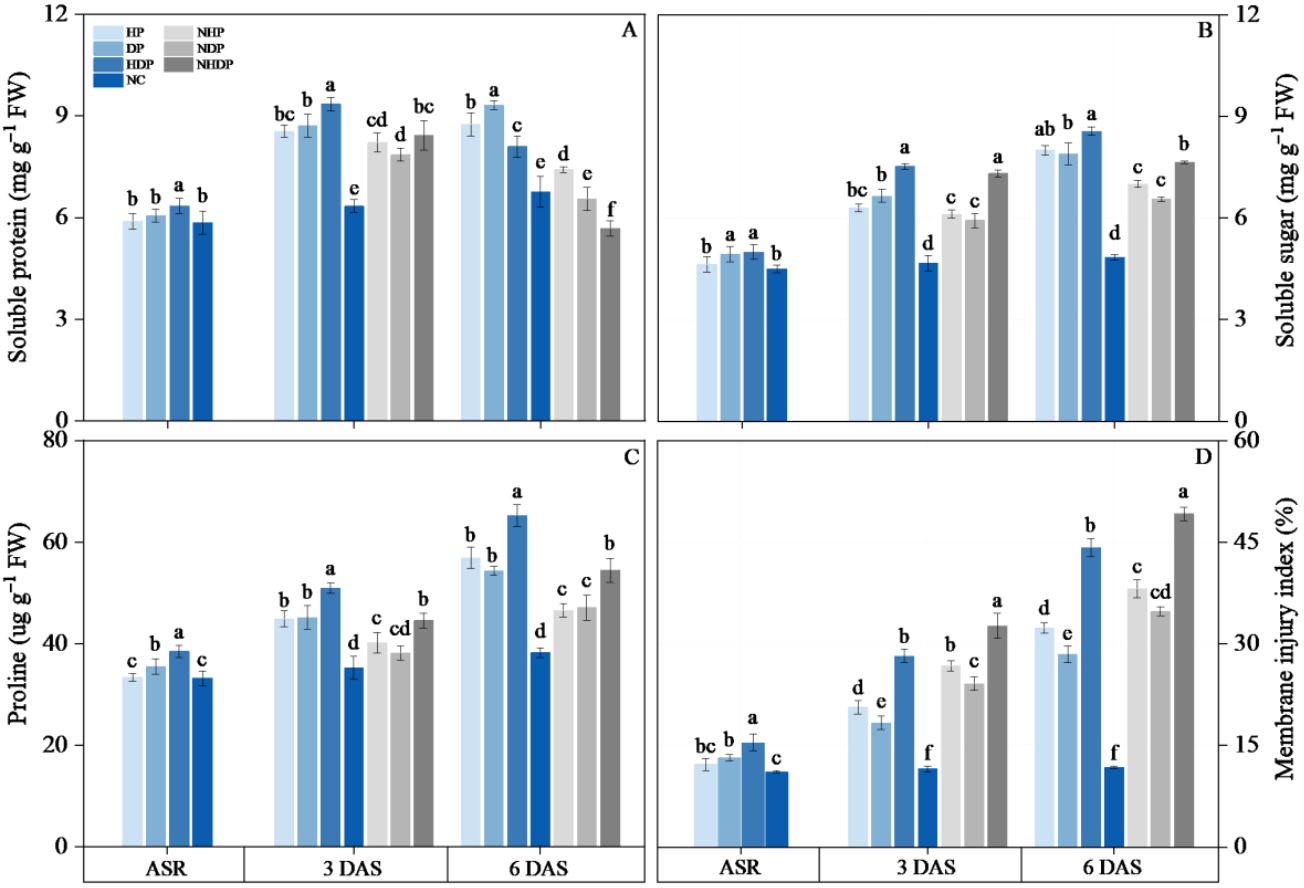


Figure 7. Effects of stress priming on soluble protein (SP, **A**), soluble sugar (SS, **B**), proline (Pro, **C**) contents, and membrane injury index (MII, **D**) of seedlings under stress conditions. HP, heat priming + later heat stress; DP, drought priming + later drought stress; HDP, combined stress priming + later combined stress; NHP, no heat priming + later heat stress; NDP, no drought priming + later drought stress; NHDP, no combined stress priming + later combined stress; NC, no stress priming + no later stress. The bars represent means ± SD (n = 5), where different letters designate significant differences at the 0.05 level.

3.7. Responses of Physiological Traits to Stress Priming

A heatmap analysis was performed to assess the changes in physiological traits between the N and NP seedlings (Figure 8A). Compared with no priming, stress priming markedly enhanced the antioxidant capacity of seedlings, which could be demonstrated by improved antioxidant enzyme activities, thereby reducing the levels of O_2^-, MDA, and MII. Stress priming effectively improved cellular osmoregulation, as indicated by increased SP, SS, and Pro contents. Furthermore, photosynthetic pigment contents and P_n were significantly elevated under stress-priming conditions. Overall, combined heat and drought priming was more effective than heat and drought stress priming in improving antioxidant enzyme activities.

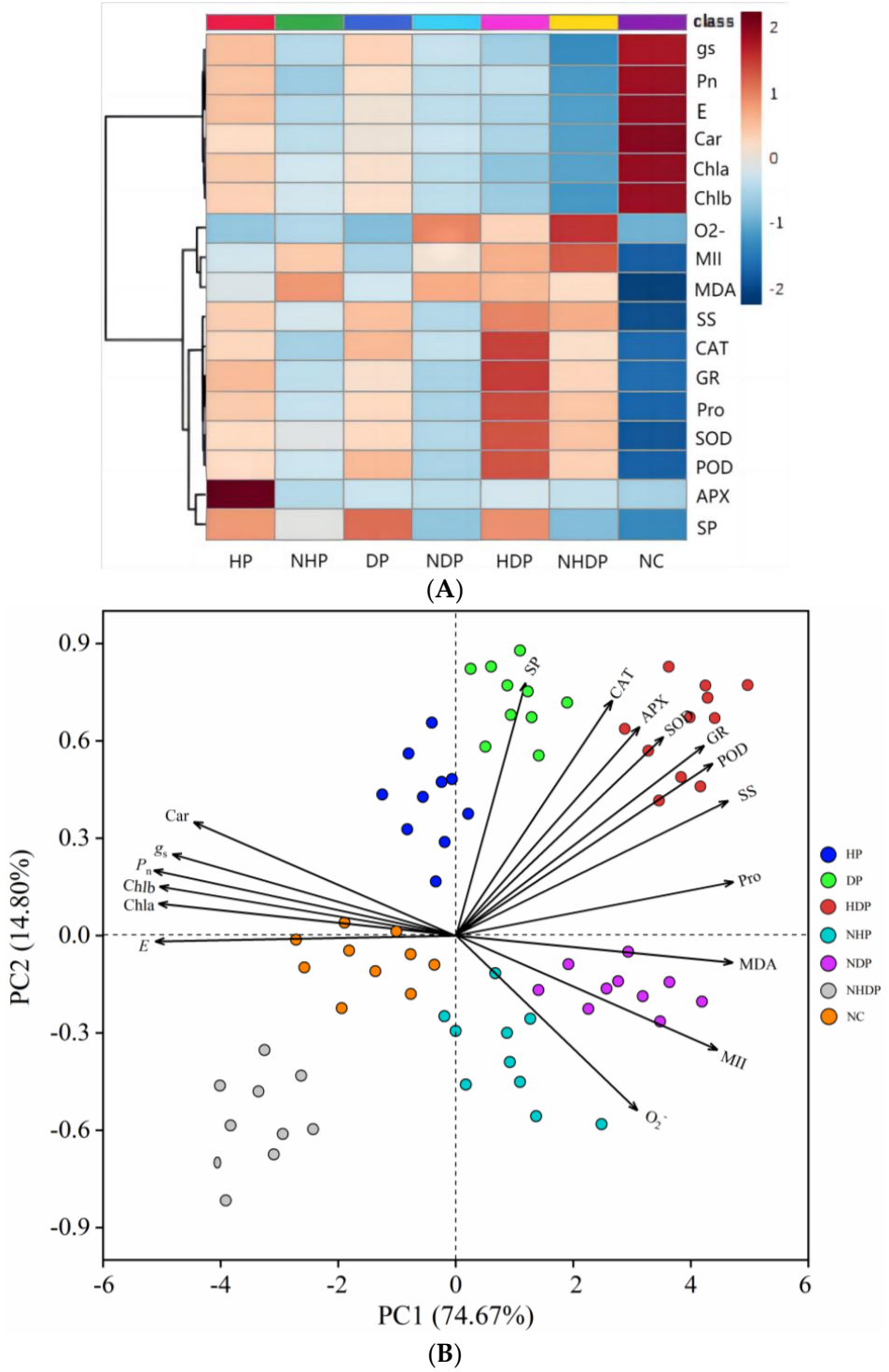
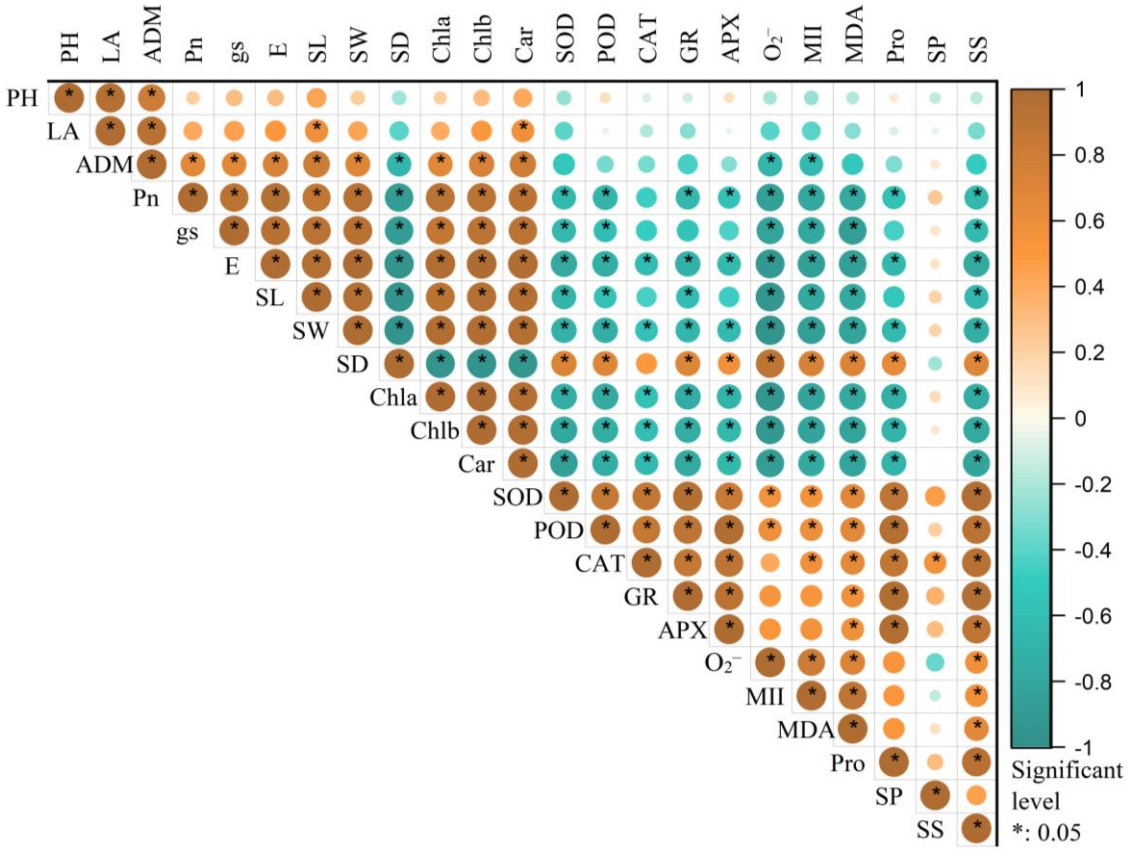


Figure 8. *Cont*.



(**C**)

Figure 8. (**A**) Heatmap analysis of physiological traits. (**B**) Principal component analysis of physiological traits. (**C**) The relationships between growth and physiological traits.

Principal component analysis (PCA) was conducted on physiological traits (Figure 8B). PC1 (74.67%) and PC2 (14.80%) accounted for 89.47% of the cumulative contribution. Gas exchange parameters and leaf pigments were concentrated as a group on the left horizontal axis of the biplot and segregated opposite from antioxidant enzymes, osmoregulatory substances, MDA, O_2^-, and MII. Interestingly, the data points of the HDP treatments were concentrated above the PC1 axis and closer to the vectors of antioxidant enzymes and osmoregulatory substances than the NHDP seedlings, suggesting that the P seedlings can respond positively to later combined stresses by improving ROS detoxification and osmoregulatory capacity. The data coordinates of the NHDP treatment were located on the lower left of the PC1 axis. This indicated that combined stress severely disrupted the physiological and metabolic activity of seedlings, thereby inhibiting the ability of the antioxidant detoxification system to scavenge superoxide radicals. All correlations between growth and physiological traits are shown in Figure 8C. We observed that P_n was positively correlated with aboveground dry mass and leaf pigment contents. There were significant positive correlations among stomatal length, stomatal width, and gas exchange parameters. Stomatal density was negatively correlated with gas exchange parameters and leaf pigment contents. Gas exchange parameters and leaf pigment contents were negatively correlated with the levels of MII, MDA, and O_2^-. Antioxidant enzyme activities increased linearly with increasing O_2^- and MDA contents.

3.8. ROC Analysis of Physiological Traits

The ROC (receiver operating characteristic) analysis was performed to examine whether antioxidant enzymes and osmoregulatory substances can identify later individual and combined stresses in maize plants (Table 2). AUC values are used to evaluate the

diagnostic capability of traits for different stresses, and greater values indicate greater diagnostic capability. The results indicated that antioxidant enzymes (0.772–0.971) had significant reliability in determining whether maize plants were subjected to heat stress, especially SOD and POD (0.941–0.971). Heat stress suffered by maize plants was more accurately detected by SS and Pro (0.829–0.838), whereas the capability of SP to detect this was restricted. Compared with other target traits, GR and SS (0.919–0.958) showed greater accuracy in detecting maize plants that experienced drought stress, and the ability of APX and SP to diagnose drought stress was relatively weak. The sensitivity of most physiological traits to combined heat and drought stresses reached significant levels, with the AUC values of SOD, GR, and Pro all above 0.90.

Table 2. The results of ROC in diagnosing heat, drought, and combined stress.

Traits	Heat Stress			Drought Stress			Combined Stress		
	AUC	YI	BT	AUC	YI	BT	AUC	YI	BT
SOD	0.941 **	0.829	54.88	0.751 *	0.576	50.35	0.907 **	0.845	65.17
POD	0.971 **	0.874	49.38	0.889 **	0.74	52.50	0.830 *	0.723	57.35
CAT	0.891 **	0.825	32.32	0.827 *	0.694	35.41	0.749 *	0.701	39.70
GR	0.824 *	0.699	7.44	0.958 **	0.886	7.20	0.934 **	0.815	8.31
APX	0.772 *	0.633	2.91	0.69 ns	0.576	3.02	0.759 *	0.631	3.25
SP	0.683 ns	0.423	7.57	0.705 ns	0.667	7.71	0.702 ns	0.583	8.11
SS	0.829 *	0.722	5.91	0.919 **	0.774	6.08	0.866 **	0.729	7.28
Pro	0.838 *	0.732	41.21	0.802 *	0.684	41.71	0.958 **	0.833	45.56

YI represents Youden's index; BT represents best threshold. ns: non-significance in statistics; '*' $p < 0.05$; '**' $p < 0.01$.

4. Discussion

Drought and heat stress accelerate the decomposition of pigments in maize leaves, leading to reduced chlorophyll content [43]. Here, the contents of Chla, Chlb, and Car decreased markedly in response to later drought and heat stress, and a combination of heat and drought stress resulted in greater reductions than individual stresses. Nevertheless, higher Chla and Chlb contents in the P seedlings may compensate for this loss caused by later stresses, suggesting that heat and drought priming were linked to enhanced synthesis and slower decomposition of chlorophyll. Interestingly, the primed plants showed a significant higher accumulation of Chla and Chlb compared with non-primed plants. The ability of the primed plants to maintain higher chlorophyll may allow plants to deliver sufficient energy to deal with the energy-consuming adaptations to individual heat and drought and their combined stress [8]. Here, the observed higher Chla and Chlb contents could potentially lead to an increase in the transfer of excitation energy to the PSII and a higher capacity of light harvesting, which directly leads to a higher P_n. This is well supported by the finding that the photosynthetic rate is positively related to Chla and Chlb contents in plants [44]. Moreover, a combination of stress priming effectively mitigated the degradation of Car, as evidenced by a higher Car content in the P seedlings than in the NP seedlings. Similarly, improved chlorophyll and carotenoid contents were observed in wheat plants with heat priming [24] and olive plants with drought priming [12].

The reduction in photosynthesis caused by drought is primarily due to decreased stomatal conductance and an impaired photosynthetic apparatus [45]. In this study, P_n, g_s, and E were markedly depressed by later heat, drought, and combined stress (Figure 4). When soil moisture is moderately deficient and the ambient temperature is too high, the leaves choose to close the stomata [46], which also reduces the inflow of CO_2 into the leaves. The decreased availability of CO_2 affects the photosynthesis electron transport pathway of chloroplasts, resulting in a reduced photosynthetic rate [47]. Higher P_n in the combined stress-primed seedlings indicated effective alleviation of photoinhibition (Figure 4). Leaf stomata are the key to controlling the transportation of CO_2 and water vapor. Combined stress-primed plants had greater stomatal length and width than the

primed plants during the subsequent stress. We observed that the combined stress-primed plants had larger guard cells than the non-primed plants, which resulted in significantly higher stomatal lengths in the primed plants than in the non-primed plants; meanwhile, the greater stomatal width was mainly due to the greater curvature of the guard cells, which led to a larger pore between them. Therefore, the primed plants had greater stomatal openings in leaves (Figures 3 and 4). The stomatal opening is a mechanism for leaf cooling, which means that an increase in stomatal openings leads to a decrease in leaf temperature [48]. Researchers have demonstrated that one strategy for enhancing plant tolerance to heat and drought stress is to increase transpiration by improving stomatal size, which reduces leaf temperature, thereby improving leaf cooling capacity [20,49].

Stress priming was effective in alleviating the negative impacts of later stresses on maize plants, as indicated by improved aboveground growth traits in the P seedlings. The reduction in the injury index further confirmed the improvement in maize growth. For terrestrial plants, one of the main advantages of an induced priming state is the activation of the stress-tolerance mechanism, which enables the formation of larger individuals to resist subsequent stress conditions [50]. We also found that the growth traits of maize were closely related to photosynthesis (Figure 8C). The HP, DP, and HDP seedlings had better photosynthetic and growth performances than the NP seedlings, indicating that stress priming could mitigate the adverse effects of single and combined stresses on maize growth by improving photosynthesis. Our findings are supported by a study by Engels [51], who believed that a higher photosynthetic rate increases carbohydrate production, which contributes to improving biomass accumulation and source–sink capacity.

Reactive oxygen species (ROS) remarkably accumulate when plants are exposed to droughts and high temperatures, resulting in increased lipid peroxidation, thereby reducing membrane integrity and damaging the cellular structure [52]. Here, subsequent combined stress significantly increased the MDA level as a consequence of the excessive accumulation of O_2^-. Nonetheless, the HDP seedlings maintained a lower O_2^- content than the NHDP seedlings, which greatly alleviated the oxidative injury induced by drought and heat stress. To reduce ROS damage to cell membranes, it is essential to increase the activity of antioxidant enzymes that scavenge ROS [13,53]. Subsequent heat and drought stress increased a variety of antioxidant enzyme activities, and a combination of stresses contributed to a greater rise in antioxidant enzyme activities than individual stresses within a certain stress range. Stress priming further strengthened this increase mechanism. The antioxidant enzyme activities mentioned above in the HDP seedlings were higher than those in the NHDP seedlings. Comparable responses were also noted in experiments with wheat plants [22]. Most micronutrients act as cofactors in antioxidant enzymes taking part in the antioxidant defense system of plants [54]. The higher antioxidant enzyme activities in primed plants may be attributed to the increased uptake of most micronutrients by the primed plants. The upregulated expression of Cu/Zn-SOD, Mn-SOD, POD, and CAT in the primed plants contributes to the reduction in O_2^- content and better maintenance of membrane stability in response to subsequent combined heat and drought stress [55,56]. Notably, the HDP seedlings exhibited higher antioxidant enzyme activities at 6 DAS than the NHDP seedlings, which contributed to reducing the oxidative damage induced by combined stress on maize plants. This can be well evidenced by the significantly lower contents of O_2^- and MDA and MII of HDP seedlings compared to NHDP seedlings (Figure 5). The increased antioxidant capacity under stress-priming conditions reduced the concentration of ROS and restricted damage to cell membranes. Our findings are supported by Abid [22] and Chakraborty [57], who found that effective antioxidant enzyme activities in plants primed with drought and heat stress showed their greater potential to mitigate later drought and heat stress. The ROC results showed that greater SOD and GR activities may be primarily responsible for improved tolerance to a combination of heat and drought (Table 2).

Proline plays a crucial role not only in osmoregulation in the cytoplasm of plants, but also in stabilizing the structure of biomolecules and regulating cellular redox [58]. Under

stress conditions, plants actively accumulate soluble sugar (SS) to reduce osmotic potential, and the accumulation of soluble protein (SP) enhances the water retention capacity of cells and provides protection for biofilms [59,60]. In this experiment, heat and drought stress caused considerable increases in the contents of SP, SS, and Pro. Combined stress priming significantly increased SP and SS contents compared with no priming (Figure 7), which contributed to the capability of maize seedlings to withstand later combined stress (Table 2). This is probably because soluble sugar can prevent protein denaturation by the interaction of hydrogen bonds with proteins and membranes [61], which is beneficial to maintaining effective antioxidant enzyme activity to some extent. The results also revealed that combined stress priming improved proline content to reduce the osmotic potential, which is a manifestation of the plant's acclimation to heat and drought environments. At 6 DAS, a more significant increase in Pro content was observed in the HDP plants than in SS and SP contents, indicating that during subsequent stresses, the accumulated Pro in the HDP plants makes a greater contribution to regulating cellular permeability than SP and SS. As demonstrated by ROC analysis, Pro content (AUC = 0.958) was more sensitive to later combined stress. Our findings are supported by Lehr [62], who reported that Pro content of leaves was markedly elevated only under combined stress compared with single drought and heat stress, and Pro content was a possible marker for combined heat and drought stress. However, a previous study suggested that Pro may be harmful to the cell structure of Arabidopsis under combined heat and drought stress [63], which was not in agreement with our findings. This is probably associated with the stress tolerance of plant species and stress duration.

5. Conclusions

Results demonstrated that combined heat and drought stresses resulted in greater reductions in stomatal size, photosynthesis, chlorophyll content, and aboveground growth traits when compared with individual stresses. However, maize seedlings pre-exposed to heat and drought stress retained a long-lasting stress memory that triggered a more efficient stress scavenging mechanism towards subsequent severe combined stress. Stress priming strengthened antioxidant defense mechanisms and osmoregulation capacity, as evidenced by increased SOD, POD, CAT, GR, and APX activities, as well as improved SS and Pro contents. Maize seedlings with combined stress priming enhance adaptation to subsequent more severe stresses, which was largely ascribed to the improved leaf pigment contents and photosynthesis, as well as enhanced antioxidant and osmoregulatory capacities. The lower O_2^- and MDA contents in the combined stress-primed plants demonstrated the mitigation of oxidative damage. Moreover, combined stress priming effectively improved photosynthesis, leading to less aboveground growth reduction and an injury index increase in maize plants. ROC analysis showed that SOD, GR, and Pro were the more critical physiological traits of stress tolerance, and that they were able to respond positively to combined heat and drought stress. Our study enhances the understanding of the response of maize plants to combined stress after pre-exposure to stress conditions. Combined heat and drought priming can be a strategy for improving plant performance during recurrent heat and drought stress.

Author Contributions: C.R., X.H. and W.W. designed the experiments, C.R. performed the research and data analysis, D.C. provided guidance on the determination of indicators, and C.R. wrote the manuscript with contributions from all authors. All authors have read and agreed to the published version of the manuscript.

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