



Nitrogen Modulates the Effects of Heat, Drought, and Combined Stresses on Photosynthesis, Antioxidant Capacity, Cell Osmoregulation, and Grain Yield in Winter Wheat

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Abstract

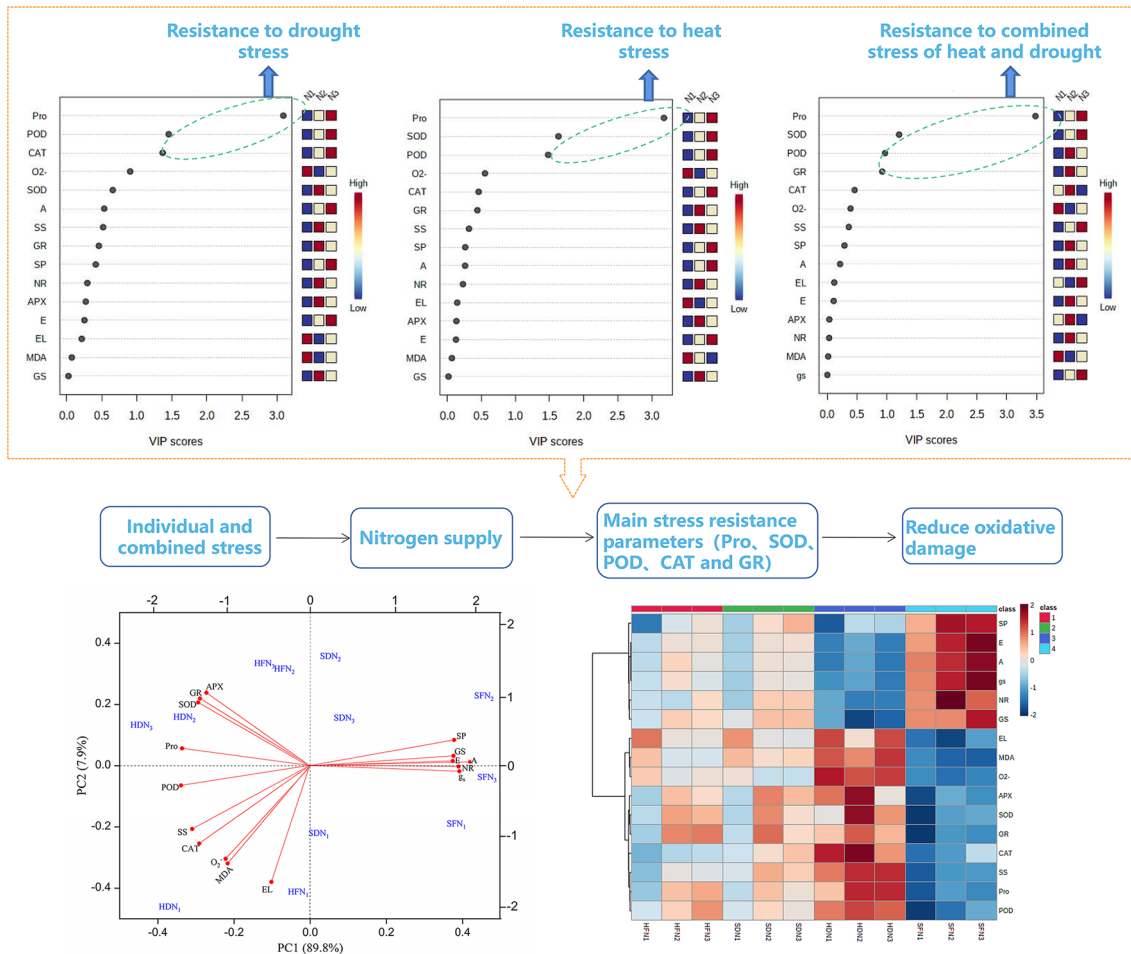
Longer and more intense heat and drought stresses will occur in terrestrial ecosystems in the future. Although the effects of individual heat or drought stress on wheat plants have been largely explored, the regulatory effect of nitrogen (N) on winter wheat under heat, drought, and combined stresses and whether N alleviates damage to wheat plants caused by these stresses remain unclear. Therefore, the objective of the present study was to investigate the growth, photosynthesis, antioxidant enzyme and N metabolism-related enzyme activity, cell membrane system, osmoregulatory substance, and yield responses to heat, drought, and combined stresses in wheat plants and to clarify the regulatory effects of N on the growth, physiological and biochemical characteristics, and yield of wheat plants under stress conditions. The results showed that wheat plant exposure to individual heat or drought stress reduced photosynthesis and N metabolism-related enzyme activities and increased antioxidant enzyme activities, electrolyte leakage (EL), and the contents of MDA (malondialdehyde) and O_2^- (superoxide anion). The above parameters showed typical superposition effects under combined stress. Under individual heat or drought stress, wheat plants treated with a medium (N_2) or high (N_3) N supply maintained higher photosynthesis and N metabolism-related enzyme activities than did those treated with a low N supply (N_1). Enhanced heat and drought tolerance in wheat plants under an appropriate N supply may be attributed to improved antioxidant capacity, as exemplified by increased activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), glutathione reductase (GR) and ascorbate peroxidase (APX), and to enhanced osmoregulation capacity, as signified by increased contents of soluble sugar (SS), soluble protein (SP), and proline (Pro). Variable importance in projection (VIP) analysis indicated that efficient SOD, POD, CAT, and GR activities and an increased Pro content had superior potential to alleviate heat, drought, and combined stress stresses in wheat plants, and the improvements in growth and grain yield in wheat plants further confirmed the oxidative stress alleviation and stress tolerance enhancement. However, positive effects of N on wheat growth and grain yield under combined stress were usually observed under a low N supply. These results may facilitate future research on the effects of N fertilizer on the stress resistance of winter wheat.

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Graphical Abstract



Keywords Abiotic stress · Antioxidant system · Cell osmotic adjustment · Nitrogen management · Photosynthesis · Grain yield

Introduction

Heat and water shortages have a negative impacts on crop growth and production in many agricultural areas worldwide (Semenov et al. 2014). Climate prediction models predict that heat waves and drought stress will become more intense and frequent in the future (Rosenzweig et al. 2014) and that the global temperature will rise by 1.8–4.0 °C by the end of this century (Ji et al. 2017). Extreme climate events seriously threaten global food security and production (Semenov et al. 2014; Schmidt et al. 2020).

Wheat is one of the most widely planted crops in the world (Fao 2020). Extreme heat and drought stresses are the two major constraints on wheat growth and production worldwide (Schmidt et al. 2020). It was reported that under heat and drought stresses, stomatal limitations, disorder of

chloroplast functions, and low photosystem efficiency are the main limiting factors that produce a lower photosynthetic rate (Mathur et al. 2014; Mu et al. 2021). Heat or drought stress directly damages the photosynthetic process and results in oxidative stress via the generation of reactive oxygen species (ROS) in plant cells, which damage membranes, proteins, nucleic acids, and other molecules (Khan et al. 2019; Neves et al. 2019). Compared to individual stresses, the combination of drought and heat stresses may have additional detrimental effects on plants. Combined stress (heat and drought) in wheat could induce lower leaf water potentials and chlorophyll contents and higher osmoregulatory substance contents (Sattar et al. 2020). Qaseem et al. (2019) reported that heat stress in combination with drought markedly reduced average yields by 44.66% in 108 wheat genotypes. Winter wheat often encounters combined heat

and drought stress during the growing season in the Guanzhong Plain in Northwest China (Li et al. 2020b; Zheng et al. 2020; Ju et al. 2021). Applying mitigation measures to alleviate yield losses caused by heat and drought stresses is of great significance.

Enhancing the ability of crops to resist heat and drought stresses is essential. Most studies on this topic have focused on ascertaining genetic sources of enhanced resilience (Devasirvatham et al. 2016; Frey et al. 2016), and little attention has been given to plant ecology (Slafer and Roxana 2018). Nitrogen (N) fertilizer is one of the most widely used fertilizers in crop production (Ata-UI-Karim et al. 2016). Increasing the N supply can usually improve plant growth and photosynthesis (Zhang et al. 2017), reduce MDA contents, and increase osmoregulatory substance contents to affect plant physiological characteristics (Saneoka et al. 2004; Agami et al. 2018; Shehab and Guo 2020). It was previously reported that an appropriate N supply could stimulate plant growth, improve water use efficiency, and alleviate the effects of drought stress (Brueck et al. 2010). N improved antioxidant enzyme activities and cell osmoregulation by inducing proline (Pro) accumulation to alleviate physiological damage caused by drought stress (Li et al. 2020a). In addition, N plays a vital role in heat stress tolerance. Higher temperatures affected plant nutrient absorption and negatively impacted plant growth, while appropriate N management effectively reduced the destructive effect of heat on crops (Waraich et al. 2012). The withdrawal of N caused a deterioration of plant protective mechanisms against heat damage or the malfunction of normal plant processes due to deficiency (Pérez-Jiménez et al. 2019). On the other hand, N could also directly and independently regulate reproductive development, and a reasonable N supply improved the development of spikes and increased the grain number per spike and 1000-grain weight (Kubar et al. 2021). The growth rate decline and impaired flower development caused by N deficiency could aggravate the effect of heat stress on yields (Ferrante et al. 2020). However, field experiments performed by Elía et al. (2018) concluded that a high N supply exacerbated the effects of heat stress, and part of this conclusion was based on experiments performed in controlled environments and field studies in which both “high N” and “heat stress” were extreme.

In contrast to the abundant information available on plant responses to heat and drought stresses, to our knowledge, studies on N regulation of the effects of heat and drought stresses on plants have primarily focused on individual stresses (heat or drought). However, the regulatory effects of N on the growth, physiological characteristics, and yield of wheat plants under combined heat and drought stress remain unclear. Analyzing possible interactions between factors may facilitate the formulation of more appropriate fertilization strategies. Furthermore, an overall evaluation of the

effects of N on growth, photosynthesis, the antioxidant system, N metabolism, the cell membrane system, osmotic regulators, and grain yield in winter wheat under heat, drought, and combined stresses has not been provided. Quantitative screenings of physiological parameters that provide effective resistance to heat, drought, and combined stresses under reasonable N regulation have rarely been reported.

Therefore, the present study hypothesized that a reasonable N supply would have a positive impact on wheat plants under heat, drought, and combined stresses. The main objectives were (1) to examine the effects of different N supplies on the growth, photosynthesis, antioxidant and N metabolism-related enzyme activities, cell membrane stability, cell osmoregulation, and grain yield in wheat plants under heat, drought, and combined stresses and (2) to quantitatively screen the physiological parameters that provide effective resistance to heat, drought, and combined stresses with the addition of an appropriate N supply.

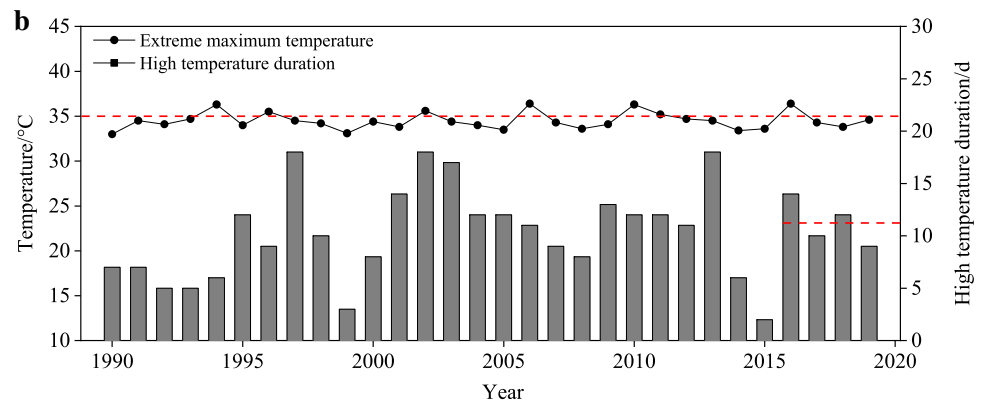
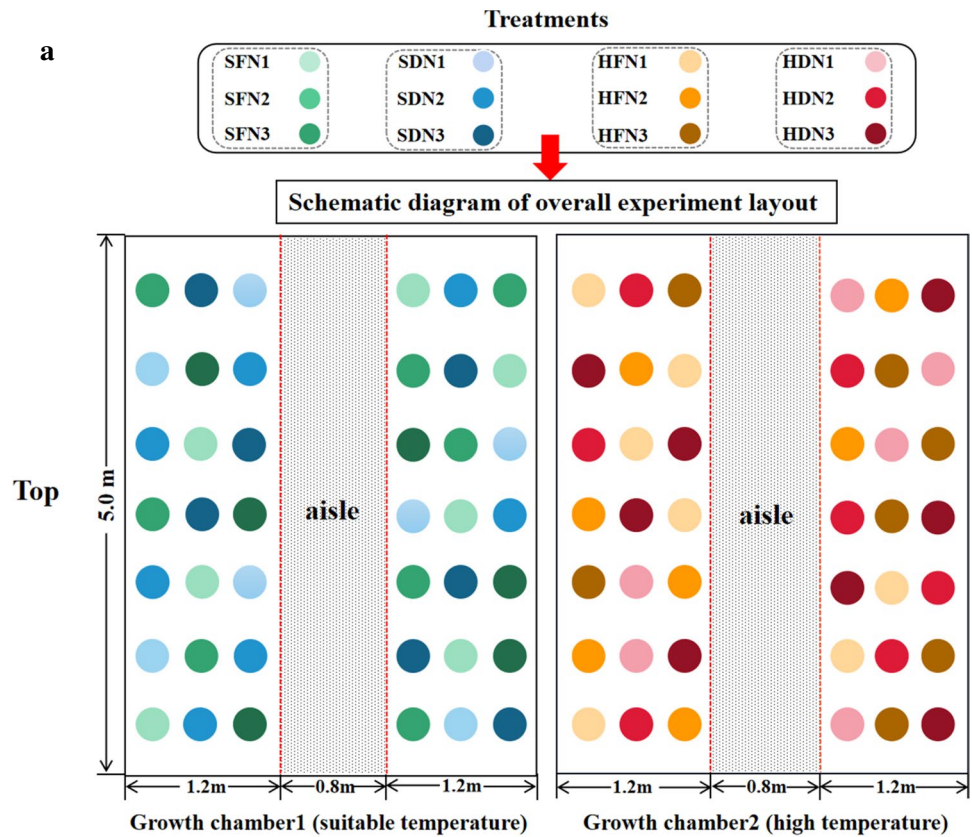
Materials and Methods

Experimental Design

The experiment was performed in an artificial growth chamber at the Key Laboratory of Agricultural Water and Soil Engineering in Arid and Semiarid Areas, Northwest A&F University (108.04°E, 34.20°N). Each pot was 30 cm high and 28 cm in diameter, with seven holes at the bottom, although no water leaching was observed during the treatment period. A mesh strainer and gravel were laid under the soil to ensure air permeability. Nineteen kilograms of sieved (0.5 mm) dry soil was added to each pot, and the soil type was heavy loam. The soil had the following basic physical and chemical properties: the pH was 7.61; the organic matter content was 10.02 g/kg; the total N content was 0.62 g/kg; the total phosphorus content was 0.55 g/kg; the total potassium (K₂O) content was 16.8 g/kg; the alkali-hydro N content was 36.07 mg/kg; and the available phosphorus content was 17.64 mg/kg. The soil water holding capacity (SWHC) was 27.13%, and the average soil volume mass was 1.47 g/cm³. Perlite (1 cm in thickness) was laid on the soil surface to reduce soil water evaporation. A high-yield winter wheat cultivar (*Triticum aestivum* L. cultivar ‘Xiaoyan 22’) was selected for the study. Plump seeds of uniform size were selected. The seeds were soaked in 20% H₂O₂ for 10 min for disinfection and then fully washed with distilled water. Seeds were sown after soaking in distilled water for 12 h. In total, 20 seeds were sown in each pot on October 17, 2019, and the pots were thinned to ten seedlings at the wheat trilobal stage.

The treatments were arranged in a split-plot design with two temperature levels as the main plot and two watering levels and three N supply levels as the subplot (Fig. 1a).

Fig. 1 a Schematic diagram of the overall experiment layout. **b** Extreme maximum temperature after winter wheat anthesis in the main production areas in Guanzhong Plain in China from 1990 to 2019



According to the meteorological data of the main wheat-producing areas in the Guanzhong Plain in Northwest China (<http://data.cma.cn>), the extreme maximum temperature after anthesis from 1990 to 2019 was approximately 35 °C (Fig. 1b). Therefore, the heat stress (H) treatment involved temperatures of 36 °C/26 °C/31 °C (daily maximum temperature/minimum temperature/average temperature). The suitable temperature (S) treatment included temperatures of 26 °C/16 °C/21 °C. Daily temperature changes in the artificial growth chamber were simulated based on the daily temperature changes of the external environment. Two watering

treatments were established under the two temperature levels: a drought stress (D) treatment, in which the pots were irrigated to 45–55% SWHC_s and a full irrigation (F) treatment, in which the pots were irrigated to 75–85% SWHC. Three levels of N (relative values) were applied under the heat and drought treatments: low N (N₁), medium N (N₂), and high N (N₃) supplies. Basal fertilizer was applied before sowing, with the same amount of phosphate (P), potassium (K), and N in all treatment pots. Before sowing, 0.74 g (N₁), 1.10 g (N₂) or 1.84 g (N₃) urea, 1.35 g diamine phosphate, and 1.15 g potassium chloride were homogeneously mixed with the soil in each pot, and the remaining 50% of

N fertilization was applied at the jointing stage. The P, K, and N fertilizers were diamine phosphate ($P_2O_5 = 46\%$), potassium chloride ($K_2O = 50\%$), and urea ($N = 46\%$), respectively. Other crop management methods used were consistent with local high-yield cultivation practices, and no major insect pests or diseases were observed. Two artificial growth chambers (RQS-15, Hengyu Instrument & Equipment Manufacturing Co., Ltd. Nanjing, China) were used to maintain plant growth and perform heat stress treatments. Excluding temperature, the relative humidity (60–65%), photoperiod (8:00–20:00), and photosynthetic photon flux density ($1100 \text{ mol}\mu\text{m}^2/\text{s}$) were consistent between the two growth chambers.

After sowing, all potted plants were grown in a canopy under natural outdoor conditions, and the soil moisture content of the potted plants was maintained at 70–80% of SWHC. The canopy was closed before rainfall to avoid the impact of natural rainfall on the soil moisture of potted plants. Before the drought treatment began, the soil moisture of the drought-treatment pots was gradually adjusted to the designed range to ensure that the pots treated with the two different watering levels were simultaneously subjected to the temperature treatments. All plants were acclimatized to S conditions for three days in a growth chamber. According to the growth process of wheat, heat and drought stresses were imposed 7 days after anthesis (DAA). The relatively stable duration of recent extreme high temperatures was approximately 11.25 days (Fig. 1b). Therefore, heat and drought stresses were applied simultaneously for 12 days to simulate the potentially growing trend of increased days of high temperatures in northwest China occurring during the wheat grain-filling period. The combination of the weighing method and soil moisture meter measurements (HM-SW, Shandong Shian Biotechnology Co., Ltd., China) was used to determine the soil moisture contents of the potted plants. When the potted plants were in the outdoor environment, all potted plants were weighed every 2 days. During the stress treatments, all potted plants were weighed daily. Weighing was performed between 18:00 and 20:00, and watering was performed according to the upper limit of the pot weights for the corresponding treatment. The theoretical upper and lower limits (g) of pot weight under the drought treatment were the pot weight + the estimated seedling weight in the pots + the perlite and gravel weight in the pots + the total dry soil weight in the pots ($1 + \text{SWHC} \times \text{upper and lower limits of the designed relative water content}$). Supplementary water was slowly injected into the rhizosphere along the cracks in the soil around the seedlings. To reduce differences in light and temperature among plants in different locations in the growth chamber during heat stress, the positions of the inner and outer pots were exchanged daily. All pots were transferred to natural conditions after heat stress until the plants were mature. A total of 12 treatments were performed

after stress: HFN₁, HFN₂, and HFN₃ (heat stress + full irrigation + N₁/N₂/N₃); SDN₁, SDN₂, and SDN₃ (suitable temperature + drought stress + N₁/N₂/N₃); HDN₁, HDN₂, and HDN₃ (heat stress + drought stress + N₁/N₂/N₃); and SFN₁, SFN₂, and SFN₃ (control treatments, suitable temperature + full irrigation + N₁/N₂/N₃). Seven replicates (pots) were set for each treatment, with a total of 84 pots.

Plant Sampling

During the stress treatment time, each potted plant had approximately 30 fully expanded leaves. At 19 DAA, five wheat plants were sampled to determine the aboveground dry weight (ADW), green leaf area (GLA), plant height (PH) and root–shoot ratio (R/S). The last fully expanded leaves under each treatment were detached at 10, 13, 16, and 19 DAA, and other leaves were immediately sent to the university laboratory for the determination of electrolyte leakage (EL). Additional leaves were wrapped in aluminum foil, immediately frozen in liquid nitrogen and stored at -80°C for further analysis, including antioxidant and N-metabolizing enzyme activity, MDA content, superoxide anion content, and cell osmoregulatory substance content analyses. For the above growth and biochemical parameter measurements, five independent replications were performed. At maturity, three pots (replicates) of wheat under each treatment were harvested to calculate yield and yield components.

Growth Parameters

Five wheat plants were randomly selected from each treatment to measure the plant height with from the potted soil surface to the top of the spike with a calibration ruler (length: 1 m, accuracy: 0.5 mm). Five plants from each treatment were cut at the base of the stem to obtain the whole crown. All green leaves of a single plant were cut with scissors and tiled onto a scanning plate (LIDE-400, Beijing Canon Co., Ltd., China). The leaves of each plant were scanned at a resolution of 600 DPI and saved as pictures. ImageJ software (V1.8.0, National Institutes of Health, USA) was used to scan the green leaf area (GLA, cm^2/plant) in the images, and the average value of the results was retained.

After sampling the wheat crown, the roots were sampled until no fine hairy roots could be observed by eye, and then, the root samples were cleaned and dried with nitrogen-free absorbent paper. The aboveground and root parts were dried to constant weight at 75°C in an oven to obtain the dry weights of the aboveground and root parts. The R/S ratio was calculated using the following formula. The growth parameters were measured in five independent replications.

$$\frac{R}{S} = \frac{\text{Root dry weight at maturity}}{\text{Aboveground dry weight at maturity}} \quad (1)$$

Photosynthesis Parameters

Photosynthetic parameters were measured in the middle of the last fully expanded leaves of wheat plants at 10, 13, 16, and 19 DAA. The net photosynthesis rate (A), stomatal conductance (g_s), and transpiration rate (E) were measured using an LI-6800XT portable photosynthesis measurement system (LI-COR, USA) from 9:00 to 11:30. Ten leaves (replicates) per treatment were selected for each measurement, and these leaves were selected from distinct pots under the same treatment for each measurement. A red and blue light source was used, and the photosynthetically active radiation (PAR) was set to 1100 mol μ m/s. The average value of the results was retained.

Activities of Antioxidant Enzymes and N Metabolism-Related Enzymes

Crude enzymes were extracted using 50 mmol/L phosphate buffer (pH 7.8, 1 mmol/L EDTA-2Na, 5 mmol/L MgCl₂, 1% polyvinylpyrrolidone, and 0.3% Triton-X100). The leaf samples (0.5 g) were ground in a frozen mortar with precooled extraction medium. The homogenates were centrifuged at 8000 rpm at 4 °C for 20 min, and the filtrate was used as the enzyme extract for the following superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) assays.

SOD (EC 1.15.1.1) and POD (EC 1.11.1.7) activities were determined according to the methods of Tan et al. (2008). The reaction mixture contained phosphate buffer (pH 7.8, 50 mmol/L), 130 mmol/L methionine, 0.75 NBT, 0.02 mmol/L riboflavin, 0.1 mmol/L EDTA-2Na, and the enzyme extract. The tubes were exposed to 4000 lx illumination for 20 min. The reaction was stopped by placing the tubes in the dark. The absorbance of the reaction mixture was measured at 560 nm. POD activity was measured by the guaiacol oxidation method. The reaction mixture contained sodium phosphate buffer (pH 7.0, 50 mmol/L), guaiacol and 30% H₂O₂. The reaction was initiated by adding the enzyme extract. Absorbance readings at 470 nm were recorded at 1-min intervals for 3 min after the start of the reaction. CAT (EC 1.11.1.6) activity was measured following the protocol described by Aebi (1984). The reaction mixture contained phosphate buffer (pH 7.0, 50 mmol/L), 0.3% hydrogen peroxide and the enzyme extract. The decomposition of H₂O₂ was observed at 240 nm for at least 3.5 min.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured following previous methods (Nakano and Asada 1980). The leaf samples (0.5 g) were ground and extracted

by adding precooled 50 mmol/L K₂HPO₄-KH₂PO₄ 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 μ L of potassium phosphate buffer (pH 7.5, 80 mmol/L), 100 μ L of 5 mmol/L ascorbate, 100 μ L of 1 mol/L EDTA, 100 μ L of 1 mmol/L H₂O₂, and 50 μ L 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. Glutathione reductase (GR, EC 1.6.4.2) activity was measured according to the methods of Schaedle and Bassham (1977) 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 (pH 7.8, 50 mmol/L), 2 mmol/L Na₂EDTA, 0.15 mmol/L NADPH, 0.5 mmol/L GSSG (glutathione oxidized), and 225 μ L 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.

Nitrate reductase (NR, EC 1.6.6.1) activity was measured following the method of Gaudinová (1990) with some modifications. Leaf samples (0.5 g) were incubated in 2 mL of potassium phosphate buffer (pH 7.5, 0.1 mol/L) containing 25 mmol/L KNO₃ and 4% n-propanol at 30 °C in the dark for 1 h, and then, 0.5 ml of 0.3% 1-naphthylamine and 0.5 ml of 1% sulfonamide were added to the reaction mixture. After 15 min, the optical density of the solution was measured at 540 nm. Glutamine synthetase (GS, EC 6.3.1.2) activity was measured by referencing the method of Zhang et al. (1997). Leaf samples (0.5 g) were homogenized with 5 mL of buffer solution (pH 7.5, 0.1 mol/L Tris-NaOH, 6 mmol/L MgCl₂, 2 mmol/L EDTA, and 40 mmol/L β -mercaptoethanol) precooled in ice, followed by centrifugation at 8000 rpm at 4 °C for 20 min. The supernatant was used for enzyme determination. After the mixture was cultured at 37 °C for 15 min, FeCl₃ (2% (w/v) trichloroacetic acid (TCA) and 3.5% (w/v) FeCl₃ in 2% HCl) were added to terminate the reaction. The production of glutamyl hydroxamate was determined at 540 nm.

Electrolyte Leakage and MDA and Superoxide Anion Contents

EL was measured as described by Chen et al. (2015). Leaf samples (0.2 g) were cut into 5-mm long pieces and placed in a test tube containing 20 mL of distilled deionized water in a water bath maintained at a constant temperature of 32 °C. After 2 h, the initial conductivity of the medium (EC₁) was measured. The sample was then boiled at 100 °C for 20 min. Afterward, the sample was cooled to 25 °C, and the conductivity (EC₂) was measured. EL was calculated by the formula $EL = EC_1/EC_2 \times 100\%$.

Leaf samples (0.5 g) were homogenized in 10 mL of 10% TCA. The homogenate was centrifuged at 4000 rpm at 4 °C for 10 min. Two milliliters of 0.6% thiobarbituric acid (TBA) was added to the supernatant, and the mixture was incubated at 100 °C for 15 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 MDA content was calculated according to the formula of Du and Bramlage (1992).

The superoxide anion (O_2^-) content was measured using the methods of Elstner and Heupel (1976) with some modifications. Leaf samples (0.5 g) were homogenized with 3 mL of phosphate buffer (pH 7.8, 50 mmol/L). The homogenate was centrifuged at 8000 rpm at 4 °C for 10 min. Then, 1 mL of supernatant and 3 mL of hydroxylamine hydrochloride were combined, and the mixture was stored at 25 °C for 30 min. Afterward, 1 mL of the above mixture was mixed with 1 mL of aminobenzene sulfonamide and 1 mL of α -naphthylamine. The reaction solution was placed in a water bath (30 °C) for 30 min, and the absorbance was measured at 530 nm.

Soluble Protein, Soluble Sugar, and Proline Contents

The soluble protein (SP) content was measured according to the methods of Bradford (1976) with some modifications. Bovine serum albumin (BSA) was used as the protein standard. Leaf samples (0.5 g) were homogenized with 5 mL phosphate buffer (pH 7.8, 50 mmol/L). The homogenate was centrifuged at 8000 rpm at 4 °C for 15 min, and the supernatant was retained as the protein extract. Then, 0.1 mL of the protein extract was mixed with 5 mL of Coomassie brilliant blue G-250 solution. After the mixture stood for 2 min, the absorbance was measured at 595 nm, and the SP content was calculated according to the standard curve.

The soluble sugar (SS) content was measured according to the anthrone method described by Sadasivam and Manickam (1992). Leaf tissue samples (0.2 g) were boiled with 10 mL of 80% (v/v) ethanol in a water bath (95 °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. One 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 Pro content was determined according to the method of Bates et al. (1973) with some modifications. Leaf samples

(0.5 g) were placed into 5 mL of 3% sulfosalicylic acid, extracted in a boiling water bath for 15 min, and cooled and centrifuged at 3000 rpm for 10 min. The supernatant was retained as the Pro extract. A mixture of 1 ml Pro extract, 1 mL glacial acetic acid, 1 mL water, and 2 mL acid ninhydrin reagent was heated in a boiling water bath for 40 min. After cooling, 3.5 mL toluene was added to the mixture, and the mixture was vortexed for 30 s. The absorbance of the toluene layer was measured at 520 nm.

Yield and Yield Components

After maturity, three pots of wheat (three repetitions) were randomly selected from each treatment to analyze the grain number per spike (SGN), seed setting rate (SSR), 1000-grain weight (TGW) and grain yield. First, the panicles were threshed manually, and then full, half-full and empty grains were counted and dried to constant weight in an 80 °C oven. The moisture content of the grains was adjusted to 13% to determine the yield. Finally, SGN, SSR and TGW were calculated.

Data Analysis

The treatments were arranged in a split-plot design with two temperature levels as the main plot and two watering levels and three N supply levels as the subplot (Fig. 1a). The number of replicates (pots) per treatment was seven. There were approximately 30 plants in each plant pot after anthesis. At least three replicates were assessed for each measurement, and the results are shown as the mean \pm standard error. The analysis of variance (ANOVA), correlation analysis, and regression analysis in this study were performed using SPSS 19.0 (IBM, Chicago, IL, USA). Before all analyses, the data were tested to determine whether the assumptions of ANOVA, normal distribution, error independence, and variance homogeneity were met. Nonnormal data were transformed (log-transformed and arcsine-transformed) to meet ANOVA assumptions. Duncan's method was used to test the significance of differences ($P < 0.05$). Multiway analysis of variance (ANOVA) was used to test the main effects and interaction effects of temperature (T), watering (W), nitrogen supply (N), and stress duration (D) on the examined parameters ($P < 0.05$). The relationships between the net photosynthetic rate and other parameters were analyzed using linear regression. Pearson correlations between antioxidant enzymes, Pro, and other physiological parameters were examined. Partial least squares discriminant analysis (PLS-DA) and principal component analysis (PCA) were performed using MetaboAnalyst 5.0 software (Worley and Powers 2013).

Results

Effects on Wheat Growth Parameters

Compared to the control (SF), the ADW, GLA, and PH of wheat significantly decreased under heat, drought, and combined stresses (Table 1). The ADW in the SD, HF, and HD treatments decreased by 16.60%, 14.84%, and 27.27%, respectively, compared to the control, and combined stress showed a typical decreasing superposition effect. Similar laws were also found for the GLA and PH. The R/S of wheat increased when exposed to individual heat and drought stresses, but no superimposed effects were observed under combined stress. Compared with N₁, the ADW in the SD and HF treatments under the N₂ supply was significantly higher than under the N₁ supply, but N₃ supply significantly exacerbated the decrease in the ADW under the HD treatment. There were differences in the response of the GLA to N between the stress treatments. Compared to N₂, N₃ supply limited the increase in the GLA under the HF treatment, and the GLA in the SD treatment increased with the increase of N supply. To a

certain extent, the PH under individual stress treatments (HF and SD) increased with increasing N supply, and the PH in the HD treatment under the N₁ supply was significantly higher than that with N₃ supply. Overall, the R/S showed a decreasing trend with increasing N supply. The effects of T, W, and N on growth parameters were more significant than those of the interactions between factors.

Effects on Leaf Photosynthetic Parameters

Compared to that under the SF conditions, the reduction in A, g_s, and E caused by combined heat and drought stress was significantly higher than those under the individual stress (Fig. 2). At 10 and 13 DAA, A in the SD and HF treatments increased significantly with the increase of N supply, and A of N₃ supply was significantly greater in the SD and HF treatments than that of N₁ supply. During the entire stress period, N₂ supply was conducive to maintaining a higher A under combined stress (Fig. 2a). The N₃ supply had the best regulatory effect on g_s under the SD and HF treatments at 10 DAA. With the extension of stress time, the N₃ supply became no longer conducive to the increase in g_s under individual stresses (Fig. 2b). E under all stress treatments

Table 1 Effects of the N supply on wheat growth parameters under heat, drought and combined stresses

Treatments	ADW (g/plant)	GLA (cm ² /plant)	PH (cm)	R/S	
S	DN ₁	2.90 ± 0.045dbbF	56.24 ± 1.88dbcdE	62.17 ± 1.23dbcbG	0.076 ± 0.001aaaA
	DN ₂	3.10 ± 0.029cbaD	67.65 ± 1.97cbabc	64.07 ± 0.88cdbaEF	0.073 ± 0.001bcabb
	DN ₃	2.99 ± 0.077cdbbE	71.02 ± 2.78caaC	64.65 ± 1.23cbabEF	0.071 ± 0.002cacBCD
	FN ₁	3.45 ± 0.045babB	89.55 ± 1.98aaaA	66.89 ± 2.10babcbC	0.067 ± 0.001ddcEF
	FN ₂	3.61 ± 0.120aaaA	90.04 ± 2.92aaaA	67.67 ± 1.55ababB	0.064 ± 0.001eddG
	FN ₃	3.72 ± 0.082aaaA	82.49 ± 2.63babB	69.01 ± 0.77aaaA	0.059 ± 0.004fceH
H	DN ₁	2.61 ± 0.039cdacdGH	64.50 ± 1.19bbbcD	61.80 ± 1.20ccbcG	0.069 ± 0.003bcdDE
	DN ₂	2.69 ± 0.60cacG	58.93 ± 2.11ccbcDE	60.25 ± 1.83cccH	0.067 ± 0.001cdcEF
	DN ₃	2.53 ± 0.021dcdH	49.44 ± 1.35dcdF	58.56 ± 1.35cdI	0.066 ± 0.002dbefG
	FN ₁	2.95 ± 0.051bbceEF	60.37 ± 1.93bcdDE	63.86 ± 2.43bbeF	0.072 ± 0.001abaBC
	FN ₂	3.21 ± 0.101abcC	68.70 ± 1.44abcC	65.89 ± 0.67abcdCD	0.069 ± 0.002bbdDE
	FN ₃	3.02 ± 0.084bbdeDE	65.40 ± 1.54abbcD	65.18 ± 1.78abdDE	0.070 ± 0.001babCD
ANOVA	T	***	***	***	**
	W	**	**	***	**
	N	**	*	*	**
	T×W	*	**	**	*
	T×N	*	*	**	ns
	W×N	**	ns	*	*
	T×W×N	ns	***	**	ns

The values are the means ± SEs, n = 5.

Black lowercase letters for comparisons between treatments under S and H conditions; Blue, red and green lowercase letters for comparisons between treatments under the N₁, N₂ and N₃ supplies, respectively; Pink and orange lowercase letters for comparisons between treatments under D and F conditions, respectively; Black uppercase letters for comparisons between all treatments combined by T, W and N. ‘*’, ‘**’ and ‘***’ designate significant differences at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. *ns* indicates no significance.

ADW aboveground dry weight, GLA green leaf area, PH plant height, R/S root/shoot ratio, S suitable temperature, H high temperature, F full watering, D drought, T temperature, W watering, N nitrogen.

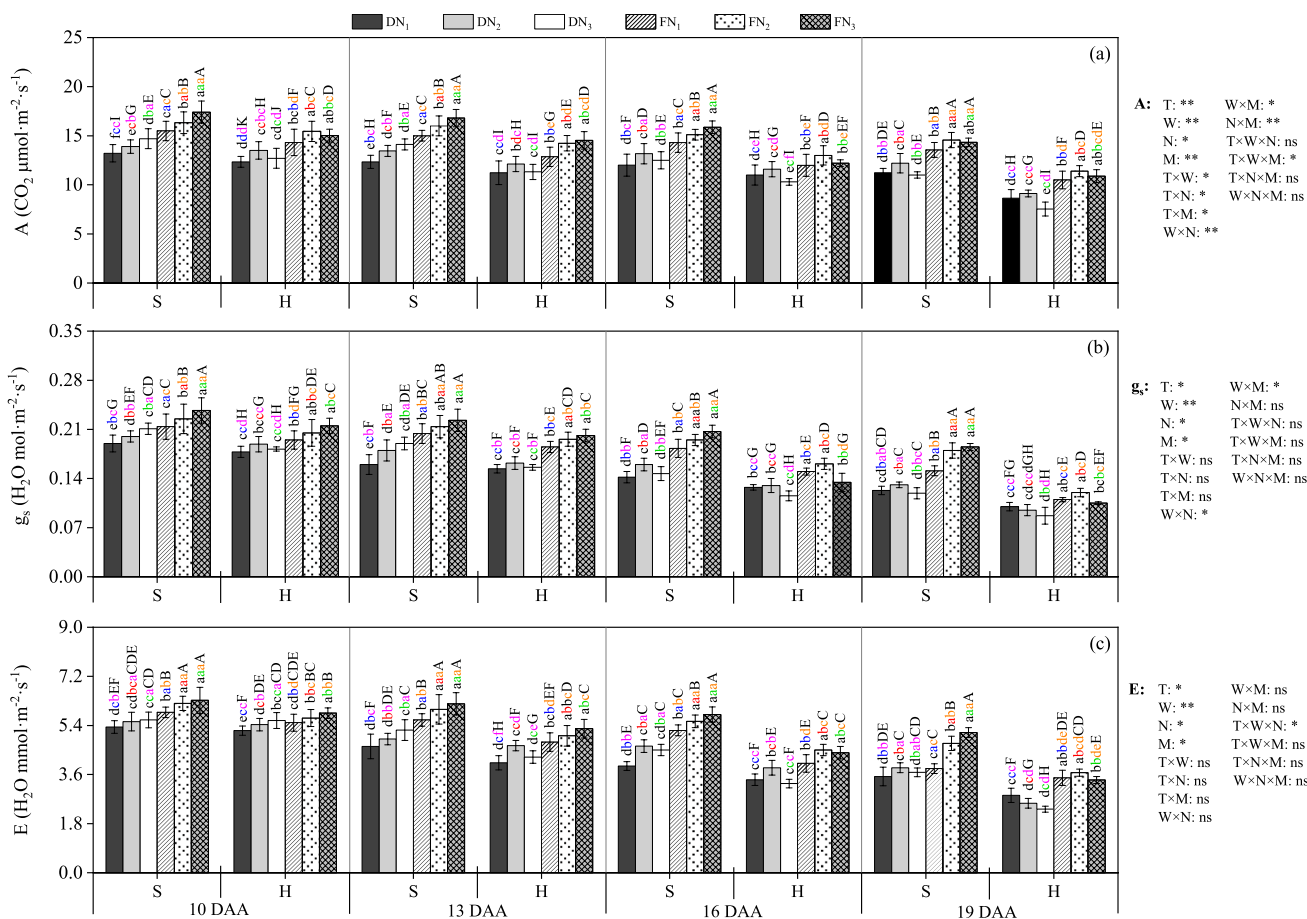


Fig. 2 Effects of the N supply on the net photosynthesis rate (A, **a**), stomatal conductance (g_s , **b**) and transpiration rate (E, **c**) in wheat leaves under heat, drought, and combined stresses. *S* suitable temperature, *H* high temperature, *F* full watering, *D* drought, *T* temperature, *W* watering, *N* nitrogen, *M* measurement date. The values are the means \pm SEs, $n=10$. Black lowercase letters for comparisons between treatments under *S* and *H* conditions; Blue, red, and green lowercase

letters for comparisons between treatments under the N_1 , N_2 , and N_3 supplies, respectively; Pink and orange lowercase letters for comparisons between treatments under *D* and *F* conditions, respectively; Black uppercase letters for comparisons between all treatments combined by *T*, *W*, and *N*. ‘*’, ‘**’, and ‘***’ designate significant differences at $P<0.05$, $P<0.01$, and $P<0.001$, respectively. ns indicates no significance (Color figure online)

maintained the maximum value with N_2 supply at 16 DAA. It is worth noting that *E* in the *HD* treatment under the N_1 supply was significantly higher than under the N_2 and N_3 supplies at 19 DAA (Fig. 2c). The effects of *T*, *W*, *N*, and *M* on *A*, g_s and *E* were significant, and the interaction effect of each factor was much lower than those of single factors.

Effects on Antioxidant Enzyme and N Metabolism-Related Enzyme Activities

The SOD, POD, and CAT activities in leaves significantly increased under heat, drought, and combined stresses (Fig. 3). Notably, the SOD and CAT activities constituted the initial response to heat and drought stress. The ability of SOD and CAT to scavenge ROS may have been inhibited at 16 DAA, and this scavenging ability may have been enhanced by increased POD activity. The regulatory effects

of N on SOD activity under the *SD* and *HD* treatments changed significantly at 13 DAA (Fig. 3a), and the SOD activity in response to N_2 supply was significantly higher than that in response to N_1 and N_3 supplies. At the later stress stage (19 DAA), the SOD activity under each treatment with N_2 supply was the highest compared with N_1 and N_3 supplies. Increasing the N supply promoted the increase in POD activity under all treatments at 10 DAA (Fig. 3b). Compared to N_1 and N_2 supplies, the positive effect of the N_3 supply on POD activity under the *SD* and *HF* treatments was most obvious at 16 DAA. A high N supply inhibited POD activity in leaves under the *SD* and *HD* treatments at 19 DAA. For CAT, the activity increased with the increase of N supply under the *SF* and *HF* treatments (Fig. 3c). After 13 DAA, the positive effect of increasing the N supply on CAT activity under the *SD* treatment gradually diminished. The N_2 supply maintained higher CAT activity under the

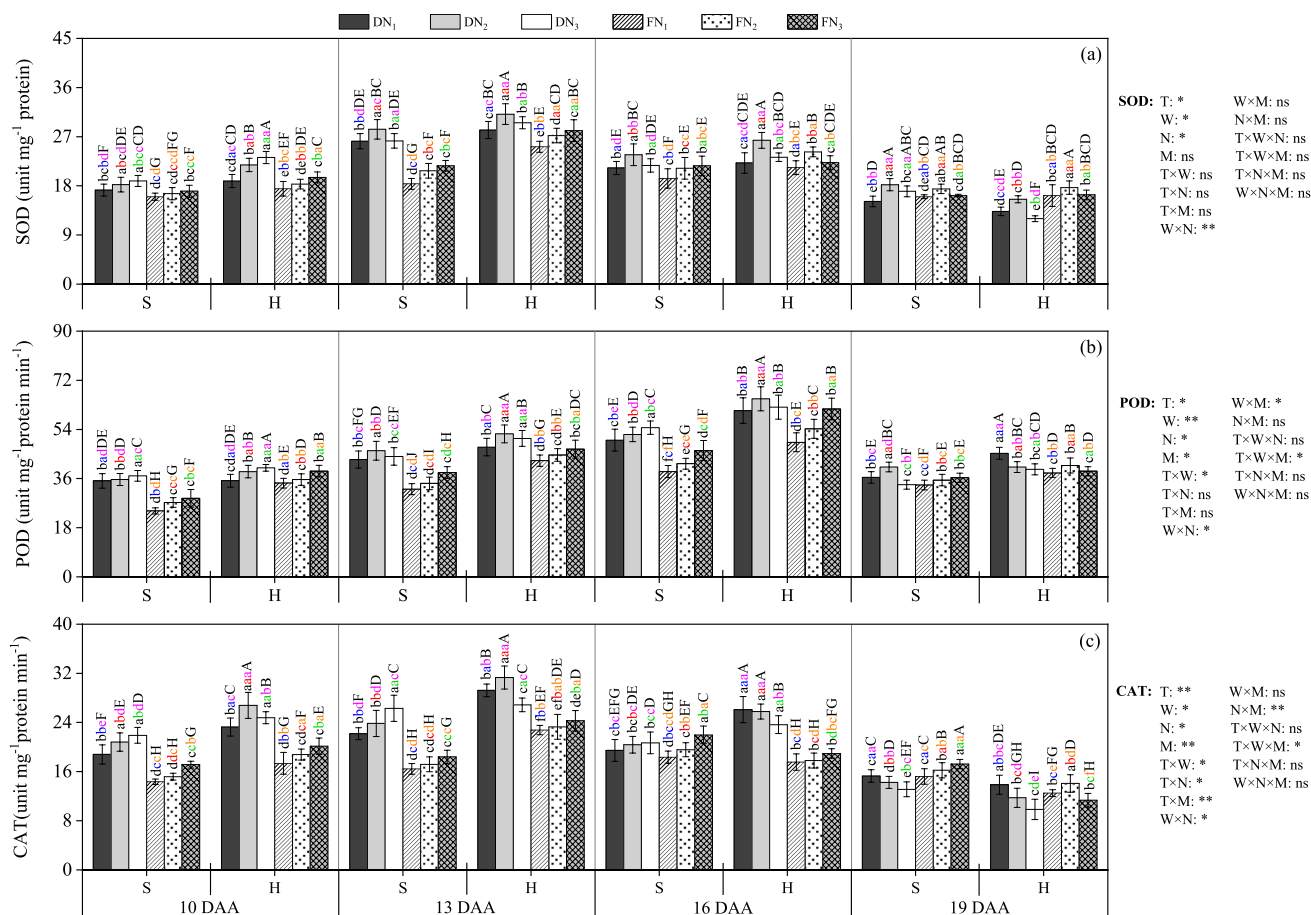


Fig. 3 Effects of the N supply on superoxide dismutase (SOD, **a**), peroxidase (POD, **b**), and catalase (CAT, **c**) activities in the leaves of wheat under heat, drought, and combined stresses. *S* suitable temperature, *H* high temperature, *F* full watering, *D* drought, *T* temperature, *W* watering, *N* nitrogen, *M* measurement date. The values are the means ± SEs, n=5. Black lowercase letters for comparisons between treatments under S and H conditions; Blue, red, and green lowercase

letters for comparisons between treatments under the N₁, N₂ and N₃, supplies, respectively; Pink and orange lowercase letters for comparisons between treatments under D and F conditions, respectively; Black uppercase letters for comparisons between all treatments combined by T, W, and N. ‘*’, ‘**’ and ‘***’ designate significant differences at P<0.05, P<0.01, and P<0.001, respectively. ns indicates no significance (Color figure online)

HD treatment at 10 d and 13 DAA, and the N₁ supply was more conducive to the increase in CAT activity under the HD treatment at the later stage of stress. The W×N interaction had a more significant effect on SOD activity than the individual factors. POD activity was mainly affected by single factors. CAT activity was primarily affected by single factors and the interaction of two factors.

Heat, drought, and combined stress significantly increased GR and APX activities compared with the control (Fig. 4). GR activity under the SD and HF treatments increased gradually during 10–16 DAA. GR activity under the HD treatment decreased relatively earlier at 16 DAA, and the activity was significantly lower than those under the individual stress treatments at 19 DAA (Fig. 4a). Except for the HD treatment, APX activity under the other treatments increased with the extension of stress time (Fig. 4b). During the stress period (except at 10 DAA), the N₂ was the best

N supply for maintaining higher GR and APX activities in the SD treatment compared with N₁ and N₃ supplies. With increasing stress duration, the positive effects of increasing the N supply on GR and APX activities under the HF treatment gradually weakened. Compared to N₁ and N₂, the inhibition of GR activity by N₃ under the HD treatment began to appear at 16 DAA, and negative effects on APX activity occurred earlier (13 DAA). The T, N, T×W, and W×N interactions had significant effects on GR activity. APX activity was most significantly affected by T, W, N, and M.

NR and GS activities tended to decrease in response to heat and drought stresses, and the interaction effects of these two stresses were more significant than the individual effects (Fig. 5). At 10 and 13 DAA, the effects of individual stresses (SD and HF) on NR activity were similar. However, the inhibitory effect of drought on NR activity was stronger than that of heat stress after 13 DAA (Fig. 5a). The

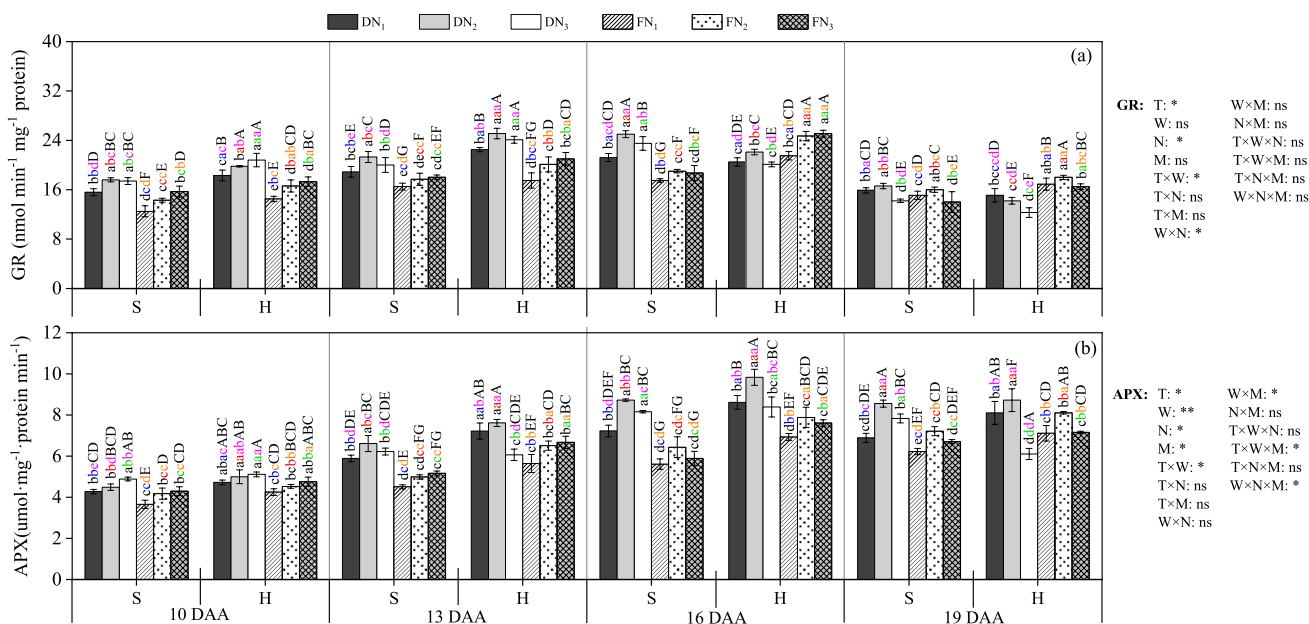


Fig. 4 Effects of the N supply on glutathione reductase (GR, **a**) and ascorbate peroxidase (APX, **b**) activities in the leaves of wheat under heat, drought, and combined stresses. *S* suitable temperature, *H* high temperature, *F* full watering, *D* drought, *T* temperature, *W* watering, *M* measurement date. The values are the means ± SEs, n=5. Black lowercase letters for comparisons between treatments under S and H conditions; Blue, red, and green lowercase letters

for comparisons between treatments under the N₁, N₂, and N₃ supplies, respectively; Pink and orange lowercase letters for comparisons between treatments under D and F conditions, respectively; Black uppercase letters for comparisons between all treatments combined by T, W, and N. ‘*’, ‘**’, and ‘***’ designate significant differences at *P*<0.05, *P*<0.01, and *P*<0.001, respectively. ns indicates no significance (Color figure online)

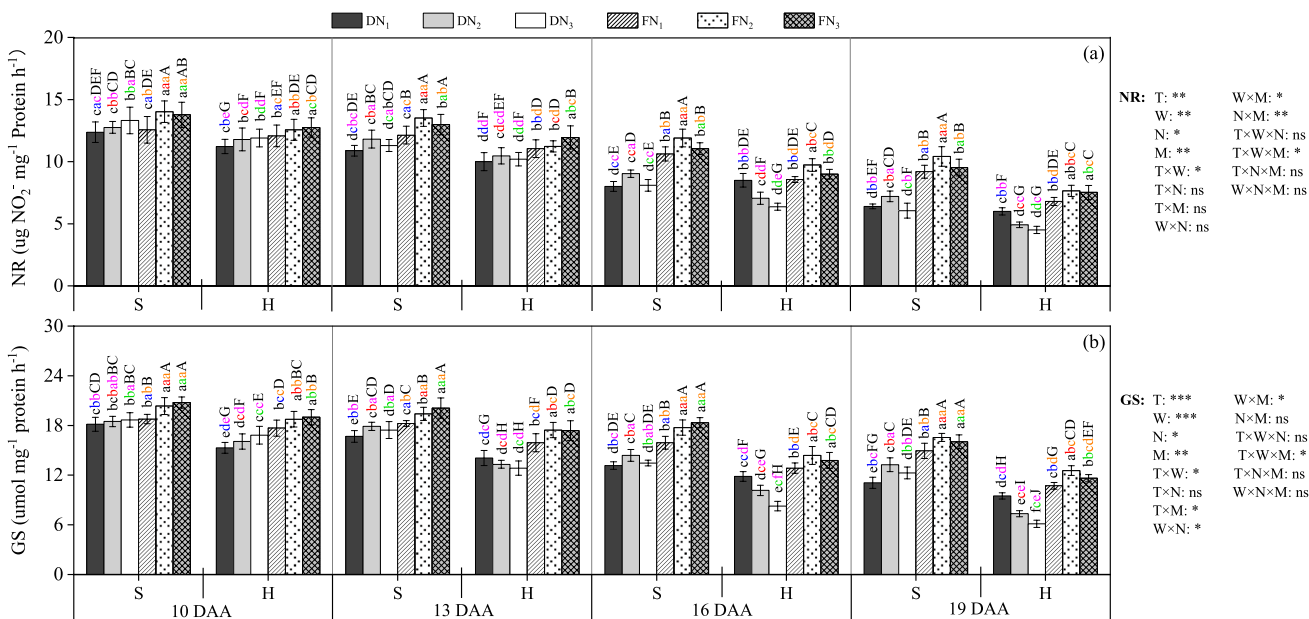


Fig. 5 Effects of the N supply on nitrate reductase (NR, **a**) and glutamine synthetase (GS, **b**) activity in wheat leaves under heat, drought, and combined stresses. *S* suitable temperature, *H* high temperature, *F* full watering, *D* drought, *T* temperature, *W* watering, *N* nitrogen, *M* measurement date. The values are the means ± SEs, n=5. Black lowercase letters for comparisons between treatments under S and H conditions; Blue, red, and green lowercase letters for compari-

sons between treatments under the N₁, N₂, and N₃ supplies, respectively; Pink and orange lowercase letters for comparisons between treatments under D and F conditions, respectively; Black uppercase letters for comparisons between all treatments combined by T, W, and N. ‘*’, ‘**’, and ‘***’ designate significant differences at *P*<0.05, *P*<0.01, and *P*<0.001, respectively. ns indicates no significance (Color figure online)

same effect was observed in GS activity (Fig. 5b). Properly increasing N supply effectively improved NR and GS activities under the SD and HF treatments. Except at 10 DAA, the N₂ was the best N supply for maintaining higher NR activity under the SF and SD treatments. Compared to the other treatments, the activities of NR and GS under the HD treatment decreased significantly after 13 DAA. At 16 and 19 DAA, NR and GS activities were significantly inhibited in response to N₂ and N₃ supplies compared with N₁ supply. Similar to NR activity, GS activity under all treatments was positively correlated with the increase in the N supply at 10 DAA. With increasing stress duration, the effect of N₃ supply on improving GS activity in the HF treatment was gradually weakened compared with N₂ supply, while N₁ supply was more conducive to maintaining higher GS activity in the HD treatment. The T, W, N, and M primarily affected the activities of NR and GS.

Effects on EL and the Contents of MDA and O₂⁻

During the entire stress period, drought stress caused a greater increase in MDA content than heat stress, and there was a significant superposition effect under combined stress (Fig. 6a). At 10 and 13 DAA, the MDA content under the HF and SF treatments decreased with increasing N supplies. N₁ or N₃ supply did not decrease the MDA content, and this result primarily occurred under the SD treatment (13 and 16 DAA), HF treatment (16 and 19 DAA), and HD treatment (10, 13, and 16 DAA). At 19 DAA, the effects of N on the MDA content under the SD treatment were opposite to those at 10 DAA, and the HD treatment yielded similar results.

EL showed a trend of first increasing and then decreasing with increasing stress time, and it reached a maximum at 16 DAA (Fig. 6b). The EL of N₃ supply under all stress treatments was significantly lower than that of N₁ supply at

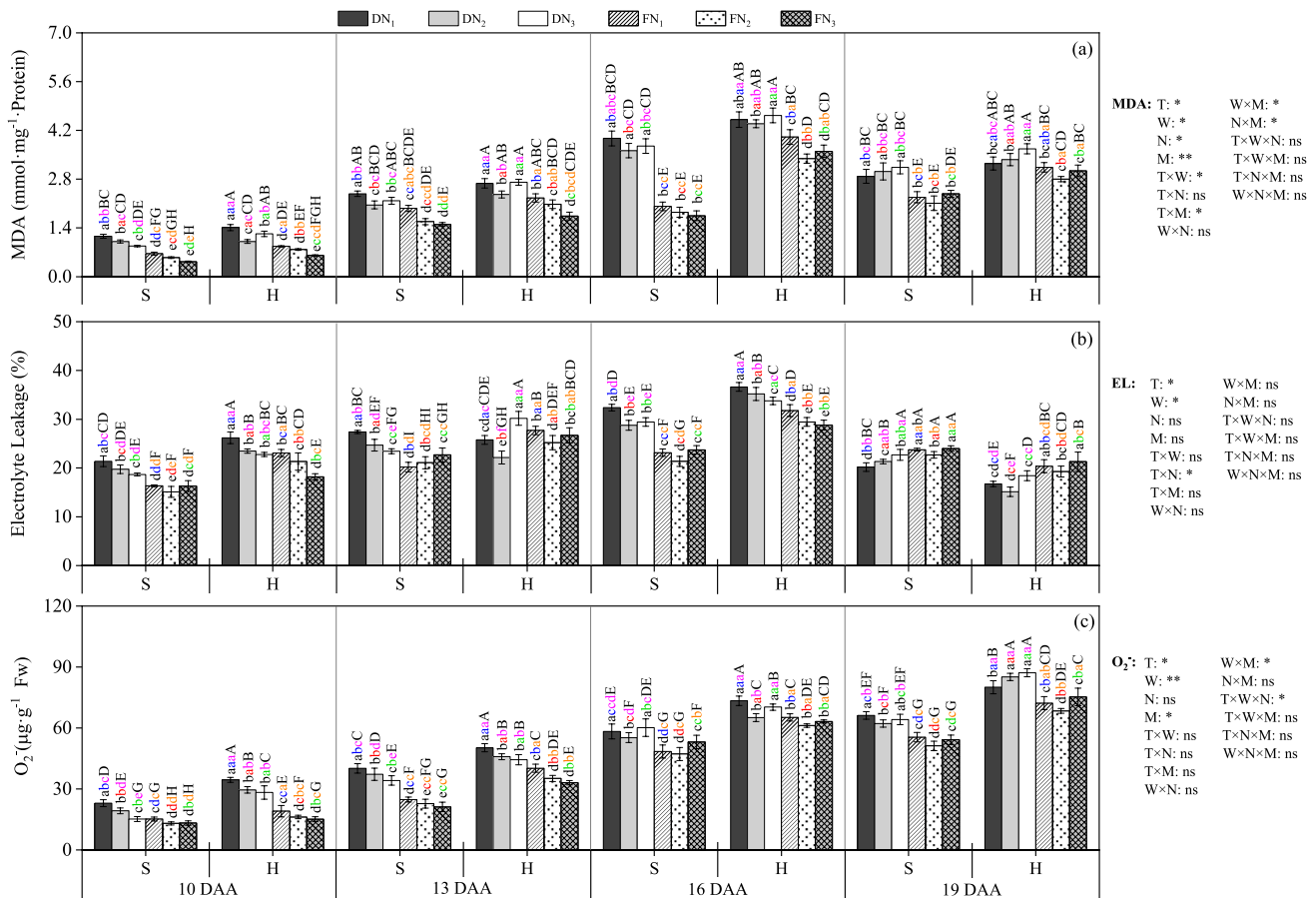


Fig. 6 Effects of the N supply on the content of malondialdehyde (MDA, **a**), electrolyte leakage (EL, **b**), and the content of superoxide anions (O₂⁻, **c**) in wheat leaves under heat, drought, and combined stresses. *S* suitable temperature, *H* high temperature, *F* full watering, *D* drought, *T* temperature, *W* watering, *N* nitrogen, *M* measurement date. The values are the means ± SEs, n=5. Black lowercase letters for comparisons between treatments under S and H conditions; Blue,

red, and green lowercase letters for comparisons between treatments under the N₁, N₂, and N₃ supplies, respectively; Pink and orange lowercase letters for comparisons between treatments under D and F conditions, respectively; Black uppercase letters for comparisons between all treatments combined by T, W, and N. *, **, and *** designate significant differences at *P* < 0.05, *P* < 0.01, and *P* < 0.001, respectively. ns indicates no significance (Color figure online)

10 DAA. At 13 DAA, N₃ supply significantly increased EL in HD-treated leaves, which was unlike the results obtained under individual stresses. However, N₂ and N₃ supplies effectively reduced the permeability of the cell membrane under the SD treatment (except at 19 DAA), and this result was also observed for the HF treatment. At 19 DAA, leaf cell membrane permeability under the HD treatment was severely reduced, which was reflected by the rapid decrease in electrolytes.

Compared to the responses to individual stresses, the increase in O₂⁻ content in response to combined stress caused more oxidative damage (Fig. 6c). At 10 and 13 DAA, the accumulation of O₂⁻ was similar between the SD and HF treatments. With increasing stress duration, the content of O₂⁻ caused by heat stress gradually increased compared with that under drought stress, the content of O₂⁻ in the SD, HF, and HD treatments increased by 19.70%, 34.21%, and

56.88% compared with the control at 19 DAA, respectively. Increasing the N supply effectively inhibited the accumulation of O₂⁻ under each treatment at 13 DAA, and the effects of N₃ supply were the strongest. The content of O₂⁻ of N₂ supply in the SD and HF treatments was lower than that of N₁ supply at 19 DAA, while N₁ supply maintained the O₂⁻ content at a low level under the HD treatment. MDA was mainly affected by single factors and the interaction of two factors. T, W, and T×N primarily affected EL. T, W, M, and the interactions of W×M and T×W×N significantly influenced the content of O₂⁻.

Effects on the SP, SS, and Pro Contents

Heat, drought, and combined stresses increased the content of SP in the leaves (Fig. 7a). The rapid increase in the SP content under the HD treatment at 13 DAA indicated the

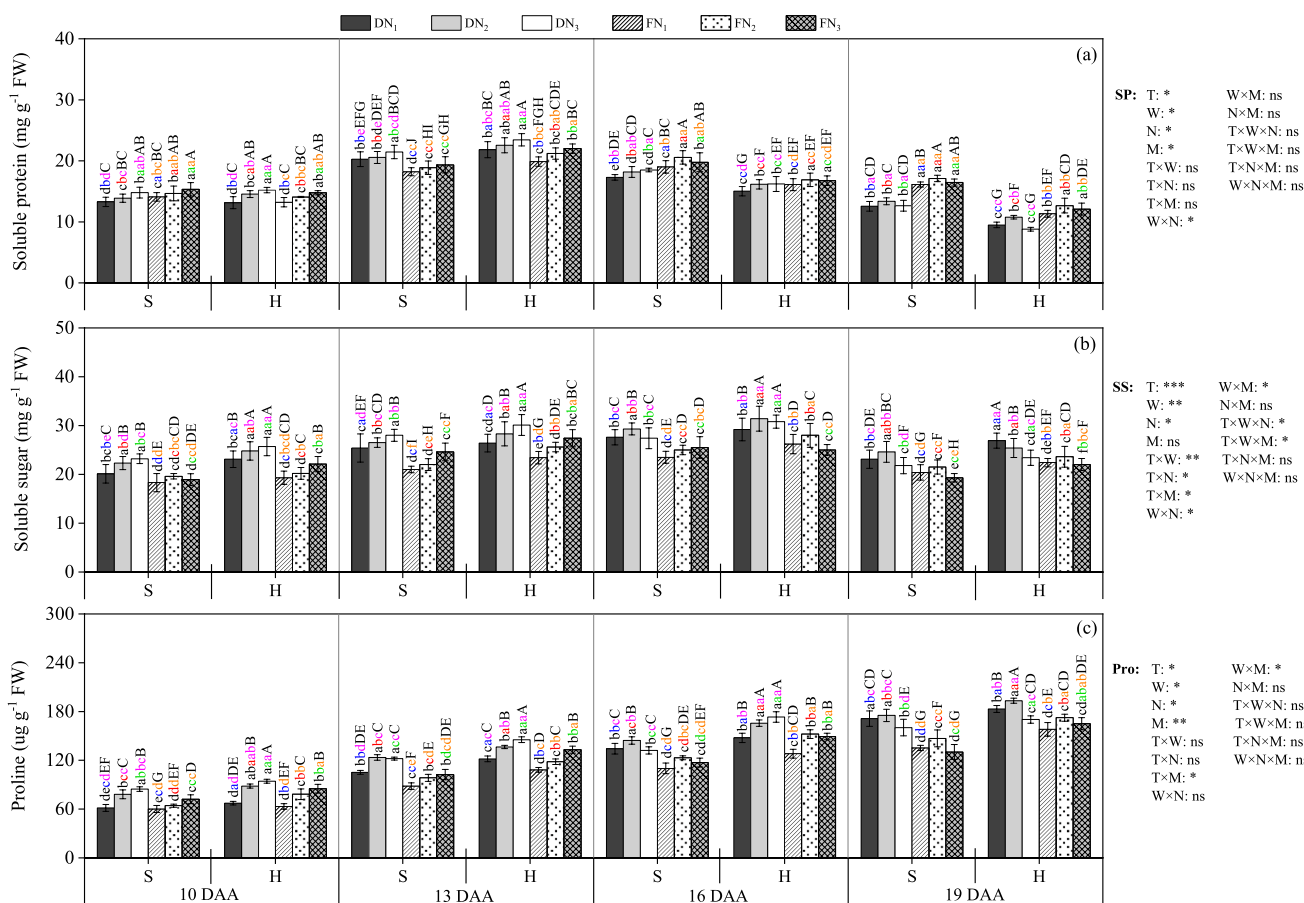


Fig. 7 Effects of the N supply on the contents of soluble proteins (SP, a), soluble sugars (SS, b), and proline (Pro, c) in wheat leaves under heat, drought, and combined stresses. S suitable temperature, H high temperature, F full watering, D drought, T temperature, W watering, N nitrogen, M measurement date. The values are the means±SEs, n=5. Black lowercase letters for comparisons between treatments under S and H conditions; Blue, red, and green lowercase letters

for comparisons between treatments under the N₁, N₂, and N₃ supplies, respectively; Pink and orange lowercase letters for comparisons between treatments under D and F conditions, respectively; Black uppercase letters for comparisons between all treatments combined by T, W, and N. ‘*’, ‘**’, and ‘***’ designate significant differences at P<0.05, P<0.01, and P<0.001, respectively. ns indicates no significance (Color figure online)

defense strategy of an active response to combined stress, unlike that under individual stresses. However, this stress response also resulted in a significant reduction in the SP content during subsequent stress. The regulatory effects of N on the SP content under all treatments were similar at 10 and 13 DAA, increasing the N supply can effectively improve the SP content under all stress treatments. At 19 DAA, the content of SP under the N₂ supply was significantly increased compared with N₁ supply, especially in the HF and HD treatments (Fig. 7b). N₂ supply at 19 DAA maintained the SS content under each treatment (except HD) at a higher level, while N₁ supply was more conducive to increasing the SS content under the HD treatment. For Pro, the N₂ supply promoted an increase in the SD and HF treatments at 16 and 19 DAA. Except at 19 DAA, the Pro content under the HD treatment significantly increased in response to increasing the N supply (Fig. 7c). SP was mainly affected by single factors. T, W, and the interaction of T × W had very significant effects on SS. The regulatory effects of T, W, N, M and the

interactions of T × M and W × M on Pro were more significant than those of other factor.

Effects on Yield and Its Components

Compared to the control, the SGN, SSR, TGW, and grain yield of wheat decreased when exposed to heat and drought stresses, and combined stress showed obvious superposition effects (Fig. 8). The reduction in yield components in drought-stressed plants was higher than that in heat-stressed plants, which led to a lower yield under the SD treatment than under the HF treatment. The SGN and SSR increased under the HF treatment with increasing N supply and reached a maximum at the N₃ supply. The SGN, SSR, and TGW under the SD treatment remained at their maximum at the N₂ supply. Notably, increasing N supply significantly inhibited the increases in the SGN, SSR, and TGW under the HD treatment, especially affecting the SGN and TGW. Compared to N₁, N₂ and N₃ supplies increased the grain

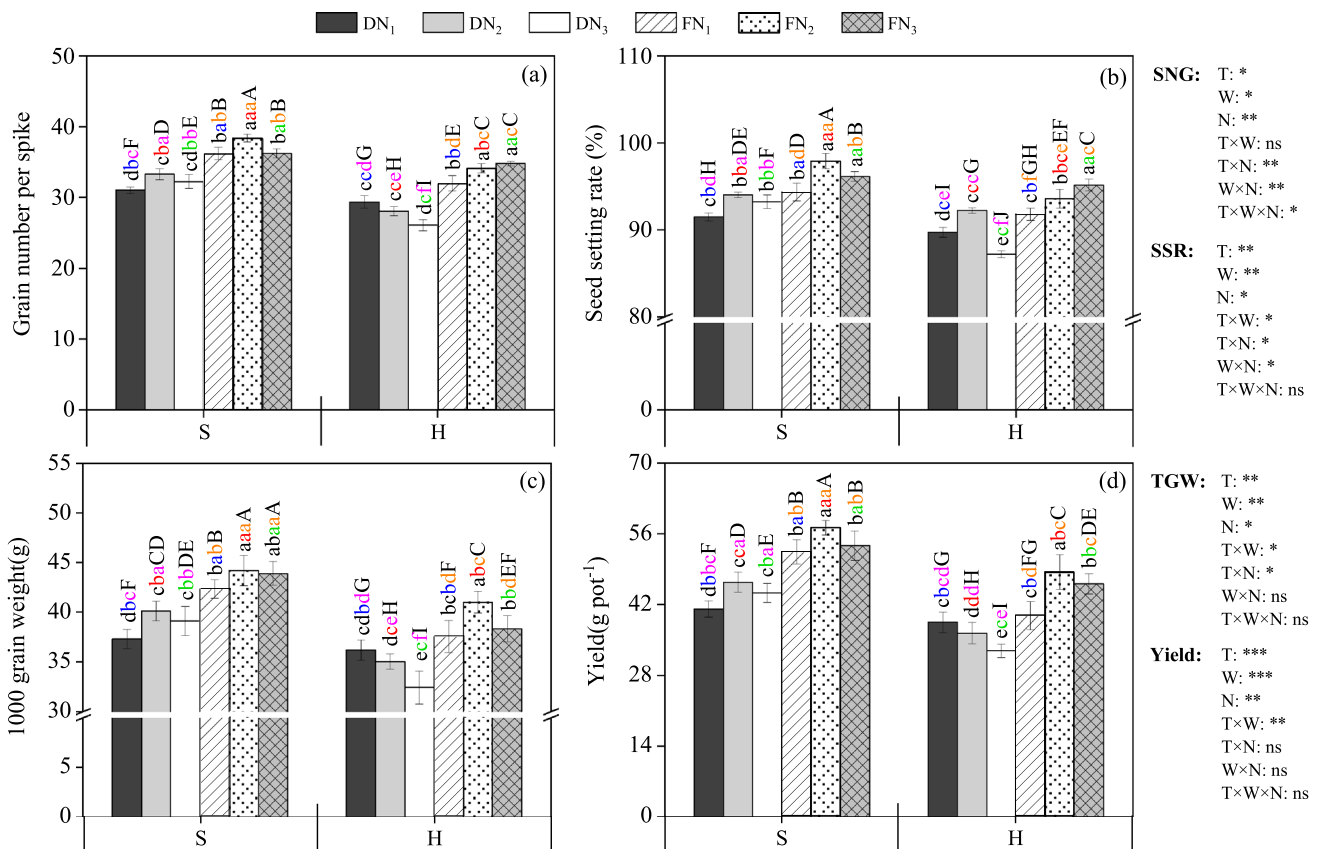


Fig. 8 Effects of the N supply on the grain number per spike (SGN, a), seed setting rate (SSR, b), 1000-grain weight (TGW, c), and yield (d) of wheat under heat, drought, and combined stresses. S suitable temperature, H high temperature, F full watering, D drought, T temperature, W watering, N nitrogen. The values are the means ± SEs, n=3. Black lowercase letters for comparisons between treatments under S and H conditions; Blue, red, and green lowercase letters

for comparisons between treatments under the N₁, N₂, and N₃ supplies, respectively; Pink and orange lowercase letters for comparisons between treatments under D and F conditions, respectively; Black uppercase letters for comparisons between all treatments combined by T, W, and N. ‘*’, ‘**’, and ‘***’ designate significant differences at P < 0.05, P < 0.01, and P < 0.001, respectively. ns indicates no significance (Color figure online)

yield under the HF and SD treatments, and the grain yield with N_1 supply under the HD treatment was 17.06% higher than that with N_3 . Except for the interaction of $T \times W$, other factors and their interactions had significant effects on the SGN. T and W had very significant effects on the SSR and TGW. T, W, N and the interaction of $T \times W$ had very significant effects on the grain yield.

Relationships of Leaf Photosynthesis with Antioxidant Enzyme and N Metabolism-Related Enzyme Activities

We evaluated the relationships of the net photosynthesis rate (A) with the activities of antioxidant enzymes (SOD, POD, CAT, GR, and APX) and N-metabolizing enzymes (NR and GS) (Fig. 9). SOD, POD, CAT, GR, and APX activities decreased linearly with increasing A, and the R^2 value of POD activity was the highest ($R^2 = 0.684$). In contrast, NR and GS activities increased linearly with increasing A. Compared to antioxidant enzyme activities, NR and GS activities had a stronger correlation with A (NR, $R^2 = 0.779$, GS, $R^2 = 0.788$).

Multivariate Statistical Analysis Results of the Different Responses of Plant Physiological and Biochemical Parameters to N Supply Levels Under Heat and Drought Stresses

PLS-DA based on a variable importance in projection (VIP) plot showed the responses of various physiological and biochemical parameters to different N supplies under

heat, drought, and combined stresses (Fig. 10a, b and c). VIP plots were used to determine VIP scores (0.0–3.0) for the top 15 components. Under drought conditions, the Pro content and POD and CAT activities increased significantly with an increase in the N supply (VIP score higher than 1) (Fig. 10a, marked in brown), and the content of O_2^- decreased significantly (Fig. 10a, marked in blue), which indicated that Pro, POD, and CAT had critical roles in wheat drought stress defense mechanisms. We found that Pro, SOD, and POD increased significantly (Fig. 10b, marked in brown), which indicated that Pro, SOD, and POD played a significant positive role in wheat heat stress defense mechanisms. In addition to Pro, SOD, and POD, GR actively participated in the defense system to provide stress resistance under the combined stress of heat and drought, but this result was not significant under individual stresses. PCA (Fig. 10d) showed that the cumulative contribution rate of principal component 1 (PC1; 89.8) and principal component 2 (PC2; 7.9%) reached 97.7%. As shown in Fig. 10d, SP, GS, E, A, NR, and g_s assembled along the abscissa axis on the right side of the biplot as a group. This grouping indicates a strong positive correlation between the abovementioned parameters. APX, GR, SOD, and Pro and other parameters segregated oppositely from SP, GS, E, A, NR, and g_s segregation, and the angle between the two groups of variables was obtuse. This result indicates that the correlation between these variables is negative (Fig. 10d).

The increases and decreases in physiological and biochemical characteristics were evaluated, and heatmap analysis was performed. A cumulative heatmap was constructed to clarify the responses of physiological and biochemical

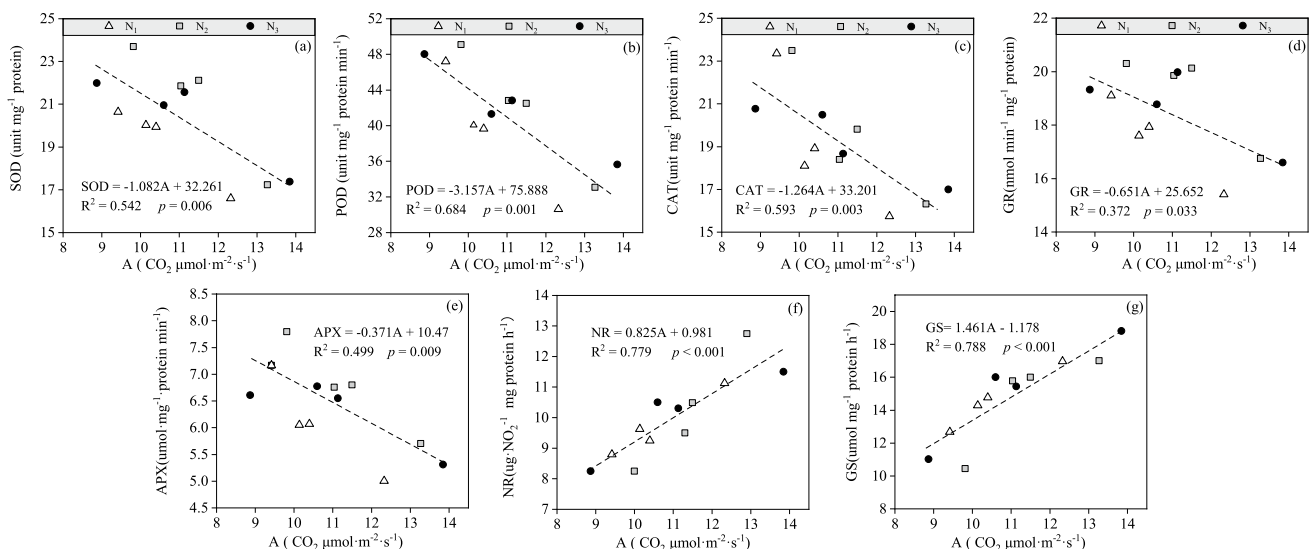


Fig. 9 Relationships of leaf photosynthesis with the activities of antioxidant enzymes (SOD superoxide dismutase, POD peroxidase, CAT catalase, GR glutathione reductase, APX ascorbate peroxidase, a, b,

c, d and e) and N metabolism-related enzymes (NR nitrate reductase, GS glutamine synthetase, f and g). The data points in the figure are the means of four measurement stages

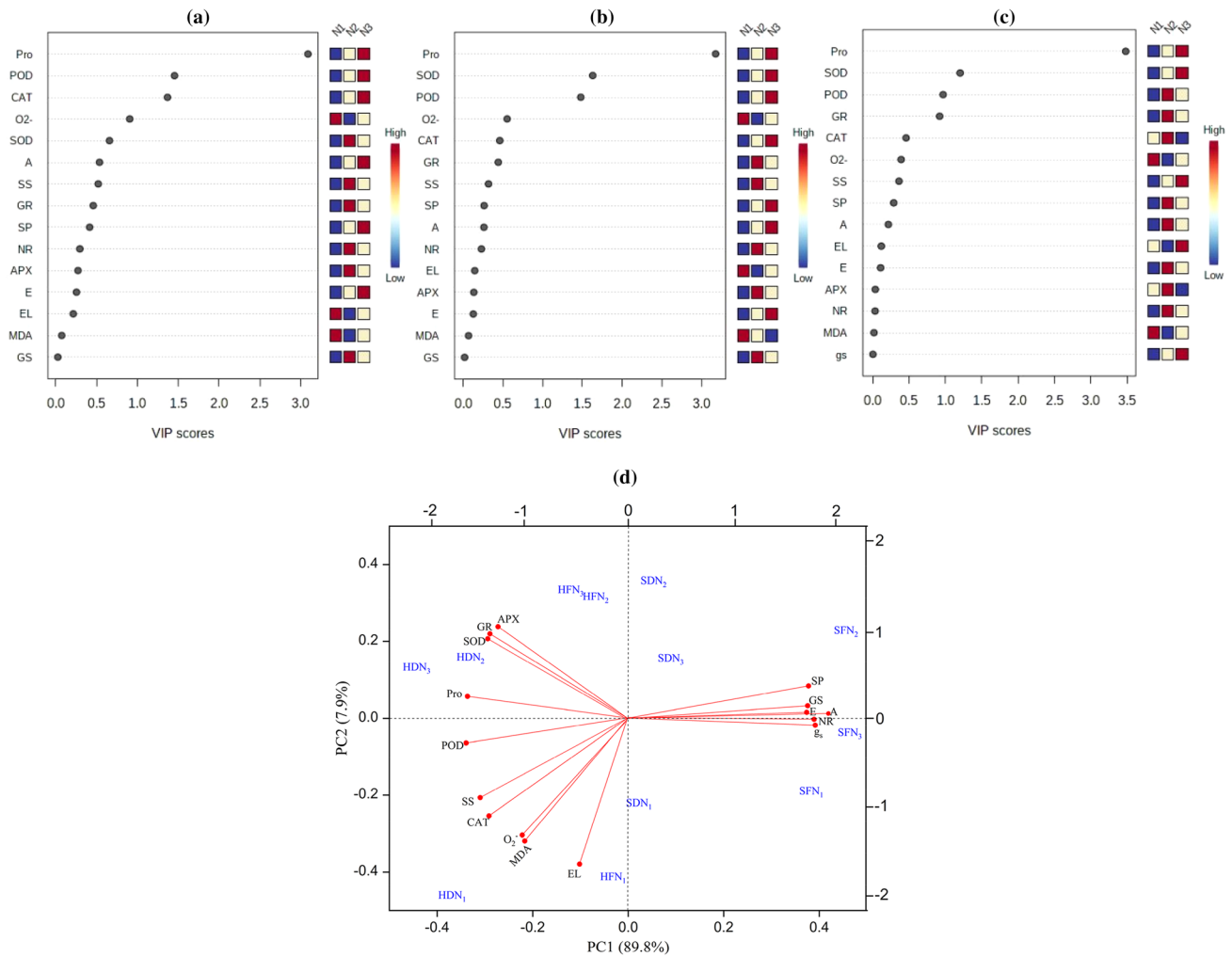


Fig. 10 **a**, **b**, and **c** VIP score plot and **d** biplot of the PCA results. **a** VIP plot of various physiological and biochemical parameters under drought stress with the addition of different N levels. **b** VIP plot of physiological and biochemical parameters under heat stress with the

addition of different N levels. **c** VIP plot of physiological and biochemical parameters under the combined stress of drought and heat with the addition of different N levels

parameters to different levels of N application (Fig. 11). Under heat, drought, and combined stresses, we found significant increases in APX, SOD, GR, CAT, and POD activities and the contents of SS and Pro and a significant reduction in the SP content, E, A, g_s , NR activity, and GS. Notably, combined stress showed a significant superposition effect. An appropriate N supply increased antioxidant enzyme activity and the osmoregulatory substance content in cells and effectively reduced damage due to O_2^- and MDA contents in plants.

According to Fig. 10a, b, and c, the main heat, drought, and combined stress resistance parameters (Pro, POD,

SOD, CAT, and GR) were selected. The Pearson correlation coefficients between the main stress resistance parameters and other physiological and biochemical parameters are shown in Fig. 12. The correlation coefficients of Pro with POD, SOD, GR, and SS were all above 0.90, but Pro showed a weak negative correlation with SP ($P > 0.05$). The correlations between POD and other physiological and biochemical parameters were significant. SOD and GR showed stronger positive correlations with Pro, APX, POD, and SS and weak correlations with SP, GS, and EL. EL, MDA, and O_2^- showed a consistent positive correlation with the increase in CAT.

Fig. 11 Cumulative heatmap of physiological and biochemical parameters under heat, drought, and combined stresses with different N supplies. (1) represents heat stress treatments, (2) represents drought stress treatments, (3) represents combined stress treatments, and (4) presents control (SF) treatments

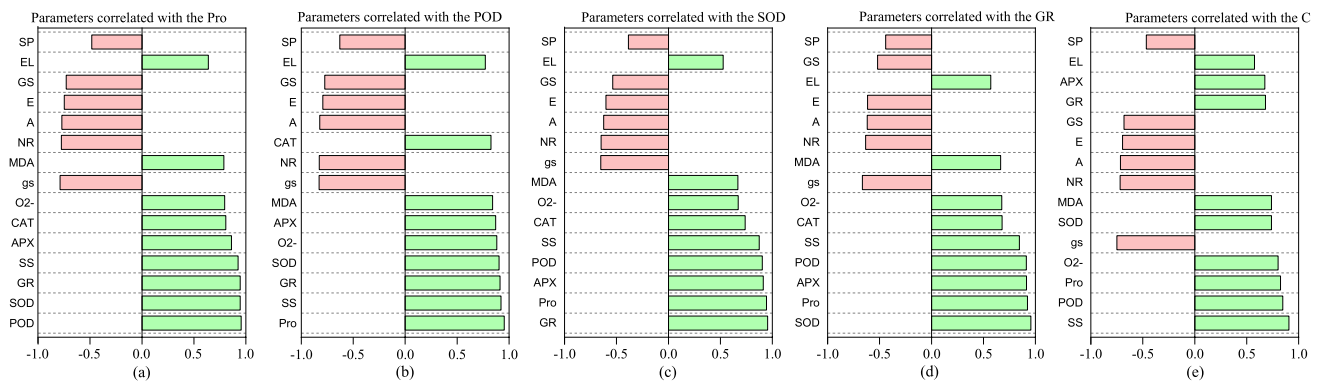
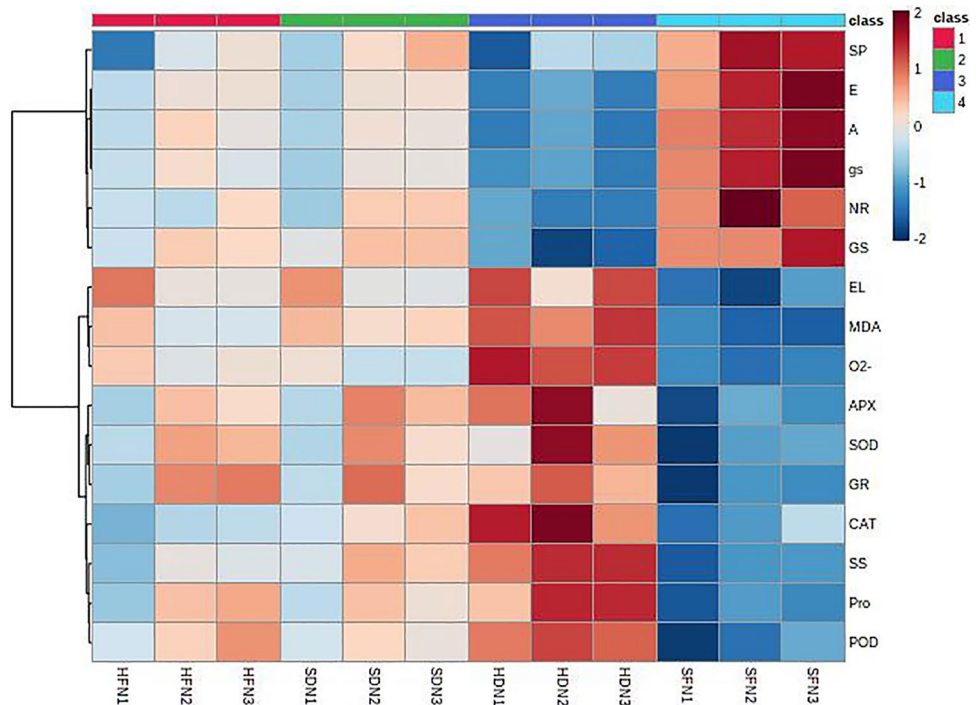


Fig. 12 Correlations of the main wheat stress resistance parameters with other physiological and biochemical parameters. Green represents a positive correlation, and pink represents a negative correlation. Correlation coefficients of proline (Pro, **a**), peroxidase (POD,

b), superoxide dismutase (SOD, **c**), glutathione reductase (GR, **d**), and catalase (CAT, **e**) with other parameters under heat, drought, and combined stresses with the addition of different N supplies

Discussion

Effects of N on the Growth and Yield of Wheat Under Heat, Drought, and Combined Stresses

Drought and heat stresses are the main abiotic stress factors affecting plant growth and development, and long-term heat or drought stress has an irreversible negative impact on plant growth (Hossain et al. 2012; Lobell et al. 2015). In our study, heat and drought stresses caused significant decreases in ADW, GLA, and PH, and combined

stress showed a typical superposition effect (Table 1). The significantly decreased ADW induced by heat and drought stresses may have been due to the decline in meristem activity and the inhibition of cell division, which led to a decrease in aboveground dry matter. Previous studies reported that temperatures above 31 °C during grain filling accelerated the senescence of wheat leaves, which resulted in leaf wilting and yellowing (Asseng et al. 2011). The decrease in the GLA under heat stress observed in the present study confirmed the previous conclusion. Previous studies have shown that increasing the N supply can reduce or aggravate the inhibitory effect of drought stress

on growth (Liu et al. 2012; Agami et al. 2018). Our results showed that a reasonable N supply effectively improved the growth of wheat under heat and drought stresses. Under individual stresses (heat and drought), medium N (N_2) or high N (N_3) increased the ADW, GLA, and PH to some extent, which is consistent with Agami et al. (2018), who suggested that N actively promoted plant growth and biomass accumulation under drought stress conditions. Similar conclusions were also found in other studies, i.e., Liu et al. (2012) believed that under drought stress, an appropriate N supply had a positive effect on plant growth and development. Waraich et al. (2012) suggested that increasing the N supply can improve plant growth traits and heat stress tolerance. The responses of plants to the N supply, soil moisture, and high temperature suggested that the N supply can effectively regulate the effects of drought and heat stresses on plant growth. It is interesting to note that increasing the N supply could not alleviate the adverse effects of combined heat and drought stress. In contrast, it aggravated the effects of combined stress and led to a decrease in plant growth. Therefore, it can be seen from the above findings that N plays a vital role in regulating the effects of heat and drought stresses on plant growth. However, its regulatory effects significantly differ due to differences in stress types.

Heat and drought stresses affect wheat crops at all growth stages, especially in the early stage of grain filling. Heat and drought stresses at this stage usually reduce the average grain number and grain yield (Semenov et al. 2014). Altenbach (2012) reported that severe drought or heat stress during the reproductive stage of wheat inhibited carbon assimilation and N absorption, which were the main reasons for the reduction in yield. In the current study, heat and drought stresses significantly reduced the wheat grain yield, which was mainly attributed to the reduction of the SGN, SSR, and TGW, while the reduction in the grain yield under combined stress was primarily attributed to the lower SGN and TGW. Previous research has illustrated that N application could significantly improve the growth traits and productivity of wheat (Agami et al. 2018). With increasing N application, the number of panicles, grains per panicle, TGW, and yield components of winter wheat first increased and then decreased (Kubar et al. 2021). We found that wheat yield and its components increased significantly under N_2 and N_3 compared to N_1 under individual heat or drought stress in the current study. This finding was previously noted in some crop plants, i.e., Lalelou and Fateh (2014) found that nitrogen supplementation compensated for the wheat yield loss and deteriorative effects of water-deficit conditions. A high N rate at panicle initiation or flowering was shown to alleviate the detrimental effects of high temperatures on grain yield formation (Duan et al. 2013). Our study also found that the yield response to N in heat-stressed plants was similar

to that in drought-stressed plants. Reduced N application was more helpful than N_2 and N_3 for maintaining a higher SGN and TGW and increased yield in wheat under combined stress. However, a low N supply effectively alleviated the negative effect of heat stress on grain formation, which indicated that the grain weight decreased slightly under a low N supply compared with a high N supply (Ordóñez et al. 2015). These findings showed that plants can better cope with one abiotic stress or several completely different abiotic stresses under an insufficient N supply (Elía et al. 2018), but the related internal physiological mechanisms need to be further explored.

Effects of Nitrogen on Leaf Photosynthetic Parameters Under Heat, Drought, and Combined Stresses

The decrease in photosynthetic activity caused by heat stress is related not only to stomatal closure, but also to the structural rearrangement of thylakoids (Mathur et al. 2014). Excessive ROS induced by drought stress causes serious structural and functional damage primarily in chloroplasts, including the overoxidation of thylakoid membranes, thereby significantly reducing photosynthesis (Mu et al. 2021). In our study, heat, drought, and combined stresses significantly decreased A in leaves. This negative impact was more significant in combined-stressed plants, which was also confirmed by Correia et al. (2018). However, Loka et al. (2020) reported that the photosynthetic rate under heat stress remained similar to that of the control, which they attributed to increases in g_s and E. We assume that the difference between the findings of these studies is due to the longer duration of the heat stress treatment in the latter because the resulting increase in the transpiration rate effectively reduces the leaf temperature and prevents the destruction of the photosynthetic mechanism.

It was previously reported that increasing the N supply can weaken crops under drought stress and that g_s decreased under a high N supply after 3 d of drought stress compared with plants supplied with medium N (Ding et al. 2018). Drought stress inhibited the photosynthetic rate in plants with a sufficient N supply, but this effect was not significant in plants with N deficiency (Zong and Shangguan 2014). A medium or high N supply increased A in leaves under stress in our study (Fig. 2a), and this regulatory effect was more pronounced in plants under individual stresses. The increase in photosynthesis may be related to the increase in the total chlorophyll content and chloroplast photochemical activity induced by increasing the N supply (Li et al. 2013). The maintenance of stomatal opening under high N is important for maintaining leaf conductance for CO_2 transpiration, photosynthetic reactions, and electron transport (Zhong et al. 2017). Here, we also observed that an excessive N supply

could not alleviate drought stress in plants. In contrast, it aggravated drought stress and decreased photosynthesis, resulting in a reduction in plant growth. Previous studies also reported that a high N supply aggravated the degree of soil water deficit, and stomatal limitations in leaves increased significantly, which led to a decrease in g_s (Araus et al. 2020). This result corresponds to our findings, in which a high N supply (N_3) did not always have a positive regulatory effect on g_s during the stress period, especially under the HD treatment at 19 DAA. Heat stress and high N application exacerbated the occurrence of drought stress, which may be the main reason for the lower g_s observed under the high N treatment.

Effects of Nitrogen on EL and the Contents of O_2^- and MDA Under Heat, Drought, and Combined Stresses

Previous studies have reported that drought and heat stresses reduced the integrity of the cell membrane, increased lipid peroxidation, and eventually led to damage to cell structures (Khan et al. 2019; Neves et al. 2019). These results correspond to our findings, in which the contents of O_2^- and MDA in heat- and drought-stressed plants increased significantly. If ROS production is not counteracted by antioxidants, it may induce cell membrane damage, protein oxidation, and lipid peroxidation (Khan et al. 2019; Neves et al. 2019). In the late stage of stress (19 DAA), the contents of O_2^- and MDA in leaves under combined stress were significantly higher than those under individual heat and drought stresses. At this time, antioxidant enzymes could not effectively remove excessive O_2^- , resulting in the destruction of the membrane structure. The significant decrease in EL further confirmed this finding.

The adverse effects of heat and drought stresses on the cell membrane structure were evaluated in the current study. An increase in N application repaired the biofilm damage caused by stress to a certain extent, which was reflected by the significant reduction in the MDA content and EL. This positive modification of the cell membrane effect may be attributed to N nutrition maintaining membrane stability. This finding was consistent with some previous results. N nutrition contributed to drought tolerance in *bentgrass* by preventing cell membrane damage and lowering MDA accumulation, as well as improving osmoregulation. In addition, Agami et al. (2018) reported that under water-deficit conditions, increasing the N supply greatly reduced the relative conductivity and effectively reduced cell membrane damage under water deficit; furthermore, N application decreased the release rates of H_2O_2 , O_2^- , and MDA in sorghum plants under drought stress (Shehab and Guo 2020). The study also found that the MDA contents under N_2 and N_3 supplies were lower than that under the N_1 level in heat-stressed plants,

which reflected the improvement of wheat heat tolerance after N application, and the damage to cell membrane lipids was reduced. Similar results were also observed in drought-stressed plants (except at 19 DAA). Compared to drought stress, the positive effect of N on membrane stability under heat stress was more pronounced, which may be related to the coordination ability of enzymatic and nonenzymatic antioxidant systems.

Effects of Nitrogen on Antioxidant Enzyme and N Metabolism-Related Enzyme Activities Under Heat, Drought, and Combined Stresses

Reducing ROS damage to the cell membrane system requires increases in the activities of enzymes involved in ROS scavenging to maintain the balance between ROS production and scavenging in plants (Neves et al. 2019). In our study, SOD, POD, CAT, APX, and GR activities increased under heat and drought stresses. Similar conclusions were found by Sattar et al. (2020). It is worth noting that the levels of ROS and MDA were higher under combined drought and heat stress than their individual stresses in wheat plants, which was consistent with the research results of Hussain et al. (2019). We also found that the changes in antioxidant enzyme activity within a certain stress duration were similar to those of the O_2^- content. This result is because the extension of the stress duration leads to O_2^- accumulation in cells, and the increased O_2^- content greatly activates antioxidant enzymes to counteract the effects of oxidative damage. The activities of antioxidant enzymes in the leaves decreased during the late stress period (Figs. 3, 4), which suggests that the ROS-scavenging system cannot completely alleviate oxidative damage caused by combined stress. It was also reported that combined drought and heat stress led to lower POD and APX activities compared to those observed under drought stress. The researchers suggested that this result was due to the accumulation of more secondary metabolites and osmolytes under high-temperature conditions. The decrease in enzyme activity under combined stress requires further exploration.

Previous studies showed that increasing the supply of N improved the activity of the cell enzymatic defense system under drought stress conditions (Agami et al. 2018). Gou et al. (2017) reported that under drought stress conditions, foliar application of urea could increase SOD and POD activities and reduce MDA content in maize. Similar conclusions were also observed in the current study. Under individual stresses and combined stress, a medium or high N supply increased the activities of antioxidant enzymes in wheat leaves, which indicates that the redox defense state of plants was greatly improved to reduce the damage caused by ROS. Previous research has illustrated that N-deprived plants generate greater accumulations of

antioxidant compounds and, consequently, greater protection against peroxidation, while the exposure of plants to excess nitrate or ammonium leads to nutritional stress (Chen et al. 2009). This is consistent with the finding corresponding to a low N supply under combined stress in our study. Moreover, the VIP scores (Fig. 10) further confirmed the above conclusion. The increased SOD, POD, and CAT activities (VIP > 1) induced by an appropriate N supply played a leading role in coping with individual heat and drought stresses and effectively reduced the oxidative damage induced by heat, drought, and combined stresses. This result strongly confirms the previous research findings, i.e., Li et al. (2020a) suggested that increasing the N supply alleviated the physiological damage caused by drought stress by enhancing the antioxidant enzyme activities and cell osmoregulation. Lin and Lai (2013) and Shafi et al. (2015) believed that the relative expression of SOD, POD, CAT, and APX isoforms was significantly elevated upon drought occurrence under sufficient N conditions but not under deficient N conditions. These expression patterns explained the N-dependent induction of antioxidant enzymes upon drought occurrence. The regression analysis in Fig. 9 shows that although there was a negative correlation ($P < 0.05$) between A and the antioxidant enzyme activities under stress conditions, the increased activities of antioxidant enzymes under a reasonable N supply improved A to a certain extent (Fig. 9), which indicates that the development of an antioxidant defense mechanism with N regulation under heat, drought, and combined stresses may contribute to the protection of photosynthetic processes. Shehab and Guo (2020) reported that the development of favorable ROS-detoxifying antioxidant systems in drought-stressed plants under higher N levels might have contributed to the improvement of photosynthesis. This finding is consistent with the results of this study.

In the current study, compared to the low N supply, GS activity under each treatment had a pronounced positive response to the N₂ supply, and a sufficient N level increased the abundance of enzymes and their activities (Gao et al. 2012). This indicates that a sufficient N supply ensures a smooth and efficient N metabolism process in leaves after anthesis and increases the level of N metabolism in plants. Plant N metabolism has a significant regulatory effect on photosynthetic capacity. The positive correlation between A and NR and GS activities (Fig. 9) indicated that the increased NR and GS activities under an increased N supply contributed to the increase in A. This result may be because N₂ or N₃ supply enhanced N absorption and transformation ability and improved the photosynthetic N utilization efficiency to increase A.

Effects of Nitrogen Cell Osmotic Adjustment in Leaves Under Heat, Drought, and Combined Stresses

In the current study, the Pro content under individual stresses increased significantly compared to that under the control conditions. This result is consistent with the findings of Kishor et al. (2005) and Hussain et al. (2018), who demonstrated that Pro was conducive to stabilizing membranes, subcellular structures, and the cellular redox potential by destroying free radicals. We also found that heat and drought stresses resulted in a significant increase in the SP content, which may be attributed to the increased production of amino acids under heat and drought stresses (Sattar et al. 2020). Nevertheless, Jing et al. (2020) reported a reduction in total protein in plants under heat stress conditions. This decrease in protein content could be due to a decrease in protein synthesis or an increase in protease activity, resulting in a higher rate of protein degradation.

N addition may induce greater biosynthesis of leaf photosynthetic pigments and consequently improve the photosynthetic process to produce more assimilates, including osmoprotectants. These osmoprotectants include SS, SP, and Pro (Agami et al. 2018). Hassanein et al. (2009) believed that SS plays a vital role in alleviating the stress impacts either by osmoregulation or by providing some desiccation tolerance to plant cells. Rahimi et al. (2013) suggested that increasing the N supply under drought stress increased the SS and Pro contents in *plantains*. In this study, appropriately increasing N application (N₂ or N₃) induced greater osmoregulatory substance (SP, SS, and Pro) contents (Fig. 7), which suggests that the increased osmoregulatory substances caused by increasing the N supply played an important role in alleviating the negative impacts of heat and drought stresses on wheat. These results were in agreement with previous findings observed in barley and wheat, in which it was found that N application improved the accumulation of SS in plant leaves and increased antioxidative defense mechanisms to reduce oxidative damage (Waraich et al. 2011). It is very interesting that the positive effect of the N₃ level on the increase in the SP, SS, and Pro contents greatly diminished at 19 DAA, which indicates that high N application dose not always have a positive effect on plants. Pro plays a variety of positive roles in stress resistance and plant protection, and there is a strong correlation between the Pro content and the capability to overcome stress (Liu et al. 2021). This study strongly supported previous research results. We found that the VIP score of Pro (VIP > 3) was the highest of all physiological and biochemical parameters under individual and combined stresses (Fig. 10a, b, and c). These results address our second study objective. The same result was obtained by Li et al. (2020a), who found that an increase in N supply can improve antioxidant enzyme activities and osmoregulation

by inducing Pro accumulation to alleviate physiological damage caused by drought stress. It has also been reported that sucrose, rather than Pro, accumulates as an osmotic protective agent under the combination of heat and drought in *Arabidopsis*, and Pro accumulates under drought stress conditions. Pro may be toxic to cells in combined-stressed plants (Rizhsky et al. 2004). This result is different than the conclusion of our study, which may be related to the stress duration and the different tolerances of plant species to stress.

Conclusion

Under individual heat and drought stresses, wheat plants treated with a medium N supply maintained higher photosynthesis, N metabolism-related enzyme activities, anti-oxidant enzyme activities, and osmoregulatory substance contents and lower MDA and O_2^- contents and EL. However, reducing N supply (N_1) had a similar positive regulatory effect on wheat plants under combined stress. The increases in Pro, SOD, POD, CAT, and GR under N regulation were more conducive to improving the resistance of wheat plants to heat, drought, and combined stresses. The current data showed that increasing the N supply can alleviate the adverse effects of heat and drought stresses on wheat growth and production by preventing cell membrane damage and enhancing antioxidant capacity and cell osmoregulation. However, under combined stress conditions, a low N supply is more useful for improving the growth and grain yield of wheat plants. Under the background of frequent heat and drought events, future studies need to accurately diagnose the demand of plants for N fertilizer to better address heat, drought, and combined stresses.

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Data Availability Datasets used and/or analyzed during the current study that are not included in the manuscript are available from the corresponding author.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest to this work. The authors declare that they do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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