

Optimizing the EBR spraying window can maintain the physiological function of wheat flag leaves under heat stress and thus grain yield

Ming Su

Ningxia University

Zhengrui Nie

Ningxia University

Yueqi Li

Ningxia University

Ziqiang Hong

Ningxia University

Xiaochun Wang

Sichuan Agricultural University

Hongliang Wu

nxuwu@nxu.edu.cn

Ningxia University

Jianhong Kang

Ningxia University

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Abstract

Post-anthesis heat stress is a major abiotic stress restricting grain filling and yield formation of spring wheat, and rational foliar application of 2,4-epibrassinolide (EBR) can alleviate these adverse impacts. To clarify the suitable spraying stage and concentration of EBR, field experiments were carried out in 2023-2024. Distilled water, 0.10 mg L⁻¹ and 0.20 mg L⁻¹ EBR were foliarly sprayed at 5, 10 and 15 d after anthesis, respectively. Subsequently, three-day consecutive heat stress (35 ± 2 °C) was imposed. We systematically analyzed flag-leaf physiological characteristics, antioxidant capacity, grain-filling dynamics, dry matter accumulation and yield responses. The results showed that compared with natural temperature, heat stress significantly reduced leaf area index, leaf biomass, SPAD value and net photosynthetic rate, inhibited antioxidant enzyme activities, increased malondialdehyde (MDA) content by 31.65% and hydrogen peroxide (H₂O₂) content by 35.45%, and disturbed endogenous hormone homeostasis. Under heat-stress conditions, the maximum grain-filling rate, mean grain-filling rate and dry-matter accumulation decreased by 8.59%, 8.89% and 12.21%, respectively, compared with natural temperature, with an average yield loss rate of 9.09%. Nevertheless, appropriate EBR spraying timing and concentration could effectively mitigate such adverse effects. Among all treatment combinations under heat stress, foliar application of 0.10 mg L⁻¹ EBR at 5 d after anthesis obtained the optimal performance. It markedly improved leaf physiological status and antioxidant capacity, and facilitated the balance of endogenous hormones. Compared with the water-sprayed control, this treatment increased the maximum grain-filling rate by 16.13%, dry-matter accumulation by 16.24% and grain yield by 12.50%, and reduced yield loss rate by 8.02%. Furthermore, random forest analysis indicated that Gmax was the key factor governing yield formation under heat stress. In summary, foliar application of 0.10 mg L⁻¹ EBR at 5 days after anthesis can effectively maintain flag-leaf function, promote grain filling, coordinate the source-sink relationship and stabilize spring wheat yield under post-anthesis heat-stress conditions.

1. Introduction

Wheat is one of the most important staple food crops worldwide, and its stable and high yield plays a fundamental role in ensuring global food security (Becker-Reshef et al., 2010). As a major wheat-producing country, China has a planting area of more than 23.5 million hectares, accounting for 10.68% of the global total harvested area and ranking third in the world, with an average yield of 5000–6000 kg ha⁻¹, contributing 18% of the global wheat production (FAO Stat, 2022; Kang et al., 2026). However, under the context of global warming, the regional extreme maximum temperature in China has increased significantly, posing a severe threat to wheat yield stability (Senapati et al., 2026). Studies have demonstrated that each 1°C rise in global mean temperature may reduce wheat yield by 2.4%–10.5% (Asseng et al., 2015; Li et al., 2025). In current field production, extreme high-temperature heatwaves mostly occur during the post-anthesis grain-filling stage. Plants possess limited self-repair capacity at this stage, and heat stress shortens the grain-filling duration and inhibits reserve accumulation, resulting in irreversible yield losses (Li et al., 2026). Post-anthesis heat stress reduced wheat grain yield by 24.80%, which was significantly higher than the 15.10% yield reduction caused by pre-anthesis heat stress (Bicego et al., 2026). These findings further demonstrate that post-anthesis heat stress acts as a critical environmental factor restricting wheat yield formation.

Northwest China accounts for approximately 10% of the national wheat planting area. In recent years, wheat frequently suffers from high temperatures above 30°C during the middle and late growth stages, accompanied by drought and high light stress, resulting in dry-hot wind that adversely affects grain filling and yield formation

(Liu et al., 2014; Zhong et al., 2026). Specifically, heat stress significantly restricts dry matter accumulation and assimilate translocation to grains, thereby reducing 1000-grain weight and causing yield losses (Sihag et al., 2024; Li et al., 2025). Post-anthesis grain filling constitutes the pivotal period for wheat yield formation. As the dominant photosynthetic source organ at this stage, flag leaf function directly determines source assimilate supply capacity (Li et al., 2025; Wang et al., 2026). Heat stress initially damages chloroplast structure, accelerates chlorophyll degradation, reduces leaf area index and photosynthetic performance, and decreases leaf relative water content (Bergkamp et al., 2018). Concurrently, excessive reactive oxygen species inhibit antioxidant enzyme activities and aggravate membrane lipid peroxidation, accelerating leaf senescence and shortening functional duration (Xu et al., 2026). In addition to oxidative damage, heat stress disrupts endogenous hormone homeostasis, disturbs source-sink coordination, and restrains grain filling and dry matter redistribution (Zhong et al., 2026).

In recent years, brassinosteroid (BR) and its analogs have emerged as critical regulatory substances for alleviating crop heat damage, owing to their dual functions in promoting plant growth and improving stress tolerance (Yin et al., 2025; Zhong et al., 2026). Foliar application of exogenous 2,4-epibrassinolide (EBR) can delay leaf senescence and sustain flag leaf function during grain filling by strengthening antioxidant defense system, maintaining cell membrane stability, protecting photosynthetic apparatus, regulating plant water status and balancing endogenous hormones (Wu et al., 2026; Zhong et al., 2026). In wheat, such regulatory effects not only stabilize photosynthetic capacity of source tissues, but also improve assimilate transport and sink organ sink strength, thereby increasing grain filling rate, prolonging effective filling duration and elevating final grain weight (Liang et al., 2023; Zhong et al., 2026). Nevertheless, most existing studies merely confirm the heat-stress-mitigation effect of exogenous BR and are mainly conducted under controlled environments (Yang et al., 2025; Zhang et al., 2023). Few spraying experiments have been performed targeting the late watery-ripe, early milk-ripe and mid-milk-ripe grain developmental stages corresponding to 5, 10 and 15 d after anthesis. Less attention has been paid to the optimal spraying timing, suitable concentration and their interactive effects after anthesis. In particular, field-based systematic evidence covering the complete physiological chain from flag-leaf function maintenance, antioxidant regulation and hormone homeostasis to grain-filling characteristics and yield formation remains insufficient.

Accordingly, this study adopted spring wheat as experimental material. Foliar spraying treatments of EBR were arranged at different post-anthesis stages combined with short-term post-anthesis heat stress. We systematically investigated the effects of spraying timing and concentration on flag leaf photosynthetic characteristics, antioxidant capacity, endogenous hormone levels, grain filling traits, dry matter accumulation and yield formation. This study aimed to screen out the optimal combination of spraying period and concentration, and clarify the physiological mechanisms by which EBR alleviates post-anthesis heat stress, delays flag leaf senescence and stabilizes as well as increases grain yield. The findings can provide theoretical basis and technical guidance for high-temperature resistant cultivation and heat-tolerant variety breeding of spring wheat.

2. Materials and methods

2.1. Site descriptions

Field experiments were conducted during the spring wheat growing seasons (February to July) of 2023 and 2024 at Pingjipu Experimental Farm in Yinchuan, Ningxia Hui Autonomous Region, China (106°01' E, 38°24' N, altitude 1110 m). This region features a temperate continental climate with an annual mean temperature of 8.5°C and annual precipitation ranging from 180 to 300 mm. The temperature and precipitation dynamics during the experimental period are shown in Fig. 1A. The experimental soil is light loamy light sierozem. The basic physicochemical properties of the 0–20 cm topsoil were as follows: soil organic matter 7.70 g kg⁻¹, Total N 0.72 g kg⁻¹, Total P 0.57 g kg⁻¹, Alkali-hydrolyzable N 31.64 mg kg⁻¹, Available P 13.87 mg kg⁻¹, Available K 198.32 mg kg⁻¹, and soil pH value of 8.46. Soil physicochemical properties were determined at the beginning of each growing season. In local conventional cultivation, spring wheat is planted via wide-narrow row pattern with wide row spacing of 15 cm and narrow row spacing of 10 cm under integrated drip irrigation and fertigation management (Fig. 1B).

2.2. Experimental design and field management

The experiment was arranged in a split-split plot design. The main plots consisted of two temperature treatments: natural temperature (NT) and heat stress (HT). The subplots included three EBR spraying timings, namely 5, 10 and 15 days after anthesis (T5, T10 and T15). The sub-subplots were assigned three EBR concentrations, including 0.00 mg L⁻¹ (CK, sprayed with distilled water), 0.10 mg L⁻¹ (C1) and 0.20 mg L⁻¹ (C2). Anthesis initiation was defined when 5%–10% of middle spike florets bloomed in late May, which was recorded as 0 day after anthesis. EBR solutions were uniformly sprayed at 7:00–9:00 on the 5th, 10th and 15th day after anthesis. A fine mist sprayer was adopted to ensure even and tiny droplets. Spraying was mainly performed on wheat spikes and upper leaves to achieve uniform coverage on the whole plants.

Heat stress treatment was subsequently imposed for three consecutive days spanning 16–18 d after anthesis. For the HT group, the mean daytime air temperature was maintained at 35 ± 2°C from 9:00 to 18:00, while night temperature remained consistent with ambient conditions. A total of eighteen treatments were established in this experiment. Each experimental plot covered an area of 20 m² with dimensions of 5×4 m, and all treatments were arranged in three biological replicates. The detachable greenhouse facilities were composed of steel frames (5.5 m in length, 4.5 m in width and 2 m in height), high-transmittance polyethylene films with light transmittance over 90%, automatic heaters, ventilation fans and humidity control systems. Ventilation windows were installed 1.0 m above the ground on both sides of the greenhouse. During the heat treatment period, canopy light intensity was measured daily using an AccuPAR LP-80 plant canopy analyzer (USA). Carbon dioxide concentration was recorded every 30 minutes via a portable WKN-GT100 detector produced in Japan, and ambient temperature as well as relative humidity were synchronously monitored at 30-minute intervals using Elmetron PWT-401 thermohygrometer. After three days of daytime heating treatment, the greenhouses were removed to allow wheat plants to grow naturally until maturity. The temperature dynamics of heat stress and natural temperature groups are presented in Fig. 2A, and other canopy microclimatic factors are shown in Fig. 2B-D.

The tested spring wheat cultivar was Ning 3015, the dominant local cultivar with high fertilizer requirement, with a standard sowing rate of 375 kg ha⁻¹. Uniform fertilization was adopted for all treatments, with nitrogen, phosphorus and potassium applied at 300, 150 and 180 kg ha⁻¹ respectively. All phosphorus and potassium fertilizers together with 40% of total nitrogen fertilizer were applied as basal fertilizer before sowing and incorporated into soil by rotary tillage. The remaining 60% nitrogen fertilizer was topdressed at seedling,

tillering, jointing, booting, heading and grain filling stages in the proportion of 1:2:2:2:1:2. Eight irrigations were performed during the whole growth season, with a total irrigation volume of 3600 m³ ha⁻¹, which was distributed to the above six growth stages at the ratio of 1:1:2:2:1:1. All other field management measures were identical to local conventional field production practices. Sowing was carried out on March 2 and harvesting was conducted on July 10 in both experimental years.

2.3. LAI, leaf biomass, SPAD and Pn

After the termination of heat-stress treatment in 2023 and 2024, five uniform wheat plants per treatment were selected for physiological measurements on sunny and windless mornings from 9:00–11:00. The net photosynthetic rate (Pn, $\mu\text{mol m}^{-2} \text{s}^{-1}$) of flag leaves was determined using a Li-6400 portable photosynthesis system (Li-COR, Lincoln, NE, USA), with 3 replicates for each treatment. Relative chlorophyll content was measured at the upper, middle and lower portions of flag leaves using a SPAD-502 (Konica Minolta, Japan) chlorophyll meter on five wheat plants per treatment, with 3 replicates for each treatment.

All sampled plants were transferred to the laboratory after field measurement. The length (L, cm) and width (W, cm) of all green leaves per plant were recorded, and individual leaf area was calculated with a shape coefficient of 0.83 to obtain plant leaf area (LA, m²). Leaf area index (LAI) was further calculated according to Formula 1 and Formula 2 (Lu et al., 2026). Subsequently, all leaf samples were deactivated at 105°C for 30 min and oven-dried to constant weight at 75°C. Leaf biomass (LB, g) was finally weighed by electronic balance, and the measurement was performed in three replicates per treatment.

$$\text{LA} = L \times W \times 0.83 / 10000 \quad (1)$$

$$\text{LAI} = \text{LA} \times \text{Planting density} / 10000 \quad (2)$$

2.4. Antioxidant capacity and MDA, H₂O₂, endogenous hormone content in flag leaves

After heat stress treatment in 2023 and 2024, fifteen flag leaves were sampled from each treatment on the next-day morning between 9:00 and 10:00, when air temperature and light intensity were nearly consistent across all treatments. The collected samples were placed into sampling boxes with ice packs and promptly transferred to the laboratory for preservation at – 80°C. Following the methods described by Kong et al. (2017) and Li et al. (2021), all leaf samples were fully ground into fine powder with liquid nitrogen. A total of 0.5 g leaf powder was placed into a 10 mL centrifuge tube and mixed with 5 mL 0.05 mol L⁻¹ phosphate buffer solution at pH 7.8. The mixture was kept static at 4°C for 10 min, and then centrifuged at 8000 rpm for 15 min in a refrigerated centrifuge. The obtained supernatant was stored at 4°C to determine the activities of superoxide dismutase (SOD, U g⁻¹ h⁻¹), peroxidase (POD, U g⁻¹ min⁻¹), catalase (CAT, U g⁻¹ min⁻¹), as well as the contents of malondialdehyde (MDA, $\mu\text{mol g}^{-1}$) in wheat flag leaves. Hydrogen peroxide content (H₂O₂, $\mu\text{mol g}^{-1}$) was determined according to the method described by Avalbaev et al. (2024).

Meanwhile, endogenous hormone extraction was conducted in accordance with the protocols of Yang et al. (2001), Xin et al. (2013) and Zhong et al. (2026). Briefly, 0.5 g ground leaf sample was transferred into a 10 mL centrifuge tube and homogenized with 4 mL extraction solution. The mixture was fully shaken and extracted at 4°C for 4 h, followed by refrigerated centrifugation at 8000 rpm for 15 min. The supernatant was collected and

its volume was recorded. The residue was re-extracted with another 1 mL extraction solution under the same conditions. All supernatants were pooled together, dried with nitrogen gas and stored at -20°C . The contents of gibberellin (GA, ng g^{-1}), abscisic acid (ABA, ng g^{-1}), cytokinin (CTK, $\mu\text{g g}^{-1}$) and brassinosteroid (BR, ng g^{-1}) in flag leaves were determined via indirect enzyme-linked immunosorbent assay (ELISA).

2.5. Grain filling characteristics

Grain filling samples were collected at 5-day intervals on the 19th, 24th, 29th, 34th and 39th day after anthesis upon the completion of heat stress treatment. Ten wheat plants were sampled per time, and their spikes were detached. All spike samples were fixed at 105°C for 30 min and then oven-dried to constant weight at 75°C . One thousand grains were randomly selected from each treatment to measure 1000-grain weight with an electronic balance. The Logistic equation was established between 1000-grain weight (GW) and days after anthesis (x) using CurveExpert 1.4 software to simulate grain filling dynamics (Zhai et al., 2024), and the specific formula was presented as follows.

$$\text{GW} = a / (1 + b \times e^{-cx}) \quad (3)$$

$$\text{Tmax} = (\ln b) / c \quad (4)$$

$$\text{Wmax} = a / 2 \quad (5)$$

$$\text{Gmax} = (c \times \text{Wmax}) / (1 - \text{Wmax} / a) \quad (6)$$

$$\text{T} = (\ln b + 4.59512) / c \quad (7)$$

$$\text{Gmean} = a / \text{T} \quad (8)$$

$$\text{P} = 6 / c \quad (9)$$

In this equation, a indicates the final 1000-grain weight, b and c are regression coefficients, and e is the base of natural logarithm. Additionally, Tmax represents the days required to reach the maximum grain filling rate (d), Wmax is the grain weight at the maximum filling rate (g), Gmax refers to the maximum grain filling rate ($\text{g 1000 grain}^{-1} \text{ d}^{-1}$), T means the effective grain filling duration (d), Gmean denotes the mean grain filling rate ($\text{g 1000 grain}^{-1} \text{ d}^{-1}$) and P is active grain filling period (d).

2.6 Dry matter accumulation and grain yield

At physiological maturity, ten individual wheat plants were randomly sampled from each treatment. All samples were killed at 105°C for 30 min and oven-dried to constant weight at 75°C to determine dry matter accumulation (DMA, g). For yield measurement, two central rows excluding field borders were selected in each plot, and wheat plants within 1-meter length of each row were harvested with three replications. Twenty wheat spikes were randomly collected for laboratory seed testing after natural air-drying to constant weight. Grain moisture content was measured using a grain moisture tester (PM8188, Kett, Japan), and final yield per unit area was calculated according to the standard conversion formula.

2.7. Statistical analysis

Variance analysis (ANOVA), least significant difference (LSD) test and multiple comparisons were performed via SPSS 22.0. Histograms and violin plots were plotted with GraphPad Prism. The partial least squares structural equation model (PLS-PM) was established using SmartPLS. Mantel test, correlation analysis and random forest model were conducted based on R language. Principal component analysis (PCA) and comprehensive score calculation were conducted with Origin 2025 following the method reported by Hong et al. (2024).

3. Results

3.1. LAI, leaf biomass, SPAD and Pn

Wheat leaf area index (LAI), leaf biomass, SPAD value and net photosynthetic rate (Pn) were significantly affected by temperature, EBR spraying timing and concentration (Fig. 3A-D). Compared with NT condition, HT markedly reduced LAI by 11.11%, LB by 16.74%, SPAD value by 10.79% and Pn by 19.49%. Rational regulation of EBR spraying timing and concentration could effectively improve basic physiological traits and delay leaf senescence. Under heat stress, LAI, LB, SPAD value and Pn gradually decreased with the delay of EBR application time, and increased firstly and then declined with the rise of EBR concentration. Specifically, the T5 treatment increased LAI, LB, SPAD value and Pn by an average of 18.62%, 14.04%, 6.63% and 18.24% relative to other spraying timings. In addition, foliar application of 0.10 mg L⁻¹ EBR at 5 days after anthesis (T5C1) significantly elevated LAI, LB, SPAD value and Pn by 19.42%, 13.74%, 6.96% and 16.71% in comparison with the water-sprayed control, which maintained favorable physiological status of leaves. Such positive effects were mainly attributed to the interactive effect between EBR spraying timing and concentration.

Correlation analysis integrating temperature, EBR spraying timing and concentration with leaf basic physiological traits revealed that both elevated temperature and delayed spraying timing were unfavorable for healthy leaf growth (LAI, LB, SPAD and Pn), whereas an appropriate EBR concentration alleviated these adverse effects (Fig. 3E).

3.2. Antioxidant capacity, MDA and H₂O₂ content

Temperature, EBR spraying timing and concentration exerted significant effects on antioxidant capacity, as well as MDA and H₂O₂ contents in wheat flag leaves (Fig. 4A-E). Compared with NT, HT notably reduced SOD, POD and CAT activities by 9.09%, 19.48% and 13.75%, respectively, and markedly increased MDA content by 31.65% and H₂O₂ content by 35.45% in flag leaves. Optimizing EBR spraying timing and concentration enhanced leaf antioxidant capacity, alleviated membrane lipid peroxidation and reactive oxygen species accumulation, and mitigated the adverse impacts of heat stress. Under heat stress, SOD, POD and CAT activities gradually declined with delayed EBR application and exhibited a trend of first increasing and then decreasing as EBR concentration rose, whereas MDA and H₂O₂ contents showed the opposite trend. Specifically, compared with other spraying timings, the T5 treatment increased SOD, POD and CAT activities by 11.97%, 21.83% and 16.75% on average, and decreased MDA content by 18.25% and H₂O₂ content by 75.59%. Meanwhile, spraying 0.10 mg L⁻¹ EBR at the T5 stage (T5C1) significantly increased SOD, POD and CAT activities by 8.29%, 21.64% and 11.13%, and reduced MDA content by 16.92% and H₂O₂ content by 16.36%, relative to the control. The interaction between

EBR spraying timing and concentration improved leaf heat tolerance and laid a foundation for photosynthate accumulation. Excessively high EBR concentration (C2) weakened these mitigating effects.

Correlation analysis in Fig. 3F showed that heat stress and delayed spraying timing suppressed the enhancement of antioxidant enzyme activities in flag leaves and promoted the accumulation of the membrane-lipid peroxidation product MDA and reactive oxygen species H_2O_2 .

3.3. Endogenous hormone content

Endogenous hormones serve as crucial indicators coordinating leaf responses to environmental stress and delaying leaf senescence. As shown in Fig. 5A-D, the contents of GA, ABA, CTK and BR in wheat were significantly regulated by temperature, EBR spraying timing and concentration. Compared with NT, HT significantly decreased GA content by 10.97%, CTK content by 16.00% and BR content by 8.40%, while increasing ABA content by 15.42%. Rational combination of EBR spraying timing and concentration could optimize endogenous hormone balance in leaves and exert positive effects on delaying leaf senescence. Under heat stress, the contents of GA, CTK and BR gradually decreased with delayed EBR application, and presented a trend of rising first and then falling with the increase of EBR concentration, whereas ABA content showed the opposite tendency. Specifically, T5 treatment increased the average contents of GA, CTK and BR by 7.64%, 15.00% and 9.41%, and reduced ABA content by 12.53% compared with other spraying timings. Furthermore, application of 0.10 mg L^{-1} EBR at 5 days after anthesis (T5C1) remarkably increased GA, CTK and BR contents by 12.50%, 11.11% and 16.67%, and decreased ABA content by 6.88% relative to the control group. It demonstrated that the interaction between EBR spraying timing and concentration facilitated endogenous hormone homeostasis, maintained favorable leaf physiological status, and alleviated premature leaf senescence induced by heat stress.

Correlation analysis showed that temperature was significantly negatively correlated with GA and BR contents, and positively correlated with ABA content; spraying timing had a significant negative correlation with GA, CTK and BR contents. EBR concentration was positively correlated with GA, CTK and BR contents, but negatively correlated with ABA content (Fig. 5E). These results indicated that rising temperature reduced GA and BR contents in wheat leaves, thereby triggering plant stress responses. Delayed EBR application lowered leaf GA and CTK levels, while appropriate increase in EBR concentration could elevate GA, CTK and BR contents and reduce ABA content, so as to regulate endogenous hormone balance.

3.4. Grain filling characteristics and photosynthate accumulation

The growth-curve equations provided a good fit to the interactive effects of EBR spraying timing and concentration on the wheat grain-filling process under different temperature treatments, with all coefficients of determination higher than 0.987 (Table 1). The EBR treatments effectively promoted dry matter accumulation (DMA) (Fig. 6A). Compared with NT, HT decreased W_{max} , G_{max} , G_{mean} , P and DMA by 4.17%, 8.59%, 8.89%, 3.10 d and 12.21%, respectively. Optimizing EBR spraying timing and concentration could improve grain-filling rate and final dry matter accumulation. Under heat stress, W_{max} , G_{max} , G_{mean} , P and DMA gradually declined as EBR application was delayed, whereas these variables increased first and then decreased with increasing EBR concentration. Specifically, the T5 treatment increased W_{max} , G_{max} , G_{mean} and DMA by 5.01%, 6.63%,

11.49% and 9.28%, respectively, and prolonged P by 2.40 d relative to the other spraying-timing treatments. Moreover, spraying 0.10 mg L⁻¹ EBR at 5 days after anthesis (T5C1) increased Wmax, Gmax, Gmean and DMA by 7.07%, 16.13%, 10.87% and 16.24%, respectively, and prolonged P by 5.47 d relative to the control. These findings suggest that the interaction between spraying timing and concentration exerts positive effects on optimizing grain-filling characteristics and enhancing final dry-matter accumulation. In addition, elevated temperature and delayed EBR application reduced Wmax, Gmax, Gmean and DMA and shortened P (Fig. 6B). An appropriate increase in EBR concentration could accelerate the grain-filling rate, prolong the active grain-filling period, and further facilitate improvements in DMA and final grain yield.

Table 1
Effects of EBR spraying time and concentration on grain filling characteristics under heat stress

Year	Temperature	Spraying time	Concentration	R ²	Grain filling parameters				
					Tmax (d)	Wmax (g)	Gmax (g 1000 grain ⁻¹ d ⁻¹)	Gmean (g 1000 grain ⁻¹ d ⁻¹)	P (d)
2023	HT	T5	CK	0.987	16.21	20.17	6.66	0.92	27.91
		T5	C1	0.993	15.60	21.88	7.88	1.06	35.36
		T5	C2	0.996	16.41	20.56	7.73	1.01	33.33
		T10	CK	0.995	18.30	20.11	6.39	0.85	28.09
		T10	C1	0.996	18.28	20.71	7.74	0.97	33.74
		T10	C2	0.997	19.35	20.51	7.06	0.89	30.88
		T15	CK	0.997	19.15	18.50	6.33	0.80	24.43
		T15	C1	0.992	19.22	19.89	7.36	0.90	32.09
		T15	C2	0.999	22.33	19.33	6.80	0.80	28.09
	NT	T5	CK	0.997	17.25	20.94	7.66	0.99	33.87
		T5	C1	0.993	17.41	22.75	8.19	1.06	37.67
		T5	C2	0.996	16.97	21.54	8.01	1.03	35.48
		T10	CK	0.987	16.73	20.87	7.05	0.95	30.89
		T10	C1	0.995	17.36	21.71	8.12	1.04	35.75
		T10	C2	0.997	17.09	20.54	7.31	0.96	33.78
		T15	CK	0.996	18.83	19.12	6.92	0.86	29.84
		T15	C1	0.997	15.99	20.49	7.83	1.02	34.46
		T15	CK	0.997	14.43	19.00	7.64	1.02	31.55
2024	HT	T5	CK	0.996	17.06	19.69	6.97	0.92	30.41
		T5	C1	0.998	18.65	20.80	7.95	0.97	33.90
		T5	C2	0.995	16.83	20.16	7.22	0.95	33.52
		T10	CK	0.999	21.23	19.05	6.86	0.81	27.33
		T10	C1	0.997	17.20	20.23	7.24	0.94	33.52
		T10	C2	0.997	18.98	19.39	6.94	0.87	32.35
		T15	CK	0.997	18.06	18.31	6.66	0.85	26.97

NT	T15	C1	0.998	19.25	19.91	7.21	0.89	33.15
	T15	C2	0.999	20.29	18.83	6.74	0.82	29.52
	T5	CK	0.997	18.43	20.62	7.71	0.96	35.63
	T5	C1	0.999	16.58	21.86	8.65	1.10	38.59
	T5	C2	0.998	17.85	20.83	7.96	0.99	34.55
	T10	CK	0.999	18.37	20.39	7.54	0.94	32.84
	T10	C1	0.997	14.90	21.21	8.52	1.12	36.68
	T10	C2	0.999	19.57	20.34	8.05	0.95	33.33
	T15	CK	0.995	17.56	19.84	6.78	0.89	30.70
	T15	C1	0.999	18.70	20.46	7.78	0.95	33.60
	T15	C2	0.999	24.18	20.52	7.10	0.81	31.15

HT represents heat stress and NT represents natural temperature. T5, T10 and T15 represented 5, 10 and 15 days after anthesis, respectively. CK represented spraying water, C1 and C2 represented EBR spraying at 0.10 mg L⁻¹ and 0.20 mg L⁻¹, respectively. Tmax represents the time to reach the maximum grain filling rate, Wmax represents the weight of maximum grain filling rate, Gmax represents maximum grain-filling rate, Gmean represents average grain-filling rate, P represents active grain filling period.

3.5. Grain yield

As shown in Fig. 7A, grain yield and yield reduction rate (YRR) were significantly affected by temperature, EBR spraying timing and concentration. Relative to natural temperature, heat stress caused an average grain yield reduction of 9.10%, with a maximum value of 13.07% (Fig. 7B). The interaction of EBR spraying timing and concentration effectively increased grain yield and declined YRR. Under heat stress, grain yield in T5 treatment was increased by an average of 5.92% and YRR was reduced by 30.00% compared with other spraying timings. Furthermore, foliar application of 0.10 mg L⁻¹ EBR at 5 days after anthesis (T5C1) significantly raised grain yield by 12.50% and decreased yield reduction rate by 8.02% in contrast with the control. The results indicated that the combined regulation of EBR application timing and concentration could effectively alleviate heat-induced yield loss. Correlation analysis revealed that high temperature and delayed EBR application markedly reduced grain yield, and later spraying time led to higher yield reduction rate.

3.6. Mandel test analysis and random forest model

Mantel test analysis results revealed that compared with natural temperature treatment, heat stress weakened the positive correlations among leaf physiological traits, antioxidant enzyme activities, endogenous hormone contents and grain filling characteristics. Meanwhile, heat stress generally reduced the positive associations between the above indices and dry matter accumulation as well as grain yield (Fig. 8A).

Random forest importance analysis further verified that under natural temperature, leaf area index, leaf biomass, SPAD value and grain filling rate indicators including Gmax and Gmean were the core factors affecting

dry matter accumulation and grain yield. Under heat stress, the contribution order to dry matter accumulation was CAT > SPAD > Gmax > CTK > LB. In terms of yield formation, the contribution sequence was Gmax, Gmean, P, SPAD, GA, LB, Wmax and CTK. Overall, grain filling rates Gmax and Gmean acted as the common dominant factors determining wheat yield across two temperature regimes, and temperature variation obviously reshaped the regulatory pathways of yield formation. Under natural temperature, maintaining leaf basic physiological traits and photosynthate accumulation was critical for yield formation. By contrast, yield formation under heat stress was synergistically modulated via multiple pathways involving antioxidant system, endogenous hormones and grain filling process (Fig. 8B).

3.7. Comprehensive evaluation and partial least squares path modeling analysis(PLS-PM)

Principal component analysis (PCA) was adopted to screen the optimal EBR foliar application scheme for wheat at anthesis under heat stress. The first two principal components collectively explained 92.10% of total phenotypic variation. Comprehensive evaluation scores confirmed that spraying 0.10 mg·L⁻¹ EBR at 5 days after anthesis (T5C1) was the optimal measure to alleviate heat stress damage (Fig. 9A).

The partial least squares path model (PLS-PM) with favorable goodness-of-fit was applied to further clarify the regulatory mechanism of T5C1 treatment. The results showed that heat stress markedly inhibited basic physiological functions and antioxidant capacity of wheat leaves. Appropriate spraying timing and concentration could effectively regulate endogenous hormone homeostasis, optimize leaf physiological status and enhance antioxidant defense capacity. The improvement of these physiological indices significantly optimized grain filling characteristics, promoted plant dry matter accumulation and ultimately increased grain yield. In conclusion, rational EBR application via the interaction of spraying timing and concentration could mitigate heat stress damage to wheat by regulating multiple physiological pathways, thereby stabilizing grain yield and improving crop performance under heat stress (Fig. 9B).

Under heat stress, foliar spraying of 0.10 mg L⁻¹ EBR at 5 days after anthesis could effectively alleviate stress injury. On the one hand, it increased LAI, SPAD value and Pn, enhanced the activities of SOD, POD and CAT, reduced MDA and H₂O₂ content, and maintained cell membrane stability. On the other hand, it regulated endogenous hormone balance by elevating GA, CTK and BR contents and decreasing ABA level, thus delaying leaf senescence. These combined effects optimized grain filling process, increased Gmax, dry matter accumulation and grain yield, and ultimately reduced heat-induced yield loss. Additionally, maximum grain filling rate was identified as the pivotal factor determining wheat yield under heat stress (Fig. 10).

4. Discussion

4.1. EBR alleviated heat stress-induced flag leaf senescence by stabilizing photosynthetic apparatus and activating antioxidant system

As the major photosynthetic source organ during grain filling, wheat flag leaves maintain physiological functions under heat stress mainly depending on the integrity of photosynthetic system and reactive oxygen

species scavenging capacity (Hassan et al., 2021; Fei et al., 2022). In this study, short-term post-anthesis heat stress markedly reduced LAI, SPAD values and Pn, inhibited the activities of SOD, POD and CAT, and markedly increased MDA and H₂O₂ content compared with natural-temperature conditions (Fig. 3A-D, Fig. 4A-E). Correlation analysis revealed that temperature was significantly negatively correlated with LAI, SPAD, Pn, SOD, POD and CAT, and positively correlated with MDA and H₂O₂ content (Fig. 3E, Fig. 4F). The underlying mechanisms were explained as follows. Firstly, heat stress directly damaged chloroplast thylakoid structure, inhibited the activity of photosystem II reaction centers and accelerated chlorophyll degradation (Ristic et al., 2007; Djanaguiraman et al., 2020). Secondly, heat stress triggered excessive accumulation of reactive oxygen species, induced membrane lipid peroxidation and caused irreversible damage to cell membrane systems, eventually resulting in premature leaf senescence and decreased photosynthetic rate (Abdelrahman et al., 2020).

The protective effects of EBR on flag leaves under heat stress are mainly reflected in stabilizing photosynthetic system and activating antioxidant system (Zhong et al., 2026). The present study confirmed that rational EBR application (T5C1) obviously improved leaf basic physiological characteristics, enhanced antioxidant capacity and reduced the accumulation of membrane lipid peroxidation products under heat stress (Fig. 9B). The underlying mechanisms are summarized as follows. Firstly, proper EBR spraying improves the scavenging efficiency of reactive oxygen species via activating antioxidant enzyme system, alleviates membrane lipid peroxidation, maintains the integrity and fluidity of cell membranes, and thereby alleviates high-temperature-induced cellular structural damage (Suresh et al., 2025). Secondly, EBR protects photosystem II reaction centers and electron transport chain to sustain photochemical efficiency, alleviates the destruction of chloroplast ultrastructure under high temperature, and ensures stable carbon assimilation (Lai et al., 2024). Thirdly, EBR upregulates the expression of antioxidant enzyme-related proteins to strengthen enzymatic defense system, and stabilizes metabolic pathways involved in chlorophyll synthesis (Alam et al., 2018; Ogwen et al., 2008).

In addition, the alleviating effect of EBR on plant heat stress exhibits distinct threshold effects in terms of spraying timing and concentration (Zhang et al., 2014). No substantial physiological damage occurs in wheat plants at the early stage of heat stress, so timely EBR application at T5 can optimize leaf physiological status and strengthen stress tolerance foundation. Meanwhile, EBR has an optimal concentration range of C1; excessive concentration tends to weaken antioxidant capacity and chlorophyll synthesis ability, and further induces physiological inhibition.

4.2. EBR maintain the physiological activity of flag leaf under heat stress by regulating the interaction of endogenous hormones

This study showed that compared with natural temperature, heat stress significantly reduced the contents of CTK, GA and BR while increased ABA content in wheat flag leaves (Fig. 5A-D). Correlation analysis further revealed that temperature was significantly negatively correlated with GA, BR content and positively correlated with ABA content (Fig. 5E). These results indicated that heat stress disrupted the synthesis and metabolic balance of endogenous hormones in wheat. On the one hand, heat stress markedly inhibited the activities of enzymes related to CTK, GA and BR synthesis and accelerated the degradation of these two growth-promoting hormones, leading to a sharp decline in their contents (Chen et al., 2023). On the other hand, heat stress

activated plant stress response pathways, upregulated the expression of key genes involved in ABA synthesis, and thus promoted massive ABA accumulation (Zhang et al., 2022; Chen et al., 2023).

Further results demonstrated that proper EBR application markedly increased GA, BR and CTK contents and decreased ABA content in flag leaves under heat stress, thereby optimizing the balance between growth-promoting hormones and stress-responsive hormones. The underlying mechanisms were as follows. Firstly, exogenous EBR alleviated heat-induced cellular oxidative damage, sustained leaf physiological activity, preserved endogenous GA, BR and CTK levels, and restrained stress-triggered excessive ABA accumulation. It delayed premature senescence of flag leaves by enhancing stress resistance through endogenous hormone balance, rather than maintaining persistently high ABA levels (Wang et al., 2025; Zhong et al., 2026). Secondly, Exogenous EBR activates the BR signaling pathway and triggers nuclear translocation of BZR1/BES1 transcription factors. BZR1/BES1 can directly bind to the promoters of ABA and GA biosynthetic genes, repress ABA biosynthesis and up-regulate the transcription of GA biosynthetic genes, thereby remodelling hormone homeostasis (Unterholzner et al., 2015; Moon et al., 2021; Guo et al., 2024).

Moreover, the regulatory effects of EBR on heat-stressed wheat were obviously dependent on spraying timing and concentration, and T5C1 treatment, namely foliar application of 0.10 mg L⁻¹ EBR at 5 d after anthesis, exhibited the best alleviating effect. The period of 5 d after anthesis is critical for wheat grain sink formation. EBR application at this stage could optimize endogenous hormone ratio in advance and enhance heat resistance of flag leaves. In the middle and late grain filling stages, heat stress had already initiated irreversible plant senescence, which greatly weakened the regulatory efficiency of exogenous EBR (Wang et al., 2025). In addition, excessive EBR concentration disrupted endogenous hormone metabolism in flag leaves, inhibited CTK synthesis and promoted ABA accumulation, aggravated leaf senescence, and was unfavorable to the maintenance of normal plant physiological status under heat stress (Li et al., 2021).

4.3. Maintenance of flag-leaf function and grain-filling capacity constitute the core by which EBR alleviates heat stress and stabilizes grain yield

The wheat grain-filling process is highly dependent on flag-leaf physiological activity and grain sink capacity. By damaging leaf photosynthetic apparatus, disturbing endogenous hormone homeostasis and triggering massive reactive-oxygen-species accumulation, heat stress blocks grain filling and reduces grain weight, representing a critical physiological cause of yield loss (Zhong et al., 2026). Our study showed that short-term post-anthesis heat stress markedly suppressed grain-filling rate and decreased dry-matter accumulation compared with natural temperature, and weakened positive correlations among leaf physiological performance, grain-filling rate, dry-matter accumulation and grain yield. These results indicated that impaired flag-leaf function and diminished grain-filling capacity were key limiting factors for yield formation under heat stress (Li et al., 2025).

Firstly, EBR maintained relatively high LAI, SPAD value and Pn in flag leaves, ensuring sustained and stable photosynthate supply for grain filling (Zhong et al., 2026). This effect stemmed from optimal EBR application, which enhanced antioxidant system function, alleviated membrane lipid peroxidation damage and reactive-oxygen-species accumulation, and delayed leaf functional decline (Wang et al., 2025). Secondly, EBR improved accumulation efficiency of grain assimilates, accelerated grain-filling rate, prolonged effective filling duration, and relieved heat-stress-mediated inhibition of grain development (Zhong et al., 2026). This intrinsic

mechanism lay in that appropriate EBR spraying sustained flag-leaf endogenous hormone homeostasis and guaranteed continuous photosynthate supply (Lai et al., 2024; Wang et al., 2025).

In addition, this study confirmed that heat stress markedly reduced dry matter accumulation and grain yield and enlarged yield loss compared with natural temperature. Effective application of 0.10 mg L⁻¹ EBR at 5 days after anthesis mitigated yield loss by improving leaf physiological performance and grain filling traits (Fig. 9A-B). Random forest importance analysis revealed that maximum grain filling rate (Gmax), flag leaf SPAD value, CTK content and leaf biomass were the common key factors regulating dry matter accumulation and grain yield (Fig. 8B). Collectively, EBR alleviates heat stress damage not via a single physiological pathway, but mainly through coordinating source-sink physiology. It simultaneously strengthens source functions including LAI, SPAD, Pn, antioxidant capacity and endogenous hormone levels, and improves sink accommodation capacity reflected by grain filling characteristics. Such regulation ensures efficient and stable grain filling, and consequently increases dry matter accumulation and reduces heat-induced yield loss (Li et al., 2021; Zhang et al., 2023; Li et al., 2025; Wang et al., 2025; Zhong et al., 2026).

5. Conclusion

Compared with natural temperature, heat stress severely disrupts photosynthetic and antioxidant systems of wheat flag leaves, accelerates leaf senescence, restrains grain filling and causes a sharp yield decline. Foliar application of 0.10 mg L⁻¹ EBR at 5 days after anthesis (T5C1) can distinctly improve photosynthetic efficiency, strengthen antioxidant capacity, balance endogenous hormones, delay plant senescence and optimize source-sink relationship, so as to effectively alleviate heat stress damage. This regulation strategy synchronously promotes photosynthate supply of source organs and assimilate accumulation of grain sinks, accelerates grain filling rate and ultimately stabilizes wheat yield. This study not only enriches the source-sink theory of wheat concerning the interactive effects of EBR spraying timing and concentration under heat stress, but also provides technical guidance for rational EBR application to mitigate post-anthesis heat stress. In future research, further exploration is still required to clarify the molecular mechanisms underlying EBR-regulated source-transport-sink coordination in heat-stressed wheat, as well as quantitative studies on the effects of spraying regimes on dynamic hormone changes and assimilate translocation.

Declarations

Author Contribution

Su Ming was responsible for manuscript drafting, data source investigation, and figure visualization. Nie Zhengrui conducted data source investigation. Li Yueqi performed figure visualization. Hong Ziqiang completed data processing and partial figure preparation. Wang Xiaochun carried out manuscript review and quality oversight. Wu Hongliang was responsible for funding acquisition and manuscript oversight. Kang Jianhong undertook manuscript oversight and data verification.

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Figures

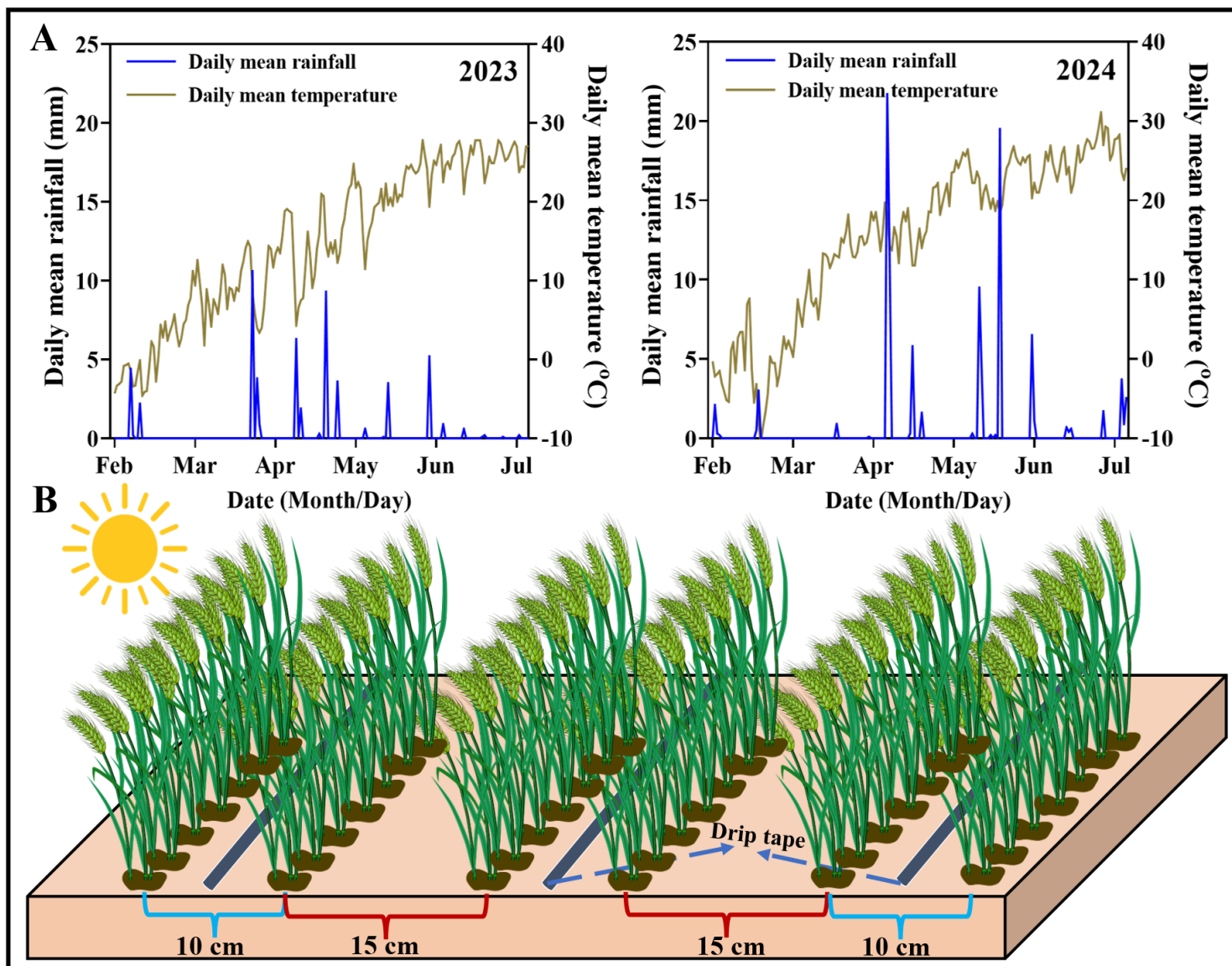


Figure 1

The temperature and precipitation of the test site (A) and the wheat planting pattern map (B)

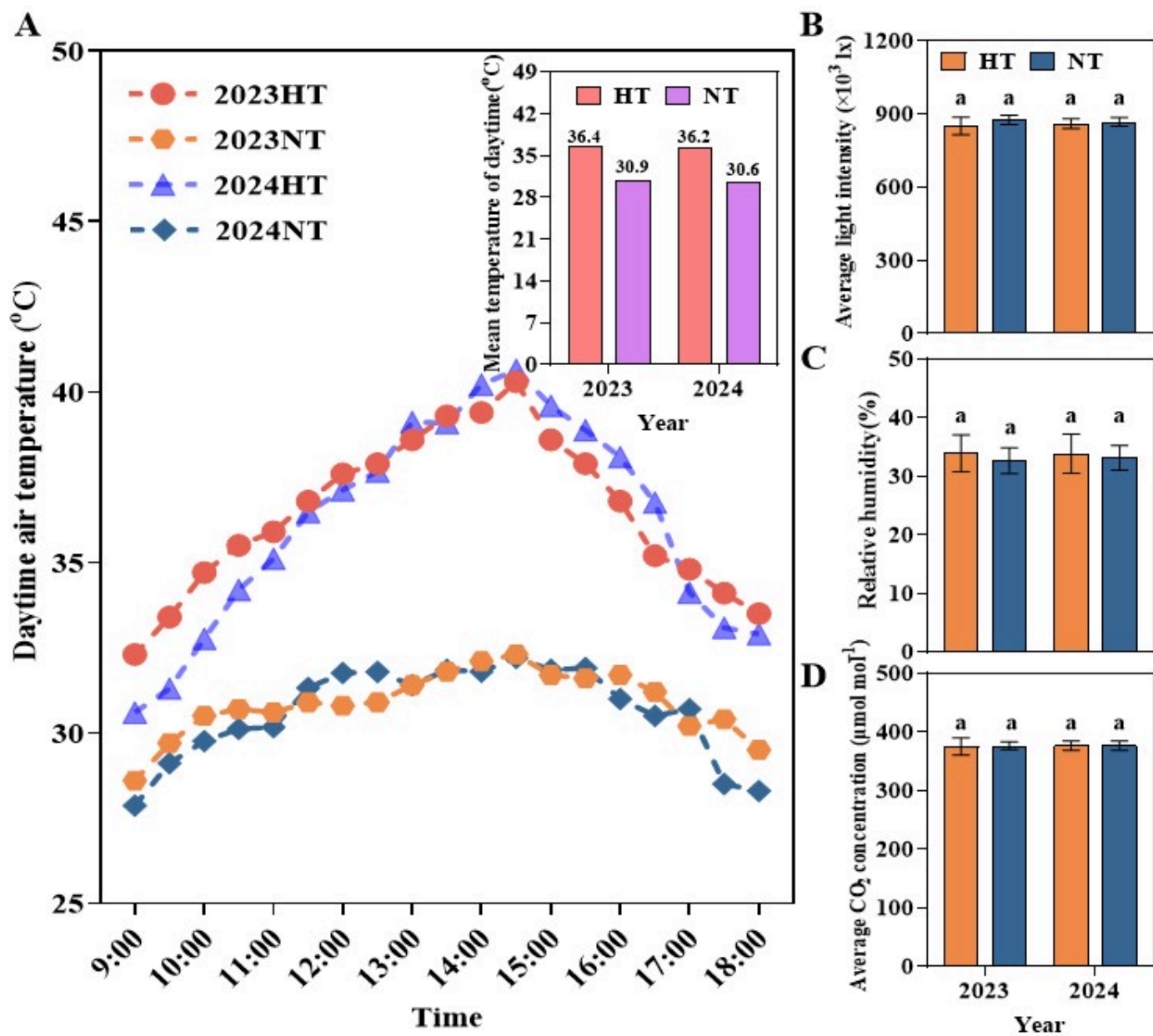


Figure 2

Temperature change during daytime warming (A) and wheat canopy microclimate during treatment (B-D). HT represents heat stress and NT represents natural temperature. The lowercase letters on the column indicate a significant difference at the $P < 0.05$ level.

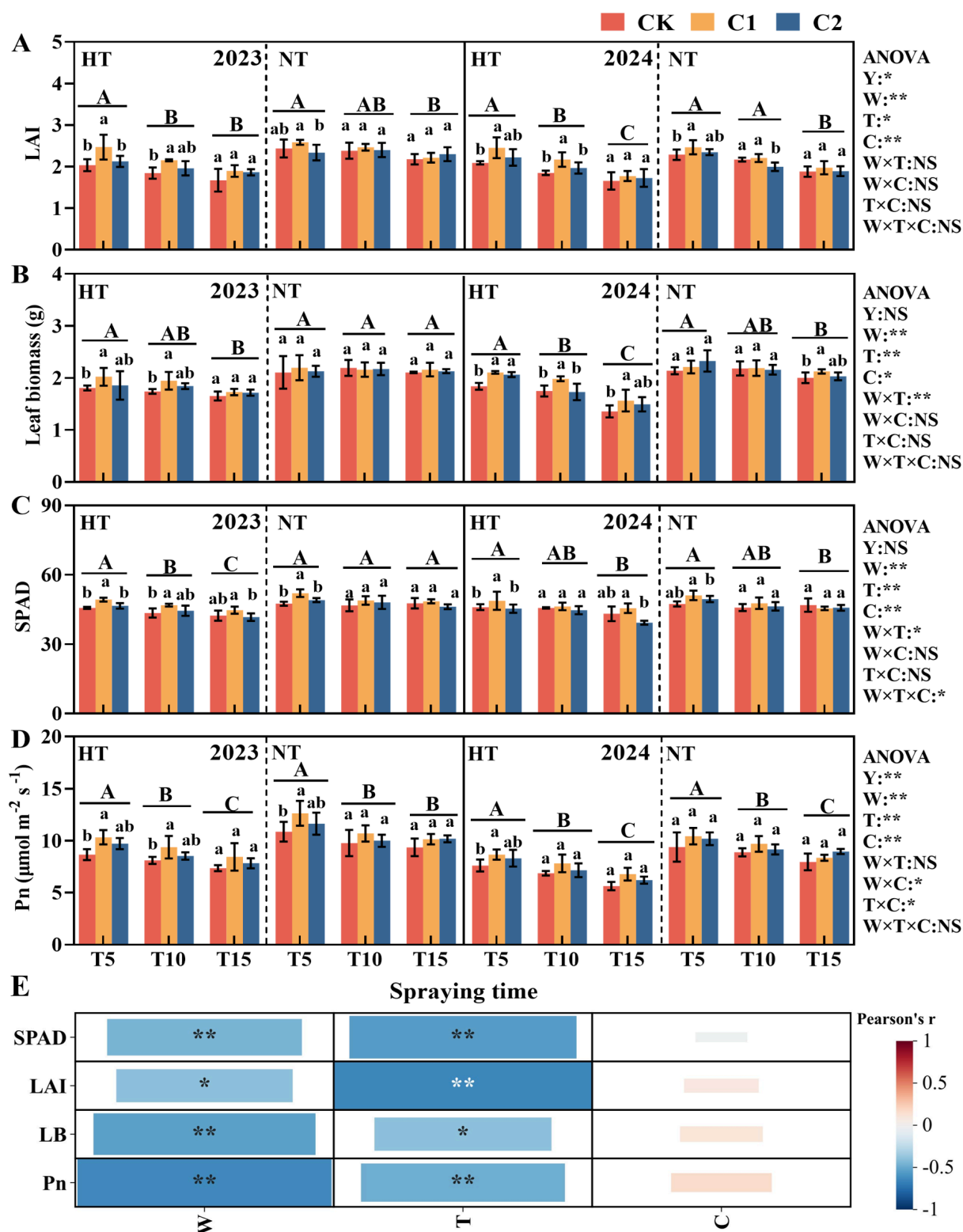


Figure 3

The effects of EBR spraying time and concentration on LAI, leaf biomass, SPAD and Pn under heat stress (A-D) and their correlation analysis (E). HT represents heat stress and NT represents natural temperature. T5, T10 and T15 represented 5, 10 and 15 days after anthesis, respectively. CK represented spraying water, C1 and C2 represented EBR spraying at 0.10 mg L^{-1} and 0.20 mg L^{-1} , respectively. Y, W, T and C represent year, temperature, spraying time and spraying concentration, respectively. * and ** indicated $P < 0.05$ and $P < 0.01$,

respectively, and NS indicated no significant difference. The lowercase letters indicate the difference of different spraying concentrations, and the uppercase letters indicate the difference of different spraying time.

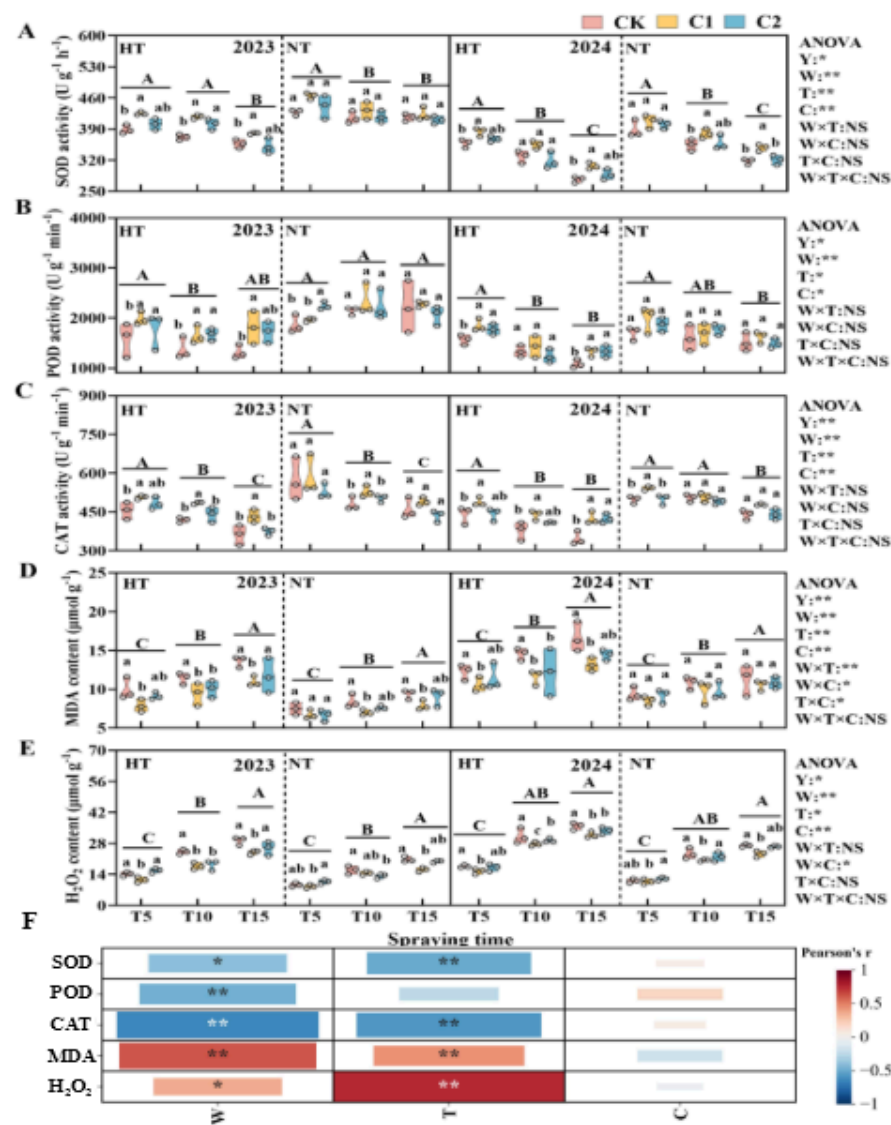


Figure 4

Effects of EBR spraying time and concentration on antioxidant capacity, MDA and H₂O₂ content (A-E) and their correlation analysis (F) in flag leaves under heat stress. HT represents heat stress and NT represents natural temperature. T5, T10 and T15 represented 5, 10 and 15 days after anthesis, respectively. CK represented spraying water, C1 and C2 represented EBR spraying at 0.10 mg L⁻¹ and 0.20 mg L⁻¹, respectively. Y, W, T and C represent year, temperature, spraying time and spraying concentration, respectively. * and ** indicated *P* < 0.05 and *P* < 0.01, respectively, and NS indicated no significant difference. The lowercase letters indicate the difference of different spraying concentrations, and the uppercase letters indicate the difference of different spraying time.

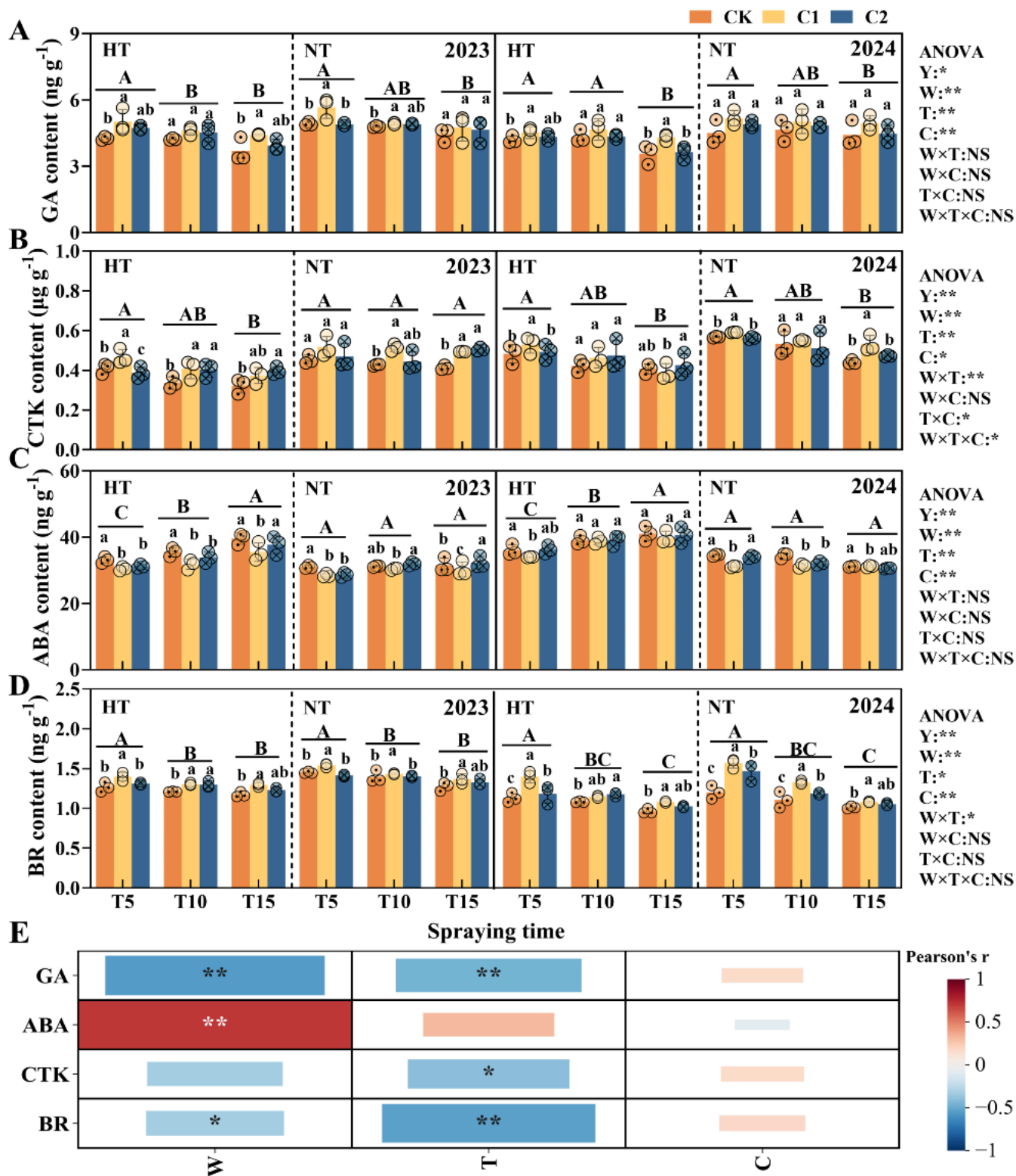


Figure 5

The effect of spraying time and concentration of EBR on endogenous hormone content in flag leaves under heat stress (A-D) and its correlation analysis (E). HT represents heat stress and NT represents natural temperature. T5, T10 and T15 represented 5, 10 and 15 days after anthesis, respectively. CK represented spraying water, C1 and C2 represented EBR spraying at 0.10 mg L⁻¹ and 0.20 mg L⁻¹, respectively. Y, W, T and C represent year, temperature, spraying time and spraying concentration, respectively. * and ** indicated $P < 0.05$

and $P < 0.01$, respectively, and NS indicated no significant difference. The lowercase letters indicate the difference of different spraying concentrations, and the uppercase letters indicate the difference of different spraying time.

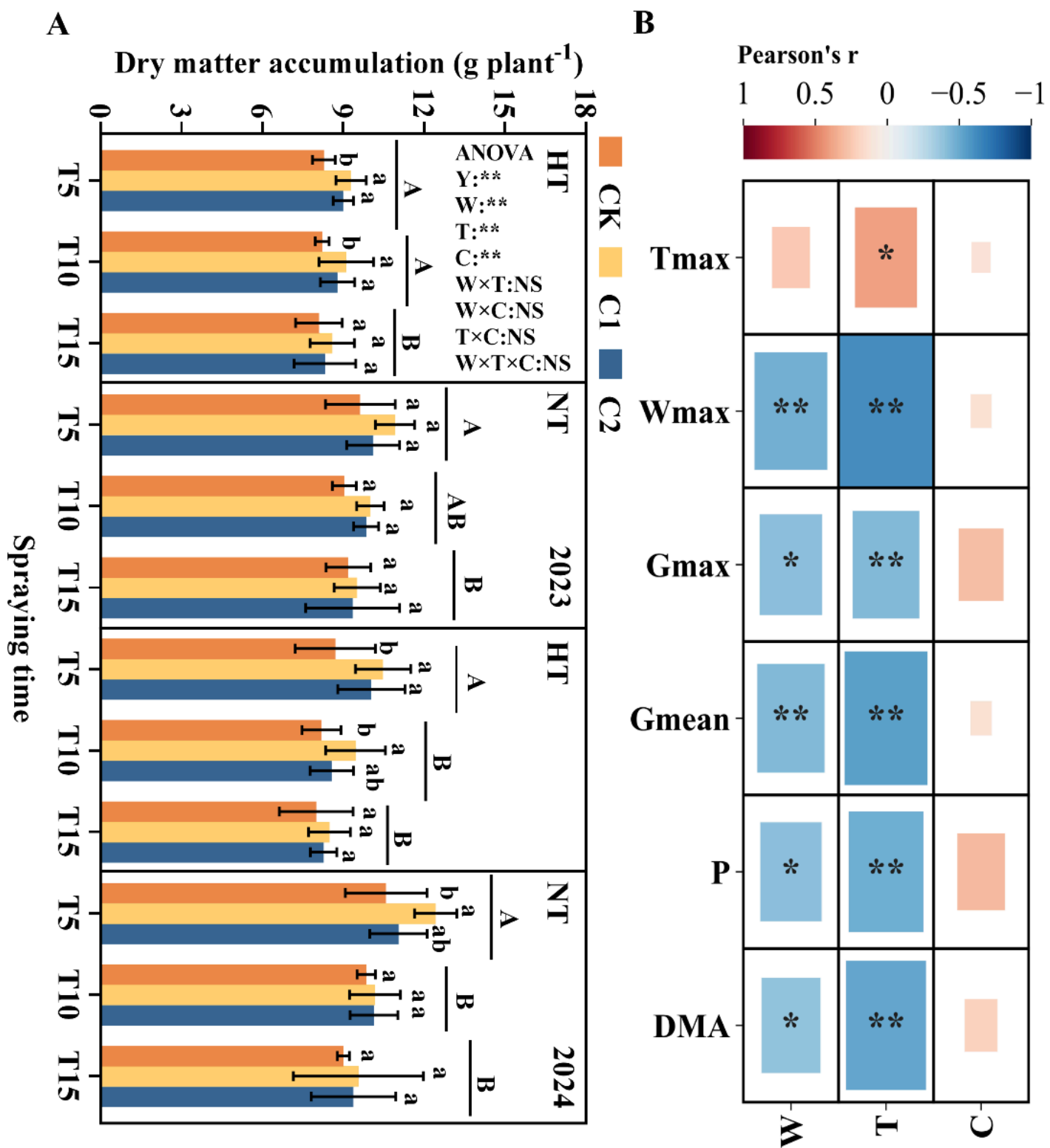


Figure 6

The effects of spraying time and concentration of EBR on dry matter accumulation under heat stress and the correlation analysis between filling characteristics and temperature, concentration and time were analyzed. HT

represents heat stress and NT represents natural temperature. T5, T10 and T15 represented 5, 10 and 15 days after anthesis, respectively. CK represented spraying water, C1 and C2 represented EBR spraying at 0.10 mg L⁻¹ and 0.20 mg L⁻¹, respectively. Y, W, T and C represent year, temperature, spraying time and spraying concentration, respectively. * and ** indicated $P < 0.05$ and $P < 0.01$, respectively, and NS indicated no significant difference. The lowercase letters indicate the difference of different spraying concentrations, and the uppercase letters indicate the difference of different spraying time. Tmax represents the time to reach the maximum grain filling rate, Wmax represents the weight of maximum grain filling rate, Gmax represents maximum grain-filling rate, Gmean represents average grain-filling rate, P represents active grain filling period and DMA represents dry matter accumulation.

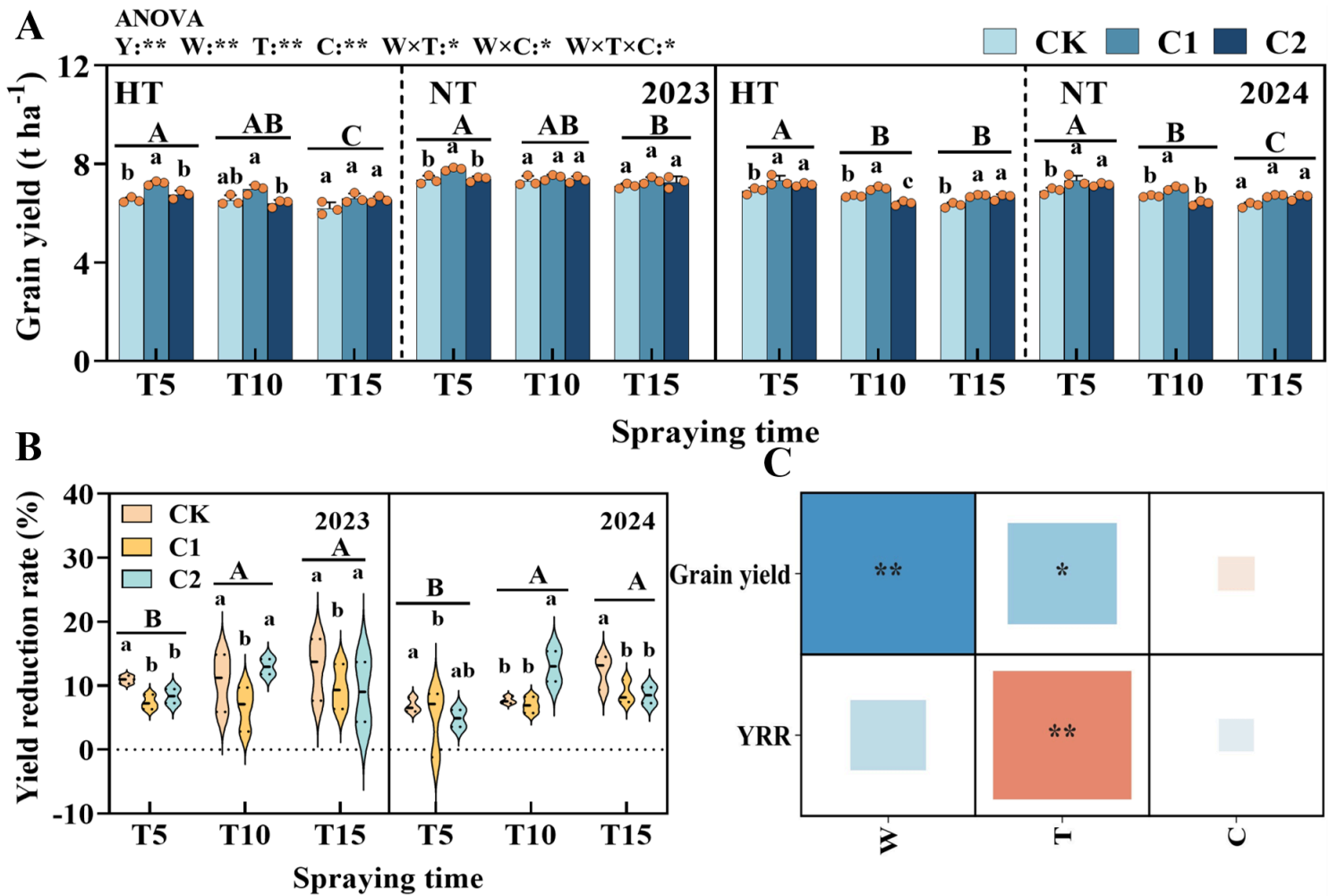


Figure 7

Effects of spraying time and concentration of EBR on grain yield and yield reduction rate under heat stress and their correlation analysis. HT represents heat stress, NT represents natural temperature and YRR represents yield reduction rate. T5, T10 and T15 represented 5, 10 and 15 days after anthesis, respectively. CK represented spraying water, C1 and C2 represented EBR spraying at 0.10 mg L⁻¹ and 0.20 mg L⁻¹, respectively. Y, W, T and C represent year, temperature, spraying time and spraying concentration, respectively. * and ** indicated $P < 0.05$ and $P < 0.01$, respectively, and NS indicated no significant difference. The lowercase letters indicate the difference of different spraying concentrations, and the uppercase letters indicate the difference of different spraying time.

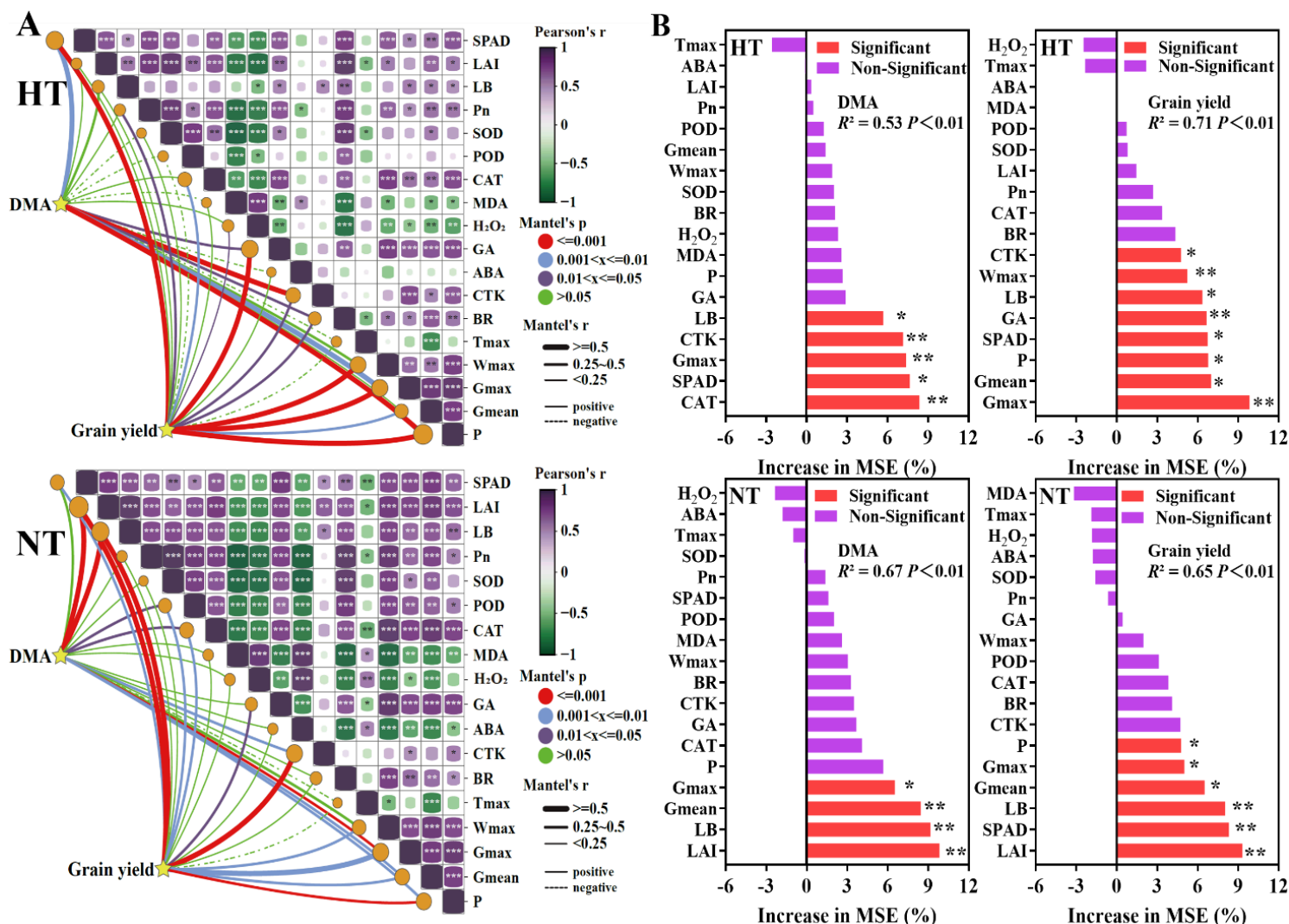


Figure 8

The correlation analysis of wheat indexes on biomass and yield under different temperature treatments (A) and the importance of various factors to it (B). HT represents heat stress and NT represents natural temperature. SPAD: relative chlorophyll content, LAI: leaf area index, LB: leaf biomass, Pn: net photosynthetic rate, SOD: superoxide dismutase activity, POD: peroxidase activity, CAT: catalase activity, MDA: malondialdehyde content, H₂O₂: hydrogen peroxide content, GA: gibberellin content, ABA: abscisic acid content, CTK: cytokinin content, BR: brassinolide content, DMA: dry matter accumulation, Tmax: the time to reach the maximum grain filling rate. Wmax: the weight of maximum grain filling rate, Gmax: maximum grain-filling rate, Gmean: average grain-filling rate, P: active grain filling period. * and ** indicated $P < 0.05$ and $P < 0.01$.

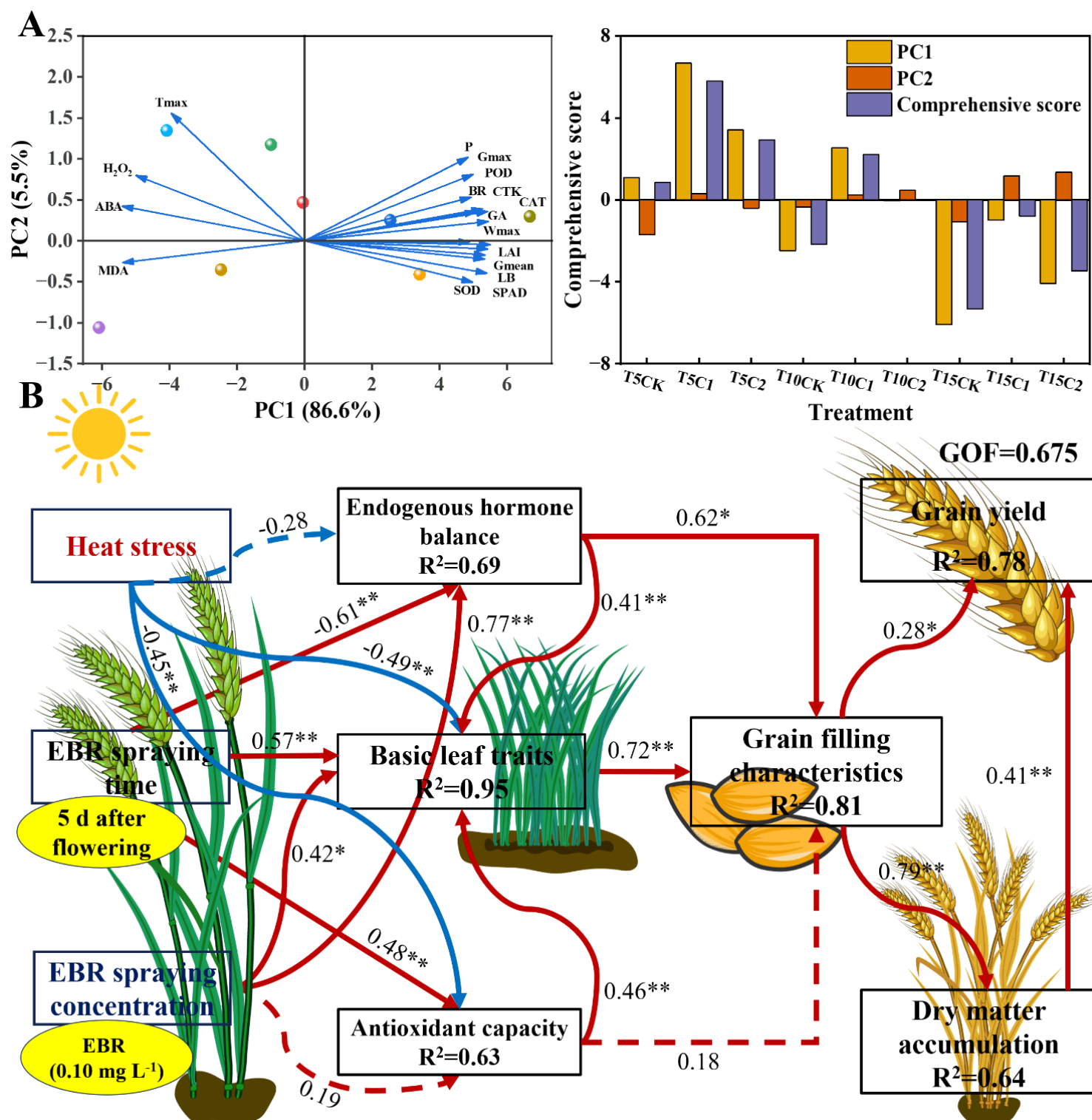


Figure 9

The determination of the best results of the interaction between time (A) and concentration of EBR spraying under heat stress and partial least squares path analysis (PLS-PM) showed that the best time and concentration of EBR spraying under heat stress at wheat flowering stage improved grain filling characteristics and increased yield by affecting leaf quality, antioxidant capacity and balancing endogenous hormones (B). T5, T10 and T15 represented 5, 10 and 15 days after anthesis, respectively. CK represented spraying water, C1 and C2 represented EBR spraying at 0.10 mg L⁻¹ and 0.20 mg L⁻¹, respectively. SPAD: relative chlorophyll content,

LAI: leaf area index, LB: leaf biomass, Pn: net photosynthetic rate, SOD: superoxide dismutase activity, POD: peroxidase activity, CAT: catalase activity, MDA: malondialdehyde content, H₂O₂: hydrogen peroxide content, GA: gibberellin content, ABA: abscisic acid content, CTK: cytokinin content, BR: brassinolide content, DMA: dry matter accumulation, Tmax: the time to reach the maximum grain filling rate. Wmax: the weight of maximum grain filling rate, Gmax: maximum grain-filling rate, Gmean: average grain-filling rate, P: active grain filling period. Red and blue arrows represent positive and negative paths, respectively, and dotted lines represent non-significant paths. Goodness-of-Fit (GOF) was used to measure the overall prediction performance of the model.

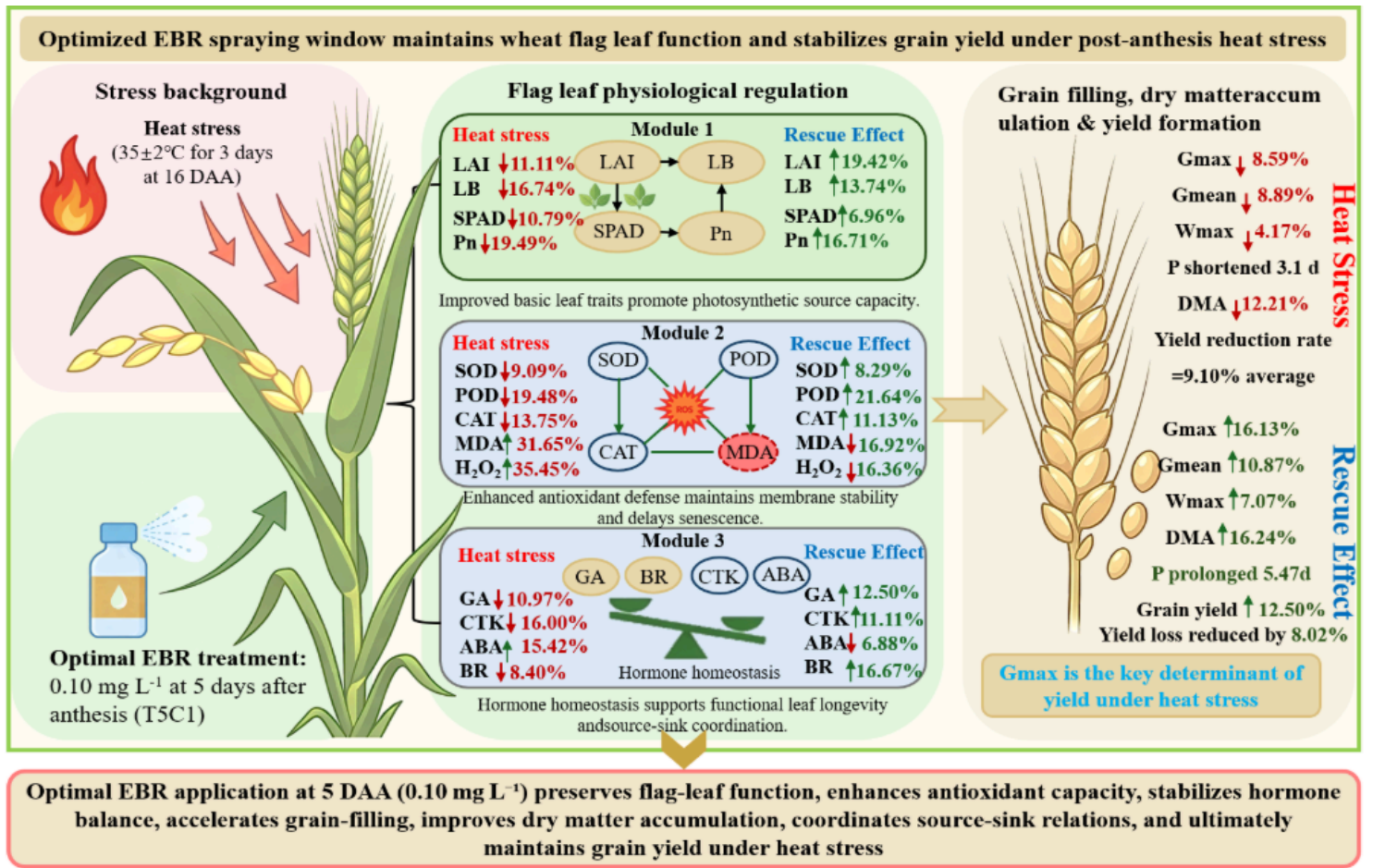


Figure 10

Physiological mechanism of spraying optimal concentration of EBR on 5 d after anthesis to alleviate heat stress and maintain flag leaf function and grain yield of wheat. SPAD: relative chlorophyll content, LAI: leaf area index, LB: leaf biomass, Pn: net photosynthetic rate, SOD: superoxide dismutase activity, POD: peroxidase activity, CAT: catalase activity, MDA: malondialdehyde content, H₂O₂: hydrogen peroxide content, GA: gibberellin content, ABA: abscisic acid content, CTK: cytokinin content, BR: brassinolide content, Wmax: the weight of maximum grain filling rate, Gmax: maximum grain-filling rate, Gmean: average grain-filling rate, P: active grain filling period.

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