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The Exogenous Application of Brassinosteroids Confers Tolerance to Heat Stress by Increasing Antioxidant Capacity in Soybeans

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Abstract: Heat stress is an important factor affecting soybean yield. Brassinosteroids (BRs) play a crucial role in plant growth, development, and defense. In the present study, the regulatory effects of 24-epibrassinolide (EBR, one of the bioactive BRs) on heat tolerance in soybeans, and its underlying physiological mechanisms were investigated. The results show that foliar spraying with EBR significantly alleviate heat stress-induced water loss and oxidative damage in soybean leaves. The activities of antioxidant enzymes (superoxide dismutase, catalase, and peroxidase) and the contents of antioxidant substances (ascorbic acid and reduced glutathione) were markedly increased in EBR-treated leaves compared with water-treated leaves, which contributed to maintaining reactive oxygen species homeostasis and relieving oxidative injury under heat stress. However, EBR-treated leaves showed a significant decrease in free proline and total soluble sugar content under heat stress compared to water-treated leaves. In addition, EBR treatment showed obviously higher photosystem II activity under heat stress, and higher net photosynthetic rate and biomass accumulation after recovery from heat stress compared to water treatment. Collectively, these results indicated that EBR could significantly improve the capacity of antioxidant defense systems to protect photosynthetic apparatus under heat stress, thereby effectively alleviating heat stress-induced growth inhibition in soybean plants.

Keywords: reactive oxygen species; chlorophyll fluorescence; photosynthesis; osmolyte; biomass

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1. Introduction

Soybean (*Glycine max* L.) is an important agricultural crop worldwide, which not only provides protein and edible oil for human consumption but can also be used as fodder and biofuel [1]. Heat stress is one of the most important abiotic stresses limiting soybean yield and productivity [2,3]. An increase of 1 °C during the growing season may lead to roughly a 17% relative reduction in soybean yield [4]. Furthermore, the frequency and severity of abiotic stresses, including heat, are forecast to increase owing to global climate change [5], which seriously threatens the production of soybean. To guarantee world food security in the setting of global climate change, there is an urgent need to explore new cultural practices for enhancing the heat tolerance of soybeans.

Heat events cause the excessive evaporation of water, leading to a reduction in stomatal conductance and photosynthesis rates [6], ultimately resulting in a decreased yield. Plants can quickly accumulate osmolytes (e.g., soluble sugar, free amino acid, and soluble protein) in response to heat stress, which play an important role in supporting osmotic adjustment

and maintaining stomatal openings [7]. Apart from stomatal limitation, the damage of photosynthetic apparatus is also a crucial mechanism of the heat stress-induced decline in photosynthesis [8]. Under heat stress conditions, thylakoid membranes become over-energized due to the imbalance in light energy absorption and consumption [9]. One of the consequences of this over-energized state is photodamage, mainly caused by the over-accumulation of reactive oxygen species (ROS) [6]. To alleviate the ROS-induced oxidative damage, the plant's antioxidant defense systems are activated to scavenge the over-produced ROS, including non-enzymatic (e.g., ascorbic acid, glutathione and phenols) and enzymatic (e.g., superoxide dismutase, catalase, and peroxidase) systems [10].

Brassinosteroids (BRs) are a group of naturally existing steroids in the plant kingdom, and are important signaling molecules regulating plant growth and development, plant type and defense response [11]. A series of studies have shown that exogenous BRs can enhance resistance to abiotic and biotic stresses in many plant species [12,13]. Previous studies indicated that BRs treatment significantly increased the content of total soluble sugar and the capacity of the antioxidant defense system to maintain stomatal openings and protect photosynthetic apparatus in tomato and rice plants under heat stress [14,15]. However, the effects of BRs on the heat tolerance of the soybean plant and its underlying mechanisms are unclear.

In the present study, we hypothesized that BRs can enhance the accumulation level of osmolytes and activate antioxidant systems to alleviate the heat stress-induced reduction in photosynthesis occurring in soybeans. To verify this hypothesis, we first screened the optimal spray concentration of BRs for inducing heat tolerance in soybeans, and then investigated the effects of BRs on antioxidant capacity, osmolyte levels, chlorophyll fluorescence parameters, photosynthetic traits, and biomass accumulation in soybeans under normal and heat conditions.

2. Materials and Methods

2.1. Plant Materials and Treatments

The experiment was performed on the campus of Nanjing Agricultural University, Nanjing, Jiangsu Province, China. Uniform seeds of soybean (*Glycine max* L. cv. Nannong 99-6) were selected and surface-sterilized with 0.1% sodium hypochlorite for 10 min. After being washed several times with sterile distilled water, the seeds were sown in well-drained pots (10 cm in height and 10 cm in diameter) filled with moist soil and moved into a growth chamber (GXZ–800D, Jiangnan Co. Ltd., Ningbo, China). The growth conditions were as follows: a 14 h photoperiod, temperature of 25 °C/20 °C (day/night), illuminance of 30,000 Lux, and relative humidity of 80%.

Experiment 1: Identifying the optimal concentration of BRs for inducing heat tolerance in soybeans. One month after sowing, the seedlings with similar growths were selected and sprayed with different concentrations of 24-epibrassinolide (EBR; Sigma–Aldrich, St. Louis, MO, USA) solutions (0, 0.05, 0.1, 0.25, 0.5 and 1 μM, containing 0.1% ethanol and 0.2% tween 80). Heat stress (40 °C at light for 8 h) was applied at 3 h after EBR pretreatment. In total, seven treatments were established: CC (0 μM EBR + no heat stress), CH (0 μM EBR + heat stress), 0.05EH (0.05 μM EBR + heat stress), 0.1EH (0.1 μM EBR + heat stress), 0.25EH (0.25 μM EBR + heat stress, 0.5EH (0.5 μM EBR + heat stress) and 1EH (1 μM EBR + heat stress). At the end of the heat stress application, the last fully expanded leaves following each treatment were used for the measurement of relative water and malonaldehyde (MDA) contents to identify the heat tolerance.

Experiment 2: Exploring the physiological mechanism of EBR-induced heat tolerance in soybeans. One month after sowing, the seedlings with similar growths were randomly divided into two groups. One group was sprayed with 0.25 μM EBR, the other group was sprayed with water. Both EBR and water solutions contained 0.1% ethanol and 0.2% tween 80. After 3 h of pretreatment, 1/2 of each group of the seedlings were challenged with heat stress, as previously described, while the other 1/2 were kept at the control temperature. Finally, four treatments were composed: CC (water + no heat stress), CH

(water + heat stress), EC (0.25 μM EBR + no heat stress) and EH (0.25 μM EBR+ heat stress). After the application of heat stress, the seedlings were recovered at normal growth condition for 3 days. The last fully expanded leaves following each treatment were collected at the end of the heat stress application for physiological analysis, and the photosynthetic rate and biomass were determined during the recovery stage.

2.2. Leaf Relative Water Content

The leaf relative water content was tested according to Yue et al. [16]. The calculation formula was leaf relative water content = (leaf fresh weight – leaf dry weight)/(leaf fresh weight at full turgor – leaf dry weight) × 100%.

2.3. Osmolyte Content

The total soluble sugar content was measured with the anthrone method as described by Sairam et al. [17]. Free proline content was determined using the ninhydrin coloring method [18].

2.4. Antioxidant System

The content of MDA was determined as described by Wang et al. [19]. The ascorbic acid (ASA) and reduced glutathione (GSH) contents were tested according to the method of Ahanger et al. [20]. The activities of superoxide dismutase (SOD; EC 1.15.1.1) and catalase (CAT; EC1.11.1.6) were tested according to the methods of Dong et al. [21]. Guaiacol peroxidase (POD; EC 1.11.1.7) activity was measured as described by Ding et al. [22].

2.5. ROS Histochemical Detection and Quantification

The histochemical staining of hydrogen peroxide (H_2O_2) and superoxide anion ($O_2 \cdot^-$) was performed as described by Xia et al. [23] using 3,3′–diaminobenzidine (DAB) and nitro blue tetrazolium (NBT), respectively. The quantification of H_2O_2 and $O_2 \cdot^-$ was determined as described by Xia et al. [23] and Wang et al. [8], respectively.

2.6. Chlorophyll Fluorescence Analysis

Chlorophyll fluorescence parameters (Fv/Fm, the maximum photosystem II (PSII) quantum yield; Fv′/Fm′, efficiency of excitation energy capture by open PSII reaction centers; ETR, electron transport rate) were detected with an imaging pulse amplitude modulated fluorometer (CF Imager, Technologica Ltd., Colchester, UK) as described in our previous study [24,25].

2.7. Leaf Net Photosynthetic Rate

The net photosynthetic rate was determined after a 12 h recovery from heat stress with a portable photosynthesis system (LI–6400, LiCor Inc., Lincoln, NE, USA), as described by Si et al. [26].

2.8. Plant Dry Mass

For the dry mass measurement, 15 plants from each treatment were harvested after 3 d of recovery from heat stress. The aboveground and underground parts were firstly treated at 105 °C for 30 min, and dried at 70 °C to a constant weight to obtain the dry mass.

2.9. Statistical Analysis

All the data are expressed as the mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (SPSS18.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effects of EBR Treatment on Soybean Heat Tolerance

After a few hours of heat treatment, severe wilting symptoms could be visually observed in water-treated leaves (Figure 1A). Leaves treated with EBR (0.05~1 μM) showed less wilting after heat treatment, compared to water-treated leaves (Figure 1A). As shown in Figure 1B, heat treatment caused a significant reduction (24.58%) in leaf relative water content. However, EBR-treated leaves showed significantly higher leaf relative water content compared to water-treated leaves under heat stress (Figure 1B). As compared with the control treatment, MDA content was dramatically increased by heat stress, whereas the increase was more moderate in EBR-treated leaves compared with water-treated leaves (Figure 1C). In addition, we observed that the 0.25 μM EBR treatment had better mitigative effects than other concentration treatments (Figure 1A–C). Therefore, 0.25 μM EBR was selected as the efficient concentration to improve the capacity of heat tolerance in soybeans.

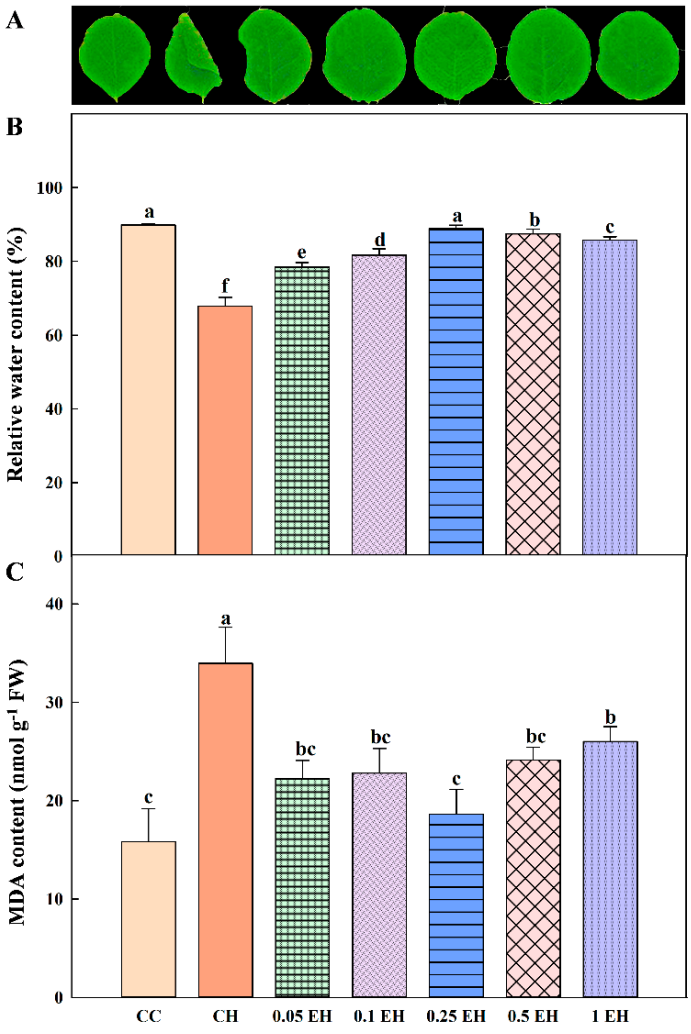

Figure 1. Heat tolerance phenotypes in different concentrations of EBR-treated soybean leaves. (**A**) Phenotypes of EBR-treated leaves under heat stress. (**B**) Leaf relative water content. (**C**) Leaf MDA content. The picture of representative leaves and the measurement of relative water content and MDA was taken at the end of heat treatment. For (**B**,**C**), each value is the mean of three biological replicates (±SD), and the different lowercase letters indicate statistically significant differences at $p < 0.05$ level. CC water + no heat stress, CH water + heat stress, 0.05EH EBR (0.05 μM) + heat stress, 0.1EH EBR (0.1 μM) + heat stress, 0.25EH EBR (0.25 μM) + heat stress, 0.5EH EBR (0.5 μM) + heat stress, 1EH EBR (1 μM) + heat stress.

3.2. Effects of EBR Treatment on ROS Level in Soybean Leaves

The levels of ROS during each treatment were detected by histochemical staining and quantitative measurements. As shown in Figure 2, the heat stress treatment (CH) dramatically increased the H_2O_2 content and $O_2 \cdot^-$ production rate in leaves, compared to the control treatment (CC). However, the EBR treatment under heat stress (EH) significantly inhibited the increase in H_2O_2 content (45.31%) and $O_2 \cdot^-$ production rate (38.35%), compared to the CH (Figure 2). These results suggest that EBR can alleviate the over-accumulation of ROS in soybean leaves under heat stress. In addition, EBR treatment under normal temperature (EC) markedly enhanced the H_2O_2 content (76.82%), and moderately increased the $O_2 \cdot^-$ production rate (24.18%), compared to the CC.

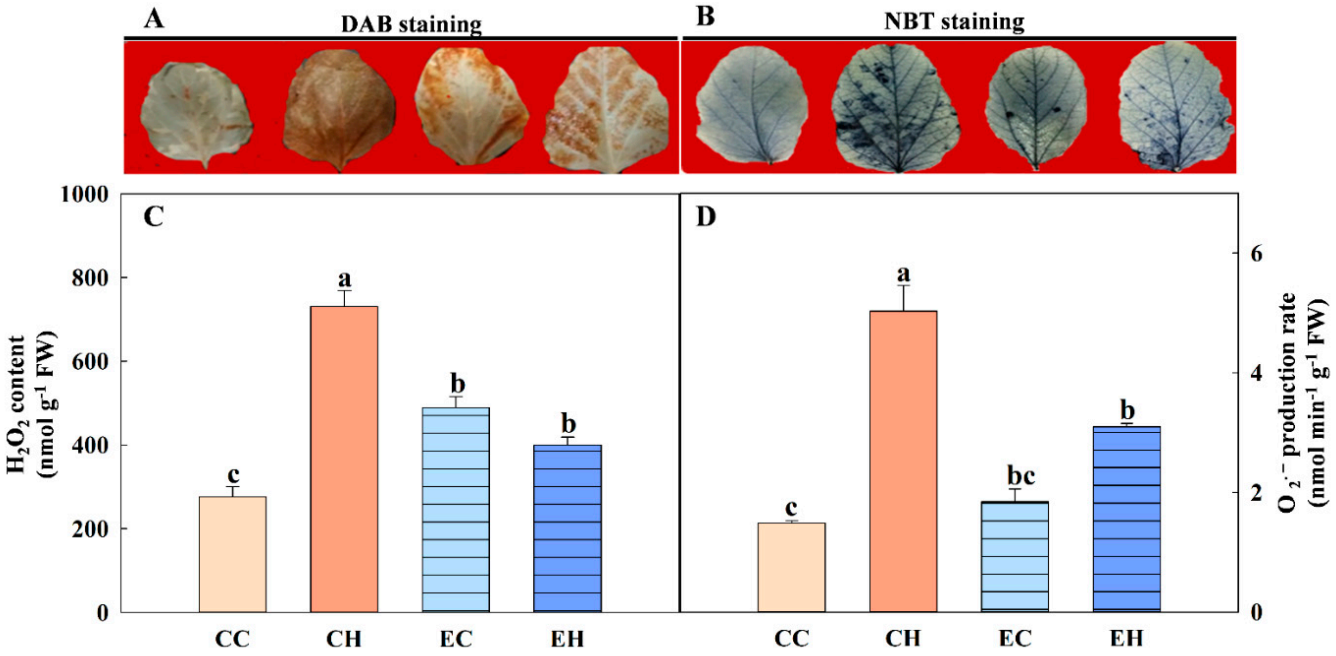

Figure 2. Effects of EBR on ROS level in soybean leaves under heat stress. (**A**) DAB staining. (**B**) NBT staining. (**C**) H_2O_2 content. (**D**) $O_2 \cdot^-$ production rate. DAB and NBT staining were used to detect the presence of H_2O_2 and $O_2 \cdot^-$ in leaves. For (**A**,**B**), the tawny and blue spots indicate the location of H_2O_2 and $O_2 \cdot^-$ in leaves, respectively. The leaves were harvested at the end of heat treatment and stained immediately. For (**C**,**D**), each value is the mean of three biological replicates (±SD), and the different lowercase letters indicate statistically significant differences at $p < 0.05$ level. CC water + no heat stress, CH water + heat stress, EC EBR (0.25 μM) + no heat stress, EH EBR (0.25 μM) + heat stress.

3.3. Effects of EBR Treatment on Antioxidant Capacity in Soybean Leaves

To analyze the underlying physiological mechanisms for EBR-inhibited ROS accumulation under heat stress, we examined the effects of EBR on the activities of antioxidant enzymes and the contents of antioxidant substances in soybean leaves. As compared with the CC treatment, the activities of SOD, CAT, and POD increased by 29.93%, 24.58% and 122.3%, while the contents of ASA and GSH decreased by 36.23% and 42.70% under the CH treatment, respectively (Table 1). The EC and EH treatments significantly enhanced the activities of SOD, CAT and POD and the contents of ASA and GSH compared to the CC and CH treatments, respectively (Table 1).

Table 1. Effects of EBR on the activities of antioxidants in soybean leaves under heat stress.

Items	CC	CH	EC	EH
SOD (U mg^{-1} protein)	98.59 ± 4.010 ^{d}	128.1 ± 7.021 ^{c}	144.9 ± 9.387 ^{a}	175.3 ± 4.569 ^{b}
CAT (U mg^{-1} protein)	1.566 ± 0.045 ^{d}	1.951 ± 0.186 ^{c}	3.257 ± 0.301 ^{a}	2.650 ± 0.029 ^{b}
POD (U mg^{-1} protein)	22.78 ± 1.729 ^{d}	50.65 ± 2.582 ^{b}	46.86 ± 3.298 ^{c}	56.86 ± 3.272 ^{a}
ASA (μmol g^{-1} FW)	1.057 ± 0.035 ^{b}	0.674 ± 0.071 ^{c}	1.657 ± 0.035 ^{a}	1.092 ± 0.032 ^{b}
GSH (μmol g^{-1} FW)	0.274 ± 0.005 ^{b}	0.157 ± 0.007 ^{d}	0.326 ± 0.012 ^{a}	0.226 ± 0.012 ^{c}

Note: The measurements were taken at the end of heat stress. Each value is the mean of three biological replicates (±SD). The different lowercase letters indicate statistically significant differences at $p < 0.05$ level. CC water + no heat stress, CH water + heat stress, EC EBR (0.25 μM) + no heat stress, EH EBR (0.25 μM) + heat stress.

3.4. Effects of EBR Treatment on Osmolytes in Soybean Leaves

As shown in Figure 3, the CH treatment significantly increased the contents of free proline (129.2%) and total soluble sugar (64.66%) compared to the CC treatment. There was no significant difference in the free proline content between the CC and EC treatments. However, the EC treatment showed a remarkably higher total soluble sugar level than the CC treatment. As compared with the CH treatment, the contents of free proline and total soluble sugar decreased by 35.45% and 20.88% under the EH treatment (Figure 3).

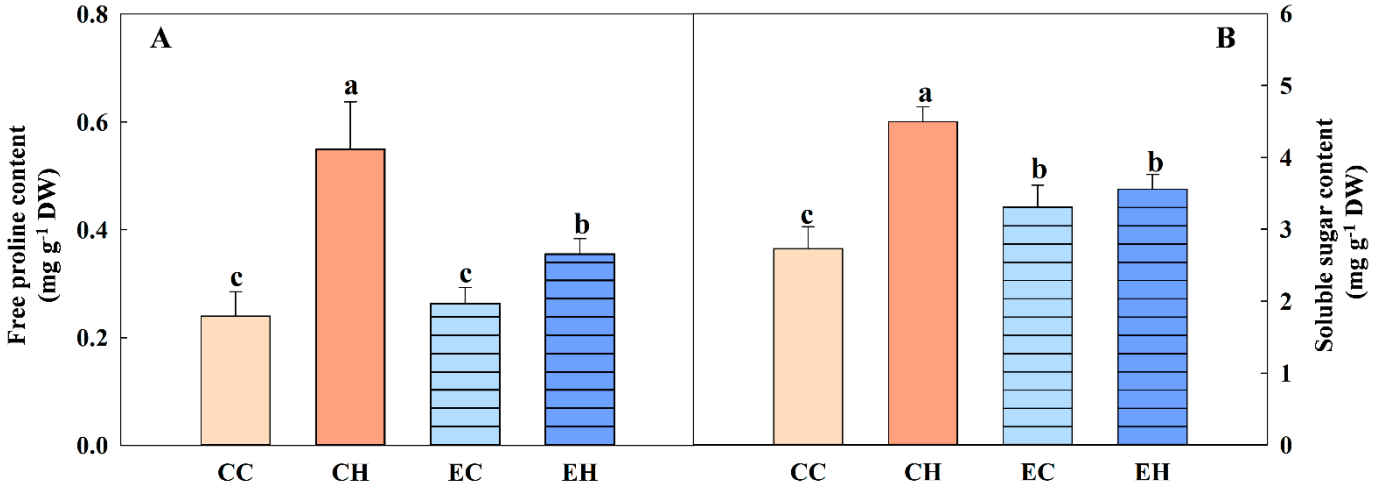

Figure 3. Effects of EBR on osmolyte levels in soybean leaves under heat stress. (**A**) Free proline content. (**B**) Total soluble sugar content. The measurement was taken at the end of heat treatment. Each value is the mean of three biological replicates (±SD). The different lowercase letters indicate statistically significant differences at $p < 0.05$ level. CC water + no heat stress, CH water + heat stress, EC EBR (0.25 μM) + no heat stress, EH EBR (0.25 μM) + heat stress.

3.5. Effects of EBR Treatment on Chlorophyll Fluorescence in Soybean Leaves

Fluorescence images and the average value of Fv/Fm showed that the CH treatment resulted in a significant reduction in Fv/Fm (6.469%) compared to the CC treatment, while the EH treatment notably alleviated the decrease in Fv/Fm (Figure 4A,B). Moreover, the EH treatment showed significantly higher Fv′/Fm′ (8.041%) and ETR (5.811%) compared to the CH treatment (Figure 4C,D). There were no significant differences in Fv/Fm, Fv′/Fm′ and ETR between the CC and the EC treatment.

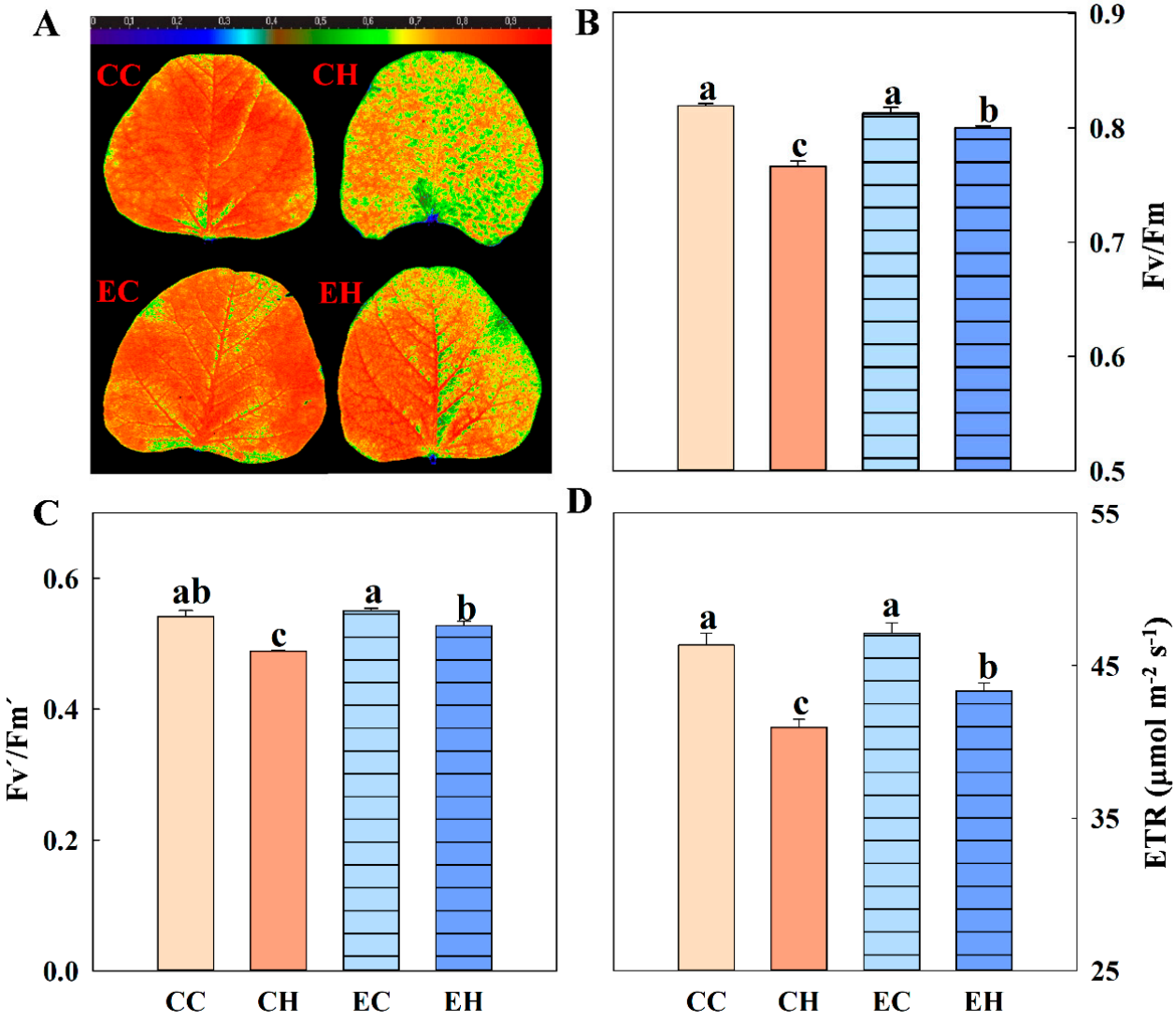

Figure 4. Effects of EBR on chlorophyll fluorescence parameters of soybean leaves under heat stress. (**A**) Image of Fv/Fm. (**B**) Average Fv/Fm values. (**C**) Average Fv′/Fm′ values. (**D**) Average ETR values. The measurement was taken at the end of heat treatment. The color code depicted on the top of the image (**A**) ranges from 0 (blue) to 1.0 (red). For (**B**–**D**), each value is the mean of three biological replicates (±SD). The different lowercase letters indicate statistically significant differences at $p < 0.05$ level. CC water + no heat stress, CH water + heat stress, EC EBR (0.25 μM) + no heat stress, EH EBR (0.25 μM) + heat stress.

3.6. Effects of EBR Treatment on the Photosynthesis and Biomass Accumulation of Soybean

The CH treatment significantly decreased the net photosynthesis rate (12.91%) and total biomass (18.12%) compared to the CC treatment (Figure 5A). The EC and EH treatments notably increased the net photosynthesis rate (19.21% and 20.15%) compared to the CC and CH treatments, respectively (Figure 5A). Accordingly, the total biomass increased by 12.53% and 15.21% in the EC and EH treatments compared to the CC and CH treatments, respectively (Figure 5B). The EC treatment simultaneously enhanced the overground and underground biomass of plants compared to the CC treatment, whereas the EH treatment only significantly increased the overground biomass compared to the CH treatment (Figure 5B).

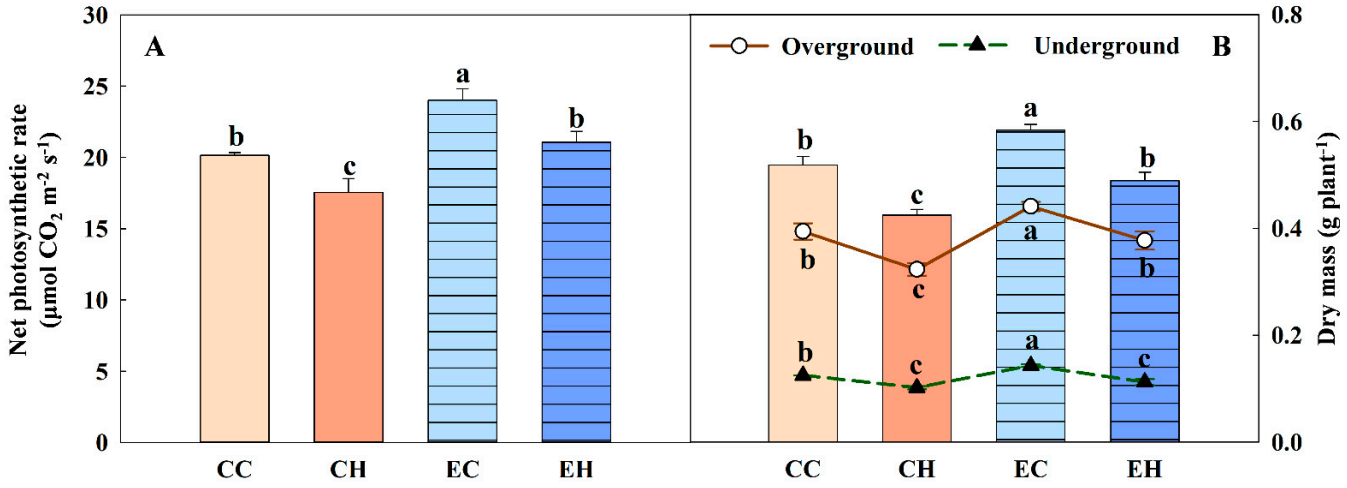

Figure 5. Effects of EBR on the net photosynthetic rate and dry mass of soybean plants after recovery from heat stress. (**A**) Net photosynthetic rate. (**B**) Dry mass. The net photosynthetic rate of leaves was measured after 12 h recovery from heat stress, and the dry mass of soybean plants were determined after 3 days recovery from heat stress. Each value is the mean of three biological replicates (±SD). The different lowercase letters indicate statistically significant differences at $p < 0.05$ level. CC water + no heat stress, CH water + heat stress, EC EBR (0.25 μM) + no heat stress, EH EBR (0.25 μM) + heat stress.

4. Discussion

Many effects of BRs on plant growth, development, and defense responses have been extensively recorded [11,12,27], but the regulatory effects of BRs on the heat tolerance of soybeans is far from clear. In the present study, foliar application of EBR (0.05~1 μM) obviously alleviated the heat stress-induced decrease in relative water content and the increase in MDA content in soybean leaves (Figure 1). The relative water content was used to determine plant–water status under heat stress conditions [15], and MDA is an important indicator of oxidative damage in plants [28]. From this, the application of EBR could significantly mitigate heat stress-induced dehydration and peroxidation damage, thereby enhancing heat tolerance in soybeans (Figure 1). Similar findings have been reported in EBR-treated tomato, *Ficus concinna*, and *Arabidopsis* plants [12,29–31]. These results indicated that BRs signal play an important role in plant responses to high temperature stress. Phytohormones are usually trace amounts and efficient, and the regulatory effects of plant growth regulators on plant growth and development usually occur in a dose–dependent manner [32]. Xia et al. [23] found that 0.1 μM was the most effective concentration of EBR to improve chilling tolerance in cucumber plants. Our results show that 0.25 μM of EBR would be the optimum concentration for increasing heat tolerance in soybean seedlings. Disruptions in plant ROS homeostasis is a general consequence of abiotic stresses [33]. Chloroplasts are major sources of ROS generation in plants under stress conditions [34]. The excessive accumulation of ROS in chloroplasts can result in the peroxidation of the thylakoid membrane and degradation of D1 proteins in PSII and stromal enzymes, such as ribulose–1,5–bisphosphate carboxylase oxygenase (Rubisco), which causes more ROS generation and further damage [10]. Chlorophyll fluorescence is an effective method to accurately assess the activity of PSII and quantify stress damage [35]. The current study showed that heat stress remarkably increased the accumulation level of H_2O_2 and production rate of $O_2 \cdot^-$ in soybean leaves (Figure 2), accompanied by a significant reduction in Fv/Fm, Fv′/Fm′ and ETR (Figure 4), indicating that the excess accumulation of ROS induced by heat stress might cause damage to the photosynthetic apparatus of soybean leaves. Accordingly, heat stress treatment significantly decreased the leaf net photosynthesis rate and plant biomass during the recovery stage (Figure 5). These results were consistent with the previous studies that determined that heat stress significantly increased ROS levels and decreased the photosynthetic capacity and grain yield [36–38]. Although the activity of antioxidant

enzymes (SOD, CAT, and POD) and contents of antioxidant substances (ASA and GSH) were enhanced, the accumulation level of ROS was still significantly increased under heat stress, suggesting that the production rate of ROS under heat stress exceeded its clear rate in this study.

Previous studies found that EBR treatment significantly increased the activities of SOD, CAT, GR (glutathione reductase) and APX (ascorbate peroxidase), as well as the ratios of GSH/GSSG (oxidized glutathione) and ASA/DHA (oxidized ascorbate), which conferred tomato plant heat tolerance [12,23]. In this study, EBR treatment also effectively improved the activities of SOD, CAT, and POD and the levels of ASA and GSH in soybean leaves, accompanied by a significant decrease in H_2O_2, $O_2 \cdot^-$ and MDA levels compared to treatment under heat stress without EBR (Table 1 and Figures 1 and 2). In addition, EBR treatment significantly increased leaf Fv/Fm, Fv′/Fm′, ETR and the net photosynthesis rate under heat stress (Figures 4 and 5A). These results suggest that EBR treatment can maintain ROS homeostasis under heat stress in soybean leaves by enhancing the antioxidant capacity, which might be beneficial to alleviate the damage to the photosystem caused by heat stress, and finally promote plant growth (Figure 5B). Our results are consistent with those obtained by Thussagunpanit et al. [15], who reported that BR treatment effectively reduced MDA and H_2O_2 content, and increased the leaf net CO_2 assimilation rate and grain yield in rice under heat stress conditions. Ribeiro et al. [39] and Soliman et al. [40] also found that BRs had a significant regulatory effect on the antioxidant system in soybeans under water deficit and salt stresses. Previous studies showed that EBR could upregulate the expression of genes encoding antioxidant enzymes, such as *Cu/Zn-SOD*, *CAT1*, *cAPX* and *GR1* in tomato and cucumber seedlings [12,23], suggesting that EBR regulated antioxidant capacity on the transcriptional level. Our study, as well as previous ones, indicated that maintaining ROS homeostasis is an important physiological mechanism for EBR to improve plant abiotic stresses tolerance.

It is interesting to note that EBR treatment obviously increased the level of ROS, especially the level of H_2O_2 under normal conditions in soybean leaves (Figure 2). The role of ROS in plants is twofold, where high levels of ROS cause cell damage and low levels of ROS have regulatory roles in plant stress responses [23,33]. From this, we speculated that the ROS signal might be involved in EBR-induced improvement of the antioxidant capacity and heat tolerance in soybean plants. Cui et al. [41] and Zhou et al. [12] found that EBR induced apoplastic ROS accumulation by increasing NADPH oxidase activity, and this ROS could act as a system signal to activate the antioxidant system in cucumber and tomato plants. These results imply that ROS is a key signal molecule in mediating EBR-regulated abiotic stress tolerance in plants.

In the present study, although EBR-treated leaves significantly decreased free proline and the total soluble sugar content compared with water-treated leaves under heat stress, EBR treated leaves still showed an obviously higher relative water content (Figures 1 and 3). These results suggested that EBR-alleviated water loss might not be dependent on the accumulation level of osmolytes in soybean leaves under heat stress. Xia et al. [42] reported that EBR could induce stomatal closure by crosstalk with H_2O_2 and abscisic acid signals in tomato plants. Shi et al. [43] also showed that treatment with EBR significantly closed the stomata of *Arabidopsis*. Therefore, in the present study, it can be observed that EBR treatment might reduce water evaporation rates under high temperature conditions by decreasing the stomatal opening. In addition, EBR treatment significantly increased the total soluble sugar content under normal conditions (Figure 3), which could be important for EBR-treated leaves adaptation to heat stress. Altogether, these results reveal that EBR treatment is beneficial for soybean plants to cope with subsequent high temperature stress.

5. Conclusions

In summary, heat stress causes severe oxidative damage and dehydration injury, and significantly decreases photosystem activity and suppresses the photosynthesis of soybean leaves, resulting in plant growth inhibition. The application of EBR to the foliage

of soybean plants regulated soybean heat tolerance in a dosage–dependent manner, and 0.25 μM was the optimum concentration of the plants that were tested. EBR treatment significantly increased the activities of antioxidant enzymes (SOD, CAT, and POD) and the contents of antioxidant substances (ASA and GSH), thereby effectively alleviating the heat stress-induced reductions in photosystem activity, photosynthetic rate, and biomass. The ROS signal might be involved in EBR-induced soybean heat tolerance in this study. However, the physiological mechanism of EBR alleviating heat stress-induced water loss in soybean leaves needs further investigation. This study contributes to the physiological understanding of EBR-improved heat tolerance, and should provide effective strategies to alleviate heat stress-induced grain yield reductions in soybeans.

Author Contributions: H.J. designed and supervised the research; W.W., Y.X. and C.L. analyzed the data and wrote the manuscript; Y.X. and W.W. performed pot and laboratory experiments. All authors have read and agreed to the published version of the manuscript.

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