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24-Epibrassnolide Alleviates the Adverse Effect of Salinity on Rice Grain Yield through Enhanced Antioxidant Enzyme and Improved K^+/Na^+ Homeostasis

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Abstract: Previous researchers have focused on the role of 24-epibrassnolide (EBR) in alleviating stresses in plants, whereas the effect of EBR on rice grain yield formation from the perspective of the whole growth stage remains less concerned. To further confirm the optimal application concentration and application periods of EBR in rice (*Oryza sativa* L.) under salt stress, a seed germination experiment, a seedling experiment, and a pot experiment were designed and conducted. Results showed that EBR treatment significantly enhanced germination indicators and seedling morphological traits, and the effects varied among different EBR concentrations, which were 0.5 > 1.0 > 0.1 > 0.05 mg L^{-1}. Under 0.5% salt treatments, 0.5 mg L^{-1} EBR spraying significantly enhanced the seedling height, root length, above-/under-ground fresh weight, and above-/under-ground dry weight by 9.2%, 15.9%, 48.0%, 19.5%, 29.3%, and 12.5%, respectively. The spraying of EBR at different periods enhanced rice yield by 6.7% to 94.4% under salt stress. The relatively higher panicle number (increased by 42.9%) and spikelet number per panicle (increased by 96.1%) were the main reason resulting in higher grain yield under the S+T5 (EBR sprayed at both transplanting and heading stage) treatment. Compared to those under S treatment, catalase (CAT) activity was significantly enhanced by 25.0%, while malondialdehyde (MDA) content was dramatically decreased by 37.3% under the S+T5 treatment. The S+T5 treatment significantly enhanced the K^+ content in rice root and leaf and decreased the Na^+ content in rice root (by 30.4%), thereby leading to higher K^+/Na^+ under salt stress. The current study concluded that 0.5 mg L^{-1} was the optimal concentration of EBR in alleviating the adverse effect of salinity. Spraying (transplanting + heading) of EBR twice displayed the best alleviating effect under salt stress, which was realized through enhanced antioxidant enzyme, higher K^+ maintenance in leaves, and lowered absorption of Na^+ in rice root.

Keywords: rice (*Oryza sativa* L.); salt stress; yield; 24-epibrassnolide

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1. Introduction

As an important reserve cultivated land resource, about 1125 million hectares of saline land exist in the whole world [1]. In China, about a quarter of arable land has been identified as saline soils and is underutilized [2]. Rice (*Oryza sativa* L.) is a major staple crop species, which is more prone to soil salinity compared with wheat, barley, and other cereal crops [3]. Numerous research papers have clarified the adverse effects of salinity on rice yield and grain quality formation, where the intracellular osmotic stress and ion toxicity are considered as the direct reasons leading to the subsequent shielding responses of the morphological and physiological traits, as well as related molecular events [4–6].

The application of plant growth regulators has been one of the promising approaches for plants to enhance stress tolerance. Grove and his partners discovered steroid hormones, brassinolides (BRs) [7]. Although the contents in plants are very small, brassinolides have strong physiological activity; thus, the 16th International Year of Growth Substances listed it as the sixth-most-important hormone in plants. 24-epibrassnolide (EBR), an active by-product from brassinolide biosynthesis, has the ability to stimulate different plant metabolic processes such as photosynthesis [8] and protein and nucleic acid biosynthesis [9]. It has been reported that EBR can help plants mitigate the adversities of different abiotic stresses including drought, cold, salinity, and heavy metal stress [10–13].

Several previous studies indicated a potential role of EBR in alleviating salt stresses [11,14,15]. EBR application significantly enhanced seed germination and seedling establishment under salinity stress [16]. EBR treatment mitigated salt stress in pea plants by improving the fresh weight, dry weight, and leaf area [17]. Foliar application of 0.1 μM EBR increased the activity of catalase and, thereby, eliminated reactive oxygen species (ROSs) in peanut [18].

EBR has also been reported to be effective in alleviating the adverse effects of salinity in rice plants. It has been indicated that seed application of EBR improved seedling growth, alleviated lipid damage, and decreased proline accumulation caused by salt stress in a salt-sensitive rice variety, IR-28 [19]. Supplementation of the saline solution with EBR considerably reduced the inhibitory effect of salinity on seed germination, and the promotion of growth in rice seedlings by EBR under saline conditions was associated with enhanced levels of nucleic acids and soluble proteins [20]. EBR applied to rice seedlings showed an improvement in growth, levels of protein and proline content, and antioxidant enzyme activity under NaCl stress [21], which displayed improved shoot length, root length, root number, and rice fresh/dry weight.

Although there have been reports on EBR alleviating salt stress in rice, most of these studies focused on the effect of EBR on rice seed germination and mainly focused on seedling stages. Thus, the current study aimed to (a) determine the appropriate EBR concentration in alleviating salt stress in rice, (b) the effect of EBR spraying for rice yield formation under salt stress from the whole growth stage perspective, and (c) the most effective spraying period for alleviating salt stress for rice and the relative mechanisms.

2. Materials and Methods

2.1. Plant Materials and Experimental Design

In the current study, a seed germination experiment and a seedling experiment were designed to determine the optimal application concentration of EBR, after which a pot experiment was designed and conducted in 2021 to further determine the optimal application periods of EBR in rice under salt stress.

Germination experiment: The germination experiment contained three salinity levels (0, 100, and 150 mmol L^{-1} NaCl) and five EBR concentrations (0, 0.05, 0.1, 0.5, and 1.0 mg L^{-1}), with three replicates for each treatment. Rice cultivar Nanjing 9108 was adopted, and plump seeds were selected and soaked in 5% sodium hypochlorite solution for 20 min. The sterilized rice seeds were well washed with distilled water and subsequently placed in a petri dish with a diameter of 9 cm, with two layers of filter paper covering each petri dish. A 15 mL solution for each treatment was added to the petri dishes. The seeds were cultured in an artificial climate chamber (HP 1000GS-B by Wuhan Ruihua Instrument & Equipment Co., Ltd., Wuhan, China) for 3 days in the dark, after which the seeds were further cultivated with a photoperiod of 12 h/12 h light/dark (light: 8:00–18:00, 29 °C; dark: 18:00–8:00, 20 °C).

Seedling experiment: Plump rice (Nanjing 9108, China) seeds were selected and soaked at 38 °C to promote germination, and uniformly germinated seeds were further selected and sown in a 96-hole black hydroponics box with a nutrition solution [22]. Then, the boxes were transferred into the growth chamber with a photoperiod of 12 h/12 h light/dark (light: 8:00–18:00, 29 °C; dark: 18:00–8:00, 20 °C). Seedlings at the three-leaf stage were sprayed with four EBR concentrations (0, 0.05, 0.1, 0.5, and 1.0 mg L^{-1}) for three

consecutive days. Subsequently, the seedlings were treated with three salinity levels (0, 0.5, and 1% NaCl). The height, root length, and fresh and dry weight of seedlings were measured at 10 days after treatment.

Pot experiment: The pot experiment was conducted at the experimental base of Yangzhou University, Jiangsu Province, China (32°30′ N, 119°25′ E), in 2020. All pots were arranged in the field, covered with a transparent waterproof top during the rice-growing season. Rice cultivar Nanjing 9108 was adopted in this experiment and synchronously arranged with two salt concentrations (0 (S0) and 3.0 (S2) g kg^{-1} NaCl; the conductivity of the water layer was equivalent to 0 and 5.82 mS cm^{-1}, respectively) and two EBR concentrations (0 and 0.5 mg L^{-1}). The treatments are shown in Table 1. The pot was 25 cm in diameter and 30 cm in height, filled with 15 kg of sieved fine soil and planted with 16 seedlings evenly in 4 hills. All treatments had twenty pots as replicates. Rice seeds were sown in May 15, and rice seedlings were transplanted in June 18 and harvested in October 26.

Table 1. Treatment designs in the pot experiment.

Salt Concentration (%)	EBR Concentration (mg L^{-1})	EBR Spraying Period	Denotation
0	0	/	CK
0.3	0	/	S
0.3	0.5	Transplanting	S+T1
0.3	0.5	Panicle initiation	S+T2
0.3	0.5	Heading	S+T3
0.3	0.5	Transplanting + panicle initiation	S+T4
0.3	0.5	Transplanting + heading	S+T5
0.3	0.5	Panicle initiation + heading	S+T6
0.3	0.5	Transplanting + panicle initiation + heading	S+T7

EBR, 24-epibrassnolide.

2.2. Determination of Germination Indicators

The germination energy (GE), germination capacity (GC), and germination index (GI) were calculated using the formulas below:

The GE was calculated at 3 day: GE = G3/T × 100%;

The GC was calculated at 7 day: GC = G7/T × 100%.

G3 and G7 mean the number of germinated seeds at 3 days and 7 days, respectively, and T is the total number of seeds.

GI = $\sum$ (Gt/Dt).

Gt indicates the number of germinated seeds at 3 days, and Dt indicates the relative days of germination. The relative GE or GC was calculated as the percentage of the GE or GC values for treatments compared to the GE or GC values for the control.

2.3. Determination of LAI and Leaf Area Decay Rate

At the heading and full-ripening stages, the rice leaf area index (LAI) was measured through a Leaf Area Meter (LI-3100C, Lincoln, NE, USA). The efficient LAI was determined with the LAI of the top three leaves. The leaf area decay rate was further calculated by the changing percentage between the LAI at heading and full-ripening stages.

2.4. Leaf Na^+ and K^+ Content

Dry samples of 0.3 g were crushed and digested with HNO_3 and $HClO_4$ (4:1, *v*/*v*) and subsequently concentrated using a microwave oven (Mars, CEM Inc., New York, NY, USA). The final Na^+ and K^+ concentrations were determined with atomic absorption spectrometry (PinAAcle 900, PerkinElmer Life and Analytical Sciences, Inc., Shelton, CT, USA).

2.5. Leaf Antioxidant Enzyme Activities, MDA, and Proline Content

About 9 rice flag leaves were collected from 9:00–10:00 a.m. at 20 days after heading, which were subsequently transferred immediately to an ice bath, frozen in liquid nitrogen, and then, stored at −80 °C for subsequent analysis.

Frozen leaf samples (0.3 g) were crushed in 5 mL of 0.1 M Tris-HCl buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiothreitol, and 1% polyvinyl pyrrolidone, and the obtained homogenate was subsequently centrifuged at 20,000× *g* for 20 min at 4 °C. The clear supernatant was obtained and used to measure enzyme activities. The peroxidase (POD; EC 1.11.1.7) activity was determined by analyzing the guaiacol oxidation at 470 nm according to Jiang et al. [23], and the catalase (CAT; EC 1.11.1.6) activity was assayed as the absorbance decrease at 240 nm that accompanied H_2O_2 consumption following the method of Panda et al. [24].

The determination of the MDA content was performed by a testing company (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) following the modified method of Kramer et al. [25]. The chloroplast supernatant (0.5 mL) was reacted with 2 mL of 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA), followed by boiling at 95 °C for 30 min, and the reaction was terminated in a rice bath. The samples were centrifuged at 10,000× *g* for 10 min, after which the absorbance was recorded at 440, 532, and 600 nm. The proline content was determined as described previously [26].

2.6. Leaf Photosynthetic Potential, Crop Growth Rate, and Net Assimilation Rate

Leaf photosynthetic potential, crop growth rate, and the net assimilation rate were analyzed according to the LAI and plant dry weight using the following formulas [27]:

Leaf photosynthetic potential = 1/2 (LAI1 + LAI2) × (t2 − t1);

Crop growth rate = (W2 − W1)/(t2 − t1);

Net assimilation rate = [(In(LAI1)/(LAI2 − LAI1)] × [(W2 − W1)/(t2 − t1)].

The numbers following by LAI, t, and W indicate the heading and full-ripening stage, respectively; t means the date of the rice growth stage, and W means the dry weight of the rice plant.

2.7. Rice Grain Yield and Yield Components

The panicle number and spikelet number per panicle were counted from 6 pots (2 pots as a replicate). Panicles were hand-threshed, and the filled and unfilled spikelets were separated by submerging them in tap water. All filled spikelets were air-dried and weighed, and the filled spikelets were calculated by counting the three subsamples of 30 g of filled spikelets. All unfilled spikelets were also air-dried and counted to determine the number of unfilled spikelets. Grain yield was determined from 6 pots (2 pots as a replicate) in each plot and adjusted to the standard moisture content of 14%.

2.8. Statistical Analysis

The data were subjected to the analysis of variance with SPSS ver. 19.0. The least significant difference (LSD) test was performed to identify differences at a significance level of 5% and 1%. All figures were drawn using the Origin 9.0 software program.

3. Results

3.1. Rice Seed Germination Indicators (Germination Experiment)

As indicated in Table 2, the GE, relative GE, GC, relative GC, and GI notably decreased with the increase of the salt concentration. The GE, GC, and GI decreased by 49.2%–64.6%, 17.1%–28.3%, and 34.9%–49.3% under 100 salt stress, respectively, compared to those under 0 salt and 0 EBR treatment. Under non-salt treatments, all seed germination indicators were well improved under different EBR treatments. Under salt stresses, EBR treatment significantly enhanced germination indicators, and the effect varied among different EBR concentrations, which were 0.5 > 1.0 > 0.1 > 0.05 mg L^{-1}. Under salt stress, the GE, relative GE, GC, relative GC, and GI of rice seeds increased by 7.5%–23.4%, 10.4%–32.3%,

12.5%–20.0%, 13.3%–21.1%, and 32.9%–33.0% under the 0.5 mg L^{-1} EBR treatment, respectively, compared to those without EBR treatment.

Table 2. Rice seed germination indicators.

Salt Concentration (mmol L^{-1})	EBR Concentration (mg L^{-1})	GE (%)	Relative GE	GC (%)	Relative GC	GI
0	0	72.5 b	100.0 b	94.6 a	100.0 a	21.5 c
	0.05	80.8 a	111.4 a	95.8 a	101.3 a	22.6 ab
	0.1	78.3 a	108.1 a	92.9 ab	98.3 ab	22.1 ab
	0.5	79.2 a	109.2 a	96.3 a	101.8 a	22.7 a
	1.0	79.6 a	109.9 a	93.3 ab	98.7 ab	22.0 bc
100	0	23.3 f	32.1 f	77.5 f	81.9 f	14.0 g
	0.05	33.8 e	46.6 e	82.1 e	86.8 e	16.3 f
	0.1	38.3 de	53.0 d	86.7 cd	91.6 cd	17.6 e
	0.5	46.7 c	64.4 c	90.0 bc	95.2 bc	18.6 d
	1.0	42.1 cd	58.1 d	88.3 cd	93.4 cd	17.7 e
150	0	7.9 h	10.9 h	66.3 h	70.1 h	10.9 i
	0.05	13.8 g	19.0 g	71.7 g	75.8 g	12.1 h
	0.1	14.2 g	19.6 g	76.3 f	80.6 f	12.7 h
	0.5	15.4 g	21.3 g	86.3 cd	91.2 cd	14.5 g
	1.0	14.6 g	20.2 g	85.4 e	90.3 de	14.0 g

EBR, 24-epibrassnolide, GE, germination energy; GC, germination capacity; GI, germination index. The values in the same column followed by different letters indicate statistical significance at the 0.05 probability level.

3.2. Rice Seedling Morphological Traits (Seedling Experiment)

As shown in Table 3, salt stress significantly inhibited the plant height, root length, and fresh/dry weight of the rice seedlings. EBR spraying displayed significant alleviated effects of salt stress on rice seedling morphological traits, where the 0.5 mg L^{-1} concentration showed the best effect, especially under 0.5% salt treatments. Under 0.5% salt treatments, 0.5 mg L^{-1} EBR spraying significantly enhanced the seedling height, root length, above-/under-ground fresh weight, and above-/under-ground dry weight by 9.2%, 15.9%, 48.0%, 19.5%, 29.3%, and 12.5%, respectively.

Table 3. Morphological traits and biomass of rice seedlings.

Salt Concentration (%)	EBR Concentration (mg L^{-1})	Plant Height (cm)	Root Length (cm)	Above-Ground Fresh Weight (mg $plant^{-1}$)	Under-Ground Fresh Weight (mg $plant^{-1}$)	Above-Ground Dry Weight (mg $plant^{-1}$)	Under-Ground Dry Weight (mg $plant^{-1}$)
0	0	14.33 a	13.90 abc	71.0 a	74.3 ab	11.8 a	7.9 abc
	0.1	13.30 b	12.73 e	63.1 b	68.4 cd	10.2 b	7.8 abc
	0.5	13.98 a	14.53 a	70.4 a	74.4 ab	12.0 a	8.7 a
	1	12.48 cd	14.37 ab	67.9 a	77.5 a	11.4 a	8.3 ab
0.3	0	11.63 e	13.37 cde	45.7 e	65.6 de	8.2 c	6.8 de
	0.1	12.48 cd	12.73 e	51.7 d	67.8 cd	9.4 b	7.1 cde
	0.5	12.80 bc	13.60 bcd	53.8 cd	71.1 bc	9.5 b	7.5 bcd
	1	12.32 cde	13.37 cde	52.9 d	67.3 cd	9.2 b	7.3 bcd
0.5	0	10.87 f	11.30 f	32.2 g	54.4 f	7.5 c	6.4 e
	0.1	10.9 f	12.90 de	36.9 f	54.5 f	7.6 c	6.4 e
	0.5	11.87 de	13.10 cde	47.5 e	64.9 de	9.7 b	7.2 cde
	1	10.95 f	11.03 f	38.5 f	62.9 e	7.8 c	7.0 cde

EBR, 24-epibrassnolide. The values in the same column followed by different letters indicate statistical significance at the 0.05 probability level.

3.3. Rice Plant Height (Pot Experiment)

As shown in Figure 1, salt stress inhibited rice plant height, which was decreased by 37.1% compared to that of CK. Overall, spraying EBR was conducive to increased rice height, and the effect was best under the S+T4 treatment. Compared to that of the S treatment, rice plant height increased by 17.2% under the S+T4 treatment.

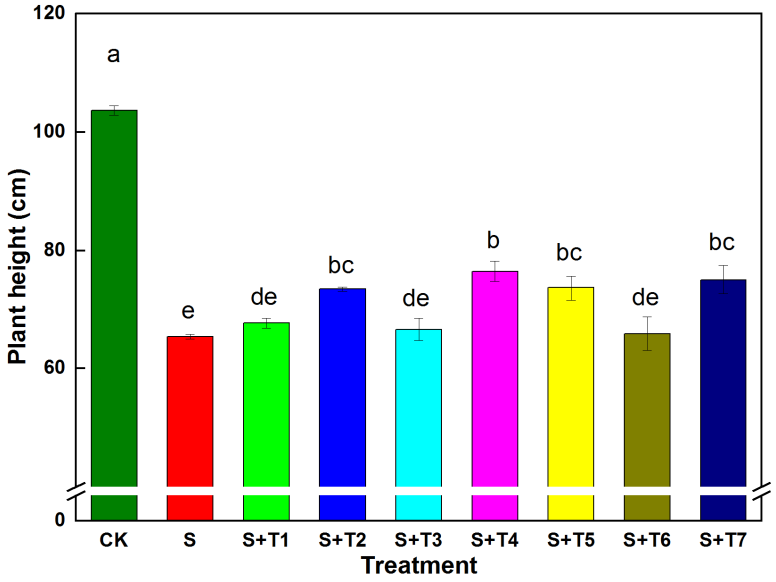

Figure 1. Effects of EBR spraying on rice plant height at full-ripening stage under salt stress. S indicates salt concentration of 3.0 g kg^{-1} NaCl; S+T1 to S+T7 indicate the EBR spraying at transplanting, panicle initiation, heading, transplanting + panicle initiation, transplanting + heading, panicle initiation + heading, and transplanting + panicle initiation + heading stages, respectively, under salt stress. The data (n = 3) are mean values ± SD, calculated from three independent experiments. The different letters indicate significant differences at $p < 0.05$.

3.4. Rice Leaf Area Index, Efficient LAI, and Leaf Area Decay Rate

Under 0.3% salt stress, the LAI and efficient LAI of rice leaves at the heading stage were dramatically decreased by 66.5% and 62.2%, while the LAI at the full-ripening stage decreased by 74.6% (Table 4). The spraying of EBR at different stages notably increased the LAI and efficient LAI of the rice, especially under the S+T5 treatment. Compared to those under the S treatment, the LAI and efficient LAI of rice leaves at the heading stage were enhanced by 57.7% and 48.8%, respectively, and the LAI at the full-ripening stage increased by 72.4% under the S+T5 treatment.

Table 4. Rice LAI, efficient LAI, and leaf area decay rate.

Treatment	LAI (Heading Stage)	Efficient LAI (Heading Stage)	LAI (Full-Ripening Stage)	Leaf Area Decay Rate (%)
CK	6.23 a	4.15 a	3.93 a	36.45 f
S	2.15 e	1.57 f	1.00 f	52.63 bc
S+T1	2.39 e	1.72 ef	1.28 de	47.55 d
S+T2	2.84 d	1.95 de	1.60 bc	42.02 e
S+T3	2.43 e	1.77 ef	1.12 ef	55.18 b
S+T4	3.37 bc	2.30 bc	1.33 d	60.17 a
S+T5	3.39 b	2.33 b	1.72 b	50.34 cd
S+T6	3.07 cd	2.17 bcd	1.44 cd	52.39 bc
S+T7	3.11 bcd	2.09 cd	1.62 bc	47.29 d

LAI, rice leaf area index, S indicates salt concentration of 3.0 g kg^{-1} NaCl; S+T1 to S+T7 indicate the EBR spraying at transplanting, panicle initiation, heading, transplanting + panicle initiation, transplanting + heading, panicle initiation + heading, and transplanting + panicle initiation + heading stages, respectively, under salt stress. The values in the same column followed by different letters indicate statistical significance at the 0.05 probability level.

3.5. Rice Plant Dry Weight at Heading and Full-Ripening Stage

As shown in Table 5, salt stress notably decreased the dry weight of rice stem, leaf, spike, and whole plant at the heading and full-ripening stage. The decrease of rice dry weight under salt stress was significantly alleviated after spraying EBR at different stages.

At the full-ripening stage, the alleviation effect was the best for stem dry weight (increased by 41.8%) under the S+T7 treatment, whereas it peaked under the S+T5 treatment for the dry weight of rice leaf, spike, and total plant (increased by 125%, 101.9%, and 73.6%, respectively), compared to those under other treatments.

Table 5. Rice dry weight at different growth stages.

Treatment	Heading Stage				Full-Ripening Stage			
	Stem Dry Weight (g hill^{-1})	Leaf Dry Weight (g hill^{-1})	Spike Dry Weight (g hill^{-1})	Total Dry Weight (g hill^{-1})	Stem Dry Weight (g hill^{-1})	Leaf Dry Weight (g hill^{-1})	Spike Dry Weight (g hill^{-1})	Total Dry Weight (g hill^{-1})
CK	19.61 a	7.95 a	5.03 a	32.59 a	18.58 a	8.06 a	25.91 a	52.05 a
S	4.33 g	2.41 f	0.98 e	7.72 f	4.31 e	1.76 e	5.14 g	11.21 f
S+T1	4.75 f	2.78 def	0.92 e	8.44 e	4.59 de	2.07 de	5.77 fg	12.43 ef
S+T2	5.53 e	3.04 cde	1.30 d	9.87 d	4.77 de	2.89 c	6.97 d	14.62 d
S+T3	4.70 f	2.72 ef	1.41 d	8.83 e	4.91 cde	2.31 cde	6.26 ef	13.47 de
S+T4	6.42 b	3.38 bc	1.32 d	11.11 c	4.72 de	2.15 de	9.61 c	16.47 c
S+T5	6.40 b	3.59 b	1.82 c	11.81 b	5.13 cd	3.96 b	10.38 b	19.46 b
S+T6	6.06 c	3.26 bcd	2.00 c	11.32 bc	5.63 bc	2.60 cd	6.52 de	14.74 d
S+T7	5.81 d	3.15 bcde	2.45 b	11.40 bc	6.11 b	3.70 b	9.41 c	19.21 b

S indicates salt concentration of 3.0 g kg^{-1} NaCl; S+T1 to S+T7 indicate the EBR spraying at transplanting, panicle initiation, heading, transplanting + panicle initiation, transplanting + heading, panicle initiation + heading, and transplanting + panicle initiation + heading stages, respectively, under salt stress. The values in the same column followed by different letters indicate statistical significance at the 0.05 probability level.

3.6. The Photosynthetic Potential, Crop Growth Rate, and Net Assimilation Rate of Rice

Salt stress was not conducive to the photosynthetic potential, crop growth rate, and net assimilation rate of rice, which were significantly decreased by 69.0%, 82.1%, and 40.4% (Table 6). Under salt stress, the photosynthetic potential was the highest under the S+T5 treatment, with an increase of 62.4% compared to that of the S treatment. The rice crop growth rate and net assimilation were enhanced the most under the S+T5 and S+T7 treatments, which increased by 119.7%–124.0% and 33.8%–46.9%, respectively.

Table 6. The photosynthetic potential, crop growth rate, and net assimilation rate of rice.

Treatment	Heading Stage—Full-Ripening Stage		
	Photosynthetic Potential (m^2 dm^{-2})	Crop Growth Rate (g m^{-2} d^{-1})	Net Assimilation Rate (g m^{-2} d^{-1})
CK	243.54 a	15.59 a	3.13 a
S	75.50 e	2.79 d	1.87 cde
S+T1	88.13 d	3.19 cd	1.81 cde
S+T2	106.44 c	3.81 cd	1.77 de
S+T3	84.95 d	3.72 cd	2.21 bcd
S+T4	112.82 c	4.30 c	1.96 cde
S+T5	122.64 b	6.13 b	2.50 abc
S+T6	107.92 c	2.74 d	1.28 e
S+T7	113.53 c	6.25 b	2.74 ab

S indicates salt concentration of 3.0 g kg^{-1} NaCl; S+T1 to S+T7 indicate the EBR spraying at transplanting, panicle initiation, heading, transplanting + panicle initiation, transplanting + heading, panicle initiation + heading, and transplanting + panicle initiation + heading stages, respectively, under salt stress. The values in the same column followed by different letters indicate statistical significance at the 0.05 probability level.

3.7. Rice Grain Yield, Yield Components, and Panicle Traits

As shown in Table 7, salinity stress significantly decreased rice yield by 86.2%, whereas the spraying of EBR enhanced rice yield by 6.7% to 94.4% under salt stress, and the S+T5 treatment displayed the best alleviating effect. Under salinity stress, the relatively higher panicle number (increased by 42.9%) and spikelet number per panicle (increased by 96.1%)

were the main reason resulting in higher grain yield under the S+T5 treatment. Salinity stress was not conducive to the formation of rice panicle traits (Table 8), with a significant decline in panicle length, number of primary and secondary branches, and the grain number of the primary and secondary branch by 39.4%, 51.0%, 82.4%, 58.5, and 85.0%, respectively. The rice panicle traits with EBR at different stages were significantly improved under salt stress, especially under the S+T5 treatment.

Table 7. Rice grain yield and its components.

Treatment	Panicle Number (pot^{-1})	Spikelets per Panicle	Filled Kernel Percentage (%)	1000-Grain Weight (g)	Yield (g pot^{-1})
CK	36.0 a	148.14 a	81.33 a	27.41 a	116.72 a
S	25.2 d	40.49 g	73.84 c	23.71 f	16.08 g
S+T1	24.0 d	63.56 de	67.84 d	23.48 g	17.16 g
S+T2	28.0 c	55.33 f	82.02 a	24.08 e	23.42 e
S+T3	28.0 c	53.81 f	78.58 b	22.76 i	21.52 f
S+T4	32.0 b	72.42 c	75.15 c	24.95 b	28.74 c
S+T5	36.0 a	79.41 b	73.47 c	24.58 c	31.26 b
S+T6	29.2 c	62.23 e	63.85 e	24.20 d	26.44 d
S+T7	32.0 c	66.04 d	78.68 b	23.36 h	27.33 d

S indicates salt concentration of 3.0 g kg^{-1} NaCl; S+T1 to S+T7 indicate the EBR spraying at transplanting, panicle initiation, heading, transplanting + panicle initiation, transplanting + heading, panicle initiation + heading, and transplanting + panicle initiation + heading stages, respectively, under salt stress. The values in the same column followed by different letters indicate statistical significance at the 0.05 probability level.

Table 8. Rice panicle traits.

Treatment	Panicle Length (cm)	Number of Primary Branches	Grain Number of Primary Branch	Number of Secondary Branches	Grain Number of Secondary Branch
CK	16.82 a	11.59 a	73.00 a	25.00 a	75.15 a
S	10.19 e	5.68 e	30.29 f	4.40 e	11.26 e
S+T1	11.60 c	7.00 cd	41.66 cd	8.00 cd	21.89 cd
S+T2	11.10 d	6.95 cd	37.52 de	6.86 d	17.81 d
S+T3	11.09 d	6.81 cd	35.14 ef	7.57 d	18.66 d
S+T4	12.33 b	7.42 bc	43.21 bc	10.79 b	29.59 b
S+T5	12.28 b	8.33 b	47.93 b	11.40 b	31.85 b
S+T6	10.86 d	6.33 de	35.25 ef	7.69 cd	20.86 cd
S+T7	12.06 bc	7.50 bc	41.13 cd	9.00 c	24.92 c

S indicates salt concentration of 3.0 g kg^{-1} NaCl; S+T1 to S+T7 indicate the EBR spraying at transplanting, panicle initiation, heading, transplanting + panicle initiation, transplanting + heading, panicle initiation + heading, and transplanting + panicle initiation + heading stages, respectively, under salt stress. The values in the same column followed by different letters indicate statistical significance at the 0.05 probability level.

3.8. Leaf Antioxidant Enzyme Activities, MDA, and Proline Content

As shown in Figure 2, the activity of POD and CAT and the content of MDA and proline in rice leaves were significantly enhanced by 10.0%, 108.9%, 93.4%, and 17.0%, respectively, under salt stress. The activity of POD and CAT with EBR at different stages was significantly improved, and the POD activity was the highest under the S+T3 treatment, while the CAT activity was the highest under the S+T5 treatment. Under salt stress, the MDA content with EBR was notably declined, and the proline content with EBR was overall enhanced, especially under the S+T3 treatment. Compared to those under the S treatment, the CAT activity was significantly enhanced by 25.0%, while the MDA content dramatically decreased by 37.3% under the S+T5 treatment.

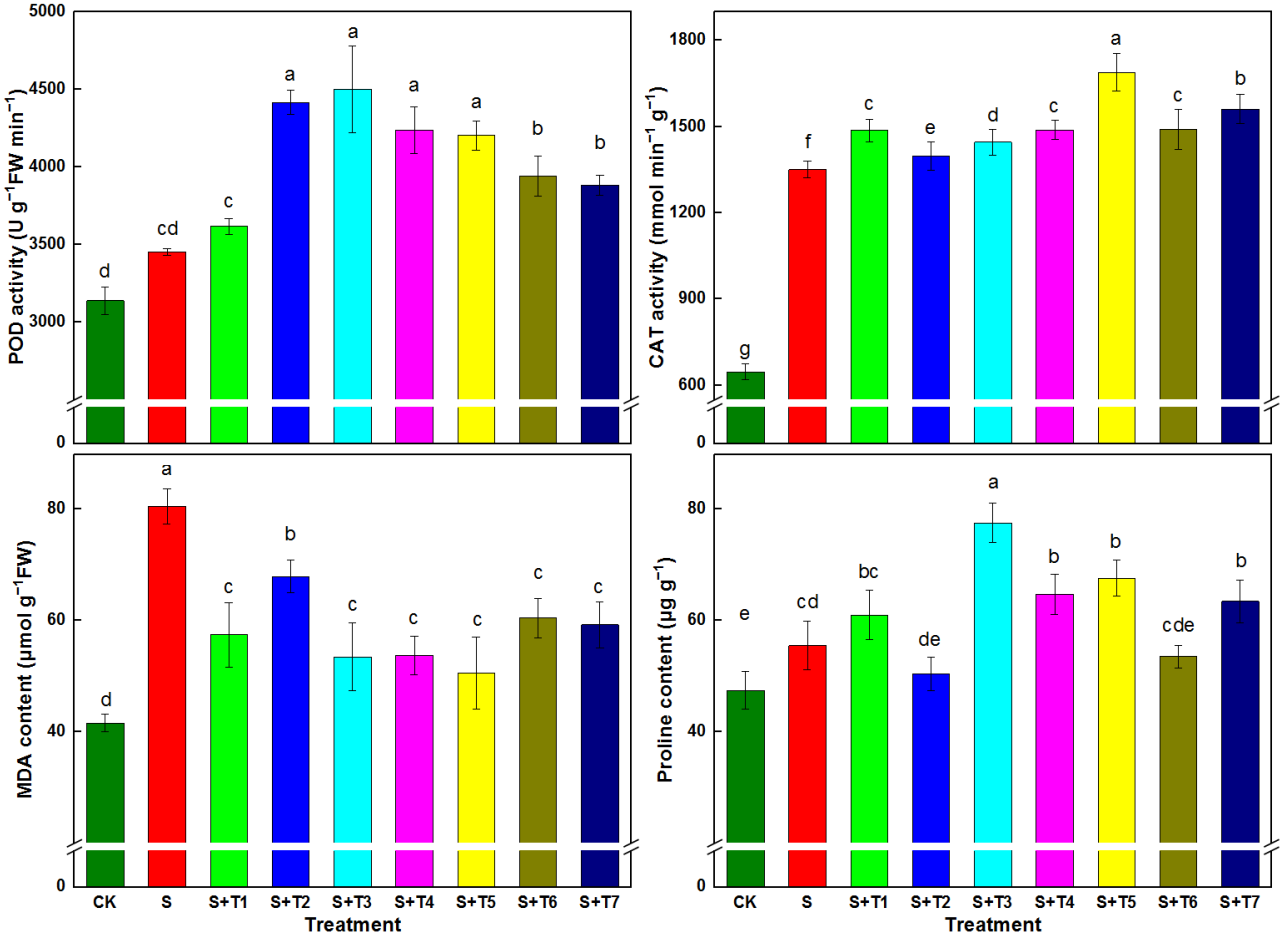

Figure 2. Effects of EBR spraying on POD and CAT activity and MDA and proline content in rice leaves under salt stress. S indicates salt concentration of 3.0 g kg^{-1} NaCl; S+T1 to S+T7 indicate the EBR spraying at transplanting, panicle initiation, heading, transplanting + panicle initiation, transplanting + heading, panicle initiation + heading, and transplanting + panicle initiation + heading stages, respectively, under salt stress.. The data (n = 3) are the mean values ± SD, calculated from three independent experiments. The different letters indicate significant differences at $p < 0.05$.

3.9. Na$^+$ and K$^+$ Content in Rice Organs

The content of Na^+ and K^+ in rice organs at the full-ripening stage were displayed in Table 9. Salinity resulted in relatively higher Na^+ content and lower K^+ content and K^+/Na^+ in rice root, stem, and rice leaves, whereas the spraying of EBR significantly improved K^+/Na^+ homeostasis in rice organs under salt stress. The K^+ content in spikes was significantly enhanced under the S+T1 and S+T2 treatments and displayed no significant change under other EBR treatments. The Na+ content in spikes was reduced notably under the S+T4, S+T6, and S+T7 treatments. Overall, the K^+/Na^+ in spikes was enhanced under the EBR treatments. The S+T5 treatment significantly enhanced the K^+ content in rice root and leaf and decreased the Na^+ content in rice root (by 30.4%), thereby leading to higher K^+/Na^+, compared to those under the S treatment.

Table 9. Na^+ and K^+ content in rice organs at full-ripening stage.

Treatment	Root			Stem			Leaf			Spike		
	K^+ (mg g^{-1})	Na^+ (mg g^{-1})	K^+/Na^+	K^+ (mg g^{-1})	Na^+ (mg g^{-1})	K^+/Na^+	K^+ (mg g^{-1})	Na^+ (mg g^{-1})	K^+/Na^+	K^+ (mg g^{-1})	Na^+ (mg g^{-1})	K^+/Na^+
CK	9.59 a	30.65 e	0.31 a	53.69 a	25.35 e	2.12 a	36.63 e	13.88 g	2.64 a	6.16 d	8.29 d	0.74 a
S	1.73 ef	50.15 bcd	0.03 ef	30.01 cd	90.29 c	0.33 cd	17.97 g	53.19 d	0.34 c	9.11 c	18.84 ab	0.48 c
S+T1	0.70 f	44.33 d	0.02 f	26.82 de	114.35 a	0.24 f	40.87 d	91.79 a	0.45 bc	15.45 a	23.29 a	0.66 ab
S+T2	4.88 bc	48.77 cd	0.10 c	27.40 de	116.65 a	0.24 f	49.55 b	88.34 ab	0.56 bc	11.85 b	22.93 a	0.52 bc
S+T3	2.48 de	48.01 cd	0.05 de	32.13 bc	107.58 b	0.30 de	57.89 a	83.84 bc	0.69 b	10.36 bc	21.92 a	0.47 c
S+T4	2.53 de	60.69 a	0.04 ef	33.97 b	73.69 d	0.46 b	21.53 f	43.03 f	0.50 bc	8.37 c	13.59 c	0.62 abc
S+T5	5.03 b	34.90 e	0.14 b	30.11 cd	103.94 b	0.29 e	44.92 c	79.58 c	0.57 bc	10.22 bc	21.82 a	0.47 c
S+T6	3.68 cd	53.86 bc	0.07 d	25.88 e	89.12 c	0.29 e	12.53 h	44.23 ef	0.28 c	9.03 c	15.77 bc	0.57 bc
S+T7	5.9 b	55.18 ab	0.11 c	29.57 cd	86.96 c	0.34 c	23.28 f	50.33 de	0.46 bc	8.74 c	14.34 c	0.61 abc

S indicates salt concentration of 3.0 g kg^{-1} NaCl; S+T1 to S+T7 indicate the EBR spraying at transplanting, panicle initiation, heading, transplanting + panicle initiation, transplanting + heading, panicle initiation + heading, and transplanting + panicle initiation + heading stages, respectively, under salt stress. The values in the same column followed by different letters indicate statistical significance at the 0.05 probability level.

4. Discussion

With the development of scientific research, more and more attention has been paid to chemical regulation technology. The use of exogenous plant growth regulators to improve crop salt tolerance has gradually become a research hotspot. As a highly active sterol lactone compound, 24-epibrassinolide (EBR) could improve the resistance of plants to adversities such as: high/low temperature, drought, salt, disease, etc. [18].

As the first stage for plant growth, seed germination would be notably affected by salinity stress, leading to inhibited plant growth and development. Previous studies addressed that EBR improved the seed germination in maize, oat, tomato, cucumber, alfalfa, etc., under salt stress [11]. However, the optimal concentration of EBR to regulate seed germination of different plants under salt stress is varied. Larré and his partners studied the effect of EBR soaking on rice seed germination using salt-sensitive and salt-tolerant rice as materials under 100 mM NaCl [28]. The results showed that different concentrations of EBR (0.01, 0.1, and 1.0 mM) alleviated the germination rate and vigor index of salt-sensitive rice. EBR soaking promoted the germination potential and vigor index of rice seeds, and the effect was not significant at 0.1 mM, while the high concentration of 1.0 mM significantly reduced the germination rate and vigor index. Similarly, in the current study, different concentrations of EBR soaking can significantly improve the germination potential, relative germination potential, germination rate, relative germination rate, and germination index of seeds under the conditions of 100 and 150 mM salt stress, and 0.5 mg L^{-1} EBR displayed the best. It has been reported that a 10^{-7} M EBR treatment significantly enhanced the values of rice growth parameters such as shoot length, root length, root number, and fresh and dry weight under 100 mM NaCl stress [21]. Here, 0.5 mg L^{-1} EBR spraying displayed the most distinct alleviating effect on seedling height, root length, above-/under-ground fresh weight and above-/under-ground dry weight under salt stress.

Salinity inhibits rice growth, delays the rice growth process, and accelerates plant senescence. Previous studies have shown that under salt stress, rice plant height, plant biomass, and the leaf area index decreased, leading to lower grain yield [29,30]. In the current study, the spraying of EBR at different periods enhanced rice yield by 6.7% to 94.4% under salt stress (Table 7). Among all the EBR spraying periods, the transplanting + heading combination displayed the best alleviating effect. The higher panicle number and spikelet number per panicle were the two main reasons resulting in higher grain yield after spraying EBR. Similarly, Shahid [31] reported that exogenous spraying of EBR increased the number of pea seeds, the 1000-grain weight, and the yield of peas under salt stress. Besides, spraying EBR at different periods relieved the inhibition of salt stress on the growth of rice leaves, slowed the senescence of leaves from heading to maturity, increased the leaf area index of rice, and then, increased the photosynthetic potential [32]. The dry matter accumulation from heading to maturity of rice under the EBR+S treatment was relatively high, which ensured sufficient grain filling and helped to increase the yield. Under salt stress, the crop growth rate of the S+T5 treatment from heading to maturity was the highest,

which was beneficial to the accumulation of dry matter and the formation of photosynthetic products in rice, as well as promoted the population growth rate, thereby increasing the yield (Tables 5 and 6).

Under salt stress, Na^+ is competitively absorbed by root and accumulated in rice plants, which provokes ion toxicity and the imbalance in Na^+/K^+ homeostasis. The ion toxicity causes intracellular osmotic stress and the production of reactive oxygen species (ROSs) such as superoxide radicals, hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^·$), and so on [5,33]. These oxygen radicals affect proteins and lipids, trigger the occurrence of oxidative stress, and cause great damages to the cell plasma membrane, leading to cell injury and death [1]. However, plants have evolved an antioxidant enzyme system to reduce ROS content, protect the cell membrane structure, reduce cell membrane structure damage, and improve salt tolerance [34]. Peroxides (PODs), catalase (CAT), and superoxide dismutase (SOD) are among the enzymatic antioxidant systems that regulate the production and scavenging of ROSs within organisms. During stresses, the antioxidant genes can be activated by signals to up-regulate the activity of PODs, CAT, and SOD for self-protection in plants [33]. Consistent with previous research, our results showed that 0.3% salt stress significantly enhanced the activity of CAT and PODs by 10.0% and 108.9%, respectively, in rice leaves (Figure 2). EBR has been reported to reduce ROS production and limit oxidative damage under salt stress previously [18,35]. In the current study, exogenous spraying of EBR at different periods under salt stress resulted in higher activities of CAT and PODs in rice leaves compared with those under the S treatment, which was conducive for the further scavenging of reactive oxygen species and improving the salt tolerance in rice. The generation of ROSs increases in plant parts under salt stress, which ultimately causes oxidative stress via lipid peroxidation, electrolyte leakage, etc. In general, MDA content directly reflects the degree of lipid peroxidation of the cell membrane. In our results, the MDA content was dramatically enhanced by 93.4% under the S treatment compared to that of CK, indicating the high degree of lipid peroxidation of the cell membrane of rice under salt stress. However, under EBR spraying compared to that of the S treatment, the MDA content obviously decreased after spraying EBR, especially under the S+T5 treatment, which indicated that the EBR treatment effectively alleviated the injury of salinity on the cell membrane in rice. This was also consistent with the findings of López-Gómez et al. [36], who reported that foliar treatment with EBR decreased MDA content and displayed a protective effect against salt stress in *Medicago truncatula*. Abiotic stresses usually lead to the accumulation of an array of metabolites, particularly amino acids. Proline is one such amino acid, which, besides its role in protein synthesis, has long been known to act as a compatible osmolyte to counteract salinity and drought [37]. Proline accumulation under salinity conditions has been correlated with salt tolerance, and its concentration has been shown to be generally higher in salt-tolerant than in salt-sensitive species [38]. In the current study, the proline content in rice leaves was enhanced by 17.0% under the S treatment compared to that of CK, whereas it was further enhanced after spraying with EBR, especially under the S+T3 and S+T5 treatments. This further indicated that EBR could protect the cell membrane through regulating the osmolytes under salt stress [39,40].

Imbalanced Na^+/K^+ homeostasis acts as a major reason leading to growth inhibition and yield decrease in salt-stressed plants. Excess Na^+ in the cytoplasm interferes with K^+ uptake and K^+ function, while K^+ retention in leaf mesophyll cells is reported to be essential to salinity tolerance and premature leaf senescence regulation [41–43]. EBR spraying notably enhanced K^+/Na^+ in rice organs (Table 9). Under the S+T5 treatment, root Na^+ was significantly decreased and K^+ was enhanced, leading to improved K^+/Na^+ in rice root, which was beneficial for the nutrition absorption of rice. Besides, the K^+ content in rice leaves was dramatically enhanced, which would be conducive for the maintenance of leaf function and normal photosynthesis. Overall, EBR spraying ensured higher rice yield under salt stress through the enhanced antioxidant enzyme and K^+/Na^+ regulation. In our study, the concentration of 0.5 mg L^{-1} and spraying EBR twice (transplanting + heading) would be recommended to achieve higher grain yield in saline conditions. The

study further clarified the application method and internal mechanism of EBR in alleviating salt stress in rice, which could provide a theoretical basis for high-yield cultivation of rice in saline soil.

5. Conclusions

Although there have been reports on EBR alleviating salt stress in rice, most of these studies have focused on the effect of EBR on rice seed germination and mainly focused on seedling stages, whereas the effect of EBR on grain yield formation from the whole growth stage remains less concerned. The current study focused on the determination of the optimal application concentration and application periods of EBR in rice under salt stress. By a seed germination experiment, a seedling experiment, and a pot experiment, it was concluded that 0.5 mg L^{-1} was the optimal concentration of EBR to alleviate the adverse effect of salinity, where seed germination indicators and seedling morphological traits were shown to be the best. Under the present conditions, spraying EBR twice (transplanting + heading) resulted in the highest grain yield under salt stress, which was realized through enhanced antioxidant enzymes, higher K^+ maintenance in leaves, and lowered absorption of Na^+ in rice root. However, even though EBR spraying enhanced the grain yield under salt stress, the absolute grain yield was still very low. Thus, more efforts are still encouraged for high crop production in saline soil in the future.

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