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RESEARCH ARTICLE

Role of exogenous 24-epibrassinolide in enhancing the salt tolerance of wheat seedlings

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Abstract

To understand the functions of exogenous 24-Epibrassinolide (EBR) in enhancing the salt tolerance of wheat (*T. aestivum* L.) seedlings to salt stress, a hydroponic experiment was performed to investigate the effects of EBR on chlorophylls, root activity, H^+-ATPase, malondialdehyde (MDA), electrolyte leakage, free proline, soluble protein, reactive oxygen species (ROS), antioxidant enzymes and minerals content in wheat plants subjected to non-stress conditions or salt stress (120 mM NaCl) with foliar application of EBR (1, 10 and 100 nM). Results showed that spray of low concentrations EBR (1 and 10 nM) under non-stress conditions could promote wheat plant growth. 120 mM NaCl induced osmotic stress, oxidative stress and imbalance in mineral nutrients absorption. However, EBR enhanced the ability of resistance to osmotic stress by increasing free proline and soluble protein content, and enhanced the ability of resistance to oxidative stress by increasing antioxidant enzymes activities. As a result of increase of chlorophyll content, root activity and H^+-ATPase activity, the inhibition of K, Ca, Mg, Fe and Zn uptake was ameliorated and consequently, decline in plant growth induced by NaCl stress was alleviated. Based on these results, we conclude that EBR had a positive role in regulating wheat growth and development under salt stress, and spray of 10 nM EBR had the most significant alleviating effect against NaCl toxicity.

Keywords: Salt stress, wheat, EBR, antioxidative systems, ion accumulation

1. Introduction

Salinity stress is one of the most serious abiotic stress factors limiting crop productivity, and it is a menace for the cultivation of plants across the globe and causes drastic changes in plant growth and development (Roy *et al.,* 2014; Hussain *et al.,* 2016). NaCl-specific damage is associated with the accumulation of Na^+ in leaf tissues and results in necrosis of older leaves, starting at the tips and margins and working back through the leaf. Growth and yield reductions occur as a result of the shortening of the lifetime of individual

leaves, thus reducing net productivity and crop yield. Salt stress causes a number of changes in plant metabolism. Of them, ion toxicity, osmotic stress and production of ROS are most prominent (Tian *et al.,* 2015). Salinity also induces water deficit even in well watered soils by decreasing the osmotic potential of soil solutes, thus making it difficult for roots to extract water from their surrounding media. ROS are highly reactive and in the absence of any protective mechanism they can seriously cripple normal metabolism through oxidative damage to lipids, proteins and nucleic acids (Athar *et al.,* 2008), which results in premature leaf senescence and loss of photosynthetic efficiency leading to reduced carbon assimilation and ultimately crop yield. To alleviate these oxidative effects, plants generate different kinds of antioxidants such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) (Tian *et al.,* 2015). Salinity causes the accumulation of low molecular weight compounds called compatible solutes such as proline and glycine betaine (Chen *et al.,* 2007). High salinity adversely affects growth, physiology and productivity by increasing ion toxicity, and decreasing protein synthesis and lipid metabolism (Geissler *et al.,* 2009). In addition, further investigations regarding the approaches to alleviating salinity toxicity are needed.

Brassinosteroids (BRs) play a prominent role in various physiological processes in plants, like cell division and expansion, xylem differentiation, stem elongation and root growth (Choudhary *et al.,* 2012, Zheng *et al.,* 2016). Moreover, BRs are also reported to have an ameliorative effect on plants subjected to environmental stress such as drought stress (Hua *et al.,* 2013), cold stress (Hu *et al.,* 2010), heat stress (Zhang *et al.,* 2013) and heavy metals (Hayat *et al.,* 2010, Fariduddin *et al.,* 2015). Karlidag *et al.* (2011) demonstrated that BRs can mitigate the adverse effects of salt stress on stomatal conductance, membrane permeability, and leaf water content, ionic composition in salt stressed strawberry, and Derevyanchuk *et al.* (2015) also indicated that BRs can stimulate total respiration rate in *Arabidopsis thaliana* leaf, particularly alternative respiratory pathway, under salt stress conditions. BR-enhanced stress tolerance is associated with the regulation of ROS metabolism and the increase in the antioxidant enzyme activity, as well as with elevated content of ascorbic acid, glutathione, carotenoids, abscisic acid, etc. (Mazorra Morales *et al.,* 2014). Fariduddin *et al.* (2015) suggested that BRs affect the synthesis of proteins or enzymes by involving the specific gene expression that improves the overall metabolic activities of plants. Moreover, Ali *et al.* (2008) demonstrated that BRs also modified the plasma membrane, increased the nutrient uptake and assimilation and facilitated the translocation of photosynthates to the sink, besides improving metabolic activities, under stress conditions. All of these results indicated the importance of BRs in protection against stress-induced deleterious effects. Although some studies demonstrated that BRs could alleviate salinity toxicity (Sharma *et al.,* 2013; Derevyanchuk *et al.,* 2015), the mechanisms of EBR action on salt tolerance in plants are still far from being completely understood.

Wheat is an important cereal crop, and it is a moderately salt-tolerant crop (Tian *et al.,* 2015). The growth and grain yield of wheat are significantly affected by soil salinity. Recently, wheat salt-responsive proteins or the molecular mechanisms of salt tolerance in wheat (Li *et al.,* 2013) were evidenced. Many studies demonstrated that BRs could enhance salt tolerance in multiple plants, such as rice (Sharma *et al.,* 2013), strawberry (Karlidag *et al.,* 2011) and *Arabidopsis thaliana* (Derevyanchuk *et al.,* 2015), but limited information is available on the role of brassinosteroids (BRs) in response of wheat plants to salt stress. Therefore, this experiment focuses on the effect of the exogenous application of 24-EBR as a foliar spray on the plant growth, some physiological parameters and chemical content of wheat plants under saline conditions, and determines the mechanism of the application of exogenous 24-EBR could alleviate salinity toxicity and increase salt-tolerance of wheat plants.

2. Materials and Methods

2.1. Plant material and culture conditions

Seeds of common wheat (*T. aestivum* L. cv. Shannong 22) were surface sterilized with 10 % (v/v) sodium hypochlorite solution for 10 min, then vigorously rinsed with distilled water. Sterilized seeds were sown in plug tray and arranged in FPG-300C-30D illumination incubator (25/20 ºC; day/night, light intensity 150 μmol m^{-2} s^{-1}, 14 h photoperiod, 60 % relative humidity). Two weeks after sowing, two fully expanded leaves were maintained, plants at the same growth stage were selected and transplanted to glass containers (6.0 × 3.5 × 10.0 cm^3) filled with Hoagland solution. Plants were sprayed with water or with EBR. The experimental design is given as follows: (1) 0 mM NaCl + 0 nM EBR (CK); (2) 120 mM NaCl + 0 nM EBR (NaCl); (3) 0 mM NaCl + 100 nM EBR (EBR1); (4) 0 mM NaCl + 10 nM EBR (EBR2); (5) 0 mM NaCl + 1 nM EBR (EBR3); (6) 120 mM NaCl + 100 nM EBR (NaCl+EBR1); (7) 120 mM NaCl + 10 nM EBR (NaCl+EBR2); (8) 120 mM NaCl + 1 nM EBR (NaCl+EBR3). The nutrient solution was adjusted to pH 6.5–6.8. The treatment solution was changed daily to maintain constant NaCl concentration. The plants were sampled at 15 d after treatment.

2.2. Determination of plant growth and relative water content

After harvest, shoots and roots of wheat seedlings were separated and carefully rinsed with distilled water and blot dried with tissue papers before fresh weight (FW) was recorded. Plant samples were oven-dried at 80 °C to a constant mass before the dry weight (DW) was recorded and relative water content (RWC) was calculated as follow:

RWC (%) = (FW - DW)/FW × 100.

2.3. Determination of chlorophyll content

The chlorophyll content was determined according to the method of Song *et al.* (2016). Fresh wheat leaf (0.5 g) was extracted in 2 mL 95 % ethanol for 24 h in the dark, and the extracted solution was analyzed. The amounts of chlorophyll a, b and carotenoid were determined using a spectrophotometer (SHIMADZU UV-2450, Kyoto, Japan), by reading the absorbance at 665, 649 and 470 nm respectively. The chlorophyll content results are expressed as unit's mg per gram-fresh weight (mg g^{-1} FW).

2.4. Determination of root activity

The root activity was expressed in the amount of triphenyl formazan (TTF) deoxidized by triphenyltetrazolium chloride (TTC), as described by Duncan and Widholm (2004) with some modifications. Briefly, roots were washed thoroughly with distilled water and finally with de-ionized water and cut into small pieces. Root samples (0.5 g each) were placed into test tubes, 5 ml 0.4 % TTC and 5 ml 1/15 mM phosphatic buffer solution (pH 7.0) were added to each tube and reacted for 2 h at 37 °C. Then 2 ml of 1 M H_2SO_4 was added to stop the reaction. The TTF was extracted by 95 % ethanol for 24 h till the root fade the red color.

2.5. Determination of H^+-ATPase in PMs

A membrane fraction enriched in plasma membrane vesicles was prepared as described by Song *et al.* (2016) with minor modifications. Excised roots were homogenized (1/2, w/v) with a mortar and pestle in ice containing: 25 mM HEPES-Tris (pH 7.2), 250 mM mannitol, 5 mM EDTA, 1 mM DTT and 1.5 % (w/v) PVP. The whole isolation procedure was carried out at 4 °C. The homogenate was filtered through four-layer cheesecloth and centrifuged at 560 × *g* for 12 min, then the supernatant was centrifuged at 10,000 × *g* for 15

min, and the obtained supernatant was then centrifuged at 60,000 × *g* for 30 min to yield a crude membrane fraction. The resulted pellet was re-suspended with 1 ml in a gradient buffer containing: 20 mM HEPES-Tris (pH 7.5), 5 mM EDTA, and 0.5 mM EGTA. The supernatant was layered on top of a step gradient consisting of 1 ml of 45, 33 and 15 % (w/w) sucrose, respectively, and then centrifuged for 2 h at 70,000 × *g*.

ATP hydrolysis assays were performed as described by Wang *et al.* (2016). 0.5 ml of the reaction medium containing: 36 mM Tris-Mes (pH 6.5), 30 mM ATP-Na_2, 3 mM $MgSO_4$, 1 mM NaN_3, 50 mM KNO_3, 1 mM Na_2-MoO_4, 0.02 % (v/v) Triton X-100, in the presence or absence of 2.5 mM Na_3VO_4. The reaction was started by adding 50 ml PM vesicles. After 30 min incubation at 37 °C, the reaction was quenched by the addition of 55 % (w/v) TCA. The H^+-ATPase activity was determined by measuring the release of Pi (Wang *et al.,* 2016).

2.6. Determination of lipid peroxidation and membrane Permeability

Lipid peroxidation was evaluated by measuring malondialdehyde content (MDA), as described by Heath and Dong *et al.* (2015). About 100 mg of the frozen plant materials (roots and leaves) was ground in 1.5 ml of 0.1 % trichloroacetic acid (TCA) and centrifuged. An aliquot of 0.5 ml of the supernatant was reacted with 0.5 ml 20 % TCA containing 0.5 % thiobarbituric acid (TBA) at 90 °C for 20 min, then cooled in an ice bath. The resulting mixture was centrifuged at 12,000 rpm for 10 min and the absorbance of the supernatant was measured at 532 nm. Measurements were corrected for unspecific turbidity by subtracting the absorbance at 600 nm. The amount of MDA was expressed as nmol g^{-1} FW.

Wheat leaves were harvested and cut into 3–4 mm pieces. The leaves were washed in deionized water to remove surface-adhered electrolytes and then were placed in Petri dishes with 15 ml of deionized water at 25 °Cfor 3 h. Electrical conductivity in the bath solution was determined (EC_1). Then, the samples were heated at 100 °C for 2 h and conductivity of the bath solution was read again (EC_2). Relative ion leakage (%) = $EC_1/EC_2 \times 100$.

2.7. Determination of proline and protein content

Proline concentration was determined using the method of Tian *et al.* (2015). After extraction at room temperature with 3% 5-sulfosalicylic acid solution, the proline content was determined from a standard curve and calculated on fresh weight basis. Soluble protein content was determined according to Tian *et al.* (2015) using the Coomassie brilliant blue G-250 reagent with bovine serum albumin (BSA) as standard.

2.8. Determination of $O_2^{\cdot-}$ generation rate and H_2O_2 concentration

Superoxide anion radical generation rate was measured by the sulfanilamide method (Tian *et al.,* 2015). Samples were homogenized in 0.05 M phosphate buffer (pH 7.8) by grinding with a mortar and pestle under chilled condition. The homogenate was filtered through four-layers muslin cloth and centrifuged at 12,000 × *g* for 10 min at 4 °C. The absorbance was measured at 530 nm, and $O_2^{\cdot-}$ generation rate was calculated from a standard curve of $NaNO_2$ reagent.

For determination of H_2O_2 content, 1 g fresh leaves, roots were homogenized in 2 cm^3 ice-cold acetone centrifuged at 4 000*g* for 15 min. Titanium reagent (2 % $TiCl_2$ in conc. HCl) was added to a known volume of extract supernatant to give a Ti (IV) concentration of 2 %. The Ti-H_2O_2 complex, together with unreacted Ti, was then precipitated by adding

0.2 cm^3 17 M ammonia solution for each 1 cm^3 of extract, then centrifuged at 4 000*g* for 15 min, after centrifugation the supernatant was discarded. The precipitate was washed five times with ice acetone by resuspension, drained in 1 M H_2SO_4 (3 cm^3).The absorbance of the solution was measured at 410 nm against blanks, which had been similarly prepared but without plant tissue (Simaei *et al.,* 2011).

2.9. Enzyme extraction and enzyme assays

For the extraction of antioxidant enzymes, fresh samples were homogenized with 50 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 7.8) containing 0.2 mM EDTA and 2% insoluble polyvinylpyrrolidone in a chilled mortar and pestle. The homogenate was centrifuged at 12,000 × *g* for 20 min and the resulted supernatant was used for determination of enzyme activities. The whole extraction procedure was carried out at 4° C. All spectrophotometric analysis was conducted on a spectrophotometer. Superoxide dismutase (SOD) activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium following the method of Tian *et al.* (2015). Peroxidase (POD) activity was measured by the increase in absorbance at 470 nm due to guaiacol oxidation (Dong *et al.,* 2016). Catalase (CAT) activity was measured as the decline in absorbance at 240 nm due to the decrease of extinction of H_2O_2 according to the method of Tian *et al.* (2015).

2.10. Determination of ion concentration

For estimation of plant dry matter and concentrations of mineral nutrients in plant, the plant samples were oven-dried for 30 min at 105 ° C, then at 80 ° C till the materials reach their constant weights. Approximately 0.1 g dried material of the leaves and roots were completely digested with 5 ml 98 % H_2SO_4 at 200 ° C, supplemented with a few drops of H_2O_2 (30 %, v/v). After digestion, each sample was brought up to a 25 ml final volume with distilled-deionized water. Na, K, Ca, Mg, Zn and Fe contents were measured by atomic absorbance spectrometry (Persee TAS-990, Beijing, China).

2.11. Statistical analysis

The analysis of variance (ANOVA) was performed using SAS software (SAS Institute, Cary NC). Differences between treatments were separated by the least significant difference (LSD) test at a 0.05 probability level.

3. Results

3.1. Plant growth and and RWC

Compared to the control (CK), spray of 10 nM and 1 nM EBR under non-stress conditions significantly increased the fresh and dry weight of wheat shoots and roots, but the 100 nM EBR had no significant effect. 120 mM NaCl stress significantly decreased plant growth ($P \leq 0.05$), with a reduction of 30.90 and 50.63 % of shoots and roots FW, respectively (Table 1). The RWC of shoots and roots treated with 120 mM NaCl was obviously lower than CK. Spray of EBR reversed the decline, with The NaCl+EBR2 (10 nM EBR) treatment had the best effect of alleviation. Taken together, the alleviating effect of EBR under salt stress was found in a general trend of 10 nM EBR > 1 nM EBR > 100 nM EBR.

Table 1. Effects of exogenous 24-Epibrassinolide on the fresh and dry weight [g (10 plants)$^{-1}$] of wheat shoots and roots under salt stress. Means SD, $n = 3$. Different letters after means within the same column indicate significant differences at $P \leq 0.05$.

Treatments	Shoots			Roots		
	Fresh weight	Dry weight	RWC (%)	Fresh weight	Dry weight	RWC (%)
CK	4.01±0.11cd	0.51±0.02cd	87.27±0.70a	0.87±0.04bc	0.09±0.01ab	89.65±0.22ab
NaCl	2.77±0.18f	0.40±0.02g	85.40±0.47b	0.43±0.03e	0.05±0.00d	87.31±0.51c
EBR1	4.30±0.27bc	0.54±0.04c	87.41±0.48a	0.89±0.09bc	0.09±0.01ab	89.82±0.69ab
EBR2	5.56±0.44a	0.66±0.02a	88.15±1.03a	1.06±0.09a	0.10±0.01a	90.72±1.31 a
EBR3	4.55±0.46b	0.60±0.01b	86.67±1.25ab	0.95±0.13ab	0.10±0.01a	89.85±1.71ab
NaCl+EBR1	2.93±0.01f	0.43±0.01fg	85.47±0.14b	0.46±0.04e	0.06±0.00d	87.34±1.40c
NaCl+EBR2	3.58±0.33de	0.47±0.03de	86.65±2.11ab	0.77±0.02c	0.08±0.01bc	89.45±0.43ab
NaCl+EBR3	3.45±0.20e	0.46±0.04ef	86.62±0.73ab	0.62±0.04d	0.07±0.01c	88.24±0.81bc

3.2. Chlorophyll content

Table 2 showed the spray of EBR under non-stress conditions increased the total chlorophyll, chl *a*, chl *b* and carotenoids concentrations in leaves of wheat seedlings compared with CK, especially 10 nM EBR. The total chlorophyll, chl *a*, chl *b* and carotenoids contents showed a significant decrease on NaCl-alone treatment (20.12, 20.63, 20.51 and 25.93 % respect to CK). However, when treated with EBR (100, 10 or 1 nM) in the presence of salt stress, this inhibition was alleviated. The NaCl + EBR1, NaCl+EBR2 and NaCl + EBR3 treatments increased total chlorophyll by 10.69, 21.37 and 17.56 % than NaCl treatment. And the best of alleviating effect on increasing total chlorophyll content was NaCl+EBR2 treatment. Similar findings were found for the chl *a*, chl *b* and carotenoids contents.

Table 2. Effects of exogenous 24-Epibrassinolide on the chlorophyll contents [mg g^{-1} (f.m.)] in leaves of wheat seedlings under salt stress. Means SD, $n = 3$. Different letters after means within the same column indicate significant differences at $P \leq 0.05$.

Treatments	Total Chl contents	Chl *a*	Chl *b*	Carotenoids *x.c*
CK	1.64±0.02c	1.26±0.01c	0.39±0.01bc	0.27±0.01bc
NaCl	1.31±0.05f	1.00±0.03f	0.31±0.01e	0.20±0.03d
EBR1	1.65±0.03c	1.27±0.03c	0.38±0.01bc	0.28±0.01b
EBR2	1.89±0.08a	1.45±0.07a	0.43±0.01a	0.31±0.02a
EBR3	1.75±0.05b	1.34±0.01b	0.41±0.03ab	0.28±0.02b
NaCl+EBR1	1.45±0.07e	1.12±0.05e	0.33±0.02de	0.25±0.01c
NaCl+EBR2	1.59±0.01cd	1.23±0.01cd	0.37±0.00c	0.27±0.01bc
NaCl+EBR3	1.54±0.02d	1.18±0.02de	0.36±0.02cd	0.26±0.01bc

3.3. Root activity and H^+-ATPase activity

Spray of EBR under non-stress conditions increased root activity and H^+-ATPase activity of wheat seedlings compared with CK, especially 10 nM EBR (Figure 1). Treatment with NaCl decreased root activity by 52.10 % (Figure 1A) and H^+-ATPase activity by 42.99 % (Figure 1B) than CK. However, this inhibition was alleviated with spray of EBR, and NaCl+EBR2 treatment had the best alleviating effect. The foliar application of 10 nM EBR increased root activity by 89.88 % and H^+-ATPase activity by 65.49 % than NaCl-alone treatment.

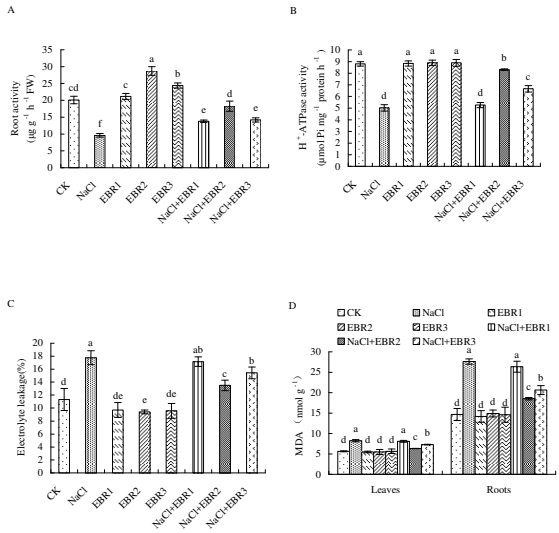

Figure 1. Effects of exogenous 24-Epibrassinolide on root activity (A) , H^+-ATPase activity (B) , Electrolyte leakage (C) and lipid peroxidation (D) of wheat seedlings under salt stress.Values represent mean ± SD (n = 3). Different small letters within the same row indicate significant difference at $P \leq 0.05$.

3.4. Electrolyte leakage and lipid peroxidation

Figure 1 showed that electrolyte leakage and MDA content in leaves or roots of wheat seedlings on EBR1, EBR2 and EBR3 treatments had no significant changes compared with CK. The salinity caused electrolyte leakage and MDA accumulation in plants. Electrolyte leakage in leaves of wheat plants seedlings increased dramatically after 15 days of NaCl treatment relative to CK, while the increase was lower in plants treated with 1 and 10nM EBR (Figure 1C). The electrolyte leakages on NaCl+EBR2 and NaCl+EBR3 treatments were lower by 24.07 % and by 13.15 % than in NaCl-treated plants. However, the 100 nM EBR had no significant effect.

Malondialdehyde content was measured as an index of lipid peroxidation. NaCl-alone treatment induced a significant increase in MDA content of wheat seedlings, which was increased by 45.97 % in leaves and by 88.37 % in roots compared with CK (Figure 1D). However, the application of exogenous EBR reduced MDA accumulation; and the 10 nM EBR markedly reduced NaCl-induced MDA accumulation; MDA content decreased by 23.24 % in leaves and by 32.92 % in roots compared with NaCl treatment.

3.5. Osmotic regulator accumulation

The level of proline exhibited an increase in response to salinity stress, both in the leaves and roots of wheat plants, compared to CK (Figure 2A). The NaCl treatment increased proline content by 58.12 % in leaves and by 90.75 % in roots as compared to those of control. The quantity of proline was higher in leaves than the roots. The spray of EBR on unstressed plant brought about a significant decrease in the level of proline in leaves, but could not bring about a significant change in roots. However, the application of EBR under salinity stress elevated the quantity of proline, both in leaves and roots. The maximum quantity of proline was found in the plants which were subjected to both NaCl stress and subsequently sprayed with 10 nM EBR, and the 100 nM EBR could not bring about a significant change in the level of proline. Compared to NaCl treatment, the proline content in leaves and roots increased by 62.17% and by 16.14 % under the NaCl+EBR2 treatment.

The spray of EBR on unstressed plant could not bring about a significant change in the level of protein (Figure 2B). Soluble protein concentration decreased in leaves but increased in roots after NaCl treatment. However, higher protein content was detected in plants treated with EBR under salt stress both in leaves and roots as compared to only salt-treated plants. The increase was significant especially in the plants treated with 10 nM EBR.

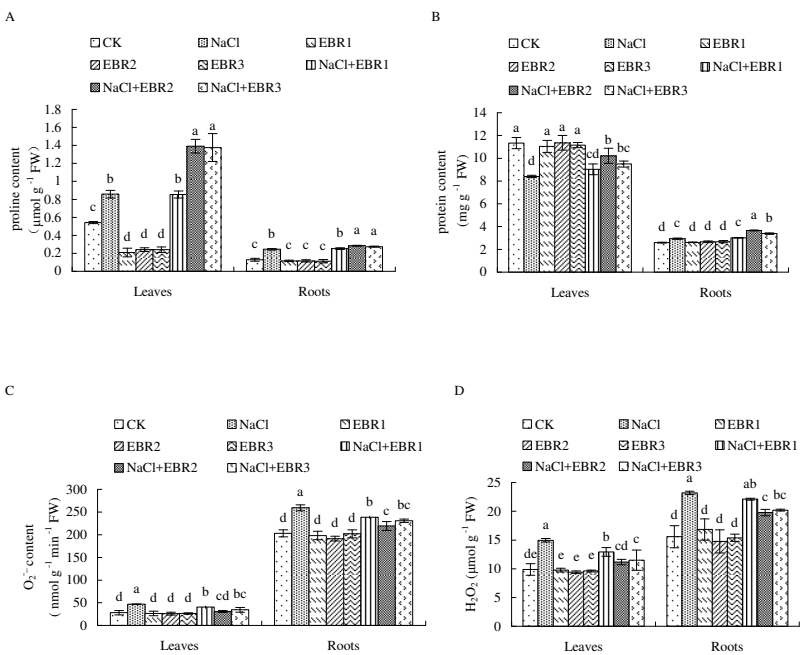

Figure 2. Effects of exogenous 24-Epibrassinolide on proline (A) , protein content (B), $O_2^{\cdot-}$ generation rate (C) and H_2O_2 content (D) of wheat seedlings under salt stress. Values represent mean ± SD (n = 3). Different small letters within the same row indicate significant difference at $P \leq 0.05$.

3.6. $O_2^{\cdot-}$ generation rate and H_2O_2 content

As shown in Figure 2C and Figure 2D, the spray of EBR on unstressed wheat seedlings could not bring about a significant change in the levels of $O_2^{\cdot-}$ generation rate and H_2O_2 content. 120 mM NaCl treatment induced a dramatic increase in $O_2^{\cdot-}$ and H_2O_2 production in leaves and roots of wheat seedlings compared with CK. Spray of EBR significantly diminished NaCl induced ROS accumulation in leaves and roots, especially 10 nM EBR. The $O_2^{\cdot-}$ generation rate and H_2O_2 content under NaCl+EBR2 treatment were decreased by 34.43, 25.30 % in leaves and by 15.39, 14.76 % in roots compared with NaCl treatment.

3.7. Antioxidant enzymes

Results in Figure 3 demonstrated that the spray of EBR under non-stress conditions increased the activities of SOD, POD, and CAT in leaves and roots of wheat seedlings compared with CK, especially 10 nM EBR. Salt stress significantly decreased the activity of SOD in the wheat leaves and roots (Figure 3A), but application of EBR alleviated the decline of the SOD activity under salt stress. The 10 nM EBR had the best alleviating effect.

Compared to CK, the POD activity under salt stress decreased significantly in leaves; but it increased markedly in roots (Figure 3B). When treated with EBR in the presence of salt stress, the POD activity exhibited an increase, both in the leaves and roots, especially 10 nM EBR.

Salt stress increased the activity of CAT in the wheat leaves and roots (Figure 3C), and application of EBR under salt stress elevated the increase of CAT activity, especially 10 nM EBR.

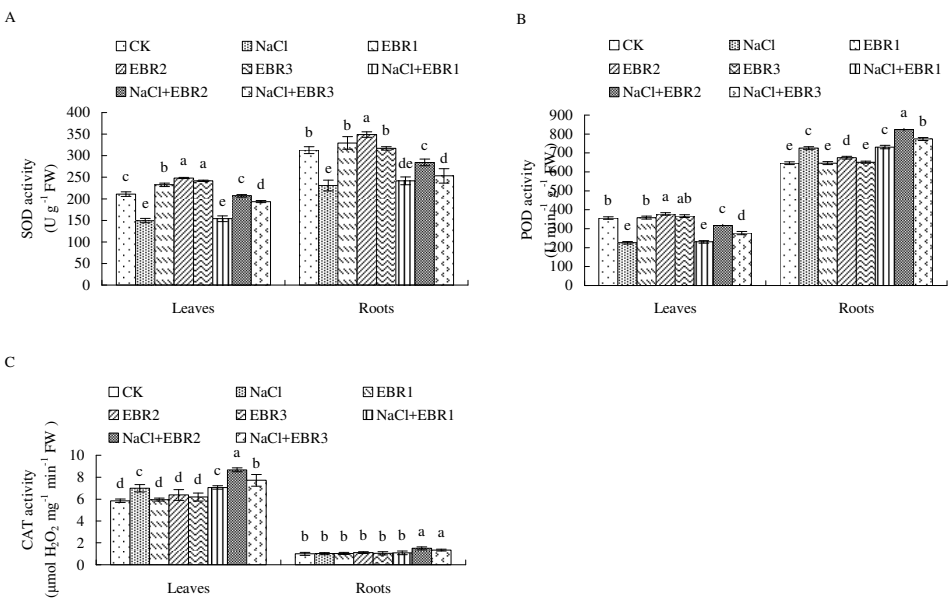

Figure 3. Effects of exogenous 24-Epibrassinolide on SOD (A), POD (B) and CAT (C) content in leaves and roots of wheat seedlings under salt stress. Values represent mean ± SD (n = 3). Different small letters within the same row indicate significant difference at $P \leq 0.05$.

3.8. Mineral nutrient concentration

As showed in Table 3, the spray of low concentration EBR (1 and 10nM EBR) on unstressed plant could increase the contents of mineral nutrients (K, Ca, Mg, Fe and Zn). A high concentration of Na was observed in the treatment of NaCl alone, and concentrations of all the mineral nutrients (K, Ca, Mg, Fe and Zn) under NaCl treatment were lower than those of CK. However, the application of EBR under salt stress alleviated the decline, especially 10 nM EBR. When treated with 10 nM EBR in the presence of salt stress, the concentrations of K, Ca, Mg, Fe and Zn were increased by 7.30, 54.61, 7.56, 30.31, and 19.19 % in leaves and by 27.78, 64.88, 46.94, 82.00, 33.26 % in roots, respectively as compared to NaCl-alone treatment. However, the 100 nM EBR had no significant effect. The K/Na and Ca/Na ratios significantly decreased in wheat seedlings under saline conditions (Table 4). However, the spray of EBR increased K/Na and Ca/Na ratios in leaves and roots, especially 10 nM EBR.

Table 3. Effects of exogenous 24-Epibrassinolide on the concentrations of K, Na, Ca, Mg, Fe [g kg^{-1} (d.m.)], Zn [mg kg^{-1} (d.m.)] in the leaves and roots of wheat seedlings under salt stress. Means SD, $n = 3$. Different letters after means within the same column indicate significant differences at $P \leq 0.05$.

Element	Tissue / Treaments	CK	NaCl	EBR1	EBR2	EBR3	NaCl+EBR1	NaCl+EBR2	NaCl+EBR3
K	Leaves	20.45±0.89a	17.94±0.35d	20.02±0.87ab	19.99±0.38ab	20.03±0.62ab	18.52±0.43cd	19.25±0.38bc	19.23±0.64bc
	Roots	21.75±0.61c	11.77±0.48e	25.94±0.43a	23.37±1.09b	25.97±0.86a	12.73±0.51e	15.04±1.49d	14.33±0.73d
Na	Leaves	4.32±0.61c	9.06±0.22a	3.72±0.25d	3.49±0.12d	4.02±0.25cd	8.50±0.18b	8.11±0.37b	8.00±0.18b
	Roots	9.24±1.06c	14.22±0.63a	8.64±0.55cd	7.54±0.11d	7.38±1.63d	14.05±0.26a	12.64±0.50b	13.25±0.52ab
Ca	Leaves	5.25±0.49b	3.42±0.69c	5.36±0.65b	7.06±1.50a	8.15±0.41a	4.74±0.69bc	5.28±1.38b	5.25±1.28b
	Roots	3.16±0.25b	1.56±0.08d	2.74±0.09bc	4.14±0.87a	2.75±0.34bc	2.44±0.18c	2.57±0.16bc	2.25±0.09c
Mg	Leaves	0.83±0.09b	0.74±0.01c	0.85±0.05b	0.94±0.03a	0.94±0.05a	0.80±0.02bc	0.80±0.03bc	0.79±0.02bc
	Roots	0.83±0.05bc	0.52±0.04e	0.81±0.02bc	1.02±0.08a	0.86±0.02b	0.63±0.01d	0.77±0.04c	0.64±0.07d
Fe	Leaves	1.24±0.10a	0.89±0.07c	1.25±0.26a	1.26±0.17a	1.26±0.07a	0.94±0.05bc	1.16±0.13ab	1.14±0.18ab
	Roots	4.60±0.24a	1.74±0.20d	4.65±0.17a	4.69±0.17a	4.66±0.15a	2.19±0.34cd	3.16±0.51b	2.42±0.29c
Zn	Leaves	67.06±2.53a	46.04±6.52c	65.99±3.19a	69.14±1.82a	66.51±4.48a	52.94±5.27bc	54.87±6.32b	53.26±5.52bc
	Roots	100.10±8.72a	73.54±6.30c	95.94±8.10a	101.82±8.63a	99.43±5.68a	83.67±8.95bc	98.00±3.09a	94.26±2.97ab

Table 4. Effects of exogenous 24-Epibrassinolide on K/Na and Ca/Na ratios in the leaves and roots of wheat seedlings under salt stress. Means SD, $n = 3$. Different letters after means within the same column indicate significant differences at $P \leq 0.05$.

Parameters	Tissue/Treaments	CK	NaCl	EBR1	EBR2	EBR3	NaCl+EBR1	NaCl+EBR2	NaCl+EBR3
K/Na	Leaves	4.78±0.56c	1.98±0.01d	5.39±0.32ab	5.72±0.08a	5.00±0.38bc	2.18±0.09d	2.38±0.15d	2.40±0.06d
	Roots	2.37±0.23c	0.83±0.06d	3.01±0.14b	3.10±0.12b	3.61±0.61a	0.91±0.05d	1.19±0.12d	1.08±0.09d
Ca/Na	Leaves	1.24±0.31b	0.38±0.07c	1.44±0.09b	2.03±0.49a	2.03±0.04a	0.56±0.09c	0.65±0.14c	0.66±0.17c
	Roots	0.34±0.04b	0.11±0.01c	0.32±0.03b	0.55±0.11a	0.38±0.10b	0.17±0.02c	0.20±0.02c	0.17±0.01c

4. Discussion

As demonstrated, exogenous application of very low BRs concentrations influences multiple plant growth and development processes, while micromolar concentrations cause inhibitory effects (Choudhary *et al.,* 2012). In the present study, spray of EBR under non-stress conditions increased the fresh and dry weight of wheat shoots and roots compared with CK, especially 10 nM and 1 nM EBR (Table 1). This study indicated that the low concentrations of EBR could promote wheat plant growth. Wheat seedlings under NaCl stress exhibited a significant decline in all the growth parameters compared to CK plants (Table 1). These results were in agreement with that of Li *et al.* (2013) who showed marked reduction in growth parameters of wheat subjected to NaCl stress. It has been proposed that this growth inhibition caused by salinity could partly be due to the shortage of energy because processes involved in salt transport and salt damage repair on membrane or proteins are energy consuming (Tian *et al.,* 2015). Tian *et al.* (2015) also indicated that reduction in the plant growth has been attributed to reduced water absorption due to osmotic effect, nutritional deficiency on account of the ionic imbalance and decrease in many metabolic activities. However, this reduction in growth was alleviated with spray of EBR (Table 1).

Foliar application of low concentrations EBR resulted in significant increases in growth parameters of plants under 120mM NaCl treatment compared to CK plants. In the present study, the most effective dose of EBR under saline conditions was found to be 10 nM EBR. Similar results were observed in Medicago truncatula (López-Gómez *et al.,* 2016) and strawberry (Karlidag *et al.,* 2011), which was found that foliar application of 24-EBL was effective in improving growth, in terms of increasing plant fresh and dry biomass under both non-saline and saline conditions. RWC is considered as an alternative measure of plant water status, reflecting the metabolic activity in plant tissues (Tian *et al.,* 2015). Table 1 showed that salt stress significantly declined RWC in leaves and roots compared to CK treatment. Spray of EBR under salt stress increased the RWC compared with only NaCl treatment, especially 10 nM EBR. Increased RWC by BRs application had been reported for *Brassica juncea* (Hayat *et al.,* 2010) under cadmium stress and *Vigna radiata* (Ali *et al.,* 2008) under aluminium stress. It is known that at non-stress conditions various BRs are proposed to regulate photosynthesis. Salt stress significantly decreased the total chlorophyll, chl *a*, chl *b* and carotenoids concentrations in leaves of wheat seedlings compared to CK (Table 2). Similarly, Sharma *et al.* (2013) indicated that NaCl stress caused a significant reduction in the chlorophyll concentration. This decrease in chlorophyll concentration may be attributed to the increase in the activity of chlorophyll degrading enzyme chlorophylase, under saline conditions (Tian *et al.,* 2015). However, application of EBR under salt stress elevated the chlorophyll content (Table 2). This positive effect can be reasoned from the possibility of BRs-induced impact on transcription and/or translation in the synthesis of pigments (Bajguz, 2000). These results were in agreement with some earlier reports in which it has been observed that 24-EBL could ameliorate the decrease in chlorophyll content caused by aluminium stress (Dong *et al.,* 2008) and weak light stress (Wang *et al.,* 2010).

In the present study, the spray of EBR on unstressed plant could promote wheat plant growth by increasing root activity and H^+-ATPase activity (Figure 1A, B), which might be responsible for root activity can directly affect the shoot growth, nutrition status, and H^+-ATPase plays a major role in the activation of ion and nutrient transport (Frédéric *et al.,* 2007). NaCl treatment significantly lowered root activity and H^+-ATPase activity, whereas spray of EBR mitigated the

salt effect, and NaCl+EBR2 treatment had the best effect (Figure 1A, B). Khripach *et al.* (2000) have demonstrated that EBR could regulate cell elongation and divisional activities by activating cell wall loosening enzymes. Song *et al.* (2016) also indicated that EBR could induce H^+-ATPase activity, which may be attributed to EBR increasing absorption of Ca, Mg under NaCl stress.

Electrolyte leakage of wheat plants was higher in 120 mM NaCl compared to the nonsaline conditions (Figure 1C). Dong *et al.* (2015) reported that salt stress led to a significant increase in the level of electrolyte leakage in many crops. In the present study, EBR treated plants under salt stress had less electrolyte leakage compared to only NaCl treatment. Moreover, BRs are reported to modify the membrane structure/stability under stress conditions. In this present study, NaCl treatment significantly increased the level of malondialdehyde in wheat seedlings (Figure 1D). Egbichi *et al.* (2013) reported that salt stress induced oxidative damage to membrane lipids, as revealed by the amount of malondialdehyde produced in salt-treated soybean root nodules. However, spray of EBR under salt stress could decrease production of malondialdehyde in leaves and roots of wheat seedlings (Figure 1D), and the 10 nM EBR had the best effects. However, the data obtained from this study suggests that application of EBR can protect wheat plants from the effect of salt induced-membrane damage. Ali *et al.* (2008) also demonstrated that application of 24-Epibrassinolide could decrease membrane lipid peroxidation and protect against the stress generated by salinity and nickel in *Brassica juncea*. But there was no marked difference in the lipid peroxidation level between the NaCl-alone treatment and NaCl+EBR1 treatment. This result indicated that the appropriate concentrations of EBR could alleviate salt stress induced oxidative damage to membrane lipids.

Salinity causes a lot of physiological and morphological changes including the accumulation of low molecular weight compounds called compatible solutes such as proline (Chen *et al.,* 2007). In this present study, salt stress significantly increased proline content in leaves and roots of wheat seedlings compared with CK (Figure 2A). Proline, under stress conditions acts as osmoprotectant, membrane stabilizer and ROS scavenger (Dong *et al.,* 2015). Osmotic adjustment is the main part of the physiological machinery by which plants respond to salt stress. Accumulation of protein in the cytosol and other organelles helps in the osmotic adjustment of plants. Recent studies demonstrated that EBR could alleviate the detrimental effects of different stresses on the plant growth by improving photosynthesis in leaves, mainly due to upregulation of the levels of protein and proline content (Sharma *et al.,* 2013; Zhang *et al.,* 2013). These results were in agreement with the present study in which it has been observed that Spray of EBR increased the proline (Figure 2A) and protein content (Figure 2B), thus resulted in an increase in the ability of tolerance to NaCl.

To maintain metabolic functions under stress conditions, the balance between generation and degradation of ROS is required, otherwise oxidative injuries may occur. Overaccumulation of ROS in plant cells is the main effect of NaCl toxicity (Rady 2011). The accumulation of $O_2^{\cdot-}$ and H_2O_2 during salinity stress can arise as a result of the imbalance in the rate of production and removal of ROS (Egbichi *et al.,* 2013). In the present study, samples treated with 120 mM NaCl recorded the highest level of $O_2^{\cdot-}$ generation rate and H_2O_2 content (Figure 2C, D). The result was in agreement with some earlier reports in which it had been observed that salt stress could increase H_2O_2 content in rice and barley (Li *et al.,* 2008). Spray of EBR decreased the $O_2^{\cdot-}$ generation rate and H_2O_2 content (Figure 2C, D), and reversed the salinity-induced stress. Ahammed *et al.* (2013) also indicated that EBR sig-

nificantly decreased harmful ROS accumulation and lipid peroxidation through the induction of antioxidant enzymes activity.

Plant detoxification pathways consisted of several metabolic processes which include the activation of antioxidant enzymes (Ahammed *et al.,* 2013). SOD is a first line defence enzyme involved in the dismutation of $O_2^{\cdot-}$. Again, H_2O_2 is converted into water and oxygen by the action of POD, CAT and APX (Song *et al.,* 2016). In the present study, salt stress significantly inhibited SOD and POD activities in leaves and SOD activity in roots of wheat seedlings (Figure 3A, B and C). A decrease in antioxidant enzyme activity suggests that generation of $O_2^{\cdot-}$ and H_2O_2 exceeds the elimination ability of enzymes (Song *et al.,* 2016). Inhibition in antioxidant enzyme activity is consistent with the increased $O_2^{\cdot-}$ and H_2O_2 production and subsequent lipid peroxidation in the current study. However, spray of EBR under NaCl stress could increase the antioxidant enzyme activity not only in leaves but also in roots (Figure 3A, B and C), thus elevate stress tolerance. EBR could increase the antioxidant enzyme activity, thus elevate stress tolerance. Some early studies also demonstrated that EBR could increase the antioxidant enzyme activity, thus elevate stress tolerance (Mazorra Morales *et al.,* 2014).

Mineral contents of the leaves and roots of the wheat plants except Na drastically decreased under salt stress (Table 3). It has been shown that high concentrations of NaCl may disorder nutrient-ion activities, causing plants to be susceptible to osmotic and specific-ion injury as well as to nutritional disorders that result in reduced yield and quality. Mineral nutrients such as K and Ca are essentially required for the activities of enzymes, protein synthesis and integrity of cell wall and plasma membrane (Song *et al.,* 2016). NaCl not only reduces Ca and K availability, but reduces Ca and K transport and mobility to growing regions of plants. The results were in agreement with the view that plants growing under saline conditions suffer ionic imbalance, nutrient deficiency and specific ion toxicity (Dong *et al.,* 2015). Foliar application of EBR altered the accumulation of mineral contents in the leaves and roots (Table 3). Karlidag *et al.* (2011) also demonstrated that 24-EBL application significantly increased K, Ca, Mg, Fe and Zn content in strawberry plants under salt stress. Foliar application of EBR under salt stress increased K/Na and Ca/Na ratios compared to only NaCl treatment (Table 4). Similarly, Shahbaz and Ashraf indicated that foliar spray with 0.0125 mg L^{-1} EBL increased K/Na ratio in salinized plants of MH-97 wheat plants (Song *et al.,* 2016). EBR-induced increase in growth was accompanied with corresponding increase in K/Na and Ca/Na ratios in the salt stressed plants. Growth promotive effect of BRs might have also been due to its role in ion homeostasis, which is necessary for various biochemical or physiological processes controlling growth (Karlidag *et al.,* 2011).

5. Conclusions

Salt stress induced lower plant growth, relative water conter (RWC) and mineral contents of wheat seedlings. However, with spray of exogenous 24-Epibrassinolide (EBR) in wheat plants, the deleterious effects of salinity stress were alleviated. The potential mechanisms include: (1) EBR could increase content of chlorophyll contents, soluble proteins, and free proline; (2) EBR could regulate activities of key antioxidant enzymes to eliminate ROS; (3) EBR could help the wheat plants to improve cell membrane stability and nutrient uptake under salinity stress. Therefore, EBR could improve wheat plant growth under salt stress, and the optimal concentration appears to be 10 nM concentration. The foliar EBR applications could offer a simple application in wheat production in saline soil but further studies are required in order

to determine the efficiency of these materials under natural field condition.

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