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Effect of Glutathione, 24-Epibrassinolide and Proline on Wheat Growth and Antioxidant Enzymes Activity Under Salt Stress

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Abstract: Plants are sessile organisms that affect both biotic and abiotic stress factors. Adverse effects of sodium chloride (NaCl; 2000 and 4000 ppm) were investigated in wheat (Giza 168). NaCl salt decreased the plant fresh and dry weights, plant height, number of tillers and flag leaf area of plants at tillering and flowering stages. Also, the yield productivity including spikes number/ plant, spiklets number/spike, weight of 1000 grains and weight of yield/plant strongly reduced with increasing salt stress. Salinity decreased the relative water content (RWC), salt tolerance index and increased the osmotic potential of flag leaf cells sap under stress. Salt stress affects various aspects of plant system including antioxidant enzymes activity. Plants exposed to high NaCl concentration showed a higher increasing in superoxide dismutase (SOD) and phenylalanine ammonia lyase (PAL) activities, while, the activity of polyphenol oxidase (PPO) was decreased. Exogenous application with glutathione (GSH), 24-epibrassinolide (EBL) and proline mitigate the damage effect of NaCl in promoting growth and enhancing the activities of antioxidant enzymes under NaCl stressful conditions. GSH exhibited a maximum activity of guaicol peroxidase (POD), ascorbate peroxidase (ASP) and glutathione reductase (GR). The activity of PPO positively increased with EBL. Proline acts as an osmolyte and ameliorated the activity of POD, PPO, ASP and GR enzymes under salt stress.

Key words: Wheat · Salt stress · Glutathione · 24-epibrassinolide · Proline · Antioxidant enzymes · Stress tolerance

INTRODUCTION

According to Sairam *et al.* [1], salinity is one of the serious abiotic stress factors that negatively affects various aspects of plant growth, biochemical and molecular changes in plant tissues due to accumulates excessive concentrations of salt, especially sodium chloride (NaCl). Sodium ions replace potassium ions causing a disturbing in the activity of major cytosolic enzymes, osmotic stress due to limiting water absorption from saline soil leading to ionic toxicity [2]. Under salt stress condition plant cell suffered from oxidative stress due to exceeding production of a signalling molecules of reactive oxygen species (ROS) as an attempt to regionalize plant with adverse abiotic stress conditions. These ROS are produced as a by- product through an aerobic metabolism and accumulated in various subcellular compartments [1]. Wheat (*Triticum aestivum*) is a widely cultivated grass for its grain, which is a worldwide indispensable food [3]. It considers the 2nd most produced cereal crop after maize, it cultivates in 220.4 million hectares on world land area, which produce about 749 million tons in 2016 [4, 5]. It is an important source of carbohydrate and it is rich in vegetal protein (about 13%) in human food [6]. Increased plant acclimation to salinity often associated with over production of ROS such as superoxide anion (O_2^-), hydroxyl radicals (OH^-), hydrogen peroxide (H_2O_2) and singlet oxygen (O_2) which have a devastating effect on plant growth, loss of vigor and cause membrane damage leading to plant cell death. Anjum *et al.* [7] reported that ROS production is enhanced under salinity stress through the disruption of electron transport and oxidizing metabolic reactions occur in the chloroplast, mitochondria and other cell microbodies. The antioxidant enzymes are involved in ROS scavenging and play a protective role against the potentially deleterious effects of these toxic components [8]. The interplay between ROS production and antioxidant enzymes scavenging system measure the steady-state level of ROS in the cell [9]. Tripeptide

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glutathione (GSH; □-glutamate- cysteine- glycine) is sulfur containing non-protein thiol, synthesized in the plastid and translocate through phloem to other sub-cellular compartments and other cells to act as electron acceptor and donor for many biochemical reactions [10]. Foyer and Noctor [11] added that the higher water solubility and stability of GSH make it an ideal antioxidant compound to plants against ROS. In the normal metabolical function, reduced GSH is oxidized into oxidized glutathione disulphide (GSSG) due to ascorbate-glutathione (AsA- GSH) cycle but it should be rapidly converted back into GSH in the presence of NADPH to maintain the balance between both GSH and GSSG [12]. Moreover, Anjum *et al.* [7] reported that an increase in the cellular ROS can shift the form of GSH towards a more of GSSG form. Most plant phytohormones can play dual role in development growth and implicate abiotic stress responses. Brassinosteroids (BRs) are steroidal hormones that regulate plant growth and productivity by elevating seed germination, pollen tube growth, vascular differentiation, activation of proton pump, nucleic acids and protein biosynthesis [13]. In addition, enhancing reproductive growth which leading to increase production of flowers and fruits. Also, Anuradha and Roa [14] recommended that exogenous application of BRs mitigate the damage effect of salt stress. Zhang *et al.* [15] stated that 24- epibrassinolide (EBL) treatment modulates both enzymatic and non-enzymatic antioxidants in plants exposed to salt stress. Armengaud *et al.* [16] reported that L- proline is a highly soluble organic compound, has a low molecular weight and accumulates in large quantities without any toxic effect in response to salt stress. It is synthesized in the cell through glutamic acid pathway for stress-induced proline accumulation. Proline is a compatible solute provides plant tolerance against salt stress by contribute the cellular adjustment, detoxifying ROS, maintain protein integrity, enzymes stabilization and elevating the activity of different antioxidant enzymes [17].

Thus, the objectives of this study revealed the following:

- Showed the adverse effect of salt stress against wheat plant growth and yield at tillering, flowering and harvest stages.
- Plant antioxidant enzymes activity assessment under stressed and unstressed conditions.
- Ameliorated salt stress adverse effect on plant growth, yield production and activation of antioxidant enzymes defense system by exogenous treatments of glutathione; through its role as an antioxidant compound, 24- epibrassinolide; which is within the plant growth regulators and has an indirect impact in motivating the plant tolerance and proline; which has an effective role as an osmoregulator.

MATERIAL AND METHODS

To assess the effect of reduced glutathione (GSH), 24-epibrassinolide (EBL) and proline as a salinity relief organic compounds on the growth and yield of wheat variety Giza 168 under salt stress, a pot experiment was conducted during winter seasons of 2015-2016 and 2016-2017, in a wire-house of Experimental Farm of Faculty of Agriculture, Ain Shams University, Shoubra El Kheima, Qalyubia Governorate, Egypt. Certificated seeds were purchased from Field Crop Research Institute, Agricultural Research Centre, Giza.

Experimental Design and treatments: Plastic pots 30 x 40cm with drainage holes in bottom were filled with 15 kg acid washed sandy soil. Ten seeds were sown in each pot at 15th October of 2015-2016 and 2016-2017 seasons and then irrigated with tap water to keep seeds wet enough until germination. The germinated seedlings were thinned out to five uniform seedlings / pot after three weeks of germination. Plants were irrigated with Hoagland nutrient solution, one strength twice a week till full maturity of the main shoot spike [18]. Treatments were arranged in a split-plot design with three replicates for each treatment. Each replicate contains 5 pots with 5 plants per pot.

Two concentrations of NaCl (2000 and 4000 ppm) were applied in the main plot in addition to non-saline treatment (control). These treatments were prepared by dissolving separately calculated amounts of NaCl in tap water. The saline and non-saline water were added on the soil surface with irrigating immediately after thinning.

Plants at 30 days-old were sprayed separately on foliage, in subplots, five times with one-week intervals, by four different exogenous spraying applications.

- Control (sprayed with distilled water)
- Glutathione at 5 mM
- 24- epibrassinolide at 0.2 mg/l
- Proline at 5 mM.

Tween 20 (0.1%) was added to the spraying solutions as a surface wetting agent.

Vegetative Growth Parameters: Three plants from each replicate of all treatments were detached randomly at the tillering stage (45 days after sowing) to obtain:

- Plant height (cm) from the base to the top of the stem using a meter scale.
- Shoot fresh weight (g)
- Shoot dry weight (g), fresh shoot was dried in an oven at 70°C to the constant weight.
- The number of tillers per plant were recorded at this stage.
- Also, at the flowering stage (80 days after sowing) another three plants were taken randomly from each replicate to measure
- Plant height (cm).
- Shoot fresh and dry weights.
- Flag leaf area (cm^2) was calculated by the formula of Gardner *et al.* [19] as Length x Width x 0.75

Yield Determinations: Spikes number/plant, spikelet number/spike, number of grains/spike and the weight (g) of 100 grains were recorded and then multiplied by 10 to calculate the weight (g) of 1000 grains to evaluate the adverse effect of salinity on the yield productivity at 120 days after sowing (DAS) and estimate the efficiency of the under studied biostimulants on improving these products under salinity.

Leaf Water Status Measurements: Flag leaf relative water content (LRWC) was determined according to the method of Kaya *et al*. [20] to study plant water status at flowering stage (80 DAS). For the estimation of LRWC, 20 leaf discs (10 mm in diameter) were taken with a cork borer from the flag leaf of three plants per replicate and placed in a reweighed Petri dish to determine fresh weight (f.wt.), then discs were floated for 24 hours in distilled water inside a closed petri dish until the discs became fully turgid. Discs were weighed periodically after gently wiping the water from the leaf surface with a filter paper to determine turgid weight (t.wt.). Finally, the leaf discs were placed in a pre-heated oven at 70°C to a constant weight (almost 24 h) and weighed again to obtain dry weight (d.wt.). LRWC % was calculated using the equation of LRWC (%) = [(f.wt.-d.wt.) / (t.wt. - d.wt.)] × 100

Osmotic Potential (Ψ_s): The flag leaf of three repetitions were collected from all treatments under non-saline and stress conditions with all foliar treatments to determine the leaf osmotic potential (Ψ_s) as described by Pérez-López *et al.* [21]. The collected flag leaf immediately frozen for 1 week to break the cell walls. Then the frozen samples were thawed and the cell sap was extracted by grinding in mortar, thereafter the extracts were centrifuged at 10000 rpm for 5 min, then the osmotic potential (OP) was directly determined in the leaf cell sap using the Osmomat (030, Genotech GMBH, Berlin) and calculated by (-MPa).

Biochemical Analyses

Antioxidant Enzymes Activities Assay

Preparation of Crude Enzymes Extract: One-gram fresh flag leaf tissues (80 DAS), already being frozen in liquid nitrogen to prevent proteolytic reactions, were homogenized in 10 cm^3 of 100 mM cold potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% polyvinyl pyrrolidone (PVP) (w/v) at 4°C. The homogenate was centrifuged at 15000 rpm for 15 min at 4°C. Supernatant was used to measure the activities of superoxide dismutase (SOD, EC 1.15.1.1), guaicol peroxidase (POD, EC 1.11.1.7), phenylalanine ammonia lyase (PAL, EC 4.3.1.5), polyphenol oxidase (PPO, EC 1.14.18.1), ascorbate peroxidase (ASP; EC 1.11.1.11) and glutathione reductase (GR; EC 1.6.4.2). All enzymes activity was calculated as unit mg $protein^{-1}$ min^{-1}.

The activity of guaicol peroxidase was assayed by the method of Hammerschmidt *et al*. [22]. The reaction mixture consisted of 0.25 % (v/v) guaicol in 10 mM potassium phosphate buffer (pH 6 containing 10 mM hydrogen peroxide, H_2O_2). Volume of 100 µl of the crude enzyme extract was added to initiate the reaction which was measured spectrophotometrically (CT 200 spectrophotometer) at 470 nm min^{-1}. One international unit (IU) of enzyme activity was expressed as Δ OD= 0.01.

The activity of superoxide dismutase was assayed by the method of Beauchamp and Fridovich [23] by measuring the ability of enzyme to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). A reaction mixture containing 40 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 2 µM riboflavin, 0.1 mM EDTA and 100 µl of the crude enzyme extract was shaken and placed 30 cm below light source consisting of 15 W fluorescent lamp. The absorbance was recorded at 560 nm. One unit of SOD activity is the amount of protein required to inhibit 50% initial reduction of NBT under light.

Phenylalanine ammonia lyase (PAL) activity was quantified by the method of Beaudoin-Eagan and Thorp [24]. One unit of enzyme activity was defined as the amount of enzyme that caused an increase in absorbance of 0.01 $hour^{-1}$ at 290 nm.

Polyphenol oxidase (PPO) activity was measured according to Benjamin and Montgomery [25]. One unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.001 per min at 420 nm.

Ascorbate peroxidase (ASP) was assayed according to the method of Nakano and Asada [26]. Reaction mixture (3 ml) consists of 50 mM potassium phosphate buffer (pH=7.5), 0.5 mM ascorbic acid, 0.1 mM EDTA, 1.5 mM H_2O_2 and 100µl crude enzyme extract. The reaction was started when H_2O_2 was added to the reaction mixture to record the reduction in absorbance between 1-60 s at 290 nm due to the decrease in ascorbic acid concentration.

To assay glutathione reductase (GR) activity, 66.67 mM potassium phosphate buffer (pH=7.5), 0.333 mM EDTA, 0.5 mM 5, 5-dithiobis-2-nitrobenzoic acid (DTNB) dissolved in 0.01M potassium phosphate buffer (pH=7.5), then 0.0667 mM NADPH, 100 µl crude enzyme extract and 0.667 mM oxidized glutathione were added to initiate the reaction. GR activity determined at 412 nm by recording the increase in absorbance during a period of 5 min [27]. All enzymes activities were recorded by using spectrophotometer (UV-Vis spectrophotometer UV 9100 B, LabTech).

Statistical Analysis: Data were statistically analyzed using analysis of variance in Statistix (8^{th} edition, Analytical Software, USA) by Steel *et al*. [28]. Significance between means was tested by Duncan's multiple range test at 5% probability according to the method described by Gomez and Gomez [29].

RESULTS AND DISCUSSION

Tables (1) and (2) indicated that all tested vegetative parameters at 45 and 80 days after sowing negatively affected by NaCl salinity stress. The highest reduction in plant growth parameters was shown with NaCl (4000 ppm). This rise in NaCl concentration resulted in a significant reduction in plant height, shoot fresh and dry weights, number of tillers and flag leaf area. These results are consistent with Asgari *et al.* [30], who revealed that increase salinity levels causing a clear reduction in plant morphological traits, also they noticed a marked decrease in the tillers number/plant under salt stress. The reduction in morphological growth parameters gradually increased with increasing the salinity level [31]. Tuna *et al.* [32] found that salinity condition reduced the dry matter of wheat plants. These results were explained by Kumar *et al*. [33] who suggested that the reduction in plant growth under salt stress is due to lack of water uptake and reduction in essential nutrients which happen as a reason of accumulation of sodium ions in the root zone which may cause an ion imbalance and excessive ion uptake causing a specific ion toxicity. Shahid *et al*. [34] and Kumar *et al*. [33] added that, the decline in growth was a result of inhibition of cytokinins synthesis which stimulate the growth, cell division, cell expansion and cell enlargement parallel with increasing in the production of plant growth inhibitors.

Raza *et al*. [35] mentioned that, the reduction of shoot height may be due to dehydration of protoplasm, less relative turgidity that associated with turgor loss and decreased cell expansion and cell division.

Foliar spraying with GSH (5 mM), EBL (0.2 mg/l) and proline (5 mM) have improved all wheat growth measurements under both salt stress concentrations and non-saline stress. 24-epibrassinolide (EBL) has shown a better motivates growth to unstressed circumstances. While, the alleviate role of GSH and proline was shown with a higher degree in plants exposed to NaCl saline water, particularly, with the high salinity level. Foliar spraying with GSH stimulated plants tolerant against high salinity stress. The most critical vegetative characters promoted by GSH at 45 days old plants were shoot fresh and dry weights with NaCl (4000 ppm) in the two experimental seasons. Insignificant differences were shown in tillers number/plant with both treatments of GSH and proline under the concentration of 4000 ppm NaCl. Almost the same path with spraying treatments was shown with NaCl salinity stress at 80 days old plants in the first season. Foliar spraying with GSH under NaCl (4000 ppm) was more efficient in promote growth parameters in the first season, but there are insignificant differences between GSH, proline and EBL in increasing plant fresh and dry weights with NaCl (4000 ppm) in the second season. Various authors have proposed that exogenous spraying with antioxidants, phytohormones and osmoprotectants have mitigate the detrimental effect of salinity[36-38]. Anuradha and Rao [14] stated that soaking rice grains in EBL solution stimulates the germination, growth and dry mass accumulation under saline stress.

They commented that, this activation in growth was associated with the ability of EBL to ameliorate high levels of nucleic acid and soluble protein biosynthesis which motivate the ill effects of salt stress on plant growth performance and dry mass. Glutathione is a low molecular weight antioxidant improved growth under salt stress [11]. Mahboob *et al.* [39] indicated that foliar applied proline

Table 1: Effect of reduced glutathione (GSH), 24-epibrassinolide (EBL) and proline on some wheat morphological traits, at 45 days after sowing under NaCl salt stress during the two seasons (2015/2016 and 2016/2017)

	Plant height (cm)	Shoot fresh weight (g)	Shoot dry weight(g)	No. of tillers	Plant height (cm)	Shoot fresh weight (g)	Shoot dry weight(g)	No. of tillers
Foliar treatments	1st season				2nd season			
NaCl at 0 ppm								
Control	41.53c	15.22b	2.69ab	4.00be	40.37cd	14.31ce	2.54bc	4.67ab
GSH (5mM)	41.63c	16.23a	2.72ab	4.67ac	43.17bc	14.9bc1	2.73ac	5.33a
EBL (0.2mg/l)	46.67a	16.31a	2.88a	5.33a	47.13a	16.80a	2.91a	5.33a
Proline (5mM)	43.83b	14.63c	2.76ab	5.00ab	42.10bc	15.34b	2.73ac	4.67ab
Mean	43.42A	15.67A	2.76A	4.75A	43.19A	15.34A	2.72A	5.00A
NaCl at 2000 ppm								
Control	38.37d	8.44f	1.71e	3.33de	30.10f	8.41g	1.32e	3.33c
GSH (5mM)	42.13bc	16.01a	2.42bd	4.67ac	44.10ab	16.54a	2.84ab	4.00bc
EBL (0.2mg/l)	41.03c	14.91bc	2.23cd	4.33ad	41.73bc	13.87e	2.41cd	3.33c
Proline (5mM)	40.67c	15.28b	2.62ac	4.00be	37.97d	14.81bd	2.61ac	4.00bc
Mean	40.55B	13.66B	2.24B	4.08B	38.47B	13.41B	2.30B	3.67B
NaCl at 4000 ppm								
Control	24.30g	6.82g	0.88f	3.00e	24.97g	7.01h	0.87f	3.00c
GSH (5mM)	32.43e	12.56d	2.89a	3.67ce	34.43e	14.00de	2.74ac	3.33c
EBL (0.2mg/l)	30.10f	10.98e	1.38e	3.00e	28.43f	12.72f	2.16d	3.33c
Proline (5mM)	33.40e	10.82e	2.12d	3.00e	30.63f	12.60f	2.54bc	3.00c
Mean	30.06C	10.29C	1.82C	3.17C	29.62C	11.58C	2.08C	3.17A
Mean of foliar treatments under NaCl salinity levels								
Control	34.73B	10.16D	1.76C	3.44B	31.81C	9.912C	1.58C	3.67A
GSH (5mM)	38.73A	14.93A	2.67A	4.33A	40.57A	15.15A	2.77A	4.22A
EBL (0.2mg/l)	39.27A	14.07B	2.17B	4.22A	39.1A	14.46B	2.50B	4.00A
Proline (5mM)	39.30A	13.58C	2.50A	4.00AB	36.9B	14.25B	2.63AB	3.89A

Means followed by different letters are significantly different at P ≤ 0.05 level; Duncan's multiple range test; capital letters for mean of salinity or exogenous spraying treatments, whereas lowercase letters for interaction.

Table 2: Effect of reduced glutathione (GSH), 24-epibrassinolide (EBL) and proline on some wheat morphological traits, at 80 days after sowing under NaCl salt stress during the two seasons (2015/2016 and 2016/2017)

	Plant height (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Flag leaf area (cm^2)	Plant height (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Flag leaf area (cm^2)
Foliar treatments	1st season				2nd season			
NaCl at 0 ppm								
Control	71.60b	31.20d	6.24d	47.50b	76.77a	40.25b	8.05b	53.17bc
GSH (5mM)	71.33b	33.50bc	6.70bc	50.33ab	77.00a	40.87b	8.17b	55.50b
EBL (0.2mg/l)	81.27a	39.03a	7.81a	54.67a	76.41a	43.70a	8.74a	69.17a
Proline (5mM)	71.27b	35.17b	7.03b	47.33b	77.53a	34.87d	6.97d	55.83b
Mean	73.87A	34.73A	6.94A	49.96A	76.93A	39.92A	7.98A	58.42A
NaCl at 2000 ppm								
Control	60.77de	18.63h	3.73h	32.77e	64.47d	28.17f	5.63f	39.33de
GSH (5mM)	68.27c	31.55cd	6.31cd	53.33a	75.53ab	40.23b	8.04b	51.83bc
EBL (0.2mg/l)	62.83d	31.71cd	6.34cd	46.33bc	72.47bc	38.30c	7.66c	51.17c
Proline (5mM)	61.37de	25.87f	5.17f	42.83c	72.20c	34.18d	6.83d	42.33d
Mean	63.31B	26.94B	5.39B	43.82B	71.17B	35.22B	7.04B	46.17B
NaCl at 4000 ppm								
Control	31.33h	10.06i	2.01i	21.50f	48.37f	19.05g	3.81g	23.67f
GSH (5mM)	59.57e	28.64e	5.73e	37.83d	65.87d	31.58e	6.31e	54.33bc
EBL (0.2mg/l)	46.17g	23.51g	4.70g	32.67e	58.80e	30.30e	6.06e	36.67e
Proline (5mM)	52.03f	24.10fg	4.89fg	34.00de	64.67d	31.82e	6.36e	43.17d
Mean	47.28C	21.58C	4.33C	31.50C	59.43C	28.19C	5.63C	39.46C
Mean of foliar treatments under NaCl salinity levels								
Control	54.57D	19.97C	3.99C	33.93D	63.20C	29.16C	5.83C	38.72A
GSH (5mM)	66.39A	31.23A	6.24A	47.17A	72.80A	37.56A	7.51A	52.33A
EBL (0.2mg/l)	63.42B	31.42A	6.28A	44.56B	69.23B	37.43A	7.48A	52.33A
Proline (5mM)	61.56C	28.38B	5.70B	41.39C	71.47A	33.63B	6.72B	47.11B

Means followed by different letters are significantly different at P ≤ 0.05 level; Duncan's multiple range test; capital letters for mean of salinity or exogenous spraying treatments, whereas lowercase letters for interaction

significantly increased root and shoot lengths, fresh and dry weights of wheat seedlings under various salinity levels. Similarly, exogenous application of proline exhibited maximum plant height, flag leaf area, dry weight, number of spikes and grain yield [40]. The positive effect of proline was attributed to its role as an osmolyte to maintain cell turgor, array osmotic balance, prevent membrane electrolyte leakage and contribute in stabilizing the concentrations of reactive oxygen species (ROS) to enhance stress tolerance of plant under salt stress condition [41].

Derived from the following data in Table (3), yield parameters characterized by spikes number/plant and spiklets number/spike significantly reduced with presence of NaCl salinity. This reducing increased clearly with increasing NaCl concentration from 2000 to 4000 ppm at the two seasons. As a result of these spike and spiklets reduction, a clear decline in the grains number /spike and the weight of 1000 grains was observed, which eventually led to reduce total yield weight/plant. These results agree with that obtained by Asgari *et al.* [30] who mentioned that number of spikes/plant, number of spiklets/spike, thousand grain weight and grain yield of all wheat tested genotypes significantly decreased with increasing salinity levels. Munns [42] indicated that endogenous plant salt combat drastic reduction in growth by causing premature senescence of old leaves and decrease supply of assimilates to productive parts.

In the present investigation, 24-epibrassinolide has exceeded yield productivity with non-stressed plants. Adding NaCl (2000 ppm) with foliar GSH spraying gave the best results of spiklets number per spike, whereas, EBL improved the number of grains for each spike and weight of 1000 grains.

It is also noted that GSH treatment resulted in increasing the weight of 1000 grains and the final weight of the harvest yield of each plant exposed to NaCl (2000ppm). High salt concentration (NaCl 4000 ppm) with spraying biostimulants (GSH, EBL and proline) significantly increased the productivity of plants subjected to salt stress when compared with unsprayed plants under the same salinity conditions. This increase enhanced to more than two folds. Proline enhanced the efficiency of stressed plants to produce more number of spikes/plant, number of spiklets/spike, weight of 1000 grains and the weight of grains/plant in the first season. In the second season, the three tested biostimulants increased these latest characters without significant differences.

Anjum *et al.* [7] considered that, diverse plant taxa have the capacity for adapting metabolically to salinity throw elevating the synthesis of sulphur rich peptide compounds as glutathione and synthesis and accumulation of low molecular weight nitrogenous and proteogenic amino acids compounds as proline. Application with antioxidants, plant growth substances and osmoregulators achieved an osmotic adjustment and improved the wheat crop performance to mitigate the damage effect of salt stress and enhance plant growth and yield [43]. 24-epibrassinolide has pivotal roles on regulation of ion uptake. Moreover, EBL mediate stress tolerance through acting as synergist to IAA and GA and antagonist to ABA leading to promote growth. BRs-mediated stress tolerance in Arabidopsis was linked act as synergists to GA and IAA during hypocotyl elongation of Arabidobsis [44]. Proline is one of the two major compatible solutes in plant, glutathione is a strong reductant in cell and can react with oxidizing agents under salt stress through increased endogenous reduced glutathione (GSH) and decrease disulphide oxidized glutathione (GSSG) leading to increase the GSH/GSSG ratio and prevent the oxidized form from having the chance to damage anything in the cell [45, 46].

It was shown in this study that increasing vegetative growth parameters and yield by the tested biostimulant compounds under salt stress conditions is due to the ability of spraying plants to maintain high flag leaf relative water content percent (LRWC) and the equilibrium of the cell sap osmotic potential (OP) of the flag leaf, leading to the increment of salt tolerance index of these stressed plants (Table 4). Likewise, LRWC decreased in salt stressed plants. This decrease was more pronounced under high salinity level. More negative leaf osmotic potential (Ψ_s) was observed with plants faced the salt stress condition, while less negative OP was recorded at 5 mM Glutathione. All biostimulants treatments under salt stressful condition improved leaf water status due to decreasing the flag leaf osmotic potential. These results are in line with Farouk [43] who revealed that salinity can alter the plant-water relations. The salt stress (2000 ppm NaCl) significantly reduced the leaf relative water content (LRWC) and increased the cell negative values of osmotic potential [46]. 24-epibrassinolide treatment regulates ion uptake under salt stress [15]. Sharma *et al.* [47] indicated that 24-epibrassinolide resulted in increased proline concentration in rice seedlings subjected to salinity which increased the leaf-water relations due to increasing the LRWC and water potential under salt stress. Ashraf and Foolad [48] reported that exogenous spraying proline sustained osmoprotection and buffering the cellular redox potential in plants exposed to salinity. Also, Nahar *et al.* [46] showed that exogenous application with GSH increased the leaf relative water content in salt stressed plants.

Table 3: Effect of reduced glutathione (GSH), 24-epibrassinolide (EBL) and proline on some yield measurements, at 120 days after sowing under NaCl salt stress during the two seasons (2015/2016 and 2016/2017)

	Spikes no. /plant	Spiklets no. /spike	Grains no. /spike	Weight of 1000 grains (g)	Yield (g) /plant	Spikes no. /plant	Spiklets no. /spike	Grains no. /spike	Weight of 1000 grains (g)	Yield (g) /plant
Foliar treatments	1st season					2nd season				
NaCl at 0 ppm										
Control	5.33a	22.33bc	52.00b	47.00bc	12.70b	6.00ab	23.33ad	51.33b	46.87b	14.32b
GSH (5mM)	5.00ab	24.00ab	51.67bc	45.90bc	12.89b	5.00bc	24.00ab	46.67be	45.33bc	14.64b
EBL (0.2mg/l)	5.33a	26.00a	65.67a	50.17a	17.74a	7.33a	26.00a	60.33a	54.43a	23.33a
Proline (5mM)	5.00ab	24.00ab	50.33bc	47.80bc	11.89bc	5.33bc	24.67ab	48.33be	47.13b	13.93b
Mean	5.17A	24.08A	54.92A	47.72A	13.80A	5.92A	24.50A	51.67A	48.44A	16.55A
NaCl at 2000 ppm										
Control	2.33c	20.33cd	37.67de	33.70f	4.49e	3.67cd	20.67ce	36.67fg	35.63f	5.8cd
GSH (5mM)	5.00ab	23.67ab	49.67bc	45.77c	12.39b	5.67ab	24.00ab	48.67bd	44.70bd	13.42b
EBL (0.2mg/l)	5.00ab	22.33bc	54.67b	48.17ab	12.90b	5.00bc	23.67ac	49.00bc	44.23bd	12.60b
Proline (5mM)	4.33ac	22.00bd	44.33cd	43.43d	10.95bd	6.00ab	23.67ac	46.33be	45.33bc	13.04b
Mean	4.17AB	22.08B	46.58B	42.77B	10.31B	5.08A	23.00A	45.17B	42.48B	11.22B
NaCl at 4000 ppm										
Control	2.33c	19.33cd	36.33e	33.27f	3.76e	2.67d	19.00e	33.00g	35.30f	3.91d
GSH (5mM)	4.00ac	21.67bd	39.67de	40.53e	7.25de	4.67bc	20.33de	40.67dg	40.53de	7.98c
EBL (0.2mg/l)	3.00bc	19.00d	40.33de	32.93f	5.85e	4.33bd	19.33e	41.33cf	39.47ef	7.41c
Proline (5mM)	4.33ac	22.00bd	39.67de	42.10de	7.96ce	4.33bd	21.67ce	40.33eg	41.33ce	7.94c
Mean	3.42B	20.50C	39.00C	37.21C	6.21C	4.00B	20.08B	38.83C	39.16C	6.81C
Mean of foliar treatments under NaCl salinity levels										
Control	3.33B	20.66B	42.00C	37.99B	6.98B	4.11B	21.00B	40.33C	39.27C	8.01C
GSH (5mM)	4.67A	23.11A	47.00B	44.07A	10.84A	5.11AB	22.78A	45.33B	43.52B	12.01B
EBL (0.2mg/l)	4.44AB	22.44AB	53.56A	43.76A	12.16A	5.56A	23.00A	50.22A	46.04A	14.45A
Proline (5mM)	4.56AB	22.66A	44.78BC	44.44A	10.27A	5.22A	23.33A	45.00BC	44.60AB	11.64B

Means followed by different letters are significantly different at P ≤ 0.05 level; Duncan's multiple range test; capital letters for mean of salinity or exogenous spraying treatments, whereas lowercase letters for interaction.

Table 4: Effect of reduced glutathione (GSH), 24-epibrassinolide (EBL) and proline on leaf water status measurements and salt stress index, at 80 days after sowing under NaCl salt stress during the two seasons (2015/2016 and 2016/2017

	Control	GSH	EBL	Proline	Mean	Control	GSH	EBL	Proline	Mean
Treatments	1st season					2nd season				
Leaf relative water content (%)										
NaCl 0 ppm	80.71ac	79.93ac	82.87a	79.63ac	80.78A	79.80bc	81.60	85.60a	79.90bc	81.72A
NaCl 2000 ppm	71.23e	79.23ac	81.33ab	77.57cd	77.34B	72.17d	81.67b	80.00bc	79.17bc	78.25B
NaCl 4000 ppm	65.53f	78.13bc	74.03de	77.07cd	73.70C	64.33e	79.40bc	72.47d	77.90c	73.52C
Mean	72.50B	79.10A	79.41A	78.09A		72.10C	80.89A	79.36AB	78.99B	
Salt tolerance index (%)										
NaCl 0 ppm	100.00f	107.52ef	125.3de	114.48ef	111.82C	100.00g	101.59g	108.56f	86.66h	99.20C
NaCl 2000 ppm	100.00f	169.10c	170.10c	138.88d	144.52B	100.00g	142.85c	135.99d	121.39e	125.06B
NaCl 4000 ppm	100.00f	285.93a	234.30b	243.58b	215.95A	100.00g	165.85a	159.16b	167.16a	148.04A
Mean	100.00D	187.52A	176.57B	165.65C		100.00C	136.76A	134.57A	125.07B	
Osmotic potential (-Mpa)										
NaCl 0 ppm	0.83ef	0.78h	0.82fg	0.80gh	0.80C	0.80ef	0.79f	0.80ef	0.79f	0.79C
NaCl 2000 ppm	0.94a	0.85dc	0.87c	0.86cd	0.88B	0.88c	0.81e	0.83d	0.82de	0.83B
NaCl 4000 ppm	0.96a	0.86cd	0.89b	0.87c	0.89A	0.94a	0.86c	0.92b	0.86c	0.90A
Mean	0.91A	0.83C	0.86B	0.84C		0.87A	0.82C	0.85B	0.82C	

Means followed by different letters are significantly different at P ≤ 0.05 level; Duncan's multiple range test; capital letters for mean of salinity or exogenous spraying treatments, whereas lowercase letters for interaction

Wheat plants can combat abiotic stresses *via* activate the antioxidant defense system to maintain the cell structural integrity and alleviate the severely effect of oxidative damage [49]. Also, they mentioned that wheat plants have ability to alter the activity of various antioxidant enzymes.

From Figure 1, it can be noticed that superoxide dismutase (SOD), phenylalanine ammonia lyase (PAL), ascorbate peroxidase (ASP) and glutathione reductase (GR) activities were raised under salt stress. SOD and PAL activities were showed more increment in activities with increasing the concentration of NaCl. No differences were recorded in guaicol peroxidase (POD) and poly phenol oxidase (PPO) between unstressed plants and others subjected to NaCl (2000 ppm) and insignificant reduction in PPO were showed with increasing NaCl salinity to 4000 ppm in compared to control salinity. These results were agreement with Heidari [50] who showed that POD and PPO activities decreased under salt stress and Sairam *et al.* [1] who found an increase in the activities of SOD,

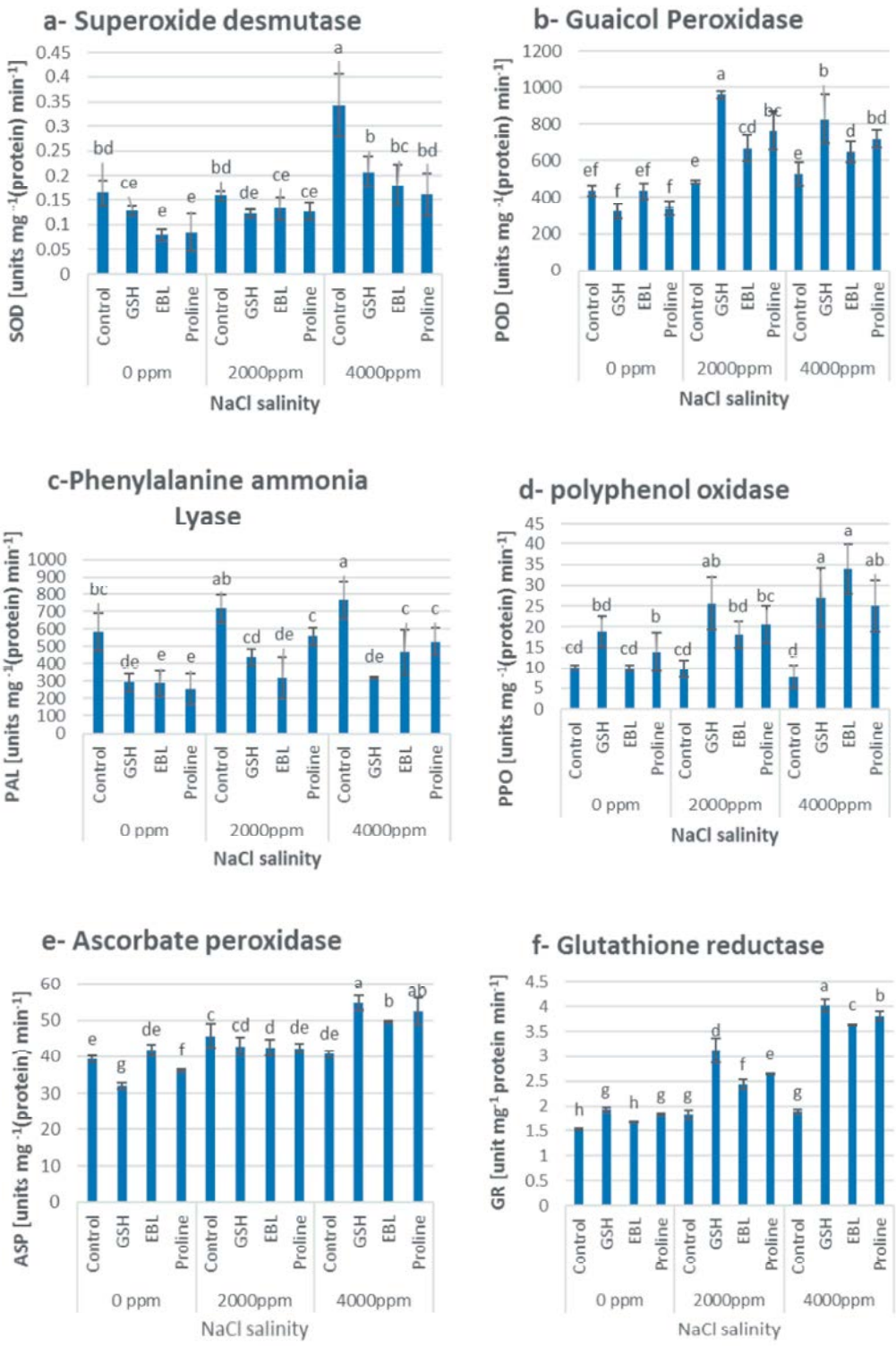

Fig. 1: Effect of foliar application of reduced glutathione (GSH), 24-epibrassinolide (EBL) and proline on SOD, POD, PAL, PPO, ASP and GR activities in wheat flag leaf under salt stress, values represent mean ± SD of two seasons; three replicates in each; values marked with different superscript letters are significantly different at P = 0.05 level; Duncan's multiple range test

ASP and GR with saline condition. SOD constitutes a frontline in scavenging superoxide radicals (O_2^-) and providing its dismutation into H_2O_2 which is in turn detoxified through ascorbate-glutathione cycle (AsA-GSH cycle) or scavenged by catalase (CAT) or other several peroxidases into water and dioxygen [51]. Ushimaru [52] reported that POD, PPO, ASP and GR are crucial antioxidant enzymes following SOD to complete neutralization of hydrogen peroxide. Ibrahim and Abdellatif [53] showed a significant increase in ASP activity with increasing stressful condition. In the present study, foliar spraying with EBL, GSH and proline increased the activity of PPO, ASP and GR enzymes activity under salt stress. Exogenous application with EBL increased the activity of POD to become equal to comparable plants under non-stressful condition, while GSH increased the activity of POD, SOD under 4000 ppm NaCl. Under saline condition both POD and PPO activities increased with foliar spraying of biostimulants. In the same time, there is a reduction in SOD and PAL activities in sprayed plants under stress condition. The activities of POD, PPO ASP and GR antioxidant enzymes with exogenous GSH, EBL and proline supplemented plants under salt stress were highly more than the activity within non-sprayed. Enzymatic antioxidants include guaicol peroxidase (POD), superoxide dismutase (SOD), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), ascorbate peroxidase (ASP) and glutathione peroxidase (GR) are represented in subcellular compartments, i.e., chloroplasts, mitochondria, peroxisomes in addition to cytosol as a detoxifying system in plant cells [9, 54]. In this regard, Zhang *et al.* [15] stated that exposed seedlings to NaCl stress exhibited significant changes in the levels of antioxidant enzymes. Also, they showed that seedlings obtained from seeds treatment with 24-epibrassinolide significantly increased the activities of antioxidant enzymes under salinity. Despite these controversies Nahar *et al.* [46] found that the activities of SOD, CAT, ASP and GR increased under salinity condition. Furthermore, they reported that exogenous application of GSH decreased H_2O_2 content and O_2^- generation rate. Zhang *et al.* [55] mentioned that exogenous proline supplemented at a low concentration increased the plant tolerance against salt stress by detoxifying the regenerated ROS by osmoregulation rather than improving the antioxidant enzymes defense system. On the other hand, Hong *et al.* [56] reported that, the protective role of proline as a ROS scavenger is more efficient than its role as an osmoregulator. Also, they reported that proline significantly increasing the activity of POD in plants exposed to salinity. May and Leaver [57] imposed that under oxidative stress, the existing of reduced glutathione (GSH) converted into oxidized glutathione (GSSG) and the biosynthesis of GSH is stimulated. These results of (GSH-GSSG) estimation with increasing in GSH synthesis is a response of drastic reduction of GSH concentration due to the reaction of GSH with the generated oxyradicals within simultaneous increment in GSSG as an induction of oxidative stress. So exogenous application of GSH regulate the redox state of the glutathione pool (GSH/GSSG) and ROS to have an important role as environmental sensors and/or modulators of global patterns in development and defense [58]. Exogenous proline application efficiently regulated the osmotic potential and decreased the toxic effect of salinity by activating the antioxidant enzyme system [59]. Hoque *et al.* [60] reported that, proline upregulates the activity of enzymes linked in ascorbate-glutathione cycle due to elevating the activities of ASP and GR enzymes which are the components of the cycle and enhancing the cytoplasmic acidosis by maintaining NADP/NADPH ratio compatible with the cytoplasmic metabolism. Ascorbate peroxidase (ASP) and glutathione reductase (GR) are the key of the main antioxidant enzymes linked ascorbate-glutathione cycle (AsA-GSH cycle) which plays a main role in ROS scavenging through H_2O_2 detoxification system. ASP is responsible for reducing H_2O_2 into water by utilizing ascorbate "derived from a chloroplast during water-water cycle" as a specific electron donor during oxidative and osmotic stress [61, 9, 7]. GR regenerate reduced glutathione (GSH) due to NAD(P)H- depended reaction as an electron donor to dismutate oxidized glutathione (GSSG) into reduced glutathione (GSH) via AsA- GSH cycle [11]. So, Yousuf *et al.* [62] indicated that GR maintains a highly ratio of GSH / GSSG which sustains the reduced form of GSH that provide plants a high tolerance to oxidative stress. The balance between SOD antioxidant enzyme and other H_2O_2 scavenging enzymes in the cell determine the steady- state level of O_2^- and H_2O_2 [63].

From these obtained results, it can be concluded that ameliorating the adverse effects of salinity on the growth and productivity of wheat plants can be achieved by using glutathione, 24-epibrassinolide and proline due to increasing the activities of antioxidant enzymes under salt stress conditions.

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