

## Glycine-betaine induced salinity tolerance in maize by regulating the physiological attributes, antioxidant defense system and ionic homeostasis

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### Abstract

The plants are exposed to different abiotic stresses, including the salinity stress (SS) that negatively affect the growth, metabolism, physiological and biochemical processes. Thus, this study investigated the effect of diverse levels of foliar-applied GB (0 control, 50 mM and 100 mM) on maize growth, membrane stability, physiological and biochemical attributes, antioxidant enzymes and nutrients accumulation under different levels of SS (i.e., control, 6 dS m<sup>-1</sup>, 12 dS m<sup>-1</sup>). Salt stress diminished the root and shoot length, root and shoot biomass, chlorophyll contents, photosynthetic rate (*Pn*), stomatal conductance (*gs*), relative water contents (RWC), soluble proteins (SP) and free amino acids; (FAA); and increased activities of antioxidant enzymes, electrical conductivity (EC) and accumulation of malondialdehyde (MDA), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), Na<sup>+</sup> and Cl<sup>-</sup> ions. GB application significantly increased root and shoot growth, leaves per plant, shoots length, chlorophyll contents, *gs*, *Pn* and membrane stability by reducing MDA and H<sub>2</sub>O<sub>2</sub> accumulation. Moreover, GB also increased the SP, FAA accumulation, activities of antioxidant enzymes and Na<sup>+</sup> and Cl<sup>-</sup> exclusion by favouring Ca<sup>2+</sup> and K<sup>+</sup> accumulation. In conclusion, the foliar-applied GB increased *Pn*, *gs*, ant-oxidants activities, and accumulation of SP and FAA; and reduced the accretion of Na<sup>+</sup> and Cl<sup>-</sup> by favouring the Ca<sup>2+</sup> and K<sup>+</sup> accretion which in turns improved growth under SS.

**Keywords:** antioxidants; growth, glycine-betaine; nutrients accumulation; photosynthesis; salt stress; soluble proteins

**Abbreviation:** APX: ascorbate peroxide, AsA: ascorbic acid, Ca: calcium, Cl<sup>-</sup>: chloride, CAT: catalase, DAP: di-ammonium phosphate, EC: electrical conductivity, FAA: free amino acids, FW: fresh weight, H<sub>2</sub>O<sub>2</sub>:

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hydrogen peroxide, K: potassium, KI: potassium iodide, LPP: leaves per plant, MDA: malondialdehyde, mM: milli molar Na: sodium, OS: oxidative stress, Pn: photosynthetic rate, POD: peroxidase, ROS: reactive oxygen species, RL: root length, RWC: relative water contents, gs: stomatal conductance, SL: shoot length, SP: soluble protein, SS: salinity stress, TCA: trichloroacetic acid, WUE: water use efficiency.

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## Introduction

Salinity stress (SS) is one of the critical factors of abiotic stress that substantially diminishes crop growth, development, and production (Mbarki *et al.*, 2018; Seleiman and Kheir, 2018; Seleiman, 2019; Seleiman *et al.*, 2020). Globally, more than 20% cultivated lands and 33% irrigated agriculture lands are facing salinity stress problems. Additionally, it has been predicted that more than 50% of world arable lands will be salinized by the end of 2050 (Jamil *et al.*, 2011; Shrivastava and Kumar, 2015). The effects of salt stress have been reported in most of the world's crops (Seleiman and Kheir, 2018; Al-Ashkar *et al.*, 2020), including maize, which is the most imperative staple food of many nations. Salinity stress has a drastic effect on plant morphology and physiology due to the physiologically mediated osmotic stress. This can result imperfections in plant water relations and ionic balance that eventually leads to ionic toxicity of plant metabolic processes (Semida *et al.*, 2016; Al-Ashkar *et al.*, 2019; Seleiman *et al.*, 2020). Moreover, SS can induce the overproduction of reactive oxygen species (ROS), which triggers the oxidative stress (OS) in different plant tissues, and causes chlorophyll degradation and oxidation of significant molecules including lipids, proteins and DNA (Radi, 2018).

Additionally, elevated SS can reduce the photosynthetic efficiency, plants growth and productivity and can induce the accumulation of toxic ions (Abd El-Mageed *et al.*, 2017; Taha *et al.*, 2021). Therefore, to mitigate the negative impacts of SS, plants have different mechanisms to protect themselves from the effects of OS by inducing the activities of various enzymatic and non-enzymatic antioxidants (Semida *et al.*, 2014). Furthermore, plants can accumulate different solutes and have ion homeostasis mechanisms that can protect them from the negative effects of SS (Zhu, 2002). Therefore, to support the endogenous plant mechanisms, different efforts have been made to mitigate the negative impacts of SS. Among these, foliar application of osmo protectants is an important approach to ensure the crops survival and improve the production in salt-affected soils (Semida *et al.*, 2017; Rady *et al.*, 2018; Seleiman *et al.*, 2020).

The variable osmolytes, including the proline, soluble sugar, amino acids and GB are endogenously produced in plants which protect them from salt- and heavy metals stresses (Hoque *et al.*, 2007; Aamer *et al.*, 2018; Ali *et al.*, 2020; Seleiman *et al.*, 2020; Seleiman *et al.*, 2021). The accumulation of these substances also maintains the subcellular structures and diminishes the oxidative effects of ROS in high salt stress (Slama *et al.*, 2015). GB is synthesized in plants as a result of SS; however, GB synthesis and accumulation largely depends upon the degree of SS tolerance (Sakamoto and Murata, 2000). Plants can decrease the accumulation of GB; therefore, exogenous applied GB can improve SS tolerance in plants (Kaya *et al.*, 2013; Alasvandyari *et al.*, 2017). GB can maintain the osmotic regulation and support the diverse transporters for the optimum functioning under SS (Gadallah, 1999).

GB can be applied to the crops as foliar spray and seed priming to mitigate the adverse effect of different stresses (Ali *et al.*, 2020). The foliar application of GB can improve the growth and activity of antioxidant enzymes which can mitigate the adverse effects of SS (Ma *et al.*, 2006; Alasvandyari *et al.*, 2017). The GB application also can improve the stomatal conductance (gs), chlorophyll contents, RWC, membrane stability and water use efficiency (WUE) which can cause an improvement in crop performance under SS conditions (Rady *et al.*, 2018). Moreover, the GB differentiates Na<sup>+</sup> against K<sup>+</sup> under SS conditions (Mansour 1998), and improves the root cells' efficacy to accumulate more Na<sup>+</sup> in plants grown under SS conditions (Rahman *et al.*, 2002). Additionally, GB application can improve the K<sup>+</sup> accumulation, and reduce Na<sup>+</sup> and malondialdehyde (MDA) accumulation in plant under SS conditions (Hu *et al.*, 2012), favouring overall plant growth. Maize is

an imperative crop, and it is globally cultivated for food, feed and bioenergy purposes (Seleiman *et al.*, 2013). However, the SS can have devastating impacts on maize growth and production.

The mechanisms lying behind the reduction of SS effects as a result of GB application in maize are still poorly understood. Therefore, they should be adequately elucidated. In the current investigation, we hypothesized that GB application would improve the salt tolerance in maize crop by improving antioxidant system activities, accumulation of  $K^+$  and  $Ca^{2+}$ , and different physio-biochemical processes. Thus, the present investigation was performed to investigate the GB's effects on maize growth, photosynthetic attributes, ROS, antioxidant activities, and ions accumulation under SS conditions.

## Materials and Methods

### *Experimental location*

The pot study was performed to evaluate foliar-applied GB's impact on maize crop performance under different SS at the Agriculture Faculty, University of Agriculture, Faisalabad, Pakistan. The upper 1-10 cm layer of the soil was properly collected from the field, adequately sieved and finally stored in the laboratory. The various soil, physio-chemical properties were determined using the standard methods as described by Homer and Pratt (1961). The soil was loamy with pH 7.6, EC 1.04 dSm<sup>-1</sup>, organic matter 0.82%, available N 0.035%, available P 11 mg kg<sup>-1</sup> and available K 181 mg kg<sup>-1</sup>.

### *Imposition of salinity and experimental treatments*

After adding the distilled water (dH<sub>2</sub>O); the soil was well mixed and left for 2h to reach the equilibrium. Moreover, the extract was attained by filtering the soil paste with filter paper and saturation % was calculated as given by the formula:

$$\text{Saturation (\%)} = \frac{\text{Loss in soil weight on drying}}{\text{Weight of soil after drying}} \times 100$$

The experiment consisted of different SS levels (i.e., control, 6 dS m<sup>-1</sup> and 12 dS m<sup>-1</sup>) and foliar application of GB (i.e. control, 50 mM and 100 mM). The various SS treatments were applied to the soil before sowing. The concentration of salts required to attain each SS level was calculated by given below formula:

$$\text{NaCl required } \left(\frac{g}{kg}\right) = \frac{\text{TSS} \times 58.5 \times \text{Saturation (\%)}}{100 \times 1000}$$

The plastic pots having a capacity of 8 kg with a diameter of 28 cm was used for the study. In total 27 pots were used for the study. The soil for each pot was taken, and salt was appropriately mixed in soil. Next, the pots were filled with the soil. Moreover, ten seeds of maize were sown in each pot. Uniform irrigation was given to each pot when water was required to avoid the drought conditions. To enhance the nutrient uptake, urea and DAP (di-ammonium phosphate) were used twice during the experiment. After 15 days, GB was applied as a foliar spray with handheld sprayer according to the different treatments, whereas water was sprayed into control pots.

### *Growth parameters*

After 15 days of GB application, five plants from each pot were uprooted, and roots were separated from the base. The length of root and shoots was measured and averaged. Similarly, the root and shoot's fresh weight was taken and averaged. Then, roots and shoots were oven-dried to determine the dry weight. In addition, the leaves of the same plants were counted and averaged.

### *Determination of relative water contents*

RWC was determined after 10 days of the GB foliar spray according to Mostofa and Fujita (2013). Firstly, leaf samples were weighed (FW) and then submerged in H<sub>2</sub>O in a disposable cup for 24 hours. The

excess water from the samples was removed with a paper towel. Then, the turgid water (TW) was immediately determined. Afterwards, samples were placed in the oven for 48 h at 70 °C, and then dry weight was recorded. Finally, the leaf RWC was determined by the following formula:

$$RWC (\%) = \frac{FW - DR}{TW - DR} \times 100$$

#### *Electrical conductivity*

For electrical conductivity (EC): fresh leaf samples were taken and washed with dH<sub>2</sub>O to remove contamination. The leaves were placed in stopper vials containing 10 mL dH<sub>2</sub>O and incubated (25 °C) on a rotary-shaker. First, EC (E<sub>1</sub>) of the solution was recorded after 24 hours. Then, samples were autoclaved for 20 min at 120 °C, and last EC (E<sub>2</sub>) was recorded upon equilibrium at 25 °C. The EC was determined using a given equation:

$$E.C = \frac{E1}{E2} \times 100$$

#### *Chlorophyll and carotenoid contents*

Chlorophyll a, b and carotenoid were recorded according to Lichtenthaler (1987). The leaf samples were washed to remove the contaminations before extraction. After that, one g of the leaves was taken and homogenized in a 90% acetone using the mortar and pestle. The extracts were centrifuged. The absorbance was recorded at 663, 645, 470 nm using a spectrophotometer.

#### *Malondialdehyde and H<sub>2</sub>O<sub>2</sub> determination*

Malondialdehyde was recorded according to Rao and Sresty (2000). About 0.5 g frozen sample was homogenized in a 5 mL of trichloroacetic acid (TCA), and then was centrifuged for 15 min at 12,000 on 4 °C. The mixture containing supernatant was added with 5 mL of thiobarbituric acid (TBA) and heated at 100 °C for 30 min. Then it was quickly cooled at 40 °C in ice baths. After that, the supernatant value was read at 532 and 600 nm, and MDA contents were expressed in μmol/g FW. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) concentration was recorded according to Velikova *et al.* (2000). Plant sample (0.5 g) was ground in a 5 mL of TCA and was centrifuged. Then, it was placed into the test tube, and 1M potassium iodide (KI) and 100 μL potassium phosphate buffer was added. It was maintained for 30 min at the room temperature. Then, the absorbance was measured at 390 nm and later was expressed as μmol/g FW basis.

#### *Antioxidant enzymes*

The catalase (CAT) contents were determined by the described method of Aebi (1984). The test tube contained a 100 μL of H<sub>2</sub>O<sub>2</sub> (5.9Mm) and 1000 μL buffer along with the 100 μL of plant extract. The absorbance of samples was recorded at 240 nm using spectrophotometer. Peroxidase (POD) was determined by the procedure of Zhang (1992). The combinations of reactants containing 100 μL extract enzyme + 2700 μL of 50 mM potassium buffers + 100 μL guaicol and H<sub>2</sub>O<sub>2</sub> 100 μL was used for the analysis. The plant sample (0.5 g) was homogenized using 5-mL potassium phosphate buffer (50 mM) with 7.0 pH under ice-cold conditions and centrifuged at 15,000. The absorbance of the extract was recorded at 470 nm for 2 min. For ascorbate peroxidase (APX) determination, the mixture contained 100- μL enzymes extracts, 100 μL ascorbate (7.5-mM), 100 μL H<sub>2</sub>O<sub>2</sub> (300 mM), and 2.7 mL potassium buffer (25 mM), 2-mM CA having 7.0 pH. The activity of APX was determined at 290 nm wavelength using spectrophotometer. Ascorbic acid (AsA) was determined by the described method of Mukherjee and Chouduri (1983). The plant sample (0.5 g) was standardized at 5 mL of 10% tri-chloroacetic acid solution. The samples were centrifuged at 8000 rpm for 10 min. Then, 0.5 mL of DTC reagent was added in 2 mL supernatant and incubated for 3 h cooled. Then, 2 mL of sulfuric acid was added as dropwise and slightly shaken. The mixture was kept for 30 min at 30 °C, and the absorbance was recorded at 520 nm using spectrophotometer.

#### *Determination of total soluble protein and amino acids*

Total SP was determined by the method published in Bradford (1976). Samples of leaves (0.5 g) were ground with 5 mL phosphate buffer (pH 7.8) and centrifuged at 15000 rpm for 15 min. Then, 1 mL of plant extraction was transferred in test tubes with 3 mL Bradford reagent, and were left for 15 min at room temperature. The concentration of the total SP was recorded at 595 nm using spectrophotometer. Total free amino acid (FAA) was analyzed using method of Hamilton and Van Slyke (1943). Then, 1 mL extract was taken and placed into the test tubes with 1 mL of ninhydrin and pyridine. The samples were placed into the water bath for 30 min at 90 °C. Afterwards, their volume was maintained to 25 mL by adding dH<sub>2</sub>O, and the total FAA concentration was recorded 570 nm using spectrophotometer.

#### *Determination of ion accumulation*

The plants' samples (roots and leaves) were washed with dH<sub>2</sub>O to remove any of the contamination. Then, plant samples were oven-dried (65 °C) and milled to get the powder. The powdered samples (0.5 g) were digested with 1:2 of HCL and HNO<sub>3</sub> for 10 min at 180 °C, filtered and diluted with a distilled water. The Na<sup>+</sup>, Cl<sup>-</sup>, K<sup>+</sup> and Ca<sup>2+</sup> concentrations were analyzed using flame photometer (Jones and Case, 1990).

#### *Experimental design and data analysis*

The study was performed in a completely randomized design with the factorial arrangement, and each treatment was replicated three times. The data were statistical analyzed using two-way ANNOVA and least significant difference test (LSD) was employed to determine difference between different treatments at  $P \leq 0.05$  (Steel *et al.*, 1997). The PCA and heat map were made using R-studio software.

## **Results**

#### *Growth and biomass accumulation*

The different SS levels resulted in a significant reduction in growth attributes than the control treatment (Table 1). The reduction in growth traits was noted under all SS levels, but the maximum reduction was recorded with the highest SS level (i.e. 12 dS m<sup>-1</sup>) (Table 1). However, the foliar-applied GB appreciably increased the growth traits of plants grown under all SS levels and control treatment. The maximum root length (RL; 13 cm) and shoot length (SL; 57.7 cm) was noticed in plants grown under the control treatment with the foliar applied GB of 100 mM, and the lowest RL (8.6 cm) and SL (44.4 cm) were noticed with the highest SS level without GB application (Table 1). Similarly, the root and shoot biomass were decreased with increasing SS levels. However, the highest foliar-applied GB (i.e. 100 mM) markedly enhanced root and shoot fresh and dry biomass of plants grown under all SS levels (Table 1). The maximum LPP (6) was recorded from plants grown in control with the application of 100 mM GB, and lowest LPP (3) was recorded from plants grown with the highest SS level (i.e. 12 dS m<sup>-1</sup>) without foliar-applied GB (Table 1).

#### *Photosynthetic attributes*

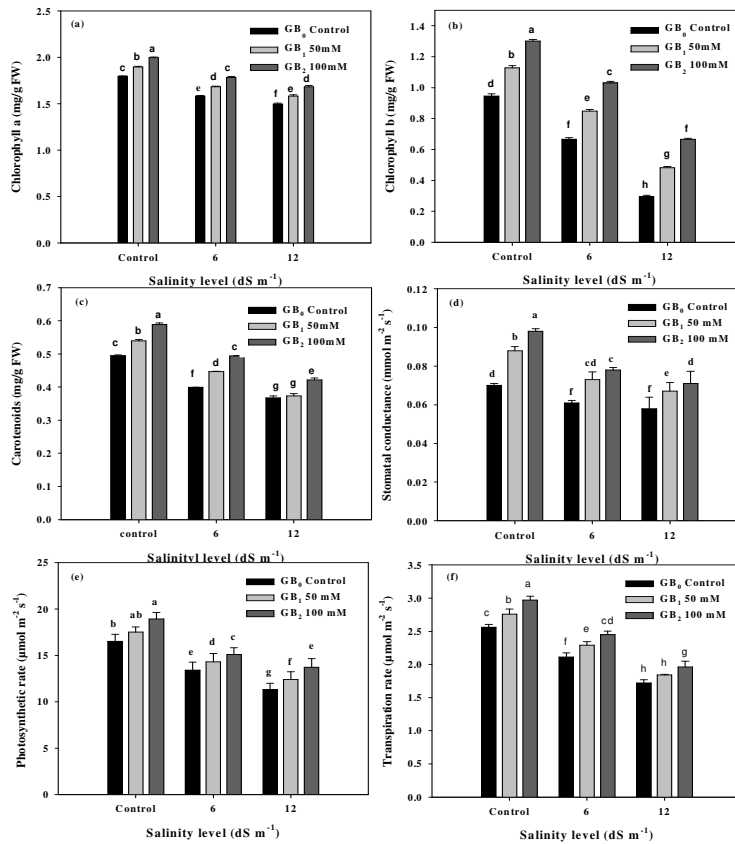
Salt stress significantly decreased the chlorophyll and carotenoid contents (Figure 1). Chlorophyll a content was decreased by 14% under the 6 dS m<sup>-1</sup> and by 21% under the 12 dS m<sup>-1</sup> SS compared to those grown in control treatment. Moreover, foliar application of GB with 50 mM and 100 mM increased the chlorophyll a content by approximately 9% and 16% under 12 dS m<sup>-1</sup> SS, respectively (Figure 1). The similar response was observed for chlorophyll b. For example, the reductions in chlorophyll b contents were 16% and 32% in plants grown under SS of 6 dS m<sup>-1</sup> and 12 dS m<sup>-1</sup>, respectively (Figure 1). The carotenoid also was decreased by 11% and 19% at 6 dS m<sup>-1</sup> and 12 dS m<sup>-1</sup> SS level, respectively. Moreover, GB application at 100 mM considerably increased the carotenoid contents compared to the application of 50 mM GB and control treatment (Figure 1). The SS significantly reduced *g<sub>s</sub>*, *P<sub>n</sub>* and transpiration rates. However, GB application remarkably increased

the gs, *Pn* and transpiration rates in plants grown with different SS treatments (Figure 1). The gs and *Pn* was decreased by 31% and 41% at 12 dS m<sup>-1</sup> SS level, respectively. However, GB application of 100 mM increased the gs and *Pn* by 21% and 22% under 12 dS m<sup>-1</sup>, respectively (Figure 1). The transpiration rate was decreased by 21% and 50% in 6 dS m<sup>-1</sup> and 12 dS m<sup>-1</sup>, whereas the GB application with 100 mM increased the transpiration rate by 16% and 14% in both aforementioned SS levels, respectively (Figure 1).

**Table 1.** Effect of GB application on growth attributes of maize grown under salt stress

Treatments	RL (cm)	SL (cm)	RFW (g)	SFW (g)	RDW (g)	SDW (g)	LPP	
Control	Control	10.33±1.31cd	51.66±3.86bc	3.72±0.09a	11.36±0.39b	1.66±0.02	2.31±0.05cd	4.00±0.81bc
	50mM	12.00±0.21ab	53.66±1.25b	3.75±0.04ab	11.62±0.47ab	1.71±0.01	2.40±0.04bc	5.00±0.81ab
	100mM	13.00±0.81a	57.66±2.62a	3.82±0.08a	12.94±0.13a	1.80±0.01	2.53±0.07a	6.00±0.81a
6dSm <sup>-1</sup>	Control	10.46±0.38cd	47.00±1.63de	3.35±0.01e	8.81±0.79cd	1.46±0.02	2.22±0.02def	3.00±0.81cd
	50mM	11.36±0.32bc	49.66±1.25cd	3.44±0.05d	11.45±1.15ab	1.55±0.03	2.34±0.05bcd	4.00±0.81bc
	100mM	11.53±0.16bc	49.00±1.63cd	3.63±0.04c	11.58±1.21ab	1.63±0.02	2.44±0.04ab	5.00±0.81ab
12dSm <sup>-1</sup>	Control	8.63±0.26e	44.44±1.70e	3.04±0.02g	8.67±0.94d	1.35±0.01	2.10±0.08f	2.00±0.81d
	50mM	9.66±0.47de	48.66±2.05cd	3.18±0.05f	8.37±0.46d	1.44±0.02	2.15±0.03ef	3.00±0.81cd
	100mM	10.66±0.33cd	44.33±2.05e	3.36±0.00e	10.25±0.30bc	1.56±0.01	2.25±0.10de	4.00±0.81bc

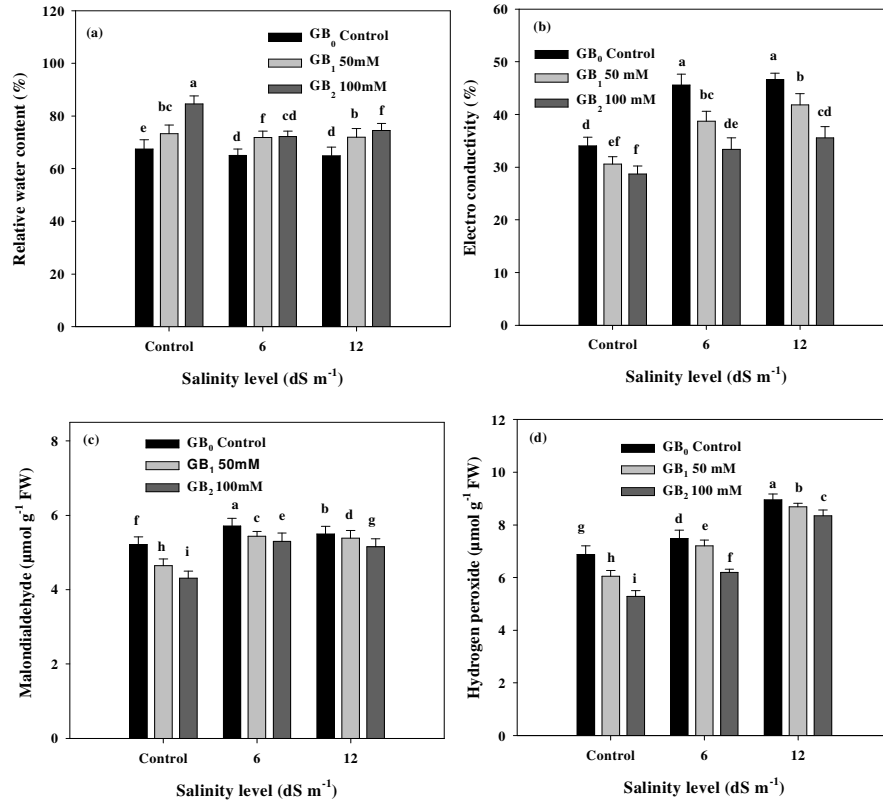
The values shown in the table contain the mean value of three replications ± S.E and different values show significant differences (P ≤ 0.05) according to LSD test. RL: root length, SL: shoot length, RFW: root fresh weight, SFW: shoot fresh weight, RDW: root dry weight, SDW: shoot dry weight, LPP: Leaves per plant



**Figure 1.** Effect of GB on (a) chlorophyll a, (b) chlorophyll b, (c) carotenoid, (d) stomatal conductance, (e) photosynthetic rate and (f) transpiration rate under salinity stress. Error bars show the mean value of three replications ± S.E and different values shows significant differences (P ≤ 0.05) according to LSD test.

*Relative water content and electrical conductivity*

RWC was reduced by 31% and 36% at 6 dS m<sup>-1</sup> to 12 dS m<sup>-1</sup>, respectively. The exogenously applied GB (i.e. 50 mM and 100 mM) markedly increased the RWC; however, the maximum increase in RWC was reported by 100 mM GB application that increased the RWC by 29% at 12 dS m<sup>-1</sup> (Figure 2). EC of maize plants substantially increased with the increment in salt stress. An increase of 29% and 37% in EC was noticed under 6 dS m<sup>-1</sup> and 12 dS m<sup>-1</sup>. Additionally, the exogenous application of GB at 50 mM and 100 mM significantly decreased the EC by 12% and 22% under 12 dSm<sup>-1</sup> SS, respectively (Figure 2).



**Figure 2.** Effect of GB on (a) RWC, (b) EL, (c) MDA and (d) H<sub>2</sub>O<sub>2</sub> under salinity stress. Error bars show the mean value of three replications ± S.E and different values shows significant differences (P ≤ 0.05) according to LSD test.

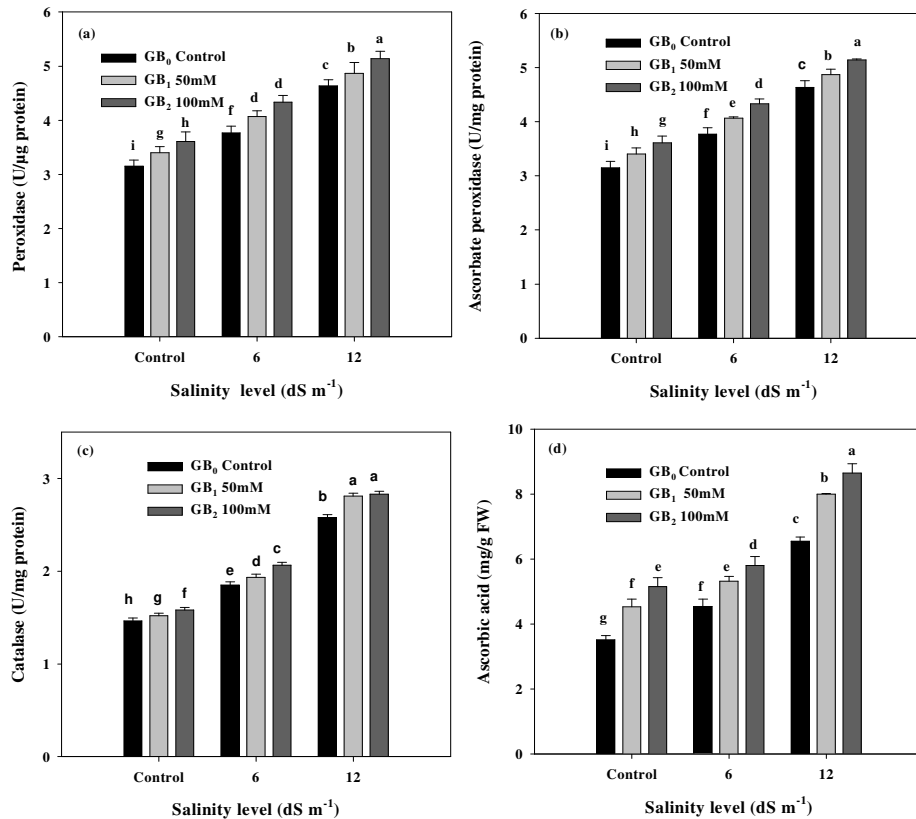
*MDA and H<sub>2</sub>O<sub>2</sub> contents*

MDA content was increased by 11% and 15%, while H<sub>2</sub>O<sub>2</sub> was increased by 21% and 38% in plants grown with 6 dS m<sup>-1</sup> and 12 dS m<sup>-1</sup>, respectively. However, the foliar applied GB reduced the MDA and H<sub>2</sub>O content (Figure 2). A reduction of 7% and 11% in MDA content, and a reduction of 6% and 9% in H<sub>2</sub>O<sub>2</sub> were observed at 50 mM and 100 mM GB at 12 dS m<sup>-1</sup>, respectively (Figure 2).

*Antioxidant enzymes*

APX, POD and CAT activities in maize plants were significantly affected by SS (Figure 3). The POD and CAT activities were enhanced in plants grown under all SS levels, whereas both rates of 50 mM and 100 mM GB substantially increased the POD and CAT activities (Figure 3). However, foliar-applied GB (i.e. 100 mM) remained at the top, and significantly increased the POD and CAT activities by 19% and 17%, respectively (Figure 6). The APX and ascorbic acid contents were increased in plants grown with 6 dS m<sup>-1</sup> and 12 dS m<sup>-1</sup> SS. Moreover, GB application with 50 mM and 100 mM significantly increased the APX and ascorbic

acid in plants grown with both levels of SS (Figure 3). Similarly, AS content was increased in SS. Moreover, GB increased the AsA content, which can be a clear indication for the increment of antioxidant activities due to GB application.



**Figure 3.** Effect of GB on the activity of (a) POD, (b) APX, (c) SOD and (d) ascorbic acid under salinity stress

Error bars show the mean value of three replications  $\pm$  S.E and different values shows significant differences ( $P \leq 0.05$ ) according to LSD test.

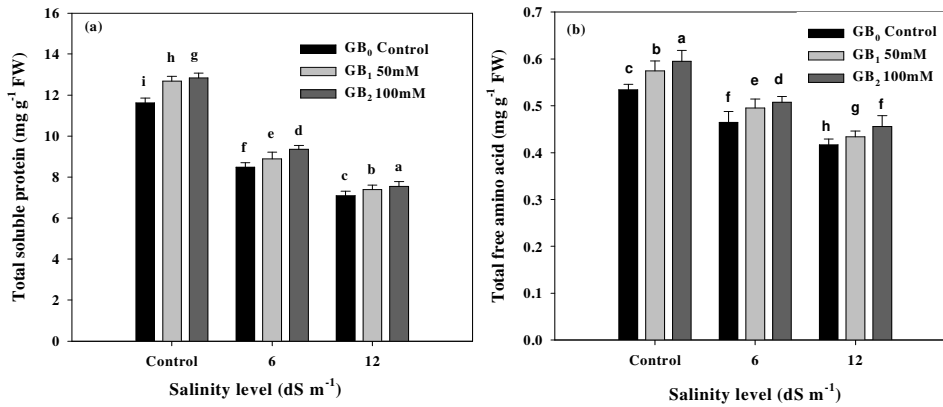
#### *Soluble proteins and amino acids*

Total SP and FAA in maize plants were significantly influenced by SS (Figure 4). The total SP was decreased by 13% and 25% under 6 dS m<sup>-1</sup> and 12 dS m<sup>-1</sup> stress condition, whereas FAA showed a reduction of 13% and 23% under the same SS (Figure 4). Both GB levels at 50- and 100-mM applications increased total SP by 11% and 16% under SS 12 dS m<sup>-1</sup> compared to control, respectively (Figure 4). The similar response was observed for FAA, and was increased by 9% and 17% with 50 mM and 100 mM GB application, respectively. Anthocyanin was decreased by 13% at 6 dS m<sup>-1</sup> and by 21% at 12 dS m<sup>-1</sup> SS (Figure 4).

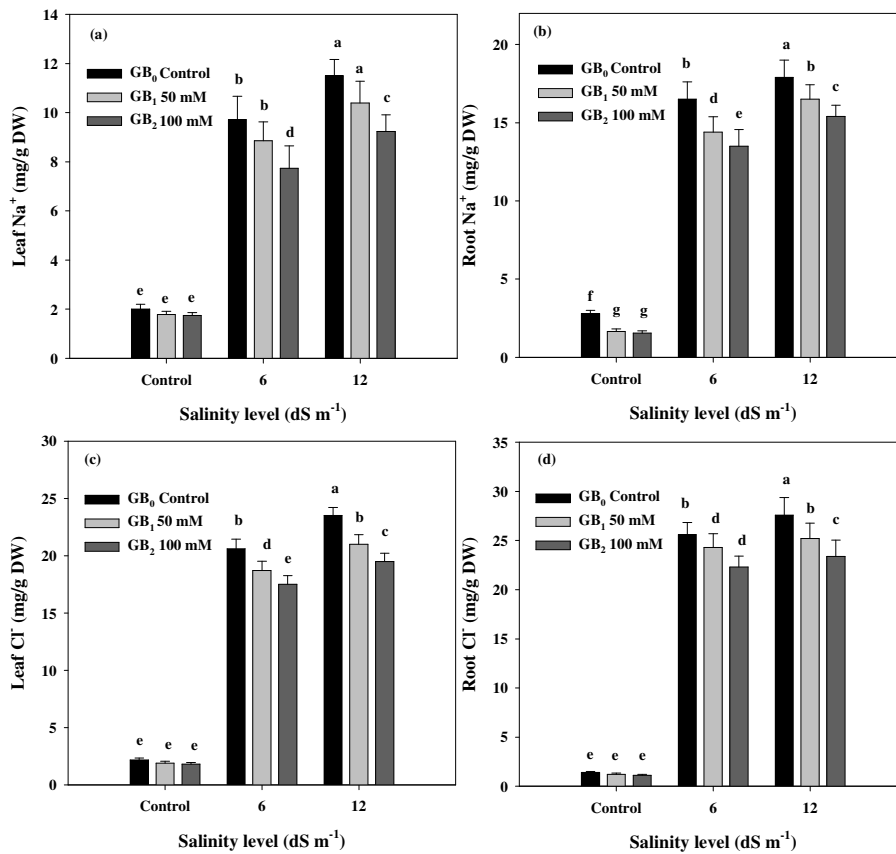
#### *Ion accumulation*

The variable SS levels and GB application (50 mM and 100 mM) had significant differences for the root and leaf Na<sup>+</sup>, Cl<sup>-</sup>, K<sup>+</sup> and Ca<sup>2+</sup> contents (Figure 5 and 6). Na<sup>+</sup> and Cl<sup>-</sup> in roots and leaves was significantly increased with increasing SS levels (Figure 5), whereas the K<sup>+</sup> and Ca<sup>2+</sup> contents were decreased with increase in SS levels (Figure 5). Moreover, both levels of foliar-applied GB significantly decreased the Na<sup>+</sup> and Cl<sup>-</sup> accumulation, however, the maximum reduction in Na<sup>+</sup> and Cl<sup>-</sup> accumulation was recorded with 100 mM GB under both SS levels (Figure 5). Additionally, both GB application levels increased the accumulation of K<sup>+</sup> and Ca<sup>2+</sup> in maize roots and shoots (Figure 6). However, 100 mM GB remarkably increased the K<sup>+</sup> accumulation

by 13% and 39%, and increased the  $\text{Ca}^{2+}$  accumulation by 55% and 37% in roots and leaves under  $12 \text{ dS m}^{-1}$  SS, respectively (Figure 6).



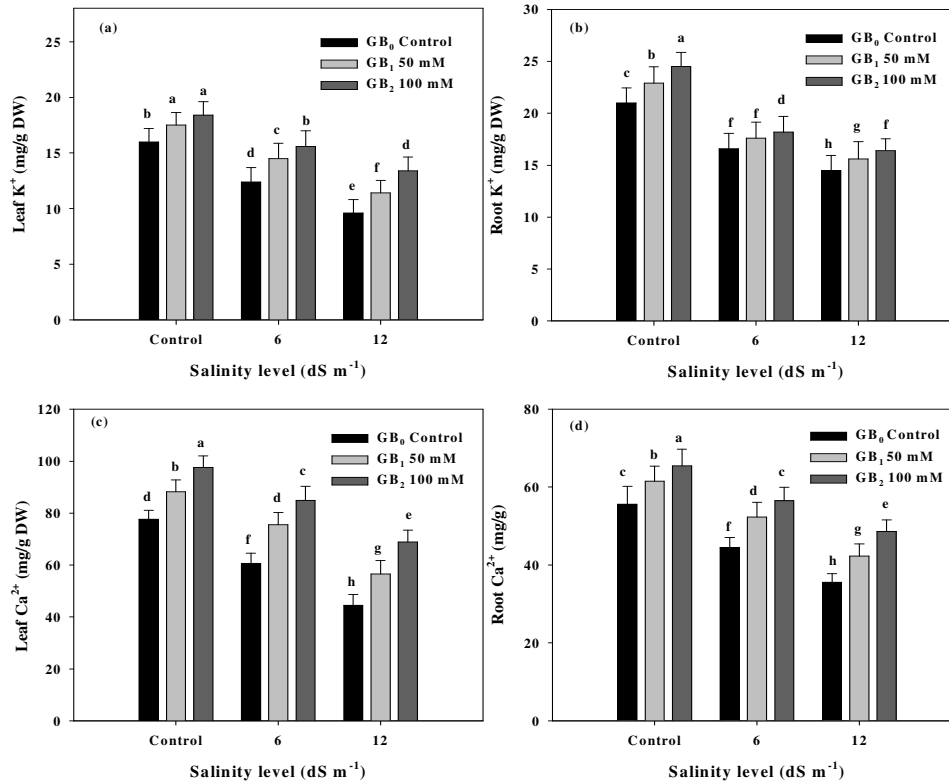
**Figure 4.** Effect of GB on (a) soluble proteins and (b) free amino acids under salinity stress. Error bars show the mean value of three replications  $\pm$  S.E and different values show significant differences ( $P \leq 0.05$ ) according to LSD test.



**Figure 5.** Effect of GB on (a) leaf Na<sup>+</sup>, (b) root Na<sup>+</sup>, (c) leaf Cl<sup>-</sup> and root Cl<sup>-</sup> under salinity stress. Error bars show the mean value of three replications  $\pm$  S.E and different values show significant differences ( $P \leq 0.05$ ) according to LSD test.

*Principal component analysis (PCA)*

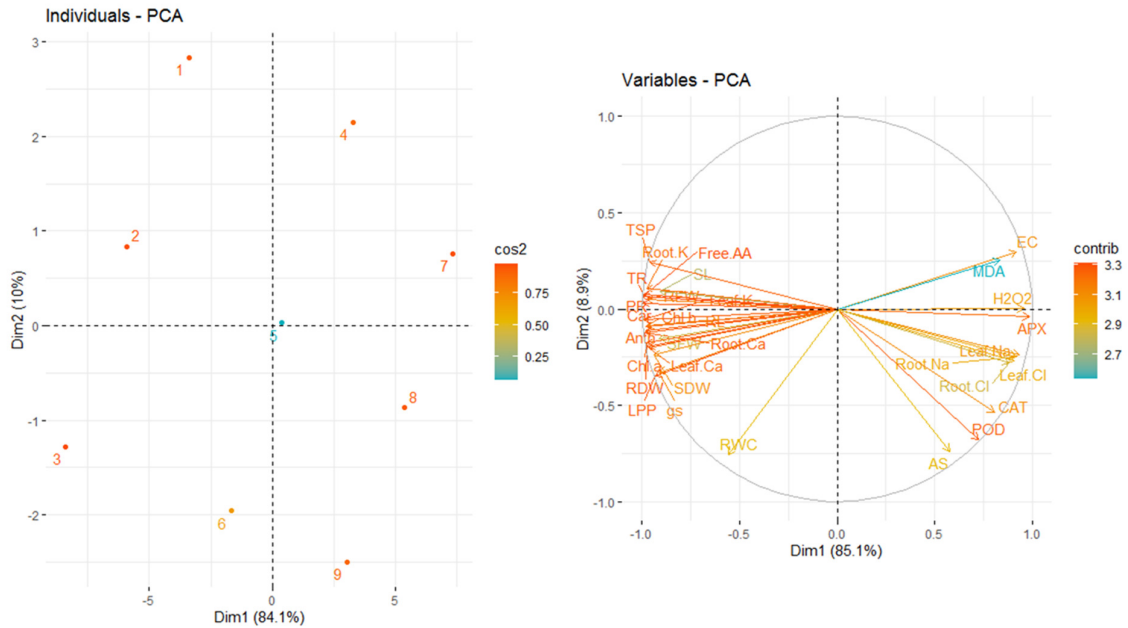
The data set were subjected to PCA for checking the relationship among the treatments and different parameters. The two components (i.e. PC1 and PC2) showed a 95% total variance in which PC1 had a share of 85.1%, and PC2 had a share of 8.9% (Figure 7). The SS at 12 dS m<sup>-1</sup> had more destructive effects; likewise, GB applied at 100 mM significantly ameliorated the effects of SS compared to the control and 50 mM GB treatments. The first group of variables of PC1 indicated the positive correction, whilst the second group of variables with PC2 indicated the negative relationship.



**Figure 6.** Effect of GB on (a) leaf K<sup>+</sup>, (b) root K<sup>+</sup>, (c) leaf Ca<sup>2+</sup> and root Ca<sup>2+</sup> under salinity stress. Error bars show the mean value of three replications  $\pm$  S.E and different values shows significant differences ( $P \leq 0.05$ ) according to LSD test.

**Discussion**

Salinity stress is one of the significant problems that can affect field crops in all climates worldwide, which has the negative impacts on the crop productivity (Seleiman *et al.*, 2018; Seleiman *et al.*, 2020). In the present study, different levels of SS negatively affected the growth and biomass production of maize (Table 1). SS adversely affect the water absorption by the roots which can cause drought/osmotic stress due to the ion toxicity. The nutritional imbalances including the reduction in K<sup>+</sup> absorption is considered as an imperative osmo-protectant for plants to face the abiotic stress (Taamalli *et al.*, 2004; Taha *et al.*, 2020). The salinity stress induced the reductions in the water and nutrients absorption diminish assimilates production, which resulted in a significant reduction in the root and shoot growth and biomass accumulation (Table 1).



**Figure 7.** Scores (left) and loading plots (right) of principal component analysis (PCA) on diverse studied traits of maize

The separation of scores plots (1-9) representing the treatments. RWC: relative water content, MDA: malondialdehyde, SDW: shoot dry weight, RDW: root dry weight, gs: stomatal conductance, Car: carotenoids, FAA: free amino acids, RFW: root fresh weight, TR: transpiration rate, SL: shoot length, Anth: Anthocyanin, TSP: total soluble proteins, LPP: leaves per plant, SFW: shoot fresh weight, RL: root length, PR: photosynthetic rate, EC: electrical conductivity, APX: Ascorbate peroxidase, H<sub>2</sub>O<sub>2</sub>: hydrogen peroxide, AS ascorbic acid, POD: peroxidase, CAT: catalase

Moreover, the foliar GB application appreciable improved the seedling growth and biomass production under SS conditions (Table 1). The present increase in growth as well as biomass accumulation could be due to the ameliorating effects of GB on the process of photosynthesis under SS (Figure 1). Consequently, this can result in an increment for producing more assimilates and their translocation into growing plant parts that can favore the growth and biomass accumulation (Caparrós *et al.*, 2020). LPP was significantly affected by both SS and GB levels. The SS reduced the leaves due to the inhabited assimilates production, whilst foliar-applied GB improved the production, nutrients and water absorption (Yildirim *et al.*, 2015; Cirillo *et al.*, 2016; Caparrós *et al.*, 2020), which contributed towards the production of more leaves (Table 1).

The lowest RWC in plants indicates the loss of turgor caused by the restricted availability of water which is necessary for the cell enlargement. GB application ameliorated the decrease in RWC caused by SS (Figure 2). The GB application prevents the salinity induced reduction in K<sup>+</sup> (Meloni and Martinez, 2009). Therefore, this can indirectly mediate the water retentions in plant tissues (Hu *et al.*, 2012). The improvement in plant water relation as a result of GB applications can led to a better growth (Nawaz and Ashraf, 2007) as also noticed in the current study (Table 1). SS significantly increased the EC, accumulation of MDA and H<sub>2</sub>O<sub>2</sub>, (Figure 2), which are considered essential systems of SS induce damages (Moustakas *et al.*, 2011). SS considerably changed the membrane integrity, which can be escorted by the increase in the electrolyte leakage (EL) from the plant cells (Ahmed *et al.*, 2019). In the present study, SS significantly decreased the membrane stability accompanied by increases in EC and accumulation of MDA and H<sub>2</sub>O<sub>2</sub> contents (Figure 2).

Nonetheless, the GB application reduced SS's damaging effects and improved membrane stability as indicated by a reduction in EC and accumulation of MDA and H<sub>2</sub>O<sub>2</sub> (Figure 2). SS can cause undesirable changes in the photosynthetic efficiency and synthesis of photosynthetic pigments (Maxwell and Johnson, 2000), as also reported in the current study (Figure 1). Nonetheless, foliar feeding of GB appreciably increased

the chlorophyll and carotenoid contents in maize seedling grown in SS conditions (Figure 1). The increment in the photosynthetic pigments by GB application might be due maintenance of endogenous water availability. Additionally, GB can protect the photo-synthetic machinery under SS by stabilizing the proteins activity under SS (Hoque *et al.*, 2007). Such increase in the chlorophyll and carotenoid contents are in agreement with different authors who reported a significant increase in the photo-synthetic pigments with GB application on plants grown under SS (Sakr *et al.*, 2012). SS significantly decreased the  $g_s$ ,  $P_n$  and transpiration rates (Figure 1), whilst, foliar-applied GB improved the  $g_s$ ,  $P_n$  and transpiration in both control and SS conditions (Figure 1). The higher RWC and improved  $g_s$  (Figure 2) by GB application was responsible for the better  $P_n$  and mitigation of deleterious impacts of SS (Blum, 2017; Ahmed *et al.*, 2019). The exogenously applied GB increased the proportions of water bound in the cell owing to its hydrophilic feature, which in turns improve the turgor pressure in guard cells and thus resulting in increase in  $g_s$  (Blum, 2017). Moreover, GB application in this study increased the chlorophyll contents (Figure 1), which gives an indication that GB application can delay the senescence and increases the  $P_n$  in plants grown under stress conditions (Mahmood *et al.*, 2009; Abbas *et al.*, 2010). The increase in the accumulation of FAA in plants grown under SS is considerably a significant to improve the salt tolerance in cereals (Livia *et al.*, 2002). In this investigation, the SS reduced the accumulation of FFA; conversely the foliar applied GB (50 mM and 100 mM) appreciably improved the accumulation of FFA (Figure 3). The increase in the accumulation of FFA in plants can serve as an imperative compatible cytoplasmic solute for maintaining the osmotic balances under SS, which in turns can improve the growth (Table 1) and photosynthetic efficiency (Ranganayakulu *et al.*, 2013).

The concentration of total SP substantially decreased with increasing the SS (Figure 3). Moreover, the foliar applied GB at 100 mM significantly increased the total SP under SS. Likewise; Habib *et al.* (2012) noticed a significant increase in SP with a foliar spray of GB under SS. The SP can improve the cell turgor, stomatal conductance, CO<sub>2</sub> intake, and water uptake, resulting in a significant improvement in plants performance under SS (Habib *et al.*, 2012).

The salt stress increased ROS production (Lee *et al.*, 2001), which can cause the oxidative damages to lipids, proteins, and DNA (Apel and Hirt, 2004). The plants protect themselves from the damaging effects of SS by activating antioxidant defense system (Mittler, 2002). The exogenous GB has the protect effects on the activities of antioxidant system under SS (Hoque *et al.*, 2007). Foliar applied GB appreciably increased the activities of POD, CAT, APX and AsA under the SS (Figure 4). The foliar-applied GB alleviated the adverse impacts of SS by scavenging the ROS and protecting the antioxidant enzymes (Hoque *et al.*, 2007). Similarly, the increase in activity of CAT under SS due to GB application has been also reported in rice (Demiral and Turkan 2004). Additionally, GB also increased the APX activity in plants grown in SS which indicate its ROS scavenging role (Hasanuzzaman *et al.*, 2014) and thus resulted in better growth under SS (Table 1).

The salt tolerance in plants is linked with an increase in the Na<sup>+</sup> exclusion and K<sup>+</sup> accumulation and maintenance of optimum K<sup>+</sup>/Na<sup>+</sup> ratio in plants (Raza *et al.*, 2007). The K<sup>+</sup>/Na<sup>+</sup> and Ca<sup>2+</sup>/Na<sup>+</sup> ratio can be a valid criterion to assess the SS in diverse crops (Ashraf, 2004). Therefore, K<sup>+</sup> and Ca<sup>2+</sup> maintenance and acquisitions are imperative contributors to SS tolerance. In the present investigation, Na<sup>+</sup> accretion in roots and leaves of maize significantly increased whilst, accumulation of K<sup>+</sup> and Ca<sup>2+</sup> considerably decreased (Figure 6). Moreover, foliar-applied GB reduced the accumulation of Na<sup>+</sup>, an increase in K<sup>+</sup> and Ca<sup>2+</sup> (Figure 5 and 6). Therefore, the improvement in the maize growth under SS with GB applied could be due to discrimination of Na<sup>+</sup> against K<sup>+</sup> and Ca<sup>2+</sup>.

The GB maintained membrane integrity under different abiotic stresses (Sakamoto and Murata, 2002). Moreover, GB also protects the diverse transporters to work typic generally under SS (Mansour, 1998). Therefore, it can be advocated that GB has simple protective effects in discriminating Na<sup>+</sup> against K<sup>+</sup> and Ca<sup>2+</sup> under SS. Additionally, GB also increased the vacuole's efficiency in plant roots to accumulate more Na<sup>+</sup> (Rahman *et al.*, 2002). These vacuoles stored the Na<sup>+</sup> in roots and decreased the transportation of Na<sup>+</sup> to shoots and leaves. It is quite evidence in this study, Na<sup>+</sup> was less partitioned too leaves due to GB application under SS (Figure 5).

Moreover, the  $\text{Ca}^{2+}$  accumulation also increased the roots due to GB application and the better accumulation of  $\text{Ca}^{2+}$  maintained the membrane integrity and enzymatic activities under SS (Munns and Tester, 2008) which reduced the EL and MDA and therefore, improved the RWC (Figure 1) and growth of maize (Table 1). The foliar-applied GB also increased the  $\text{K}^+$  accumulation (Figure 6), which contributes to favouring the osmotic adjustment favouring the overall plant growth under SS (Munns and Tester, 2008). Additionally, SS also reduced the  $\text{Na}^+$  accumulation (Figure 6) and maintained higher  $\text{K}^+/\text{Na}^+$  which appreciably improved the growth and salt tolerance.

## Conclusions

The salt stress adversely affected the maize growth, and biomass production and these effects were appreciably reversed by the foliar-applied GB. This amelioration was a due to increase in photosynthetic pigment,  $g_s$ ,  $P_n$ , membrane stability, activities of antioxidant enzymes, accumulation of free amino acids and proteins, and decrease in the accumulation of MDA and  $\text{H}_2\text{O}_2$ . For example, MDA content was increased by 11% and 15%, while  $\text{H}_2\text{O}_2$  was increased by 21% and 38% in plants grown with 6  $\text{dS m}^{-1}$  and 12  $\text{dS m}^{-1}$ , respectively. However, a reduction of 7% and 11% in MDA content, and a reduction of 6% and 9% in  $\text{H}_2\text{O}_2$  were observed at 50 mM and 100 mM GB at 12  $\text{dS m}^{-1}$ , respectively. Additionally, GB reduced the ionic toxicity due to the reduction in the  $\text{Na}^+$  and  $\text{Cl}^-$  accumulation and the increase in the accumulation of  $\text{K}^+$  and  $\text{Ca}^{2+}$ . Therefore, it is suggested that GB application can be a promising approach to mitigate the salt stress effects in maize plants. However, field studies are direly needed before making a recommendation for the farmers. Moreover, additional studies should be conducted to understand how GB application mediates the hormonal cross talks under SS.

## Authors' Contributions

Conceptualization: IK, MUC, M.F.S., MUH and ZD. Formal analysis: IK, MUC, M.F.S., MUH, R.S.J., B.A.A. and ZD. Investigation: ZD; Methodology: IK, MUC, ZD, MSF, E.A., Y.R., R.S.J and B.A.A. Writing - original draft: IK, MUC, M.F.S., E.A., MUH and ZD. Writing - review and editing: M.S.F, E.A., Y.R., B.A.A., R.S.J. All authors read and approved the final manuscript.

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## Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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