Glycine betaine enhancing plant growth and antioxidant activity of fenugreek (*Trigonella foenum-graecum* L.) under salt stress

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Abstract

The effect of an exogenous foliar spray of Glycine Betaine (GB) on vegetative growth and some biochemical aspects of fenugreek (*Trigonella foenum-graecum* L.) in the presence of salt was studied in a pot experiment. In addition to the control, four GB concentrations (0, 25, 50, and 75 mM) were used under salt concentration, 150 mM NaCl. Salt stress reduced all growth parameters and had a negative impact on photosynthetic pigments, relative water content, and proteins. The foliar spray of GB, on the other hand, raises these parameters, particularly at concentrations of 50 mM. Carbohydrates, total phenolics, flavonoids, and antioxidant activity all increased in response to salt stress compared to controls; additionally, GB foliar spray induced their accumulation. Furthermore, increasing the concentration of GB increased these parameters. GB, more than others, reduces the entry of harmful ions (Na^+ and Cl^-), while increasing the accumulation of Ca^{2+} and K^+. These findings suggest that foliar spraying with GB can improve fenugreek growth and tolerance under saline conditions.

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Key words: Biochemical aspects; foliar spray; photosynthetic pigments; osmoprotectant; saline condition.

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Introduction

Fenugreek (*Trigonella foenum-graecum* L.) is a common and annual herb that is used for a variety of purposes. It is one of the most common species in the Leguminosae family, and it is grown for its medicinal properties in many parts of the world.[1] Because fenugreek aerial parts have a strong flavor and aroma, they are frequently used in cooking as a spice and as an aromatic component for medicinal purposes.[2] Salinity reduces fenugreek crop yield because it is a very sensitive plant to salt stress, which negatively affects growth.[3] Stress can potentially reduce crop growth and productivity because it has a devastating effect on various biochemical, physiological, and metabolic processes that inhibit vegetative growth and reproduction.[4] Salinity is expected to affect more than 50% of lands and threaten agricultural production worldwide. The drastic effect of salinity on plant metabolic processes is caused by an imbalance in plant water potential and ionic concentration.[4,5] Salinity causes oxidative stress on proteins and DNA, disrupting normal assimilation, starch metabolism, and respiration. The responses and tolerance to salt stress of five different plant species vary significantly.[6]

Glycine Betaine (GB) is a non-toxic natural product of microorganisms and plants that is high in humus and nitrogen compounds, allowing it to promote assimilation by increasing water uptake and nutrient transport.[7] GB is applied to various crops to reduce the negative effects of salt stress by accumulating K^+ and decreasing Na^+, thereby enhancing growth.[8] Furthermore, exogenous GB application can improve growth rate under salinity by increasing stomatal conductance (gs), Relative Water Content (RWC), and chlorophyll content.[9] Previous research has shown that salt-tolerant plants can endogenously synthesize GB in excess to adjust salinity.[10] Exogenous application of GB to plants, such as adding to a nutrient solution or spraying for salinity resistance, is not capable of GB accumulation.[11]

Salt stress has a negative impact on morphological and physiological attributes, resulting in a loss of agricultural production. Furthermore, GB serves as an osmoprotectant for plants in saline

environments. Thus, the purpose of this study is to investigate the effect of GB on fenugreek in the presence of salt stress and to determine the optimal concentration of GB for better growth and higher values of biochemical aspects under saline conditions.

Materials and Methods

Plant material and experimental design

Fenugreek seeds (Giza 30) were sterilized with 70 % ethyl alcohol then by sodium hypochlorite and finally washed with distilled water several times and dried. The pot experiment was conducted during the spring season in the greenhouse where the average temperature was 21.5 ± 2.7 °C and relative humidity was 40.8 ± 1.7%. The cultivation of 15 seeds occurred in pots with a diameter of 30 cm. The clay soil was chosen for this study according to its physical and chemical properties,[12] the soil was with pH 8.2, EC 3.4 dSm^{-1}, organic matter 1.2%, total P 0.69%, and available N 0.037%. The seedlings were thinned to five per pot after one week of emergence. Before sowing, the soil was applied with 150 mM NaCl as a salt stress treatment. Fenugreek plants were sprayed during the four weeks of growth period by different concentrations of GB (25, 50, and 75 mM) in addition to control. Hence, the experiment consisted of 5 treatments, control, 150 mM NaCl, 150 mM NaCl + 25 mM GB, 150 mM NaCl + 50 mM GB, and 150 mM NaCl + 75 mM GB and each treatment was replicated 3 times. Three plants from each pot and ten leaves from each plant were collected at 4 weeks of sowing and then kept for further analysis.

Growth characteristics like shoot length (cm), shoot fresh weight (g), shoot dry weight (g), leaf area (cm^2) and number of leaves per plant were recorded in three plants from each pot.

Determination of elements content

The concentration of elements (Na^+, K^+, and Ca^{2+}) was determined in the whole plant of fenugreek.[13] Shoot samples were cleaned with fresh and distilled water; then they were dried in an oven at 65°C for 24 h and digested in 10 mL acids mixture (1HNO_3 + 3 HCl). The elements were measured by flame photometer Shimadzu Model AA 640 F (Japan). The concentration of Cl^- was determined by a chloride analyzer (model 926, Sherwood Scientific, Cambridge, UK).

Determination of pigments and Relative water content

The content of photosynthetic pigments like chlorophyll a, chlorophyll b, and carotenoids were determined in fresh leaves by 90 % acetone.[14] The absorbance was measured by a spectrophotometer at 663, 645, and 470 nm.

Relative Water Content (RWC) was determined in the second top leaf by using three samples and repeated three times.[15] Fresh, turgid and dry weights of leaf samples were recorded by using the following formula:

$$RWC\ (\%) = [(FW-DW)/(TW-DW)]\times 100$$

Where: FW is fresh weight, DW is dry weight and TW is turgid weight.

Estimation of protein and carbohydrates contents

The content of total protein was estimated by 10 mL of a 25 mM borate buffer solution (pH 8.5) for protein extraction and Coomassie brilliant blue (G250) as a protein reagent. The absorbance was measured and Bovine Serum Albumin (BSA) was used as a standard for the calculation of protein concentrations in terms of mg g^{-1} DW.[16] Total carbohydrates content was determined by phenol sulphuric acid at 490 nm absorbance.[17] A glucose standard curve was used for calculating the amount of total soluble carbohydrates as mg g^{-1} DW.

Estimation of total phenolics, flavonoid, and antioxidant activity

Total phenolic content was measured by ethanol and folin reagent and the absorbance was recorded at 650 nm.[18] Total flavonoid content was estimated by the colorimetric method using aluminum chloride and absorbance at 510 nm.[19]

The antioxidant activity of fenugreek plant was determined by using the Free Radical Scavenging Activity (FRSA) against 2,2-difenil-1-picrylhydrazyl (DPPH) and converting the color from dark violet to light yellow. The reduction of DPPH was recorded by a spectrophotometer at 517 nm.[20]

Statistical analysis

F-test represents the one-way ANOVA which was performed for analyzing data using SPSS program version 23 depending on means and Standard Deviation (SD) values and followed by post hoc test using Duncan Multiple Range (DMR) test for comparison between means at the 5% probability level (p value at 0.05).

Results

Growth parameters

In comparison to the control, salinity significantly reduced fenugreek growth (Table 1). Salt stress reduced all of the studied growth parameters, including shoot length, shoot fresh weight, shoot dry weight, leaf surface area, and leaf number. However, the GB foliar spray had a significant effect on fenugreek growth parameters under salt stress. Under salt stress, the maximum growth parameters were recorded at 50 mM GB (Table 1). Furthermore, the growth parameters (shoot length, fresh weight, number of leaves, and leaf area) have a significant negative correlation with Na^+ in Pearsons' correlation (Table 2). It can be concluded that increasing the concentration of Na^+ can slow fenugreek growth by lowering the growth parameters.

Element content

Table 3 showed the concentration of the four investigated elements (Na, K, Ca, and Cl) in fenugreek plant. Salinity and different treatments of GB under salt stress showed relevant variations in the content of these elements in the whole plant. The concentration of Na^+ and Cl^- was significantly increased under salt stress compared to control, while K^+ and Ca^{2+} concentrations decreased under salinity (Table 3). Moreover, the foliar application of GB reduced Na^+ and Cl^- under salinity. The maximum reduction was 43.9 and 21.5% respectively which was detected at 50 mM GB. Whereas, the K^+ and Ca^{2+} contents were enhanced by GB foliar spray and the maximum accumulation was 69.2 and 28.9% which was observed at 50 mM GB under salinity (Table 3). Pearsons' correlation (Table 2) shows that Na^+ and Cl^- were highly significant positive correlated at $p \leq 0.05$ ($r^2 = 0.95$). Also, Ca^{2+} showed a highly significant positive correlation with K^+ ($r^2 = 0.97$). Whereas, Na^+ and Cl^- showed a highly significant negative correlation with K^+ and Ca^{2+} which indicate that these elements are antagonistic to each other.

Pigments and RWC

Salinity significantly decreased the three pigments and relative water content (Figure 1). Under salt stress, Chlorophyll a, b, and carotenoid showed the lowest values (1.160, 0.597, and 0.325 mg g^{-1} FW respectively). The addition of GB determined a noticeable recovery of pigment contents under salinity especially at treatment 50 mM GB, the maximum levels of the three pigments were 2.167, 1.247, and 0.659 mg g^{-1} FW respectively (Figures 1A, B, C). Similarly, the relative water content was reduced by 13% under salt stress compared to the control (Figure 1D). Moreover, a significant increase in RWC by 27, 37.6, and 28.6% were observed at GB foliar application of 25, 50, and 75 mM. In the present study, chlorophyll a was found to be negatively correlated with Na^+ and Cl^- at $p \leq 0.05$ ($r^2 = -0.27$ and -0.04 respectively; Table 2). Also, chlorophyll, carotenoid, and RWC were found to be negatively correlated with Na^+. It can be suggested from the present study that Na^+ can reduce the photosynthetic pigments by reducing the contents of Chlorophyll a, b, and carotenoid and affect the percent of RWC by altering the water flow to the plant.

Total protein and carbohydrates

The total protein content of fenugreek was curbed by salinity

Table 1. Effect of different levels of glycine betaine on growth parameters of fenugreek under salt stress.

Treatments	Shoot length (cm)	Shoot FW (g/plant)	Shoot DW (g/plant)	Leaves (no./plant)	Leaf area (cm^2/plant)
Control	21.877±0.69^c	2.527±0.11^d	0.910±0.03^c	8.280±0.15^c	3.490±0.18^c
150 NaCl+0 GB	18.893±0.23^d	1.837±0.06^e	0.623±0.01^d	7.067±0.19^d	2.817±0.29^d
150 NaCl+25 GB	25.523±0.37^b	3.163±0.05^c	1.323±0.09^b	10.327±0.18^b	4.173±0.1^b
150 NaCl+50 GB	32.583±0.43^a	4.370±0.12^a	2.083±0.06^a	12.897±0.49^a	5.283±0.18^a
150 NaCl+75 GB	26.493±0.34^b	3.590±0.13^b	1.317±0.14^b	10.903±0.5^b	4.353±0.19^b
F-test	***	***	***	***	***

Mean± standard deviation based on ANOVA analysis. Means in the same column followed by the same letter are not significantly different from each other at the 5-percent probability level (p value at 0.05) according to Duncan Multiple Range Test (DMRT). *** F-test is highly significant.

Table 2. Pearsons' correlation among the different parameters.

Variables	1	2	3	4	5	6	7	8	9	10	11	12	13
1- Shoot length	1												
2- Shoot FW	0.97*	1											
3- Shoot DW	0.95*	0.94*	1										
4- Leaves number	0.96*	0.95*	0.94*	1									
5- Leaf area	0.94*	0.93*	0.91*	0.96*	1								
6- Na^+	-0.04*	-0.1*	0.00	-0.02*	-0.09*	1							
7- K^+	0.15	0.24	0.1	0.16	0.21	-0.96*	1						
8- Ca^{2+}	0.03	0.1	-0.01	0.02	0.08	-0.99*	0.97*	1					
9- Cl^-	0.18	0.13	0.19	0.21	0.13	0.95*	-0.83*	-0.93*	1				
10- Chlo. a	0.88*	0.85*	0.81*	0.9*	0.91*	-0.27*	0.38	0.25	-0.04	1			
11- Chlo. b	0.95*	0.89*	0.95*	0.95*	0.92*	-0.05*	0.12	0.02	0.15	0.87*	1		
12- Carotenoid	0.93*	0.88*	0.94*	0.91*	0.91*	-0.01*	0.11	0.01	0.19	0.81*	0.96*	1	
13- RWC	0.94*	0.95*	0.9*	0.96*	0.95*	-0.17*	0.29	0.16	0.06	0.91*	0.88*	0.83*	1

*significant, P< 0.05.

Table 3. Element concentration (mg g -1DW) in the whole plant of fenugreek at different levels of GB under salt stress.

Treatments	Na^+	K^+	Ca^{2+}	Cl^-
Control	3.450±0.28^e	24.200±0.62^a	77.290±0.53^a	4.643±0.33^e
150 NaCl+0 GB	20.860±0.26^a	12.990±0.26^e	51.867±0.38^e	35.323±0.51^a
150 NaCl+25 GB	17.920±0.44^b	15.133±0.24^d	54.957±0.32^d	32.363±0.46^b
150 NaCl+50 GB	11.693±0.21^d	21.980±0.14^b	66.860±0.39^b	27.697±0.57^d
150 NaCl+75 GB	14.527±0.24^c	18.010±0.26^c	60.670±0.21^c	29.893±0.8^c
F-test	***	***	***	***

Mean± standard deviation based on ANOVA analysis. Means in the same column followed by the same letter are not significantly different from each other at the 5-percent probability level (p value at 0.05) according to Duncan Multiple Range Test (DMRT). *** F-test is highly significant.

(Figure 2A). Salt stress significantly decreased the content of total protein by 28.2% compared to the control. However, GB foliar spray improved the protein content by 63.8, 104, and 74% at 25, 50, and 75 mM. Whereas, carbohydrates showed an accumulation under salinity, compared to control (Figure 2B). A further accumulation of carbohydrates was observed after GB foliar application, this accumulation increased by increasing the levels of GB.

Total phenolics, flavonoid, and antioxidant activity

There was a noticeable increase in total phenolics, flavonoid, and antioxidant activity of fenugreek under salt stress (Figure 3). Total phenolics, flavonoid and antioxidant activity increased by 7.5, 41.6, and 4.5% under salt stress, compared to the control. The foliar application of GB resulted in further increases in their contents at 25, 50, and 75 mM. The maximum value of total phenolics, flavonoid and antioxidant activity was 27.5, 31.4,

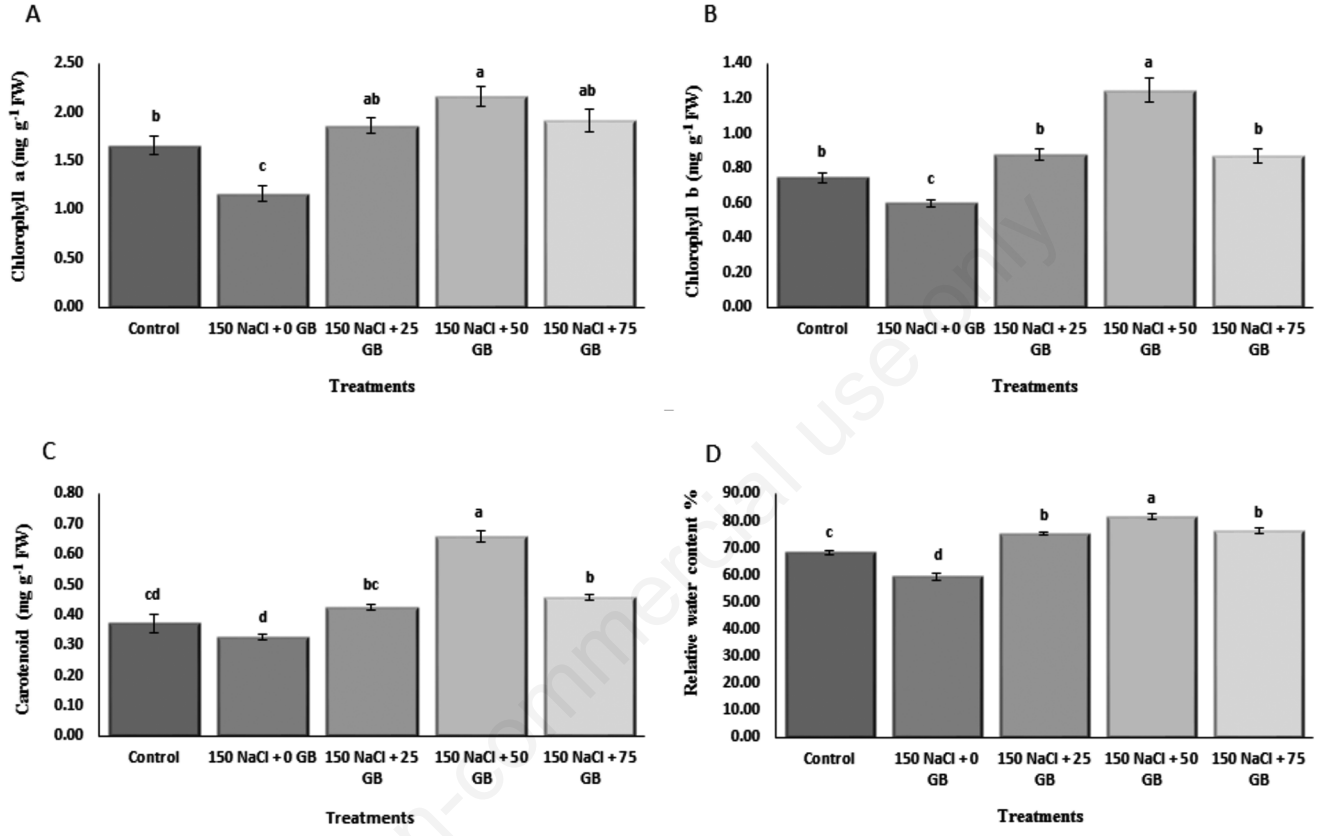

Figure 1. Impact of different levels of GB on the content of: (A) chlorophyll a; (B) chlorophyll b; (C) carotenoid and (D) RWC (%) under salt stress. Error bars indicate the standard deviation (SD) of three replicates.

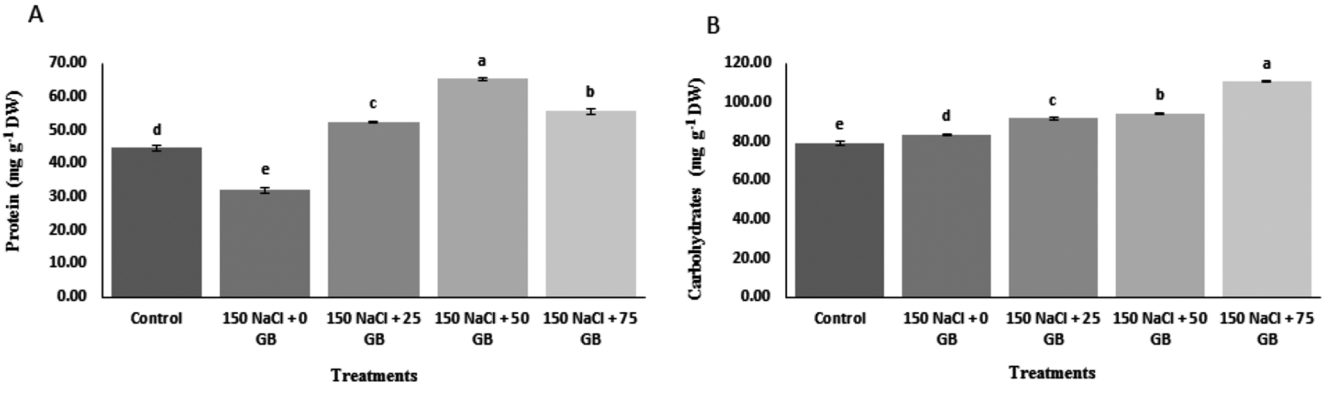

Figure 2. Impact of different levels of GB on: (A) protein; (B) carbohydrates under salt stress. Error bars indicate the standard deviation (SD) of three replicates.

and 24.6% respectively which were detected at 75 mM GB (Figure 3).

Discussion

Because of their transportation nature and hormonal activity, osmoprotectants such as glycine betaine have recently become an interesting topic against stress conditions.[21] Salt stress had a negative impact on all of the growth parameters studied. According to Athar *et al.*,[22] salt stress reduces plant growth and yield, and this decrease may be related to the toxic effects of noxious ions like Na^+ and Cl^-, which accumulate under salinity and reduce photosynthesis rate. The results of leaf area and leaf number, in particular, agree with Yildirim *et al.*,[23] who recorded similar data in lettuce. Nonetheless, GB mitigated the negative effects of the saline condition by inducing the expression of genes and enzymes that scavenge Reactive Oxygen Species (ROS) as well as membrane property protection.[24] Furthermore, Hamani *et al.*,[9] demonstrated the importance of GB foliar application in cotton seedling stomatal conductance, transpiration rate, water use efficiency, photosynthesis rate, and photosystem II effectiveness under salinity. The increase in growth parameters as a result of foliar application of GB may be attributed to its ability to overcome the negative effect of salts in photosynthesis and to the increased production of assimilation units that promote growth.[25] The highest growth parameter values were obtained at 50 mM GB. The plant dry weight results agreed with those of other researchers on tomato,[26] eggplant,[27] and okra.[28]

Plant survival under salinity conditions is linked to maintaining an optimal K:Na ratio, which leads to improved growth.[8] In the current study, GB application alleviates the negative effects of salinity by lowering the concentrations of Na^+ and Cl^- while increasing the accumulation of K^+ and $Ca2^+$. By the way, Na and K are important in plant growth because their interaction is essential for many physiological processes.[29] Furthermore, $Ca2^+$ can improve salt tolerance and alleviate negative effects by controlling the flow of Na through the ionic channels.[30] Overall, GB application improved fenugreek growth and salt tolerance, which could be attributed to the recognition of K^+ and $Ca2^+$ against Na^+ and Cl^-.

Under salt stress, the pigment content (chlorophyll a, chlorophyll b, and carotenoids) of fenugreek decreased, which is consistent with the findings of Abdel-Fattah *et al.*,[31] in cowpea plants under the same conditions. The pigment content decrease is related to the sensitive effect of salinity on chloroplasts and the degradation of the pigment-protein complex.[32] When compared to salt stress, foliar spray of GB increased the level of pigment content. Dustgeer *et al.*,[33] obtained similar results in maize, which is because GB protects the assimilating unit by inhibiting ROS production. RWC is critical in determining the extent of salt damage and assessing plant tolerance to salts. Under salt stress, RWC decreased in fenugreek; the high osmotic pressure caused by salt stress leads to limited water uptake, resulting in low RWC, which is critical for cell development. GB can restore RWC to a level

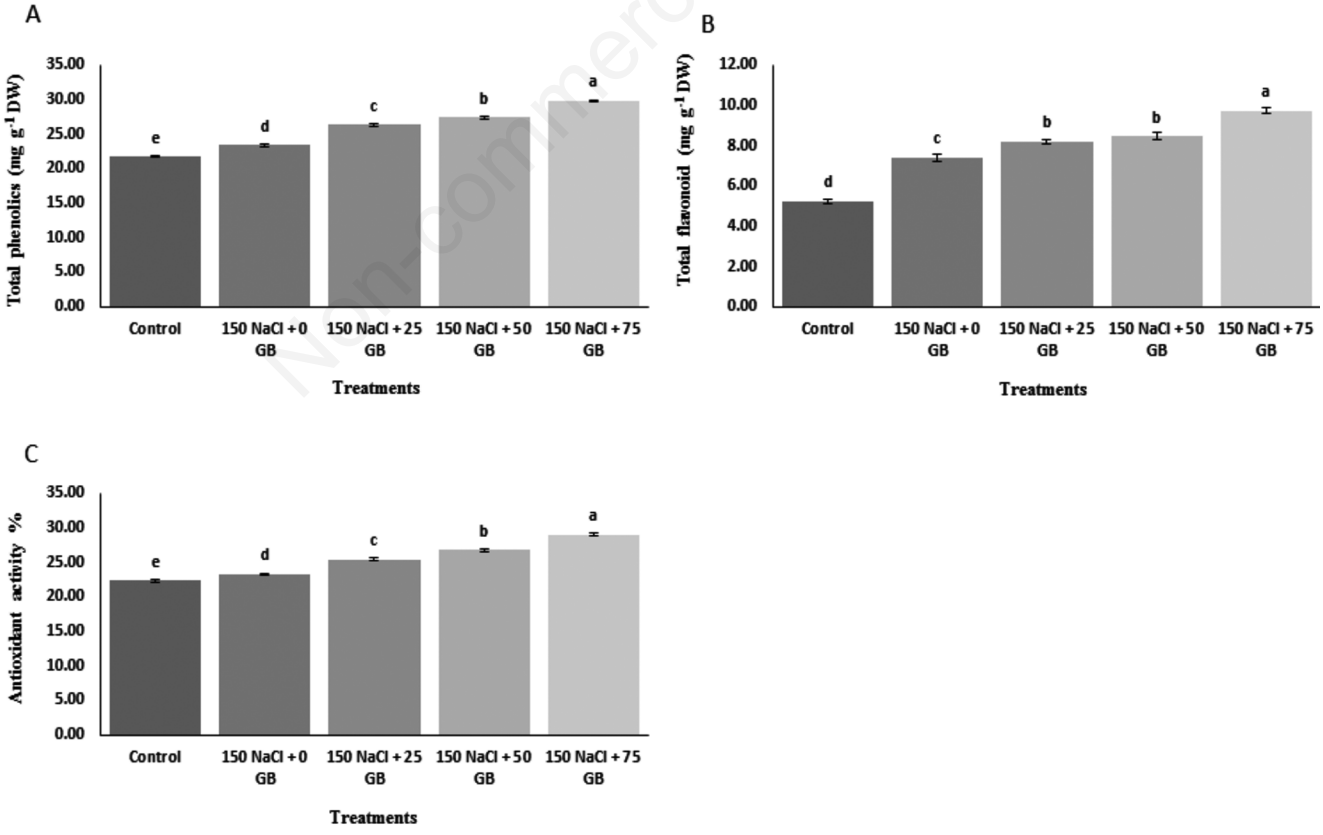

Figure 3. Impact of different levels of GB on: (A) total phenolics; (B) flavonoid; (C) antioxidant activity under salt stress. Error bars indicate the standard deviation (SD) of three replicates.

comparable to the control, resulting in improved cell membrane properties.[33] This could be attributed to the accumulation of K^+ caused by GB treatment, which is required to maintain osmotic pressure and turgor potential.[34] Furthermore, GB application can increase water flow from roots to shoots, which increases turgor pressure in guard cells and thus stomatal conductance.[35]

The GB foliar spray controls the production and accumulation of soluble proteins as well as the carbohydrate content. The increase in carbohydrate content in fenugreek as a result of salt stress and GB application may be attributed to carbohydrates, such as starch and sugars, which act as both nutrients and signal molecules in stress reduction.[3] Carbohydrate results are consistent with previous studies on lettuce[36] and cowpea[37] under salt stress. There is a decrease in protein content in the current study, which could be due to a defect in biosynthesis under salt stress. This could be related to ion toxicity caused by the accumulation of noxious ions (Na^+ and Cl^-), which caused protein synthesis to be disrupted and plant growth to be altered.[38] GB application increases protein concentration, particularly at 50 mM GB, which is consistent with the findings of Habib *et al.*[28] in the okra plant.

In the current study, GB foliar application improves total phenolic, flavonoid, and antioxidant activity accumulation; Abdelhameed *et al.* reported a similar increase under saline conditions.[39] Phenolic compounds are secondary metabolites that protect cellular membranes and have antioxidant activity in plants under biotic stress by scavenging ROS.[40] Furthermore, Hoque *et al.*[10] demonstrated that the GB application boosts growth under salinity stress by activating antioxidant enzymes like POD, CAT, and APX.

Conclusions

All of the studied growth parameters and photosynthetic pigments decreased under saline conditions, proving that fenugreek is a salt-susceptible plant. Under salt stress, GB can be used as a growth regulator and osmoprotectant. GB mitigates the negative effects of salt stress and promotes growth by increasing relative water content and photosynthetic pigments. The best GB foliar spray concentration was 50 mM. Furthermore, GB promotes carbohydrate, phenolic, flavonoid, and antioxidant accumulation. As a result, this study suggests that GB plays an important role in fenugreek's response to salinity stress. However, more research on the GB is needed to determine the molecular mechanism of growth and antioxidant activity under salt stress.

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