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Role of glycine betaine in mitigating salt-induced oxidative stress in *Vigna radiata*

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Abstract: The impact of exogenously applied glycine betaine (GB; 0, 5, 10, 20 and 50 mmol) was evaluated in preventing *Vigna radiata* from the adverse effects of salt (100 mmol NaCl) stress. Salinity reduced growth parameters, such as plant height and fresh and dry weight of plants, while GB application significantly alleviated the decline. Salinity stress led to a decline in total chlorophylls and carotenoids, as well as a reduction in the net photosynthetic rate and gas exchange attributes, including stomatal conductance, transpiration rate, and intercellular CO_2. However, GB supplementation significantly alleviated this decline. Salinity stress increased the accumulation of hydrogen peroxide, superoxide and methylglyoxal, while as applied GB reduced their accumulation, causing a significant decline in the lipid peroxidation. Application of GB, at all concentrations, increased the activity of the antioxidant enzymes under normal and salinity stress treatments with 10 and 20 mmol concentrations, imparting the highest increase. Increase in the radical scavenging activity due to GB application was also supported by increased total antioxidant activity assays measured as percent DPPH and ABTS radical scavenging. In addition, GB-supplemented plants exhibited an apparent increase in the activities of glyoxalase I and glyoxalase II enzymes. Accumulation of osmotic compounds like proline, sugars and GB increased significantly due to GB application and showed a further increase in salt-stressed plants. More importantly, the GB-treated plants exhibited a considerable decline in sodium accumulation, causing a decline Na/K in them. Glycine betaine was effective in mitigating the deleterious effects of salinity.

Keywords: abiotic stress; legume; mung bean; osmolytes; salt stress; tolerance mechanisms

Salt stress is a significant abiotic stress factor that impacts growth and sustainable productivity worldwide. The percentage of salt-affected agricultural soils is increasing daily, thereby posing a significant threat to agricultural productivity. Excessive use of mineral fertilisers polluted and saline water for irrigation, and the lack of proper drainage in agricultural fields significantly contribute to salt-affected soils (Nuruzzaman et al. 2025). It has been reported that salinity stress affects germination, seedling growth, chlorophyll and photosynthesis, therefore affecting the yield potential of plants (Hamani et al. 2020, Attia et al. 2022, Alamer and Attia 2022, Azeem et al. 2023). Increasing salt concentrations in soils have resulted in the conversion of productive agricultural land into unproductive wasteland. Excess salinity triggers physiological and biochemical alterations in plants, leading to considerable changes in metabolism and cellular functioning (Hameed et al. 2021). Salinity leads to excessive generation of reactive oxygen radicals that seriously affect the normal functioning of cellular organelles and the whole plants as well (Hasanuzzaman et al. 2014, Azeem et al. 2023, Rehman et al. 2024). Excessive radicals induce oxidative damage to membranes and proteins, affecting photosynthesis, electron transport,

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enzyme functioning, etc. Plant cells are equipped with several mechanisms that are implemented to mitigate the adverse effects resulting from stress (Qin et al. 2020). The antioxidant system, glyoxalase cycle and the accumulation of osmotic components contribute to preventing the adverse effects of stresses (Hasanuzzaman et al. 2014, Lu et al. 2023). It has been reported that the efficient functioning of these mechanisms is reflected in better growth performance through improved protection of cellular metabolism. In addition, the compartmentation and sequestration of toxic ions like Na into the vacuole or apoplastic spaces also contributes to greater salinity tolerance (Ahanger and Agarwal 2017). Among the compatible osmotic components are proline, glycine betaine, sugars, and others, which are accumulated in large concentrations (Islam et al. 2021, Kaur et al. 2024).

Glycine betaine (GB), N, N, N-trimethyglycine, is an important osmolyte which is synthesised in chloroplasts, peroxisomes and cytoplasm from choline and has a key role in alleviating stress-induced deleterious effects (Hernandez-Leon and Valenzuela-Soto 2023). It has been observed that plants accumulating increased GB content display better growth and adaptation to stresses, thereby leading to improved performance (Zhang et al. 2020). It is involved in growth regulation, protection of photosynthesis and chlorophyll synthesis, enzyme function, proper protein folding, and gene expression, among other processes (Hernandez-Leon and Valenzuela-Soto 2023). Exogenous treatment of GB to *Solanum melongena* L. has been reported to improve photosynthesis and growth by modulating phytohormone metabolism, amino acid synthesis and the key enzymes of the Calvin cycle (Niu et al. 2023). In addition, treatment of GB up-regulates the functioning of tolerance mechanisms, including the antioxidant system and triggers osmolyte accumulation, resulting in the prevention of damage to membrane functioning (Singh et al. 2022). Recently, Dong et al. (2024) have demonstrated that exogenous treatment of GB prevented the oxidative effects of salinity in *Glycyrrhiza uralensis* Fisch by up-regulating key tolerance mechanisms and inducing significant improvement in endogenous GB metabolism. However, much needs to be done to get a thorough understanding of the GB-mediated salinity tolerance in plants. In this context, the present study was conducted to assess the impact of varying concentrations of exogenously applied GB on specific key physiological and biochemical characteristics in *Vigna radiata* L.

Vigna radiata L. is an important legume crop grown worldwide and is rich in proteins, vitamins and carbohydrates. It is a minor crop with high nutritional value, enabling small farmers and households to enhance both profitability and sustainability. Agricultural productivity must rise significantly to meet the demands of a growing population. However, several stress factors influence the growth and metabolism of *Vigna radiata* L., posing a significant impact on its yield. Therefore, the present study hypothesised that exogenous treatment of GB can improve growth and salinity stress tolerance in *Vigna radiata* by improving the tolerance mechanisms.

MATERIAL AND METHODS

Seeds of *Vigna radiata* L. var. *radiata* were disinfected by using 0.001% $HgCl_2$ for 5 min. The sterilised seeds were thoroughly washed with distilled water, and the adhering water was rinsed by blot drying. Ten healthy seeds were sown in the earthen pots that were filled with reconstituted soil containing soil, compost and sand (4:1:1). All the pots were wetted by applying Hoagland nutrient solution to full saturation and thereafter 100 mL nutrient solution was applied every alternate day. Two weeks after germination, the number of seedlings per pot was thinned to five and pots were divided into two separate groups. One group of pots was irrigated with normal Hoagland solution, while another group was irrigated with modified Hoagland nutrient solution containing 100 mmol NaCl. In both groups, the application of GB (0, 5, 10, 20, and 50 mmol) was also initiated on the foliage using Teepol (0.1%) as a surfactant, and GB (10 mL per pot) was applied once a week. The composition of nutrient solution used was: 3 mmol KNO_3, 2 mmol $Ca(NO_3)_2$, 2 mmol $MgSO_4$, 1 mmol $NH_4H_3PO_4$, 50 µmol KCl, 25 µmol H_3BO_4, 2 µmol $MnCl_2$, 20 µmol $ZnSO_4$, 0.5 µmol $CuSO_4$, 0.5 µmol $(NH_4)_6Mo_7O_{24}$, and 20 µmol Na_2Fe-EDTA (Ahanger et al. 2017). Pots were arranged in green house under natural climatic conditions. After five weeks of treatment with NaCl and GB, plants were uprooted carefully and washed to remove the adhering soil. Tissue was frozen in liquid nitrogen and stored in a deep freezer. The summary of all treatments is as: (*i*) control; (*ii*) 5 mmol GB; (*iii*) 10 mmol GB; (*iv*) 20 mmol GB; (*v*) 50 mmol GB; (*vi*) 100 mmol NaCl; (*vii*) 5 mmol GB + 100 mmol NaCl; (*viii*) 10 mmol GB + 100 mmol NaCl; (*ix*) 20 mmol + 100 mmol NaCl GB, (*x*) 50 mmol GB + 100 mmol NaCl. The

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detailed protocols used for working with different parameters are described below:

Growth parameters. The plant height was measured manually by using a scale. The fresh weight of the whole plant was measured immediately after uprooting. Thereafter, the same plants were dried in an oven set at 60 °C for 72 h for the measurement of dry weight.

Estimation of total chlorophylls, carotenoids, photosynthesis and gas exchange parameters. The content of total chlorophylls and carotenoids was extracted by homogenising 100 mg of leaf tissue in 80% acetone. After centrifugation of the homogenate, the absorbance of the supernatant was measured at 480, 645, and 663 nm against a blank acetone solution (Arnon 1949). The measurement of net photosynthetic rate (P_n), intercellular CO_2 concentration (C_i), stomatal conductance (g_s) and transpiration (E) were measured using a portable photosynthetic apparatus Li-6400 (LI-COR Inc., Nebraska, USA).

Estimation of proline, glycine betaine and sugars. The content of proline was extracted by macerating the 300 mg dry plant material in 3% sulphosalicylic acid. After centrifugation, the supernatant was mixed with acid ninhydrin reagent and incubated for 1 h at 100 °C. Thereafter, proline was separated using toluene, as described by Bates et al. (1973). Glycine betaine content was estimated according to the method of Grieve and Grattan (1983). Briefly, plant material was extracted in distilled water, and the extract was mixed with 2 mol/L H_2SO_4. Thereafter, the formation of betaine-periodide complex was determined at 365 nm after adding the cold KI-I2 reagent. The anthrone method, as described by Shields and Burnet (1960), was used to determine the sugar content in leaves, with absorbance measured at 585 nm.

Measurement of hydrogen peroxide, superoxide and lipid peroxidation. The content of hydrogen peroxide (H_2O_2) in all treatments was estimated following Velikova et al. (2000). Fresh leaf tissue was macerated using trichloroacetic acid in a pestle and mortar. After centrifuging the homogenate, the supernatant was added to a potassium phosphate buffer (pH 7.0), followed by the addition of potassium iodide. Thereafter, the absorbance of the mixture was taken at 390 nm.

The method described by Yang et al. (2011) was used to determine the superoxide in normal and stressed plants. Leaf tissue was extracted in 65 mmol potassium phosphate buffer (pH 7.8), and after centrifuging the extract at 5 000 g for 10 min, 10 mmol hydroxylamine hydrochloride was added to the supernatant. After 20 min, sulfanilamide and naphthylamine were added, and incubation was done at 25 °C for 20 min. Absorbance was taken at 530 nm.

Lipid peroxidation was determined according to the method of Heath and Packer (1968). Briefly, fresh tissue was macerated in trichloroacetic acid, and the supernatant was incubated with thiobarbituric acid at 95 °C for 30 min. After cooling on ice, the samples were centrifuged for 5 min at 5 000 g. Absorbance was taken at 532 and 600 nm.

The method described by Wild et al. (2012) was employed to measure the content of methylglyoxal in salinity and GB-treated plants. Leaf tissue was extracted in 5% perchloric acid, and centrifugation was done for 10 min at 11 000 *g*. The supernatant was decolourised by adding charcoal and subsequently neutralised using potassium carbonate. Thereafter, sodium dihydrogen phosphate and N-acetyl-L-cysteine were added to the supernatant and allowed to stand for 10 min. Absorbance was taken at 288 nm.

Total antioxidant activity. The total antioxidant activity in normal and stressed plants was measured in terms of DPPH and ABTS radical scavenging potential. Following the methods described by Brand-Williams et al. (1995) and Re et al. (1999), respectively. In the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging potential assay, methanolic extract was reacted with DPPH and the optical density was taken at 515 nm. However, in the ABTS [2, 2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid] radical scavenging assay, the methanol extract was reacted with ABTS and potassium persulfate. The optical density was recorded at 734 nm.

Measurement of antioxidant system functioning. Both the enzymatic and non-enzymatic components of the antioxidant were measured. For the extraction of antioxidant enzyme, 500 mg fresh leaf tissue was extracted in a cold 100 mmol phosphate buffer (pH 7.8) supplemented with 1% polyvinyl pyrolidine, 0.1 mmol phenylmethylsulfonyl fluoride and 1 mmol EDTA. After centrifuging the homogenate at 12 000 *g* for 15 min, the supernatant was collected and used as an enzyme source for assaying the activities of antioxidant enzymes. The activity of superoxide dismutase (SOD) was assayed following Bayer and Fridovich (1987). After incubating the samples under fluorescent light for 15 min, the absorbance was recorded against the non-illuminated blank at 560 nm. The amount of protein that caused 50% photoreduction was considered 1 unit of enzyme activity and has been expressed as enzyme unit (EU) per milligram of protein. Activity of peroxidase (POD) was determined

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following Zhou and Leul (1998), and the change in absorbance was recorded at 470 nm for 2 min. The method of Nakano and Asada (1981) was used for assaying the activity of ascorbate peroxidase (APX), and the optical density was taken at 290 nm for 3 min. For assaying the activity of dehydroascorbate reductase (DHAR), the method described by Nakano and Asada (1981) was followed, and the optical density was taken at 265 nm for 2 min. The activity of catalase (CAT) was measured according to Aebi (1984), and the change in optical density was monitored at 240 nm for 2 min. The activity of glutathione reductase (GR) was assayed using the Foyer and Halliwell (1976) method, and the absorbance was measured for 2 min at 340 nm. Protein was estimated according to Lowry et al. (1951).

Among the non-enzymatic antioxidants, ascorbic acid (AsA) and reduced glutathione (GSH) were determined. For measuring the content of ascorbic acid and reduced glutathione, the methods described by Mukherjee and Choudhuri (1983) and Ellman (1959) were followed, respectively. AsA was extracted by homogenising 100 mg tissue in trichloroacetic acid and the supernatant was incubated with dinitrophenylhydrazine and thiourea for 15 min. After adding cold H_2SO_4, the optical density was taken at 530 nm. However, GSH was extracted by homogenising 100 mg tissue in phosphate buffer (pH 8.0) and the supernatant was mixed with 5, 5-dithiobis-2-nitrobenzoic acid. After 10 min, the optical density was taken at 412 nm.

Activity of glyoxalase cycle enzymes. The activity of both glyoxalase I and II was assayed, which were extracted by macerating the leaf tissue in potassium phosphate buffer (pH 7.0) containing β-mercaptoethanol, ascorbate, glycerol and potassium chloride. The homogenate was centrifuged at 11 500 *g*, supernatant was collected and used for assaying the activity of glyoxalases. The methods described by Hasanuzzaman et al. (2011) and Principato et al. (1987) were used for measuring the activity of glyoxylase I and glyoxalase II, respectively.

Estimation of sodium and potassium. The content of sodium and potassium ions in leaves and root tissues was determined by digesting dried material in acid. After diluting the digested samples with distilled water, the content of Na and K was determined using a flame photometer (Staffordshire UK).

Statistical analysis. The mean (± SE) of three replicates has been presented and to determine the significance of data, Duncan's multiple range test was performed and least significant difference (*LSD*) was calculated at $P < 0.05$.

RESULTS

The results showing the effects of salinity stress and the applied GB on the height, fresh and dry weight of the plant were given in Figure 1. Relative to the control, declines of 30.91, 32.52, and 38.67% were observed in height, fresh weight, and dry weight, respectively, due to the NaCl treatment. However, the application of GB at all concentrations increased these parameters and significantly alleviated the decline caused by NaCl. In normally grown plants, the highest increases in height, fresh weight, and dry weight were 17.62, 18.35,

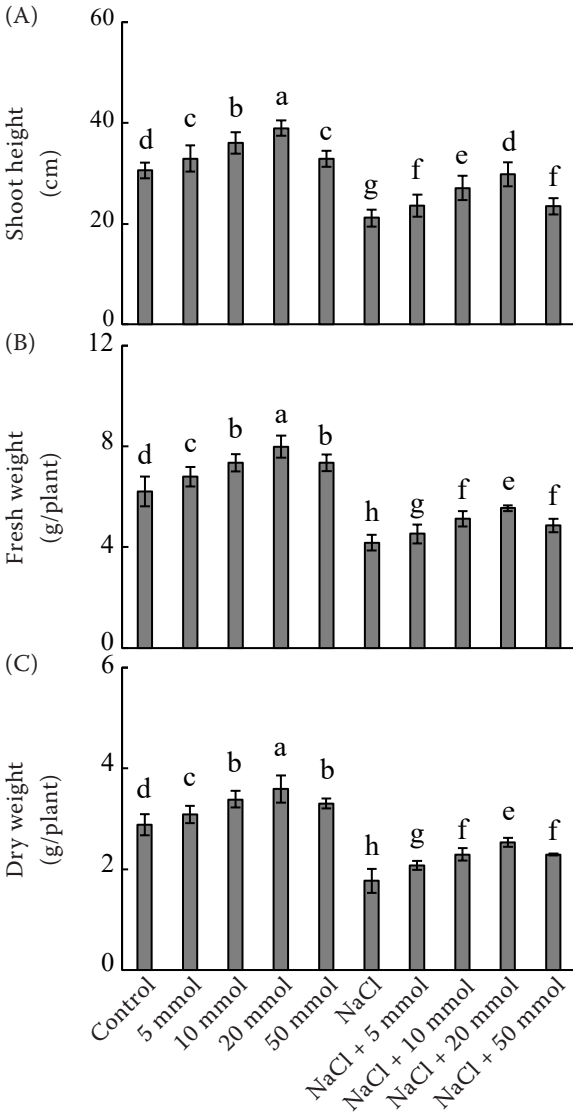

Figure 1. Effect of salinity stress (100 mmol NaCl) on (A) shoot length; (B) shoot fresh weight, and (C) shoot dry weight of *Vigna radiata* treated with different concentrations of glycine betaine (0, 5, 10, 20 and 50 mmol). Data is the mean of three replicates, and different letters on graphs indicate a significant difference at $P < 0.05$

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and 17.77%, respectively, due to 10 mmol GB, and 27.42, 28.66, and 24.73%, respectively, due to 20 mmol GB, compared to the control. All applied GB concentrations resulted in alleviation of the decline in height, fresh, and dry weight; however, 10 and 20 mmol GB resulted in obvious and significantly more pronounced effects. Compared to NaCl stressed plants, the increase in height was 28.21% and 41.11%, in fresh weight was 22.43% and 32.45% and in dry weight was 22.80% and 43.18% (Figure 1).

Salinity treatment resulted in a decline of 40.20% and 44.31% in total chlorophyll and carotenoids, respectively, compared to the control plants. Exogenous treatment of GB increased the pigment content and mitigated the decline caused by salinity stress. Relative to control, an increase of 6.17, 15.00, 24.39 and 6.42% was observed in total chlorophylls, while carotenoids increased by 8.15, 19.62, 32.06 and 19.42% due to 5, 10, 20 and 50 mmol GB, respectively. Compared to NaCl stressed plants, the content of total chlorophylls and carotenoids increased by 11.94% and 16.01% in NaCl + 5 mmol GB, by 26.92% and 38.57% in NaCl + 10 mmol GB, by 46.34% and 58.61% in NaCl + 20 mmol GB and by 17.66% and 37.07% in NaCl + 50 mmol GB treated plants (Figure 2).

In addition, a decline of 33.77% in net photosynthesis, 32.06% in intercellular CO_2, 24.74% in stomatal conductance, and 35.53% in transpiration rate was observed due to NaCl stress compared to the control. In unstressed plants, the treatment with 20 mmol GB caused the highest increases of 32.27, 19.97, 21.12%, and 42.97% in net photosynthesis, intercellular CO_2 concentration, stomatal conductance, and transpiration rate compared to the control. The application of GB alleviated the decline, and it was observed that the alleviatory effect of 10 and 20 mmol GB was more pronounced. Compared to NaCl-stressed plants, an increase of 36.40% and 46.74% in net photosynthesis, 27.94% and 35.27% in intercellular CO_2 concentration, 17.90% and 18.90% in stomatal conductance and 28.20% and 28.84% in transpiration rate was observed in NaCl + 10 mmol GB and NaCl + 20 mmol GB treated plants (Figure 3).

Treatment of GB caused an increase in the accumulation of proline, sugars and GB in normal and the NaCl-stressed plants. In normal grown plants, the content of proline, sugar and GB increased by 12.51, 9.50 and 59.59% due to 5 mmol GB, by 29.14, 32.14 and 94.69% due to 10 mmol GB, by 29.82, 37.85 and 58.87% due to 20 mmol GB and by 25.69, 9.50 and 104.11% due to 50 mmol GB contrary to the control. The increase in proline was 72.31%, sugars were 73.57%, and GB was 32.81% due to NaCl stress compared to the control. Further increase was exhibited in the accumulation of proline, sugars and GB due to the treatment of exogenous GB to NaCl-stressed plants. The highest increases of 131.81% in proline, 115.46% in sugars, and 244.24% in GB were observed in NaCl + 20 mmol GB-treated plants compared to the control (Figure 4).

Salinity stress increased the oxidative stress parameters, including hydrogen peroxide, superoxide (O_2^-), methylglyoxal, and lipid peroxidation; however, GB application resulted in a decline in these parameters. Relative to control, H_2O_2, O_2^-, methylglyoxal and lipid peroxidation showed an increase of 142.09, 87.21, 109.33 and 104.89% respectively, due to NaCl stress.

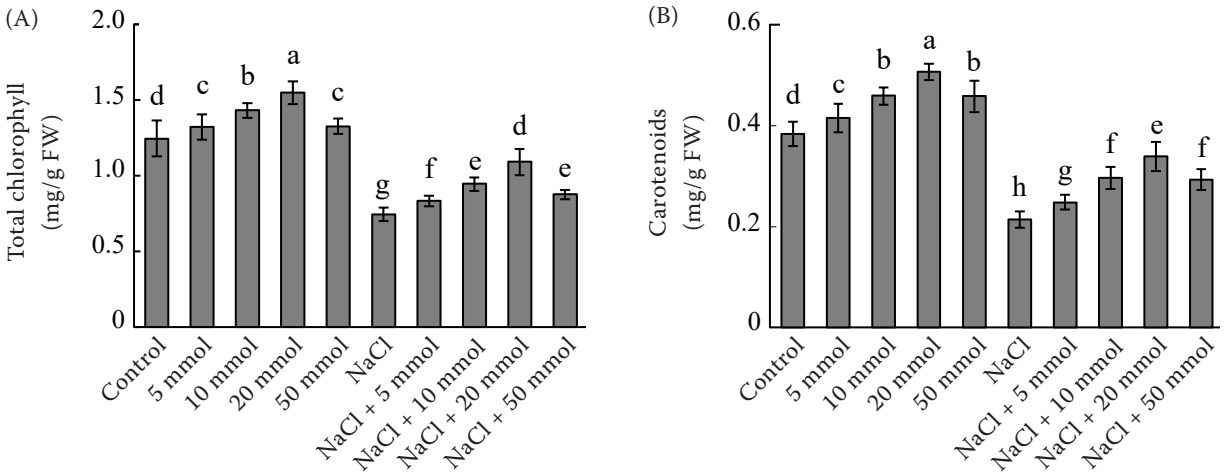

Figure 2. Effect of salinity stress (100 mmol NaCl) on (A) total chlorophylls and (B) carotenoids in *Vigna radiata* treated with different concentrations of glycine betaine (0, 5, 10, 20 and 50 mmol). Data is the mean of three replicates, and different letters on graphs indicate a significant difference at $P < 0.05$. FW – fresh weight

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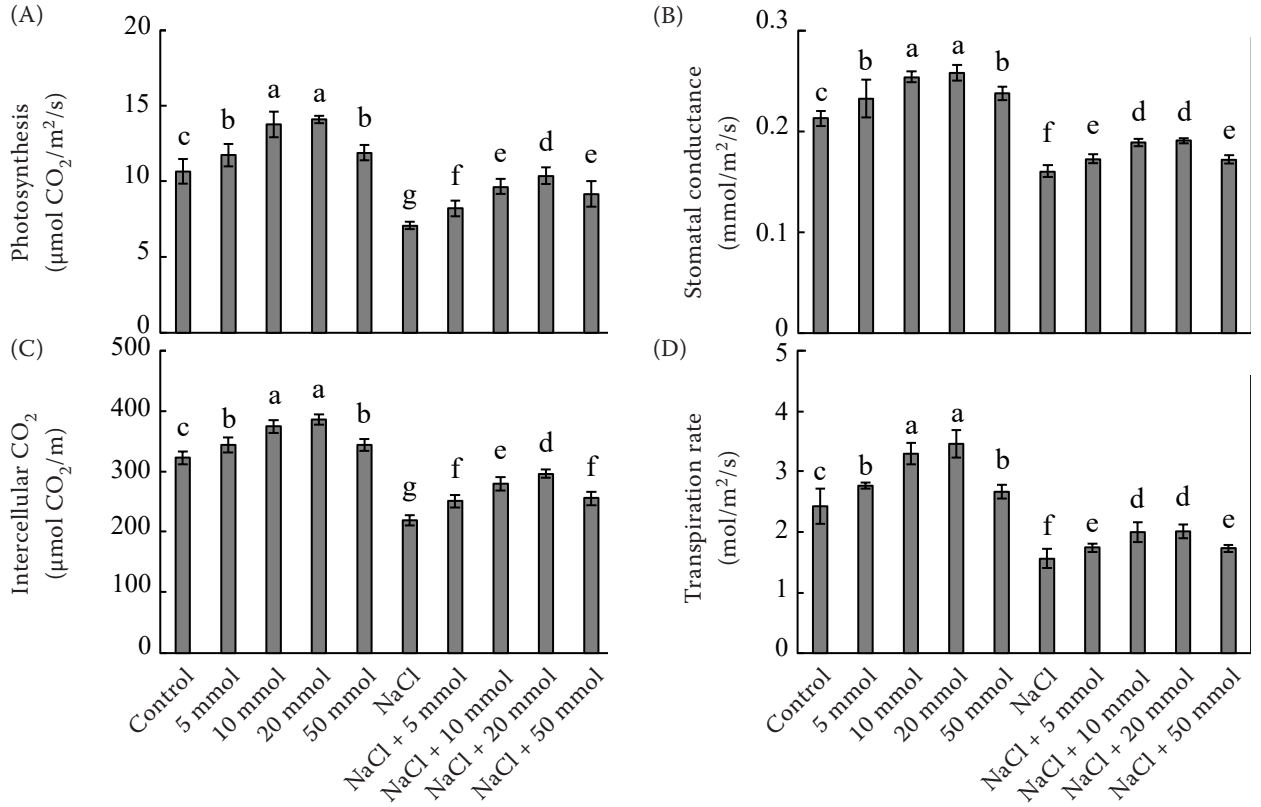

Figure 3. Effect of salinity stress (100 mmol NaCl) on (A) photosynthesis; (B) stomatal conductance; (C) intercellular CO_2 concentration, and (D) transpiration rate of *Vigna radiata* treated with different concentrations of glycine betaine (0, 5, 10, 20 and 50 mmol). Data is the mean of three replicates, and different letters on graphs indicate a significant difference at $P < 0.05$

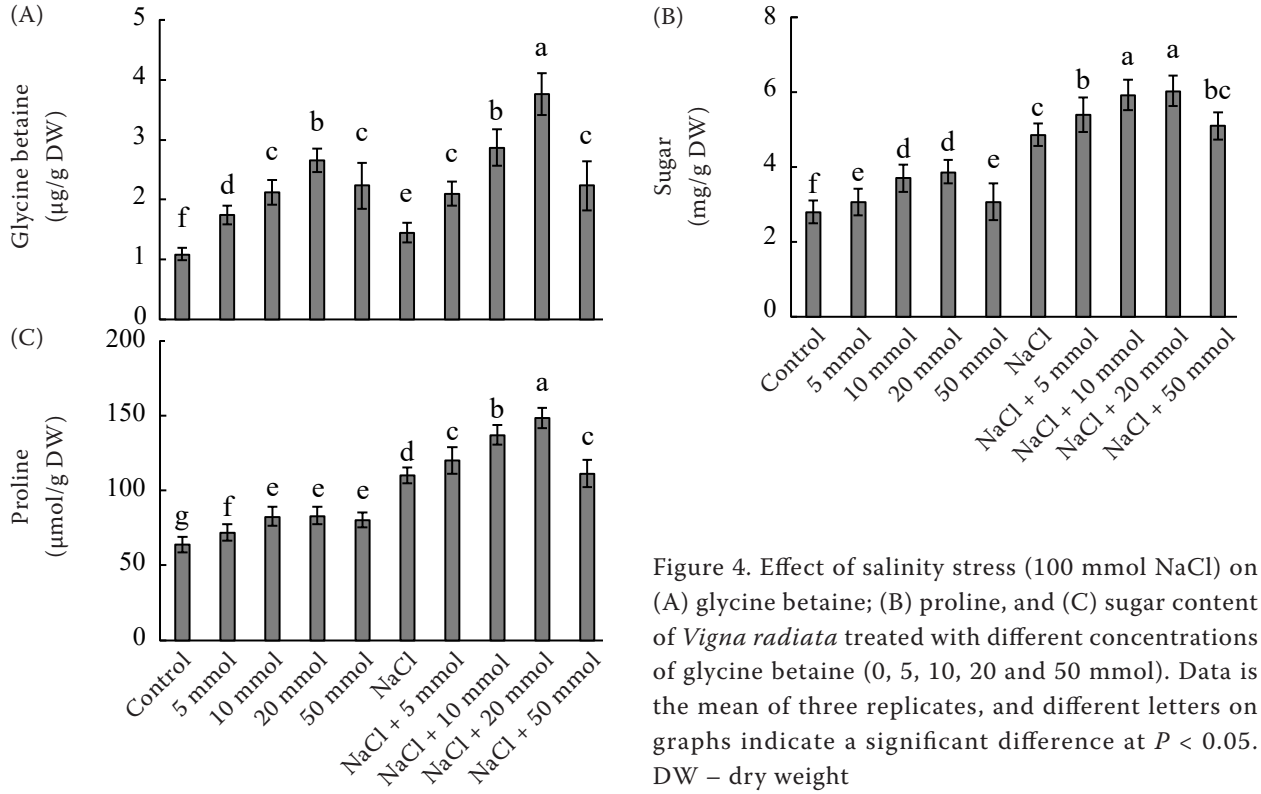

Figure 4. Effect of salinity stress (100 mmol NaCl) on (A) glycine betaine; (B) proline, and (C) sugar content of *Vigna radiata* treated with different concentrations of glycine betaine (0, 5, 10, 20 and 50 mmol). Data is the mean of three replicates, and different letters on graphs indicate a significant difference at $P < 0.05$. DW – dry weight

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Treatment of GB to NaCl-stressed plants caused a decline in these parameters at all concentrations. Compared to NaCl-stressed counterparts, the highest decline of 38.60, 36.67, 24.14 and 39.63% in H_2O_2, O_2^-, methylglyoxal and lipid peroxidation was observed in NaCl + 20 mmol GB-treated plants. In unstressed plants, treatment of 20 mmol GB resulted in reduction of 40.50, 36.80, 25.53 and 41.25% in H_2O_2, O_2^-, methylglyoxal and lipid peroxidation as compared to the control (Figure 5).

The activities of antioxidant enzymes like SOD, POD, CAT, APX, DHAR and GR increased due to the exogenous treatment of GB at all concentrations. In unstressed plants, the activity of SOD, POD, CAT, APX, DHAR and GR was increased by 17.24, 1.91, 3.88, 9.38, 4.45 and 5.19% due to 5 mmol GB, by 37.22, 15.86, 11.60, 23.47, 22.26 and 11.03% due to 10 mmol GB, by 34.48, 15.86, 21.75, 23.47, 25.12 and 12.98% due to 20 mmol GB and by 13.18, 1.91, 3.99, 10.79, 15.71 and 3.89% due to 50 mmol GB contrary to control. In contrast to control plants, the activities increased by 83.16% for SOD, 70.17% for POD, 60.47% for CAT, 57.74% for APX, 89.21% for DHAR, and 70.11% for GR in NaCl-stressed plants. At all concentrations, application of GB to NaCl-stressed plants resulted in a further increase in the activity. The activities of SOD, POD, CAT, APX, DHAR and GR exhibited the highest increase of 138.94, 142.06, 95.65, 87.79, 146.40 and 115.39% respectively in NaCl + 20 mmol GB treated plants, contrary to the control (Figure 6). The content of AsA and GSH increased due to treatment with GB, and in unstressed plants, the highest increases of 16.10% in AsA and 14.34% in GSH were observed in 20 mmol GB-treated plants, compared to the control. Salt stress resulted in increases of 43.07% and 66.08% in AsA and GSH, respectively, compared to the control. When applied to NaCl-stressed plants, GB caused a further increase in AsA and GSH, and the highest increase of 20.58% and 18.84% in AsA and GSH was observed in NaCl + 20 mmol GB-treated plants (Figure 7).

The activities of glyoxalase I and II enzymes increased in the presence of NaCl. Relative to control, the activity of glyoxalase I increased by 61.88% and glyoxalase II increased by 53.28% due to NaCl stress.

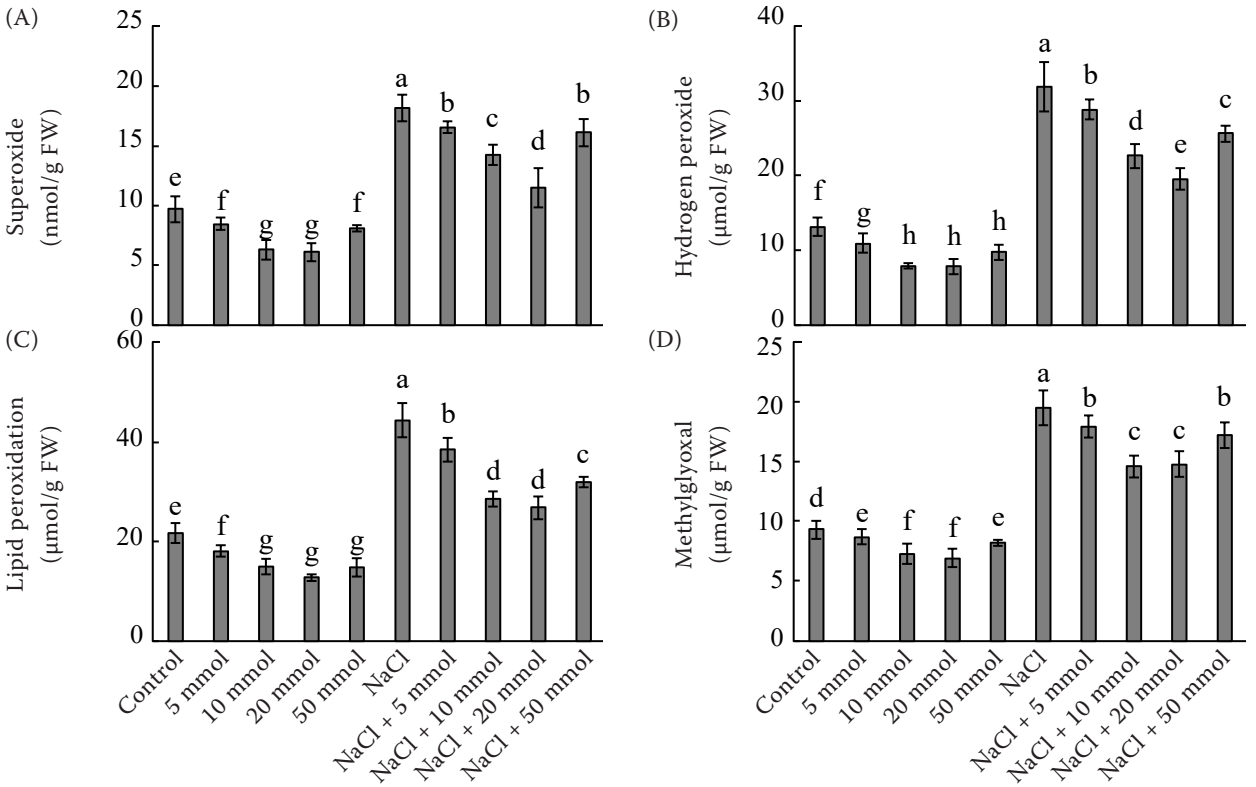

Figure 5. Effect of salinity stress (100 mmol NaCl) on (A) superoxide; (B) hydrogen peroxide; (C) lipid peroxidation, and (D) methylglyoxal content of *Vigna radiata* treated with different concentrations of glycine betaine (0, 5, 10, 20 and 50 mmol). Data is the mean of three replicates, and different letters on graphs indicate a significant difference at $P < 0.05$. FW – fresh weight

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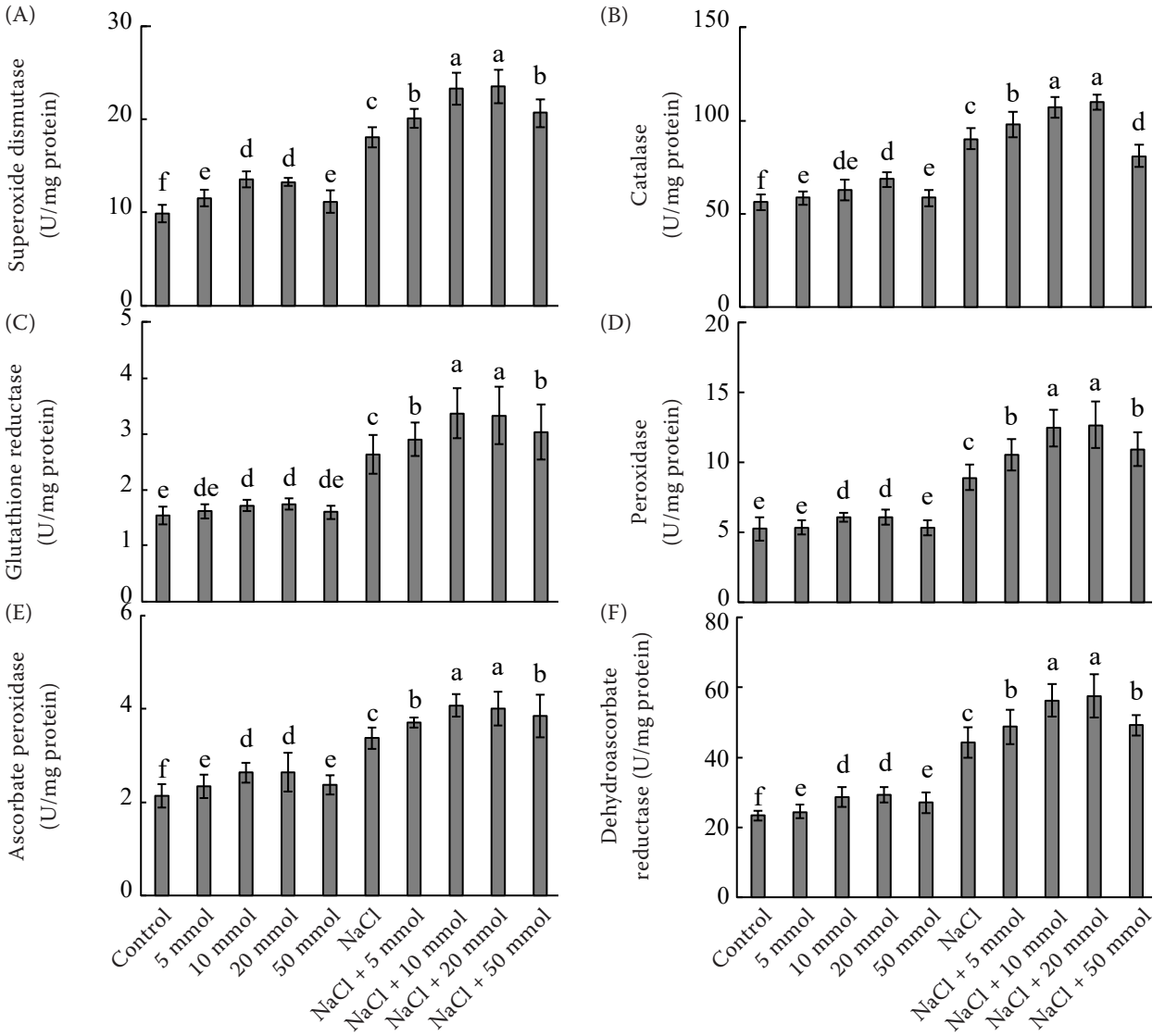

Figure 6. Effect of salinity stress (100 mmol NaCl) on the activity of (A) superoxide dismutase; (B) catalase; (C) glutathione reductase; (D) peroxidase; (E) ascorbate peroxidase, and (F) dehydroascorbate reductase of *Vigna radiata* treated with different concentrations of glycine betaine (0, 5, 10, 20 and 50 mmol). Data is the mean of three replicates, and different letters on graphs indicate a significant difference at $P < 0.05$

Application of GB to NaCl resulted in a further increase in their activities. Contrary to the NaCl-stressed plants, an increase of 8.63% and 11.10% in glyoxalase I and glyoxalase II was observed due to NaCl + 5 mmol GB, 17.19% and 24.14% due to NaCl + 10 mmol GB, 27.62% and 29.69% due to NaCl + 20 mmol GB and 11.30% and 11.48% due to NaCl + 50 mmol GB. In unstressed plants, exogenous GB treatment caused an increase of 5.66% and 6.11% at 5 mmol GB, 14.71% and 15.22% at 10 mmol GB, 21.50% and 21.34% at 20 mmol GB and 13.86% and 9.70% at 50 mmol GB in the activity of glyoxalase I and glyoxalase II, respectively (Figure 8).

Salinity stress resulted in increased total antioxidant activity, and the application of GB further increased this activity. As compared to the control, per cent DPPH and ABTS scavenging increased by 64.37% and 52.74% due to NaCl. The highest increases of 115.63% in DPPH and 103.45% in ABTS scavenging were observed in NaCl + 20 mmol GB-treated plants, as compared to the control. In unstressed conditions, the applied GB also increased DPPH and ABTS scavenging (Figure 9).

The content of Na increased in both leaf and root, while K was decreased significantly due to NaCl treatment. Relative to control, Na exhibited an increase

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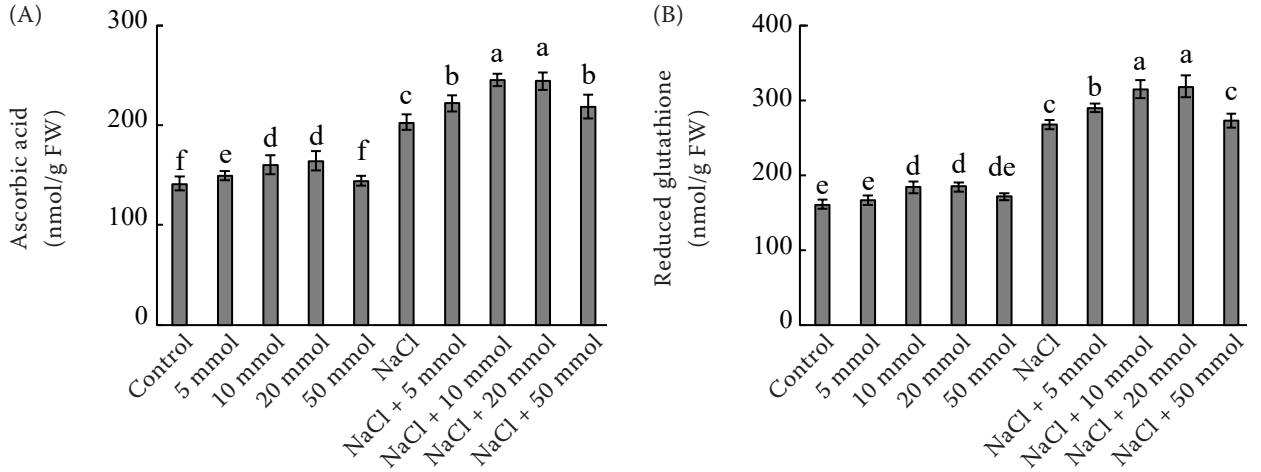

Figure 7. Effect of salinity stress (100 mmol NaCl) on the content of (A) ascorbic acid and (B) reduced glutathione of *Vigna radiata* treated with different concentrations of glycine betaine (0, 5, 10, 20 and 50 mmol). Data is the mean of three replicates, and different letters on graphs indicate a significant difference at $P < 0.05$. FW – fresh weight

of 125.21% and 174.80% in root and leaf, while K content declined by 40.06% and 43.21% due to NaCl stress. The application of GB reduced the Na content in both roots and leaves. Contrary to NaCl-treated plants, a decline of 8.68% and 11.46% in Na content of root and leaf was observed in NaCl + 5 mmol GB, 21.69% and 20.34% in NaCl + 10 mmol GB, 25.48% and 32.95% in NaCl + 20 mmol GB and 23.93% and 20.91% in NaCl + 50 mmol GB. However, the GB application increased K content in normal plants and alleviated the decline caused by NaCl. Relative to the control, K exhibited the highest increases of 39.75% and 24.59% in root and leaf, respectively, due to 20 mmol GB in unstressed plants. Contrary to NaCl stressed plants, application of GB increased K by 11.45% and 10.60% in root and leaf respectively in NaCl + 5 mmol GB, by 27.00% and 22.51% in NaCl + 10 mmol GB, by 40.68% and 37.00% in NaCl + 20 mmol GB and by 24.10% and 20.66% in NaCl + 50 mmol GB (Figure 10).

DISCUSSION

Various elements contribute to the increasing salinity in soils, resulting in considerable yield and productivity losses. In this context, the present study aimed to evaluate the impact of applied GB in strengthening the indigenous salinity tolerance mechanisms in *Vigna radiata* L. Glycine betaine is considered an important and compatible osmotic, having an immense role in plant stress protection. Different concentrations of GB were used, and it

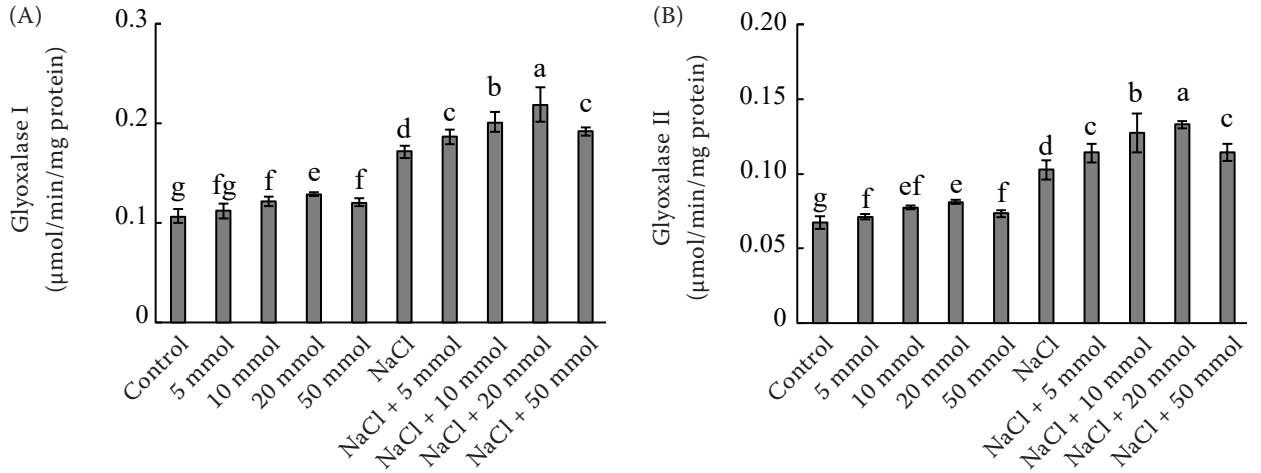

Figure 8. Effect of salinity stress (100 mmol NaCl) on the activity of (A) glyoxalase I and (B) glyoxalase II of *Vigna radiata* treated with different concentrations of glycine betaine (0, 5, 10, 20 and 50 mmol). The data represent the mean of three replicates, and different letters on the graphs indicate a significant difference at $P < 0.05$

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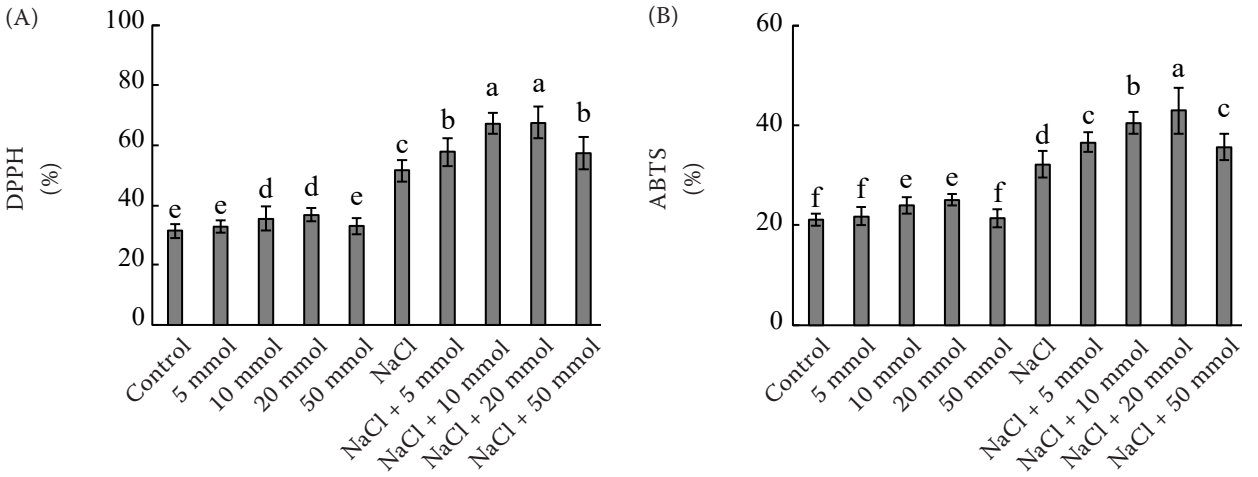

Figure 9. Effect of salinity stress (100 mmol NaCl) on the percent scavenging of (A) DPPH (2,2-diphenyl-1-picrylhydrazyl) and (B) ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) of *Vigna radiata* treated with different concentrations of glycine betaine (0, 5, 10, 20 and 50 mmol). The data represent the mean of three replicates, and different letters on the graphs indicate a significant difference at $P < 0.05$

was observed that the applied GB increased growth attributes, such as plant height and fresh and dry weight, in a concentration-dependent manner. In addition, treatment of GB to salinity-stressed plants alleviated the decline to significant levels. Salinity considerably reduces germination, seedling emergence, growth and biomass production by inducing osmotic and ionic stress (Arif et al. 2020). Salinity stress hinders the cell cycle functioning, therefore influencing division and tissue proliferation (Oney-Birol 2019). In addition, several reports have shown that reduced growth and biomass production under salinity are attributed to decreased tissue water, reduced mineral uptake, and assimilation (Qin et al. 2020, Alamer 2023a). Treatment of GB alleviated the decline in growth and fresh and dry weight of plants, achieving the maximal increase at 10 and 20 mmol concentrations. Treatment of GB alleviated the salinity stress-induced decline in plant height, leaf area, and weight of maize, as reported by Zhu et al. (2022). In addition to its role in osmoregulation, the increased protection in GB-treated plants is attributed to the maintenance of ion homeostasis (Zhu et al. 2022) and improved water use efficiency (Hamani et al. 2021). Excess Na availability drastically affects the uptake of essential nutrients, such as K, thereby posing significant alterations in key developmental events (Ahanger and Agarwal 2017). The reduced Na accumulation due to GB treatment is reflected in the maintenance of a low Na/K ratio, which results from the increased expression of transporter genes that mediate the compartmentation and exclusion of toxic ions (Zhu et al. 2022). Besides, a significant increase was observed in the content of K due to GB treatment, resulting in a decline in the Na/K ratio and eventually preventing the oxidative and ill effects of excess Na on key metabolic pathways. Maintaining relatively high K concentrations enables plants to regulate several key functions, including enzyme activity, osmoregulation, and tolerance mechanisms (Ahanger et al. 2017, Huang et al. 2024). Plants that received exogenous GB treatment exhibited a significant enhancement in K content in both root and leaf tissues, reflecting its beneficial role in preventing the damaging effects of NaCl.

Reduced growth in *Vigna radiata* L. under salinity stress was accompanied by a significant decrease in photosynthetic pigments and photosynthetic gas exchange parameters. It was evident that exogenous GB alleviated the decline in these parameters caused by NaCl. Earlier, reduced synthesis of chlorophyll pigments, a decline in the rate of photosynthesis, and altered gas exchange parameters have been reported in wheat (Ahanger et al. 2019), *Dianthus caryophyllus* (Kwon et al. 2019), and *Robinia pseudoacacia* (Lu et al. 2023). Salinity affects photosynthetic functioning and related attributes by reducing chloroplast size, altering the organisation of lamellae, accumulating lipids and starch, and disrupting cross-membrane transportation (Hameed et al. 2021). Additionally, the activity of genes involved in chloroplast development is downregulated in response to salt stress (Lu et al. 2023). Salt stress triggers chlorophyll degradation and interferes with chlorophyll synthesis by reduc-

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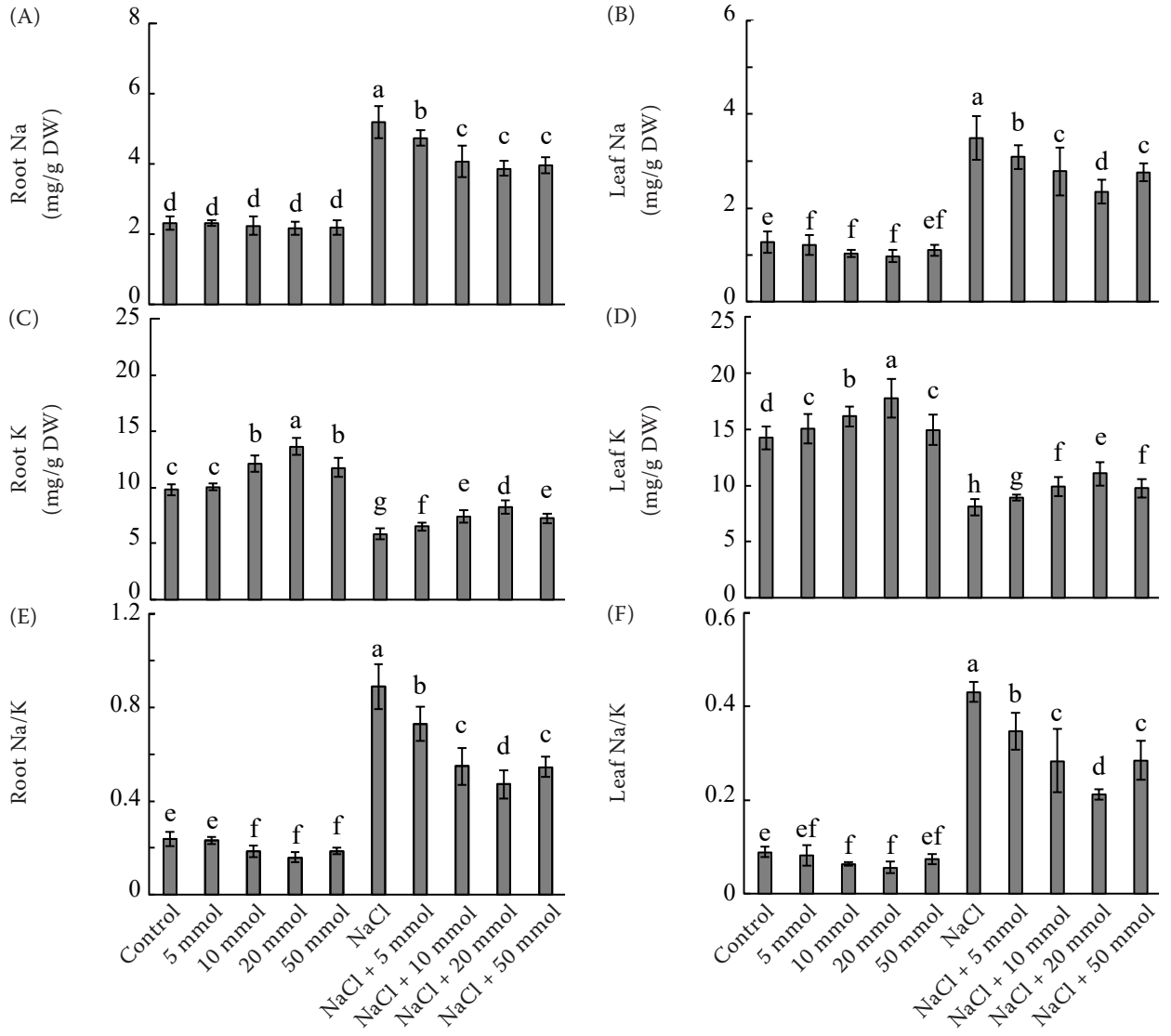

Figure 10. Effect of salinity stress (100 mmol NaCl) on the content of (A) root Na; (B) leaf Na; (C) root K; (D) leaf K, the ratio of (E) root Na/K and (F) leaf Na/K of *Vigna radiata* treated with different concentrations of glycine betaine (0, 5, 10, 20 and 50 mmol). The data represent the mean of three replicates, and different letters on the graphs indicate a significant difference at $P < 0.05$. DW – dry weight

ing the activity of enzymes regulating chlorophyll biosynthesis (Qin et al. 2020, Al-Mushhin 2022). Applied GB not only increased the photosynthetic parameters but also alleviated the decline caused by NaCl, with 10 and 20 mmol GB imparting a much obvious impact. Increased photosynthesis and the chlorophyll pigments in GB-treated plants may be ascribed to its role in protecting chloroplast structure, maintaining osmotic balance and ROS homeostasis. In *Gossypium hirsutum* L. (Hamani et al. 2020) and *Zea mays* L. (Shemi et al. 2021), the treatment of GB has been reported to alleviate the decline in chlorophyll pigments and the stomatal parameters of photosynthesis under salinity and drought stress. Applied GB may have upregulated the chlorophyll synthesis pathway, improved water and mineral availability, and also reduced the accumulation of toxic radicals, leading to greater protection of the photosynthetic apparatus. Further studies can be interesting.

The accumulation of osmolytes, including proline, sugars and GB itself, increased significantly due to the application of exogenous GB. Osmolytes play a crucial role in mitigating the negative effects of stress by maintaining cellular water content, regulating enzyme activity, scavenging radicals, and stabilising the structure and function of membranes (Choudary et al. 2023). Increased accumulation of osmolytes, including proline, sugar and GB under salt stress has also been

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reported by Ahanger et al. (2019), Alamer (2023a), Jin et al. (2024) and Su et al. (2024) in different plant species. Maintaining osmotic adjustment is crucial for cell turgidity and membrane stability, as it regulates protein folding to mediate stress signalling mechanisms and maintains thylakoid membrane stability, leading to photoprotection (Kaur et al. 2024). Exogenous treatment of GB further increased the accumulation of osmolytes, thereby protecting key structural and functional cellular components and metabolic pathways. Increased proline accumulation due to exogenous GB treatment has been reported to enhance RWC and improve membrane stability in *Phaseolus vulgaris* plants, thereby significantly reducing oxidative effects (Sofy et al. 2020). The increased accumulation of osmolytes directly results from modulations in their metabolic pathways, wherein biosynthetic pathways are upregulated, and catabolic pathways are downregulated (Alamer 2023b, Qin et al. 2024). Exogenously applied GB may also have influenced the accumulation of osmolytes by modulating the metabolism. Moreover, the increased accumulation of osmolytes contributes to enhanced photosynthetic performance, reduced photorespiration, and greater stability of the oxygen-evolving complex (Khalid et al. 2022).

The application of GB resulted in increased activity of the antioxidant enzymes. The activities of the antioxidant enzymes are up regulated in plants exposed to stress growth conditions. There are numerous reports available demonstrating a significant increase in the activity of antioxidant enzymes upon exposure to salt stress in various crop species (Ahanger and Agarwal 2017, Qin et al. 2020, Alamer 2023a, Methela et al. 2024). In *Amaranthus caudatus*, Tebini et al. (2025) have recently demonstrated that genotypes exhibiting higher antioxidant activity also show better growth and tolerance to salinity. The application of GB effectively enhanced the activity of the antioxidant enzymes assayed and further increased their activity when applied to salt-stressed plants. Though, GB caused an increase at all concentrations, however, 10 and 20 mmol concentrations imparted maximal enhancement. Earlier, Hasanuzzaman et al. (2014) also observed a significant increase in the activities of antioxidant enzymes, resulting in the maintenance of growth and redox balance in rice under salinity stress. Increase in the activity of SOD, CAT, POD, and the components of the ascorbate-glutathione cycle due to GB application reflects the beneficial role in preventing oxidative effects of salinity. The up-regulated functioning of the antioxidant system facilitates the rapid elimination of toxic radicals, thereby maintaining their concentration at a low level, which protects major cellular structures and their functioning (Hasanuzzaman et al. 2014, Tebini et al. 2025). Plants exhibiting an enhanced antioxidant system function show greater adaptability to stressful conditions and display improved growth and photosynthetic performance (Foyer and Shigeoka 2011). Islam et al. (2021) have also demonstrated a considerable increase in the antioxidant activity of *Brassica juncea* due to exogenous GB application, resulting in improved growth and photosynthetic functioning under salt stress. Additionally, the accumulation of GSH and AsA was also enhanced by the GB treatment. Both GSH and AsA are key antioxidant molecules that act as redox components and contribute actively to several developmental events in plants (Mishra et al. 2023). These non-enzymatic antioxidants scavenge toxic radicals, mediate several enzymatic reactions and form key components of the ascorbate-glutathione cycle; therefore, they also contribute to the maintenance of electron transport by maintaining the NADP/NADPH ratio. Increased GSH and AsA content in GB-treated plants has been reported by Hasanuzzaman et al. (2014) in salt-stressed rice and Islam et al. (2022) in chromium-stressed *Cicer arietinum*. The increased functioning of antioxidant enzymes and the accumulation of AsA and GSH, resulting from GB application, showed a close relationship with the accumulation of toxic radicals and lipid peroxidation. Both reactive radicals, including hydrogen peroxide and superoxide, exhibited a significant reduction in accumulation due to the addition of GB. Stresses, including salinity, trigger oxidative damage by causing a manifold increase in the accumulation of radicals as has been reported in wheat (Alamer 2023a), moringa (Azeem et al. 2023) and peanut (Singh et al. 2025). Reduced radical accumulation and lipid peroxidation in GB-supplemented plants reflect in improved cellular and membrane stability. Similar to these results, Hasanuzzaman et al. (2014) and Dai et al. (2024) have also observed a decline in radical accumulation and lipid peroxidation in rice and tomato under salinity and cold stress upon the application of exogenous GB. In addition, the supplementation of GB resulted in increased radical scavenging, as determined by DPPH and ABTS assays. Salinity increases total antioxidant activity assayed as percent DPPH and

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ABTS scavenging, as observed in moringa (Azeem et al. 2023) and *Amaranthus caudatus* (Tebini et al. 2025). However, the impact of exogenously applied GB on DPPH and ABTS scavenging potential under salinity stress has not been reported.

The activity of glyoxalase enzymes registered an increase with the GB application under normal as well as NaCl stress. Environmental stresses, including salinity, increase the production of toxic methylglyoxal, which can have adverse effects on growth and metabolic processes (Talaat and Todorova 2022). Methylglyoxal is a cytotoxic molecule that provokes detrimental effects by triggering the formation of radicals and advanced glycation end production. Also, it weakens the antioxidant system, while at lower concentrations it can act as a stress signal showing crosstalk with calcium, reactive oxygen species and phytohormones like ABA (Li 2022). The up-regulated activity of glyoxalase enzymes facilitates the rapid detoxification of methylglyoxal within cells, thereby preventing its adverse effects on cells and plants as a whole (Li 2022). Applied GB caused a significant increase in the activities of glyoxalase I and II, resulting in a substantial decline in methylglyoxal accumulation. This reduced accumulation of methylglyoxal in GB-treated plants confirms its beneficial role in protecting cellular functioning and metabolism from adverse stress effects. In rice, exogenous treatment with GB has been reported to decrease methylglyoxal accumulation under salinity stress by upregulating the activity of glyoxalase cycle enzymes (Hasanuzzaman et al. 2014).

Conclusively, it can be said that the application of GB alleviated the adverse effects of salinity by up-regulating key tolerance mechanisms, such as the antioxidant system, glyoxalase cycle, and compatible solute accumulation. These mechanisms contributed to the elimination of toxic radicals, thereby protecting major cellular functions, including photosynthesis. In addition, GB treatments reduced Na accumulation, thereby contributing to ion homeostasis and preventing the damaging effects of Na, accompanied by a concomitant increase in K. Oxidative effects were mitigated by the application of GB, more evidently at 10 and 20 mmol concentrations, as reflected by reduced radical accumulation and lipid peroxidation. Hence, the application of GB can enhance the growth and productivity of *Vigna radiata* L. by regulating key physiological and biochemical mechanisms to mitigate the adverse effects of salinity.

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