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Alleviating cadmium toxicity in maize plants: role of glycine betaine in enhancing growth, photosynthetic efficiency, water status, and antioxidant defense mechanism

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Abstract: The issue of heavy metals (HMs) contamination poses a significant challenge in the environment, exerting a severe impact on the growth and productivity of crops. Cadmium (Cd) is specifically identified as the seventh heavy metal among the top 20 pollutants, primarily due to its elevated phytotoxicity and its solubility in water. In the current study, foliar application of glycine betaine (GB) (500 µmol) investigated the toxic effects of cadmium in maize plants subjected to two Cd concentrations (50 and 100 µmol) as $CdCl_2$. The maize plants exposed to Cd stress exhibited a massive reduction in growth, biomass, photosynthetic pigments [chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), carotenoids, and total pigments], gas exchange parameters [transpiration rate (T_r), net photosynthetic rate (P_n), intracellular CO_2 concentration (c_i), and stomatal conductance (g_s)], relative water content (RWC), and organic osmolytes content [total soluble protein (TSS), and total soluble sugar (TSS)]. These impacts were significant with the 100 µmol $CdCl_2$ treatment. Moreover, Cd led to remarked increase in proline, nonenzymatic antioxidants levels [ascorbic acid (AsA) and glutathione (GSH)] as well as the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR). On the other hand, GB application efficiently relieved the Cd toxic impacts on maize and maintained higher growth criteria, gas exchange parameters, photosynthetic pigments, RWC, and organic osmolytes. In addition, the exogenous application of GB added more enhancement to the antioxidative system (enzymatic and nonenzymatic). These results imply that GB could significantly preserve maize growth under Cd toxicity conditions by maintaining photosynthetic characteristics, water status, and antioxidant system. This suggests an enhancement in the plant's resilience to stress induced by heavy metals.

Keywords: *Zea mays* L.; environmental pollutants; water homeostasis; physiological parameters; stomatal behaviour; adaptation

Heavy metal (HM) toxicity poses a dual threat, affecting both living organisms and the global environment. Cadmium (Cd), chromium (Cr), zinc (Zn), aluminum (Al), nickel (Ni), and metalloids like arsenic (As) contribute to diminished plant growth and development, promoting metabolic changes in plants (Riyazuddin et al. 2022). The involvement of HMs in oxidation/reduction processes forms the foundation of their roles in plant metabolism (Zulfiqar et al. 2022). Cd, a toxic non-essential transition metal, causes health risks to both animals and plants, originating from natural sources, as well as industrial and agricultural activities (Zhao et al. 2021). In addition, Cd has a lasting impact on the environment, persisting for many years (Zulfiqar et al. 2022). Cereal crop cultivars, crucial for human sustenance, can accu-

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mulate high Cd concentrations in their grains, with more than 40% absorbed by plants and subsequently influencing human health either indirectly (through animals) or directly (through grains) (Zulfiqar et al. 2022). High concentrations of Cd impede seed germination, root elongation, alter chloroplast ultrastructure, promote chlorosis, and disrupt antioxidant enzymes activity (Riyazuddin et al. 2022). Cd interacts with various components of the photosynthetic apparatus, reducing the efficiency of electron transport, causing damage to the photosynthetic organs, and changing the structure of chlorophyll, which inhibits PSII stimulated by light energy capture and power efficiency (Zulfiqar et al. 2022). To combat heavy metal stress, plants employ strategies such as synthesising osmolytes, chelating agents, enzyme antioxidants, and nonenzymatic antioxidants (Zhao et al. 2021). These mechanisms play a crucial role in minimising the stress induced by heavy metal exposure. Glycine betaine (GB) stands out as a quaternary ammonium compound, playing a significant role as a compatible solute for various plants facing diverse environmental stresses like heat, drought, salinity, and HMs (Kumar 2021). When applied to plants exposed to stress from HM, GB shows a successful enhancement of growth by promoting nutrient uptake, increasing chlorophyll content, elevating photosynthetic rate, boosting antioxidant enzyme activities, and mitigating excessive HM uptake and oxidative stress (Ali et al. 2020). Previous studies have investigated the significant impacts of GB on alleviating HM toxicity in various plant species, including tobacco, cauliflower, and maize (He et al. 2019, Ahmad et al. 2020, Zhang et al. 2020). Furthermore, GB acts as an effective scavenger for toxic reactive oxygen species (ROS) (Ali et al. 2020). It maintains optimal osmotic pressure and revitalises the antioxidant machinery during abiotic stress, contributing to the plant's resilience in challenging conditions (Kumar 2021).

Maize (*Zea mays* L.) is a member of the Poaceae family and is useful as human food, animal feed, and a raw material for many industries (Amin et al. 2024). On the other hand, it is a great source of photochemical chemicals and nutritional components. It has over 3 500 items, such as specialty maize like QPM (quality protein maize), which has almost twice the amount of lysine and tryptophan (two amino acids necessary for human consumption) (Kumar et al. 2022). Consequently, the goal of the current study is to determine how successful foliar spraying of GB is in relieving the destructive influence of Cd toxicity on growth parameters (plant height and root length) biomass (fresh and dry weights), Cd content, water status, photosynthetic pigments, gas exchange parameters, some organic osmolytes content [proline, total soluble protein (TSS), total soluble sugar (TSS)], non-enzymatic antioxidant levels [ascorbic acid (AsA) and glutathione (GSH)], and antioxidant enzyme activity [superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR)], in maize plants.

MATERIAL AND METHODS

Plant culture. This study was conducted in Egypt (31°25'E, 30°06'N) at the beginning of May/2021. Maize seeds (*Zea mays* L., cv. Giza 2) were supplied by the Egyptian Agriculture Ministry. A homogeneous group of healthy maize seeds was sterilised in a 70% ethanol solution for 30 s, then transferred to 6% NaOCl for 15 min before being repeatedly washed with distilled H_2O. The sterilised seeds were soaked for 24 h in distilled water under fluorescent white light in a germinator. The seeds were allowed to germinate in a sporadic mist perlite medium until their first true leaves grew. Hoagland's solution (No. 1) quarter-strength or distilled water was used to irrigate the plants. After washing the roots to remove residual perlite, the selected plants were placed in polyethylene containers with adequate aeration and in 2 L of modified Hoagland's solution at a quarter strength.

Plants were transferred to full-strength modified Hoagland's solutions After pre-cultured for 10 days. The composition of the nutrient solution was (in mmol/L): 0.10 KH_2PO_4, 2.0 $Ca(NO_3)_2{\cdot}4\,H_2O$, 0.10 KCl, 0.50 $MgSO_4{\cdot}7\,H_2O$, 0.70 K_2SO_4, and (in µmol/L): 0.50 $ZnSO_4{\cdot}7\,H_2O$, 0.50 $MnSO_4{\cdot}H_2O$, 10 H_3BO_3, 0.01 $(NH_4)_6Mo_7O_{24}{\cdot}4\,H_2O$, 0.20 $CuSO_4{\cdot}5\,H_2O$, 100 Fe-EDTA. The plants were classified into two sets; the 1st set continued to be cultured in Hoagland's nutrient solution and was subclassified into two subgroups named control (cont.) and control + glycine betaine (cont. + GB). The 2nd set was cultured with Hoagland's nutrient solution plus Cd as $CdCl_2$ (50 and 100 µmol/L) and divided into four subgroups: 50 µmol $CdCl_2$ (50 µmol); 100 µmol $CdCl_2$ (100 µmol); 50 µmol $CdCl_2$ + glycine betaine (50 µmol + GB), and 100 µmol $CdCl_2$ + glycine betaine (100 µmol + GB). The plants were sprayed with glycine betaine (10 mmol) weekly during the experiment. Each treatment was applied randomly in a complete block design, with three rep-

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lications of each treatment. Aeration and nutrition solution replacement were done every three days. By daily adjustments with 0.1 mol/L NaOH or 0.1 mol/L HCl, the pH was maintained at 6. The experiment was conducted in a greenhouse with natural lighting throughout the day and average day and night temperatures of 30 ± 4 and 24 ± 5 °C, respectively. The greenhouse also had a relative humidity of 70% to 62%. At the V6 ("V" refers to vegetative) developmental stages, for all treatments, where the 6th leaf of the maize plant fully emerged and the leaf collar is visible, the plants were harvested from the control as well as the treatments for all measurements.

Plant growth and biomass measurements. Shoot and root fresh weight were used to measure plant growth. Plants were separated into shoots and roots and dried for 48 h at 80 °C to determine the dry weight (DW).

Cd content. The samples from the fresh tissues of stressed and unstressed plants were dried in an oven and ground into a fine powder. After soaking the samples in HNO_3-$HClO_4$ (3:1, *v*/*v*), the concentration of Cd in shoots and roots was determined using an atomic absorption spectrophotometer (Schimadzu AA-7800, Tokyo, Japan) according to Woodis et al. (1977). For each sample, the Cd concentration was calculated using a standard curve generated from a series of known Cd concentrations.

Photosynthetic pigments. Fresh discs from the 6th leaf of all treatments were used to extract chlorophylls and carotenoid pigments by grinding in cold acetone (80%) and centrifuged at 5 000 *g* for 10 min. The chlorophyll and carotenoid contents were estimated using an atomic absorption spectrophotometer (Schimadzu AA-7800) at wavelengths of 480, 644 and 663 nm according to Lichtenthaler and Wellburn (1983). The absorbance of purified pigments (mg/g DW) was detected at 470, 646, and 663 nm using a spectrophotometer against blank of pure 80% aqueous acetone.

Gas exchange parameters. Net photosynthetic rate (P_n); transpiration rate (T_r); intracellular CO_2 concentration (c_i), and stomatal conductance (g_s) were determined for photosynthetic gas exchange traits between 10:00 and 11:00 AM for the 6th leaf of all treatments using portable gas exchange system, LCA-4 (Analytical Development Company Ltd, Hoddeston, UK). Measurements were carried out with a 5.32 cm^2 leaf area, chamber temperature of 30 °C and leaf chamber CO_2 concentration of 370 g at photosynthetic photon flux density (PPFD) of 1 800 µmol photons/m^2/s.

Relative water content. The RWCs of stressed and unstressed maize 6th leaves were measured as described by Schonfeld et al. (1988). A scalpel cut the leaves at the base, and the fresh weights (FW) were determined immediately. After that, the leaves were let to soak for 24 h at room temperature in distilled H_2O. Tissue papers were used to dry the leaves before measuring their turgid weights (TW). The leaves were then maintained in an oven set at 80 °C for 48 h, during which their dry weights were calculated. The following formula was used to determine each treatment's RWC:

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

Determination of the total soluble protein. The Bradford (1976) method extracted and measured TSP in both treated and untreated maize 6th leaves. Saline phosphate buffer was prepared by combining 10 mmol/L Na_2HPO_4, 2.7 mmol/L KCl, 2 mmol/L KH_2PO_4, and 1.37 mmol/L NaCl. To maintain pH 7.2, 62.5 mmol/L of Tris HCl was used. 0.5 g of fresh-weight leaves were extracted and placed in saline phosphate buffer to determine TSP. The supernatant is extracted by centrifuging in the solution. After dissolving the dye stock to the same volume as the supernatant and swirling, it was incubated for 30 min. The absorbance was measured using an atomic absorption spectrophotometer (Scilogex SCI-UV1100, Hong Kong, China) set at 595 nm. For the standard curve, a series of 0 to 100 µg/mL bovine serum albumin was used.

Determination of total soluble sugar. Applying Yoshida et al. (1976) method, TSS were extracted and assessed from treated and untreated maize 6th leaves. Dry tissue was immersed in 10 mL of 80% (*v*/*v*) ethanol at 25 °C to extract TSS. For the entire night, shaking occasionally. TSS was determined by heating 0. l mL of alcoholic extract in a boiling water bath for 10 min, reacting it with 3.0 mL of freshly prepared anthrone reagent. The samples were then measured at 625 nm using a Spectronic 21D spectrophotometer (Thermo Fisher Scientific, Waltham, USA). A series of 0 to 100 µg/mL glucose for the standard curve was used to estimate the TSS concentration.

Estimation of proline. According to Lee et al. (2018), proline was evaluated using the ninhydrin-based colourimetric method in both treated and untreated maize 6th leaves. Following grinding the fresh leaves (0.5 g), 20 µL of 1% (*w*/*v*) sulfosalicylic acid was added per mg FW tissue. After centrifuging at 15 000 *g* for 5 min at 4 °C, the supernatant was

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extracted and mixed with acidic ninhydrin (1.25% [*w*/*v*] ninhydrin in 80% [*v*/*v*] acetic acid) in a 1:2 ratio. The mixture was then incubated at 95 °C for 30 min. An atomic absorption spectrophotometer (Schimadzu AA – 7800, Tokyo, Japan) was used to detect the absorbance at 510 nm. A series of 0 to 100 µg/mL proline was used for the standard curve to estimate the proline content.

Estimation of reduced glutathione. Ellman's (1959) method was used to determine reduced glutathione (GSH) levels. After 500 mg of fresh tissues of the 6th leaf were homogenised in 15% metaphosphoric acid, they were centrifuged at 5 000 *g* for 30 min at 4 °C. Following a 30 min incubation period, 200 µL of the supernatant was combined with 2.6 mL of phosphate buffer (100 mmol/L, pH 8.0) and 200 µL of 5,5'-dithiobis (2-nitrobenzoic acid) (6 mmol). The absorbance was measured at 412 nm using an atomic absorption spectrophotometer (Schimadzu AA-7800). A series of 0 to 100 µg/mL reduced glutathione is used for the standard curve to determine the GSH level.

Estimation of ascorbate. Using the method Mukherjee and Choudhuri (1983) described, ascorbate (AsA) content was determined. A pestle and mortar were used to homogenise the 6th leaf fresh tissue in 6% (*w/v*) trichloroacetic acid. The mixture was heated for 15 min in a water bath after the extract was centrifuged at 5 000 *g* for 10 min, 2% dinitrophenylhydrazine, and 10% thiourea were added to the supernatant. 5 mL of cooled 80% H_2SO_4 were added to the samples once they had cooled, and the absorbance was measured at 530 nm using an atomic absorption spectrophotometer (Schimadzu AA-7800). The ascorbate solution (0 to 100 µg/mL) standard curve was used to determine the AsA concentration.

Antioxidant enzyme extraction and assay. Using a prechilled pestle and mortar, 1 g of the 6th leaf fresh tissue was homogenised in 50 mL of chilled phosphate buffer (100 mmol/L, pH 7.0), supplemented with 1 mL of EDTA (ethylenediaminetetraacetic acid) and 1% (*w/v*) polyvinyl pyrrolidine to extract antioxidant enzymes. The homogenate was centrifuged at 15 000 *g* for 20 min at 4 °C, and the supernatant was then utilised as a source of enzymes. The protein content of the supernatant was assessed using the Lowry et al. (1951) method, with bovine serum albumin serving as a standard by using an atomic absorption spectrophotometer (Schimadzu AA-7800).

Superoxide dismutase (SOD, EC 1.15.1.1) was assessed by using Bayer and Fridovich (1987) method and nitroblue tetrazolium (NBT) photochemical reductions were recorded at 560 nm in a 1.5 mL assay mixture containing sodium phosphate buffer (50 mmol/L, pH 7.5), 100 µL EDTA, 13 mmol L-methionine, 75 µmol NBT, 60 µmol riboflavin and 100 µL enzyme extract. The light was switched off after 15 min of incubation.

The Aebi (1984) method was applied for the catalase assay (CAT, EC 1.11.1.6). The change in absorbance was monitored for 2 min at 240 nm. An extinction coefficient of 39.4 mmol/cm was used for the calculation.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured using the method described by Nakano and Asada (1981). The absorption was recorded at 290 nm for 3 min in a 1 mL reaction mixture containing potassium phosphate buffer (100 mmol/L, pH 7.0), 0.5 mmol hydrogen peroxide, 0.5 mmol ascorbic acid, and 0.1 mL enzyme extract. The calculation of the extinction coefficient of 2.8 mmol/cm was used.

Foyer and Halliwell (1976) method was used to assess the glutathione reductase (GR; EC 1.6.4.2) activity in an assay mixture containing sodium phosphate buffer (100 mmol/L, pH 7.8), 0.5 mmol/L oxidised glutathione, 0.1 mmol/L nicotinamide adenine dinucleotide phosphate, and 0.1 mL enzyme extract. The absorption was recorded for 2 min at 340 nm, and an extinction coefficient of 6.2 mmol/cm was used for calculation.

Statistical analysis. The means ± standard errors (SEs) from three replicates were used to analyse the data. Welch's ANOVA test, followed by the Games-Howell post hoc test of less than 5% ($P < 0.05$), was used to analyse the significant differences between treatments. IBM SPSS statistics data editor 29.0 and Sigma Plot 10.0 were the programs used to conduct the analyses and create the graphs (Daniel 1995).

RESULTS

Impact of GB application and Cd toxicity on growth parameters of maize. The growth parameters of the maize shoot (plant height, shoot fresh and dry weights) showed a progressive decline as the Cd concentration increased. (Figures 1A, B, D). For instance, the reduction in the plant height, shoot fresh and dry weights were higher with the100 µmol (34.6, 32, and 31%) than the 50 µmol (22.4, 18.6, and 19%), respectively. Furthermore, the GB application alleviated the effect of Cd and showed less reduction in the plant height, shoot fresh and dry weights by 8, 9.5, and 8.7% with

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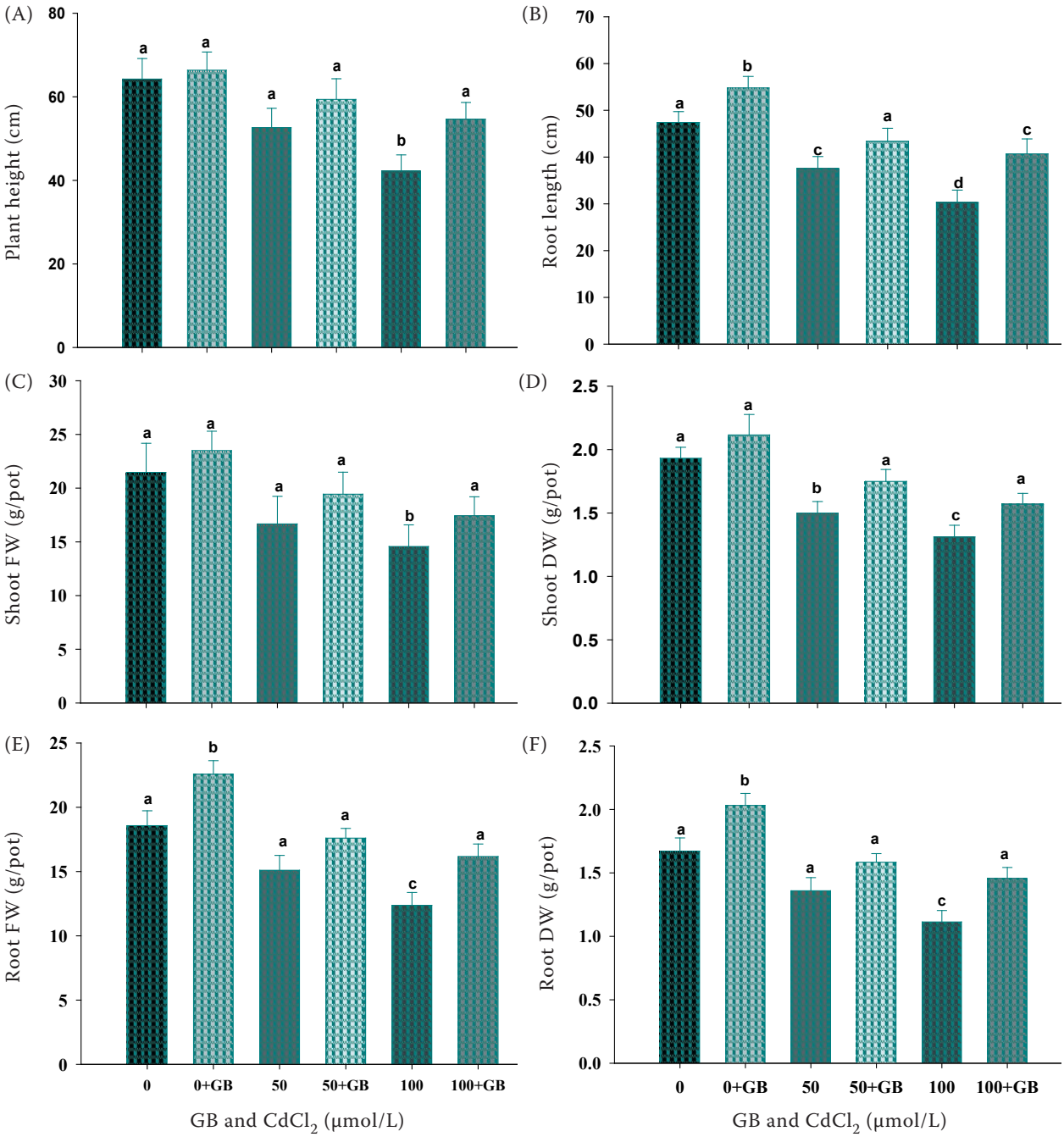

Figure 1. Impact of glycine betaine (GB) application and cadmium (Cd) toxicity on the growth parameters of maize. (A) plant height; (B) root length; (C) shoot fresh weight; (D) shoot dry weight; (E) root fresh weight, and (E) root dry weight. The mean of 3 replicates ± standard error is shown in each column. Error bars are used to show means of standard errors. Welch's ANOVA test, followed by the Games-Howell post hoc test of less than 5% ($P < 0.05$), were used to analyse the significant differences between different treatments indicated by various letters. 0 – control; 0 + GB – glycine betaine (GB); 50 – 50 µmol $CdCl_2$; 50 + GB – 50 µmol $CdCl_2$ + GB; 100 – 100 µmol $CdCl_2$; 100 + GB – 100 µmol $CdCl_2$ + GB; FW – fresh weight; DW – dry weight

50 µmol + GB and by 15.4, 18.8, and 17% with 100 µmol + GB, respectively, as compared to control plants.

Additionally, similar results have been observed for the root growth parameters (root length, fresh and dry weights) (Figures 1C, E, F). Plants treated with Cd showed less decrease in root length and fresh and dry weight with 50 µmol (20.8, 18.7, and 17%) than 100 µmol (36, 33.4, and 32%), respectively. On the other hand,

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the GB supplementation caused an enhancement in the (root length, fresh and dry weight (8.4, 5.2, and 6%) with 50 µmol + GB and (14.2, 12.9, and 12%) with 100 µmol + GB, respectively, concerning control values.

GB's role in alleviating Cd accumulation in maize shoots and roots. Cd applications led a remarkable ($P < 0.05$) accumulation in the Cd content of maize shoots and roots, as compared with untreated plants (Figures 2A, B). The Cd concentrations were higher in root (120.6 and 202.5 mg/kg) than in shoot (50.4 and 68.8 mg/kg) with 50 µmol and 100 µmol treatments, respectively. When stressed plants were treated with GB, the increase in Cd accumulation in shoots and roots was less pronounced, estimating 20.3 and 56 mg/kg with 50 µmol + GB and 32.2 and 85 mg/kg with 100 µmol + GB, respectively.

The influence of GB application and Cd stress on RWC in maize. About the untreated plants, Cd stress reduced the RWC of maize leaves (Figure 2C). This reduction was more noticeable ($P < 0.05$) with the 100 µmol (30.4%) than with the 100 µmol treatment (12%). On the other hand, GB application repaired RWC levels by (7.8 and 22.5%) under 50 and 100 µmol + GB treatments, respectively. Otherwise, the lowest (62 ± 3.26) and highest (96 ± 2.45) RWC values were recorded with 100 µmol and 0 µmol + GB treatments, respectively.

GB treatment enhanced photosynthetic pigments in maize under Cd stress. Cd application led to a remarkable reduction ($P < 0.05$) in the photosynthetic pigments (Chl *a*, Chl *b*, carotenoids, and total pigments) of maize leaves, as compared with untreated plants (Figure 3). For instance, the decrease in Chl *a* and Chl *b* was higher with 100 µmol (54% and 55.4%) than 50 µmol (18.8% and 22.8%), respectively (Figures 3A, B). When plants were treated with 100 µmol $CdCl_2$ + GB, the drop in Chl *a* and Chl *b* content was less pronounced, estimating 23% and 29.7%, respectively.

Furthermore, plants stressed by Cd showed less decrease in carotenoids and total pigments with 50 µmol (19% and 19.8%) than 100 µmol (55% and 54.5%), respectively (Figures 3C, D). Additionally, the foliar application of GB showed less reduction in the content of carotenoids and total pigments (14.5% and 13.2%) with 50 µmol + GB and (34.4% and 27%) with 100 µmol + GB, respectively, concerning control values. On the other hand, the maximum (1.64 ± 0.13 and 6.8 ± 0.55) and minimum (0.59 ±

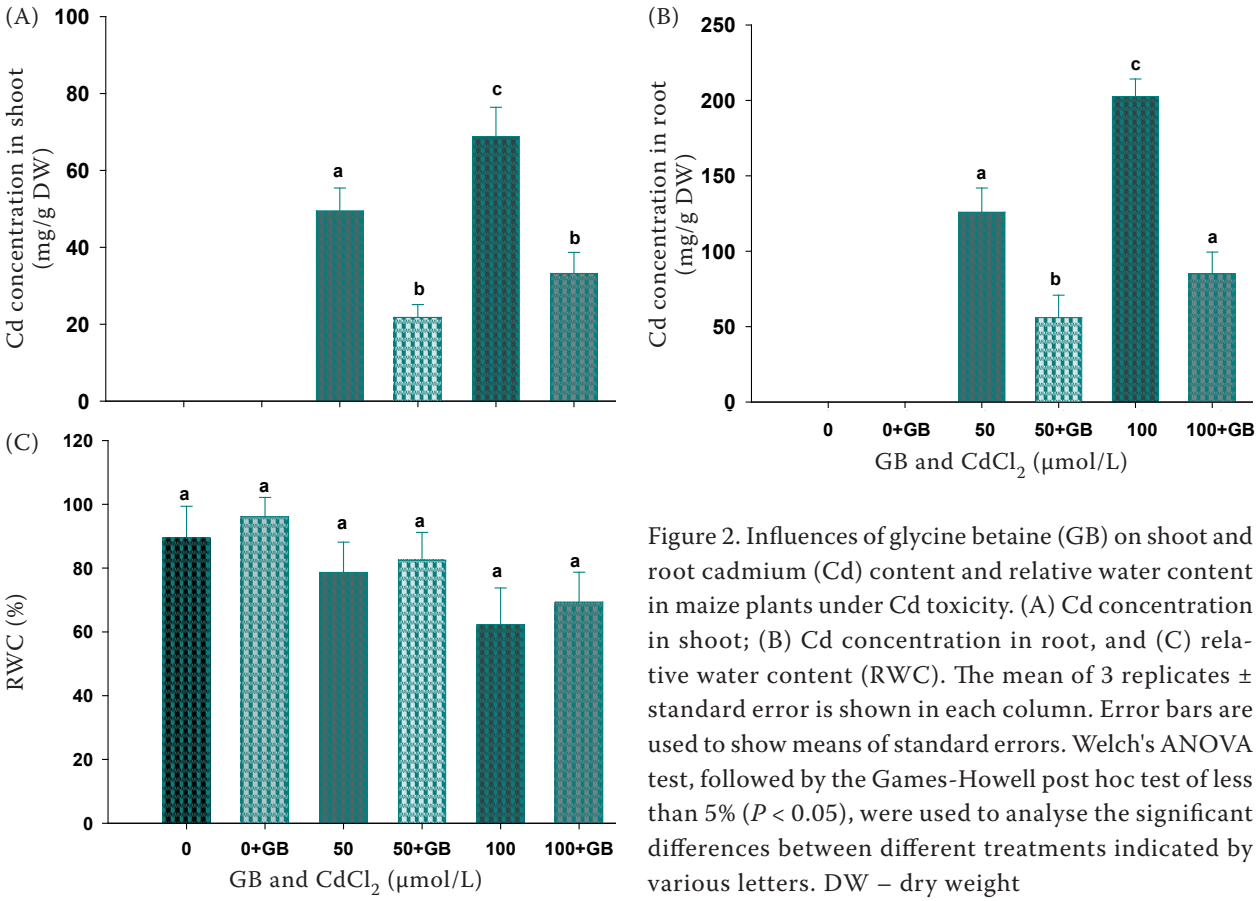

Figure 2. Influences of glycine betaine (GB) on shoot and root cadmium (Cd) content and relative water content in maize plants under Cd toxicity. (A) Cd concentration in shoot; (B) Cd concentration in root, and (C) relative water content (RWC). The mean of 3 replicates ± standard error is shown in each column. Error bars are used to show means of standard errors. Welch's ANOVA test, followed by the Games-Howell post hoc test of less than 5% ($P < 0.05$), were used to analyse the significant differences between different treatments indicated by various letters. DW – dry weight

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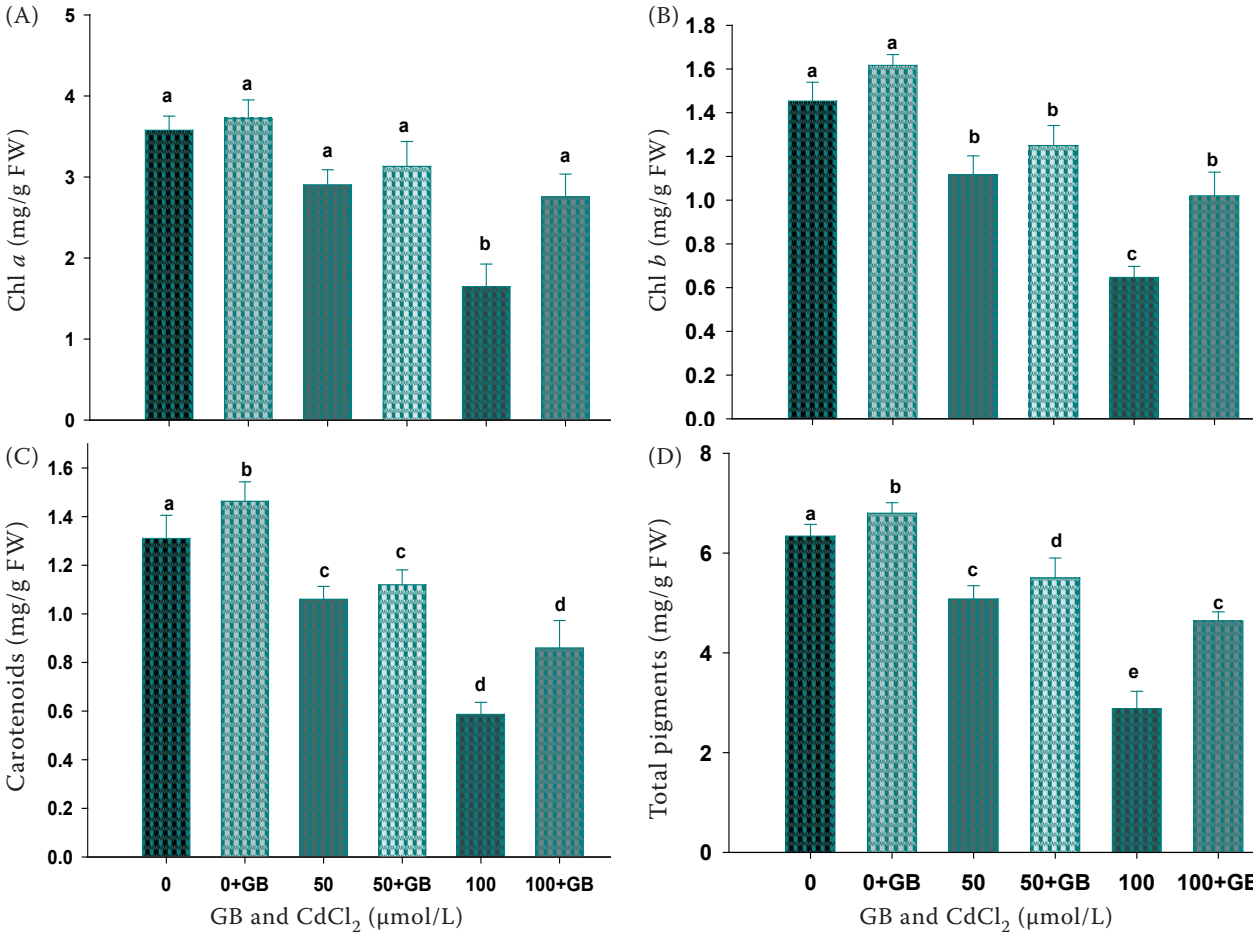

Figure 3. Effects of glycine betaine (GB) application and cadmium (Cd) stress on the photosynthetic pigments in maize leaves: (A) chlorophyl *a* (chl *a*); (B) chlorophyl *b* (chl *b*); (C) carotenoids, and (D) total pigments. The mean of 3 replicates ± standard error is shown in each column. Error bars are used to show means of standard errors. Welch's ANOVA test, followed by the Games-Howell post hoc test of less than 5% ($P < 0.05$), were used to analyse the significant differences between different treatments indicated by various letters. FW – fresh weight

0.07 and 2.88 ± 0.24) carotenoids and total pigment values were recorded with 0 µmol $CdCl_2$ + GB, and 100 µmol treatments, respectively.

GB's role in gas exchange parameter measurements in maize is under Cd toxicity. A significant reduction in net photosynthetic rate, transpiration rate, stomatal conductance, and intracellular CO_2 concentration was recorded in Cd-stressed maize, although the reduction extent was higher with 100 µmol treatment (Figure 4).

Adding Cd reduced the P_n and T_r levels in treated maize plants compared to their respective controls. The decrease was more notable ($P < 0.05$) with the 100 µmol (59% and 53%) than the 50 µmol (20% and 25.8%) in P_n and T_r levels, respectively (Figures 4A, B). Furthermore, GB foliar application to Cd-treated plants enhanced the P_n and T_r levels but remained below the control levels. Additionally, the highest values of P_n and T_r (20.7 ± 2.36 and 3.74 ± 0.23) and lowest (7.56 ± 1.35 and 1.67 ± 0.12) were detected with 0 µmol + GB and 100 µmol treatments, respectively, compared with untreated plants.

A similar pattern of results was observed for c_i and $g_{s,}$ where they were considerably ($P < 0.05$) reduced by 21.3% and 28.3% with 50 µmol and by 38.6% and 60% with 100 µmol $CdCl_2$ application, respectively, in relation to control plants (Figures 4C, D). On the other hand, GB addition seemed to improve the T_r and g_s levels by 10.6% and 16.4% with 50 µmol + GB treatment and by 16.6% and 35% under 100 µmol + GB treatment, respectively, as compared with control plants.

GB foliar application enhanced the accumulation of organic osmolyte content in Cd-stressed maize.

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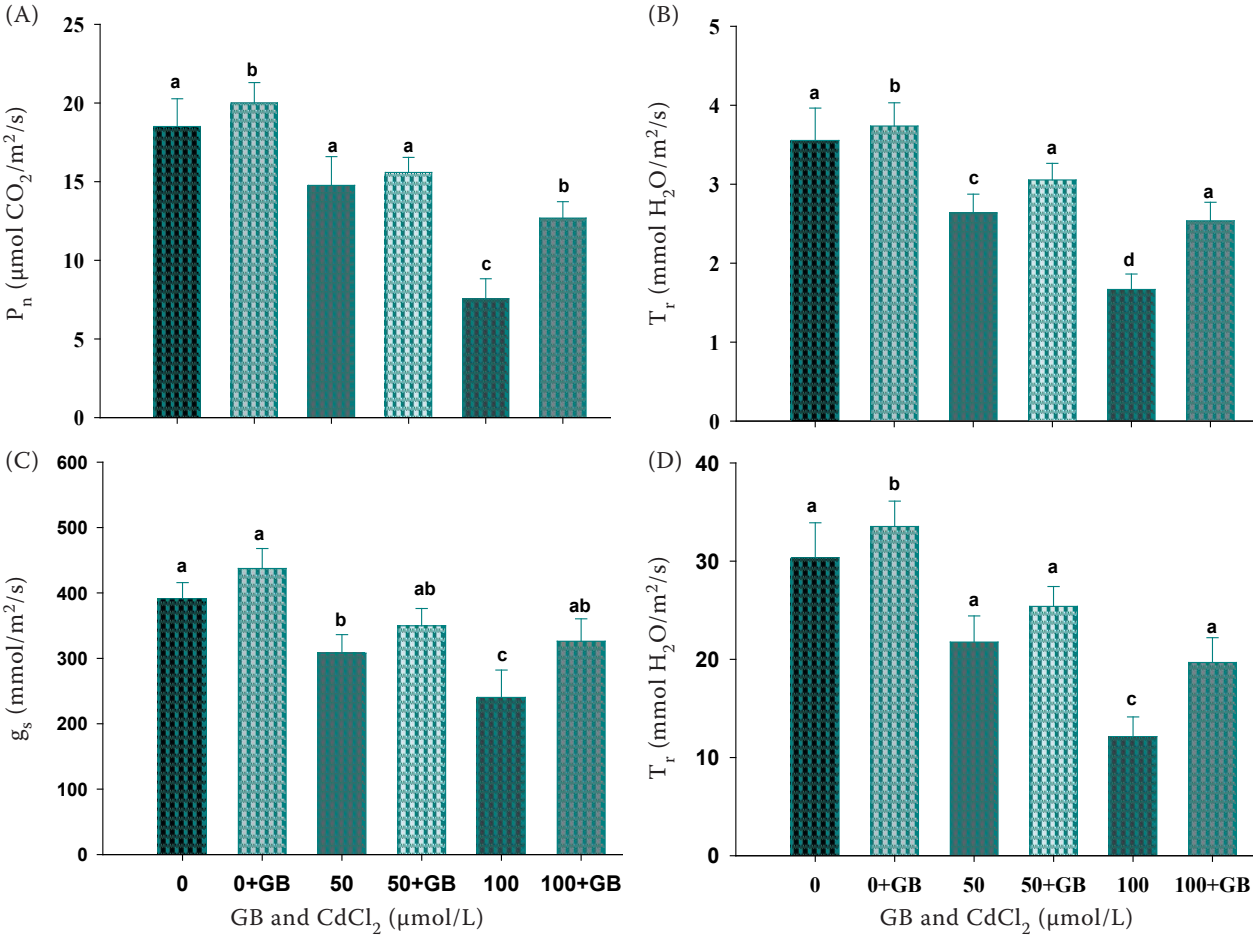

Figure 4. The impact of different $CdCl_2$ concentrations and glycine betaine (GB) on: (A) photosynthetic rate (P_n); (B) transpiration rate (T_r); (C) intracellular CO_2 concentration (c_i), and (D) stomatal conductance (g_s). The mean of 3 replicates ± standard error is shown in each column. Error bars are used to show means of standard errors. Welch's ANOVA test, followed by the Games-Howell post hoc test of less than 5% ($P < 0.05$), were used to analyse the significant differences between different treatments indicated by various letters

The effect of $CdCl_2$ and GB treatments on organic solutes in the maize leaves is shown in Figure 5. The TSS and TSP content were reduced with the increase in the Cd dose. The reduction was more significant ($P < 0.05$) with the 100 µmol (46.3% and 42%) than the 50 µmol (28.5% and 25%) in the content of TSS and TSP, respectively, compared to their respective controls (Figures 5A, B). Additionally, the TSS and TSP levels were repaired by GB treatment in Cd-stressed plants, but they remained below the control values. Additionally, the highest TSS and TSP (32.5 ± 0.36 and 12.2 ± 0.1) and lowest values (15.4 ± 0.21 and 6.4 ± 0.12) were detected with 0 µmol + GB and 100 µmol treatments, respectively.

On the other hand, the proline level was noticeably increased ($P < 0.05$) by 37.5% and 81.2% under 50 and 100 µmol, respectively (Figure 5C). Moreover, applying GB enhanced the proline content by 49.5% and 112.5% with 50 and 100 µmol + GB treatments, respectively, in relation to unstressed plants.

Changes in non-enzymatic antioxidants under GB and Cd application. The impact of $CdCl_2$ and GB treatments on the nonenzymatic antioxidants (AsA and GSH) in maize leaves is shown in Figure 6. Concerning the untreated plants, AsA and GSH levels were significantly increased ($P < 0.05$) by 11.6% and 19.6% under 50 µmol and by 19.3% and 32.3% under 100 µmol, respectively. Furthermore, applying GB induced additional accumulation in AsA and GSH levels by 33.4% and 43.5% with 100 µmol + GB treatments, respectively, on unstressed plants. The minimum and maximum values of AsA (232.7 ± 10.2

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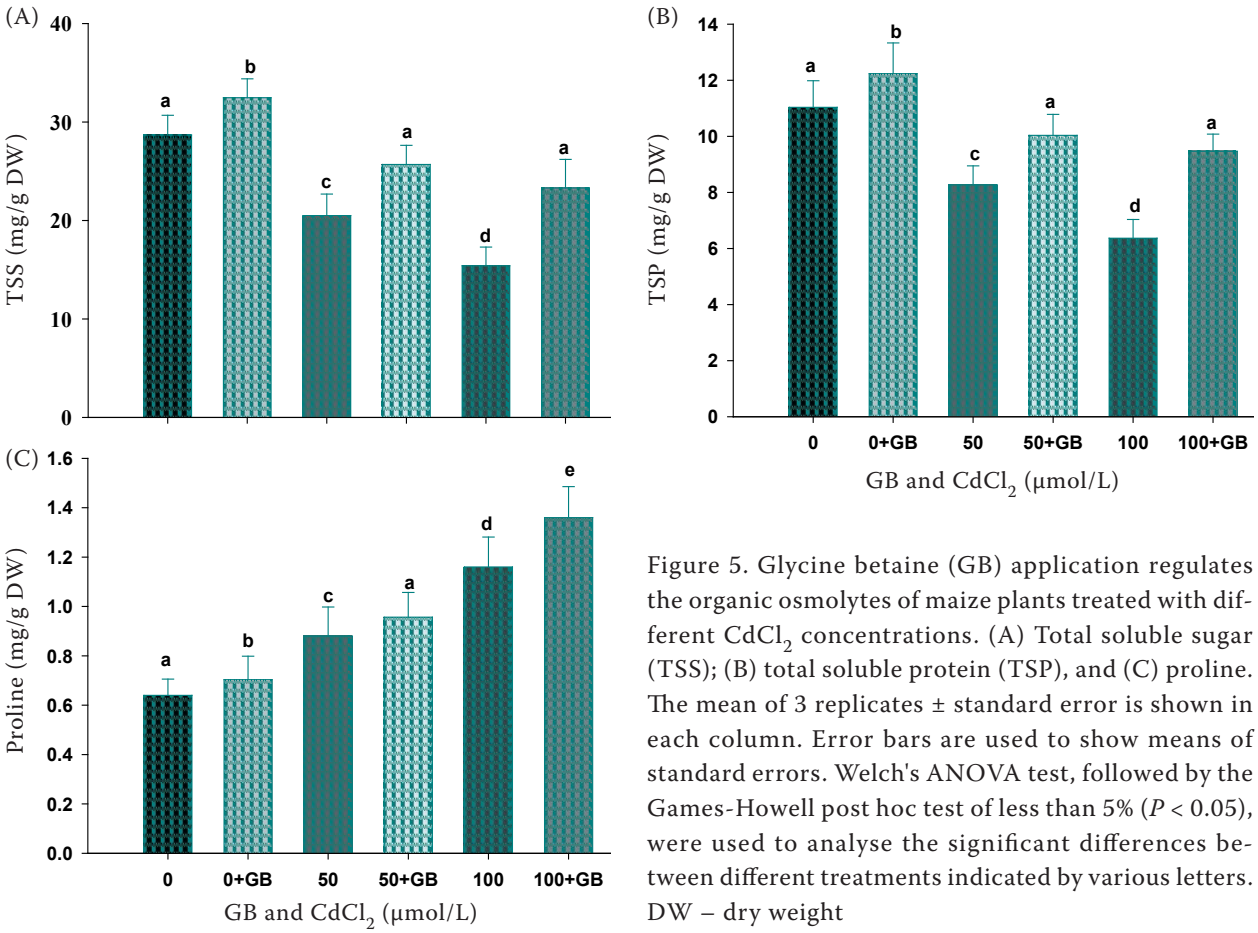

Figure 5. Glycine betaine (GB) application regulates the organic osmolytes of maize plants treated with different $CdCl_2$ concentrations. (A) Total soluble sugar (TSS); (B) total soluble protein (TSP), and (C) proline. The mean of 3 replicates ± standard error is shown in each column. Error bars are used to show means of standard errors. Welch's ANOVA test, followed by the Games-Howell post hoc test of less than 5% ($P < 0.05$), were used to analyse the significant differences between different treatments indicated by various letters. DW – dry weight

and 310.3 ± 11.4) and GSH (190 ± 8.5 and 272.7 ± 9.4) were recorded with 0 µmol and 100 µmol + GB treatments, respectively.

Impact of GB application and Cd toxicity on antioxidant enzyme activity. Cd stress increased the SOD, CAT, APX and GR activities in maize leaves

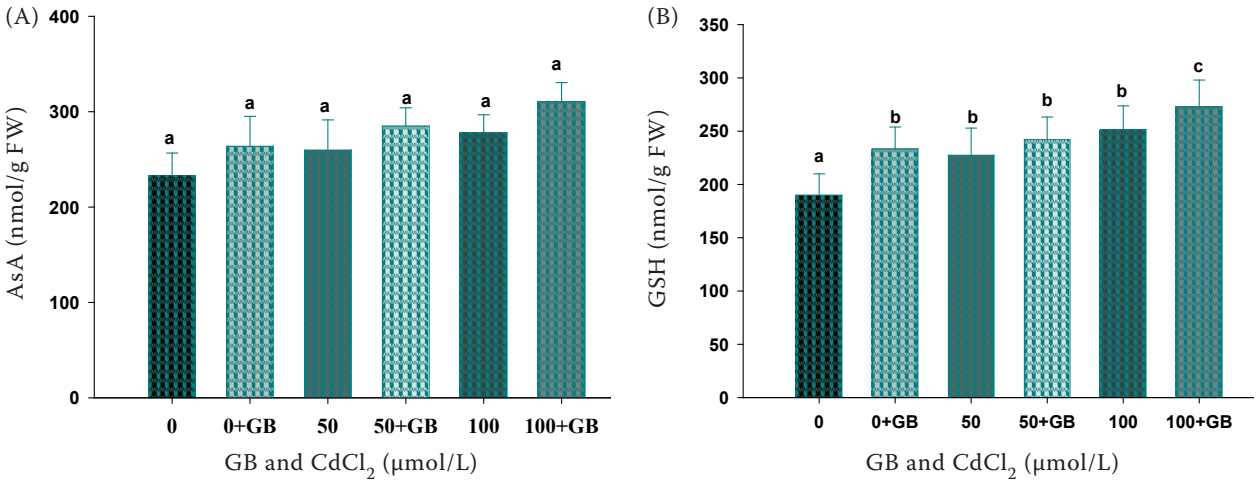

Figure 6. The impact of $CdCl_2$ and glycine betaine (GB) treatments on the nonenzymatic antioxidants in maize leaves. (A) Ascorbic acid (AsA), and (B) glutathione (GSH). The mean of 3 replicates ± standard error is shown in each column. Error bars are used to show means of standard errors. Welch's ANOVA test, followed by the Games-Howell post hoc test of less than 5% ($P < 0.05$), were used to analyse the significant differences between different treatments indicated by various letters. FW – fresh weight

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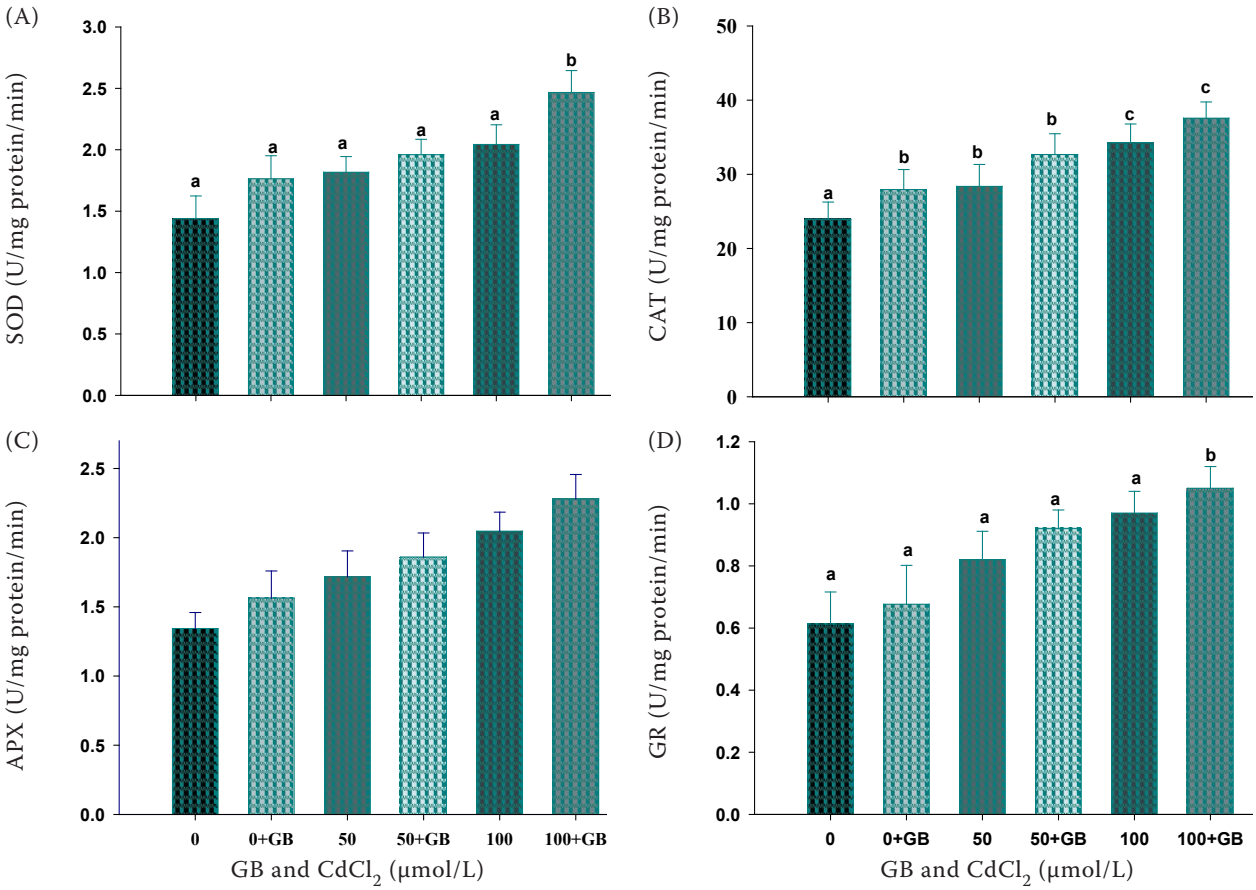

Figure 7. The impact of $CdCl_2$ and glycine betaine (GB) treatments on the enzymatic antioxidants in maize leaves. (A) Superoxide dismutase (SOD); (B) catalase (CAT); (C) ascorbate peroxidase (APX), and (D) glutathione reductase (GR). The mean of 3 replicates ± standard error is shown in each column. Error bars are used to show means of standard errors. Welch's ANOVA test, followed by the Games-Howell post hoc test of less than 5% ($P < 0.05$), were used to analyse the significant differences between different treatments indicated by various letters

(Figure 7). These increases were significant ($P < 0.05$) by 41.9, 43.2, 52, and 58.2%, respectively, under 100 µmol treatment. Furthermore, GB supplementation further enhanced SOD, CAT, APX, and GR by 71.3, 57, 69.7, and 71.2%, respectively, compared to their respective controls. The highest values (2.47, 37.6, 2.3, and 1.05 U/mg protein) for SOD, CAT, APX, and GR activities, respectively, were observed with 100 µmol + GB treatment.

DISCUSSION

Plant growth is altered by Cd stress, as seen by stunted growth, reduced fresh and dry biomass, and leaf area (Zulfiqar et al. 2022). Cd can injure plant growth at both the physiological and morphological levels (Alyemeni et al. 2018). According to Almuwayhi (2021), Cd poisoning causes leaf chlorosis, slows growth, inhibits respiration and photosynthesis, increases oxidative damage, and reduces a plant's capacity to absorb nutrients. Furthermore, Cd buildup in tissues hinders the advancement of the cell cycle and inhibits the activity of proton pumps, which results in changes to growth patterns and metabolism, according to Alyemeni et al. (2018). In the current study, Cd toxicity reduced the plant height, root length and biomass accumulation (fresh and dry) (Figure 1) confirmed other findings with various plants such as tomatoes (Alyemeni et al. 2018), tobacco (He et al. 2019), cowpea (Sadeghipour 2020), maize (Zhang et al. 2020), and sassafras (Zhao et al. 2021). In this regard, Almuwayhi (2021) investigated that the application of Cd significantly influenced the morphological parameters of leaves, stems, roots, seeds, flowers, and fruit of *Pisum sativum* L. On the other hand, it has been stated that GB supplementation can enhance plant tolerance to environmental stresses such as HMs. In this study, exogenous GB reduced

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the bioavailability of Cd in the stressed maize plant and mitigated the damage induced by this HM. GB application increased the plant height, root length and biomass accumulation (fresh and dry) of maize plants compared to Cd treatment. This finding agrees with other investigations on many plants, such as tobacco (He et al. 2019), cowpea (Sadeghipour 2020), and maize (Zhang et al. 2020).

Due to its high assimilability and mobility, Cd can enter plants through their roots and move through transporters and transpiration to reach shoots in an ionic state in the xylem and phloem (Abedi and Mojiri 2020). Under conditions of significant exposure, cadmium may reach the xylem by apoplastic or symplastic transport (He et al. 2019). After that, Cd is transferred to the shoot by being loaded into the stele's tracheids or vessel components. Solute transport *via* extracellular fluid and gas gaps between and inside cell walls is accomplished by apoplastic routes (Abedi and Mojiri 2020). Water and solutes are intracellularly transferred in symplastic pathways, travelling between cells *via* tubular channels known as plasmodesmata (Almuwayhi 2021). The amount of Cd accumulated in the roots of the maize plants in the current investigation was significantly greater than in the shoots after exposure to this metal (Figure 2). This finding is consistent with earlier studies conducted on many plant species, including tobacco (He et al. 2019), cowpea (Sadeghipour 2020), tomato (Alyemeni et al. 2018), and pea (Almuwayhi 2021). One of the plants' most significant defensive mechanisms against Cd toxicity is a higher concentration of Cd in the root than in the shoot (Sadeghipour 2020). This is probably Cd deposited in the root, which is immobilised by the cell wall and extracellular carbohydrates (Almuwayhi 2021). However, in the current experiment, the application of GB significantly reduced the translocation of Cd from the root as well as its accumulation in the root. This finding was consistent with those of previous studies (He et al. 2019, Sadeghipour 2020) and indicated that by lowering Cd absorption and translocation to the plant's above-ground parts, GB contributes significantly to reducing Cd toxicity in maize plants.

Water homeostasis is critical for plant life processes and all living organisms on the planet. Various abiotic stresses in the environment have an impact on plant tissues by decreasing the RWC of the leaves (Haider et al. 2021). RWC is a significant indicator of a plant's water status, as it is linked to the uptake of water from the roots and the transpiration rate from the leaves (Sadeghipour 2020). Through stomata closure, Cd treatment dramatically reduced the RWC of maize leaves in this study (Figure 2B). This could be due to a higher amount of Cd assimilation in the root, which can inhibit growth and prevent water transport to above-ground parts, or it could be due to a reduction in xylem conductivity caused by Cd-induced weakness of the cross-sectional area dominating water transportation (Ullah et al. 2020). According to these findings, Cd treatment lowered the RWC of tomatoes (Alyemeni et al. 2018), common beans (Sadeghipour 2018), and chickpeas (Ullah et al. 2020), and Cd could disrupt the plant water balance through an impact on water transport, stomatal conductance, and cell wall flexibility. Furthermore, in this work, supplementation with GB enhanced the water relations in terms of RWC during Cd stress by increasing stomatal conductance. Sadeghipour (2020) demonstrated that supplying proline or GB externally to cowpea seedlings treated with Cd increased the RWC, a consequence of the tissue's ability to maintain water.

Photosynthesis is considered an essential physiological process in plants. The photosynthetic apparatus is impaired by Cd toxicity, particularly the light-harvesting complex and photosystems I and II (Zulfiqar et al. 2022). Iron (Fe) aids in the improvement of chlorophyll content and as well as the synthesis of other pigments that are involved directly in photosynthetic light absorption. Cd-promote suppression of iron (Fe^{3+}) reductase induces an iron (Fe^{2+}) deficit, which has an essential role in photosynthesis and its apparatus (Haider et al. 2021). Furthermore, Cd could suppress the enzyme activity in chlorophyll synthesis and prevent electron chain transfer by substituting Mg, thus destroying the chloroplast structure (Zhang et al. 2020). The Cd also causes stomatal closure and a general reduction in photosynthesis in higher plants (Zhao et al. 2021). In the current investigation, Cd stress caused a noticeable decrease in photosynthetic pigment content and gas exchange parameters in maize as the Cd concentration increased (Figures 3 and 4). In addition, the reduction in gas exchange parameters caused by Cd toxicity could be the result of a stomatal structural abnormality. Zhao et al. (2021) similarly reported declines in P_n and T_r, as well as subsequent declines in plant biomass due to Cd stress. Previously, multiple studies have shown that Cd causes a decrease in photosynthetic rate, which is linked to a reduction in stomatal functioning,

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including conductance and Rubisco protein expression (Almuwayhi 2021). In this regard, Haider et al. (2021) investigated Cd in the soil, which causes osmotic stress in plants by reducing stomatal conductance and transpiration, leading to physiological harm to the plants. In various plants such as tomato (Alyemeni et al. 2018), cowpea (Sadeghipour 2020), maize (Zhang et al. 2020), pea (Almuwayhi 2021) and sassafras (Zhao et al. 2021), Cd toxicity suppressed photosynthetic activity.

GB is abundant in chloroplasts, preserving the thylakoid membrane and sustaining photosynthetic activities (Ahmad et al. 2020). In the current study, the GB application enhanced the photosynthetic pigments content as well as gas exchange parameters in Cd-treated and untreated maize plants. Similarly, in various studies, exogenous application ameliorated the resistance of tobacco (He et al. 2019) and cowpea (Sadeghipour 2020) to Cd stress by enhancing their transpiration rate, chlorophyll synthesis capacity, stomatal conductance, and photosynthetic capacity. Moreover, GB can enhance the photosynthesis capacity of maize by minimising its nonstomatal and stomatal limitations, which significantly impact the photosynthetic rate (Zhang et al. 2020). In this regard, Ahmad et al. (2020) demonstrated that the application of GB has been shown to activate the production of genes associated with ROS enzymes, resulting in the protection of the photosynthetic apparatus from oxidative damage. Furthermore, GB preserves the plant's photosynthetic activities by boosting stomatal conductance, preserving the ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCo) enzyme activity, and conserving the ultrastructure of chloroplast during environmental stress conditions (Sadeghipour 2020).

Under abiotic stress, plant cells typically allow various osmolytes to enter, sequester, and synthesise, then accumulate them to maintain homeostasis and preserve cell turgidity for plant growth and development. Plants use an osmotic adjustment, mediated by the generation of osmolytes, to protect their cellular machinery from pressures that could otherwise impart abiotic stress tolerance (Ghosh et al. 2021).

TSS is not only a plant photosynthesis product but also participates in the plant photosynthesis process, providing the energy for plant growth and development and playing a considerable role in plants (Zhao et al. 2021). TSS can maintain cell water balance, proper metabolism, and a reduction in osmotic potential (Elhakem 2020). In the current study, as related to unstressed plants, the TSS content decreased under different Cd concentrations (Figure 5). Previous studies have reported that Cd toxicity caused a remarked reduction in the TSS content in various plants, i.e., pea, mungbean, and sassafras (Almuwayhi 2021, Anwar et al. 2021, Zhao et al. 2021). This outcome could be attributed to the destruction of chloroplasts and subsequent reduction in photosynthesis when stress levels increased. In addition, plant cells increase their metabolic processes and consume some TSS to protect themselves from heavy metal toxicity. It is indeed probable that Cd binds to enzymes, affecting carbohydrate metabolism, which is why there's a decline in sugar content in the leaves. Anwar et al. (2021) obtained similar results. They also accord with Zhao et al. (2021) study, which found that Cd toxicity induces the stomata in the leaves to close, resulting in a reduction in photosynthesis and, as a result, a change in the leaf sugar content. On the other hand, the effect of GB treatment on maize leaf TSS was considerable in both stressed and unstressed plants. This finding agrees with what Zhang et al. concluded (2020).

The production of stress proteins and the synthesis of normal proteins can both be influenced by Cd stress. TSP can enhance the quantity of functional protein in cells for maintaining normal physiological metabolic processes, improving plant stress resistance (Zhao et al. 2021). In this experiment, the TSP content increased with 50 µmol $CdCl_2$ and then declined with 100 µmol $CdCl_2$ (Figure 5). However, as the level of Cd stress increases, the protein synthesis mechanism is disrupted to some level. Furthermore, Cd stress reduces plant photosynthesis, causing a decline in the protein content that contributes to ATP activities and leads to a decrease in the content of TSP. TSP levels were also reduced due to Cd toxicity in many plants, i.e., peas, mungbean, and sassafras (Almuwayhi 2021, Anwar et al. 2021, Zhao et al. 2021). Furthermore, the results demonstrated that foliar application of GB improves the TSP content levels in maize leaves under control and Cd stress conditions. Other studies have reported increased TSP accumulation due to HM stress, such as Cd (Zhang et al. 2020) and Cr (Kumar 2021).

Proline is a significant osmotic protective molecule accumulated in plant organs exposed to different environmental stresses (Zhao et al. 2021). The stress caused by HMs influences the water potential in plants and results in proline accumulation, which participates in the cell's osmotic adjustment

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(Sadeghipour 2020). In the current study, compared with unstressed plants, the accumulation of proline in maize plants is enhanced by increasing Cd concentration (Figure 5). The improvement in proline level noticed in Cd-stressed plants suggested an adaptive strategy for regulation of transcript levels of Cd toxicity, osmotic adjustment, stability of subcellular structure, stress proteins as well as cellular adaptation to abiotic stress, which were reported in various investigations (Hasanuzzaman et al. 2019, Anwar et al. 2021). These findings suggested that the metabolic reactions linked to the translocation pathway may be responsible for improving proline levels, which may aid in recognising different abiotic stressors and enhance physiological responses (Elhakem 2020). Moreover, Sharma et al. (2019) demonstrated that proline's role as a signalling molecule for regulating mitochondrial function affects cell proliferation by activating genes crucial for stress recovery. Additionally, an increase in proline levels may contribute to preserving membrane integrity by maintaining cellular redox potential and lowering ROS-oxidised lipid oxidation. In addition, foliar application of GB induced more proline accumulation in maize leaves. Several other studies have also investigated the enhancement of proline accumulation due to HM stress, such as Cd (Zhang et al. 2020) and Cr (Kumar 2021). Proline accumulation with GB in leaves decreased the extent of Cd-induced damage even more than in Cd-stressed plants alone. In addition, Hasanuzzaman et al. (2019) stated that there is a positive correlation between GB and proline under abiotic stresses.

The ROS generated during abiotic stress consists of hydroxyl radicals (OH•), hydrogen peroxide (H_2O_2), and superoxide ($O_2^{\bullet -}$). ROS are produced when electrons from the mitochondrial and chloroplastic electron transport systems leak and interact with oxygen without normal electron acceptors (Hasanuzzamanet et al. 2019). ROS causes uncontrollable lipid peroxidation, which damages membrane systems and causes structural distortions in proteins and nucleic acids (Marques et al. 2019). Almost all types of environmental stresses caused an increase in ROS production (Chowardhara et al. 2020). Effective scavenging of ROS could occur by non-enzymatic antioxidants like AsA, GSH, polyamines (PAs), etc. and enzymatic antioxidants like SOD, POD, CAT, APX, GR, and GPX (Riyazuddin et al. 2022). The protective effect of antioxidants against Cd toxicity differs among various experimental conditions and plant species (Marques et al. 2019). In the current study, Cd toxicity increased the antioxidant functioning (CAT, SOD, APX, GR, GSH and AsA) (Figures 6 and 7) confirmed other findings (Alyemeni et al. 2018, He et al. 2019, Chowardhara et al. 2020). Increased activity indicates improved ROS scavenging and, hence, improved survival instincts (Chowardhara et al. 2020), shielding photosynthetic electron transport from $O_2^{\bullet -}$ in the PSII reaction centre, preventing the irreversible oxidation of the D1 protein (Hasanuzzaman et al. 2019). Furthermore, regulating the antioxidant machinery during the phytoremediation process is crucial to shield the plant from Cd toxicity. Certain hyperaccumulators, for instance, have a propensity to absorb or eliminate a significant amount of Cd from the soil, but their biochemical and physiological systems remain intact. This might be a result of the hyperaccumulators' strong antioxidant enzyme activities, which enable them to survive in even the harshest environment (Riyazuddin et al. 2022). In addition, high levels of antioxidant enzyme activity, photosynthetic rate, and hormone concentrations are associated with Cd tolerance in wheat (Guo et al. 2019). In contrast, our study's results do not agree with Seifikalhor et al. (2020) and Almuwayhi (2021), who reported that Cd toxicity negatively affects the enzymatic antioxidants in wheat and pea, respectively. In this respect, Zhang et al. (2020) investigated lower concentrations of Cd (e.g., 10 mg/kg Cd) induce a moderate stress response in maize, leading to slight decreases or even temporary increases in some antioxidant enzyme activities as the plant attempts to cope with the stress. In contrast, higher concentrations of Cd (50 mg/kg and 100 mg/kg) cause severe oxidative stress, significantly inhibiting the activities of antioxidant enzymes and depleting nonenzymatic antioxidants, which indicates that the Cd level of stress can influence the specific antioxidant responses in maize.

On the other hand, the GB application led to a significant enhancement in the GPX, CAT, SOD, GR and APX activities as well as GSH and AsA levels (Figures 6 and 7). Previous research has demonstrated the beneficial effects of GB on reducing the harmful effects of ROS and mitigating the effects of Cd stress in a variety of plant species by increasing antioxidant enzyme activity in the leaves of tobacco (He et al. 2019), cauliflower (Ahmad et al. 2020) and maize (Zhang et al. 2020). Moreover, Tiwari and Lata (2018) reported that changes in gene expression have been noted in response to HM stress. In this regard,

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Ali et al. (2020) stated that in perennial ryegrass under Cd stress, GB controlled all the necessary gene expressions to produce the extra antioxidant enzymes GPX, CAT, SOD, GR, and APX and effectively scavenge the unwanted ROS. Remarkably, GB stimulates the ROS-defense system but does not itself show antioxidant activity (Ali et al. 2020). Additionally, Hasanuzzaman et al. (2019) reported that applying GB induced the activities of enzymes such as dehydroascorbate reductase (DHAR), GR, and APX involved in the AsA-GSH cycle.

In conclusion, this study demonstrates that Cd contamination significantly hampers maize growth and affects various physiological and biochemical parameters. However, the foliar application of GB mitigates these adverse effects, promoting better growth, photosynthesis, water retention and the antioxidant defence mechanisms in maize, suggesting that it can effectively bolster the plant's resilience to Cd toxicity. These findings highlight the potential of GB as a protective agent in managing heavy metal stress in crops, contributing to sustainable agricultural practices.

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