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Zinc Supplementation Enhances Glutathione-Mediated Antioxidant Defense and Glyoxalase Systems to Conferring Salt Tolerance in Soybean (*Glycine max* L.)

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Abstract: In this study, the role of zinc (Zn) in salt-affected soybean (*Glycine max* L.) was scrutinized by exposing plants to salt stress (150 mM NaCl) alone and in combination with exogenous Zn (priming and/or foliar spray with 1 mM $ZnSO_4.7H_2O$). Salt stress decreased plant growth and caused the destruction of chlorophyll and carotenoids. It also disrupted physiological processes and antioxidant defenses, resulting in an oxidative burst. The levels of the toxic metabolite methylglyoxal (MG) rose substantially under salinity. Salinity resulted in a high accumulation of Na^+ and decreased K^+ which decreased the K^+/Na^+ ratio. Zn supplementation decreased ion toxicity and improved ion homeostasis in soybean plants. Zn increased glutathione (GSH) levels, decreased glutathione disulfide levels, and increased their ratio in salt-treated soybean plants compared to salt-treated plants without Zn addition. Zn supplementation also upregulated the activities of the glutathione-dependent enzymes glutathione reductase, dehydroascorbate reductase, glutathione peroxidase, and glutathione *S*-transferase in salt-stressed plants. The enhanced GSH pool and increased activity of GSH-dependent enzymes decreased oxidative damage, as indicated by the reduced levels of H_2O_2 and malondialdehyde and lower electrolyte leakage. The increased GSH level and high activity of glyoxalase I and glyoxalase II conferred by Zn under salt stress helped to scavenge methylglyoxal. The restoration of photosynthetic pigment levels and increased proline accumulation, together with the recovery of leaf relative water content, were further signs of salt stress recovery and tolerance conferred by Zn supplementation. Our results showed that the antioxidant defense, glyoxalase system and some other physiological parameters were improved by Zn supplementation which contributed to mitigating the effects of salt stress in soybean.

Keywords: cross tolerance; ionic toxicity; micronutrient; glutathione; antioxidant defense; methylglyoxal

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1. Introduction

Salinity primarily occurs following the inundation of coastal regions by seawater. Secondary or dryland salinity can also occur due to the release of salt from rocks during weathering, while tertiary salinity or irrigation salinity is caused by anthropogenic activities related to agricultural practices [1]. These patterns of salinity indicate that salinity is not only restricted to limited regions of the world but can also be common in different areas for various reasons. Recent information from 118 countries, accounting for 85% of the global land area, shows that more than 424 million hectares of topsoil and 833 million hectares of subsoil are salt affected [2]. Saline conditions adversely affect the growth of crop

plants through their effects on germination, growth, and development because of various unpredicted effects on the physiological processes and modifications of biomolecules in plant cells.

One of the major consequences of salinity is the alteration of cellular metabolic processes in the different cellular compartments that generate oxygen radicals or their derivatives, usually termed reactive oxygen species (ROS). The types of ROS produced in plant cells are typically hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), and hydroxyl radical ($^{\bullet}OH$) [3]. Excessive generation of ROS is a common consequence under most stress conditions, including salinity [3]. ROS can cause oxidative damage to cellular components, including the cell membrane and the membranes of subcellular organelles such as chloroplasts and mitochondria, by disrupting the structure of lipids, proteins, and carbohydrates. Thus, ROS damage both the structure and functioning of cellular organelles [3]. Plants have evolved an antioxidant defense system that protects them against generated ROS, thereby enhancing their tolerance to many stresses, including salinity. Many enzymes, including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), the ascorbate-glutathione (AsA-GSH) cycle enzymes (ascorbate peroxidase, APX; monodehydroascorbate reductase, MDHAR; dehydroascorbate reductase, DHAR; and glutathione reductase GR), peroxiredoxins (PRX), glutathione peroxidases (GPX), and glutathione *S*-transferases (GST), function in this defense system, along with organic compounds, such as GSH, ascorbic acid (AsA), α-tocopherol, flavonoids, carotenoids, alkaloids, phenolic acids, and some other non-protein amino acids, are commonly studied components of the antioxidant defense systems and participate in ROS scavenging [3]. Glutathione is the most abundant thiol compound in plants [4] and regulates growth and development. It is a strong antioxidant and serves as a substrate of the GPX and GST enzymes involved in the detoxification of ROS and xenobiotics. GSH affects/improves plant adaptation to stress through the regulation of cellular redox homeostasis and signal transduction [5,6].

Stress conditions can also lead to an elevated production of methylglyoxal (MG) [7], a metabolite that can damage cellular ultrastructural components and even DNA. Glutathione functions in the glyoxalase system responsible for the detoxification of MG through the activity of glyoxalase I (Gly I) and glyoxalase II (Gly II) [8]. Methylglyoxal is first converted to *S*-D-lactoylglutathione (SLG) by GSH, and SLG is then converted to D-lactic acid by Gly II, with a concurrent regeneration of GSH [4]. Studies have confirmed that upregulation of GSH, as well as higher activities of glyoxalase system enzymes, are correlated with enhanced abiotic stress tolerance including tolerance to salinity [7,9].

Reducing the adverse effects of salinity or increasing crop productivity in saline soils are serious crop production concerns. Various agricultural approaches are now exploited to make salt-affected soils less detrimental and to increase crop plant potential under salinity. One neglected approach is the use of zinc, an essential micronutrient and structural component of several biomolecules, such as lipids and proteins. Zinc is also a co-factor of auxin activity and has a role in nucleic acid metabolism in plants [10]. Zinc can regulate carbon metabolism, and it has beneficial effects on different growth stages of plants, thereby improving yield and quality [11,12]. However, despite its potency as a plant nutrient, the function of Zn in improving GSH metabolism and Zn involvement in the antioxidant and glyoxalase systems for improving abiotic stress tolerance has not been widely studied [13,14]. We hypothesized that Zn supplementation could enhance plant defense systems against salt stress. Soybean is one of the promising oil crops in the world and it has multiple uses. However, its cultivation is limited in coastal areas due to sensitivity to salt stress. Therefore, it is one of the important tasks for plant biologists and agronomists to find ways of enhancing salt tolerance in soybean. The aim of this study was therefore to address the different effects of Zn on salt-affected soybean (*Glycine max* L.) plants, with a particular focus on GSH-mediated antioxidant defenses, the glyoxalase system, and physiological, biochemical, and growth responses.

2. Materials and Methods

2.1. Plant Materials, Growth Conditions and Treatments

Soybean (*Glycine max* L.) seeds were collected from the seed store and dried in the sun followed by cleaning. Then they were primed by soaking in 1 mM $ZnSO_4 \cdot 7H_2O$ solution and kept at 25 °C for 3 h in the dark. Safe moisture content was obtained by air-drying the seeds, followed by three washes with fresh water. The primed seeds were air-dried for about 5 h to get back the safe moisture (about 14%). the moisture content of both seeds (primed and non-primed) was the same to maintain uniform germination conditions for treated and control seeds. Both primed and non-primed seeds were sown in plastic pots with the recommended doses of organic manure and fertilizer and allowed to grow in a transparent shed house to avoid rainfall. A salinity stress treatment was applied 25 days after sowing (DAS) by irrigation with 150 mM NaCl; the control plants were irrigated with water only. Foliar applications with 1 mM $ZnSO_4 \cdot 7H_2O$ were initiated at 20 DAS at three-day intervals. The experiment was carried out in a completely randomized design (CRD) consisting of three replications. Relevant data were measured 25 days after the start of the Zn treatments.

2.2. Measurement of Growth Parameters

Soybean plant height was measured from the base to the tip of the plants using the measuring tape; the average from five plants was considered and expressed as cm.

Three full mature leaves from each plant were selected randomly to measure leaf area. Leaf photographs were taken by a digital camera; Image-J software was then considered for the calculation of leaf area [15] and it was expressed as cm^2.

Stem diameter was taken using slide calipers and expressed as mm.

2.3. Measurement of Photosynthetic Pigments

Leaves were homogenized in 80% v/v acetone (centrifuging at 5000× g), then the supernatant was considered to take absorbances with a UV-visible spectrophotometer at 663, 645 and 470 nm for determination of chlorophyll (Chl) *a*, Chl *b*, and carotenoid (Car) content, respectively. Calculation of Chl content was done following Arnon [16] while Car content was calculated following Kirk et al. [17]. The content of pigment was expressed in mg g^{-1} fresh weight (FW).

$$\text{Chl } a = \{12.7\ (A_{663}) - 2.69\ (A_{645})\} \times V \times (1000 \times W)$$

$$\text{Chl } b = \{22.9\ (A_{645}) - 4.68\ (A_{663})\} \times V \times (1000 \times W)$$

$$\text{Chl} = \text{Chl } a + \text{Chl } b = \{20.2\ (A_{645)} - 8.02\ (A_{663})\} \times V \times (1000 \times W)$$

$$\text{Car} = \{(1000 \times A_{470}) \times V/W/1000) - (1.9 \times \text{Chl } a) - (63.14 \times \text{Chl } b)\}/214$$

A = optical density; V = final volume of 80% acetone (mL) and W = fresh weight of leaf sample.

2.4. Measurement of Physiological Attributes

2.4.1. Determination of Leaf Relative Water Content

Using the method of Barrs and Weatherly [18], leaf RWC was determined. Fresh weight (FW) of leaf blade was recorded, and then that was immersed in distilled water (dH_2O) in Petri dish for 24 h by covering it with filter paper. After wiping the excess water from the leaf surface, the leaf blade was weighed again to get the turgid weight (TW). These leaf blades were dried for 48 h at 70 °C. After being cooled, the dry weight (DW) was taken. The RWC was estimated as follows:

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

2.4.2. Determination of Electrolyte Leakage

Following the method of Dionisio-Sese and Tobita [19], electrolytic leakage (EL) was determined. Leaves were put into falcon tubes dH_2O and heated at 40 °C. After being cooled at room temperature EC_1 was measured by using an electrical conductivity meter (HI-993310, Hanna, Woonsocket, RI, USA). The falcon tubes were again heated at 121 °C and after being cooled at room temperature, the final EC_2 value was taken. The formula for the determination of EL was: EL (%) = $EC_1/EC_2 \times 100$.

2.5. Determination of Proline

Following the method of Bates et al. [20] proline (Pro) content was enumerated. Leaves were extracted using sulfosalicylic acid and then centrifuged. Two mL of filtrate was taken in a test tube and 2 mL of glacial acetic acid and 2 mL of acid ninhydrin were added, mixed and then heated in the boiling water bath at 100 °C for 60 min. After being cooled, toluene was included to dissolve the chromophore. The absorbance at 520 nm was considered for measuring Pro. A standard curve of different known concentrations of Pro was used for the Pro value of different treatments in the present study. Proline content was expressed as μmol g^{-1} FW.

2.6. Determination of Lipid Peroxidation and Hydrogen Peroxide

Leaf samples were extracted with trichloroacetic acid (TCA, 20%) and centrifugation was carried out at 11,500× *g*. Thiobarbituric acid (TBA) reagent was mixed with supernatant of plant extract (4 mL of reaction buffer and 1 mL supernatant) to determine lipid peroxidation content at 532 and 600 nm spectrophotometric absorbance [21]. To obtain MDA content, extinction coefficient of 155 mM^{-1} cm^{-1} was used for calculation and finally, it was expressed in nmol mg^{-1} FW.

To determine H_2O_2 content, the technique of Yu et al. [22] was followed. Leaves were homogenized in TCA and centrifuged at 11,500× *g*. Supernatants were mixed with potassium-phosphate (K-P) buffer (pH 7.0) and potassium iodide (KI) (2.0 mL supernatant with 666.4 μL reaction mixture). Absorbance was taken at 390 nm to measure the H_2O_2 content (the unit was nmol mg^{-1} FW).

2.7. Assay of Glutathione Pool

Leaves were homogenized and extracted in meta-phosphoric with ethylenediaminetetraacetic acid. Then, centrifugation was carried out at 11,500× *g*. The supernatant was used for the investigation of glutathione [22,23]. The assay mixture of total glutathione contained 200 μL plant extract and 300 μL K-P buffer; the same assay mixture together with 10 μL 2-vinylpyridine was used for GSSG determination. The content of GSH had been computed by deducting the GSSG from the total GSH. Standard curves of known concentrations of GSH and GSSG were used for determining the desired GSH and GSSG values of plant samples and these were expressed in nmol mg^{-1} FW unit.

2.8. Determination of Protein

Leaves (0.5 g) were homogenized and extracted in a buffer solution containing 50 mM K-P buffer (pH 7.0) with KCl, AsA, β-mercaptoethanol and glycerol. The supernatants were then centrifuged at 11,500× *g*. The protein content was determined using standard bovine serum albumin following the method of Bradford [24].

2.9. Assay of the Enzymatic Activities

The activity of DHAR was measured according to Nakano and Asada [25]. Buffer solution was prepared by adding water to potassium phosphate buffer pH 7.0, GSH, and EDTA. The solution of dehydroascorbic acid (DHA) was prepared. Using an extinction coefficient 14 mM^{-1} cm^{-1}, the activity of DHAR (EC: 1.8.5.1) was assayed from the change in absorbance at 265 nm for 1 min and the unit was nmol min^{-1} mg^{-1} protein.

GR (EC: 1.6.4.2) activity was taken following the method of Hasanuzzaman et al. [23] assay buffer contained EDTA in potassium phosphate buffer (pH). The assay procedure was started by adding distilled water, buffer solution and enzyme sample. The activity of GR was initiated by adding GSSG and the decrease in absorbance was observed at 340 nm using an extinction coefficient of 6.2 mM^{-1} cm^{-1} and finally, GR activity was expressed in nmol min^{-1} mg^{-1} protein.

GPX (EC: 1.11.1.9) activity was determined by using an extinction coefficient of 6.62 mM^{-1} cm^{-1} following Elia et al. [26] and Hasanuzzaman and Fujita [27]. The reaction mixture consisted of buffer solution, H_2O_2, and enzyme. The oxidation of NADPH was recorded at 340 nm. The unit nmol min^{-1} mg^{-1} protein was used to express the GPX activity.

GST (EC: 2.5.1.18) activity was estimated according to Hossain et al. [28]. The GST activity was assayed by adding distilled water; a reaction mixture of Tris-HCl buffer (pH 6.5), GSH, 1-chloro-2,4-dinitrobenzene (CDNB); and enzyme solution (plant sample). The reaction was initiated by CDNB; the increase in absorbance was measured at 340 nm. The activity was calculated using an extinction coefficient of 9.6 mM^{-1} cm^{-1} and expressed as nmol min^{-1} mg^{-1} protein.

The activity of Gly I (EC: 4.4.1.5) and Gly II (EC: 3.1.2.6) was estimated according to the method of Hasanuzzaman and Fujita [27]. The absorbance of Gly I and Gly II was recorded at 240 and 412 nm, respectively. For Gly I, GSH solution, MG, assay buffer and enzyme were used as reaction mixture. For Gly II, Tris HCl buffer and SLG reaction mixture were used. The extinction coefficient of 3.37 and 13.6 mM^{-1} cm^{-1} was considered for Gly I and Gly II, respectively, and the activities were expressed in µmol min^{-1} mg^{-1} protein.

2.10. Determination of Methylglyoxal

Leaves were extracted with 5% perchloric acid and then were centrifuged at 4 °C for 10 min at 11,000× *g*. The supernatant was neutralized using potassium carbonate solution which was used to estimate MG including sodium dihydrogen phosphate and *N*-acetyl-L-cysteine. Then the product *N*-*α*-acetyl-*S*-(1-hydroxy-2-oxo-prop-1-yl)cysteine was estimated at a wavelength of 288 nm following the method of Wild et al. [29]. A standard curve of known concentration of MG was used to get the value of MG of the plant sample which was expressed in the unit of µmol g^{-1} FW.

2.11. Estimation of Na^+ and K^+ Content

Fresh leaves were extracted mechanically and Na^+ and K^+ contents were measured using a compact Na^+ ion meter and K^+ ion meter (Horiba, Kyoto, Japan), respectively. They were expressed in mg g^{-1} dry weight. The K^+/Na^+ ratio was obtained from the estimated values of Na^+ and K^+.

2.12. Statistical Analysis

The final calculated data were used for the analysis of variance (ANOVA) using the software CoStat v.6.400 [30]. Means were separated according to Tukey's honestly significant difference (HSD) test at $p \leq 0.05$.

3. Results

3.1. Growth Parameters

Compared to the unsprayed controls, the Zn spray treatment increased plant height, leaf area, and stem diameter, with the primed and sprayed plants showing the best results. Plant height, leaf area, and stem diameter were decreased noticeably under salt stress by 30, 26, and 20%, respectively. Priming with Zn, spraying with Zn, or their combination alleviated the adverse effects of salinity in soybean plants and improved plant growth parameters. Among the different treatments, the highest values for plant height, leaf area, and stem diameter under salt stress occurred in plants given the combined seed priming and foliar spray treatment compared to plants exposed to salt stress alone (Figure 1A–C).

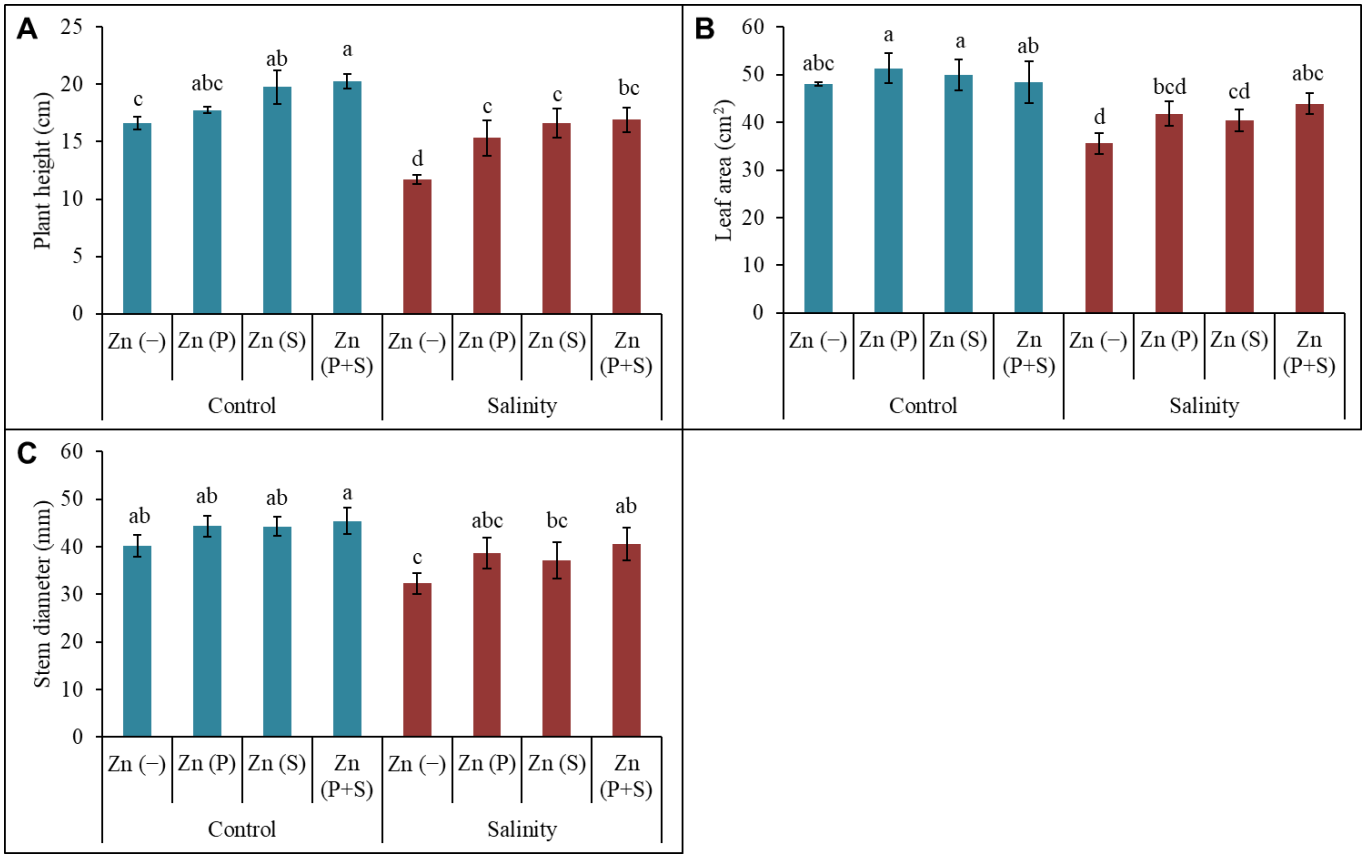

Figure 1. Plant height (**A**), leaf area (**B**) and stem diameter (**C**) of soybean plants induced by Zn supplementation. Twenty five-day-old plants were subjected to salt (150 mM NaCl) and supplemented with Zn (1 mM $ZnSO_4 \cdot 7H_2O$). P and S indicate seed priming and foliar application with Zn, respectively. Control treatments were grown with water. Mean (±SD) was calculated from three replicates for each treatment. Different letters of bars indicate significant differences at $p \leq 0.05$ applying Tukey's HSD test.

3.2. Photosynthetic Pigments

The analysis of photosynthetic pigments, including Chl *a*, Chl *b*, and Car, revealed a decreasing trend in salt-affected soybean plants compared to unstressed control plants. Salt-stressed Zn primed plants showed a 10% increase in Chl *a* content, a 16% increase in Chl *b* content, an 12% increase in Chl (*a* + *b*), and a 28% increase in Car content compared to salt-stressed plants (Figure 2A–D). The foliar Zn spray increased Chl *a*, Chl *b*, and Chl (*a* + *b*) by 15, 30, and 19%, respectively, under salt stress, compared to salt stress alone. The combination of Zn priming and spraying also increased the photosynthetic pigments to levels higher than those achieved with individual Zn applications in salt-affected plants compared to salt-stressed plants without Zn application. Under non-stressed conditions, Zn supplementation improved the status of photosynthetic pigments in some cases (Figure 2A–D).

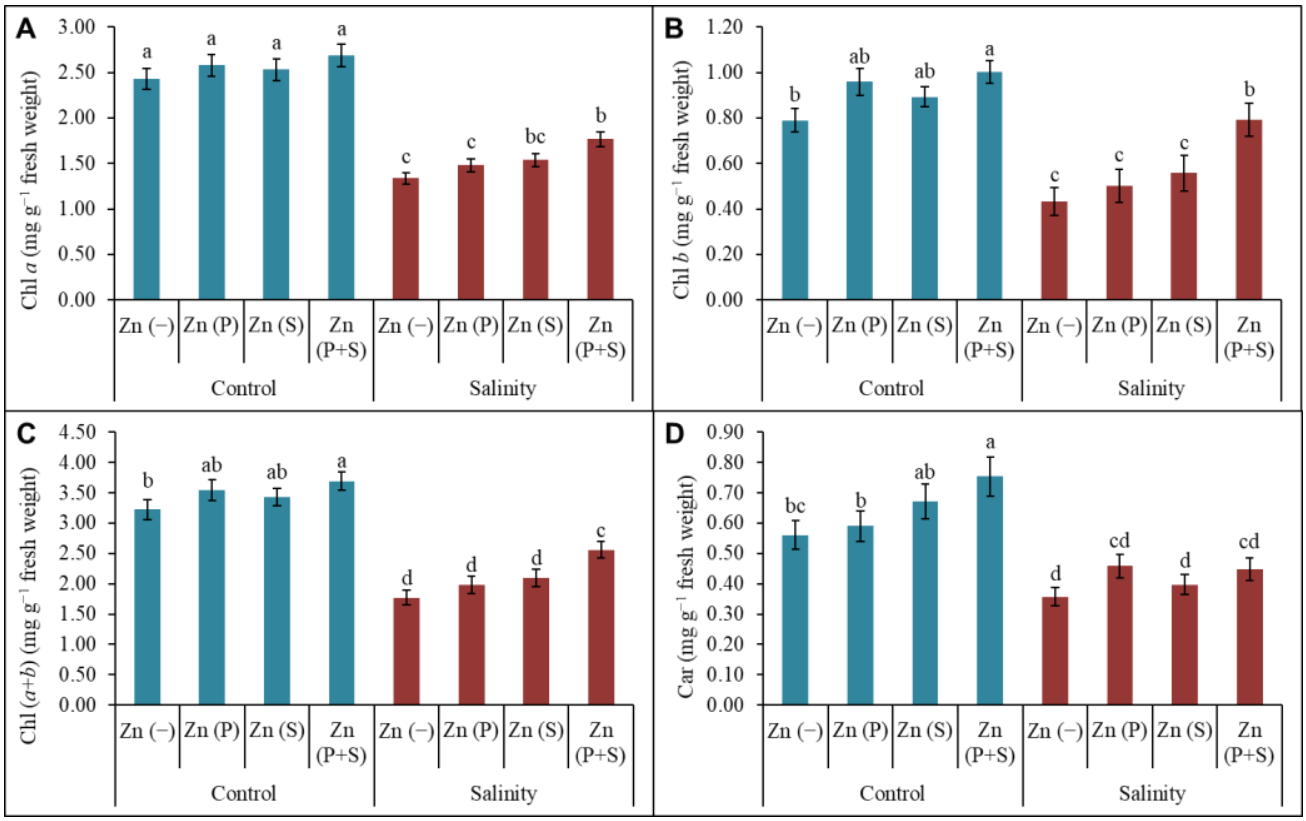

Figure 2. Chl *a* (**A**). Chl *b* (**B**), Chl (*a* + *b*) (**C**) and Car (**D**) content of soybean plants induced by Zn supplementation. Twenty five-day-old plants were subjected to salt (150 mM NaCl) and supplemented with Zn (1 mM $ZnSO_4 \cdot 7H_2O$). P and S indicate seed priming and foliar application with Zn, respectively. Control treatments were grown with water. Mean (±SD) was calculated from three replicates for each treatment. Different letters of bars indicate significant differences at $p \leq 0.05$ applying Tukey's HSD test.

3.3. Physiological Parameters

3.3.1. Relative Water Content

Salt stress decreased the leaf RWC by 32% compared to the unstressed control plants. The Zn priming treatment increased the RWC by 16%, spraying with Zn increased the RWC by 26%, and combined priming and spraying increased the RWC by 22%, compared to salt-stressed plants without Zn treatment (Figure 3A).

3.3.2. Electrolyte Leakage

A large increase in EL occurred in salt-stressed plants compared to unstressed control plants. Exogenous Zn treatment reversed this electrolyte leakage in salt-affected soybean plants compared to untreated salt-stressed plants (Figure 3B). Exogenous Zn decreased EL in salt-affected plants by 15, 14, and 25%, in the Zn-primed, Zn-sprayed, and combined Zn-treated plants exposed to salt stress compared to salt stress alone (Figure 3B).

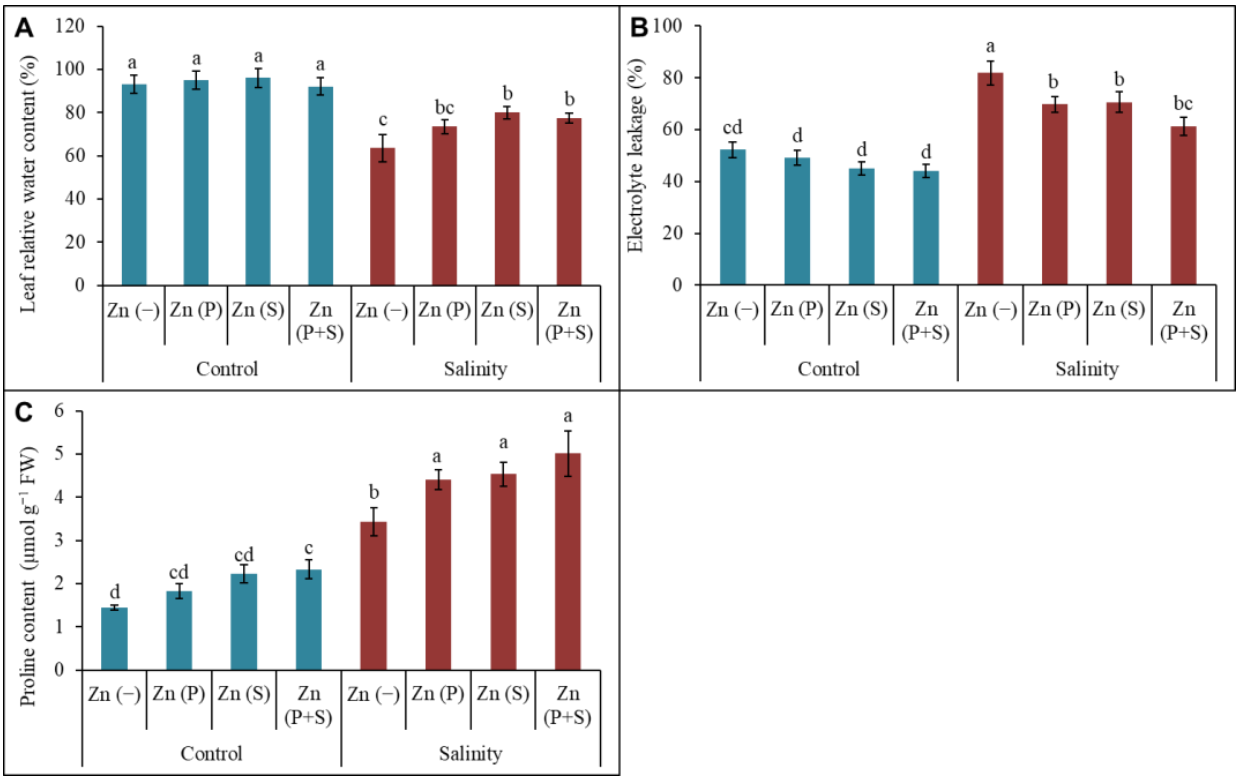

Figure 3. Leaf relative water content (**A**), electrolyte leakage (**B**), and Pro content (**C**) of soybean plants induced by Zn supplementation. Twenty five-day-old plants were subjected to salt (150 mM NaCl) and supplemented with Zn (1 mM $ZnSO_4 \cdot 7H_2O$). P and S indicate seed priming and foliar application with Zn, respectively. Control treatments were grown with water. Mean (±SD) was calculated from three replicates for each treatment. Different letters of bars indicate significant differences at $p \leq 0.05$ applying Tukey's HSD test.

3.4. Proline

Proline levels were strongly increased by 137% under salt stress compared to unstressed control plants (Figure 3C). Exogenous application of Zn increased Pro levels under salt stress compared to the salt-stressed controls, and the combination of Zn priming and spraying increased Pro to levels higher than those observed in the Zn-primed plants or the Zn-sprayed plants. The combined Zn treatment increased the Pro value by 46% compared to salt stress alone. The unstressed control plants showed no changes in Pro values in response to Zn treatment (Figure 3C).

3.5. Oxidative Stress

The levels of one of the major ROS, H_2O_2, increased by 60% under salt stress, and this partly contributed to the 79% increase in the MDA level observed in salt-stressed plants compared to unstressed controls. Both the H_2O_2 and MDA levels were decreased in Zn-supplemented salt-stressed plants compared to salt stress alone (Figure 4A,B). Zinc priming and Zn spraying both decreased the H_2O_2 and MDA levels, but a greater decrease was achieved with the combined application (Figure 4A,B).

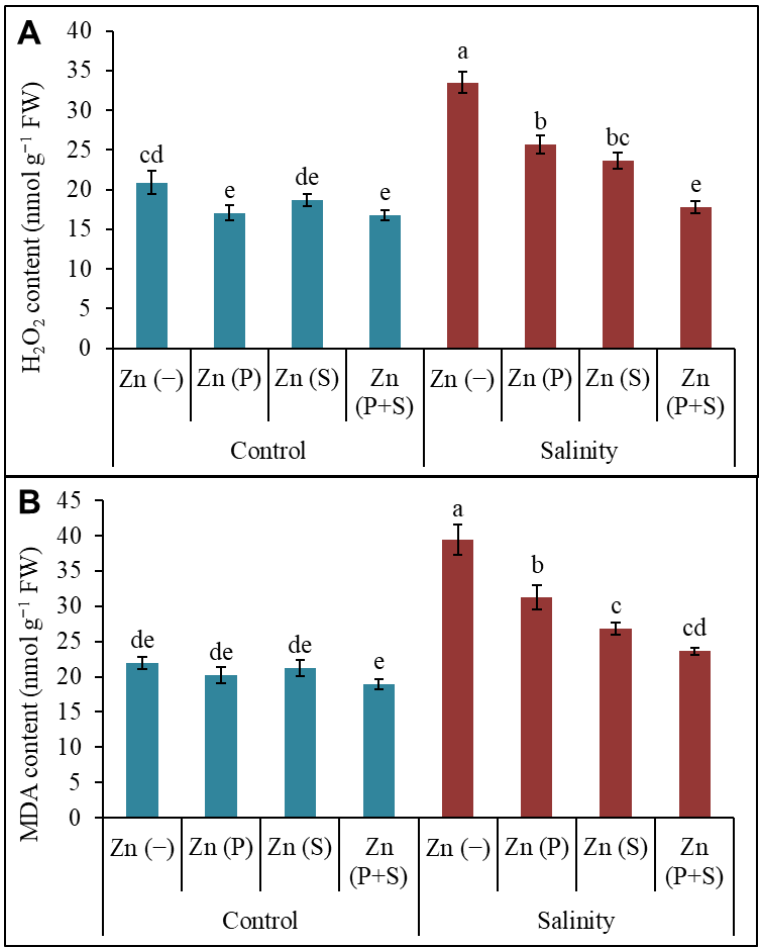

Figure 4. Content of H_2O_2 (**A**) and MDA (**B**) of soybean plants induced by Zn supplementation. Twenty five-day-old plants were subjected to salt (150 mM NaCl) and supplemented with Zn (1 mM $ZnSO_4 \cdot 7H_2O$). P and S indicate seed priming and foliar application with Zn, respectively. Control treatments were grown with water. Mean (±SD) was calculated from three replicates for each treatment. Different letters of bars indicate significant differences at $p \leq 0.05$ applying Tukey's HSD test.

3.6. Glutathione Pools

Glutathione levels decreased by 23%, GSSG levels increased by 67%, and the GSH/GSSG ratio decreased by 54% in salt-stressed plants compared to unstressed controls. The supplementation of salt-stressed plants with Zn altered different parameters of the GSH pool and increased the GSH/GSSG ratio compared to salt stress alone (Figure 5A–C). Priming, spraying, or their combination showed similar effects in salt-affected plants, but additive effects were evident with the combined application. The combined Zn priming and spraying treatments increased GSH levels by 37%, decreased GSSG levels by 29%, and increased the GSH/GSSG ratio by 94% under salt stress compared to salt stress alone (Figure 5A–C).

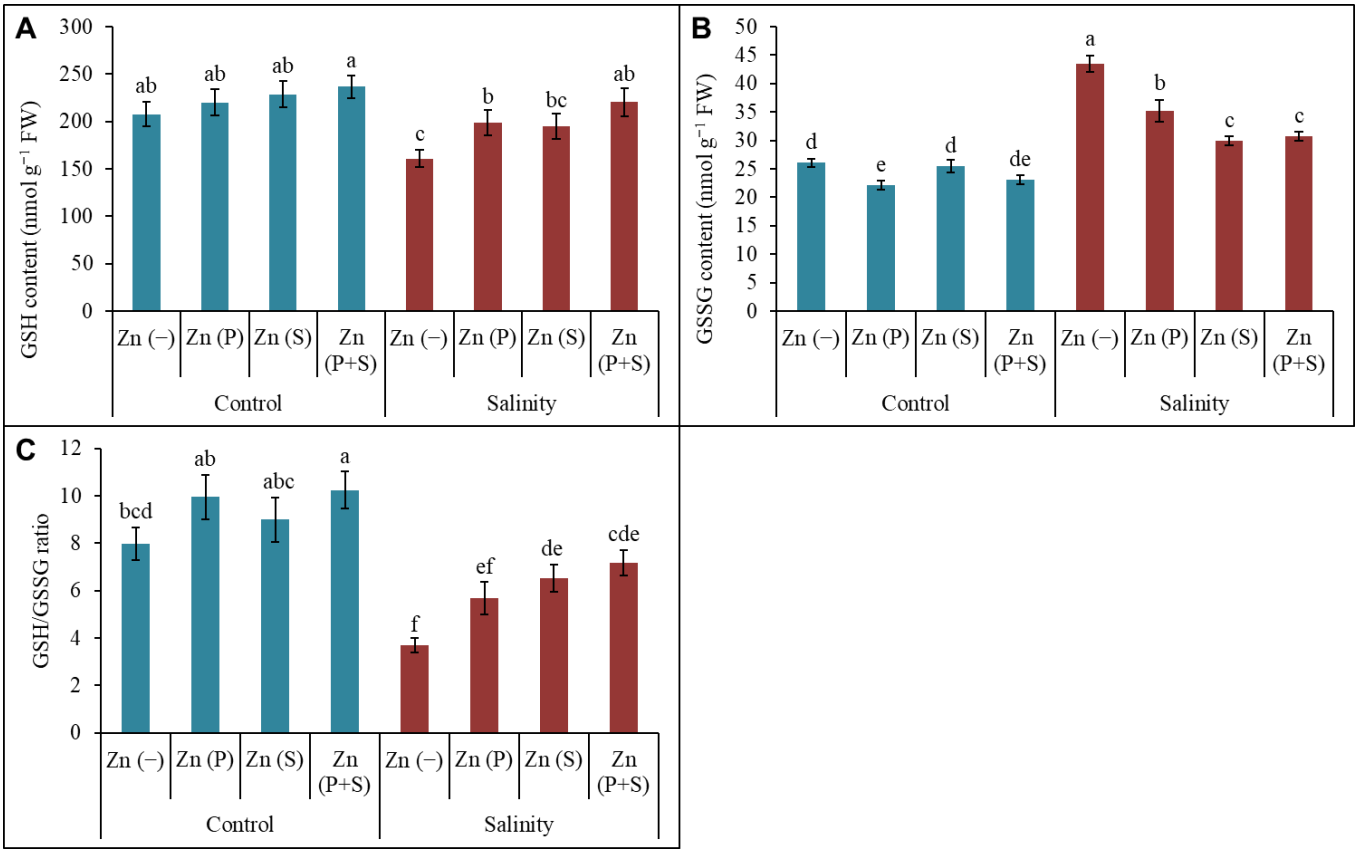

Figure 5. The GSH (**A**) and GSSG (**B**) and GSH/GSSG (**C**) of soybean plants induced by Zn supplementation. Twenty five-day-old plants were subjected to salt (150 mM NaCl) and supplemented with Zn (1 mM $ZnSO_4 \cdot 7H_2O$). P and S indicate seed priming and foliar application with Zn, respectively. Control treatments were grown with water. Mean (±SD) was calculated from three replicates for each treatment. Different letters of bars indicate significant differences at $p \leq 0.05$ applying Tukey's HSD test.

3.7. Glutathione-Dependent Antioxidant Enzymes

The activity of GR decreased by 32% under salt stress compared to unstressed control plants (Figure 6A). The activity of DHAR increased by 26% under salt stress compared to the unstressed condition (Figure 6B). The activities of GPX and GST increased by 73 and 53%, respectively, in salt-affected plants compared to unstressed control plants (Figure 6C,D). Zinc priming, spraying, or the combination treatment increased GR, GPX, and GST activities compared to salt-stressed plants. Under non-stress conditions, none of the Zn treatments caused any significant changes in these enzyme activities (Figure 6A,C,D).

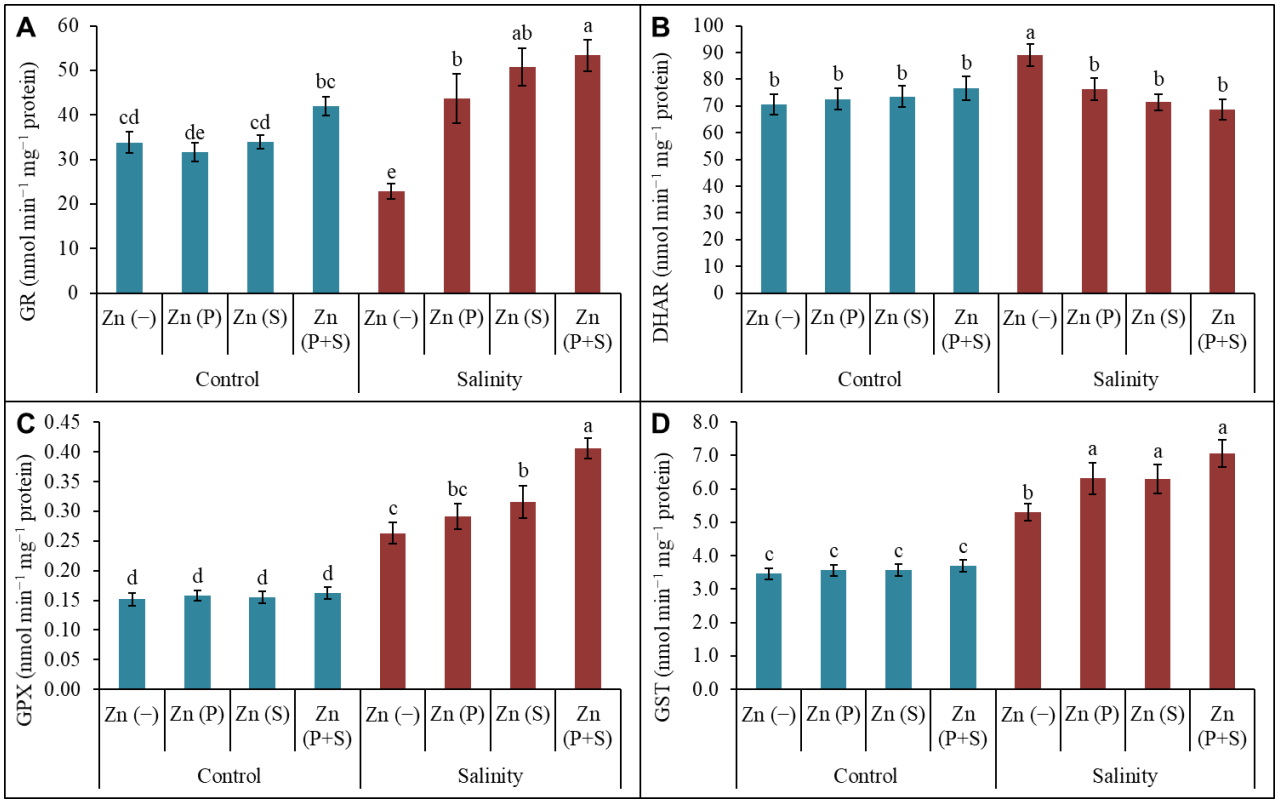

Figure 6. Activity of GR (**A**), DHAR (**B**), GPX (**C**) and GST (**D**) of soybean plants induced by Zn supplementation. Twenty five-day-old plants were subjected to salt (150 mM NaCl) and supplemented with Zn (1 mM $ZnSO_4 \cdot 7H_2O$). P and S indicate seed priming and foliar application with Zn, respectively. Control treatments were grown with water. Mean (±SD) was calculated from three replicates for each treatment. Different letters of bars indicate significant differences at $p \leq 0.05$ applying Tukey's HSD test.

3.8. Glyoxalase System

The Gly I and Gly II activities were decreased by 37 and 19% under salt stress, respectively, compared to the unstressed control plants (Figure 7A,B). Salt stress increased the MG level by 76% compared to the unstressed condition (Figure 7C). By contrast, Zn supplementation increased the Gly I and Gly II activities and decreased the MG content in salt-stressed soybean plants compared to salt stress alone. Zinc priming, spraying, and the combined treatment decreased the MG content by 20, 21, and 32%, respectively, under salt stress compared to salt stress alone (Figure 7A–C).

3.9. Ion Homeostasis

Increases in Na^+ (increased by 724%) and decreases of K^+ (decreased by 33%) resulted in a lower K^+/Na^+ ratio (decreased by 92%) under salt stress, compared to the unstressed control (Figure 8A–C). The zinc treatment of salt-stressed plants reversed this pattern and resulted in decreases in Na^+ and increases in K^+, compared to salt stress alone. The lower accumulation of Na^+ and higher accumulation of K^+ increased the K^+/Na^+ ratio in Zn-supplemented salt-treated plants compared to salt stress alone. The K^+/Na^+ ratio under salt stress increased by 95, 118, and 226% in Zn-primed, Zn-sprayed, and combined treatment plants, respectively, compared to salt stress alone (Figure 8A–C).

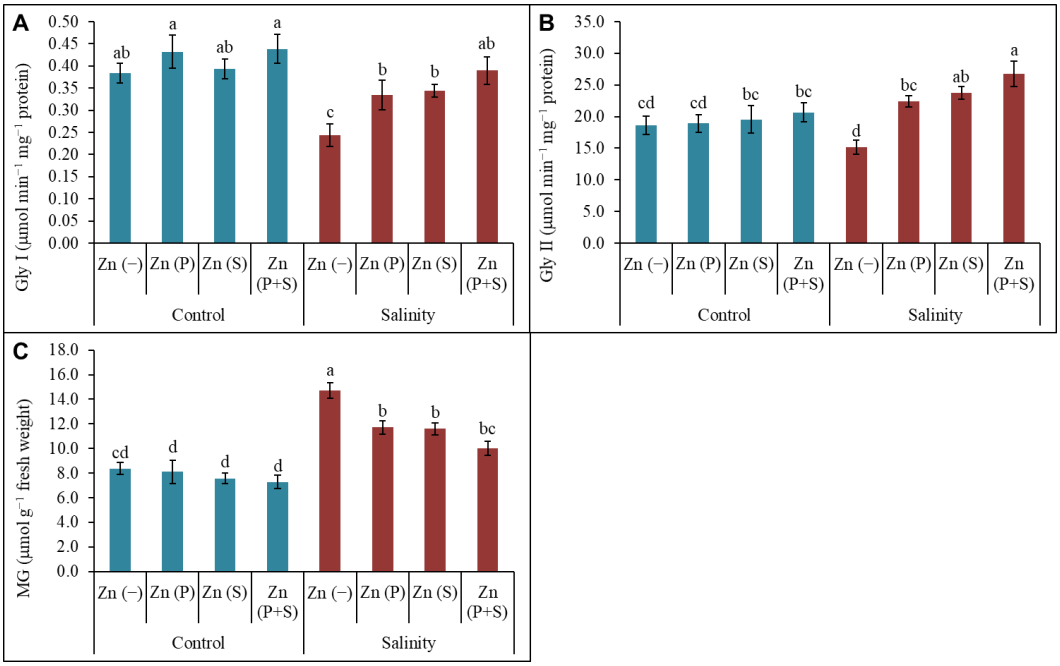

Figure 7. Activity of Gly I (**A**) and Gly II (**B**) and content of MG (**C**) of soybean plants induced by Zn supplementation. Twenty five-day-old plants were subjected to salt (150 mM NaCl) and supplemented with Zn (1 mM $ZnSO_4 \cdot 7H_2O$). P and S indicate seed priming and foliar application with Zn, respectively. Control treatments were grown with water. Mean (±SD) was calculated from three replicates for each treatment. Different letters of bars indicate significant differences at $p \leq 0.05$ applying Tukey's HSD test.

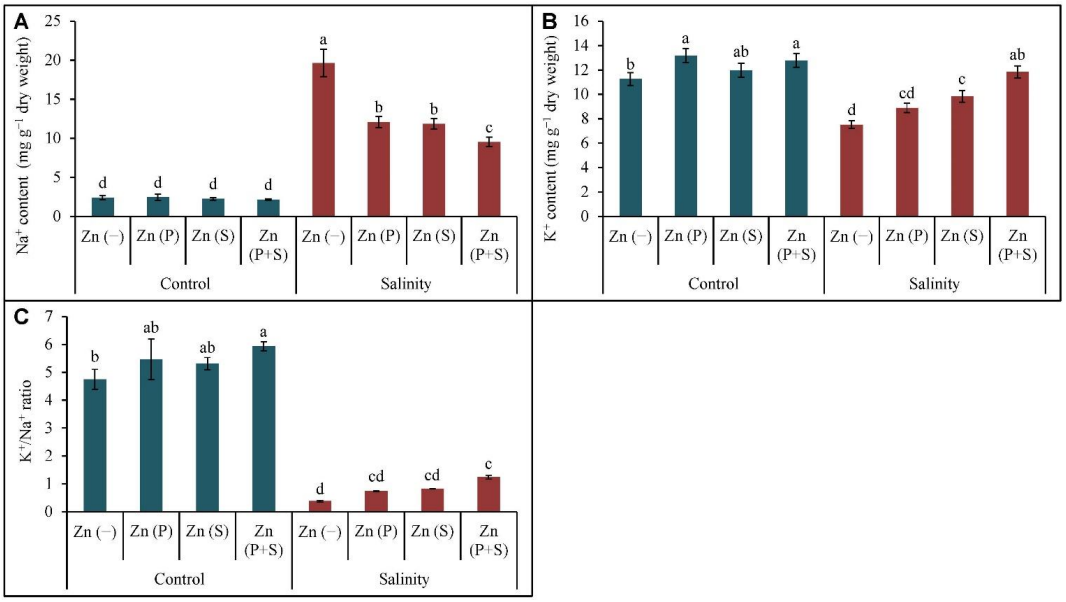

Figure 8. Content of Na^+ (**A**) and K^+ (**B**) and K^+/Na^+ (**C**) of soybean plants induced by Zn supplementation. Twenty five-day-old plants were subjected to salt (150 mM NaCl) and supplemented with Zn (1 mM $ZnSO_4 \cdot 7H_2O$). P and S indicate seed priming and foliar application with Zn, respectively. Control treatments were grown with water. Mean (±SD) was calculated from three replicates for each treatment. Different letters of bars indicate significant differences at $p \leq 0.05$ applying Tukey's HSD test.

4. Discussion

Salt stress negatively affects plants' physiological processes, including water uptake, water use efficiency, respiration, photosynthesis, and the translocation of assimilates, thereby hindering normal growth and developmental processes [3,31]. Decreases in plant height, leaf area, and stem diameter were noted in salt-stressed soybean plants in the present study. However, when Zn was exogenously applied to salinity-stressed plants, the growth was improved compared to salt stress alone. Zinc has been reported to enhance plant growth under salt stress in some research findings [32]. The prevention of oxidative damage to biomembranes and the destruction of biomolecules increases in photosynthetic pigments, and reductions in ion toxicity, together with increased nutrient uptake, improved plant–water relations and water use efficiency, and better regulation of transpiration, stomatal conductance, and photosynthesis have been documented in some published research reports, where those attributes acted in coordinately to improving plant growth performance under salt and other stresses [14,31,32].

The salt-induced breakdown of Chl is one of the most common stress responses [33,34], and the soybean plants in the present study showed reductions in Chl *a*, Chl *b*, and Chl (*a* + *b*). Diverse reasons have been proposed to explain Chl breakdown under salt stress; these include oxidative stress, inhibition of Chl biosynthesis, and activation of the Chl degradation enzyme chlorophyllase [35]. Zinc supplementation of soybean plants increased the Chl content compared to salt stress alone, in line with the findings of other studies [34]. Car photosynthetic pigments also act as antioxidants that scavenge ROS. Carotenoids participate in harvesting light energy for photosynthesis and are recognized as quenchers of triplet Chl and O_2 and as dissipators of excess energy via the xanthophyll cycle. Carotenoids stabilize chloroplast membranes and decrease membrane fluidity and vulnerability to lipid peroxidation [34,36]. The present study showed a decrease in Car content in soybean under salt stress, and this result is in line with the findings of previous studies [34]. Zinc supplementation under salinity-stress conditions increased the Car content in soybean plants, indicating a protective function of Zn [32].

The initial obstacle faced by plants under salinity is osmotic stress because of the decreased water potential outside the plant body. This osmotic stress inhibits water uptake and results in reduced water content, termed physiological drought. The reduction in the relative water content percentage observed in soybean plants under salt stress might be a result of this type of event and may activate several responses in plant tissue. One of these responses during physical or physiological drought is an accumulation of osmoregulatory molecules, which increase the chances of water entering into the plant [37]. The soybean plants in the present study showed increases in Pro levels when exposed to salt stress compared to the unstressed control plants. Some plant species are proficient in osmoregulation under abiotic stress conditions, including salinity [31,33]. A further increase in Pro was detected in response to Zn supplementation compared to salt-treated plants alone, indicating an increasing relative water content in those sets of plants and pointing toward the role of Zn in regulating the osmoprotectant molecules in some ways. Iqbal et al. [31] demonstrated that the addition of exogenous Zn regulated Pro and glycine betaine levels to confer osmoregulation in maize plants, as confirmed by water potential, osmotic potential, and turgor potential values. Ahmad et al. [38] reported increased Pro and leaf RWC in salt-stressed *Brassica juncea* (L.) in response to Zn supplementation.

Soybean plants under salt stress showed high EL compared to the unstressed controls. Electrolyte leakage occurs when the membrane is damaged and EL increases under stress conditions, including salinity, which may cause substantial damage to the cell [7]. Supplementation with Zn decreased EL in salt-affected soybean plants compared to salt stress alone. Decreases in EL under salt stress in response to exogenous Zn have also been reported in previous studies [38,39].

Soybean plants under salt stress generated H_2O_2 and MDA, indicating oxidative damage. ROS are mainly produced in the apoplast, chloroplasts, mitochondria, and peroxisomes. Under salt stress, osmotic stress, ionic toxicity, the disrupted structural

integrity of biomolecules, and altered enzymatic activity can perturb the biochemical and physiological processes that generate ROS levels in those cellular organelles beyond the scavenging capacity of the antioxidant defense system [37,40]. Zinc supplementation reduced the H_2O_2 and MDA levels in salt-stressed soybean plants, indicating that Zn is an ameliorator of oxidative stress, in line with the findings of a previous report in salt-stressed tomato, where H_2O_2 and MDA contents were decreased by Zn supplementation [39].

Salt stress decreased the GSH content while increasing the GSSG level, resulting in a decrease in the GSH/GSSG ratio (compared to the unstressed control). Glutathione is one of the major non-enzymatic antioxidants and efficiently scavenges ROS. Glutathione is also known for its signaling function, as it can actively participate in signal cascades and confer abiotic stress tolerance. The GSH/GSSG ratio is considered to play a more crucial role than GSH content alone in controlling signaling cascades [41]. Increases in GSH and the GSH/GSSG ratio were evident in soybean plants supplemented with Zn compared to salt stress alone, and this response was correlated with a higher activity of GR, as it recycles GSH from its oxidized form, GSSG. Aazami et al. [39] reported similar increases in GSH and the GSH/GSSG ratio when Zn was included in salt-treated soybeans.

Various enzymes related to GSH metabolism were assayed in the present study in salt-stressed soybean with and without Zn supplementation. The antioxidant AsA is oxidized to DHA while scavenging ROS. In the plant antioxidant system, DHAR is the enzyme involved in recycling AsA from DHA and requires GSH to catalyze this reaction [42]. Glutathione is converted to GSSG during ROS scavenging, and it is regenerated by the GR reaction in the AsA-GSH cycle. Increased GR activity is advantageous for sustaining the GSH/GSSG ratio, scavenging ROS, and improving abiotic stress tolerance [43,44]. GPX utilizes GSH to reduce H_2O_2 and organic and lipid hydroperoxides (LOOHs), thereby preventing oxidative damage to cellular components [4]. GPX can also act as an oxidative signal transducer [45]. The GST enzymes catalyze the conjugation of xenobiotic substrates with GSH [46]. GST isoenzymes are also reported to exhibit POX activity [47]; therefore, GST is a vital component that enhances abiotic stress tolerance. In the present study, the activity of GR decreased in response to salinity stress, while Zn supplementation restored its activity under salt stress. Upregulation of GPX and GR activities by Zn supplementation has been reported in salt-affected tomato [39]. In a few other studies, Zn supplementation increased the activities of other antioxidant enzymes under salt stress conditions [13,31]. These enhanced activities were involved in accelerating ROS detoxification by upregulation of GSH and GSH/GSSG, which also suggested a role in stress signal transduction for conferring abiotic stress tolerance. Therefore, the increases in GSH levels and increased activities of GSH-related enzymes, such as GR, GPX, and GST, following Zn treatment of salt-stressed plants helped to reduce ROS levels and oxidative stress.

As with other abiotic stresses, salt stress disrupts metabolic processes, such as glycolysis, to generate MG, which can also be generated from photosynthetic intermediates. MG production is increased 2–6-fold under stress conditions [7,33]. The salt-affected soybean plants in the present study showed higher MG content compared to the unstressed controls, but the MG level was decreased by Zn compared to salt stress alone. Zn addition increased GSH levels in salt-affected soybean seedlings, in agreement with the findings of Aazami et al. [39] In the present study, exogenous Zn priming or/and spraying increased Gly I and Gly II activities and GSH levels to decrease MG toxicity. Zinc-dependent Gly I was reported as a key enzyme in MG detoxification in Arabidopsis and also enhanced salt tolerance. The performance of plants overexpressing genes for Gly I was better in terms of reductions in MG levels and growth compared to the wild-type under both 150 mM and 300 mM NaCl stress [9]. The roles of Zn in regulating GSH and in neutralizing glycation end products, including MG, have been reported in various studies related to humans or animals, but more extensive studies should be conducted to understand the role of Zn in regulating GSH metabolism and the glyoxalase system in plants.

Salinity hinders the uptake of nutrients in plants due to the competition between Na^+ and ionic forms of nutrient elements [3], and the ion imbalances or toxicity under

salinity are primarily caused by Na^+. Imbalances in ion and nutrient homeostasis caused by salinity have been proven in several previous studies [7,33]. Salt depolarizes the root plasma membrane and induces the function of guard cell outward-rectifying K channels. These events result in elevated Na^+ accumulation and decreased K^+ accumulation [48]. In our study, the salt-stressed soybean plants showed decreased K^+ and increased Na^+ and consequently decreased K^+/Na^+ ratio compared to the unstressed control plants. However, Zn supplementation reversed the salt-induced changes in ion status, thereby improving the K^+/Na^+ ratio compared to salt treatment alone. These results are similar to the findings of other studies on maize plants, where a high accumulation of Na^+ and Cl^- created imbalances in K, Ca, Cu, and Zn under salt stress, but exogenous Zn application decreased Na^+ and increased K^+ and other nutrient levels [31]. Similar results for Zn in decreasing ion toxicity and maintaining nutrient homeostasis were also reported in other studies [32,34]. Zinc, through its effects on osmoregulation and its preventive/protective effects against oxidative stress, maintains the permeability of biomembranes to reduce ion toxicity and improve ion homeostasis [49]. The zinc-induced regulation of membrane (Na^+, K^+) ATPase activity was previously reported. Zinc, being a cation, competes with Na^+ for entry through biomembranes and reduces the uptake of Na^+, resulting in decreased ionic toxicity [50].

Exogenous Zn showed various regulatory roles in enhancing salt tolerance. The reduction of Na^+ accumulation and increase of K^+/Na^+ were evident in Zn-supplemented salt stressed plants. By improving the activities of glyoxalase enzymes the MG accumulation was decreased in Zn-added salt-affected plants. Exogenous Zn increased GR, GPX and GST activity; Zn addition also increased GSH level and GSH/GSSG. All these together increased the oxidative stress tolerance in salt-affected plants which was evident from reduced ROS generation, membrane lipid peroxidation and reduced electrolyte leakage. Proline biosynthesis was one of the vital effects of exogenous Zn supplementation that improved the water status in soybean plants. The photosynthetic pigments levels were also improved by Zn addition. Overall, the upregulation of the antioxidant and glyoxalase system, improved photosynthetic pigment level, osmoregulation and improved water status of plants, reduced Na toxicity and maintenance of ionic homeostasis acted together to maintain better plant performance under salt stress, compared to salt-treated plants without Zn supplementation as was demonstrated in the better growth performance of the studied soybean plants.

5. Conclusions

The results of the present study showed that salinity stress in soybean plants resulted in ion toxicity, physiological dysfunction, destruction of photosynthetic pigments, and oxidative damage resulting from the disruption of the GSH pool and activities of GSH-dependent antioxidant enzymes and MG toxicity. By contrast, supplementation with Zn reversed the salt-induced changes, thereby improving antioxidant components and alleviating oxidative stress, as indicated by the upregulation of the glyoxalase system components and decreased MG toxicity. Overall, Zn supplementation improved the physiology and growth of salt-affected soybean plants. Some ambiguity and confusion remain regarding Zn function during stress. The mechanism by which Zn regulates the antioxidant system or glyoxalase system requires further study. The interaction between osmoregulators and Zn is also not understood. Differentiating and identifying the physiological roles of Zn during a plant's developmental processes and under stress conditions is vital for exploiting Zn in the field as a stress protector or preventer. When and how Zn works as a nutrient or agent for conferring stress tolerance needs elucidation. Extensive research is necessary to establish the doses, methods, and timing of Zn applications for its use during stress conditions.

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