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Exogenous proline enhances salt tolerance in wheat: regulating osmolytes, hormonal balance, antioxidant defence, and yield performance

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Abstract: This study investigates the impacts of exogenously applied proline (Pro, 10 mmol/L) on the growth and productivity of wheat plants in saline environments. The findings indicated that increased NaCl concentrations, 60 and 120 mmol/L, further depressed the shoot and root growth parameters and flag leaf area. However, the Pro treatment ameliorated salt stress and improved all growth parameters, reducing the magnitude of such growth inhibitions compared to nontreated plants. It also enhanced the organic osmolyte accumulation, including Pro, total soluble sugars, and total soluble protein, implicated in osmotic balance and cell protection under stress. Furthermore, supplementing Pro improved ionic balance through a reduction in Na accumulation and an enhancement in the uptake of K, Ca, and Mg, thus mitigating the negative effects of salinity on nutrient availability. Pro treatment affected phytohormone levels, especially increasing auxin and gibberellins while decreasing abscisic acid under salt stress. Antioxidant enzymes such as catalase, superoxide dismutase, ascorbate peroxidase, and glutathione reductase, as well as nonenzymatic antioxidants like ascorbic acid and glutathione, were also enhanced by Pro, thereby protecting the plants against oxidative damage. Moreover, it was noticed that Pro treatment substantially improved all yield attributes of wheat plants, such as plant height, spike length, no. of spikelets/main spike, grain no./main spike, grain fresh and dry weights, and grain yield/plant through attenuation of the negative impact of NaCl. In this regard, Pro application appears to be a very promising approach toward mitigating the adversities of salinity in agriculture, especially in crop productivity in saline environments.

Keywords: *Triticum aestivum* L.; osmotic adjustment; ionic homeostasis; antioxidant enzymes system; phytohormonal regulation; yield optimisation

The complex system of soil involves interactions between biological and physical processes. Numerous man-made and natural processes contribute to climate change, which alters soil's physical and chemical properties (Zheng et al. 2023). Among environmental changes, one of the most serious threats to agricultural fields is soil salinisation (El-Beltagi et al. 2024). According to Mahboob et al. (2023), excessive salinisation limits crop productivity by negatively affecting seed germination, root development, seedling growth, flowering, and fruit setting. Furthermore, the detrimental effects of salt stress on plant growth can be attributed to nutritional imbalance, oxidative stress induction, a particular ion effect, or osmotic impact (Atta et al. 2023). Plant performance may suffer because of these effects, and membrane stability may be compromised. Furthermore, the first threat that plants may encounter in a salinised condition is the decrease in medium water potential, which results in the dehydration of tissues (Zhou et al. 2024). Salinity

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may also promote stomata closure, damaging the photosynthetic apparatus and increasing the reactive oxygen species (ROS) production (Shahzadi et al. 2024). This leads to oxidative damage of different cellular components, which manifests as DNA damage, protein degradation, lipid peroxidation, and disruption of enzyme activity (Koc et al. 2024).

Both organic (such as glycine betaine, proline, proteins, and sugars) and inorganic (like Ca^{2+}, K^{+}, Mg^{2+}, PO_4^{2-}, NO_3^{-}, and SO_4^{2-}) osmolytes are osmoprotective solutes that enhance the ability of cells to retain water while not disrupting normal metabolic processes (Mahboob et al. 2023). These osmoprotectants' primary functions include balancing ionic transport across the cell membrane, scavenging ROS, preventing membrane disintegration, and controlling enzyme activity (Zhou et al. 2024). Additionally, these osmolytes preserve plant cellular functioning, which is thought to be the fundamental way that plants adapt to stress (Ehtaiwesh et al. 2024). Additionally, prior research has investigated the impact of osmolytes under abiotic stresses, such as drought (Elhakem 2020), salinity (Atteya et al. 2022), and heavy metals (Elhakem 2024).

Phytohormones are important for a variety of physiological and biochemical functions. Their ability to reduce environmental stressors is essential for giving plants the ability to withstand challenging conditions (Atta et al. 2023). Abscisic acid (ABA) is a crucial regulator that facilitates cellular adaptation to salt stress among all phytohormones (Elhakem 2020). According to Mahboob et al. (2023), ABA may stop the stomatal activity and then start the stress signalling response. The adaptation mechanism and mitigation of the detrimental effects of salt stress have also been studied in relation to other phytohormones, including auxin (IAA), cytokinins (CK), gibberellins (GA), ethylene, and brassinosteroids (Singh et al. 2022).

Wheat is an essential staple grain and the main source of carbohydrates for humans. Moreover, it has beneficial components such as cellulose, phosphorus, magnesium, vitamins E and B, and others (Mahboob et al. 2023). However, because plant susceptibility varies depending on physiological and biochemical processes, increasing soil salt concentrations drastically lowers their production and quality (El-Beltagi et al. 2024). Wheat plants are extremely vulnerable to salinity and frequently exhibit salt sensitivity at every stage of development, especially in the early stages (Ayman et al. 2024). To create salt-tolerant wheat cultivars, it is crucial to comprehend wheat's morpho-physiological, biochemical, and hormonal reactions to salt stress.

Although several studies have addressed the adverse effects of the saline environment on crop physiology and the significant roles of osmolytes and phytohormones, a comprehensive understanding of how exogenously applied Pro simultaneously modulates osmotic adjustments, ion homeostasis, hormonal regulation, and antioxidant defence in wheat under salinity remains limited. In addition, many earlier studies have focused on biochemical or physiological responses without including a combined approach to link this mechanism to crop productivity. Furthermore, most of these studies have focused on model or horticultural plants, while essential cereals like wheat, especially at the developmental stage, are underrepresented. The present study aims to fill a significant understanding gap by assessing the various contributions of Pro to enhancing salt tolerance in wheat. The current study clarifies Pro's mechanistic role in conferring stress resilience by combining morphological, physiological, biochemical, and yield-relevant variables and offers pragmatic consequences for boosting crop production under salinity.

MATERIAL AND METHODS

Plant material growth condition. A pot experiment was carried out in Egypt (30°06'N and 31°25'E) in the winter of 2023. Wheat grains (*Triticum aestivum* L., cv. Giza168) were supplied by the Agriculture Ministry of Egypt. After being sterilised for three min with 70% ethanol, a uniform group of wheat grains was rinsed with distilled H_2O. Wheat grains were sown in pots (25 grains/pot; 25 cm height × 30 cm width) with 5 kg of soil (clay/sand ratio 2:1, *v*/*v*). The plants were exposed to natural day/night (minimum/maximum temperature and relative humidity 19.2/30.1 °C and 63/68%, respectively) and were watered up to the field's capacity with tap water. Phosphorus and nitrogen fertilisers were applied at two stages: at the bud stage (20 days from planting) and before the heading stage concerning the recommended doses of 1.5 g P/pot (potassium dihydrogen phosphate) and 1.5 g N/pot (urea), respectively. After twenty days of planting, the plants were thinned to five uniform seedlings per pot.

Salt treatment. The pots were divided into two sets at the heading stage (after eight weeks from planting). The 1st set continued to be irrigated with tap water

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and was further divided into two subgroups: control (cont.) and control + proline (cont. + Pro). The 2nd set was irrigated with NaCl solution and divided into four subgroups: 60 mmol/L NaCl (60 mmol/L), 60 mmol/L NaCl + proline (60 mmol/L + Pro), 120 mmol/L NaCl (120 mmol/L), and 120 mmol/L NaCl + proline (120 mmol/L + Pro). During the stress period, plants in the salt-treated groups were irrigated with NaCl solution (60 or 120 mmol/L) four times consecutively, followed by one irrigation with tap water to prevent salt buildup in the soil. This five-irrigation cycle was repeated throughout the stress treatment. All plants were watered to maintain field capacity. After the stress treatment period, irrigation was continued with tap water only until grain maturity for all groups. All the morphological, physiological, and biochemical analyses were conducted two weeks after the stress application (2 WASA) for all wheat plants (treated and untreated).

Proline application. The first foliar application of Pro (10 mmol/L) was applied using a hand sprayer five days before the saline water treatments, and it was repeated weekly until the grains were filled.

Growth measurements. All the treated and untreated plants' shoot and root growth parameters and flag leaf area were evaluated. The shoots' and roots' dry weights (DW) were determined by placing them in bags and drying them in an oven set to 80 °C until the weight stabilised. Five replicates were obtained to determine the mean measurement for each treatment.

Organic osmolyte measurements. Pro level in treated and untreated wheat flag leaves was measured using the ninhydrin-based colourimetric method (Lee et al. 2018). After grinding 0.5 g of fresh leaves, 20 µL of 1% (*w*/*v*) sulfosalicylic acid was applied for each mg of fresh weight tissue. Following centrifugation at 15 000 *g* for 5 min at 4 °C, the supernatant was collected and combined with acidic ninhydrin (1.25% [*w*/*v*] ninhydrin in 80% [*v*/*v*] acetic acid) in a 1:2 ratio. The solution was subsequently incubated at 95 °C for 30 min. An atomic absorption spectrophotometer (Shimadzu AA-7800, Kyoto, Japan) was employed to measure absorbance at 510 nm. A standard curve was established using a Pro concentration range of 0 to 100 µg/mL to quantify Pro content.

Bradford's (1976) method was used to extract and assess TSP in stressed and unstressed wheat flag leaves. Saline phosphate buffer was made 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 sustain a pH of 7.2, 62.5 mmol/L of Tris HCl was employed. 0.5 g of fresh-weight leaves were isolated and immersed in saline phosphate buffer to ascertain total soluble protein (TSP). The supernatant is removed by centrifugation of the solution. Following the dye stock's dissolution to match the supernatant's volume and subsequent swirling, it was incubated for 30 min. The absorbance was quantified with an atomic absorption spectrophotometer (Scilogex SCI-UV1100, Guangzhou, China) calibrated to 595 nm. A series of bovine serum albumin concentrations ranging from 0 to 100 µg/mL was utilised for the standard curve.

Following the method of Yoshida et al. (1976), total soluble sugars (TSS) were extracted and evaluated from both treated and untreated wheat flag leaves. Dry tissue was submerged in 10 mL of 80% (*v*/*v*) ethanol at 25 °C to extract total soluble solids, with periodic agitation throughout the night. TSS was assessed by heating 0.1 mL of alcoholic extract in a boiling water bath for 10 min and subsequently reacting it with 3.0 mL of freshly produced anthrone reagent. The samples were subsequently analysed at 625 nm with a Spectronic 21D spectrophotometer (Thermo Fisher Scientific, Waltham, USA). A series of glucose concentrations ranging from 0 to 100 µg/mL was utilised to estimate TSS concentration for the standard curve.

Inorganic osmolyte determination. Wolf's (1982) method assessed Na, K, Mg, and Ca ion levels in the treated and untreated wheat flag leaves. 0.5 g of dry leaf was incubated in 5 mL H_2SO_4 all night and heated in the digestion block at 350 °C for 30 min. The mixture was cooled; 1 mL H_2O_2 was added and heated once more for 20 min. These processes were repeated until a pure solution was obtained and filtered; distilled H_2O helped to make the volume up to 50 mL. The extract next underwent flame photometer (Jenway PFP-7, Bibby Scientific Ltd., Felsted, UK) Na, K, Mg, and Ca determination. Prepared for the standard curve was a standard series (10, 20, to 100 ppm of Na, K, Mg, and Ca).

Estimation of phytohormones. Phytohormones were assessed in stressed and unstressed wheat plants using the methodology of Müller and Munné-Bosch (2011). Fresh leaves (0.2 g) were extracted using a solution of acetic acid (1%), isopropanol (79%), and methanol (20%). The samples were maintained on ice for 30 min, subjected to sonication for 10 min, and then centrifuged at 4 °C for 10 min at 13 000 rpm. The supernatant was subjected to an additional extraction cycle and thereafter injected into an LC-MS/MS system Infinity Series, Agilent 1200, Agilent

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Technologies, California, USA) connected to a triple quadrupole mass spectrometer (Agilent Technologies, Model 6430, California, USA). The Agilent Eclipse Plus chromatographic column (RRHD, 2.1 × 50 mm, 1.8 µm) was used at a flow rate of 0.3 mL/min and linked to a triple quadrupole mass spectrometer. The mass spectrometer operated in alternating negative and positive modes based on the retention period of each hormone, with samples analysed in multiple reaction modes to assess ABA, IAA, and GA_3 hormones. The generated mass spectra were analysed using Mass Hunter software (California, USA) to obtain the extracted chromatograms for each transition and to determine the zone values indicating the amount of each hormone. Standard curves were utilised to convert the zone results to µg hormone/g FW (fresh weight).

Reduced glutathione and ascorbate estimation. Reduced glutathione (GSH) level was assessed using the method described by Ellman (1959). Metaphosphoric acid (15%) was used to homogenise 500 mg (FW) of the flag leaves. They were centrifuged at 5 000 *g* for 30 min at 4 °C. After that, 2.6 mL of phosphate buffer (100 mmol/L, pH 8.0) and 200 µL of 5, 5'-dithiobis (2-nitrobenzoic acid) (6 mmol) were added to the supernatant, and after 30 min of incubation. Using an atomic absorption spectrophotometer (Shimadzu AA-7800, Kyoto, Japan), the absorbance was measured at 412 nm. For the standard curve, a series of 0 to 100 µg/mL reduced glutathione is used to determine the GSH level.

The ascorbate (AsA) level was evaluated by applying the Mukherjee and Choudhuri (1983) method. The flag leaves (FW) were homogenised in 6% (*w*/*v*) trichloroacetic acid using a pestle and mortar. After centrifuging the extract for 10 min at 5 000 *g*, the mixture was heated in a water bath for 15 min, and 10% thiourea and 2% dinitrophenylhydrazine were added to the supernatant. Cooled 80% H_2SO_4 (5 mL) was added after the samples had cooled. An atomic absorption spectrophotometer (Shimadzu AA-7800, Kyoto, Japan) measured the absorbance at 530 nm. To determine the AsA concentration, the ascorbate solution (0 to 100 µg/mL) standard curve was used.

Extraction and assay of antioxidant enzymes. Using a prechilled pestle and mortar, 1 g of fresh tissue from the flag leaves was homogenised in 50 mL of chilled phosphate buffer (100 mmol/L, pH 7.0), augmented 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 subsequently employed as an enzyme source. The supernatant's protein concentration was evaluated using the Lowry et al. (1951) method, applying bovine serum albumin as a standard, with measurements taken using an atomic absorption spectrophotometer (Shimadzu AA-7800, Kyoto, Japan).

The activity of ascorbate peroxidase (APX, EC 1.11.1.11) was evaluated using the method outlined by Nakano and Asada (1981). In a 1 mL reaction mixture with 0.5 mmol hydrogen peroxide, 0.5 mmol ascorbic acid, 0.1 mL enzyme extract, and potassium phosphate buffer (100 mmol/L, pH 7.0), the absorbance was recorded for three min at 290 nm.

The Bayer and Fridovich (1987) method was conducted to assess the superoxide dismutase (SOD, EC 1.15.1.1). In a 1.5 mL assay mixture containing 100 µL enzyme extract, 100 µL EDTA, 13 mmol L-methionine, sodium phosphate buffer (50 mmol/L, pH 7.5), 75 µmol nitroblue tetrazolium (NBT), and 60 µmol riboflavin, photochemical reductions of NBT were measured at 560 nm. After incubating for 15 min, the light was turned off.

The catalase assay (CAT, EC 1.11.1.6) used the Aebi (1984) method. The absorbance was detected for 2 min at 240 nm. The calculation was performed with an extinction coefficient of 39.4 mmol/cm.

Glutathione reductase (GR; EC 1.6.4.2) activity was assessed using the Foyer and Halliwell (1976) method in an assay mixture that included 0.1 mL enzyme extract, 0.5 mmol/L oxidised glutathione, 0.1 mmol/L nicotinamide adenine dinucleotide phosphate, and 100 mmol/L sodium phosphate buffer (pH 7.8). Absorption detection was carried out at 340 nm for 2 min with an extinction coefficient of 6.2 mmol/cm.

Yield attributes. After five months of germination, five replicates for yield attributes were taken from mature treated and untreated plants and stored to obtain grains. Plant height, spike length, 100-kernel weight, no. of spikelets/main spike, grain yield/plant, grain no./main spike, and grain FW and DW parameters were recorded.

Statistical analysis. The data was examined using means ± standard errors (SEs) from a minimum of three replicates (for physiological and biochemical analysis) and five replicates (for growth and yield parameters). Significance differences were analysed using IBM SPSS Statistics 29.0 for Welch's ANOVA test, followed by the Games-Howell post hoc test of less than 5% ($P < 0.05$). In addition, Microsoft Excel

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(Office 2016, Microsoft Corporation, USA) was used for data organisation and basic chart creation. Graphs were refined and finalised using Sigma Plot 10.0 (Systat Software, Inc., Washington, USA) (Daniel 1995).

RESULTS

Effects of Pro foliar application and NaCl treatment on growth parameters of wheat. As the NaCl concentration increased, the wheat shoot's growth criteria (plant height, flag leaf area, and shoot FW and DW) progressively decreased (Figures 1A–D). For instance, compared to 60 mmol/L (19.2, 28.6, 27.2, and 28%), the decrease in all parameters was noticeably greater ($P < 0.05$) with 120 mmol/L (35.7, 40.6, 46.2, and 44%). Additionally, compared to control plants, the Pro application reduced the impact of NaCl and exhibited a lower reduction in plant height, flag leaf area, and shoot FW and DW by 8.7,

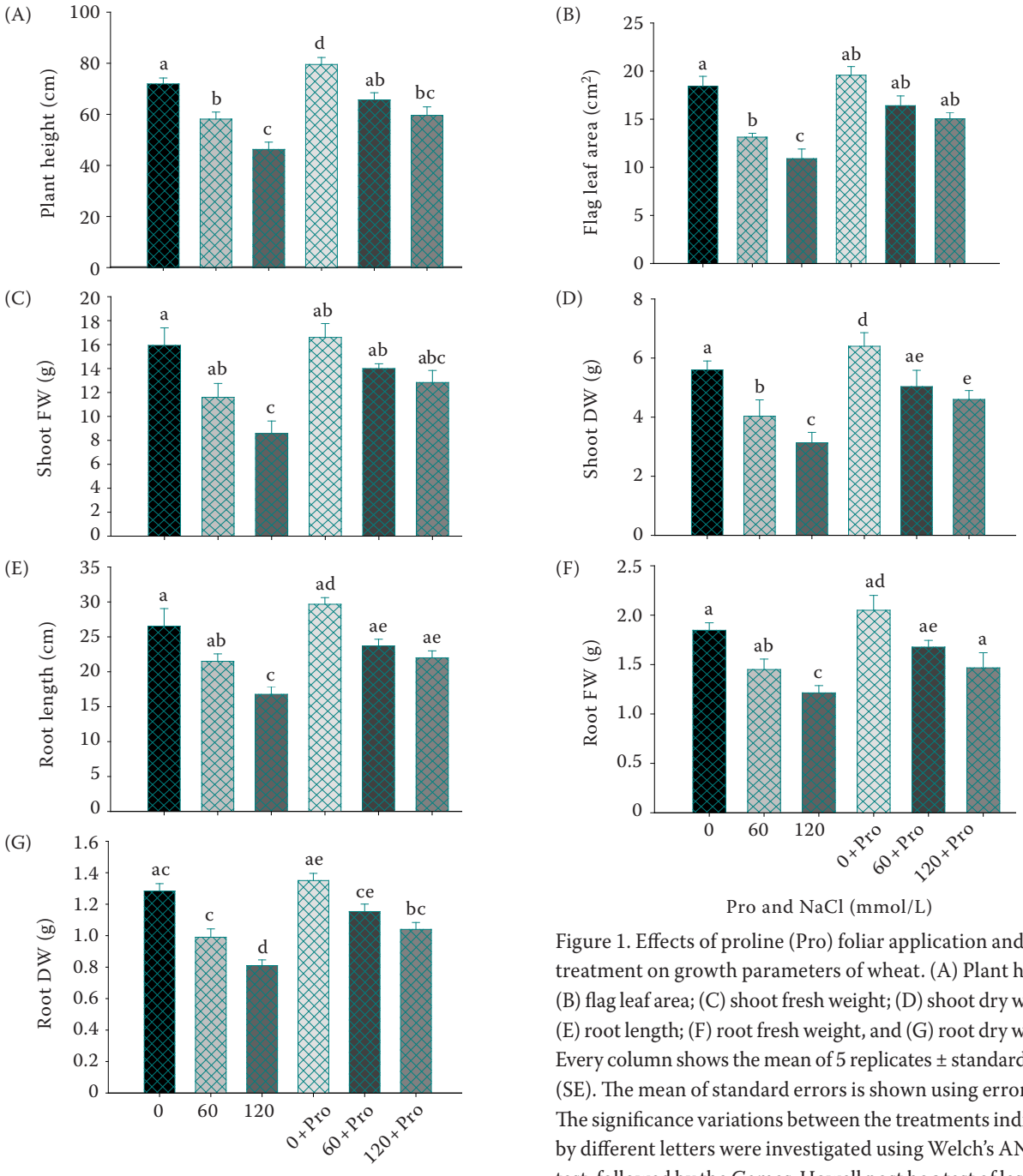

Figure 1. Effects of proline (Pro) foliar application and NaCl treatment on growth parameters of wheat. (A) Plant height; (B) flag leaf area; (C) shoot fresh weight; (D) shoot dry weight; (E) root length; (F) root fresh weight, and (G) root dry weight. Every column shows the mean of 5 replicates ± standard error (SE). The mean of standard errors is shown using error bars. The significance variations between the treatments indicated by different letters were investigated using Welch's ANOVA test, followed by the Games-Howell post hoc test of less than 5% ($P < 0.05$). FW – fresh weight; DW – dry weight

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10.9, 12, and 10% with 60 mmol/L + Pro and by 17, 18.3, 19.5, and 17.9% with 120 mmol/L + Pro, respectively.

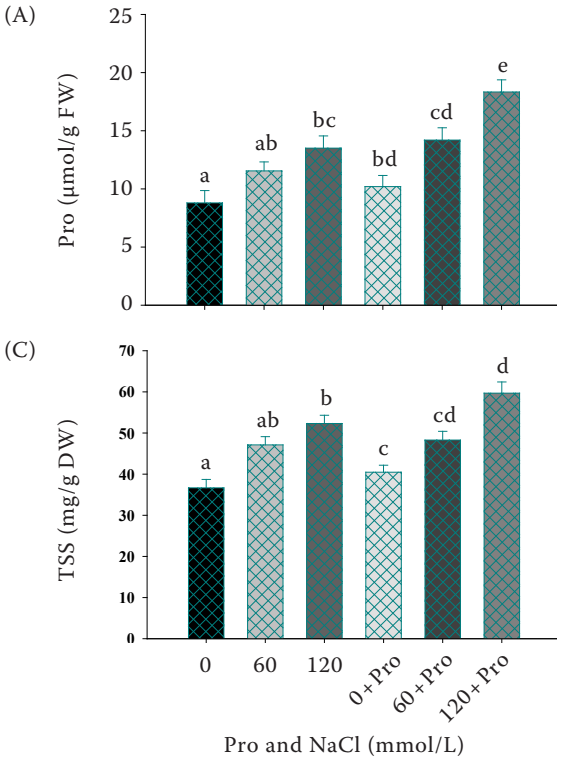
The root growth characteristics (root length and root FW and DW) have also shown comparable results (Figures 1E–G). Root length and root FW and DW decreased less in plants treated with NaCl at 60 mmol/L (19, 21.5 and 22.9%) than in plants treated with 120 mmol/L (36.8, 34.3, and 37%). However, compared to control values, the Pro addition enhanced the root length and root FW and DW by 10.7, 9.2, and 10% with 60 mmol/L + Pro and 17, 20.6, and 19% with 120 mmol/L + Pro, respectively.

Pro treatment enhanced the organic osmolyte content in wheat during NaCl stress. In comparison to their respective controls, the levels of Pro, TSP, and TSS improved more significantly ($P < 0.05$) with 120 mmol/L (53.4, 47.7, and 42.6%) than with 60 mmol/L (31.2, 25.9, and 28.4%) (Figures 2A–C). Furthermore, in plants under 120 mmol/L NaCl stress, Pro treatment caused additional enhancement in Pro, TSP, and TSS levels by 108.3, 58.8, and 61.8%. In addition, Pro, TSP, and TSS had the highest values (18.3 ± 1.3, 19.4 ± 1.5, and 59.3 ± 2.5) and the lowest values (8.8 ± 0.21, 12.2 ± 0.9, and 36.7 ± 1.6) with 0 and 120 mmol/L + Pro treatments, respectively.

Regulation of the inorganic osmolytes uptake in wheat plants by Pro application under salinity. NaCl treatment increased the Na content, and the Na/K ratio in the wheat flag leaves related to the control values. The increases were more considerable ($P < 0.05$) with 120 mmol/L (215.3 and 507%) than the 60 mmol/L concentration (102.7 and 188.6%) in Na level and Na/K ratio, respectively (Figures 3A, C). Conversely, the application of Pro in addition to salt stress exhibited less reduction in the Na content as well as the Na/K ratio with 60 mmol/L + Pro (38 and 61%) than 120 mmol/L + Pro (61.3 and 113.4%), respectively.

Furthermore, salinity stress reduced wheat flag leaves' K, Ca, and Mg concentrations. Concerning their respective controls, these reductions were more noticeable ($P < 0.05$) under the 120 mmol/L (48, 54, and 41%) than 60 mmol/L (30, 28.8, and 21%) in K, Ca, and Mg ions content, respectively (Figures 3B–E). Further application of Pro partially restored the concentrations of K, Ca, and Mg ions in the flag leaves, although they were still lower than the control levels. Additionally, the K, Ca, and Mg maximum (1.02 ± 0.03, 0.72 ± 0.02, and 11.5 ± 1.3) and minimum values (0.45 ± 0.02, 0.27 ± 0.03, and 5.3 ± 0.5) were observed with 0 mmol + Pro and 120 mmol/L treatments, respectively.

Exogenous application of Pro modulated phytohormones level in wheat plants under salt stress.



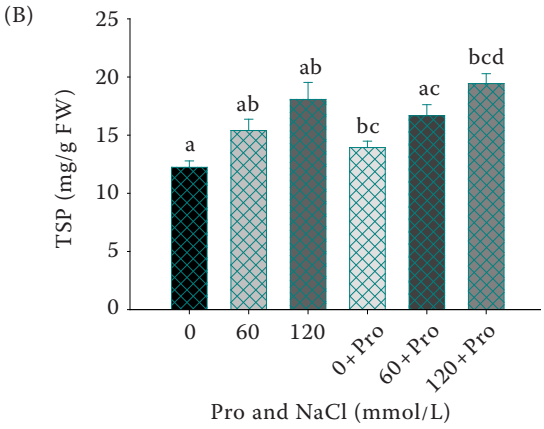


Figure 2. Proline (Pro) treatment enhanced the organic osmolyte content in wheat during NaCl stress. (A) Proline; (B) total soluble protein (TSP), and (C) total soluble sugar (TSS). Every column shows the mean of 3 replicates ± standard error (SE). The mean of standard errors is shown using error bars. The significance variations between the treatments indicated by different letters were investigated using Welch's ANOVA test, followed by the Games-Howell post hoc test of less than 5% ($P < 0.05$). FW – fresh weight; DW – dry weight

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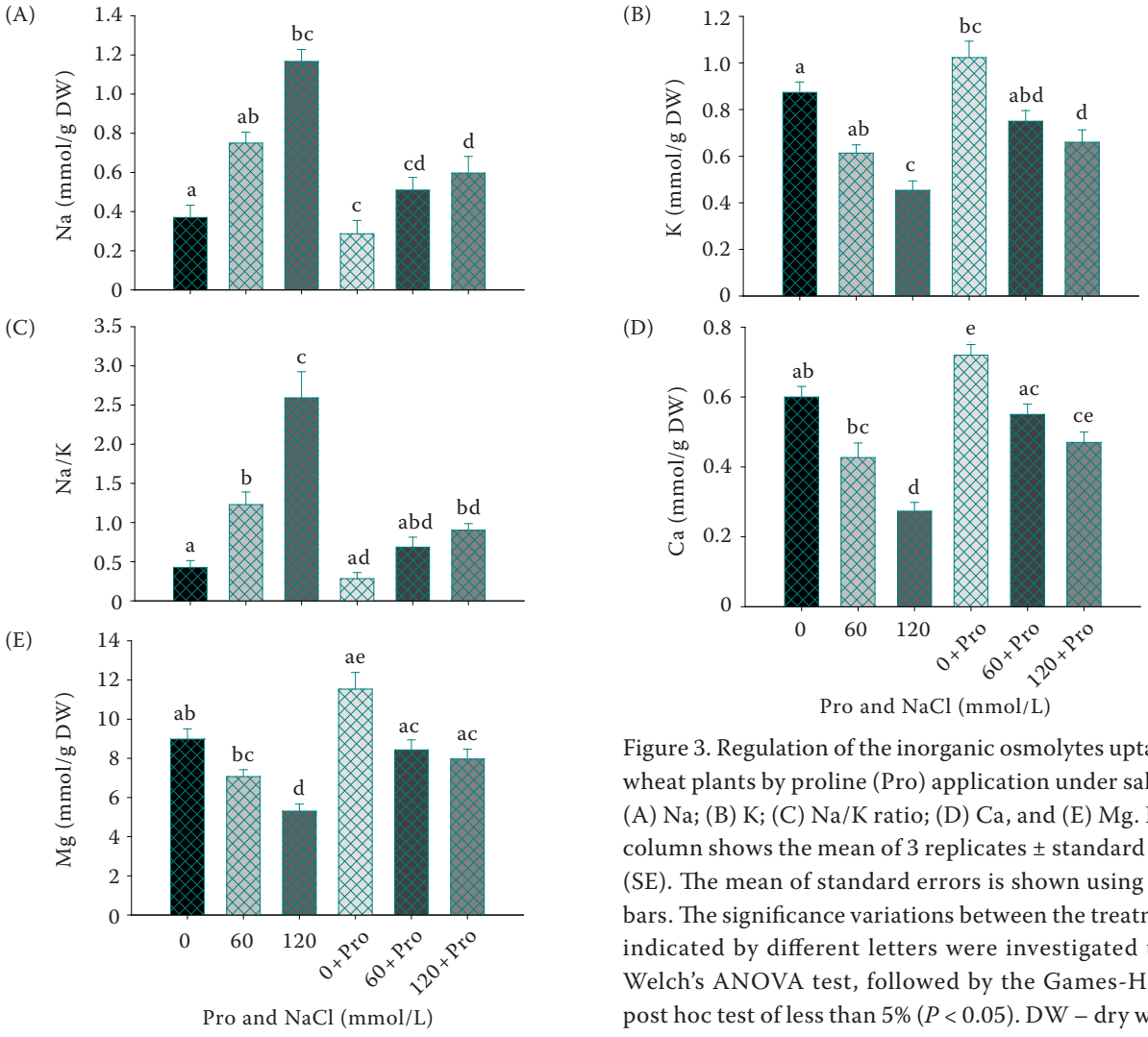

Figure 3. Regulation of the inorganic osmolytes uptake in wheat plants by proline (Pro) application under salinity. (A) Na; (B) K; (C) Na/K ratio; (D) Ca, and (E) Mg. Every column shows the mean of 3 replicates ± standard error (SE). The mean of standard errors is shown using error bars. The significance variations between the treatments indicated by different letters were investigated using Welch's ANOVA test, followed by the Games-Howell post hoc test of less than 5% ($P < 0.05$). DW – dry weight

NaCl toxicity caused a considerable reduction ($P < 0.05$) in the IAA and GAs levels by 37.2 and 28.7%, respectively, with 60 mmol/L concentration and by 53.5 and 54.3%, with 120 mmol/L treatment (Figures 4A, B). Plants treated with NaCl + Pro showed a smaller decline in IAA and GA levels by 11.4 and 15.2%, respectively, with 60 mmol/L + Pro treatment and by 27.3 and 22%, respectively, with 120 mmol/L + Pro treatment, as related to control plants.

However, under salt stress, the ABA level was significantly higher ($P < 0.05$) than in control plants, reaching 82.8% with 60 mmol/L and 207.2% with 120 mmol/L stress (Figure 4C). Pro treatment reduced the ABA level compared to salt-stressed plants without Pro by 22% under 60 mmol/L NaCl and by 58.5% under 120 mmol/L NaCl, indicating its mitigating effect on stress-induced ABA accumulation.

Response of non-enzymatic antioxidants to Pro and NaCl treatments. GSH and AsA contents showed a noticeable enhancement ($P < 0.05$) by 26.4 and 17.8% with 60 mmol/L and by 44.8 and 31.9% with 120 mmol/L concentration, respectively, concerning unstressed plants (Figures 5A, B). Pro application also led to further accumulation in GSH and AsA levels by 57.6% and 39.9% with 120 mmol/L + Pro treatment, respectively.

Impact of Pro application and NaCl toxicity on the activity of antioxidant enzymes. Salinity stress induced an improvement in the GR, SOD, CAT, and APX activities in wheat flag leaves (Figures 6A–D). Concerning control plants, these improvements were considerable ($P < 0.05$) by 56.6, 48.5, 46.4, and 53.8%, respectively, with 120 mmol/L concentration. On the other hand, Pro application caused additional enhancement in the GR, SOD, CAT, and APX activities by 46.6, 40, 37.7, and 35.3%, respectively, with 60 mmol/L + Pro treatment and by 73.8, 57.5, 53.7, and 67.7%, respectively, with 120 mmol/L + Pro treatment.

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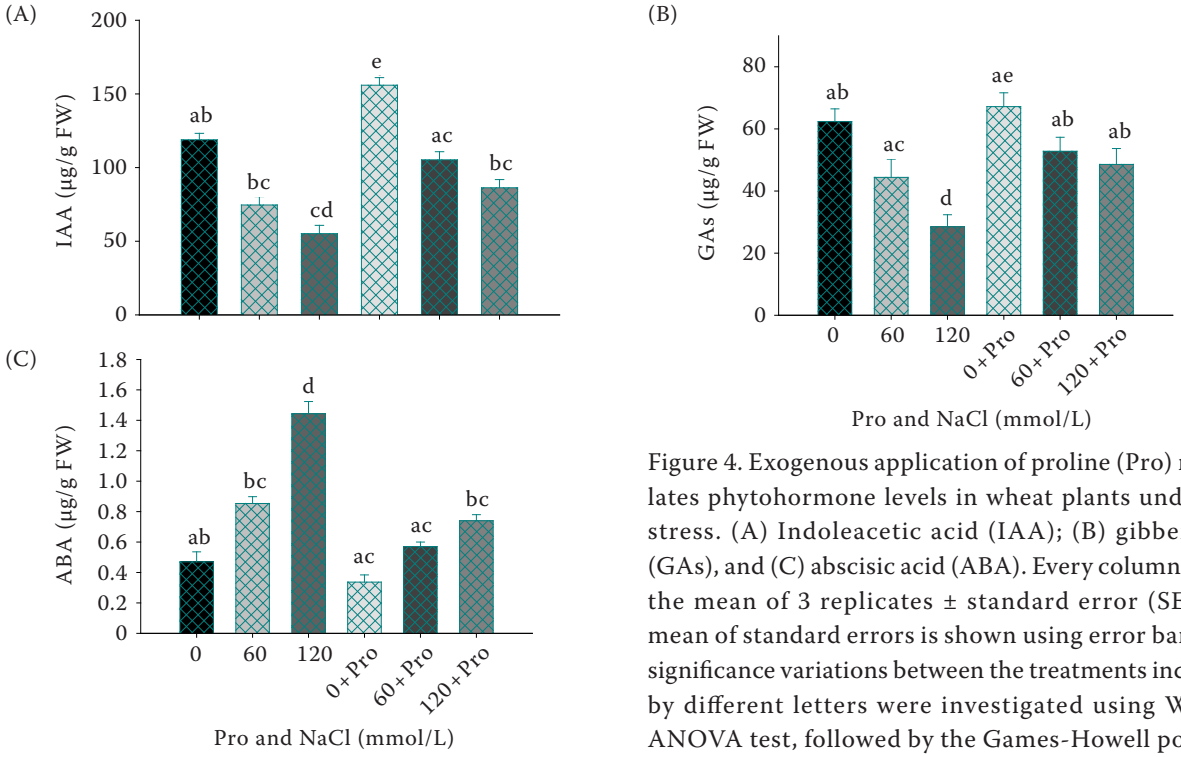

Figure 4. Exogenous application of proline (Pro) modulates phytohormone levels in wheat plants under salt stress. (A) Indoleacetic acid (IAA); (B) gibberellins (GAs), and (C) abscisic acid (ABA). Every column shows the mean of 3 replicates ± standard error (SE). The mean of standard errors is shown using error bars. The significance variations between the treatments indicated by different letters were investigated using Welch's ANOVA test, followed by the Games-Howell post hoc test of less than 5% ($P < 0.05$). FW – fresh weight

Pro treatment boosted yield attributes in wheat under salinity. NaCl application led to a significant reduction ($P < 0.05$) in all yield attributes in all wheat plants as compared with unstressed plants (Figure 7). For instance, the reduction in plant height, spike length, no. of spikelets/main spike, and 100-kernel weight was higher with 120 mmol/L (33.9, 32.3, 35.7, and 42.4%) than 60 mmol/L (16.7, 20, 17.8, and 26.5%), respectively (Figures 7A–D). When plants were treated with 120 mmol/L + Pro, the reduction in plant height, spike length, no. of spikelets/main spike, and 100-kernel weight was less obvious, estimating 15.6, 14.5, 16.9, and 18.74%, respectively.

The grain no./main spike, grain yield/plant, and grain FW and DW of wheat plants under NaCl stress also decreased less at 60 mmol/L (19, 18.3, 15.6, and

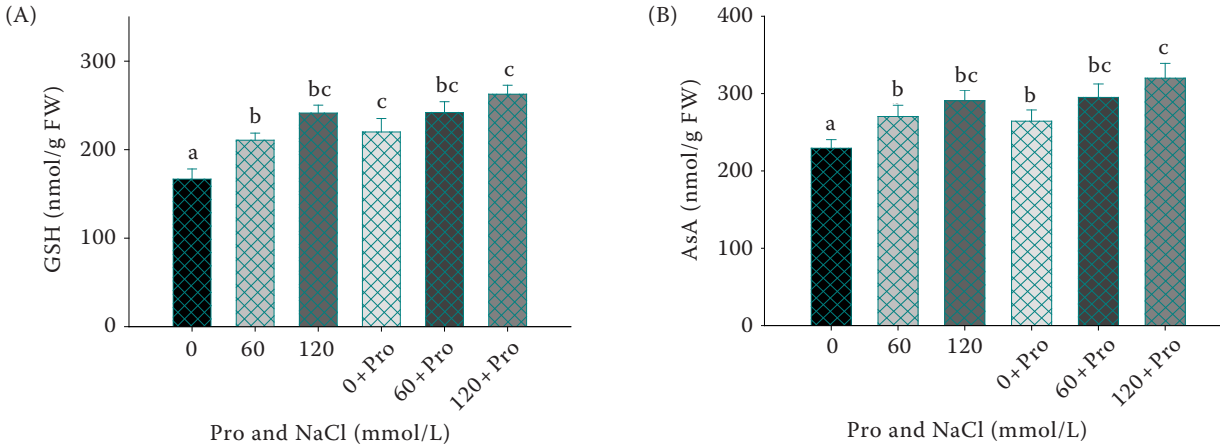

Figure 5. Response of non-enzymatic antioxidants to proline (Pro) and NaCl treatments in wheat plants. (A) Glutathione (GSH) and (B) ascorbic acid (AsA). Every column shows the mean of 3 replicates ± standard error (SE). The mean of standard errors is shown using error bars. The significance variations between the treatments indicated by different letters were investigated using Welch's ANOVA test, followed by the Games-Howell post hoc test of less than 5% ($P < 0.05$). FW – fresh weight

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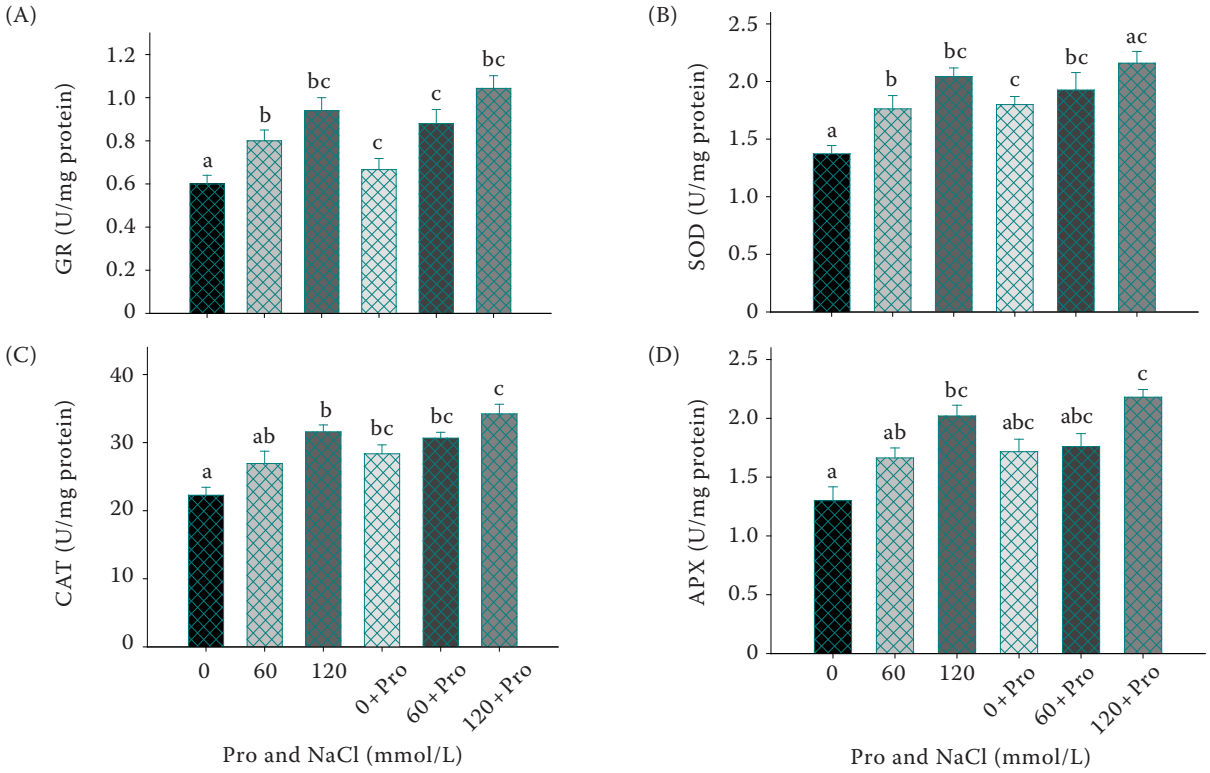

Figure 6. Impact of proline (Pro) application and NaCl toxicity on the activity of antioxidant enzymes. (A) Glutathione reductase (GR); (B) superoxide dismutase (SOD); (C) catalase (CAT), and (D) ascorbate peroxidase (APX). Every column shows the mean of 3 replicates ± standard error (SE). The mean of standard errors is shown using error bars. The significance variations between the treatments indicated by different letters were investigated using Welch's ANOVA test, followed by the Games-Howell post hoc test of less than 5% ($P < 0.05$)

18.8%) than at 120 mmol/L (37.8, 39.2, 35, and 54.5%), respectively (Figures 7E–H). Furthermore, compared to control values, the values of grain no./main spike, grain yield/plant, grain FW, and DW showed less reduction with the application of Pro by 7.9, 8.5, 6, and 8.4% with 60 mmol/L + Pro and 12, 11.5, 10.7, and 14% with 120 mmol/L + Pro, respectively.

DISCUSSION

Soil salinisation is among the important global challenges, especially crop productivity in most agricultural regions. Salinity can potentially cause damage through disrupted plant growth due to changed physiological and biochemical processes, reducing crop yields (Shahzadi et al. 2024, Zhou et al. 2024). In the current study, salt stress suppressed growth attributes in wheat plants, including shoot and root growth parameters and flag leaf area (Figure 1). The destruction impact of salinity stress on the growth criteria was reported with many plants, such as *Moringa oleifera* Lam. (Atteya et al. 2022), *Pisum sativum* L. (El-Beltagi et al. 2024), and *Triticum aestivum* L. (Ehtaiwesh et al. 2024, Shahzadi et al. 2024, Zhu et al. 2024). Under salinity conditions, osmotic stress reduces water uptake by the roots, reducing cell division and elongation (Zhou et al. 2024). Apart from that, osmotic disequilibrium results in the poor development of roots at low density of root hairs, reducing the absorption of water and nutrients (Ayman et al. 2024). A high salt level interferes with shoot development through effects on water balance and transport- both are necessary for elongation and leaf expansion (Koc et al. 2024). Meanwhile, oxidative stress and ionic toxicity due to salt further bring about an improvement in the levels of Na^+ and Cl^- ions in shoot tissues, giving way to damage to cellular structures and slow photosynthesis and reducing biomass accordingly (Atta et al. 2023). Salinity also causes nutritional deficiencies, which in turn inhibit chlorophyll synthesis and shoot vigour, canopy development, and flag leaf area, which are important for photosynthesis and grain filling (Ehtaiwesh et al. 2024). On the other hand, our findings indicated

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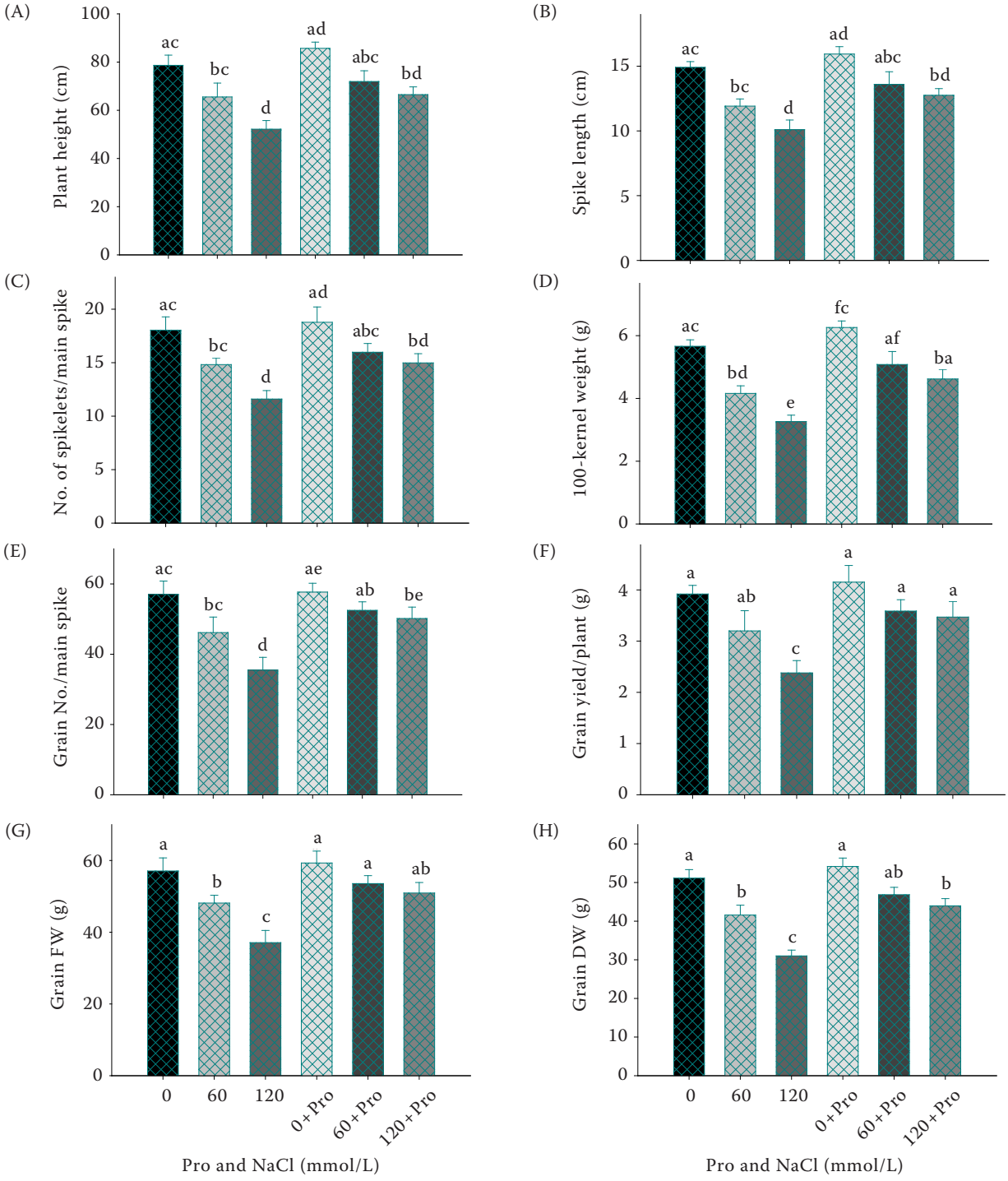

Figure 7. Proline (Pro) treatment boosted yield attributes in wheat under salinity. (A) Plant height; (B) spike length; (C) no. of spikelets/main spike; (D) 100-kernel weight; (E) grain no./main spike; (F) grain yield/plant; (G) grain fresh weight, and (H) grain dry weight. Every column shows the mean of 5 replicates ± standard error (SE). The mean of standard errors is shown using error bars. The significance variations between the treatments indicated by different letters were investigated using Welch's ANOVA test, followed by the Games-Howell post hoc test of less than 5% ($P < 0.05$). FW – fresh weight; DW – dry weight

that Pro exogenous applications improved all growth parameters of wheat plants. These findings agreed with previous studies in various plants, including maize (Alam et al. 2017), moringa (Atteya et al.

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2022), wheat (Ayman et al. 2024), pea (El-Beltagi et al. 2024), and radish (Inayat et al. 2024). In this regard, Hosseinifard et al. (2022) stated that Pro improves seed germination, photosynthetic traits, biomass, and grain yield. This improvement may be due to increased nutrient uptake, water potential, atmospheric nitrogen fixation, osmotic adjustment, and decreased oxidative damage under salinity stress (Kaur et al. 2024).

Additionally, the NaCl stress resulted in increased levels of organic osmolytes, such as Pro, TSP, and TSS, which are important for the survival and adaptation of plants under stress conditions, as represented in Figure 2. Various studies (Elhakem 2020, Ehtaiwesh et al. 2024, Shahzadi et al. 2024) have reported the same results. TSS such as glucose, sucrose, and fructan accumulate to maintain osmotic balance, protect cellular integrity, and neutralise ROS generated by salinity-induced oxidative stress (Singh et al. 2022). On the other hand, under stress conditions, TSP acts importantly in repairing cellular injury and enhancing the antioxidant defence mechanism (Inayat et al. 2024). According to Kaur et al. (2024), these osmolytes help regulate osmotic pressure, prevent oxidative damage, and maintain stable cellular processes. In addition, Pro treatment has proven to be approached to boost the accumulation of essential osmolytes under the stress of salinity (Zheng et al. 2023). The Pro application in the present study induced more Pro, TSP, and TSS accumulation compared to the treated and untreated plants. In this connection, similar results were confirmed by Rady et al. (2019), El-Beltagi et al. (2024), and Inayat et al. (2024). Applied exogenously, Pro not only enhance its concentration within the plant tissues but also takes an active part in modulating very important metabolic pathways that further result in enhanced levels of other types of osmolytes, such as TSP and TSS (Koc et al. 2024). Moreover, Hosseinifard et al. (2022) stated that Pro supplement increases the osmotic balance, strengthens cellular membrane stability, and activates stress-responsive genes that enhance the production of other osmolytes. In this regard, Atteya et al. (2022) described that moringa plants treated with Pro showed enhanced salt tolerance marked by improved osmotic adjustments, thereby reducing the magnitude of their oxidative stress and keeping growth variables such as biomass intact. Apart from that, Pro activates other stress-related signal molecules under its umbrella to enhance its protective role towards rendering salt tolerance to plants (Singh et al. 2022).

NaCl toxicity leads to toxic ion accumulation, including Na and Cl, which disrupt cellular function and cause oxidative damage to plant tissues (Zhou et al. 2024). This builds up and alters the ionic balance, reducing the uptake of essential and vital nutrients like K, Ca, and Mg (Atta et al. 2023). Figure 3 showed that NaCl stress increased the concentration of Na^+ and the Na/K ratio while decreasing K, Ca, and Mg ion uptake in wheat flag leaves. These results have been reported for various plants, including *Carthamus tinctorius* L. (Shaki et al. 2019), *Zea mays* L. (Elhakem 2020), *Moringa oleifera* (Atteya et al. 2022), and *Triticum aestivum* (Shahzadi et al. 2024, Zhu et al. 2024). Plants use some mechanisms for the attenuation of these effects *via* compartmentalisation of surplus Na in vacuoles and favouring the uptake of essential ions like K, thus maintaining enzyme and metabolic activities under salt stress (Elhakem 2020). On the other hand, under salinity stress, the disturbed ionic distribution interferes with metabolic processes in the cytoplasm. It negatively affects photosynthesis, generally impairs growth and reduces overall crop productivity (Kaur et al. 2024). On the other hand, our findings reveal that Pro plays a crucial role in maintaining ionic balance under high salinity (120 mmol/L) by restricting Na accumulation and significantly preserving K, Ca, and Mg levels compared to untreated stressed plants. This suggests that Pro may influence transporter activity or membrane stability, thereby contributing to systemic ionic homeostasis. According to Zheng et al. (2023), exogenous application of Pro strengthens the plant's efficiency in ionic balance and tight control, especially under high salinity conditions. In addition, Pro increased the activity of Na/H antiporters responsible for the transport of Na out of the cell to prevent the accumulation of injurious ions like Na and Cl (Singh et al. 2022). This promotes the Na/K ratio, which is important to maintain cellular functions appropriately. Pro also strengthens the absorption of cations, including K, Ca, and Mg; these are involved in membrane stability and protection against oxidative damage resulting from high salinity levels in plants. Multiple studies have reported comparable results (Alam et al. 2017, Rady et al. 2019, Atteya et al. 2022, Shahzadi et al. 2024, Zhu et al. 2024). Kaur et al. (2024) also associated Pro treatments with improved cellular ion sequestration and nutrient balance, which result from promoting beneficial ions and decreased levels of toxic ion accumulation. Other than that, Pro decreases membrane lipid peroxidation and

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oxidative damage maintainable through K, Ca, and Mg by stable (Singh et al. 2022). Meanwhile, Koc et al. (2024) showed that the Pro application helped maintain ion homeostasis and improved growth and productivity by mitigating the negative effect of salt stress on ion balance and distribution.

Salinity impressively disturbed the balance of interior phytohormones like IAA, GAs, CKs, and ABA, altering plant growth and stress adaptation mechanisms (Zheng et al. 2023). Our results established that when wheat plants are subjected to NaCl stress, IAA and GA levels decrease, whereas ABA levels increase (Figure 4). Previous studies by Shaki et al. (2019) and Elhakem (2020) showed that the interaction of IAA, GAs, and ABA is crucial for regulating growth inhibition and adaptation to salt stresses. On the other hand, Pro treatment has been widely recognised to modulate internal phytohormones under stressful conditions, hence increasing plant tolerance (Ayman et al. 2024). Otherwise, salinity reduced the IAA level, which Pro enhanced, improving root elongation and lateral root development responsible for water and nutrient uptakes (Singh et al. 2022). Similarly, Pro counteracts the salinity-induced decline in GAs, which allows for the continuance of critical processes like seed germination and shoot elongation. This supports previous studies that showed GAs are crucial for growth but undergo suppression under saline conditions (Atta et al. 2023). Besides, Pro modulates ABA by preventing excessive accumulation that suppresses photosynthesis and growth while maintaining its role in activating stress-responsive genes and stomatal control (Zheng et al. 2023). These results support the previous finding that ABA improves in response to salinity as a protective mechanism but that this response must be carefully regulated to prevent growth suppression (Singh et al. 2022).

The recognised hormonal changes in response to Pro treatment expose a crucial regulating pathway linking Pro metabolism with ion transport and plant stress signalling. A hallmark of stress response under salt stress is the rise in ABA and the decrease in growth-promoting hormones (IAA, GAs), which contribute to growth inhibition and help explain the reason. In the present study, foliar-applied Pro reduced the salt-induced ABA rise while restoring IAA and GAs to levels more aligned with those in unstressed controls. This hormonal rebalancing probably helped improve root development and cellular expansion, enabling more effective water and ion absorption. Our results support several studies suggesting crosstalk between Pro and phytohormones at the signalling level; they also imply that Pro may influence hormone-mediated activation of ion transporters, including Na/H antiporters. Maintaining K and Ca uptake while rejecting Na depends on these systems. Therefore, the enhanced ionic balance shown in our work is probably mediated by hormone-Pro interaction, stressing Pro's more complicated, integrated function as both an osmoprotectant and a hormonal modulator.

Our findings indicate that foliar-applied Pro substantially increased the activities of antioxidant enzymes (SOD, CAT, APX, GR) and boosted the non-enzymatic antioxidants (GSH and AsA) in wheat under both moderate and high salinity levels, implying a proactive improvement of the detoxification mechanism rather than a merely reactive response. Notably, the magnitude of increase observed in GR and APX under 120 mmol/L NaCl + Pro treatment exceeded those previously reported in similar wheat genotypes, indicating a potentially cultivar-specific sensitivity to Pro-induced redox regulation. Indeed, Pro has an essential role in improving plant enzymatic antioxidant defences against abiotic stresses through modulating activity regarding key enzymes related to the scavenging process of ROS (Koc et al. 2024). Salinity stress is characterised by increased levels of ROS, hence inducing oxidative damage to lipids and proteins, including DNA, and impairment of cellular function (Zheng et al. 2023). Pro-supplementation enhances the activities of those kinds of enzymes that might neutralise injurious ROS with increased efficiency, which can protect oxidative damage against cellular components or preserve membrane integrity (Singh et al. 2022). It has been reported by Koc et al. (2024) that the Pro-treated activity of APX and GR improves the recycling of ascorbate and glutathione, two key players in the antioxidant defence system. It allows for a strong contribution of enzymatic enhancement, maintains cellular redox homeostasis, and offers higher growth and development in the case of stressed plants by elevating various enzymatic antioxidants (Hosseinifard et al. 2022). Besides, by improving GSH and AsA, Pro thus bolsters non-enzymatic antioxidant defenses in addition to enzymes that become linked (Zheng et al. 2023). These non-enzymatic antioxidants are involved in several events necessary to maintain redox balance in the cells and detoxify active oxygen. An increased amount of GSH, through the premeditated mechanism, en-

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hances the cyclical glutathione ascorbate pathway, which helps in the decomposition of ROS. Higher amounts of AsA protect the chloroplast, as well as other structural features of the cell, against oxidative stress-mediated damage (Kaur et al. 2024). Pro acts as an osmoprotectant, maintains the structural integrity of cells or any organelle, and enhances the activities of several antioxidant molecules (Singh et al. 2022). In this regard, Hosseinifard et al. (2022) stated that Pro enhances both enzymatic and non-enzymatic antioxidants together and hence provides a strong defence against salinity stress-induced oxidative damage, fostering plant resilience and productivity under adversarial conditions.

Furthermore, NaCl stress noticeably obstructed the productivity of wheat plants by causing hormonal and osmotic imbalance, ion toxicity, and oxidative stress, inducing a reduction in all yield attributes (Figure 7). Otherwise, the Pro exogenous application has been exhibited to ameliorate these injurious effects and alleviate all yield attributes of wheat plants. In this regard, Alam et al. (2017) and Rady et al. (2019) reported that Pro treatment improved the FW and DW, seed yield, and weight of 100 seeds of *Zea mays* and *Triticum aestivum* under saline environments. In this respect, Kaur et al. (2024) demonstrated that Pro supplement alleviates salinity stress tolerance in plants by enhancing seed germination, plant growth, FW and DW, photosynthetic traits, and yield attributes by improving the acquisition of nutrients, more water uptake, atmospheric nitrogen fixation, antioxidant defence system, and ionic homeostasis (Singh et al. 2022). Furthermore, Pro acts as an osmoprotectant, preserving cellular osmotic balance and safeguarding cellular structures during stress, hence maintaining cell turgor and supporting metabolic functions (Hosseinifard et al. 2022). Moreover, our results indicated that foliar application of Pro improved organic as well as inorganic osmolytes, regulated phytohormone levels, and enhanced the antioxidant defence system, resulting in enhanced stress tolerance. This comprehensive protective mechanism improves wheat yield parameters, as evidenced by increased grain number, weight, and overall grain yield/plant. Significantly, our investigation hyperlinks physiological and biochemical changes to specific agronomic accomplishments. Unlike much previous research on stress physiology, we demonstrate that the Pro treatment produced realisable yield recovery under salty conditions, proving its practical relevance in saline agriculture. In this respect, Atteya et al. (2022) stated that Pro treatment mitigated salinity stress by enhancing osmoprotectants, antioxidant activity, and nutrient uptake, improving growth traits; stabilising osmotic potential, and reducing oxidative damage, which collectively boosted seed and oil yields in stressed *Moringa oleifera*. In another study, Pro-supplement to *Brassica juncea* cultivars along with brassinosteroid alleviated the injury effects of salinity and enhanced yield by improving photosynthesis, antioxidant activity, and leaf water potential (Wani et al. 2019). Furthermore, El-Beltagi et al. (2024) reported that zinc nanoparticles (ZnO-NPs) and Pro, individually and in combination, enhanced the growth and yield of pea plants irrigated with diluted seawater by improving osmotic adjustment, membrane stability, oxidative stress mitigation, chlorophyll preservation, and ion regulation. Therefore, exogenous Pro application serves as a viable strategy to enhance wheat resilience and productivity in saline environments.

This research presents a comprehensive perspective on how exogenously applied Pro enhances wheat tolerance to salinity stress through a tightly coordinated set of physiological and biochemical responses. The findings demonstrate that Pro contributes not only to osmotic adjustment but also plays an integrative role in maintaining ion balance, strengthening antioxidant defences, and rebalancing stress-related hormones, mechanisms that are often studied separately. Importantly, the positive effects of Pro extended to growth and yield-related traits, underscoring its agronomic relevance. While the current work focused on a single wheat cv. Giza 168, it provides a valuable reference point for understanding genotype-level responses to exogenous Pro under salinity. Using a single, well-documented concentration (10 mmol/L) allowed for targeted insights into Pro's effectiveness, aligning with previous studies while revealing unique patterns of interaction between hormones, ion regulation, and antioxidant capacity. Moreover, the fixed timing and frequency of application were based on established protocols known to be effective in stress priming, providing a consistent framework for evaluation. This research sets the stage for more nuanced studies that explore Pro's role across different cultivars, concentrations, and growth stages. Integrating molecular analyses, such as the expression of ion transporters and antioxidant-related genes, would further enrich our mechanistic understanding. Additionally, while Pro is cost-effective and easy to apply, its large-scale ag-

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ronomic potential should be validated under variable field conditions to assess consistency, scalability, and cost-benefit outcomes.

In conclusion, this study provides compelling evidence that exogenous Pro functions as a multifaceted regulator of stress adaptation in wheat under saline conditions. By enhancing osmolyte accumulation, preserving ionic balance, modulating antioxidant defence systems, and fine-tuning hormonal dynamics, Pro significantly improves plant performance from cellular function to final yield. This integrative approach advances current understanding by linking upstream physiological and biochemical changes to downstream agronomic benefits, a connection often missing in similar studies. Notably, the restoration of growth-promoting hormones (IAA, GA_3) and suppression of ABA accumulation under salt stress reflects a sophisticated role for Pro in hormonal homeostasis. These changes were associated with improved nutrient uptake and reduced oxidative damage, suggesting that Pro influences key signalling networks beyond its traditional role as an osmoprotectant. The practical implications of these findings are significant. Given Pro's affordability, simplicity of application, and biological efficacy, it holds promise as a field-ready strategy for mitigating salinity effects in wheat. Future research should expand on these results by evaluating multiple genotypes, exploring dose-response relationships, and integrating molecular tools to confirm and build upon the mechanisms identified here. Such efforts will further support the deployment of Pro-based interventions as part of climate-resilient and sustainable crop production strategies.

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