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Triacontanol-induced changes in growth, yield, leaf water relations, oxidative defense system, minerals, and some key osmoprotectants in *Triticum aestivum* under saline conditions

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Abstract: To investigate the effect of exogenous application of triacontanol (TRIA) on 2 wheat (*Triticum aestivum* L.) cultivars (S-24 and MH-97) under salt stress, an experiment was conducted in a greenhouse under natural climatic conditions. Both cultivars were grown in full strength Hoagland's nutrient solution under nonsaline (0 mM NaCl) or saline (150 mM NaCl) conditions in sand culture. Three optimized TRIA levels (0, 10, and 20 µM) were used as foliar spray at 3 growth stages, i.e. vegetative, boot, and vegetative + boot stages. Ninety-two-day-old plants were subjected to data analysis. Salinity stress adversely affected various growth, physiological, and biochemical attributes in both wheat cultivars at all growth stages. Under salt stress, activities of superoxide dismutase (SOD) and peroxidase (POD) decreased (cv. MH-97), while those of catalase (CAT) and POD (cv. S-24) increased. Contents of hydrogen peroxide (H_2O_2), malondialdehyde (MDA), Na^+, and Cl^- increased in both wheat cultivars at all growth stages. A foliar spray of 10 µM TRIA was more effective in reducing the adverse effects of salt stress on growth, yield, and leaf water relations of wheat plants when applied at the vegetative or vegetative + boot growth stages.

Key words: Triacontanol, salinity, wheat, SOD, POD, CAT, stress

1. Introduction

Salt stress–induced reductions in growth and productivity are due to a number factors including osmotic stress (Shabala et al., 2012), Na^+ and Cl^- ion toxicity or nutrient imbalance (Babu et al., 2012), oxidative stress (high ROS production) (Abbaspour, 2012), membrane damage (Farkhondeh et al., 2012), disturbed leaf water relations (Carpici et al., 2010), and hormonal imbalance (Babu et al., 2012; Iqbal and Ashraf, 2013). Adverse effects of salt stress on various growth, physiological, and biochemical attributes have been reported in crops such as wheat (Kausar et al., 2013; Kausar and Shahbaz, 2013; Perveen et al., 2013; Shahbaz and Ashraf, 2013), rice (Habib et al., 2013), mungbean (Kanwal et al., 2013), cotton (Shaheen et al., 2012), canola (Shahbaz et al., 2013), sunflower (Shahbaz et al., 2011), tobacco (Jardak Jamoussi et al., 2014), tomato (Ali et al., 2014), and vegetables (Shahbaz et al., 2012). However, tolerance to salinity stress is a multigenic response that involves regulation of myriad physiological and biochemical processes (Sairam and Tyagi, 2004). For example, accumulation of various organic osmolytes and induction of an antioxidant defense system comprising various enzymatic and nonenzymatic antioxidants are commonly occurring processes in plants under saline stress that protect plants from oxidative stress by scavenging oxygen-free radicals, thereby protecting various cytoplasmic organelles (Ashraf, 2009; Abbaspour, 2012).

Crop sensitivity to salinity stress varies at different growth stages, and wheat shows differential sensitivity to salt stress; it is more sensitive at early growth stages (Ashraf and Ashraf, 2012). Wheat grain yield is adversely affected by salt stress (Eleiwa et al., 2011), and thus improving salinity tolerance in wheat to support the rapidly growing world population is the main enigma of the present day (FAO, 2010).

Phytohormones play an important role in plant growth and development by transmitting a variety of signals between and within the cells; however, their endogenous levels undergo considerable changes under salt stress (Iqbal and Ashraf, 2013). It is now widely known that the levels of most growth up-regulators decrease in plants

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exposed to saline stress (Yurekli et al., 2004). However, deficiency in these regulators could be overcome by their exogenous application (Akram et al., 2012; Babu et al., 2012). Triacontanol (TRIA) enhances growth and yield of various crop species when applied exogenously as foliar spray at various growth stages (Singh et al., 2011). Foliar-applied TRIA ameliorates the negative effects of various abiotic stresses on growth, physiological, and biochemical processes of different plant species, e.g., *Erythrina variegata* L. seedlings (Muthuchelian et al., 2003), sweet basil (Borowski and Blamowski, 2009), common duckweed (Kilic et al., 2010), soybean (Krishnan and Kumari, 2008), maize (Ertani et al., 2012), canola (Zulfiqar and Shahbaz, 2013), and sunflower (Aziz et al., 2013). Under salt stress conditions, exogenous application of TRIA has been reported to up-regulate genes involved in the photosynthetic process while down-regulating stress-related genes, modulating activities of different metabolic and antioxidant enzymes, enhancing water and mineral nutrient uptake, and stimulating synthesis of various organic compounds through increased nitrogen metabolism (Perveen et al., 2011, 2012; Ertani et al., 2012). Although the effect of triacontanol as a seed treatment (Perveen et al., 2010, 2011, 2012a, 2012b) and foliar spray (Perveen et al., 2013) on wheat under saline conditions has been observed, the effect of this potential plant growth regulator as a foliar spray on physiological and biochemical attributes at various developmental stages is not known. It was hypothesized that foliar application of TRIA at various growth stages (vegetative, vegetative + boot, and boot growth stages) could ameliorate the negative effects of salt stress on growth, physiological, and biochemical attributes of wheat plants. Thus, the main objectives of the present study were to evaluate the effect of varying levels of foliar-applied TRIA on various growth and yield attributes, leaf water relations, enzymatic and nonenzymatic antioxidants, compatible solutes, and mineral contents at various growth stages in wheat plants under saline conditions.

2. Materials and methods

To assess the effect of exogenous application of triacontanol as foliar spray at different growth stages on wheat plants under saline and nonsaline conditions, a greenhouse experiment was conducted in the Old Botanical Garden, University of Agriculture, Faisalabad, Pakistan, under a 10 and 14 h light and dark period at 800–1100 μmol m^{-2} s^{-1} PPFD, a day and night temperature cycle of 20 and 6 °C, and mean relative humidity of 54 ± 5%. There were 2 wheat cultivars (S-24 (salt tolerant) and MH-97 (moderately salt sensitive)), 2 salinity levels (0 and 150 mM NaCl), and 3 optimized levels of TRIA (0, 10, and 20 μM) applied as foliar spray at the vegetative, boot, and vegetative + boot stages. Ten seeds per pot were sown in plastic pots containing thoroughly washed river sand. When seedlings were 10 days old, thinning was done to maintain 6 plants per pot. Plants were nourished with full-strength Hoagland's nutrient solution at the rate of 2 L of solution per pot per week. Salt (NaCl) treatment was initiated when plants were 21 days old. The desired level of NaCl was applied along with full-strength Hoagland's nutrient solution, and an aliquot of 50 mM solution per pot per day was applied until the desired level (150 mM) was attained. Foliar spray of the 3 optimized TRIA levels (0, 10, and 20 μM; solution prepared in hot, distilled water and 0.1% Tween-20 solution) was applied at the rate of 25 mL/pot when the plants were 30 (vegetative stage) or 78 (boot stage) days old. The design of the experiment was completely randomized with 4 replicates. When plants were 92 days old, data for various physiological and biochemical attributes were recorded. Two plants from each replicate were uprooted carefully, thoroughly washed with distilled water, and oven dried at 65 °C up to their constant weight. The dry weight of plants was recorded with the help of an electric balance. At maturity, data for various yield parameters, e.g., grain yield, number of grains per plant, and 100-grain weight were recorded.

2.1. Total leaf area per plant (cm^2)

Total leaf area per plant was calculated using the formula of Carleton and Foote (1965):

$$\text{leaf area } (cm^2) = \text{maximum leaf length} \times \text{maximum leaf width} \times 0.75,$$

0.75 = correction factor.

2.2. Leaf water relations

The second leaf from the top was cut with a sharp clipper from the main tiller to determine leaf water potential with a Scholander-type pressure chamber (Arimad-2, Japan) according to the method of Scholander et al. (1964). The same leaf used for water potential determination was frozen at –20 °C in a freezer for 1 week, and osmotic potential was determined using a vapor pressure osmometer (Vapro 5520, USA). Leaf turgor potential was calculated as the difference between osmotic potential and water potential values according to Nobel (1991).

2.3. Relative water contents (%)

Relative water content was determined following Jones and Turner (1978). Fresh leaf samples (0.5 g each) were weighed (Fw), kept in the dark for 24 h in deionized water, and turgid weight (Tw) was recorded. Dry weight (Dw) of the samples was recorded from samples oven-dried at 80 °C for 48 h. Percent relative water content was determined using the following formula:

$$RWC\ (\%) = [(Fw - Tw)/(Fw - Dw)] \times 100.$$

2.4. Membrane permeability (%)

Fresh leaf tissue (0.5 g of each sample) was chopped

and placed in 10 mL of distilled water, vortexed for 5 s, and the electrical conductivity (EC_0) was measured. The test tubes were kept at 4 °C for 24 h, and the electrical conductivity (EC_1) was determined. Then the test tubes containing samples were autoclaved for 1 h, cooled at room temperature, and electrical conductivity (EC_2) of dead tissues was measured. The relative membrane permeability (%) was determined using the following formula:

$$RMP\ (\%) = (EC_1 - EC_0/EC_2 - EC_0) \times 100.$$

2.5. Hydrogen peroxide (H_2O_2)

Hydrogen peroxide was determined following Velikova et al. (2000). Fresh leaf tissue (0.5 g of each sample) was homogenized in an ice bath with 5 mL of 0.1% (w/v) trichloroacetic acid (TCA) using a pre-chilled mortar and pestle. The homogenate was centrifuged for 15 min at 12,000 × *g*. To the 0.5 mL of supernatant, 0.5 mL of potassium phosphate buffer (pH 7.0) and 1 mL of potassium iodide were added. After vortexing, the absorbance of the supernatant was read at 390 nm using a UV-visible spectrophotometer (U2020 IRMECO). H_2O_2 contents were determined from a standard curve.

2.6. Malondialdehyde (MDA)

The protocol described by Carmak and Horst (1991) was used to measure the MDA contents. To 0.5 g of finely ground leaf tissue, 10 mL of 0.1% (w/v) trichloroacetic acid (TCA) solution was added, and the mixture was centrifuged for 10 min at 12,000 × *g*. To 1 mL of the supernatant, 4 mL of 0.5% thiobarbituric acid (TBA) prepared in 20% TCA was added. The reaction mixture was kept in a water bath at 95 °C for 30 min. Afterwards the samples were cooled by keeping them in an ice bath. Then the samples were again centrifuged at 12,000 × *g* for 10 min, and the absorbance of the samples was read using a spectrophotometer (U2020 IRMECO) at 2 wavelengths, 532 and 600 nm.

2.7. Extraction of antioxidant enzymes

Antioxidant enzymes were extracted by finely grinding fresh leaf samples (0.5 g of each sample) in 10 mL of phosphate buffer (50 mM, pH 7.8) in an ice bath. The homogenate was then centrifuged at 12,000 × *g* at 4 °C for 20 min and again at 15,000 × *g* for 10 min. The supernatant was stored at –20 °C for determining the activities of antioxidant enzymes.

2.7.1. Superoxide dismutase (SOD)

The protocol described by Giannopolitis and Ries (1977) was used for the determination of SOD activity. It was determined as the ability of the enzyme to inhibit photochemical reduction of nitroblue tetrazolium (NBT). The 3 mL of reaction mixture consisted of 50 mM phosphate buffer of pH 7.8, distilled water, methionine 13 mM, 50 µM of NBT, 50 µL of enzyme extract, and 1.3 µM of riboflavin. The reaction solutions were then kept under light (15-W fluorescent lamps) for 15 min at 78 µmol m^{-2} s^{-1}. The absorbance of the reaction mixture was read at 560 nm with a UV-visible spectrophotometer (U2020 IRMECO). One unit activity of SOD was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT photoreduction, as compared to the sample that lacked the plant enzyme extract.

2.7.2. Activities of catalase (CAT) and peroxidase (POD)

The method described by Chance and Maehly (1955) was used to appraise the activities of CAT and POD on a protein amount basis. The reaction solution for the CAT contained phosphate buffer and H_2O_2 of 50 and 5.9 mM, respectively. The addition of 0.1 mL of enzyme extract to the reaction mixture initiated the reaction. After every 20 s the changes in the absorbance of the reaction mixture were observed at 240 nm. The reaction mixture for POD consisted of phosphate buffer, guaiacol, and H_2O_2 with molar values of 50, 20, and 40 mM, respectively, and 0.1 mL of the enzyme extract. At 470 nm, the absorbance was taken every 20 s. The enzyme activity was assessed on a protein basis, while 1 unit of CAT was considered equivalent to 0.01 units per minute change in absorbance, and 1 unit of POD was defined as 0.01 units per minute change in absorbance.

2.8. Total soluble proteins

Fresh leaves (0.5 g of each sample) were homogenized in 10 mL of 50 mM phosphate buffer and centrifuged at 6000 × *g* for 5 min at 4 °C. The extract was used for determining total soluble proteins following Bradford (1976).

2.9. Glycine betaine determination

Glycine betaine content in fresh leaf tissues was determined following Grieve and Grattan (1983). Fresh leaf tissue (0.5 g of each sample) was finely ground in 10 mL of distilled water. The homogenate was then filtered with Whatman no. 2 filter paper. To 1 mL of the above filtrate 1 mL of 2 N H_2SO_4 was added. Then to the 0.5 mL of the above mixture, 0.2 mL of KI_3 solution was added to an ice bath, and the mixture was cooled for 90 min at 0–4 °C. Afterwards, 2.8 mL of chilled distilled water and 6 mL of 1-2-dichloroethane were added to the sample mixture. Two distinct layers formed, and the absorbance of the colored layer was read at 365 nm using a spectrophotometer (U2020 IRMECO).

2.10. Leaf free proline

Free proline contents were determined from the leaf tissues according to the method of Bates et al. (1973). Fresh leaf (0.5 g of each sample) was properly homogenized in 10 mL of sulfosalicylic acid (w/v) solution, and the filtrate was derived using Whatman no. 2 filter paper. To the 2 mL of filtrate were added 2 mL each of acid ninhydrin and glacial acetic acid, and the mixture was heated at 100 °C in a water bath for 1 h. Then the reaction mixture was placed in an ice bath to terminate the reaction. To the reaction mixture

was added 4 mL of toluene and the mixture was vigorously vortexed for 15 s. The free proline was aspirated from the chromophore layer, kept at room temperature, and the absorbance was read at 520 nm on a spectrophotometer (U2020 IRMECO). The free proline contents in the leaf tissues were calculated using the following formula:

$$\mu\text{mol proline g}^{-1}\text{ fresh weight} = \frac{\mu\text{g proline mL}^{-1} \times \text{mL of toluene}/115.5}{\text{g of sample.}}$$

2.11. Total free amino acids

Leaf free proline was determined according to the method of Moore and Stein (1957). Fresh leaves (0.5 g of each sample) were homogenized in 10 mL of citrate buffer (pH 5.0). The mixture was centrifuged at 15,000 × *g* for 10 min. The extracted samples were further processed with ninhydrin solution prepared by dissolving 2 g of ninhydrin in 100 mL of distilled water. The optical density of the solution was read at 570 nm using a spectrophotometer (U2020 IRMECO).

2.12. Total phenolics

Total phenolic contents were determined following Julkenen-Titto (1985). Fresh leaves (100 mg of each sample) were homogenized in 2 mL of 80% acetone and centrifuged at 10,000 × *g* for 15 min. The supernatant was collected in a microfuge tube and stored at –20 °C. The extract (100 µL) was diluted with 2.0 mL of distilled water in a test tube, and 0.5 mL of Folin–Ciocalteau's phenol reagent was added to it. The mixture was shaken vigorously. To the above mixture was added 2.5 mL of 20% Na_2CO_3 solution, and the final volume was brought up to 5 mL using distilled H_2O. It was then vortexed for 5–10 s. The absorbance of the extracted samples was read at 750 nm after 20 min using a spectrophotometer (U2020 IRMECO). Total phenolic contents were calculated from the standard curve obtained from different concentrations of gallic acid (0, 2, 4, 6, 8, and 10 mg L^{-1}), and total phenolics were determined on a fresh weight basis.

2.13. Mineral nutrient determination

Mineral ions (Na^+, K^+, and Ca^{2+}) in shoot and root were determined by following Allen et al. (1985). Digestion mixture was prepared by properly mixing Se (0.42 g) and $LiSO_4.2H_2O_2$ (14 g) to H_2O_2 (350 mL), slowly adding conc. H_2SO_4 (420 mL) by keeping it in an ice bath, and storing it at 2 °C and using it for plant tissue (shoot and root) digestion. Then 100 mg of dried ground shoot and root material was digested in 2 mL of digestion mixture in a digestion flask at 200 °C on a hotplate. Perchloric acid ($HClO_4$) was used to complete the digestion process. The digested mixture was diluted with distilled water up to 50 mL, filtered, and the filtrate was used for Na^+, K^+, and Ca^{2+} ion determination with the help of a flame photometer (Jenway, PFP-7).

2.14. Chloride (Cl^-) determination

For Cl^- determination 100 mg of dried ground shoot or root material was taken in a test tube, and 10 mL of distilled water was added to it. Then the material was extracted by placing the test tubes in test tube stands in an oven at 80 °C for 6 h. The concentration of Cl^- was determined with a chloride analyzer (Model 926, Sherwood Scientific Ltd., Cambridge, UK).

2.15. Statistical analysis

Four-way analysis of variance (ANOVA) of data for all attributes was performed using the MSTAT computer program (MSTAT Development Team, 1989). The least significant difference was used to compare the mean values of all treatments (Snedecor and Cochran, 1980).

3. Results

3.1. Effects on shoot and root dry weights

Root-medium–applied salinity of 150 mM (NaCl) significantly decreased shoot and root dry weights in both cultivars (Figures 1A and B). Cultivar S-24 was superior to MH-97 in shoot dry weight, while the reverse was true for root dry weights under saline stress. Foliar-applied TRIA at various growth stages markedly enhanced shoot and root dry weights of both wheat cultivars under both normal and salt-stressed conditions. A TRIA level of 20 µM was effective for shoot dry weight and 10 µM TRIA was effective for root dry weight in both cultivars under salt-stressed and nonstressed conditions. Overall, TRIA application at the vegetative + boot stage was more effective for enhancing growth of cv. S-24 under nonstress conditions, while this was true for cv. MH-97 at vegetative or boot growth stages (Figures 1A and B).

3.2. Effect on total leaf area per plant

Total leaf area per plant decreased markedly in both wheat cultivars under saline conditions. The cultivars did not differ significantly in this attribute (Figure 1C). Foliar-applied TRIA at various growth stages significantly enhanced total leaf area per plant in both cultivars under both saline and nonsaline conditions. Foliar-applied 20 µM TRIA was more effective when applied at both vegetative and boot stages for both wheat cultivars, while 10 µM TRIA was more effective when applied at the vegetative or boot stage in wheat plants, particularly under nonsaline conditions. Overall, TRIA application at the boot or vegetative + boot stages was more effective in increasing total leaf area per plant in both wheat cultivars, particularly under nonsaline conditions (Figure 1C).

3.3. Effects on yield attributes

Yield attributes, i.e. grain yield per plant (Figure 1D), number of grains per plant (Figure 2A), and 100-seed weight (Figure 2B), significantly decreased in both wheat cultivars under salinity stress. Cultivar difference among

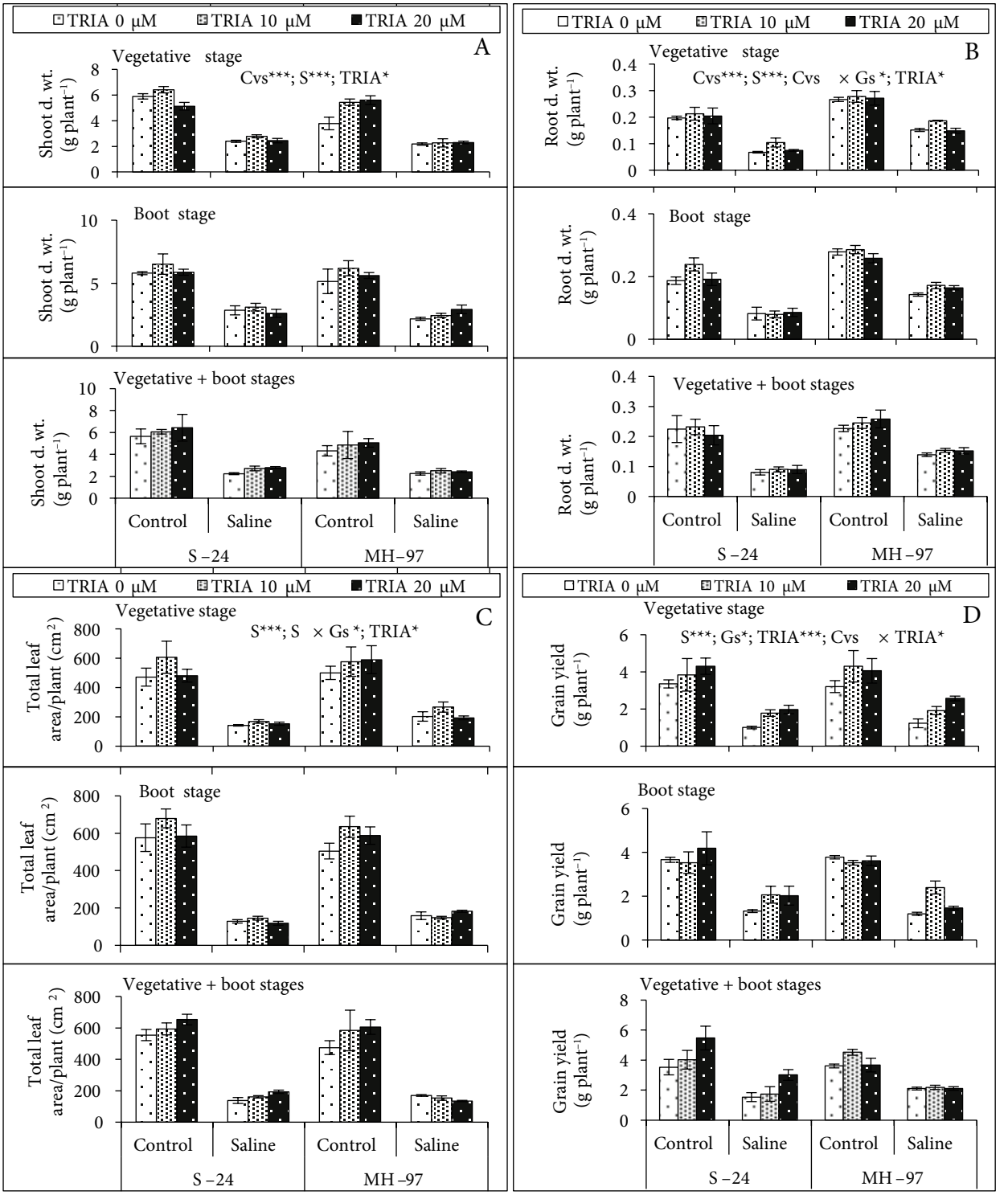

Figure 1. Shoot and root dry weights, total leaf area, and grain yield per plant of *Triticum aestivum* plants foliarly sprayed with TRIA under nonstress and salt-stress conditions at different growth stages. Cvs = cultivars, S = salinity, Gs = growth stages. *, **, and *** = significant at 0.05, 0.01, and 0.001 levels, respectively. Bars in graphs represent standard errors.

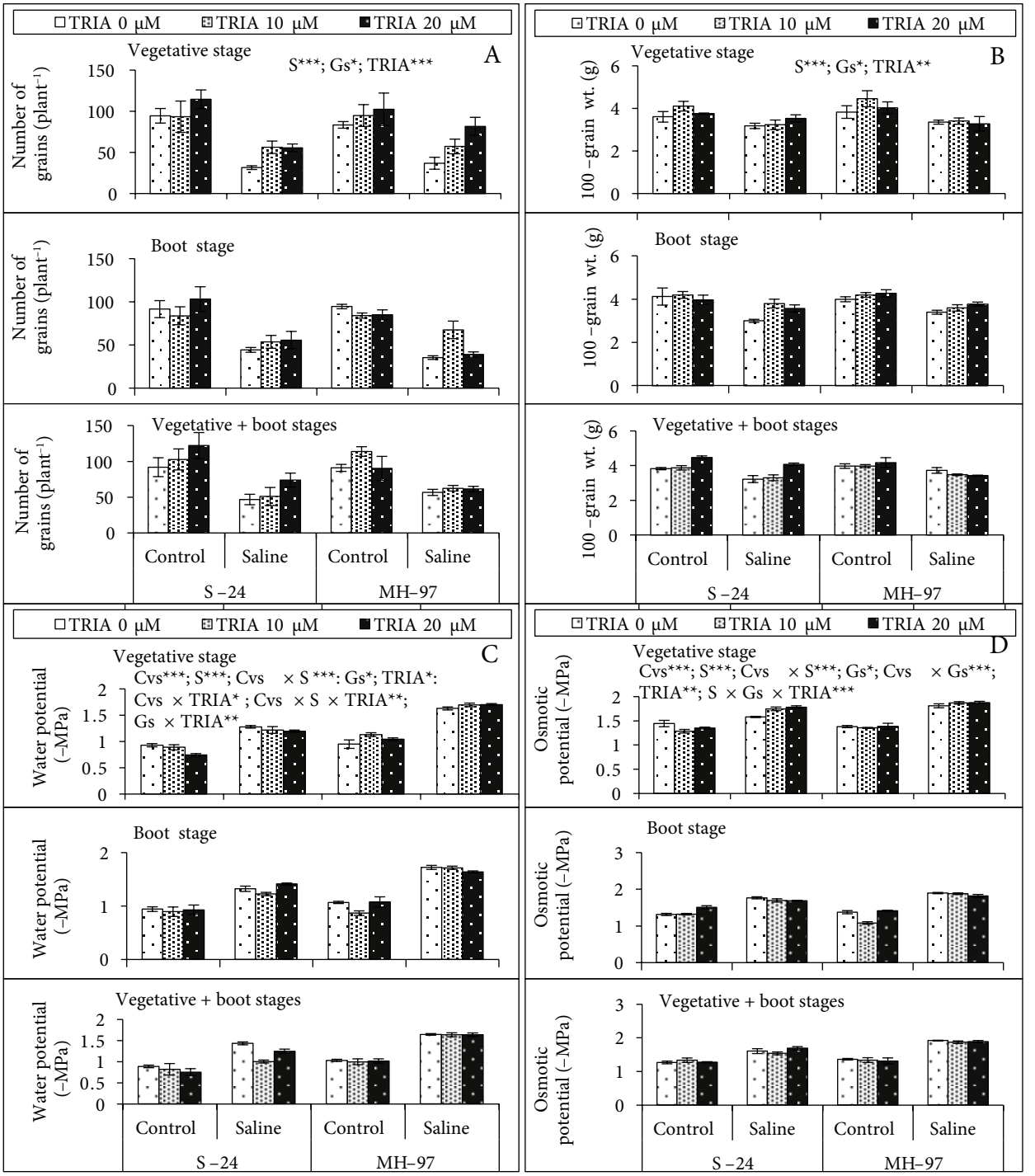

Figure 2. Number of grains per plant, 100-grain weight, and leaf water and osmotic potential of *Triticum aestivum* plants foliarly sprayed with TRIA under nonstress and salt-stress conditions at different growth stages.
Cvs = cultivars, S = salinity, Gs = growth stages. *, **, and *** = significant at 0.05, 0.01, and 0.001 levels, respectively. Bars in graphs represent standard errors.

these attributes was nonsignificant. Foliar application of TRIA at different growth stages markedly increased grain yield per plant, number of grains per plant, and 100-seed weight in both cultivars under salt-stress and nonstress conditions. Overall, TRIA application at the vegetative + boot stages was more effective in enhancing yield attributes in the salt-stressed and nonstressed plants of both cultivars as compared to TRIA application at the other growth stages.

3.4. Effects on leaf water relations

Leaf water and osmotic potentials significantly decreased in both wheat cultivars under NaCl-induced stress (Figures 2C and D). Leaf water and osmotic potentials were more negative in cv. MH-97 than in cv. S-24 under both saline and nonsaline conditions. Foliar application of TRIA significantly increased leaf water potential in both cultivars under nonsaline and saline conditions; however, the response of the cultivars to foliar-applied TRIA was variable at different growth stages, because at the vegetative growth stage leaf water potential was higher in cv. S-24 and lower in MH-97 under both salt-stress and nonstress conditions. Leaf osmotic potential slightly decreased under TRIA application at the vegetative stage, while it increased under TRIA application at the boot stage in both cultivars under salt-stress conditions.

The root-medium–applied salinity stress significantly decreased leaf turgor potential in both wheat cultivars (Figure 3A). S-24 exceeded MH-97 in leaf turgor potential under both saline and nonsaline conditions. The response of cv. S-24 to TRIA application was more positive than that of cv. MH-97 in terms of leaf turgor potential under both nonsaline and saline conditions. Increase in leaf turgor potential with increase in foliar-applied TRIA levels applied at different growth stages was consistent only in cv. S-24 under both nonstress and stress conditions. Overall, leaf turgor potential of cv. S-24 increased when TRIA was applied at the vegetative stage under both salt-stress and nonstress conditions.

Relative water content (%) significantly decreased in both wheat cultivars under saline stress, while the cultivars did not differ significantly in this attribute (Figure 3B). Foliar application of TRIA significantly increased relative water content (%) in both wheat cultivars under both nonsaline and saline conditions. Relative water content (%) was higher when TRIA was sprayed at the vegetative stage than when it was applied at the other 2 stages. Cultivar MH-97 was higher in this attribute than cv. S-24.

3.5. Effect on hydrogen peroxide (H_2O_2) content

Hydrogen peroxide (H_2O_2) significantly increased in both wheat cultivars under salt stress. Cultivar MH-97 accumulated higher H_2O_2 content than cv. S-24 under saline conditions (Figure 3C). Foliar application of TRIA reduced H_2O_2 content in the salt-stressed and nonstressed plants of both wheat cultivars when applied at various growth stages. Furthermore, H_2O_2 content decreased consistently in the salt-stressed plants of cv. MH-97 with an increase in the level of TRIA applied at the vegetative and boot stages. Overall, TRIA applied at various growth stages did not show a marked difference in H_2O_2 contents.

3.6. Effect on malondialdehyde (MDA) content

The wheat cultivars showed nonsignificant differences in malondialdehyde (MDA) content under nonsaline conditions, while content significantly increased in both wheat cultivars under saline conditions (Figure 3D). Foliar application of TRIA significantly decreased the MDA content in both wheat cultivars, and 10 μM TRIA at all growth stages was more effective in lowering MDA content in the wheat plants under both nonsaline and saline conditions.

3.7. Effect on total soluble protein content

Soluble proteins increased significantly in both cultivars under saline conditions (Figure 4A). A marked variation between the 2 cultivars was also observed. Overall, cultivar S-24 exceeded cv. MH-97 in soluble proteins, but this difference was more prominent under nonsaline conditions as compared to saline conditions. Exogenous application of TRIA did not affect soluble proteins in either cultivar.

3.8. Effects on antioxidant enzyme activity

The activity of superoxide dismutase (SOD) enzyme in both wheat cultivars showed a prominent decrease under NaCl stress (Figure 4B). The behavior of both wheat cultivars with respect to this biochemical attribute was markedly different under saline or nonsaline conditions. Exogenous application of TRIA as a foliar spray did not alter SOD activity in either cultivar under nonsaline or saline conditions.

The activity of peroxidase (POD) was not influenced by salt stress in either wheat cultivar. However, the cultivars differed significantly in POD activity under saline and nonsaline conditions. Cultivar S-24 showed higher POD activity than MH-97 under saline conditions (Figure 4C). Foliar application of TRIA significantly increased the POD activity in both wheat cultivars. A consistent increase in POD activity in both cultivars with an increasing level of TRIA was observed when TRIA was applied at the vegetative stage under both saline and nonsaline conditions. TRIA application at the boot or vegetative + boot stages caused a significant decrease in POD activity, particularly in cv. MH-97 under salt stress.

Salinity stress significantly increased catalase (CAT) activity in both wheat cultivars. The effect of foliar-applied TRIA on CAT activity was also nonsignificant; however, there was differential behavior between cultivars when TRIA was applied at the vegetative + boot stages, and CAT

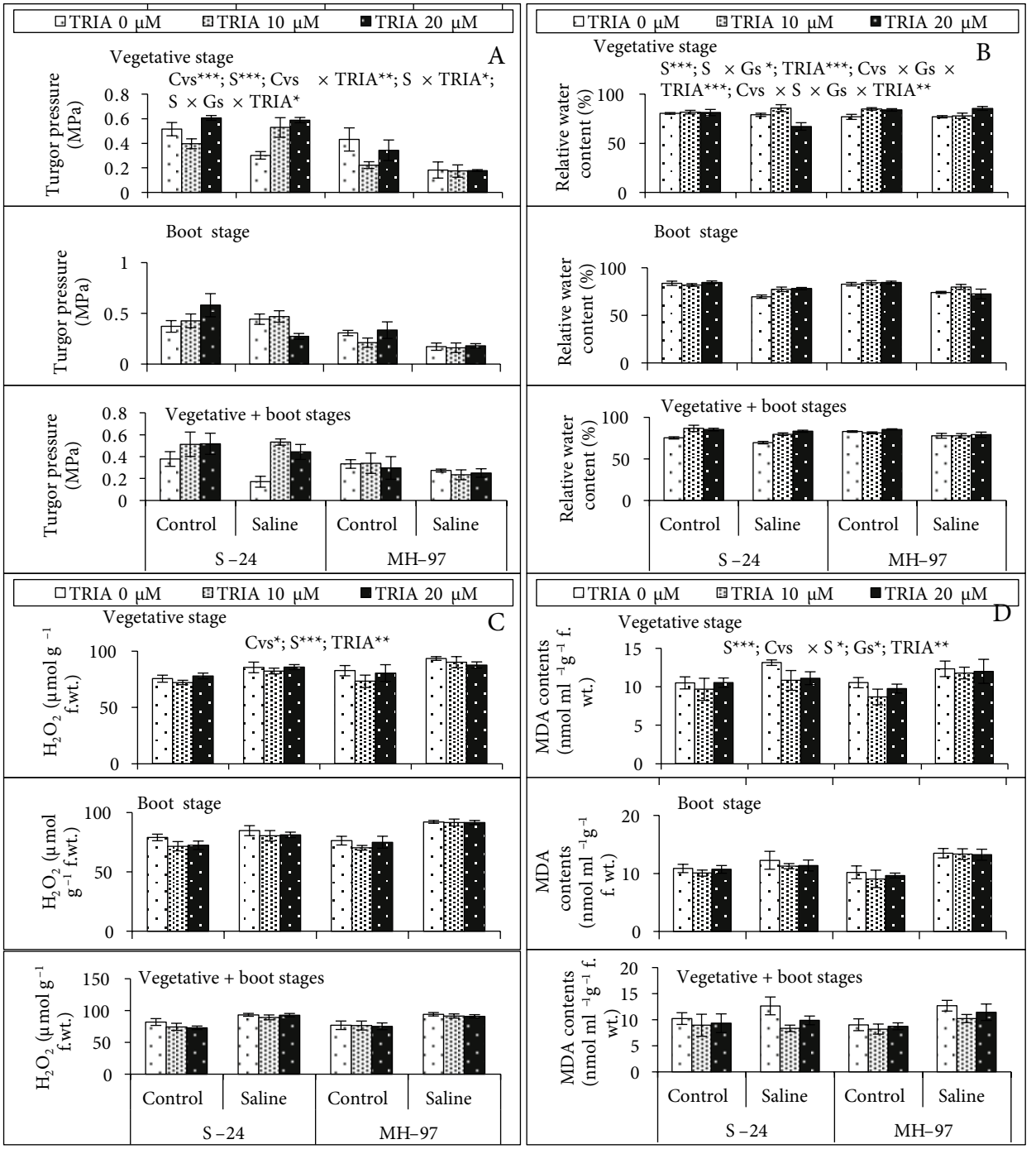

Figure 3. Leaf turgor potential, relative water content (%), hydrogen peroxide, and malondialdehyde contents of *Triticum aestivum* plants foliarly sprayed with TRIA under nonstress and salt-stress conditions at different growth stages.
Cvs = cultivars, S = salinity, Gs = growth stages. *, **, and *** = significant at 0.05, 0.01, and 0.001 levels, respectively. Bars in graphs represent standard errors.

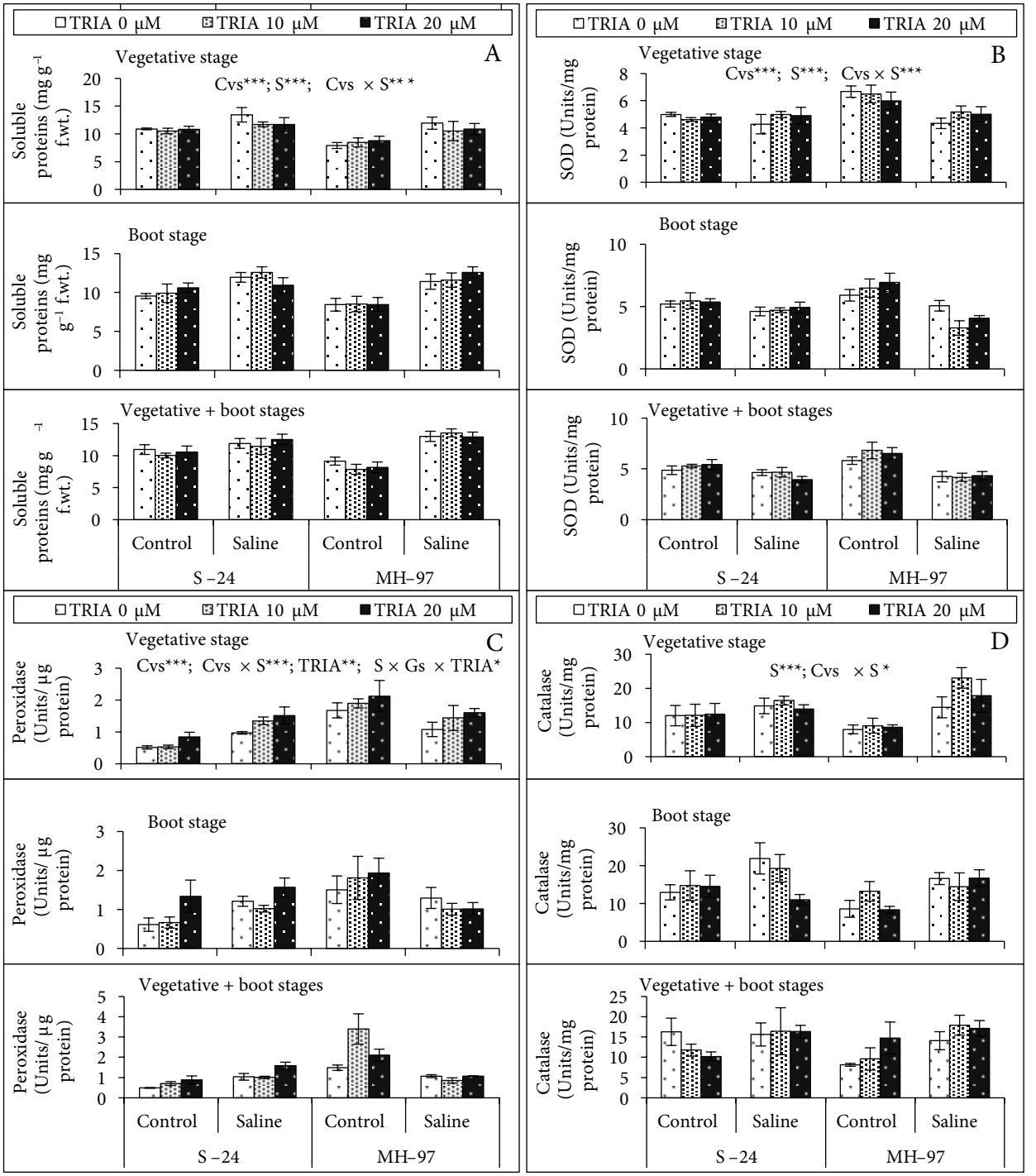

Figure 4. Soluble proteins and activity of superoxide dismutase, peroxidase, and catalase enzyme of *Triticum aestivum* plants foliarly sprayed with TRIA under nonstress and salt-stress conditions at different growth stages.
Cvs = cultivars, S = salinity, Gs = growth stages. *, **, and *** = significant at 0.05, 0.01, and 0.001 levels, respectively. Bars in graphs represent standard errors.

activity decreased significantly in cv. S-24 and increased in cv. MH-97 under nonsaline conditions (Figure 4D).

3.9. Effect on total phenolic content

Salt stress significantly increased total phenolic content in cv. S-24, while the reverse was true in cv. MH-97. The cultivars differed prominently in this attribute as salt stress increased total phenolics in cv. S-24 under TRIA application at all growth stages (Figure 5A). Furthermore, under salt stress, total phenolic contents rose when TRIA was applied at the vegetative growth stage. Foliar application of TRIA at the boot or vegetative + boot stages significantly decreased total phenolic contents in the salt-stressed plants of both cultivars. Foliar-applied 10 μM TRIA was more effective at decreasing total phenolics in both cultivars under saline conditions. However, this reduction in total phenolic content was greater in the salt-stressed plants of cv. MH-97 than in cv. S-24, particularly under TRIA application at the boot or vegetative + boot stages.

3.10. Effect on total free amino acid content

Total free amino acid content increased significantly in both cultivars under NaCl stress. Cultivar S-24 had significantly higher total free amino acids than cv. MH-97 under saline conditions as well as under TRIA application (Figure 5B). Overall, the effect of TRIA application at different growth stages on total free amino acid contents was uniform in both wheat cultivars.

3.11. Effect on leaf free proline content

Leaf free proline content increased significantly in both cultivars under NaCl stress (Figure 5C). The effect of foliar application of TRIA at different growth stages was statistically nonsignificant on leaf proline content in both cultivars.

3.12. Effect on glycine betaine content

Salt stress markedly increased the glycine betaine content in both wheat cultivars, but the cultivars did not differ significantly in this biochemical attribute (Figure 5D). The effect of foliar-applied TRIA at various growth stages was also nonsignificant on glycine betaine content in both wheat cultivars.

3.13. Effects on shoot and root mineral ion content

Shoot and root Na^+ content increased significantly in both wheat cultivars under saline conditions (Figures 6A and B). Foliar-applied TRIA at various growth stages slightly reduced shoot Na^+ content in both cultivars under saline conditions. Overall, the effect of TRIA was cultivar-specific, as 20 μM TRIA proved more effective in reducing shoot Na^+ in cv. S-24 under saline conditions and 10 μM TRIA was more effective in cv. MH-97. Comparison of various growth stages with applied TRIA showed that shoot Na^+ content decreased more sharply when applied at the boot stage under salt stress in both wheat cultivars (Figure 6A).

Shoot and root K^+ ions decreased prominently in both wheat cultivars under salinity stress. Cultivar S-24 was higher in shoot K^+ (Figure 6C) content than cv. MH-97 under both saline and nonsaline conditions, while in the roots K^+ (Figure 6D) content was higher only under nonsaline conditions. The exogenous application of TRIA at various growth stages increased shoot K^+ content in both cultivars, while it did not modulate root K^+ content under saline or nonsaline stress.

Root-medium–applied salinity stress decreased shoot and root Ca^{2+} content prominently in both wheat cultivars (Figures 7A, B). Of the 2 cultivars, S-24 was higher in shoot and root Ca^{2+} content than MH-97. Foliar-applied TRIA at various growth stages markedly increased the shoot and root Ca^{2+} content in both cultivars under both nonsaline and saline conditions. TRIA application at all growth stages showed almost uniform behavior. However, 10 μM TRIA was more effective for both cultivars under nonsaline stress, while a consistent increase in shoot Ca^{2+} content was observed at vegetative and vegetative + boot stages in cv. S-24 under saline stress.

Salinity stress caused a remarkable increase in shoot and root Cl^- content in both wheat cultivars (Figures 7C and D). The response of the 2 wheat cultivars in terms of shoot and root Cl^- content was markedly variable. Cultivar S-24 accumulated more shoot and root Cl^- content than cv. MH-97 under both salt-stressed and nonstressed conditions. Cultivar response to foliar application of TRIA in terms of shoot Cl^- content was also variable under saline or nonsaline conditions, and shoot Cl^- content increased slightly with increasing TRIA levels at all growth stages under nonsaline conditions and decreased under saline conditions in both cultivars, except at the boot stage in cv. MH-97.

4. Discussion

Salt stress, which is partially associated with hormonal imbalance, drastically reduced plant growth (Babu et al., 2012). However, exogenous application of plant growth regulators can overcome the negative effects of salt stress (Eleiwa et al., 2011). Triacontanol is known as a potential plant growth regulator like many other known growth regulators (Naeem et al., 2011). Various physiological and biochemical processes have been regulated by foliar application of TRIA under saline stress (Perveen et al., 2013).

Under saline conditions plants are unable to take up water in the presence of excess salts in the soil solution, which results in reduced growth and disorder in various metabolic processes (Munns and Tester, 2008; Tavakkoli et al., 2010). In root cells, Na^+ is readily absorbed due to its small size and is transported to all plant tissues resulting

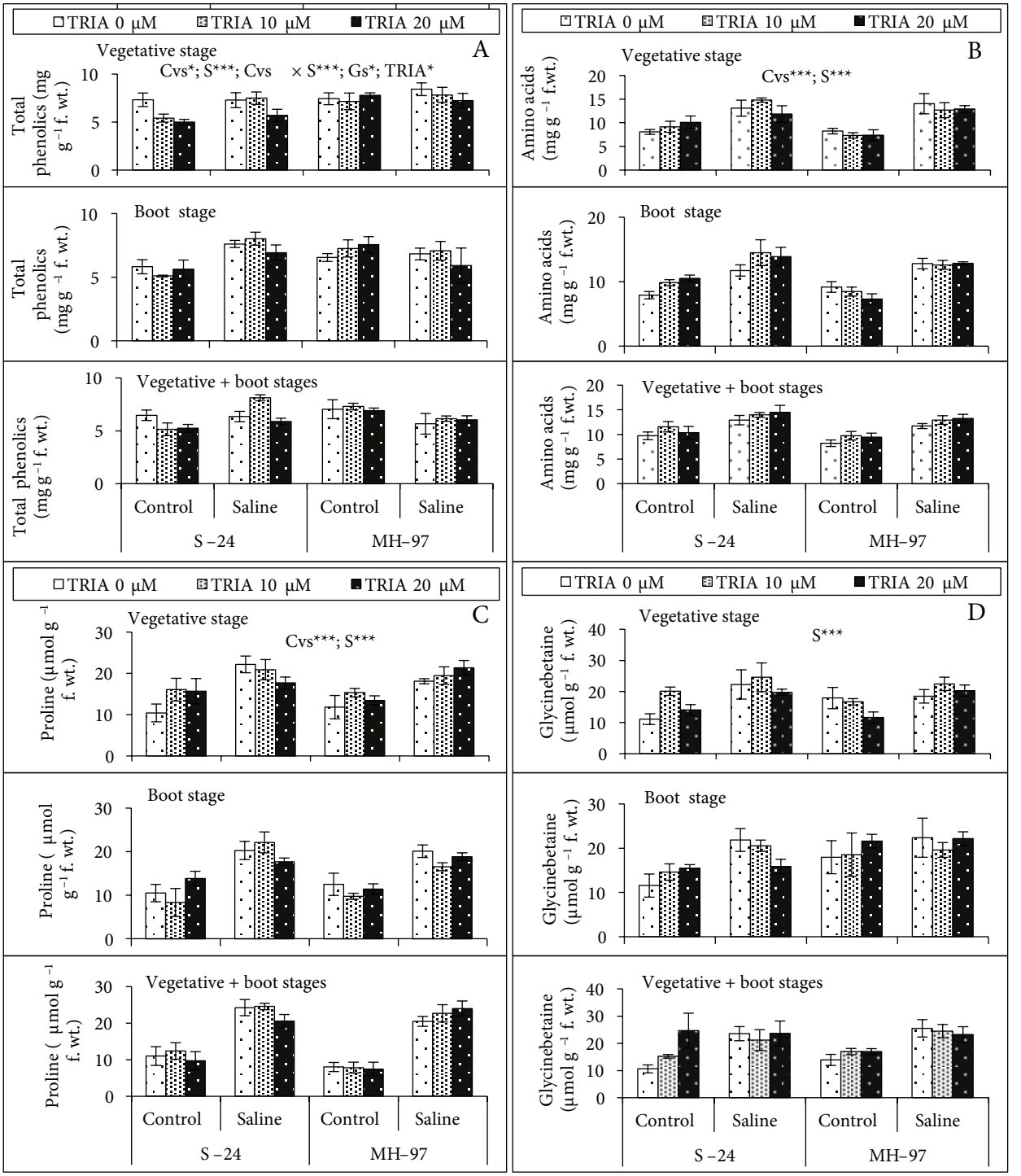

Figure 5. Total free amino acids and total phenolics, proline, and glycine betaine contents of *Triticum aestivum* plants foliarly sprayed with TRIA under nonstress and salt-stress conditions at different growth stages.
Cvs = cultivars, S = salinity, Gs = growth stages. *, **, and *** = significant at 0.05, 0.01, and 0.001 levels, respectively. Bars in graphs represent standard errors.

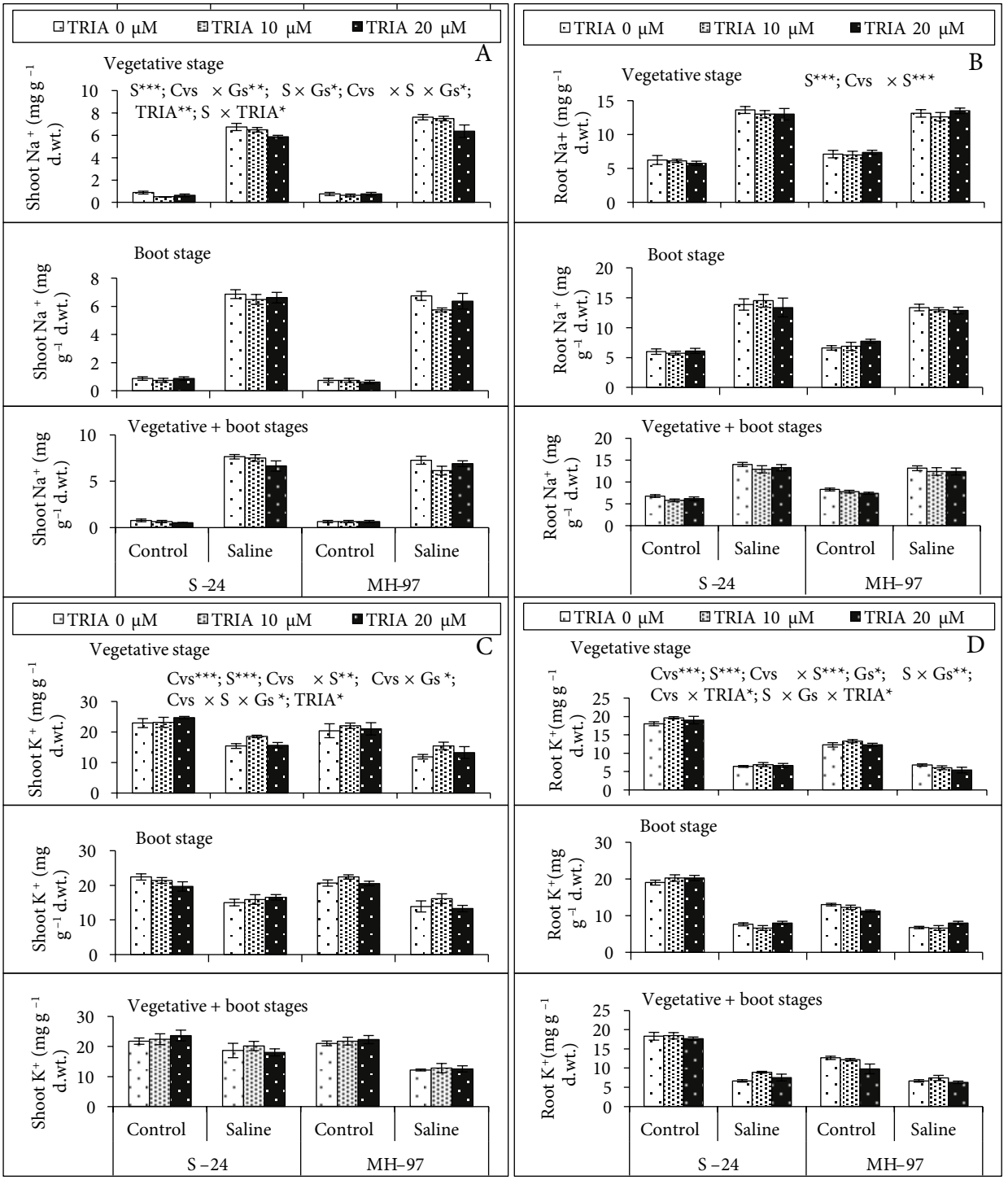

Figure 6. Shoot and root Na^+ and K^+ contents of *Triticum aestivum* plants foliarly sprayed with TRIA under nonstress and salt-stress conditions at different growth stages.

Cvs = cultivars, S = salinity, Gs = growth stages. *, **, and *** = significant at 0.05, 0.01, and 0.001 levels, respectively. Bars in graphs represent standard errors.

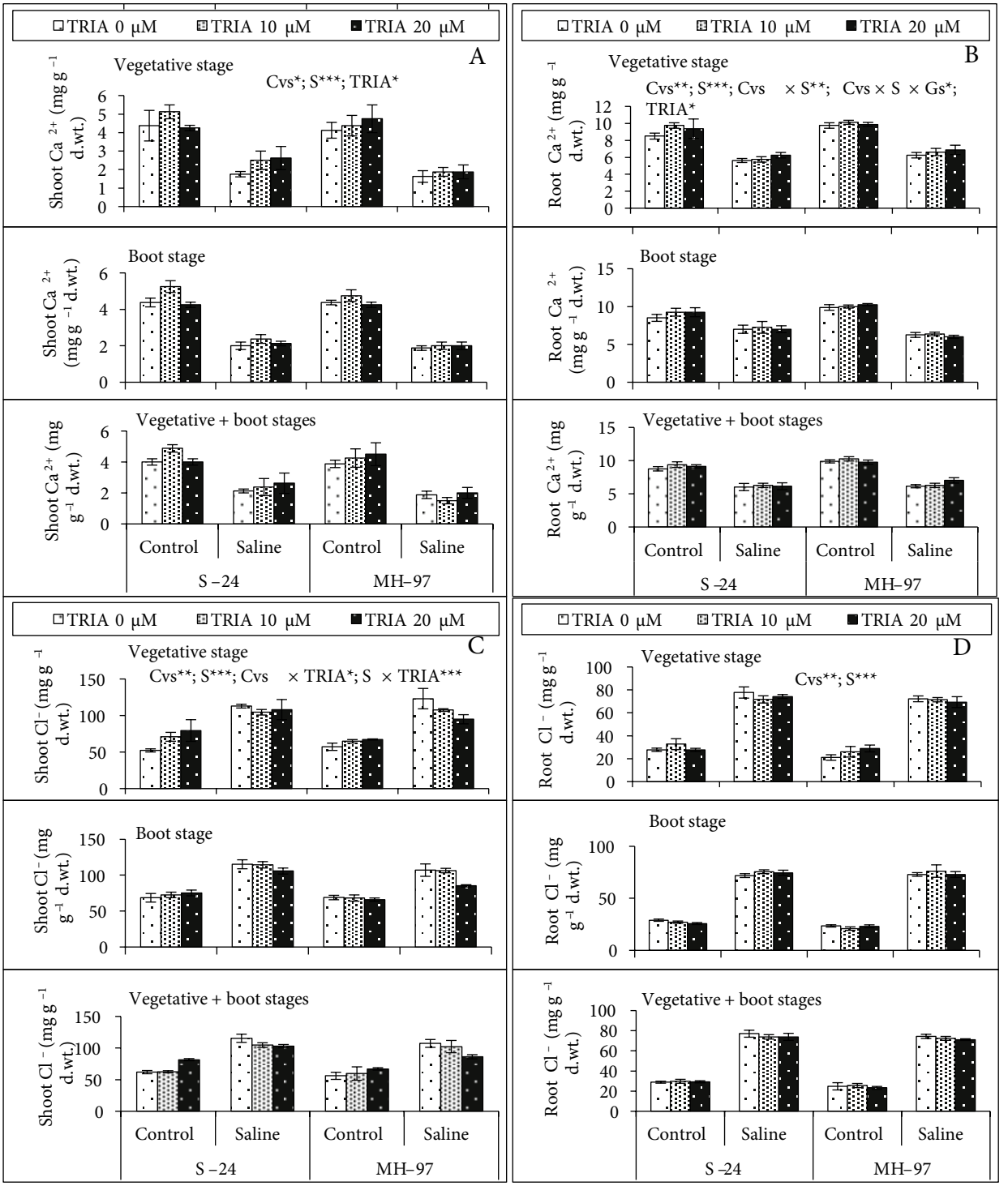

Figure 7. Shoot and root Ca^{2+} and Cl^- contents of *Triticum aestivum* plants foliarly sprayed with TRIA under nonstress and salt-stress conditions at different growth stages.

Cvs = cultivars, S = salinity, Gs = growth stages. *, **, and *** = significant at 0.05, 0.01, and 0.001 levels, respectively. Bars in graphs represent standard errors.

in ion toxicity, osmotic stress, and nutrient imbalance (Siringam et al., 2011). Shoot dry matter, leaf area, plant height, relative water content, and chlorophyll all decrease with increasing accumulation of Na^+ and Cl^- (Bybordi, 2010; Hossain et al., 2011). In addition, Na^+ and Cl^- ion toxicity leads to reduced growth and yield by altering nonstomatal factors' effect like chlorophyll degradation and impaired chlorophyll fluorescence (Nemati, 2011; Tavakkoli et al., 2011).

In the present study, foliar-applied TRIA at different growth stages significantly enhanced dry biomass (shoot and root dry weights), total leaf area per plant, and yield attributes of the salt- stressed and nonstressed plants of both wheat cultivars, particularly when TRIA was applied at the vegetative + boot stages. It has also been reported that TRIA can exert growth-stimulatory effects equally well at different growth stages (Singh et al., 2011). For example, foliar application of TRIA at early vegetative stage and at anthesis was reported to increase the growth and yield of most economic crops including wheat (Ries, 1991). In another study, foliar spray of TRIA applied 3 times at various growth stages, i.e. vegetative, flowering, and podding, was more effective in enhancing crop growth and yield in ginger (Singh et al., 2011). Furthermore, Singh et al. (1991) was of the view that at the preflowering stage TRIA is more effective at increasing seed weight, seed yield, and protein content in chickpea. In addition, TRIA can interact with other growth hormones such as cytokinins and gibberellic acid to regulate growth, yield, and metabolic processes in plants (Aftab et al., 2010). TRIA is known to induce the formation of second messenger 9-β-L (+) adenosine, which is similar in structure to cytokinins (Bonhomme et al., 2000; He and Loh, 2000). TRIA applied foliarly along with gibberellic acid at different growth stages enhanced the translocation of assimilates for pod filling in groundnut, resulting in increased yield and yield-related parameters such as pod yield, pod weight, and number of pods per plant (Verma et al., 2009).

TRIA is known to play an important role in water uptake, cell elongation, increasing cell division, and permeability of membranes (Hangarter et al., 1978). In the current study, root-medium–applied salinity stress significantly decreased leaf water relations in both wheat cultivars. However, foliar application of TRIA at different growth stages improved leaf water relations in both wheat cultivars under saline and nonsaline conditions. Krishnan and Kumari (2008) also reported an increase in relative water content and a decrease in leaf osmotic potential in TRIA-treated, salt-stressed soybean plants. TRIA-induced increase in growth could be due to maintenance of water homeostasis (water relations), which again depends on increased uptake of water, essential nutrients, and synthesis/accumulation of organic compounds by enhanced photosynthesis under salt stress. In wheat, increased levels of toxic Na^+ and Cl^- ions cause oxidative stress, which, in turn, causes membrane damage in various cytoplasmic organelles (Shabala et al., 2012). Membrane lipid peroxidation results in malondialdehyde (MDA) accumulation, which is an indication of membrane damage at the cellular level under salt stress (Weisamy et al., 2012). Peroxidation of membrane lipids occurs due to reactive oxygen species or lipooxygenases (Janmohammadi et al., 2012). In the present investigation, salinity stress increased oxidative stress in both wheat cultivars but to a greater degree in salt-sensitive cv. MH-97 than in salt-tolerant cv. S-24; however, foliar-applied TRIA at different growth stages decreased oxidative stress–induced membrane damage, which is apparent from the stress-induced reduction in MDA content (a product of lipid peroxidation) and H_2O_2 (most stable ROS in plants) in both wheat cultivars. TRIA inhibited lipid peroxidation in spinach (*Spinacea oleracea* L.) (Ramanarayan et al., 2000) and *Arachis hypogaea* L. (Verma et al., 2011) leaves and improved membrane integrity by differentially modulating membrane lipid composition (Swamy et al., 2009). In the present study, TRIA-induced improvement in growth might have been due to its effect on the performance of antioxidant enzymes like POD under salt stress (Perveen et al., 2011; Ertani et al., 2012). Increased POD activity may have taken part in the detoxification of ROS (H_2O_2 in this case), leading to a balance between ROS generation and ROS scavenging, thereby mitigating the adverse effects of salt stress on wheat plants.

Plants overcome salinity-induced osmotic effects through accumulation of inorganic or organic osmolytes/solutes such as sodium, potassium, and chloride; free proline; glycine betaine; and free amino acids by a process known as osmotic adjustment (Munns, 2005; Zhu et al., 2011) in response to decreased external water potential (Farouk, 2011). In this study, salinity stress increased total free amino acids, free proline, glycine betaine, and soluble protein content in both wheat cultivars. Foliar-applied TRIA did not alter total free amino acids, free proline, glycine betaine, or soluble protein content significantly when applied to the wheat plants at various growth stages in this study. However, increased protein content under TRIA treatment was reported by Verma et al. (2011), Kumaravelu et al. (2000), and Muthuchelian et al. (2003) in different plant species. Krishnan and Kumari (2008) reported a decrease in proline and an increase in protein content in salt-stressed soybean plants treated with triacontanol. There is no clear evidence in the literature regarding the effect of TRIA on GB accumulation in plants under stress or nonstress conditions. Phenolics act as potential antioxidant compounds that play a role in scavenging singlet oxygen ($^1O_2$) (Rice-Evans et al., 1997);

however, under salt stress the level of total phenolics usually alters depending upon the sensitivity of a plant species to salt stress (Giorgi et al., 2009). In the present investigation, salinity stress increased total phenolic content in both wheat cultivars. However, foliar-applied TRIA at various growth stages decreased total phenolic content in both cultivars and to a greater degree in the salt-sensitive wheat cultivar. This study is in agreement with Ertani et al. (2012), who showed that a TRIA-based biostimulant decreased phenolic content in salt-stressed maize plants.

Triacontanol regulates different physiological and biochemical processes including the uptake and use efficiency of different mineral ions under both normal and salt-stress conditions (Ertani et al., 2012; Perveen et al., 2012). In addition, TRIA can interact with other growth hormones such as cytokinins and gibberellic acid for regulation of growth, yield, and metabolic processes (Aftab et al., 2010). For example, TRIA induces the formation of second messenger 9-β-L (+) adenosine, which is similar in structure to cytokinins, and exogenous application of cytokinins is known to induce growth (Bonhomme et al., 2000). Treatment with TRIA increases L(+)-adenosine levels (Ries and Wert, 1988), and picomole concentrations of (+)-adenosine can enhance Ca^{2+}, Mg^{2+}, and K^+ concentrations (Ries et al., 1993). However, optimal concentrations of TRIA and plant age are among the important factors that control the growth and final yield of various plant species (Sagaral et al., 1978).

As the physiochemical properties of Na^+ and K^+ are similar, uptake of K^+ is affected by high Na^+ levels (Hossain et al., 2011), which lead to reduced potassium and Ca^{2+} uptake (Bavei et al., 2011; Tavakkoli et al., 2011). In the current study shoot and root K^+ and Ca^{2+} contents were drastically reduced under salinity stress in both wheat cultivars. Shoot and root K^+ and Ca^{2+} ions increased by exogenous foliar application of TRIA at all growth stages. Interference in essential nutrient uptake, i.e. an increase in Na^+ and Cl^- content and decrease in K^+ and Ca^{2+} ions and K^+/Na^+ ratios under NaCl stress have been reported in crops such as wheat (Shafi et al., 2010), barley (Tavakkoli et al., 2011), and maize (Turan et al., 2010). Salt-tolerant genotypes possess higher K^+ uptake due to selective absorption of K^+ rather than Na^+ (Perveen et al., 2012). Foliar application of TRIA stimulates the influx of Ca^{2+} into the cytoplasm (Ries et al., 1993), which could bind to receptor proteins such as calmodulin (Evans et al., 1991), while increased uptake of K^+ could be due to increased competition at the plasma membrane sites (Epstein, 1966) that regulate growth processes in the face of certain external stimuli (Ries et al., 1993). Overall, TRIA application at vegetative + boot stages proved more effective at increasing shoot and root K^+ and Ca^{2+} contents in both wheat cultivars, particularly under salt-stress conditions. Our findings are in accordance with Srivastava and Sharma (1990), who reported that foliar spray of TRIA at different growth stages increased shoot nutrient content. Under salt stress K^+ and Ca^{2+} content increased under foliar application of TRIA in soybean as well (Krishnan and Kumari, 2008), while a nonsignificant effect of TRIA was observed by Naeem et al. (2009). In our study, foliar-applied TRIA at various growth stages also enhanced shoot and root K^+/Na^+ ratios, particularly in salt-tolerant cv. S-24 at the vegetative stage and in both cultivars at the vegetative + boot stage. The possible mechanism of TRIA-induced alteration is a TRIA-mediated increase in membrane-bound enzyme activities, e.g., Ca^{2+}/Mg^{2+} ATPases (Lesniak et al., 1986); fluidity of membranes to several solutes by generation of an electrochemical gradient across plasma membranes; and increased uptake of the essential nutrients Ca^{2+}, Mg^{2+}, and K^+ (Ries, 1991; Ries et al., 1993). Furthermore, exogenous foliar-applied TRIA at various growth stages also reduced the accumulation of shoot Na^+ and shoot and root Cl^- in both wheat cultivars under saline conditions.

In conclusion, foliar application of TRIA ameliorated the adverse effects of salt stress on growth, yield, and leaf water relations by enhancing shoot and root dry biomass and antioxidant defense (increased POD activity) and decreasing oxidative stress (MDA and H_2O_2 content) and total phenolic content in both wheat cultivars under saline conditions. The TRIA-induced improvement in plant biomass may be due to high accumulation of shoot and root K^+ and Ca^{2+} contents and low accumulation of Na^+ and Cl^- contents. Overall, foliar application of TRIA at the vegetative as well as vegetative + boot stages was effective for increasing growth, yield, and leaf water relations in salt-stressed and nonstressed plants of both cultivars. Salt-sensitive cultivar MH-97 showed a more positive response in terms of growth and yield to 10 μM TRIA, while cv. S-24 responded more favorably to 20 μM in yield attributes under both control and NaCl stress. Salt-tolerant cultivar S-24 was higher in leaf water relations, total soluble proteins, free amino acids, proline, shoot Ca^{2+}, shoot and root K^+ content, and POD and SOD activities only under saline conditions, while salt sensitive cultivar MH-97 showed higher values for leaf osmotic potential, H_2O_2, and total phenolic content under both nonsaline and saline conditions.

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