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RESEARCH ARTICLE

Tebuconazole and trifloxystrobin regulate the physiology, antioxidant defense and methylglyoxal detoxification systems in conferring salt stress tolerance in *Triticum aestivum* L.

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Abstract Fungicides are widely used for controlling fungi in crop plants. However, their roles in conferring abiotic stress tolerance are still elusive. In this study, the effect of tebuconazole (TEB) and trifloxystrobin (TRI) on wheat seedlings (*Triticum aestivum* L. cv. Norin 61) was investigated under salt stress. Seedlings were pre-treated for 48 h with fungicide (1.375 µM TEB + 0.5 µM TRI) and then subjected to salt stress (250 mM NaCl) for 5 days. Salt treatment alone resulted in oxidative damage and increased lipid peroxidation as evident by higher malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content. Salt stress also decreased the chlorophyll and relative water content and increased the proline (Pro) content. Furthermore, salt stress increased the dehydroascorbate (DHA) and glutathione disulfide (GSSG) content while ascorbate (AsA), the AsA/DHA ratio, reduced glutathione (GSH) and the GSH/GSSG ratio decreased. However, a combined application of TEB and TRI significantly alleviated growth inhibition, photosynthetic pigments and leaf water status improved under salt stress. Application of TEB and TRI also decreased MDA, electrolyte leakage, and H_2O_2 content by modulating the contents of AsA and GSH, and enzymatic antioxidant activities. In addition, TEB and TRI regulated K^+/Na^+ homeostasis by improving the K^+/Na^+ ratio under salt stress. These results suggested that exogenous application of TEB and TRI rendered the wheat seedling more tolerant to salinity stress by controlling ROS and methylglyoxal (MG) production through the regulation of the antioxidant defense and MG detoxification systems.

Keywords Fungicides · Reactive oxygen species · Glyoxalase system · Antioxidant defense · Salt stress

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Introduction

In agriculture, salinity may cause serious problems in regions where crops are irrigated with saline water (Daliakopoulos et al. 2016). Salinity results in osmotic imbalance, by affecting the solutes of vascular transportation and electrochemical gradients (Manivannan et al. 2016), and causing ionic toxicity (Rahman et al. 2016a; Parvin et al. 2020). Excess salt hampers plant survival through ionic injury due to a higher amount of Na^+ and Cl^- ions (Assaha et al. 2015). These osmotic and ionic disruptions cause oxidative stress in plants, and therefore, seriously affect normal plant metabolism (morphological, physiological, and biochemical processes) (Mahmood et al. 2016; Mohsin et al. 2019). To survive under salinity conditions, plants

adapt by changing their cellular metabolism and activating different defense mechanisms (Ghosh et al. 2011).

Reactive oxygen species (ROS) are considered as the main source of cell damage under both biotic and abiotic stresses (Kumar et al. 2015; Parvin et al. 2019). Studies on molecular and biochemical aspects have shown that salt stress produces a significant amount of ROS such as singlet oxygen ($^1O_2$), superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$) (Hasanuzzaman et al. 2019a). These ROS are highly cytotoxic and responsible for lipid peroxidation, protein denaturing, DNA mutation, cellular damage, and programmed cell death (Quiles and López 2004; Mahmud et al. 2017). Oxidative stress depends on the balance between ROS production and ROS scavenging (Mittler et al. 2004), which in turn depends on growth conditions, stress duration, and the ability of the tissue to survive under imbalanced conditions (Costantini 2019).

To eliminate ROS, plants have developed efficient enzymatic and non-enzymatic antioxidant defense systems within their cells (Bhuyan et al. 2020). Plants activate enzymatic antioxidants including four enzymes of the ascorbate–glutathione cycle as well as non-enzymatic antioxidants such as ascorbate (AsA), glutathione (GSH), alkaloids, phenolic compounds, α tocopherols, and non-protein amino acids (Hasanuzzaman et al. 2019b). Methylglyoxal (MG) is another highly cytotoxic and reactive compound produced under salinity stress. However, glyoxalase I (Gly I) and glyoxalase II (Gly II) are two thiol-dependent enzymes, which can detoxify MG in the glyoxalase system (Hasanuzzaman et al. 2017).

To enhance abiotic stress tolerance, one of the most popular technique is exogenous application of phytoprotectants such as signaling molecules, osmolytes, plant hormones, trace elements, etc. (Hasanuzzaman et al. 2018a). The effect of fungicides against plant pathogens has already been studied; however, with the advancement of knowledge on plant signaling cascades, some fungicides at low doses have shown cellular defense such as antioxidant defense. The application of triazole and strobilurin fungicides in plants have shown different physiological and morphological variation as well as improved antioxidant activity and alkaloid production (Ruske et al. 2003, 2004; Zhang et al. 2010). Nabati et al. (1994) and Filippou et al. (2016) found that triazole and strobilurin fungicides can improve salt- tolerant mechanisms in plants. However, the role of triazole and strobilurin fungicides in reducing salt-induced damage by maintaining ion homeostasis, and the defense mechanisms of the antioxidant and glyoxalase systems has not been clearly studied. Wheat (*Triticum aestivum* L.) is a major cereal crop (Chaves et al. 2013), the growth and production of which can be severely affected by salinity (Rahaie et al. 2013). However, there are few studies that have focused on ROS metabolism and the methylglyoxal (MG) detoxification system in plants by exogenous application of fungicides. Thus, the present study was designed to explore the effect of tebuconazole (TEB, triazole fungicide) and trifloxystrobin (TRI, strobilurin fungicide) on the physiology, oxidative/antioxidative status and MG detoxification system of wheat seedlings.

Materials and methods

Plant materials and test conditions

Healthy and uniform seeds of the Japanese salt-sensitive wheat (*Triticum aestivum* L.) cultivar Norin 61 (Muranaka et al. 2002) were selected and sterilized in 70% ethanol for 10 min, then rinsed and soaked in distilled water for a further 4 h. The seeds were germinated on moistened filter paper kept in dark for 48 h, at 25 °C, and then transferred to a growth chamber maintained at 25 ± 2 °C, relative humidity of 65–70%, and 350 µmol photon m^{-2} s^{-1}; 3300-fold diluted Hyponex (Hyponex, Japan) was used as a source of nutrients. The nutrient solution contained N (8%), K (20.94%), P (6.43%), Ca (11.8%), Mg (3.08%), Mn (0.03%), Fe (0.24%), B (0.07%), Cu (0.003%), Zn (0.008%), and Mo (0.0014%). Prior to the main experiment, several trial experiments of different concentrations (from 0.01 to 100 µM) of TEB and TRI were conducted on wheat seedlings, and we selected 1.375 µM for TEB and 0.5 µM for TRI, which showed better performance under 250 mM NaCl as determined by phenotypic responses and MDA content. Three-day-old seedlings were pre-treated with fungicide (1.375 µM TEB + 0.5 µM TRI) for 48 h, and then the resulting 5-day-old seedlings were subjected to salt stress (250 mM NaCl) for the next 5 days. Control plants were grown in nutrient solution only. The nutrient solution was renewed at every 2 days interval. Afterward, physiological and biochemical data were collected from the 10-day-old seedlings. The experiment was conducted following a completely randomized design (CRD) with three replications, and repeated thrice under the same conditions.

Observation of growth parameters

Plant height was measured from the base of the shoot to the tip of the longest leaf. After harvest, fresh weight (FW) was determined immediately, and then the seedlings were dried for 48 h at 70 °C to measure dry weight (DW).

Measurement of malondialdehyde content

The lipid peroxidation level was measured in terms of malondialdehyde (MDA) content following the method of

Heath and Packer (1968) and a modification by Hasanuzzaman et al. (2018a). The fresh leaves were extracted using trichloroacetic acid (TCA) and centrifuged at 11,500 × g. The collected supernatant was mixed with thiobarbituric acid (TBA) reagent, and incubated in a hot water bath for 30 min. Thereafter, the mixture was quickly cooled to stop the reaction, and then the optical absorbance was recorded at 532 nm and corrected at 600 nm.

Determination of H_2O_2 content

Hydrogen peroxide (H_2O_2) content was determined following Hossain et al. (2020). Leaf samples were homogenized with 5% (w/v) TCA and centrifuged (11,500 × g) for 15 min. After centrifuging, the leaf extract was mixed with 10 mM K-P buffer (pH 7.0) and KI (1 M). A standard curve was used to calculate the H_2O_2 content after recording the absorbance at 390 nm.

Observation of electrolyte leakage

Electrolytic leakage (EL) was observed following the method of Dionisio-Sese and Tobita (1998). Smaller pieces of leaf tissue were put into test tubes containing deionized DH_2O and heated at 40 °C for 1 h. The test tubes were then cooled at room temperature and primary electrical conductivity (EC_1) was measured using a CON 700 conductivity meter (Eutech Instruments, Singapore). The test tubes were heated again by autoclave and after cooling, the final electrical conductivity (EC_2) was recorded. Electrical conductivity was calculated by using the following formula: EL (%) = $EC_1/EC_2 \times 100$.

Determination of photosynthetic pigment content

Photosynthetic pigment (chlorophyll, chl and carotenoid, car) was measured according to Lichtenthaler (1987). Leaf samples (0.1 g) were cut into smaller pieces and placed in small centrifuge tubes containing 100% ethanol. The samples were heated in a hot water bath for 30 min at 60 °C, and then cooled. Absorbance was measured spectrophotometrically at 664, 648, and 470 nm.

Determination of relative water content (RWC)

Leaf fresh weight (FW), turgid weight (TW), and dry weight (DW) were recorded, and RWC was calculated using following formula: RWC (%) = [(FW–DW)/(TW–DW)] × 100 (Barrs and Weatherley 1962).

Determination of proline content

Proline (Pro) content was measured following the widely used method of Bates et al. (1973). Leaf samples were homogenized with sulfo-salicylic acid (3%) and centrifuged at 11,500 × g. Leaf extracts were mixed with acid ninhydrin and glacial acetic acid, and then incubated at 100 °C for 1 h. After cooling, toluene was added and the upper toluene chromophore was subjected to spectrophotometric absorbance measurement at 520 nm.

Determination of ascorbate and glutathione content

Freshly harvested leaves (0.5 g) were extracted using TCA, and centrifuged at 11,500 × g. Total ascorbate and reduced ascorbate (AsA) contents was measured following the method described by Hossain et al. (2019). To determine total ascorbate, supernatants were neutralized with K-P buffer (0.5 M, pH 7.0), 0.1 M dithiothretitol (DTT), and distilled water to reduce the oxidized fraction of AsA; DW was used instead of DTT to measure AsA. Total ascorbate and AsA were observed spectrophotometrically at 265 nm, where the final reaction mixture contained 0.25 M, K-P buffer (pH 6.5) and ascorbate oxidase (AO). Known concentrations of AsA were used to prepare a standard curve to quantify AsA; dehydroascorbate (DHA) was calculated by subtracting AsA from total ascorbate.

Glutathione was determined according to the method of Law et al. (1983). The supernatant was mixed with K-P buffer (0.5 M, pH 7.0) and DW to be neutralized for total glutathione; 2-vinylpyridine was used instead of DW for oxidized glutathione (GSSG). Reduced glutathione (GSH) was oxidized by 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB) and reduced with NADPH in the presence of GR. The absorption changes of 2-nitro-5 thiobenzoic acid (NTB) produced from a reduction in DTNB was measured at 412 nm. Standard curves were prepared using known concentrations of GSH and GSSG to determine total glutathione and GSSG content, respectively. The content of GSH was then calculated by subtracting GSSG from total glutathione.

Protein quantification

Coomassie brilliant blue dye was used in the protein quantification method to bind with the protein. The absorbance was read at 595 nm using a spectrophotometer and the protein quantity in each sample was determined using a standard curve (Bradford 1976).

Extraction and determination of enzyme activity

Leaf samples (0.5 g) were homogenized using ice-cold extraction buffer containing 50 mM K-P buffer (pH 7.0), 1 mM AsA, 100 mM KCl, 5 mM β-mercaptoethanol, and 10% glycerol (w/v) in a chilled mortar. The resulting mixture was centrifuged (11500 × g) at 4 °C for 10 min, and the supernatants were collected separately and further used for the enzyme activity assay.

Catalase (CAT; EC: 1.11.1.6) activity was determined according to the method of Hasanuzzaman et al. (2018a). Reduction in optical absorbance was recorded at 240 nm for 1 min caused by the H_2O_2 degradation in a 700 µL reaction mixture containing 50 mM K-P buffer (pH 7.0), 15 mM H_2O_2, and enzyme solution. The activity of CAT was computed using 39.4 M^{-1} cm^{-1} as the extinction coefficient.

Ascorbate peroxidase (APX, EC: 1.11.1.11) activity was assayed according to the method of Nakano and Asada (1981), where the reaction reagent contained K-P buffer pH 7.0 (50 mM), AsA (0.5 mM), EDTA (0.1 mM), H_2O_2 (0.1 mM), and enzyme in a final volume of 700 µL. Decreased absorbance was observed at 290 nm for 1 min and the APX activity was calculated using 2.8 mM^{-1} cm^{-1} as the extinction coefficient.

Monodehydroascorbate reductase (MDHAR, EC: 1.6.5.4) activity was estimated following Hossain et al. (1984), where the reaction mixture comprised Tris-HCl buffer (50 mM, pH 7.5), AsA (2.5 mM), AO (0.5 units), NADPH (0.2 mM), and enzyme in a final volume of 700 µL. Absorbance was recorded at 340 nm for 1 min and the activity was computed using an extinction coefficient of 6.2 mM^{-1} cm^{-1}.

Dehydroascorbate reductase (DHAR, EC: 1.8.5.1) activity was determined following the method of Nakano and Asada (1981), where K-P buffer (50 mM, pH 7.0), GSH (2.5 mM), DHA (0.1 mM), and EDTA (0.1 mM) were mixed to make a reaction buffer. The activity of DHAR was estimated by observing the absorbance at 265 nm for 1 min and using 14 mM^{-1} cm^{-1} as the extinction coefficient.

Glutathione reductase (GR, EC: 1.6.4.2) activity was assayed following Hasanuzzaman et al. (2018a), where the reaction buffer solution comprised K-P buffer (0.1 M, pH 7), GSSG (1 mM), EDTA (1 mM), NADPH (0.2 mM), and enzyme. The decreasing absorbance at 340 nm for 1 min was monitored, and the activity was then estimated using 6.2 mM^{-1} cm^{-1} as the extinction coefficient.

Glutathione *S*-transferase (GST, EC: 2.5.1.18) activity was estimated according to Nahar et al. (2016), where the reaction buffer comprised Tris-HCl buffer (100 mM, pH 6.5), 1-chloro-2,4-dinitrobenzene (CDNB, 1 mM), GSH (1.5 mM), and enzyme solution. The activity was calculated by observing the increasing absorbance at 340 nm for 1 min and using an extinction coefficient of 9.6 mM^{-1} cm^{-1}.

Glutathione peroxidase (GPX, EC: 1.11.1.9) activity was assayed following the method described by Nahar et al. (2016), where the reaction buffer contained K-P buffer (100 mM, pH 7), GSH (2 mM), EDTA (1 mM), GR (1 unit), NaN_3 (1 mM), NADPH (0.12 mM), H_2O_2 (0.6 mM), and 20 µL of enzyme solution. The activity was computed by observing the absorbance at 340 nm for 1 min and using an extinction coefficient of 6.62 mM^{-1} cm^{-1}.

Glyoxalase I (Gly I, EC: 4.4.1.5) activity was determined using the method described by Hasanuzzaman et al. (2018a), where the reaction solution contained 100 mM K-P buffer (pH 7.0), 1.7 mM GSH, 15 mM $MgSO_4$, and 3.5 mM MG. Increased absorbance was observed at 240 nm for 1 min, and the activity was determined using 3.37 mM^{-1} cm^{-1} as the extinction coefficient. Glyoxalase II (Gly II, EC: 3.1.2.6) activity was measured according to Hasanuzzaman et al. (2018a) where the assay buffer contained Tris-HCl buffer (100 mM, pH 7.2), DTNB (0.2 mM), and *S*-D-lactoylglutathione (SLG, 1 mM). The activity was monitored at 412 nm for 1 min and estimated using 13.6 mM^{-1} cm^{-1} as the extinction coefficient.

Determination of methylglyoxal content

Methylglyoxal content was assayed according to the method described by Nahar et al. (2016), using perchloric acid (5%) to homogenize 0.25 g leaf samples followed by centrifugation (11,000 × g) at 4 °C for 10 min. For decolorization, the supernatant was mixed with charcoal and neutralized using sodium carbonate. The neutralized extract was further mixed with Na-P and *N*-acetyl-L-cysteine, and the final volume was 1 mL. The solution was incubated for 10 min and the formation of *N*-α-acetyl-*S*-(1-hydroxy-2-oxoprop-1-yl) cysteine was then spectrophotometrically observed at 288 nm. A standard curve of MG was used to calculate the MG content and expressed in µmol g^{-1} FW.

Determination of mineral content

Mineral nutrient (Na^+, K^+, Ca^{2+}, and Mg^{2+}) content was determined by using an atomic absorption spectrophotometer (GFA-7000A; Shimadzu, Japan). Plant samples were oven-dried at 70 °C for 48 h. The dried root and shoot samples (0.1 g) were ground and digested separately with an acid mixture at 70 °C for 48 h. The acid mixture consisted of HNO_3:$HClO_4$ (5:1 v/v).

Statistical analysis

All the recorded data were analyzed using XLSTAT v. 2020 (Addinsoft 2020) by subjecting them to analysis of variance (ANOVA). The means were compared using Fisher's Least Significant Difference (LSD) test, where $P \leq 0.05$ was considered as significant.

Results

Phenotypic appearance of seedlings

Salt stress deteriorated the growth of the wheat seedlings (Fig. 1). The seedlings treated with salt resulted in light yellowing of the leaves, and leaf rolling was also observed in some leaves. Exogenous application of TEB and TRI improved the phenotypic appearance of the wheat seedlings by reducing the salt-induced damage.

Plant growth and biomass

The application of salt, decreased the height of the wheat seedlings as well as the FW and DW of both the shoots and roots (Table 1). Salt stress decreased plant height, shoot FW, and shoot DW by 28, 48, and 36%, respectively; compared with control. Similarly, root FW and root DW also decreased by 50 and 36%, respectively. Interestingly, exogenous application of TEB and TRI to the salt-treated seedlings recovered growth inhibition and enhanced plant biomass, although no significant difference was found for root DW.

Photosynthetic pigments

The content of chl *a*, chl *b*, and car declined by 33, 23 and 24%, respectively, compared with control; accordingly, chl $(a + b)$ content decreased by 31% under salt stress. Compared with salt stress alone, significant recovery was observed in chl *a*, chl $(a + b)$, and car content in the wheat seedlings after use of TEB and TRI, but no statistical difference was found in chl *b* content (Fig. 2a–d).

Leaf water and proline content

Salt treatment in the wheat seedlings caused osmotic stress by reducing the RWC of the leaves and increasing Pro content. Compared with control, the leaf RWC of the salt-treated seedlings decreased by 50%, while Pro content increased by 672%. The application of TEB and TRI markedly restored leaf RWC and decreased Pro content in the salt-stressed seedlings (Fig. 3a, b).

Oxidative stress indicators

Salt stress caused oxidative damage as indicated by lipid peroxidation (MDA content), EL, and ROS production (H_2O_2 content) in the salt-treated wheat seedlings. Under salt stress, the content of MDA, H_2O_2, and EL significantly increased by 62, 35, and 130%, respectively, compared to control. However, exogenous application of TEB and TRI reversed the salt-induced damage by reducing MDA, EL, and H_2O_2 content (Fig. 4a–c).

Ascorbate and glutathione pool

The salt-treated wheat seedlings had decreased AsA content and AsA/DHA ratio, but increased DHA content, compared with control. However, application of TEB and

Fig. 1 Phenotypic appearance of 10-day-old wheat seedlings under different treatments. TT and S indicate 1.375 µM TEB + 0.5 µM TRI and 250 mM NaCl, respectively

Table 1 Effect of TEB and TRI on growth parameters of 10-day-old wheat seedlings under salt stress

Treatments	Plant height (cm)	Shoot		Root	
		Fresh weight (mg seedling^{-1})	Dry weight (mg seedling^{-1})	Fresh weight (mg seedling^{-1})	Dry weight (mg seedling^{-1})
Control	15.73 ± 0.57 a	119.30 ± 4.78 a	18.00 ± 1.37 a	92.93 ± 7.70 a	9.93 ± 0.75 a
TT	15.41 ± 0.46 a	107.47 ± 10.12 b	17.46 ± 1.16 a	68.53 ± 2.17 b	8.20 ± 0.45 b
Salt	11.30 ± 0.59 c	61.76 ± 3.45 d	11.43 ± 0.45 c	46.46 ± 6.67 c	6.33 ± 0.47 c
TT + salt	13.68 ± 0.72 b	88.13 ± 3.15 c	15.20 ± 0.26 b	59.13 ± 3.58 b	6.90 ± 0.20 c

Means (± SD) were calculated from three replicates for each treatment

TT indicates 1.375 µM TEB + 0.5 µM TRI

Values with different letters are significantly different at $P \leq 0.05$ applying Fisher's LSD test

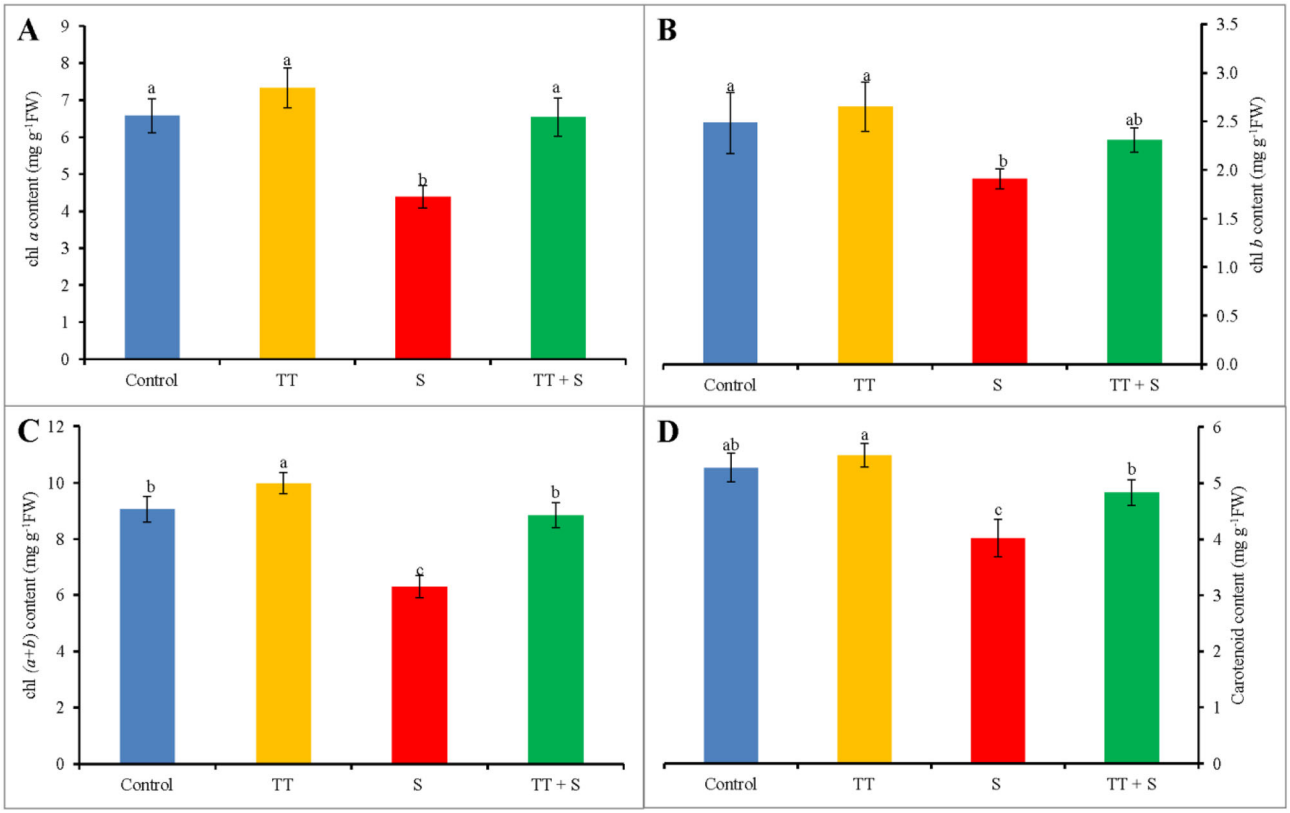

Fig. 2 Effect of TEB and TRI on chl and carotenoid contents: chl *a* (**A**), chl *b* (**B**), chl (*a* + *b*) (**C**), and carotenoid (**D**) in the leaves of 10-day-old wheat seedlings under salt stress. TT and S indicate 1.375 µM TEB + 0.5 µM TRI and 250 mM NaCl, respectively. Means (± SD) were calculated from three replicates for each treatment. Bars with different letters are significantly different at $P \leq 0.05$ applying Fisher's LSD test

TRI to the salt-exposed seedlings increased AsA content and the AsA/DHA ratio by 33 and 129%, respectively; while DHA content decreased by 41% compared with the salt-stressed seedlings (Fig. 5a–c). On the other hand, GSSG content increased under salt stress, but the content of GSH and GSH/GSSG ratio decreased compared to the control. Surprisingly, exogenous application of TEB and TRI increased the GSH content and the GSH/GSSG ratio by 53 and 98%, respectively; while GSSG content reduced by 23% compared with treatment by salt alone (Fig. 5d–f).

Antioxidant enzymes

Salt stress, increased the APX and DHAR activity by 29 and 38%, respectively; but MDHAR and GR activity decreased by 32 and 24%, respectively; in contrast to

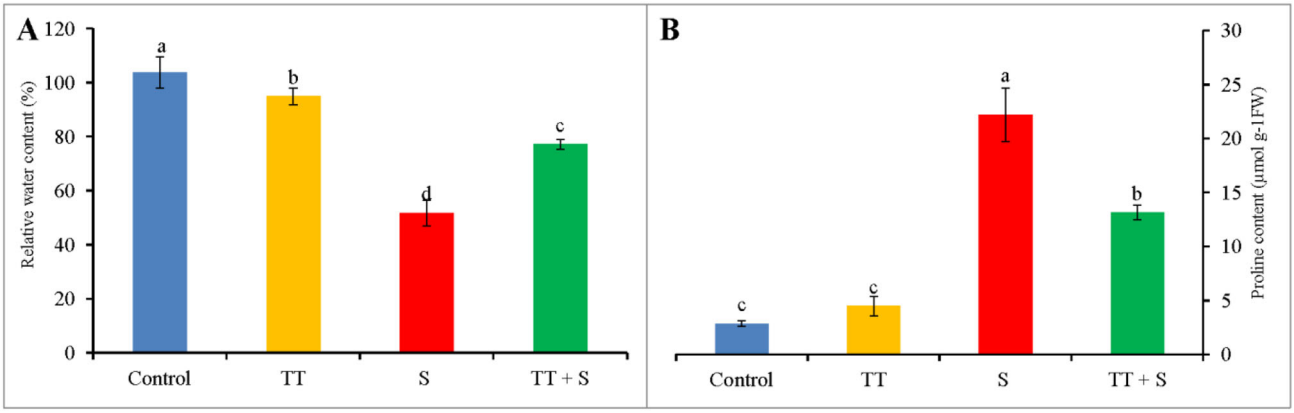

Fig. 3 Effect of TEB and TRI on leaf relative water content (RWC) (**A**) and proline content (**B**) in the leaves of 10-day-old wheat seedlings under salt stress. TT and S indicate 1.375 µM TEB + 0.5 µM TRI and 250 mM NaCl, respectively. Means (± SD) were calculated from three replicates for each treatment. Bars with different letters are significantly different at $P \leq 0.05$ applying Fisher's LSD test

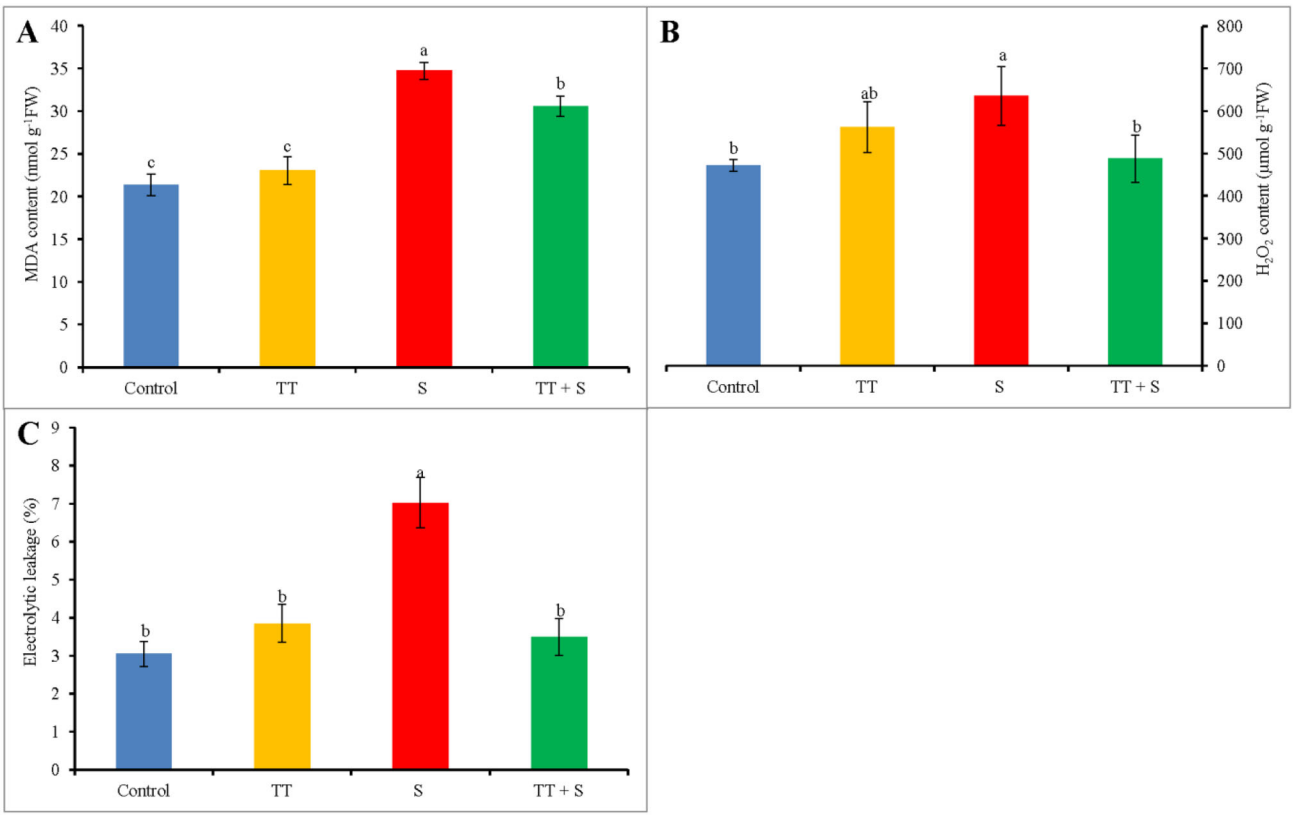

Fig. 4 Effect of TEB and TRI on lipid peroxidation: MDA content (**A**), H_2O_2 content (**B**), and electrolytic leakage (**C**) in the leaves of 10-day-old wheat seedlings under salt stress. TT and S indicate 1.375 µM TEB + 0.5 µM TRI and 250 mM NaCl, respectively. Means (± SD) were calculated from three replicates for each treatment. Bars with different letters are significantly different at $P \leq 0.05$ applying Fisher's LSD test

control. On the other hand, TEB and TRI-treated wheat seedlings decreased the activity of APX and DHAR by 20 and 48%, respectively; but MDHAR and GR activity were enhanced by 48 and 77%, respectively; compared with the seedlings subjected to salt stress alone (Fig. 6a–d).

Under salt stress, CAT activity reduced by 37% compared to control, whereas in the TEB and TRI-treated seedlings CAT activity increased by 46% compared to stress seedlings only. In contrast to control, GST activity reduced by 15% in the salt-treated seedlings, while the application of TEB and TRI increased GST activity by

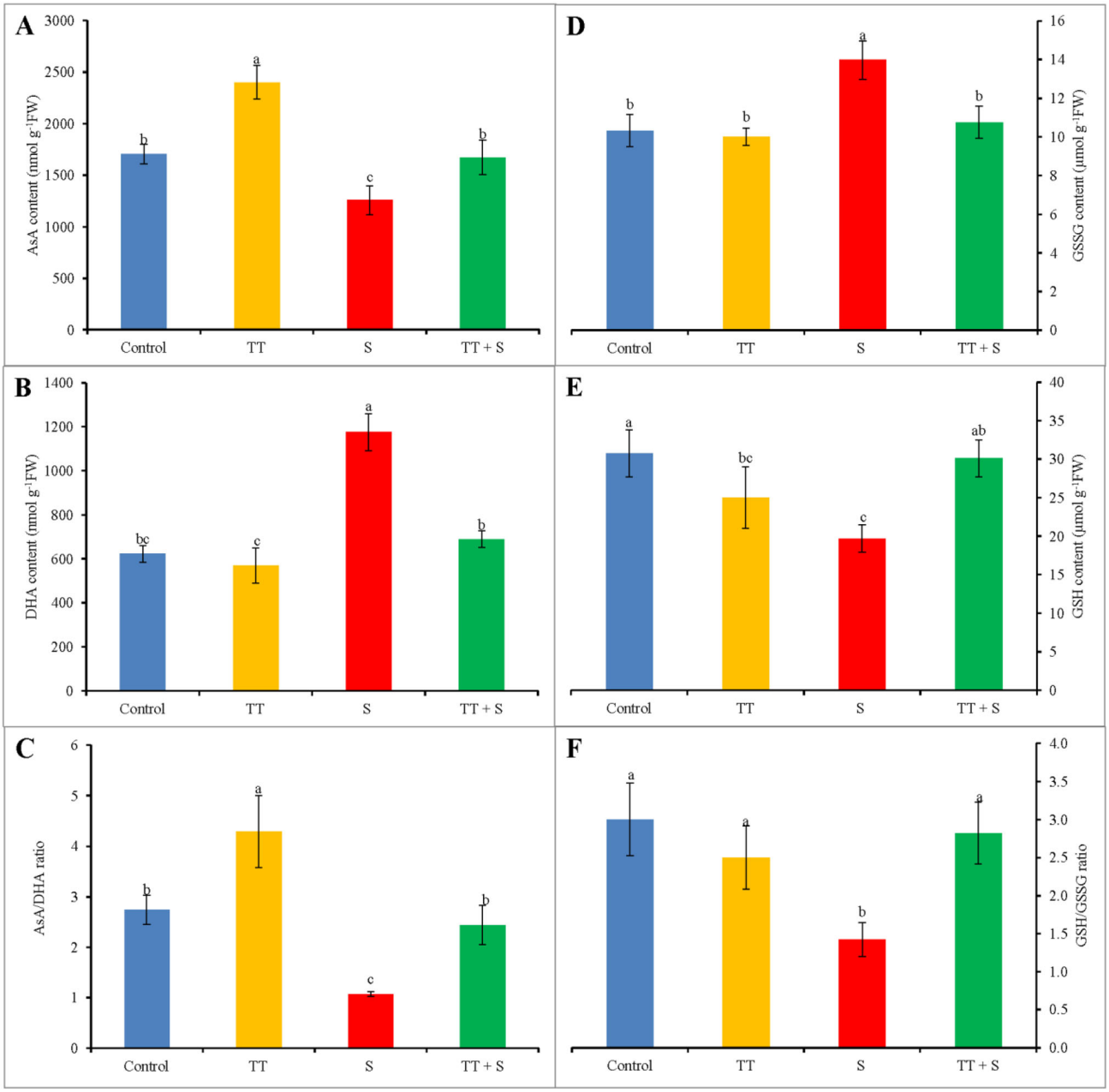

Fig. 5 Effect of TEB and TRI on non-enzymatic antioxidant: AsA content (**A**), DHA content (**B**), AsA/DHA ratio (**C**), GSSG content (**D**), GSH content (**E**), and GSH/GSSG ratio (**F**) in the leaves of 10-day-old wheat seedlings under salt stress. TT and S indicate 1.375 µM TEB + 0.5 µM TRI and 250 mM NaCl, respectively. Means (± SD) were calculated from three replicates for each treatment. Bars with different letters are significantly different at $P \leq 0.05$ applying Fisher's LSD test

64% compared with the stress alone. The activity of GPX enhanced by 13% under salt stress, in contrast to control, but after application of TEB and TRI, GPX activity reduced by 44% compared with the seedlings subjected to salt stress alone (Fig. 7a–c).

Glyoxalase system

Compared with control, salt stress reduced the activity of the Gly I and Gly II enzymes by 36 and 54%, respectively; and increased MG content by about 25%. However, TEB and TRI treatment improved the glyoxalase system as indicated by higher Gly I and Gly II activities, resulting in a reduction in MG content by 22% compared with the seedlings subjected to salt stress alone (Fig. 8a–c).

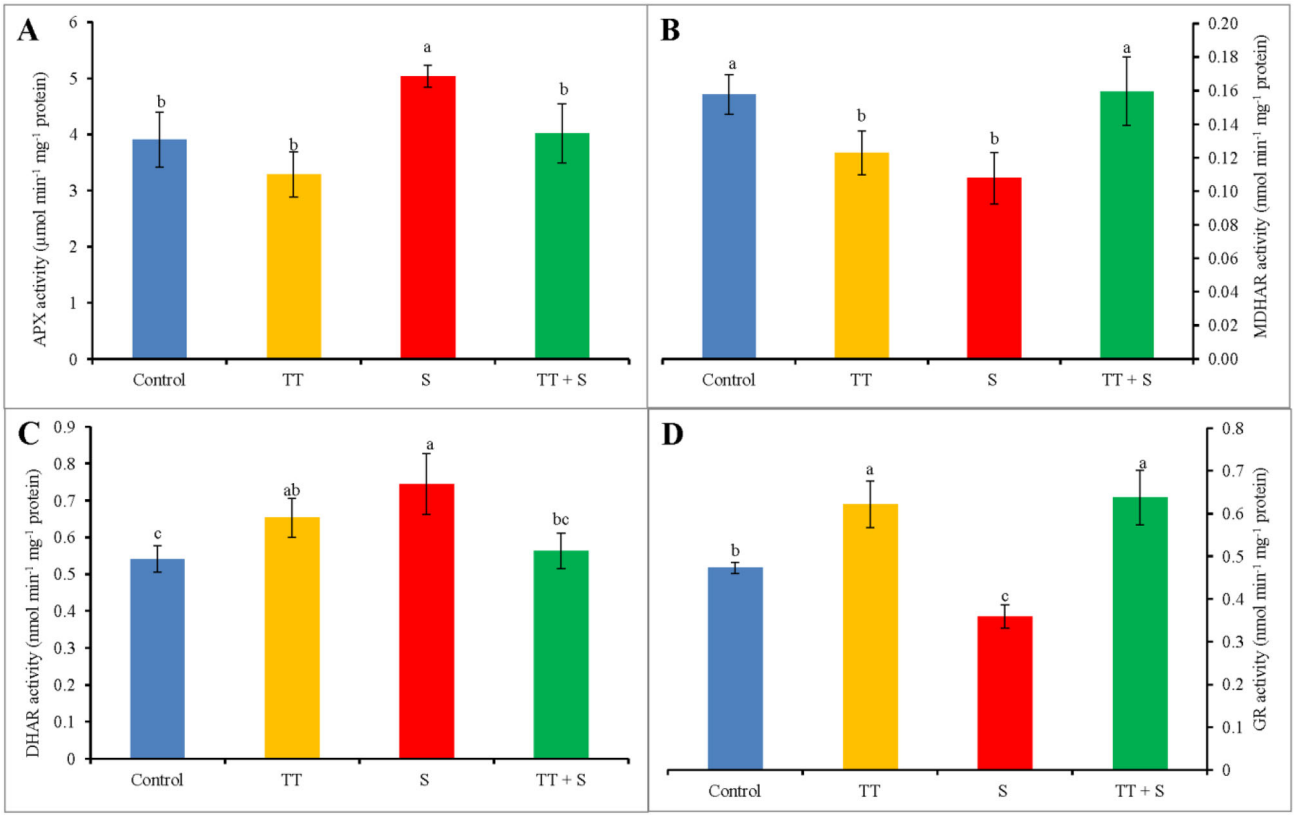

Fig. 6 Effect of TEB and TRI on the AsA–GSH pathway enzymes: APX (**A**), MDHAR (**B**), DHAR (**C**), and GR (**D**) activity in the leaves of 10-day-old wheat seedlings under salt stress. TT and S indicate 1.375 μM TEB + 0.5 μM TRI and 250 mM NaCl, respectively. Means (± SD) were calculated from three replicates for each treatment. Bars with different letters are significantly different at $P \leq 0.05$ applying Fisher's LSD test

Na^+ and K^+ homeostasis and mineral content

Salt treatment to wheat seedlings disrupted ion homeostasis by increasing Na^+ uptake and decreasing the K^+/Na^+ ratio and K^+ accumulation in both the shoots and roots, compared with the control seedlings. Exogenous application of TEB and TRI decreased Na^+ uptake and increased the K^+/Na^+ ratio as well as K^+ uptake in both the shoots and roots, compared with salt treated seedlings alone. Calcium accumulation decreased in both the shoots and roots by 48 and 64%, respectively, under salt stress, whereas improved Ca^{2+} content was observed with the exogenous application of TEB and TRI. Salt stress reduced the Mg^{2+} content in both the shoots and roots of the wheat seedlings, compared with control, while TEB and TRI application improved the Mg^{2+} content in the shoots and roots by 62 and 78%, respectively (Fig. 9a–e).

The above results show that salt treatment increased ionic toxicity in the wheat seedlings by increasing Na^+ accumulation. We also observed that leaf RWC decreased due to osmotic stress, which leads to higher Pro production and reduction in plant biomass and photosynthetic pigments. Therefore, both ionic and osmotic stress caused oxidative damage by increasing MDA, EL, H_2O_2, and MG content. However, the wheat seedlings pre-treated with exogenous TEB and TRI recovered from salt toxicity. Pre-treatment of the seedlings improved the involvement of both enzymatic and non-enzymatic antioxidants together with incremental activities of the glyoxalase enzymes, and therefore, regulated the antioxidant defense and glyoxalase systems. Finally, TEB and TRI application improved seedling growth and morphology by reducing oxidative damage.

Discussion

Salt stress disrupts ion homeostasis through accumulation of Na^+, causes osmotic stress, and inhibits plant growth and biomass (Munns 2011). In the present study, salt stress caused the reduction of wheat seedling growth, because salt-induced osmotic stress reduced plant growth and biomass by decreasing the uptake of water and the content of photosynthetic pigment (Shabani et al. 2013). It has been reported that salt stress decreases the growth and biomass of wheat plants (Yang et al. 2008; Hasanuzzaman et al.

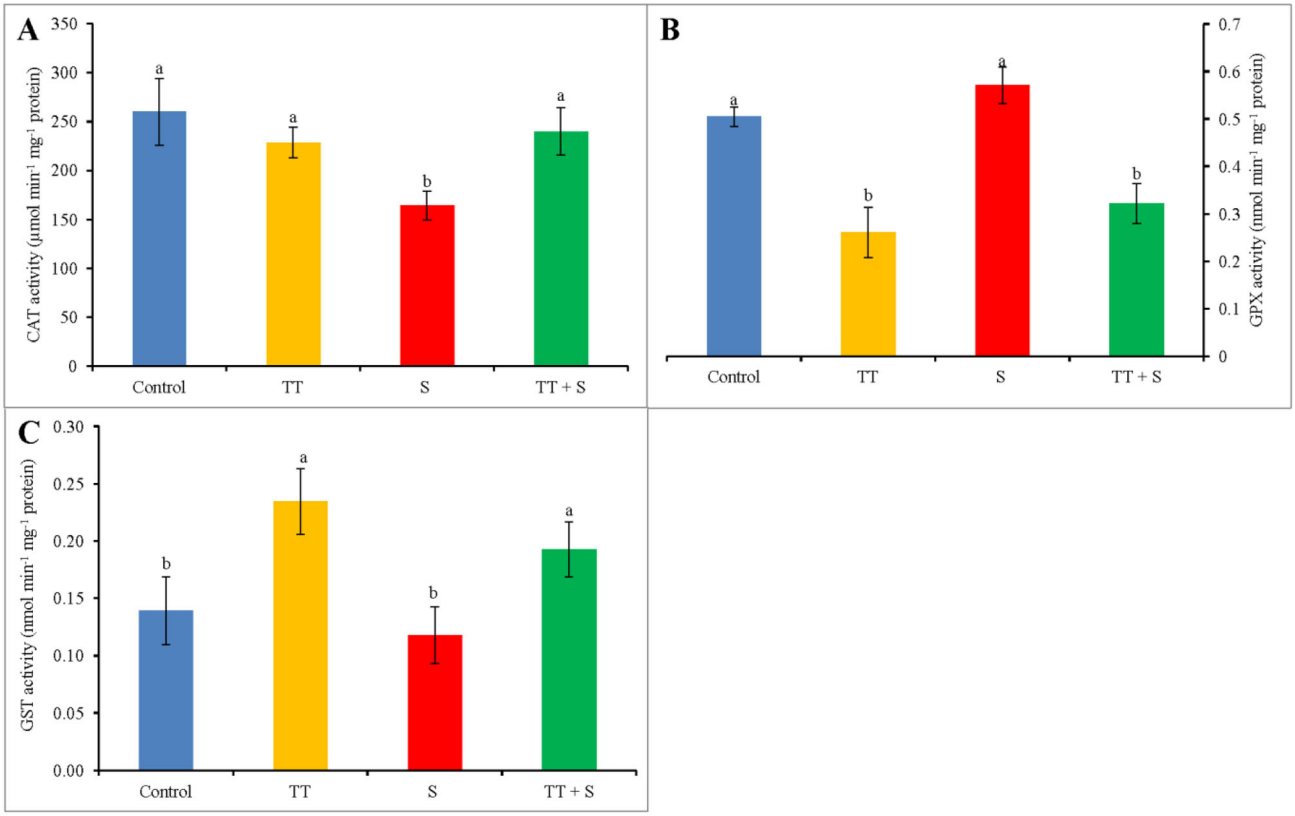

Fig. 7 Effect of TEB and TRI on CAT (**A**), GPX (**B**), and GST (**C**) activity in the leaves of 10-day-old wheat seedlings under salt stress. TT and S indicate 1.375 µM TEB + 0.5 µM TRI and 250 mM NaCl, respectively. Means (± SD) were calculated from three replicates for each treatment. Bars with different letters are significantly different at $P \leq 0.05$ applying Fisher's LSD test

2013). However, the application of TEB and TRI restored the growth parameters such as plant height, shoot FW and DW, and root FW and DW. This result might be due to the recovery of ion homeostasis and improved water status under salt stress. This result also agrees with previous reports, in which exogenous triazole fungicide restored plant growth under salinity conditions (Manivannan et al. 2008; Akbari et al. 2011). Negative results have also been found, where paclobutrazol retards the plant height but enhances root length, shoot FW and DW, and root FW and DW (Hajihashemi et al. 2007).

In our study, we found that chl and car content decreased under salt treatment, whereas adding TEB and TRI to the salt treated wheat seedlings markedly restored chl *a* (49%), chl *b* (21%), and car (37%) content. High salt concentration destabilizes the pigment–protein complex and increases chlorophyllase enzyme activity and ROS production, and as a result decreases the content of photosynthetic pigments (Saha et al. 2010; Hasanuzzaman et al. 2014). An increase in the chl and car content after treatment with TEB may be due to the increase in cytokinin production, which is responsible for stimulating the biosynthesis of photosynthetic pigment (Fletcher et al. 2000). An increase in the content of photosynthetic pigment was reported by Akbari et al. (2011) and Li et al. (2016). Furthermore, strobilurin fungicides are able to maintain higher chl content in the leaves to extend their life (McCartney et al. 2007). Similar results have also been reported by Zhang et al. (2010) and Liang et al. (2018).

Our result showed that salt treatment of the wheat seedlings resulted in higher Pro accumulation and lower RWC, which indicated that salinity disrupts the water balance and causes osmotic stress. However, exogenous TEB and TRI increased RWC and decreased the accumulation of Pro in the salt-treated wheat seedlings. Similar results have also been reported by Akbari et al. (2011) in *Brassica napus* and by Li et al. (2016) in *T. aestivum*.

Overproduction of ROS is one of the major effects of salinity (Hasanuzzaman et al. 2014), which causes lipid peroxidation, oxidation of protein, enzyme inhibition, and finally death of the plant cells (Gill and Tuteja 2010). In our experiment, the salt-treated wheat seedlings showed higher H_2O_2 content, which destroyed the higher membrane components, as indicated by higher MDA level and EL. Moreover, H_2O_2 content is regulated at the intracellular level by several antioxidant enzymes. However, adding TEB and TRI to the salt-treated wheat seedlings significantly reduced the levels of MDA (12%) and EL

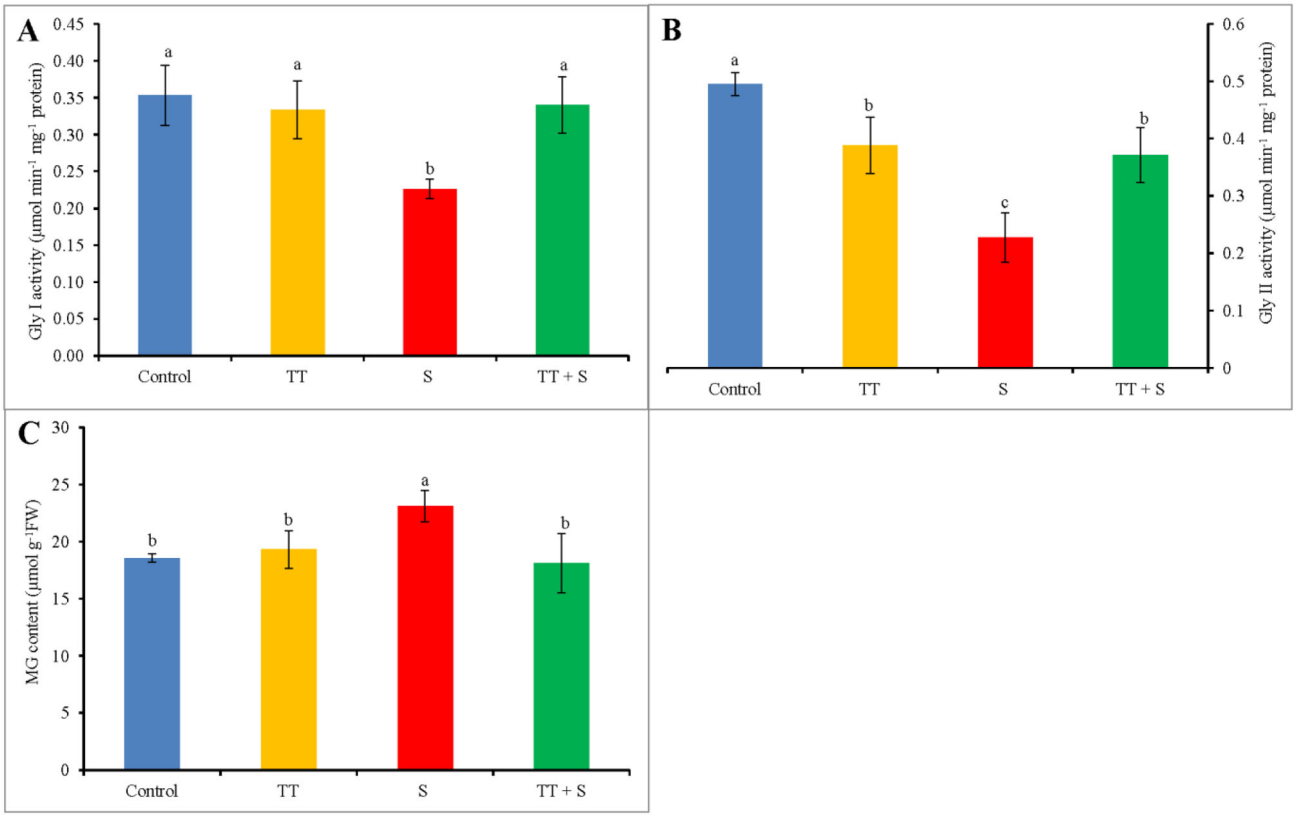

Fig. 8 Effect of TEB and TRI on glyoxalase systems: Gly I (**A**) and Gly II activity (**B**), and MG content (**C**) in the leaves of 10-day-old wheat seedlings under salt stress. TT and S indicate 1.375 μM TEB + 0.5 μM TRI and 250 mM NaCl, respectively. Means (± SD) were calculated from three replicates for each treatment. Bars with different letters are significantly different at $P \leq 0.05$ applying Fisher's LSD test

(50%), and thus decreased the H_2O_2 (23%) content, which indicated a reduction in the damaging effect caused by overproduction of ROS under salt stress. Exogenous triazole and strobilurin fungicides application increases the antioxidant activity to reduce ROS production in seedlings was reported by Li et al. (2016) in *T. aestivum*, by Liang et al. (2018) in *Panax ginseng*, and Amaro et al. (2018) in *Cucumis sativus*.

Ascorbate is the primary antioxidant, which can quench ROS directly (Gill and Tuteja 2010). In different antioxidative reactions, AsA donates electrons and protects plant cell membranes by scavenging O_2^- and OH^- directly (Hasanuzzaman et al. 2014). Glutathione is another important antioxidant that can help plants to develop stress tolerance by scavenging ROS or toxic compounds with the help of GPX and GST enzymes (Szalai et al. 2009). The AsA/DHA and GSH/GSSG ratios maintain the redox state in plant cells under unfavorable stress conditions (Hasanuzzaman et al. 2019a). In the present investigation, salt treatment reduced the AsA content (26%) and AsA/DHA ratio (61%), resulting in an increased DHA content (89%). The lower level of AsA indicated that the direct role in ROS scavenging during higher ROS production under salt stress. Exogenous application of TEB and TRI enhanced the AsA content and AsA/DHA ratio and reduced the content of DHA with increasing MDHAR activity. This finding is supported by Akbari et al. (2011), who found that exogenous hexaconazole fungicide increased the AsA content in canola leaves under salt stress. In our study, salt treatment of the wheat seedlings reduced the GSH content (36%) and GSH/GSSG ratio (53%), resulting in increased GSSG content (35%) with increased DHAR and GPX activity. However, the application of TEB and TRI decreased the GSSG content, and increased the GSH content and GSH/GSSG ratio with increasing GR activity. This finding suggested that TEB and TRI have stimulatory effects on the enzymes of the AsA–GSH cycle to increase AsA and GSH as well as control the cellular redox state of the wheat seedlings under salt stress. Triazole fungicides increasing the content of AsA and GSH has been reported by Sankar et al. (2007) in *Arachis hypogaea*, Jaleel et al. (2008) in *Withania somnifera*, and Akbari et al. (2011) in *B. napus*.

Four enzymes (APX, MDHAR, DHAR, and GR) work together with AsA and GSH in the AsA–GSH cycle to detoxify ROS and regulate the content of AsA and GSH

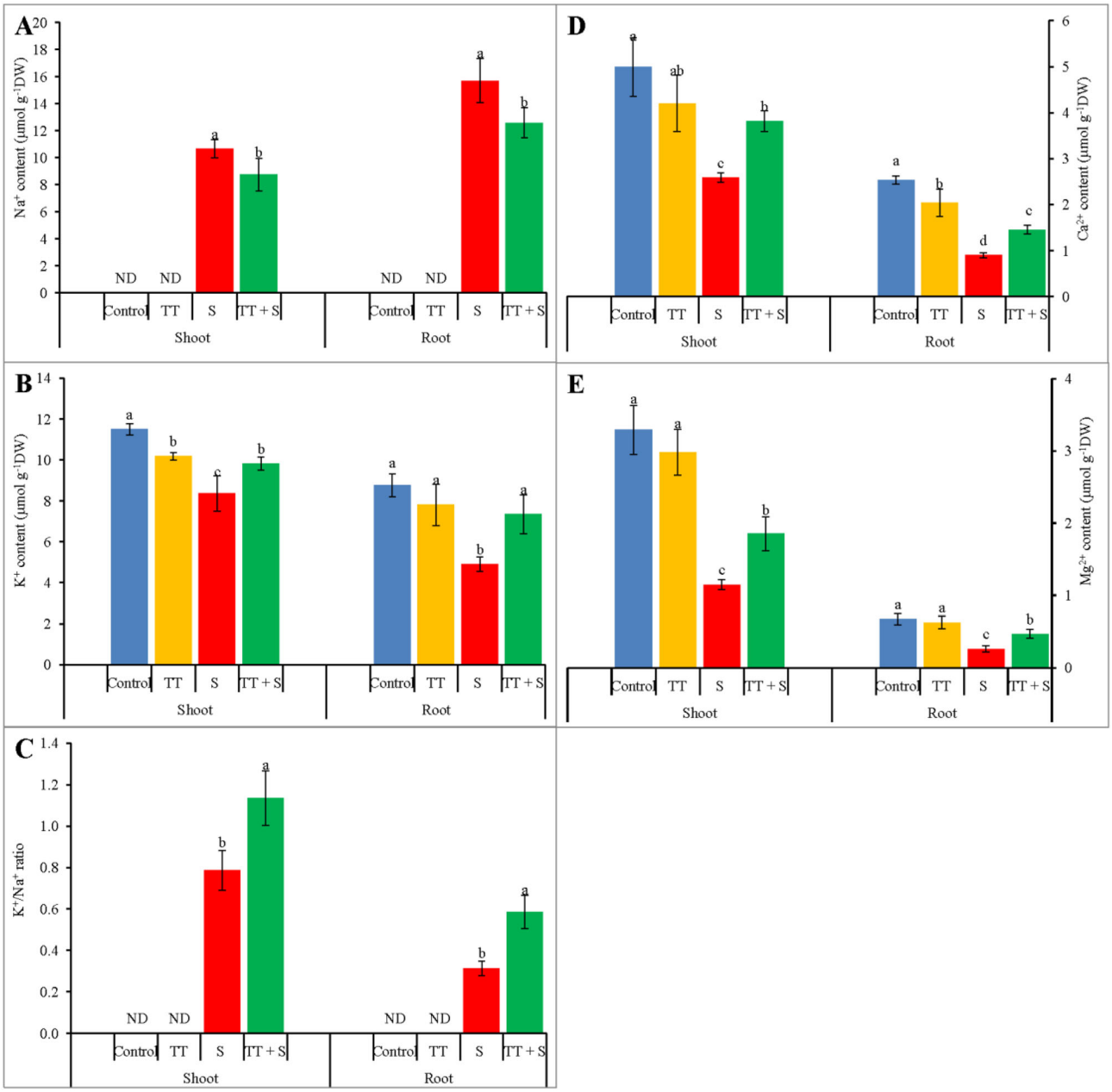

Fig. 9 Effect of TEB and TRI on ion homeostasis: Na^+ content (**A**), K^+ content (**B**), K^+/Na^+ ratio (**C**), Ca^{2+} content (**D**), and Mg^{2+} content (**E**) in the shoots and roots of 10-day-old wheat seedlings under salt stress. TT and S indicate 1.375 µM TEB + 0.5 µM TRI and 250 mM NaCl, respectively. Means (± SD) were calculated from three replicates for each treatment. Bars with different letters are significantly different at $P \leq 0.05$ applying Fisher's LSD test

(Mishra et al. 2013; Hasanuzzaman et al. 2019a). The enzyme APX converts H_2O_2 to H_2O via oxidation of AsA to produce DHA. The enzymes MDHAR and DHAR help to reproduce the AsA from MDHA and DHA using NADPH and GSH (Mishra et al. 2013). Moreover, the enzyme GR is also responsible along with MDHAR and DHAR for regenerating AsA and GSH to maintain redox balance in plant cells (Srivastava et al. 2014). In our investigation, salt stress increased APX and DHAR activity and decreased MDHAR and GR activity and AsA and GSH content. The higher APX activity might be due to the higher H_2O_2 content and the lower AsA content; accordingly, the lower GSH content may be due to the lower GR activity under salt-stress (Mishra et al. 2013; Hasanuzzaman et al. 2018b). However, exogenous TEB and TRI application to the salt-treated wheat seedlings decreased APX and DHAR activity, and increased MDHAR and GR activity and AsA and GSH content. The enzyme CAT

detoxifies H_2O_2 to H_2O and O_2 (Sanchez-Casas and Klesseg 1994). The CAT enzyme is usually generated in peroxisomes, in which different cell oxidative mechanisms (β-oxidation of fatty acids, photorespiration, and purine catabolism) are involved (Gill and Tuteja 2010). In our study, CAT activity decreased under salt-stress, which might be due to production of higher H_2O_2 content. Our results corroborate the findings of Mishra et al. (2013) and Hasanuzzaman et al. (2014), who found that salt stress significantly reduces the CAT activity. Furthermore, application of TEB and TRI to the salt-treated wheat seedlings increased CAT activity to reduce overproduction of H_2O_2. Similar results were reported by Zhang et al. (2010), Akbari et al. (2011), and Liang et al. (2018), who found that the application of triazole and strobilurin fungicides increased CAT activity to reduce oxidative stress and ROS production. The present study showed that the increase in GPX activity but decrease in GST activity under salt stress that might be due to the production of higher H_2O_2 content and inadequate detoxification of H_2O_2. This finding supports the results of Hasanuzzaman et al. (2014), who found similar GPX and GST activity under salt stress. However, application of TEB and TRI to the salt-treated wheat seedlings decreased GPX activity and increased GST activity, which might play a role in H_2O_2 detoxification.

Methylglyoxal is a highly toxic agent that results in the production of a higher amount of ROS under salt stress, damage to the cell components of plants (Hasanuzzaman et al. 2018b), and destruction of cellular proteins and DNA (Yadav et al. 2005). The Gly I and Gly II enzymes inhibit MG toxicity by converting the α-ketoaldehydes into hydroxyacids with the help of GSH, and thus increase stress tolerance in plants (Yadav et al. 2005; Singla-Pareek et al. 2008). In this experiment, we observed that salt stress increased MG production and decreased the Gly I and Gly II enzyme activities, which might be due to the depletion of the GSH pool by salinity. This result corroborates the findings of Hasanuzzaman et al. (2018b). However, these reductions in the Gly I and Gly II enzyme activities suggest that the glyoxalase system alone is not enough to detoxify MG under salt stress. However, exogenous application of TEB and TRI to the salt-treated wheat seedlings improved the detoxification of MG by enhancing the Gly I (51%) and Gly II (63%) activities. This result might be due to the increased GSH level and GSH/GSSG ratio in the TEB and TRI-treated wheat seedlings, which improved the glyoxalase system by detoxifying cellular MG under salt stress.

Maintaining ion homeostasis is one of the vital mechanisms of salt tolerance. Salt-tolerant plants often possess higher K^+/Na^+ ratios (Rahman et al. 2016a). In our study, the Na^+ content increased and K^+ content decreased under salt stress in both the shoots and roots. The accumulation of Na^+ was higher in the roots compared with the shoots. This ion imbalance under salt stress supports the findings of previous studies (Wu and Wang 2012; Rahman et al. 2016b). In the salt-affected wheat seedlings, the Ca^{2+} and Mg^{2+} content also decreased, which might be due to the activity of Na^+ displacing the Ca^{2+} and Mg^{2+}. However, the exogenous application of TEB and TRI reduced Na^+ accumulation in the shoots and roots (18 and 20%, respectively), increased the accumulation of K^+ (17 and 50%,, respectively) and K^+/Na^+ ratio (44 and 87%, respectively), and improved ion homeostasis under salt stress. This result corroborates a previous report (Hajihashemi et al. 2007), in which the application of paclobutrazol reduced Na^+ accumulation in wheat plants, but increased the contents of K^+, P and N under salt stress. Therefore, we can say that exogenous TEB and TRI increased salt tolerance in the wheat seedlings by improving ion homeostasis and reducing K^+ efflux and Na^+ influx.

Conclusion

Based on the results of this study, we conclude that salt-induced ionic and osmotic stresses negatively effected the growth and physiology of the wheat seedlings by disrupting the antioxidant defense and glyoxalase systems, and ion homeostasis, because of increased ROS and MG production and also Na^+ uptake. Higher accumulation of ROS and MG caused oxidative damage in the seedlings under salt stress, resulting in deterioration of plant growth, photosynthetic pigments, and leaf water status. The application of TEB and TRI improved enzymatic and non-enzymatic antioxidants and increased the function of the glyoxalase enzymes, and thus suppressed the overproduction of ROS and MG. In addition, TEB and TRI application also maintained ion homeostasis by increasing the K^+/Na^+ ratio under saline conditions. Therefore, the results of our study indicated that the combination of triazole and strobilurin fungicides has the potentiality to increase wheat growth by decreasing problems caused by high salinity. Thus, TEB and TRI prove their efficiency to increase salt tolerance indicating their potential for implication of these protectants to defend both abiotic and biotic stresses simultaneously as plants often face multiple stresses in field growing condition. However, further investigation is needed to elucidate the pathways, that are responsible to for making salt tolerance in TEB and TRI treated plants.

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Authors contribution SMM conceived, designed, and performed the experiment and prepared the manuscript. MH designed the experiment and analyzed the data. KN designed the experiment. MSH, MHMBB, and KP actively participated in executing the experiment. MF conceived, designed, and monitored the experiment.

Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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