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# Regulation of antioxidant activity in maize (*Zea mays* L.) by exogenous application of sulfur under saline conditions

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**Abstract:** One of the major effects of salt stress is high production of reactive oxygen species that adversely affect essential cellular metabolic pathways. To limit the elevated production of reactive oxygen species, plants stimulate various types of antioxidants. In this study, sulfur was used to induce tolerance against oxidative stress through modulation of the activities of various antioxidants. Two maize genotypes (Agatti 2003 and Pak Afgoi 2003) were subjected to different salinity (25 and 75 mM) and sulfur (40 and 80 mM) treatments and a control treatment. Various antioxidants and oxidative stress determinants in maize organs (leaf, shoot, and root) were studied. It was found that salt stress decreased ascorbic acid and tocopherol, but stimulated the production of total phenolics, carotenoids, lycopene, superoxide dismutase, peroxidase, catalase, malondialdehyde, and hydrogen peroxide. Exogenously applied sulfur decreased carotenoids, malondialdehyde, and hydrogen peroxide and increased ascorbic acid, tocopherol, total phenolics, lycopene, superoxide dismutase, peroxidase, and catalase. Agatti 2003 showed higher antioxidant activity than Pak Afgoi 2003. In conclusion, sulfur application at the 40 mM level balanced antioxidants and oxidative stress determinants to induce salt tolerance in maize plants.

**Key words:** Antioxidants, maize, oxidative stress, reactive oxygen species, sulfur, salinity

## 1. Introduction

Salt stress greatly reduces agricultural productivity all over the world. It has been reported that salinity affected 20% of the total cultivated area and 30% of irrigated land (Machado and Serralheiro, 2017). Salinity causes disturbances in various metabolic activities in plant cells and creates oxidative stress. High accumulation of $Na^+$ and $Cl^-$ ions in plant cells generates reactive oxygen species. In low concentrations reactive oxygen species play a key role in cell signaling and regulation of plant growth and development. However, in excess, reactive oxygen species cause oxidative stress. Various enzymatic (peroxidase, superoxide dismutase, catalase, and glutathione reductase) and nonenzymatic (tocopherols, phenolics, carotenoids, and lycopene) antioxidants are involved in balancing the production of reactive oxygen species through a scavenging and neutralizing mechanism (Kumari et al., 2014). An appropriate concentration of antioxidants is needed to cope with oxidative stress caused by salinity.

Among macronutrients, sulfur promotes the development of salt tolerance in plants through the limiting of oxidative stress. Sulfur is an important constituent of various antioxidants, i.e. glutathione, thioredoxin, glutaredoxin, etc. Sulfur metabolites help to protect plant cells against harmful accumulations of reactive oxygen species, thus lowering the damage caused by oxidative stress (Manna et al., 2013; Mukwevho et al., 2014; Riffat and Ahmad, 2016). Moreover, sulfur is an essential component of various proteins, nutrients, and vitamins necessary for the growth and development of plants affected by salinity (Riffat and Ahmad, 2018a). Hence, appropriate concentrations of sulfur are needed for proper activity of various antioxidants involved in scavenging the reactive oxygen species produced under salt-stress conditions.

According to one estimate, the average maize yield in Pakistan is 9.20 tons per hectare. This rate is 82% lower than the international yield rate for maize production (Aslam, 2016). Salinity is a key factor in low maize productivity, because maize is considered salt sensitive (Farooq et al., 2015). The production and quality of maize should be improved because it is a nutritionally significant crop. Application of sulfur is very helpful for improving maize productivity, because maize responds quickly to sulfur fertilization (Riffat and Ahmad, 2018b). This study focuses on improvement of the salt-tolerance potential of maize cultivars through the use of sulfur, which not only regulates the production of reactive oxygen species

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by various antioxidants but helps to increase the salt-tolerance potential of maize plants.

## 2. Materials and methods

### 2.1. Experimental plan and treatment application

The experiment was conducted in the Wire house of the University of Agriculture, Faisalabad, Pakistan. Seeds of maize cultivars were obtained from the Maize and Millet Institute, Sahiwal, Pakistan. The relative salt tolerance of maize cultivars was tested previously through screening experiments in the Environmental Biology and Plant Ecology Lab, University of Agriculture, Faisalabad, Pakistan (Riffat and Ahmad, 2016). Two maize cultivars were selected according to their salt tolerance potential (Agatti 2003 and Pak Afgoi 2003). Seed grading was performed, and uniform seeds were sown in plastic pots containing soil. The soil properties are shown in Table 1. The pots were kept in sunlight under 33/28 ± 3 °C day/night temperatures and 68/85% ± 2% relative humidity. Salinity (25 and 75 mM) was applied using sodium chloride, and the sulfur (40 and 80 mM) treatment was applied using potassium sulfate. The levels of these compounds had already been optimized in the previous screening experiment conducted by the same authors (Riffat and Ahmad, 2016). One set of plants was kept as control (0 mM salt, 0 mM sulfur). Foliar application of sulfur (40 and 80 mM) was included after 20 days of germination. For the foliar application, 40 mM and 80 mM sulfur solutions were prepared and carefully sprayed on the respective plants. Plants were harvested after 45 days of germination, and the following attributes were studied.

**Table 1.** Constituents of soil used in the experiment.

| Parameters | Concentration in soil |
|---|---|
| pH | 9.06 |
| EC | 1.210 dS/m |
| Sodium ion | 8 mg/g dry weight |
| Potassium ion | 1.2 mg/g dry weight |
| Calcium ion | 0.9 mg/g dry weight |
| Nitrate ion | 0.4268 mg/g dry weight |
| Phosphate ion | 0.098 mg/g dry weight |
| Sulfate ion | 1.5 mg/g dry weight |

### 2.2. Determination of antioxidant activity

#### 2.2.1. Ascorbic acid

Ascorbic acid contents were determined following Mukherjee and Choudhuri (1983). Plant material (0.25 g) was extracted with 10 mL of 6% trichloroacetic acid, followed by the addition of 2 mL of 2% dinitrophenyl hydrazine and a drop of 10% thiourea. The mixture was kept in a water bath for 15 min and cooled down in an ice bath. After adding 5 mL of 80% $H_2SO_4$, absorbance was noted at 530 nm using a spectrophotometer (UV-1100). A graph was plotted between standard and known concentrations of ascorbic acid, and the following formula was used for calculating ascorbic acid in plant samples.

$$\text{mg/100 g dry matter ascorbic acid} = \frac{\text{µg as read from the graph} \times \text{volume made up} \times 100}{\text{mL of sample taken} \times \text{weight of sample} \times 1000}$$

#### 2.2.2. Tocopherol

Tocopherol contents were determined by following the procedure proposed by Rosenberg (1992). Plant samples (2.5 g) were homogenized with 0.1 N $H_2SO_4$, and volume was maintained to 50 mL with 0.1 N $H_2SO_4$. The mixture was kept at room temperature overnight. The next day, the mixture was vortexed and filtered. Three centrifuge tubes were labeled as test, standard, and blank, and 1.5 mL of plant extract, standard, and water were added, respectively. After adding 1.5 mL of ethanol and xylene to each tube, the tubes were closed, shaken, and centrifuged. Then, absorbance of the mixture was noted at 460 nm using a spectrophotometer (UV-1100). Standard solution was prepared by adding 0.33 mL ferric chloride solution into the blank solution and mixing thoroughly. The optical density of the standard solution was measured at 520 nm using a spectrophotometer (UV-1100) and using water as a blank. The following formula was used for determination of tocopherol contents in plant samples.

$$\text{Tocopherol (µg)} = \frac{\text{A520 - A450}}{\text{StdA520}} \times 0.29 \times 15$$

#### 2.2.3. Soluble phenolics

Soluble phenolics were determined by following the procedure proposed by Julkunen-Tiitto (1985). After grinding the fresh plant sample (50 mg), 1 mL of 80% acetone was added. The mixture was kept at 50 °C for one h and centrifugation was done at 12,000 g for 15 min. The supernatant was collected. The volume of aliquot (100 µL) was maintained at 1 mL with distilled water, and 0.5 mL of Folin–Ciocalteu's phenol was mixed and vortexed. Then, 2.5 mL of 20% $Na_2CO_3$ was added, and the volume of the mixture was maintained at 5 mL. This mixture was vortexed for 5–10 s. The absorbance was noted at 750 nm using a spectrophotometer (UV-1100).

#### 2.2.4. Total carotenoids and lycopene

Total carotenoids and lycopene were quantified following Zakaria et al. (1979). Saponification was done by taking a plant sample (5 g) in 2.5 mL of 12% ethanolic potassium hydroxide and placing it in a water bath at 60 °C for 30 min. To this mixture 10–15 mL petroleum ether was added, and the mixture was covered with glass wool and calcium carbonate. All steps were carried out in the dark

to prevent proteolysis of carotenoids. This mixture was separated into two layers. The upper layer having petroleum ether was separated, and extraction steps were repeated for the colorless appearance of the aqueous phase. To avoid moisture, $Na_2SO_4$ was added. The absorbance was noted at 450 nm and 503 nm using a spectrophotometer (UV-1100). The following formula was used for quantification of carotenoids and lycopene expressed in mg/g tissue.

$$\text{Amount of total carotenoids} = \frac{\text{Optical density of the sample} \times 4 \times \text{volume of the sample} \times 100\text{mL of sample}}{\text{Weight of sample}} \text{ (mg)}$$

$$\text{Lycopene} = \frac{3.12 \times \text{optical density of sample} \times \text{volume of sample} \times \text{dilution} \times 100}{1 \times \text{weight of the sample} \times 1000}$$

**2.2.5. Superoxide dismutase**

Superoxide dismutase activity was measured following Giannopolitis and Ries (1977). Extraction of fresh plant samples (0.1 g) was done in 9 mL of phosphate buffer (50 mM, pH 7.8). Centrifugation was done at 15,000 × *g* at 4 °C. The supernatant was separated and stored safely for the determination of concentrations of various enzymes. The enzyme extract was treated step-wise, following Giannopolitis and Ries (1977), and superoxide dismutase activity was measured at 560 nm using a spectrophotometer (UV-1100).

**2.2.6. Peroxidase**

Peroxidase activity was determined following Chance and Maehly (1955), with modification. To the enzyme extract (0.1 mL), 50 mM phosphate buffer (pH 7.0), 20 mM guaiacol, and 40 mM $H_2O_2$ were added. Every 20 s absorbance was noted at 470 nm using a spectrophotometer (UV-1100).

**2.2.7. Catalase**

Catalase activity was determined following Chance and Maehly (1955). To the enzyme extract (0.1 mL), 50 mM phosphate buffer (pH 7.8) and 5.9 mM $H_2O_2$ were added, and final volume was maintained at 3 mL. The absorbance of the reaction mixture was noted at 240 nm every 20 s.

**2.3. Determination of oxidative stress determinants**

**2.3.1. Malondialdehyde**

Malondialdehyde contents were determined following Heath and Packer (1968). Fresh plant samples (0.25 g) were homogenized in 3 mL (1% W/V) trichloroacetic acid. Centrifugation was done at 20,000 × *g* for 15 min, and 1 mL supernatant was mixed into 1 mL (0.5% v/v) thiobutyric acid. The mixture was kept in an incubator at 95 °C for 15 min after cooling in an ice bath. The absorbance of the mixture was noted at 532 nm and 600 nm by spectrophotometer (UV-1100). The following formula was used for determination of malondialdehyde contents in plant samples.

Malondialdehyde (mmol/mL) = A532 – A600 (155000) × 106

**2.3.2. Hydrogen peroxide**

Hydrogen peroxide was determined following Velikova et al. (2000). Fresh plant samples (0.1 g) were homogenized in 1 mL of 0.1% (w/v) trichloroacetic acid in a microfuge tube placed in an ice bath. The centrifugation of the mixture was done at 12,000 × *g* for 15 min. Then, 0.5 mL supernatant, 0.5 mL potassium phosphate buffer (pH = 7), and 1 mL of 1 M potassium iodide were mixed thoroughly. The mixture was vortexed, and absorbance of the mixture was measured at 390 nm using a spectrophotometer (UV-1100).

**2.4. Statistical analysis**

The experiment was designed in completely randomized (CRD) fashion with three-factor factorial arrangement. Statistical analysis was performed by analysis of variance technique (ANOVA) (Steel and Torrie, 1986) using Co-Stat (CoHort Software, 2003, Monterey, California, USA). Statistix 8.1 was used for homogenous variation among treatment means. For graphical representation of the data, Microsoft Excel was used.

## 3. Results

**3.1. Ascorbic acid**

Salt stress decreased ascorbic acid contents in all studied organs (leaf, shoot, and root) of maize cultivars (Figure 1). It was shown from the statistically significant variety × salinity (V × Sa) interactions for leaf and root; however, ions (Table 2). A high reduction in ascorbic acid contents was found at 75 mM salt levels. However, sulfur application ameliorated the adverse effects of salinity in both maize cultivars (Agatti 2003 and Pak Afgoi 2003), as seen from statistically significant salinity × sulfur (Sa × S) interactions in maize shoots (Table 2). The effectiveness of sulfur was greatest at a 40 mM sulfur level, while higher level of sulfur (80 mM) was not very effective in improving ascorbic acid contents in both varieties (Figure 1). These findings are strengthened by statistically significant V × S interaction for root; in shoot and leaf this interaction was statistically nonsignificant (Table 2). Overall, sulfur application improved salt tolerance in both maize cultivars by improving ascorbic acid contents in maize plants (Table 2).

**3.2. Tocopherol**

Imposition of salt stress reduced tocopherol contents in maize cultivars (Agatti 2003 and Pak Afgoi 2003), as observed from statistically significant V × Sa interactions in leaf and shoot; however, in root this interaction was nonsignificant (Table 2). Application of sulfur (40 and 80 mM) improved tocopherol content in both maize varieties.

**Table 2.** Mean squares from analysis of variance (ANOVA) of the data for antioxidant content of maize subjected to different levels of salinity and sulfur.

| SOV | df | Leaf AA | Shoot AA | Root AA | Leaf Toc | Shoot Toc | Root Toc |
|---|---|---|---|---|---|---|---|
| Variety (V) | 1 | 0.0420 *** | 0.015 *** | 0.027 *** | 0.37 *** | 0.23 *** | 0.014 *** |
| Salinity (Sa) | 2 | 0.033 *** | 0.021 *** | 0.0086 *** | 0.25 *** | 0.22 *** | 0.027 *** |
| Sulfur (S) | 2 | 0.0035 *** | 0.0032 *** | 0.0048 *** | 0.061*** | 0.085 *** | 0.0080 *** |
| V × Sa | 2 | 0.0030 *** | 0.0002 ns | 0.0002 * | 0.015*** | 0.010 *** | 0.0002 ns |
| V × S | 2 | 0.00009 ns | 0.0002ns | 0.0004 ** | 0.002ns | 0.0055 *** | 0.0006 * |
| Sa × S | 4 | 0.00008 ns | 0.0003* | 0.00005 ns | 0.0065 ** | 0.0046 *** | 0.0006 * |
| V × Sa × S | 4 | 0.0001 * | 0.00005ns | 0.0005 *** | 0.002 ns | 0.0011 ** | 0.0003ns |
| Error | 36 | 0.00004 | 0.00008 | 0.00005 | 0.0015 | 0.0003 | 0.0002 |
| SOV | df | Leaf TF | Shoot TF | Root TF | Leaf TC | Shoot TC | Root TC |
| Variety (V) | 1 | 0.0019 *** | 0.0006 *** | 0.0006*** | 38.32 *** | 13.14 *** | 6.69 *** |
| Salinity (Sa) | 2 | 0.0006 *** | 0.0006 *** | 0.0002 *** | 0.93 ** | 4.59 *** | 1.51 *** |
| Sulfur (S) | 2 | 0.00007 *** | 0.00008 *** | 0.00008*** | 8.68 *** | 5.12 *** | 2.99 *** |
| V × Sa | 2 | 0.00004 ns | 0.00002ns | 0.0001 ** | 0.033 ns | 0.019 ns | 0.18 * |
| V × S | 2 | 0.000001ns | 0.000005 ns | 0.0000001 ns | 0.065 ns | 0.0082 ns | 0.18 * |
| Sa × S | 4 | 0.00001 ns | 0.00002 * | 0.00003 *** | 0.82 ** | 0.12 ns | 0.088 ns |
| V × Sa × S | 4 | 0.000004 ns | 0.000008 ns | 0.000003 ns | 0.11 ns | 0.015 ns | 0.051 ns |
| Error | 36 | 0.000004 | 0.000005 | 0.000002 | 0.13 | 0.075 | 0.051 |

*, **, *** = significant at 0.05, 0.01, and 0.001 levels, respectively. ns = nonsignificant
Abbreviations: AS = ascorbic acid, Tc = tocopherol, TF = total phenolics, TC = total carotenoids.

However, 40 mM sulfur was more effective for improving tocopherol contents. Both varieties responded differently to sulfur application. Sulfur also improved salt tolerance in the salt-sensitive variety (Pak Afgoi 2003) to some extent (Figure 2). These findings were drawn from statistically significant V × S interactions for shoot and root; however, leaf showed nonsignificant V × S interactions (Table 2). Sulfur application (40 and 80 mM) greatly improved tocopherol contents by reducing the toxic effects of salt stress. Moreover, the salt-tolerant maize cultivar (Agatti 2003) showed great improvement in tocopherol content, compared to the salt-sensitive maize genotype (Pak Afgoi 2003) (Table 2).

### 3.3. Total phenolics

Results revealed that salt stress increased total phenolics production in all studied maize organs (leaf, shoot, and root). The highest total phenolic content was found at 75 mM salt levels (Figure 3). These findings are supported by statistically significant V × Sa interactions for root; shoot and leaf of maize genotypes showed nonsignificant interactions (Table 2). Application of sulfur (40 and 80 mM) significantly increased total phenolic content at all salt levels. However, higher levels of sulfur (80 mM) improved total phenolic content to a much greater extent than lower sulfur levels (40 mM) (Figure 3). The salt-tolerant Agatti 2003 showed high total phenolic content in comparison to the salt-sensitive Pak Afgoi 2003 (Figure 3).

### 3.4. Total carotenoids

Salt stress increased the concentration of total carotenoids in both studied maize genotypes which was evident from the statistically significant V × Sa interaction for root; shoot and leaf showed nonsignificant V × Sa interactions (Table 2). At 75 mM salt levels, high total carotenoid content were found (Figure 4). Exogenously applied sulfur significantly lowered total carotenoid content in both studied maize genotypes (Agatti 2003 and Pak Afgoi 2003) at all salt levels (25 and 75 mM). Statistically significant Sa × S interactions were observed for leaf carotenoid contents, while shoot and root showed nonsignificant interactions (Table 2). The salt-tolerant maize genotype (Agatti 2003) showed high total carotenoid content compared to the salt-sensitive maize cultivar (Pak Afgoi 2003) (Figure 4).

### 3.5. Lycopene

Lycopene contents increased as salt levels increased in all studied maize cultivars. The lycopene contents showed statistically significant V × Sa interaction for leaf; root and shoot showed nonsignificant V × Sa interactions (Table 3). Sulfur applications (40 and 80 mM) improved the lycopene

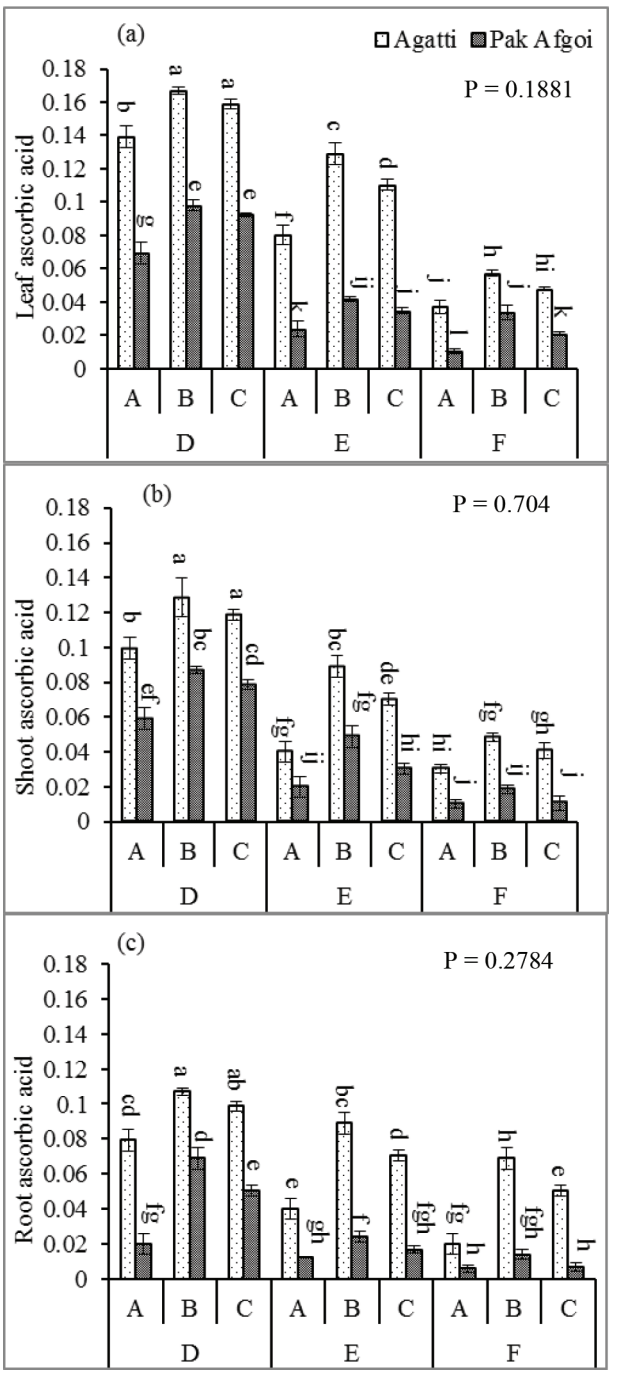


**Figure 1.** Effect of different levels of sulfur (A = 0 mM, B = 40 mM, C = 80 mM) on ascorbic acid content (µg/g fresh weight) of different maize (*Zea mays* L.) cultivars under saline conditions (D = 0 mM NaCl, E = 25 mM NaCl, F = 75 mM NaCl). Vertical lines on each bar represent standard error. Means with different letters are significantly different (Student–Newman–Keuls, $P < 0.05$).

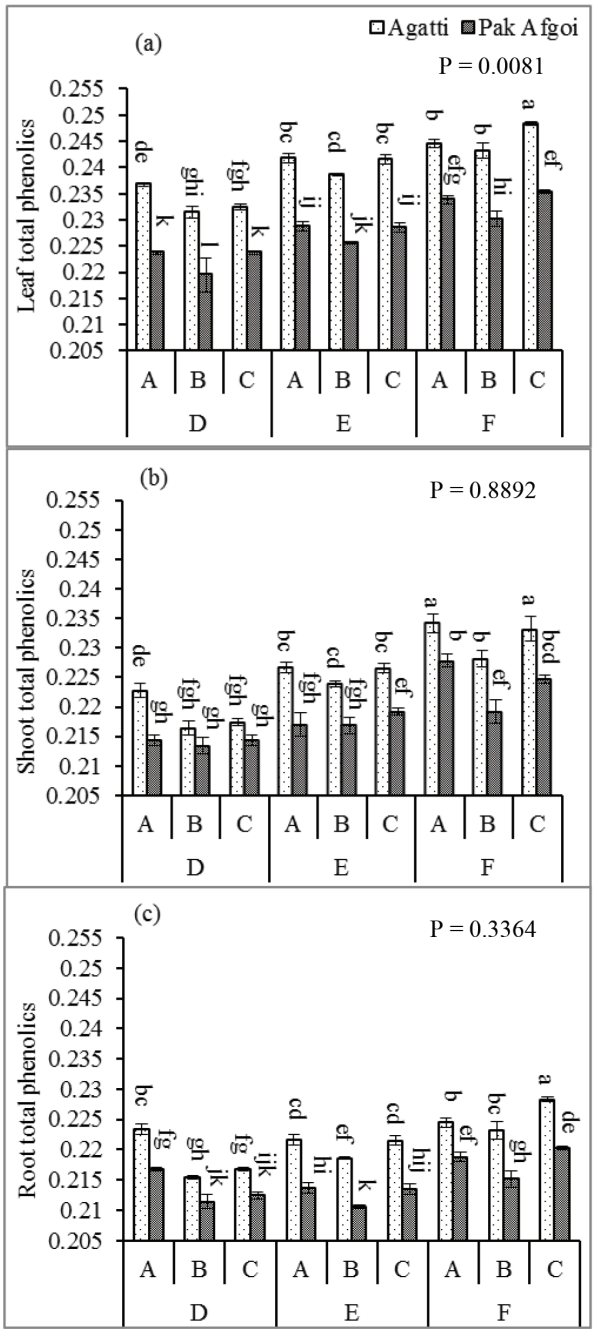


**Figure 2.** Effect of different levels of sulfur (A = 0 mM, B = 40 mM, C = 80 mM) on tocopherol content (µg/g fresh weight) of different maize (*Zea mays* L.) cultivars under saline conditions (D = 0 mM NaCl, E = 25 mM NaCl, F = 75 mM NaCl). Vertical lines on each bar represent standard error. Means with different letters are significantly different (Student–Newman–Keuls, $P > 0.001$).

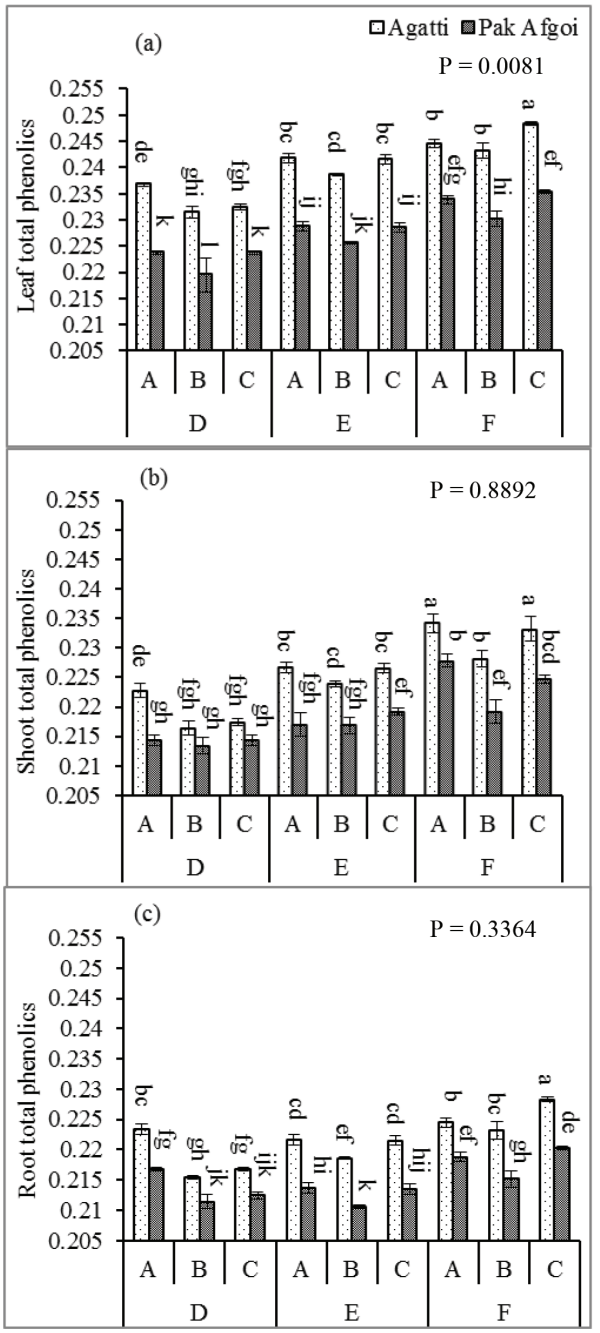


**Figure 3.** Effect of different levels of sulfur (A = 0 mM, B = 40 mM, C = 80 mM) on total phenolic content (µg/mL fresh weight) of different maize (*Zea mays* L.) cultivars under saline conditions (D = 0 mM NaCl, E = 25 mM NaCl, F = 75 mM NaCl). Vertical lines on each bar represent standard error. Means with different letters are significantly different (Student–Newman–Keuls, $P < 0.05$).

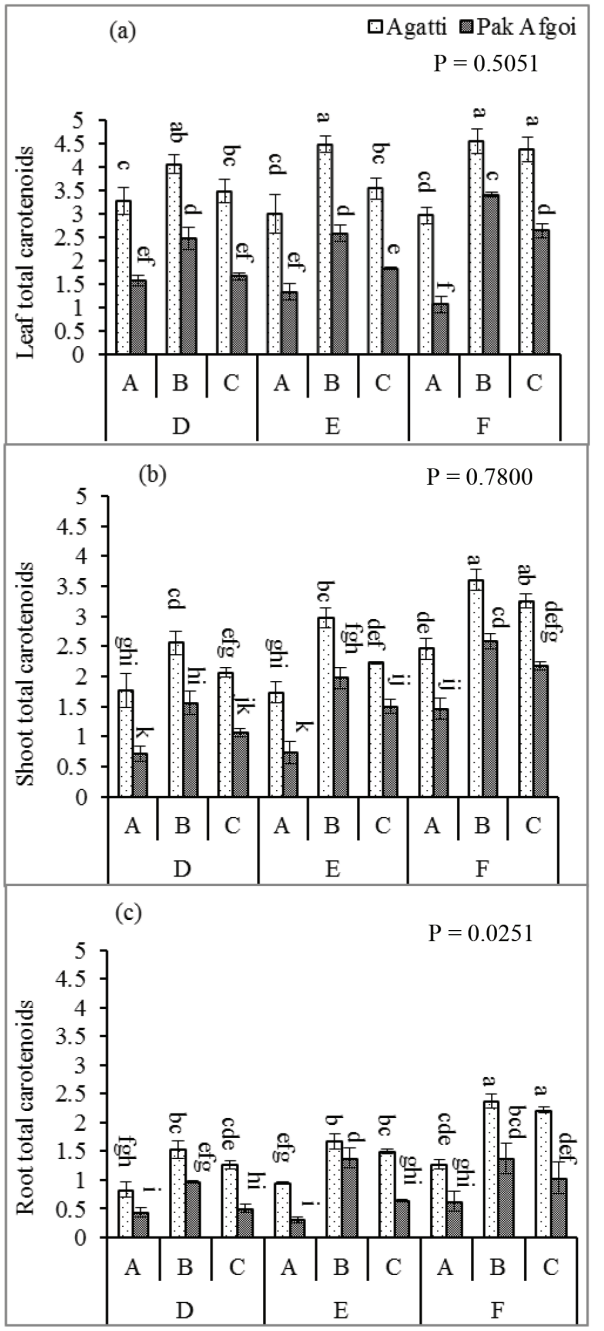


**Figure 4.** Effect of different levels of sulfur (A = 0 mM, B = 40 mM, C = 80 mM) on total carotenoid content (mg/g fresh weight) of different maize (*Zea mays* L.) cultivars under saline conditions (D = 0 mM NaCl, E = 25 mM NaCl, F = 75 mM NaCl). Vertical lines on each bar represent standard error. Means with different letters are significantly different (Student–Newman–Keuls, $P < 0.05$).

**Table 3.** Mean squares from analysis of variance (ANOVA) of the data for antioxidant content of maize subjected to different levels of salinity and sulfur.

| SOV | df | Leaf Lyco | Shoot Lyco | Root Lyco | Leaf SOD | Shoot SOD | Root SOD |
|---|---|---|---|---|---|---|---|
| Variety (V) | 1 | 26596.94 *** | 30171.73 *** | 4309.976 *** | 1568.16*** | 547.85*** | 238.22*** |
| Salinity (Sa) | 2 | 3058.47*** | 3394.11 *** | 2360.80 *** | 119.59*** | 11.12*** | 1.72 * |
| Sulfur (S) | 2 | 4392.14 *** | 4352.52 *** | 3550.32 *** | 257.72*** | 263.9 *** | 155.10 *** |
| V × Sa | 2 | 169.83 * | 218.64 * | 50.66 ns | 297.5 *** | 63.68 *** | 23.27 *** |
| V × S | 2 | 34.19 ns | 120.62 ns | 11.01 ns | 3.38 ** | 32.91*** | 8.59 *** |
| Sa × S | 4 | 36.41 ns | 56.96 ns | 45.52 * | 7.40*** | 1.07 ns | 1.59 * |
| V × Sa × S | 4 | 35.83 ns | 21.12 ns | 3.78 ns | 2.65 ** | 0.24 ns | 0.12 ns |
| Error | 36 | 40.64 | 66.85 | 15.65 | 0.638 | 0.638 | 0.427 |
| SOV | df | Leaf POD | Shoot POD | Root POD | Leaf CAT | Shoot CAT | Root CAT |
| Variety (V) | 1 | 3568.9*** | 1027.04*** | 696.96*** | 384*** | 255.67*** | 164.6 *** |
| Salinity (Sa) | 2 | 613.38*** | 43.51*** | 47.63*** | 32.05*** | 19.61*** | 15.43*** |
| Sulfur (S) | 2 | 124.22*** | 56.26*** | 37.62 *** | 28.16*** | 25.22*** | 24.07*** |
| V × Sa | 2 | 842.46 *** | 95.84*** | 65.85*** | 94.38 *** | 58.56*** | 30.7*** |
| V × S | 2 | 16.96 *** | 7.04 *** | 4.51 ** | 0.72 ns | 3.33 ** | 0.19 ns |
| Sa × S | 4 | 1.94 * | 0.61 ns | 0.65 ns | 4.97*** | 1.71* | 1.28 * |
| V × Sa × S | 4 | 3.85*** | 0.22 ns | 0.32 ns | 0.69 ns | 0.93 ns | 0.82 ns |
| Error | 36 | 0.57 | 0.43 | 0.56 | 0.62 | 0.52 | 0.35 |

*, **, *** = significant at 0.05, 0.01, and 0.001 levels, respectively. ns = nonsignificant
Abbreviations: Lyco = lycopene, SOD = superoxide, POD = peroxide, CAT = catalase.

contents at all studied salt levels (25 and 75 mM). However, lower levels of sulfur (40 mM) were more effective for improving lycopene contents than the higher sulfur level (80 mM). These findings are supported by statistically significant Sa × S interaction for root; however, shoot and leaf showed nonsignificant Sa × S interactions (Table 3). Agatti 2003 accumulated higher levels of lycopene than Pak Afgoi 2003 (Figure 5).

### 3.6. Superoxide dismutase

Results revealed that salt stress greatly increased superoxide dismutase contents in all studied maize organs (leaf, shoot, and root) of the salt-tolerant maize cultivar; however, in the salt-sensitive maize cultivar (Pak Afgoi 2003), superoxide dismutase contents decreased with increasing salt levels (Figure 6), as shown by statistically significant V × Sa interactions (Table 3). The application of sulfur (40 and 80 mM) significantly increased the superoxide dismutase contents in both maize varieties (Agatti 2003 and Pak Afgoi 2003); statistically significant V × S interactions supported these findings (Table 3). Although all studied sulfur levels improved superoxide dismutase contents, at 40 mM sulfur levels a high increase in superoxide dismutase activity was found (Figure 6). Sulfur enhanced superoxide dismutase contents at all salt levels (25 and 75 mM), as shown by statistically significant Sa × S interactions for leaf. Moreover, sulfur application increased salt tolerance in the salt-sensitive maize cultivar by raising the superoxide dismutase contents in all studied maize organs. This was revealed through statistically significant V × Sa × S interactions for maize leaf; shoot and root showed nonsignificant interactions (Table 3).

### 3.7. Peroxidase

Statistical analysis showed that salt stress increased peroxidase contents in both maize cultivars. However, in the salt-tolerant variety (Agatti 2003), peroxidase contents increased under increasing salinity; in the salt-sensitive maize variety peroxidase contents decreased under increasing salt levels (Figure 7), and this was evident from statistically significant V × Sa interaction (Table 3). Sulfur application (40 and 80 mM) significantly increased peroxidase contents in the salt-tolerant Agatti 2003. In the salt-sensitive Pak Afgoi 2003, sulfur application improved peroxidase contents in comparison to control (Figure 7). These findings are evident from the statistically significant V × S interactions for all studied maize organs (leaf, shoot, and root) (Table 3). Moreover, sulfur application reduced the toxic effects of salinity by balancing peroxidase contents in both maize cultivars (Figure 7).

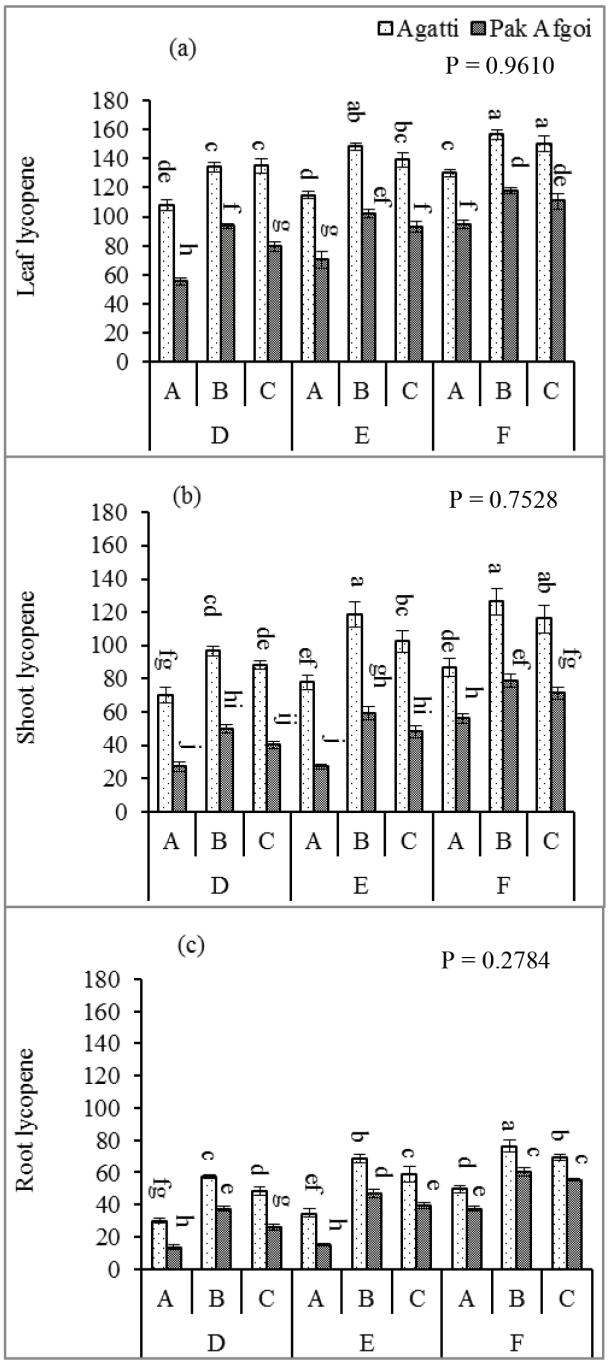


**Figure 5.** Effect of different levels of sulfur (A = 0 mM, B = 40 mM, C = 80 mM) on lycopene content (mg/g fresh weight) of different maize (*Zea mays* L.) cultivars under saline conditions (D = 0 mM NaCl, E = 25 mM NaCl, F = 75 mM NaCl). Vertical lines on each bar represent standard error. Means with different letters are significantly different (Student–Newman–Keuls, P > 0.01).

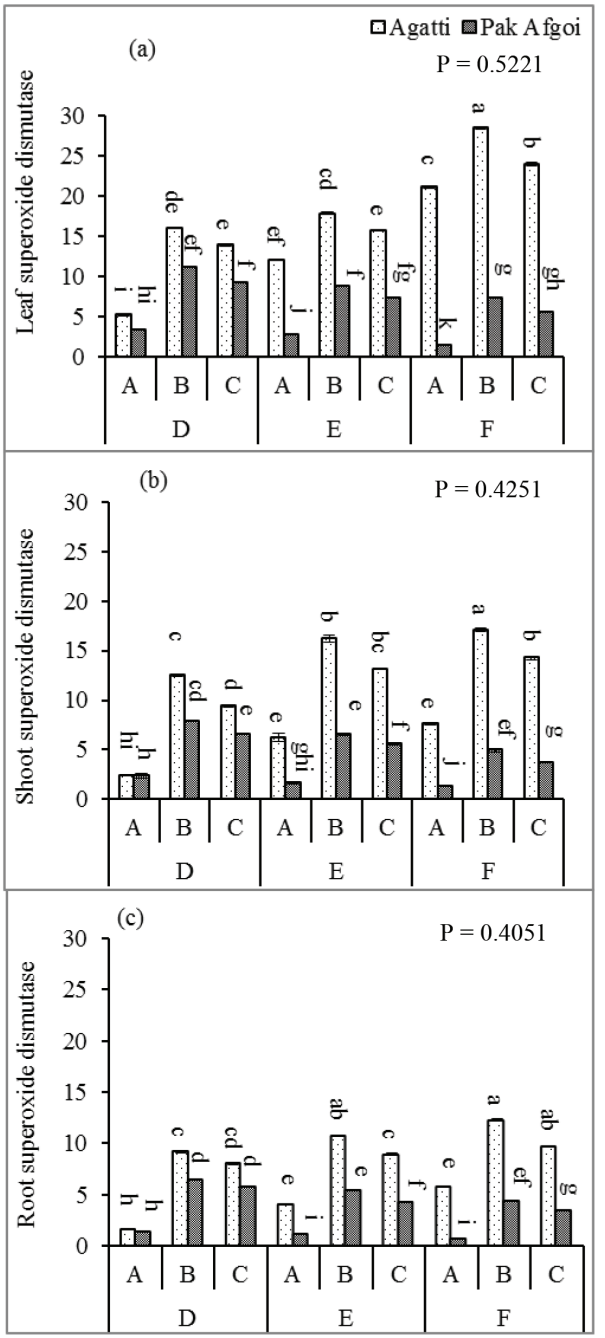


**Figure 6.** Effect of different levels of sulfur (A = 0 mM, B = 40 mM, C = 80 mM) on superoxide dismutase content (units/mg proteins) of different maize (*Zea mays* L.) cultivars under saline conditions (D = 0 mM NaCl, E = 25 mM NaCl, F = 75 mM NaCl). Vertical lines on each bar represent standard error. Means with different letters are significantly different (Student–Newman–Keuls, P < 0.05).

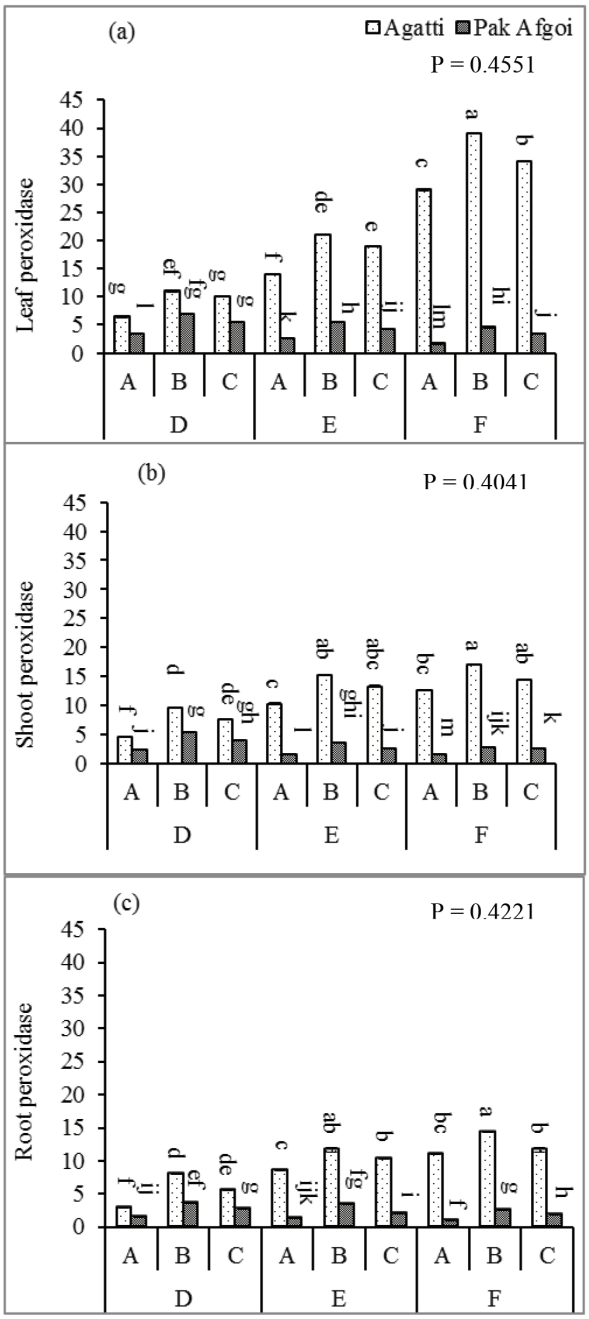


**Figure 7.** Effect of different levels of sulfur (A = 0 mM, B = 40 mM, C = 80 mM) on peroxidase content (units/mg proteins) of different maize (*Zea mays* L.) cultivars under saline conditions (D = 0 mM NaCl, E = 25 mM NaCl, F = 75 mM NaCl). Vertical lines on each bar represent standard error. Means with different letters are significantly different (Student–Newman–Keuls, $P < 0.05$).

### 3.8. Catalase

Imposition of salt stress increased catalase contents in the salt-tolerant maize variety; catalase contents decreased in the salt-sensitive variety with increasing salt levels. These findings are supported by statistically significant V × Sa interactions (Table 3). Application of sulfur improved catalase contents in both varieties; this was revealed by statistically significant V × S interactions for maize shoot; leaf and root showed nonsignificant interactions (Table 3). A statistically significant Sa × S interaction showed that sulfur application improved salt tolerance in both varieties by increasing catalase contents (Table 3). Low levels of sulfur (40 mM) were more effective for improving the catalase contents in both maize cultivars under salt-stress conditions (Figure 8).

### 3.9. Malondialdehyde

A marked increase in malondialdehyde contents was found under increasing salinity in both studied maize cultivars, as revealed from statistically significant V × Sa interactions (Table 4). At 75 mM salt levels, a higher concentration of malondialdehyde was found. Sulfur application significantly lowered malondialdehyde contents in both maize genotypes (Figure 9), as seen by statistically significant V × S interactions for leaf and root; interactions in shoot were nonsignificant (Table 4). Sulfur also lowered the toxic effects of salinity in both maize genotypes by lowering malondialdehyde contents. These findings were proven by statistically significant Sa × S interactions for leaf and shoot. A statistically significant V × Sa × S interaction showed that the salt-tolerant cultivar Agatti 2003 accumulated higher malondialdehyde contents than the salt-sensitive Pak Afgoi 2003 (Table 4).

### 3.10. Hydrogen peroxide

Imposition of salt stress elevated hydrogen peroxide contents in both maize genotypes. This was shown through a statistically significant V × Sa interaction for root; in leaf and shoot this interaction was statistically nonsignificant (Table 4). Maximum accumulation of hydrogen peroxide contents was found at 75 mM salt levels. Application of sulfur (40 and 80 mM) significantly lowered hydrogen peroxide contents in both maize cultivars and reduced the toxic effects of salinity; however, a lower level of sulfur (40 mM) was more effective (Figure 10). These results are supported by statistically significant Sa × S interactions for shoot and root; leaf showed nonsignificant interaction (Table 4). The salt-sensitive cultivar Pak Afgoi 2003 accumulated higher hydrogen peroxide contents than the salt-sensitive variety Agatti 2003. Moreover, sulfur application ameliorated the adverse effects of salinity by reducing hydrogen peroxide contents (Table 4).

## 4. Discussion

In this study it was noted that sulfur supplementation significantly improved the salt-tolerance ability of maize cultivars by developing a balance among various antioxidants and oxidative stress determinants. The maize cultivars used in this study were screened according to

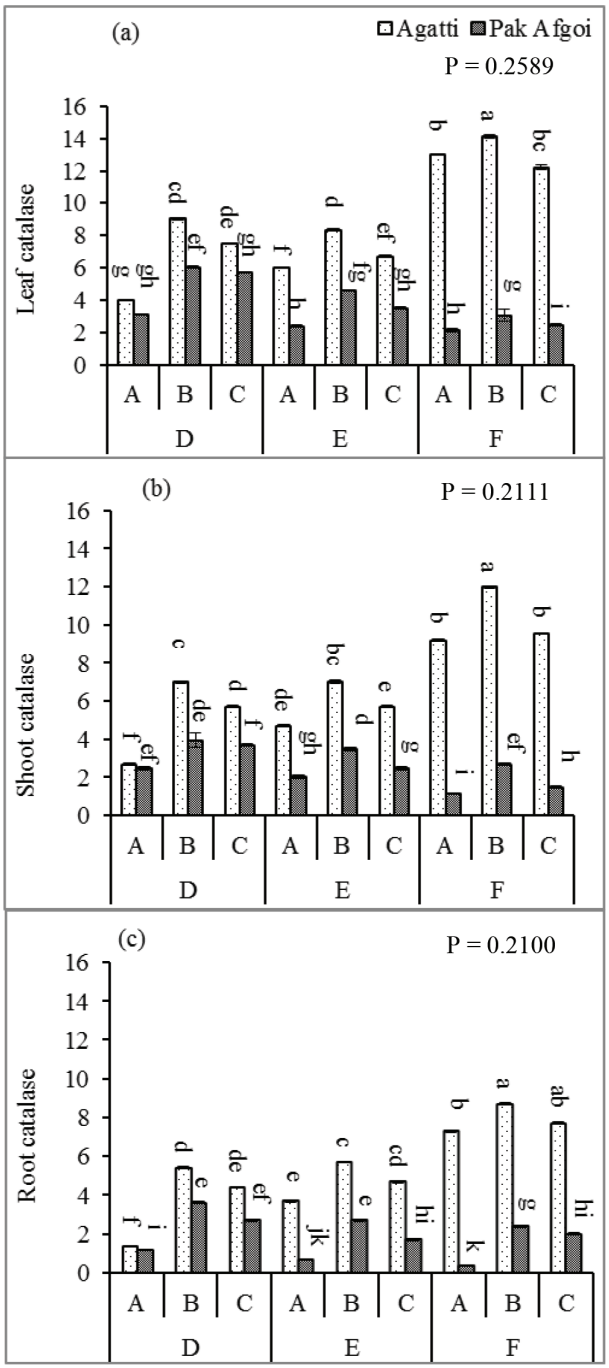


**Figure 8.** Effect of different levels of sulfur (A = 0 mM, B = 40 mM, C = 80 mM) on catalase content (units/mg proteins) of different maize (*Zea mays* L.) cultivars under saline conditions (D = 0 mM NaCl, E = 25 mM NaCl, F = 75 mM NaCl). Vertical lines on each bar represent standard error. Means with different letters are significantly different (Student–Newman–Keuls, $P < 0.05$).

their relative salt tolerance by the sequential agglomerative hierarchic and nonoverlapping (SAHN) method by the same authors. In addition, various levels of sulfur and salinity were optimized in a screening experiment (Riffat and Ahmad, 2016).

Plants have developed various mechanisms of salinity tolerance. These include ionic homeostasis, synthesis of various osmoprotectants and osmolytes, production of various antioxidants, and modulation of hormones (Gupta and Huang, 2014). The genotypes used in this study have varying responses to salt-stress conditions. These genotypes had been studied previously to test their mechanism of salt tolerance by the same authors. These studies revealed that salt-tolerant Agatti 2003 responded to sulfur fertilization by greatly improving ionic homeostasis (Riffat and Ahmad, 2018a) and biosynthesis of various osmolytes (Riffat and Ahmad, 2018b), compared to the salt-sensitive Pak Afgoi 2003. However, the application of sulfur greatly improved the salt-tolerance potential of Pak Afgoi 2003 by improving nutrient and osmolyte contents. In the current study sulfur application improved salt tolerance in both maize genotypes by balancing various antioxidants.

This study revealed that ascorbic acid contents were reduced through increasing salt stress. Seth et al. (2007) and Mandhania et al. (2010) reported that imposition of salt stress reduced ascorbic acid contents in wheat. The current study also revealed a significant increase in ascorbic acid contents after the application of sulfur; perhaps because sulfur maintains an adequate amount of ascorbic acid in plants. Under stress conditions, sulfur-containing metabolites such as glutathione serve to synthesize ascorbic acid in plants so that they can endure harsh environmental conditions (Albrecht et al., 1990).

Imposition of salinity increased tocopherol contents in maize plants. Under salt stress, tocopherol concentration in plants begins to elevate, thereby protecting the membranes via reactive oxygen species scavenging (Havaux et al., 2005). Shao et al. (2007) found that salt stress increased tocopherol concentrations. In the present study, sulfur application worked synergistically with salinity to improve tocopherol concentrations in maize plants. Glutathione is composed of sulfur which supports tocopherol in delimiting the reactive oxygen species formed during salt-stress conditions and that may be the reason for this finding (Foyer and Noctor, 2003).

The results of present study revealed that salt stress increased total phenolic content in maize plants. Navarro et al. (2006) found that salinity enhanced the activity of phenolics in pepper plants. Phenolic is a very powerful antioxidant that scavenges reactive oxygen species and retards the conversion of hydroperoxides to free radicals in salt stress conditions (Pearse et al., 2005). In this study, the application of sulfur also improved phenolic contents to induce salt tolerance in maize plants. Juan et al. (2008) reported a rise in phenolic contents after increasing sulfur concentrations in mustard plants.

**Table 4.** Mean squares from analysis of variance (ANOVA) of the data for oxidative stress determinants of maize subjected to different levels of salinity and sulfur.

| SOV | df | Leaf MDA | Shoot MDA | Root MDA | Leaf $H_2O_2$ | Shoot $H_2O_2$ | Root $H_2O_2$ |
|---|---|---|---|---|---|---|---|
| Variety (V) | 1 | 5192.79 *** | 1472.66 *** | 5704.16 *** | 0.033 *** | 0.0098 *** | 0.018 *** |
| Salinity (Sa) | 2 | 1101.41 *** | 2084.03 *** | 1294.59 *** | 0.030 *** | 0.026 *** | 0.015 *** |
| Sulfur (S) | 2 | 785.02 *** | 1447.38 *** | 979.21 *** | 0.011 *** | 0.0082 *** | 0.0073 *** |
| V × Sa | 2 | 241.51 *** | 45.055 ** | 154.16 *** | 0.000002 ns | 0.0002 ns | 0.0002 * |
| V × S | 2 | 57.46 *** | 16.16 ns | 17.16 * | 0.000008 ns | 0.0002 ns | 0.002 ns |
| Sa × S | 4 | 14.16 * | 68.24 *** | 2.82 ns | 0.0001 ns | 0.0003 ** | 0.0001 *** |
| V × Sa × S | 4 | 2.13 ns | 4.88 ns | 17.16 * | 0.000009 ns | 0.0004 ** | 0.008 ns |
| Error | 36 | 4.72 | 6.08 | 4.93 | 0.00009 | 0.00006 | 0.00004 |

*, **, *** = significant at 0.05, 0.01, and 0.001 levels, respectively. ns = nonsignificant
Abbreviations: MDA = malondialdehyde, $H_2O_2$ = hydrogen peroxide.

This study revealed that salinity increased lycopene contents in both maize genotypes. Lycopene scavenges reactive oxygen species under salt-stress conditions (Borghesi et al., 2011). Krauss et al. (2006) reported that salinity causes increases in lycopene and β-carotene in tomato fruit. Sulfur application also increased lycopene contents, inducing salinity tolerance in maize plants; perhaps because the antioxidant properties of lycopene are very important for alleviating the toxic effects of salinity (Zelená et al., 2009).

Imposition of salinity increased carotenoid contents in maize plants. Misra et al. (1997) reported that salt stress caused a rise in carotene contents in rice. Carotenoid is a very powerful antioxidant that scavenges reactive oxygen species, preventing oxidative damage under saline conditions (Abogadallah, 2010). In this study, application of sulfur helped improve carotenoid contents, inducing salt tolerance in maize plants. Kopsell et al. (2007) found that sulfur application enhanced carotenoid production in crops.

The current results revealed that superoxide dismutase activity increased under salt stress conditions in the salt-tolerant maize cultivar; in the salt-sensitive maize variety superoxide dismutase contents decreased with increasing salt levels. Tang et al. (2010) found an increase in superoxide dismutase in maize plants when they increased the stress conditions, thus implicating tolerance against abiotic stresses. Song et al. (2006) reported that superoxide dismutase contents increased at 1.5% salt concentrations; increasing salinity led to a decrease in superoxide dismutase contents in *Ulmus pumila*, demonstrating its salt sensitivity. In this study, application of sulfur increased superoxide dismutase activity in maize plants. Chandra and Pandey (2014a) reported an increase in superoxide dismutase contents as a result of increase in sulfur concentrations. However, the highest level of applied sulfur decreased superoxide dismutase contents in maize plants. Soldatinl et al. (1992) found reductions in superoxide dismutase contents through elevating sulfur concentrations.

Peroxidase contents increased in salt-tolerant maize plants and decreased under increasing salt concentrations in the salt-sensitive maize variety. Rahnama and Ebrahimzadeh (2005) found that in saline conditions, salt-tolerant potato cultivars showed high peroxidase activity. Similarly, Noreen et al. (2009) found that peroxidase activity rose due to salinity in sunflower. However, in salt-sensitive barley cultivars, increasing the salt concentration reduced peroxidase activity (M'barek et al., 2007). In the current study, adequate sulfur applications increased peroxidase activity; higher sulfur levels reduced peroxidase content in maize plants. Sulfur supply has a direct influence on peroxidase content in plants. Sulfur-stressed plants face an imbalance in antioxidant content (Chandra and Pandey, 2014b).

In this study catalase contents increased in the salt-tolerant maize variety, while catalase contents were reduced by increasing the salt levels in the salt-sensitive variety. Under salt-stress conditions catalase activity became high in order to induce salinity tolerance in plants (Mallik et al., 2011). The current results revealed that applications of sulfur significantly increased catalase concentrations in maize plants. Kumawat et al. (2006) found that sulfur increased catalase concentrations in mung bean.

Malondialdehyde was increased by increasing salt levels (Lutts et al., 1996). The application of sulfur helped to maintain the appropriate concentration of malondialdehyde in maize plants; perhaps because sulfur is an important part of various antioxidants that scavenge free radicals produced by reactive oxygen species. Glutathione is a sulfur-containing compound that

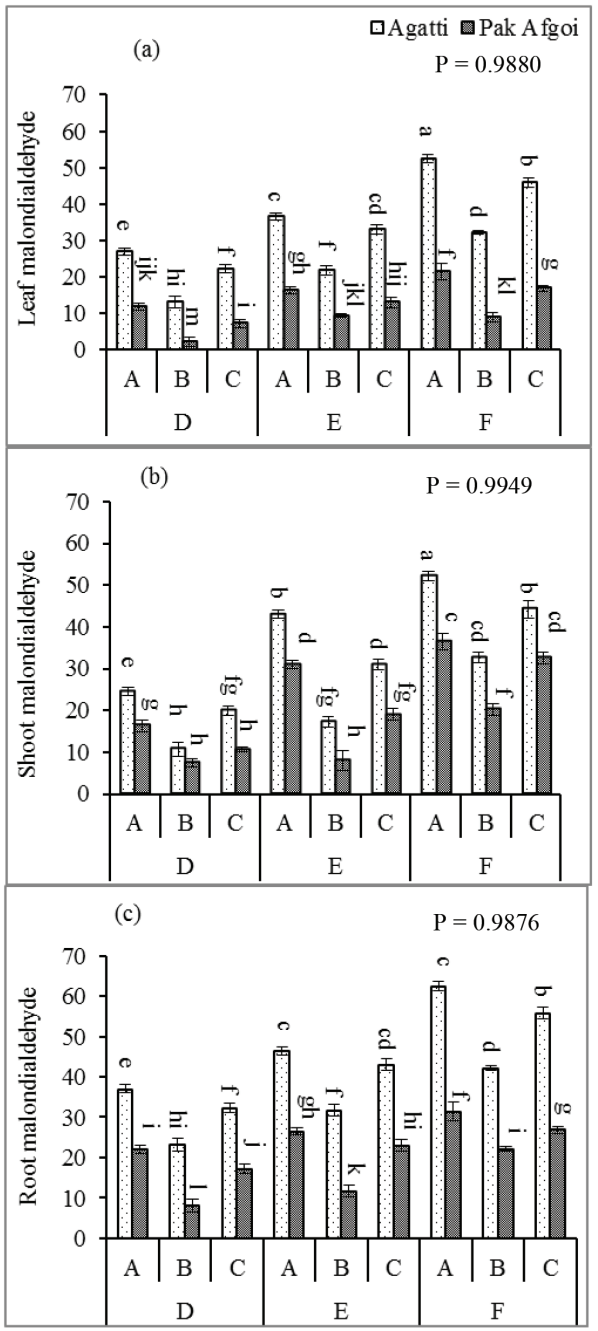


**Figure 9.** Effect of different levels of sulfur (A = 0 mM, B = 40 mM, C = 80 mM) on malondialdehyde content (nmol/g fresh weight) of different maize (*Zea mays* L.) cultivars under saline conditions (D = 0 mM NaCl, E = 25 mM NaCl, F = 75 mM NaCl). Vertical lines on each bar represent standard error. Means with different letters are significantly different (Student–Newman–Keuls, P > 0.001).

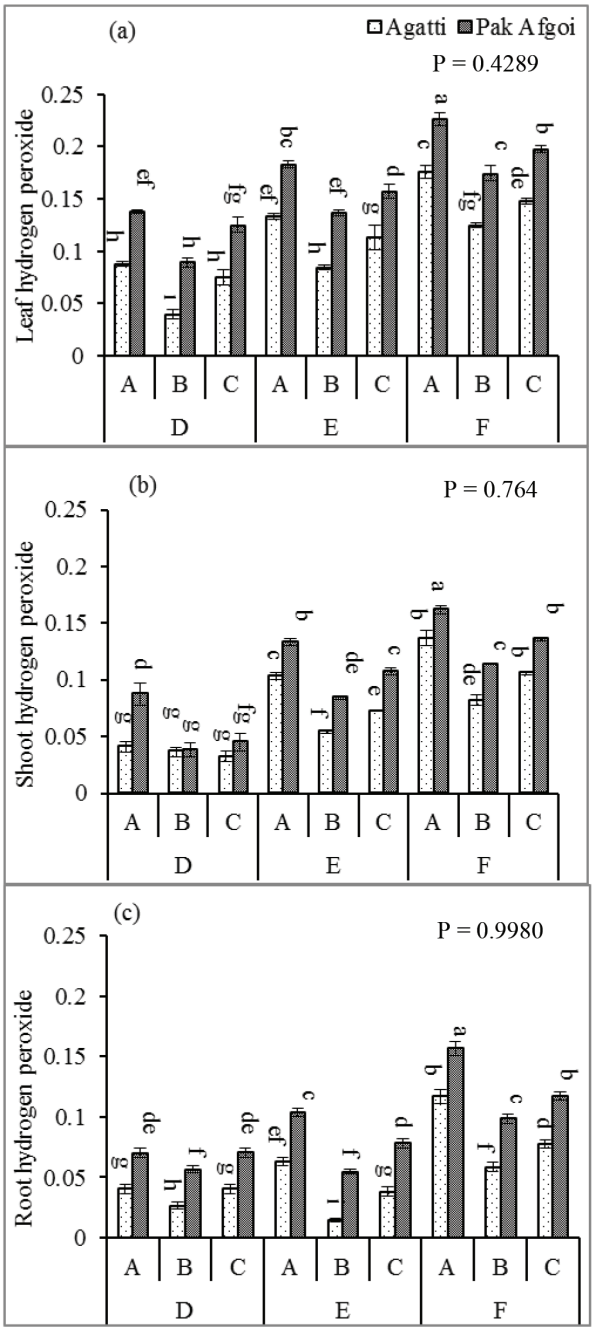


**Figure 10.** Effect of different levels of sulfur (A = 0 mM, B = 40 mM, C = 80 mM) on hydrogen peroxide content (nmol/g fresh weight) of different maize (*Zea mays* L.) cultivars under saline conditions (D = 0 mM NaCl, E = 25 mM NaCl, F = 75 mM NaCl). Vertical lines on each bar represent standard error. Means with different letters are significantly different (Student–Newman–Keuls, P > 0.001).

scavenges the reactive oxygen species synthesized during lipid peroxidation under salt-stress conditions (Most and Papenbrock, 2015).

In this study, hydrogen peroxide concentrations increased greatly under salt-stress conditions. Miller et al. (2010) and Quan et al. (2008) found that salinity greatly

increased the hydrogen peroxide contents in plants. In this study, application of a low level of sulfur (40 mM) lowered hydrogen peroxide in maize plants; perhaps because sulfur controls hydrogen peroxide concentration in plants. At high levels of sulfur, hydrogen peroxide concentrations also increased, while low concentrations of sulfur lowered the hydrogen peroxide concentrations in onion plants (Chandra and Pandey, 2014b).

From the discussion above it becomes amply clear that under salt-stress conditions, a rise in reactive oxygen species takes place, and they are scavenged by various antioxidants. However, excess antioxidants are harmful because reactive oxygen species also serve beneficial functions for plant growth. Sulfur was very helpful for balancing antioxidants in plants by lowering the production of reactive oxygen species and oxidative stress determinants. Moreover, sulfur applications also helped to develop salt tolerance in salt-sensitive maize cultivars. Hence, a low level of sulfur (40 mM) is very effective in balancing various antioxidants and supports prevention of oxidative damage in plants under saline conditions.

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