Changes in Organic and Inorganic Osmolytes of Maize (*Zea ma*ys L.) by Sulfur Application Under Salt Stress Conditions

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Abstract

Mineral nutrients have favourable potential in alleviation of salinity problem in plants. Sulfur has specific functions in regulating plant growth, metabolism, enzymatic reactions and osmolyte homeostasis in plants. Hence, an experiment was carried out to explore the role of sulfur in ameliorating salt toxicity in maize by changes in organic and inorganic osmolyte contents. A range of sulfur levels (40, 80 mM) were used to induce salinity tolerance in maize. Various treatments of salinity (25, 75 mM) were applied by using sodium chloride. Results revealed that glycine betaine, proline, total soluble sugars, total soluble proteins and total free amino acids contents were increased by applying salinity while the application of sulfur lowered the proline and increased other studied organic osmolyte contents in all studied maize organs (leaf, shoot, root). The maximum improvement in organic osmolyte contents were found at 40 mM sulfur, however, at 80 mM sulfur proline contents were reduced. Applied salinity increased leaf tissue concentration of Na^+ and decreased that of K^+, Ca^{2+}, NO_3^-, PO_4^{3-}, SO_4^{2-} leading to a severely declined in K^+/Na and Ca^{2+}/Na^+ ratio. However, application of sulfur reduced the Na^+ contents and improved K^+, Ca^{2+}, NO_3^-, PO_4^{3-}, SO_4^{2-}, K^+/Na^+ and Ca^{2+}/Na^+ ratio in the salinity grown plants. Moreover, 40 mM level of sulfur was greatly effective in osmolyte homeostasis at all levels of salinity. This indicated that use of sulfur (40 mM) ameliorated the effect of salinity by changing organic and inorganic osmolyte contents in maize plants.

Keywords: ions, maize, osmolytes, sulfur, salinity

1. Introduction

Among various abiotic stresses, salt stress has affected 20% of land used for cultivation and 33% of the irrigated land throughout the world (Machado & Serralheiro, 2017). Overall, 10 million ha of the world land has been degraded due to salinity each year (Pimentel et al., 2004). Salt stress causes disturbances in physiological, biochemical, molecular processes in the plant (Nahar et al., 2016). As a result osmotic stress, imbalance in nutrient transport and accumulation of reactive oxygen species takes place (Iqbal et al., 2014; Puniran-Hartley et al., 2014). In such conditions, plants synthesize and accumulate various organic and inorganic osmolytes or osmoprotectants. These include proline, glycine betaine, glucose, isoleucine, mannitol and proteins (Parida & Das, 2005) and various inorganic nutrients (K^+, Ca^{2+}, NO_3^-, PO_4^{3-}, SO_4^{2-}). The functions of these osmolytes are, to balance the ionic transport across the plant cell, scavenge reactive oxygen species, regulate enzyme activity and prevent membrane disintegration (Nahar et al., 2016). However, such strategies are needed that balances the concentrations of various osmolytes for maintaining plant metabolism. As higher concentration of osmolytes become toxic for plant cell.

Sulfur plays a significant role in balancing the osmolyte contents in the plants. Sulfur is a basic constituent of many important compounds that maintain plant growth and development in stress conditions. These compounds include glutathione, vitamins, phytoharmones and various co-enzymes (Spadaro et al., 2010). Sulfur helps in coordination among different physiological and biochemical processes in the plants. Hence, Sulfur improves the cellular function by balancing the organic and inorganic osmolytes that develops salt tolerance in crop plants (Taiz & Zeiger, 2006; Nazar et al., 2014; Riffat & Ahmad, 2016).

After wheat and rice, maize is very important cereal crop in the world. It is also known the 'king of crops'. It contains many types of vitamins and nutrients. Due to its nutritional importance it has become a valuable food and feed crop in many countries of world. It is used for making bread, cake and porridge. Also it is an important constitute of livestock and poultry diet (Bukhsh et al., 2011). However, the production and quality of maize is

seriously affected by salinity as maize is moderately sensitive to salinity (Farooq et al., 2015). Therefore, such methods should be devised that increase the salt tolerance of this valuable crop to meet the growing food demand.

Hence, this study focuses on the improvement in salt tolerance potential of maize by sulfur application. To maintain the balance of organic and inorganic osmolytes for development of salt tolerance is another objective of this study.

2. Method

2.1 Plan of Study

A study was conducted to determine the role of sulfur in enhancing salt tolerance by changing the osmolyte contents in maize. The seeds of maize cultivars (Agaitti, 2003; Pak Afgoi, 2003) were acquired from Maize and Millet Institute Sahiwal Pakistan. The seeds were sorted and 10 uniform seeds were sown in plastic pots filled with 10 kg soil.

2.2 Treatment Application

Salinity (25, 75 mM) was applied by using sodium chloride. Various levels of sulfur (40, 80 mM) were applied by using potassium sulfate. Both treatments were applied at sowing time. After 15 days of treatment application, sulfur (40, 80 mM) was applied as foliar spray. Then 45 days plants were harvested for the determination of various biochemical attributes.

2.3 Determination of Organic Osmolytes

2.3.1 Glycine Betaine

Grieve and Grattan (1983) proposed a procedure for the determination of glycine betaine contents. Two reagents 2N H_2SO_4 and IK-I_2 were prepared. 2N H_2SO_4 was prepared by mixing 5.6 mL of 36 M H_2SO_4 and distilled water was used for making final volume100 mL. IK-I_2 was made by mixing 20 g of potassium iodide, 100 mL water and 15.7 g of iodine. Glycine betaine contents were determined by grounding 0.5 g dried plant material in 20 mL of deionized water and shaken for 24 h at 25 °C. The extract was filtered and diluted with 2 N H_2SO_4 in 1:1 ratio. Then 0.5 mL extract was put in centrifuge tube and kept in ice cooled water for 1 hour followed by addition of 1 mL of IK-I_2, and vortexed at 0 °C at 10,000 g for 15 min. The supernatant was collected and dissolved in 9 mL of 1-2 dichloroethane. The solution was kept at room temperature for 2-2.5 h. The absorbance of glycine betaine was noted at 365 nm by using spectrophotometer (UV-1100). The values were compared with standard curve.

2.3.2 Proline

Proline contents in plants were determined by the procedure proposed by Bates et al. (1973). Firstly, some reagents were prepared. 6 M phosphoric acid was prepared by diluting 407 mL of 85 % phosphoric acid in 1000 mL distilled water. For the preparation of acid-ninhydrin, 1.25 g of ninhydrin was dissolved in 30 mL glacial acetic acid and 20 mL of 6 M phosphoric acid. 3% sulfuric acid was made by mixing 3 g of sulphosalicylic acid in 100 mL of distilled water. For the determination of proline contents in plant material 0.1 g fresh plant sample was homogenised in 10 mL of 3% sulphosalicylic acid and filtered. Then 2 mL of acid ninhydrin, 2 mL of glacial acetic acid and 1 mL of filterate was heated in water bath at 100 °C for 1 hour and then transferred to ice bath following the addition of 4 mL of toluene. The reaction mixture was vortexed, chromophore having free proline was separated in test tube, kept at room temperature and the proline contents were measured at 520 nm on spectrophotometer (UV-1100). For blank, same procedure was used by using 2 mL of 3% aqueous sulphosalycylic acid. Following formula was used for proline determination.

$$\mu moles \frac{proline}{g} \text{ fresh weight} = \frac{\mu g\ proline/mL \times mL\ of\ toluene}{(115.5\ \mu g/mole)/g\ sample/5} \tag{1}$$

2.3.3 Soluble Sugars

For the determination of soluble sugars, the procedure given by Yoshida et al. (1976) was followed. Anthrone reagent was made by mixing 1 g anthrone in 1 L conc. H_2SO_4. For the determination of soluble sugars, 0.1 g fresh plant material was boiled in 5 mL distilled water and the filtrate was diluted to 50 mL with distilled water. To 1 mL of the filtrate, 5 mL of anthrone reagent was added and heated at 90 °C for 20 min. The soluble sugar contents were determined at 620 nm by using spectrophotometer (UV-1100). For standard curve, glucose series (0, 20, 40, 60, 80 and 100 μM) was used.

2.3.4 Total Free Amino Acids

Total free amino acids in plant tissues were measured by the procedure of Hamilton and Van-Slyke (1943). 2% ninhydrine and 10% pyridine solution were prepared in the distilled water. For the determination of total free amino acids, 1 g fresh plant sample was homogenised in 10 mL of phosphate buffer (0.2 M with pH 7.2). To 1 mL of the extract, 1 mL of pyridine (10%) and 1 mL of ninhydrine (2%) were mixed and heated at 100 ^{o}C in water bath for 30 min. The volume was maintained 50 mL with distilled water and the absorbance was noted at 570 nm by using spectrophotometer (UV-1100). Following formula was used for calculating total free amino acid.

$$\text{Total amino acid (mg/g fresh weight)} = \frac{\text{Graph reading of sample} \times \text{Volume of sample} \times \text{Dilution factor}}{\text{Weight of the tissue x 1000}} \qquad (2)$$

2.3.5 Total Soluble Proteins

The concentration of total soluble protein was determined by the method given by Bradford (1976). Phosphate buffer saline was prepared by mixing 2.7 mM KCl, 10 mM Na_2HPO_4, 1.37 mM NaCl and 2 mM KH_2PO_4 and pH 7.2 was maintained by using HCl. The determination of total soluble protein was done by extracting the 0.5 g fresh plant material in phosphate buffer saline, centrifugation was done and the supernatant was collected. To equal volume of supernatant dye stock was dissolved, vortexed and kept in an incubator for 30 min. The absorbance was noted at 595 nm by using spectrophotometer (UV-1100). The standard curve was drawn by using bovine serum albumin (BSA) of the range (10 to 50 µg mL^{-1}).

2.4 Determination of Inorganic Osmolytes

2.4.1 Sodium, Potassium, Calcium (Na^+, K^+, Ca^{2+})

The dried plant sample (0.5 g) was incubated in 5 mL H_2SO_4 overnight and heated at 350 °C in the digestion block for 30 min. The mixture was cooled; 1 mL of H_2O_2 was added and again heated for 20 min. These steps were repeated until clear solution was obtained, filtered, and volume was maintained to 50 mL by using distilled water (Wolf, 1982). This extract was used for the determination of Na^+, K^+, Ca^{2+} ions by using flame photometer (Jenway PFP-7). For standard curve a series of standards (10, 20 to 100 ppm of Na^+, K^+ and Ca^{2+}) was prepared. The actual values were calculated by comparing the values from standard curve and from flame photometer.

2.4.2 Phosphate (PO_4^{2-})

The concentration of phosphate ions in plant tissues was determined by following the method of Yoshida (1976). Firstly, two reagents were prepared. For the preparation of molybdate-vanadate solution, 25 g ammonium molybdate was mixed in 500 mL of water, and 1.25 g of ammonium vanadate was mixed in 500 mL of 1N HNO_3 separately, then equal volumes of two solutions were mixed together. For the preparation of nitric acid (2 N), 10 mL of concentrated HNO_3 was mixed in 80 mL of distilled water. The phosphate content was determined by boiling 0.5 g dried plant sample in 5 mL distilled water for 1 h, filtered and 50 mL volume was prepared by using distilled water. 1 mL of extract was mixed with 2 mL of 2 N HNO_3, volume was maintained to 4 mL with distilled water, 1 mL of molybdate-vanadate reagent was added and the mixture was diluted to 10 mL with distilled water, vortexed, allowed to stand for 20 min and absorbance was noted at 420 nm by using spectrophotometer (UV-1100). For standard curve, stock solution of 25 mg/L PO_4^{3-} was prepared by mixing 0.11 g monobasic phosphate (KH_2PO_4) in 1 L distilled water and standard series was prepared by mixing 1, 2, 3, 4, 5 and 6 mL of 25 mg/L PO_4^{3-} and diluted to 8 mL with distilled water.

2.4.3 Nitrate (NO_3^-)

For the determination of nitrate contents a procedure proposed by Kowalenko and Lowe (1973) was used. The reagents were prepared. For the preparation of 0.01% TCA, 0.1% CTA stock was prepared. For this purpose, 0.247 g of chromotropic acid disodium salt (CTA) was dissolved in 100 mL of conc. H_2SO_4. Then 10 mL of CTA stock was diluted to 100 mL with H_2SO_4 for the preparation of 0.01% TCA. For the determination of nitrate contents, 0.5 g dried plant sample was boiled in 5 mL of distilled water for 1 h, filtered and diluted to 50 mL by using distilled water. 3 mL extract was mixed with 7 mL of working CTA solution, vortexed and absorbance was noted at 430 nm after 20 min by using spectrophotometer (UV-1100). Water was used for blank. For standard, 0.7216 g of KNO_3 was dissolved in 1 L distilled water for the preparation of 100 mg/L NO_3^- stock solution, then a graded series (10, 20, 30, 40, 50 and 100 mg/L NO_3^-) was prepared by diluting the stock solution.

2.4.4 Sulfate (SO_4^{2-})

For the determination of sulfate contents in plant sample, a procedure given by Tendon (1993) was used. Firstly, two reagents barium chloride/polyvinyl alcohol and acid mixture were prepared. For the preparation of barium

chloride/polyvinyl alcohol, 60 g of $BaCl_2 \cdot 2H_2O$ was dissolved in 500 mL distilled/deionized water and 2 g polyvinyl alcohol was dissolved in 400 mL distilled water separately. The two solutions were mixed, filtered and volume was maintained to 1 L by using distilled water. Acid mixture was prepared by mixing 50 mL of glacial acetic acid, 20 mL of 85% orthophosphoric acid, 6 mL of concentrated sulfuric acid/water (with a ratio of 1:1000) and 800 mL of distilled water was thoroughly mixed and diluted to 1 L by using distilled water. Sulfate contents were determined by 5 mL of the sample solution, 5 mL of the acid mixture and 5 mL of barium chloride/polyvinyl alcohol was mixed, allowed to stand for 30 seconds and aliquot was collected for the determination of absorbance at 420 nm by using spectrophotometer (UV-1100).

2.5 Statistical Analysis

The experimental design was completely randomized (CRD) with three factor factorial arrangement. The data was analyzed statistically by analysis of variance technique (ANOVA) (Steel & Torrie, 1986) by using Co-Stat software (CoHort Software, 2003, Monterey, California). Microsoft excel was used for the preparation of figures.

3. Results

3.1 Organic Osmolytes

3.1.1 Glycine Betaine

Results revealed that salt stress caused the accumulation of glycine betaine contents in both maize cultivars. The maximum concentration of glycine betaine was found at 75 mM salt level in all tissues of maize plants (root, shoot, leaf). It was evident from statistically significant V × Sa interactive effect in shoot and root while in leaf this interaction was non-significant (Table 1). Application of sulfur improved the glycine betaine contents in both maize varieties. It was shown by significant V × Sa interactive effect in leaf. However, in leaf and shoot this interaction was found non-significant (Table 1). Sulfur at 40 mM level highly improved the glycine betaine contents in root, shoot and leaf of both maize varieties at all salt levels (25, 75 mM) (Figure 1). It was evident by significant Sa × S interaction. However, in root and leaf Sa × S interaction was found non-significant (Table 1). The order of decreasing the glycine betaine contents in plant tissue was leaf > shoot > root. Salt tolerant variety (Agaitti, 2003) accumulated high glycine betaine contents as compared to salt sensitive variety (Pak Afgoi, 2003).
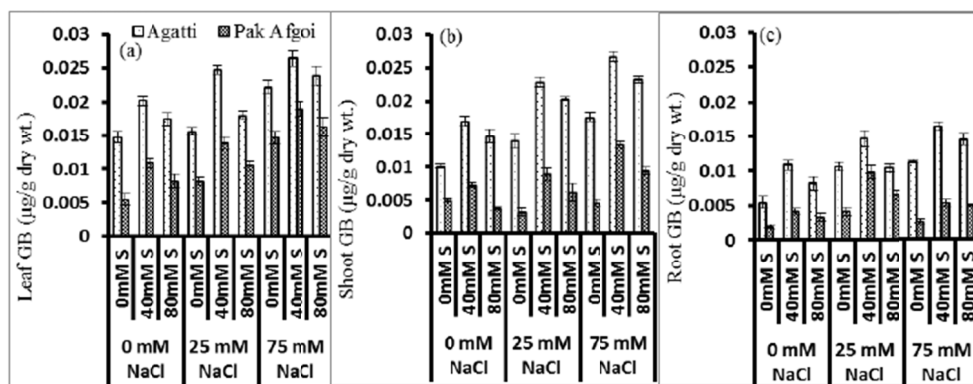

Figure 1. Effect of different levels of sulfur (S) on glycine betaine content in leaf (a) shoot (b) root (c) of different maize (*Zea mays* L.) cultivars under saline conditions

Table 1. Mean squares from analysis of variance (ANOVA) of the data for glycine betaine and proline contents of maize subjected to different levels of salinity and sulfur

SOV	Df	Leaf GB	Shoot GB	Root GB	Leaf Proline	Shoot Proline	Root Proline
Variety (V)	1	9.68e-4 ***	0.0018 ***	6.04e-4 ***	1.65e-6 ***	2.03e-6 ***	1.24e-6 ***
Salinity (Sa)	2	2.72e-4 ***	1.70e-4 ***	7.96e-5 ***	6.41e-7 ***	1.14e-7 ***	2.34e-7 ***
Sulfur (S)	2	1.46e-4 ***	2.15e-4 ***	7.96e-5 ***	5.52e-7 ***	1.36e-7 ***	2.75e-7 ***
V × Sa	2	3.71e-6 ns	3.08e-5 ***	3.49e-5 ***	1.04e-38 ns	7.47e-9 ns	3.86e-8 ***
V × S	2	1.85e-6 ns	1.38e-5 ***	2.62e-6 ns	1.04e-38 ns	7.29e-10 ns	5.37e-9 *
Sa × S	4	4.71e-6 ns	8.98e-6 ***	3.08e-6 ns	1.25e-8 *	2.56e-9 ns	1.24e-8 ***
V × Sa × S	4	1.85e-6 ns	2.55e-6 ns	2.76e-6 ns	3.32e-38 ns	1.79e-9 ns	6.40e-9 **
Error	36	2.60E-06	1.52E-06	1.37E-06	4.46E-09	3.07E-09	1.36E-09

Note. *, **, *** = significant at 0.05, 0.01 and 0.001 levels, respectively. ns = non-significant.

Abbreviation: Exponent (e), GB = Glycine Betaine.

3.1.2 Proline

Statistical analysis has shown that proline contents were increased by increasing the salinity in both maize cultivars. It was evident from significant V × Sa interaction for root, while in shoot and leaf V × Sa interaction was found non-significant (Table 1). At 75 mM salt level proline contents were high in both studied maize cultivars. The decreasing order of proline contents was leaves > shoot > root (Figure 2). Application of sulfur at 80 mM level did not much improve the proline contents significantly. However sulfur application was synergistic to the salinity effect in accumulating the proline contents in maize organs. It was shown by significant Sa × S interaction for leaf and root while in shoot Sa × S interaction was found non-significant (Table 1). Pak Afgoi (2003) accumulated low proline contents as compared to Agaitti (2003). This variation in variety is shown by significant V × Sa × S interaction for maize root while for shoot and root V × Sa × S interactive effect was found non-significant indicating that sulfur application decreased the proline contents in maize plants in both varieties at all levels of treatment (40, 60 mM) (Figure 2).
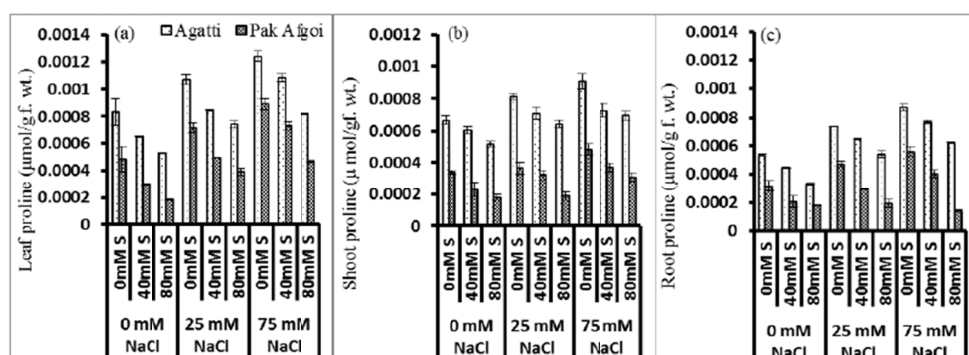

Figure 2. Effect of different levels of sulfur (S) on proline content in leaf (a) shoot (b) root (c) of different maize (*Zea mays* L.) cultivars under saline conditions

3.1.3 Total Soluble Sugar

A marked increase in total soluble sugar contents by salt application was found in both studied maize cultivars. It was shown by statistically significant V × Sa interaction for leaf, shoot and root (Table 2).
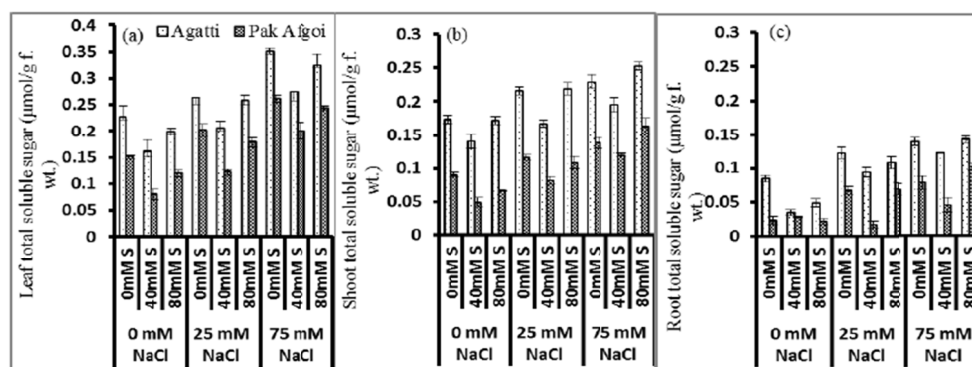
Figure 3. Effect of different levels of sulfur (S) on total soluble sugar content in leaf (a) shoot (b) root (c) of different maize (*Zea mays* L.) cultivars under saline conditions

Sulfur application (40, 80 mM) significantly improved the soluble sugar contents in all studied maize organs at all levels of salinity. It was shown by significant Sa × S interaction for shoot and root, however, for leaf Sa × S interaction was found non-significant (Table 2). Sulfur at 40 mM level significantly improved the total soluble sugar contents in both studied maize cultivars (Figure 3). It was evident form statistically significant V × Sa × S interaction in both maize varieties (Table 2).

Table 2. Mean squares from analysis of variance (ANOVA) of the data for total soluble sugars and total soluble protein contents of maize subjected to different levels of salinity and sulfur

SOV	df	Leaf TSS	Shoot TSS	Root TSS	Leaf TSP	Shoot TSP	Root TSP
Variety (V)	1	0.083 ***	0.11 ***	0.033 ***	0.021 ***	0.0053 ***	0.0042 ***
Salinity (Sa)	2	0.065 ***	0.021 ***	0.019 ***	0.0038 ***	0.0015 ***	8.14e-4 ***
Sulfur (S)	2	0.022 ***	0.0081 ***	0.0044 ***	0.0077 ***	0.0019 ***	0.0043 ***
V × Sa	2	5.39e-5 ns	1.91e-4 ns	0.0011 **	4.5e-4 ***	8.79e-5 *	1.93e-4 *
V × S	2	2.63e-5 ns	3.78e-4 ns	6.85e-4 *	1.71e-34 ns	1.67e-5 ns	7.91e-5 ns
Sa × S	4	1.18e-4 ns	6.0033e-4 *	6.84e-4 **	1.44e-4 *	1.07e-4 ***	9.33e-5 ns
V × Sa × S	4	1.45e-4 ns	9.22e-5 ns	7.72e-4 **	2.41e-34 ns	5.55e-5 *	6.88e-5 ns
Error	36	4.09E-04	1.72E-04	1.41E-04	5.14E-05	1.78E-05	5.61E-05

Note. *, **, *** = significant at 0.05, 0.01 and 0.001 levels, respectively. ns = non-significant.

Abbreviation: Exponent (e), TSS = Total Soluble Sugars, TSP = Total Soluble Protein.

3.1.4 Total Soluble Protein

Exposure to maize varieties to salinity increased the total soluble protein contents in both studied maize varieties. It was indicated by statistically significant V × Sa interaction (Table 2). At 75 mM salt level total soluble protein contents were high (Figure 4). The exogenous application of sulfur (40, 80 mM) improved the total soluble protein contents in both maize genotypes. However 40 mM sulfur level was found appropriate in improving the total soluble protein contents (Figure 4). It was shown by statistically significant Sa × S interaction for leaf and shoot and non-significant in root (Table 2). Agaitti (2003) accumulated more the total soluble protein contents as compared to Pak Afgoi (2003). It was revealed form significant V × Sa × S interaction for maize shoot. The maximum accumulation of the total soluble protein contents was found in maize leaves which decreased in shoot and root respectively (Figure 4).
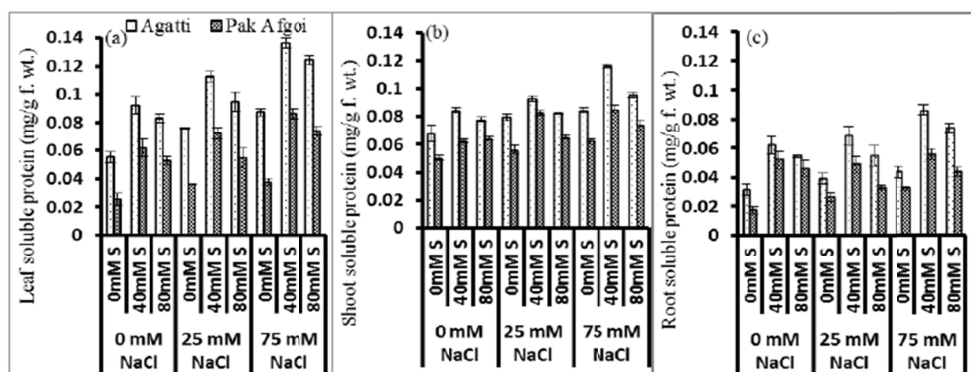
Figure 4. Effect of different levels of sulfur (S) on total soluble protein content in leaf (a) shoot (b) root (c) of different maize (*Zea mays* L.) cultivars under saline conditions

3.1.5 Total Free Amino Acid

Results revealed that salinity caused increase in total free amino acid contents in both studied maize cultivars. Maximum improvement in total free amino acid contents was found at 75 mM salt level (Figure 5). The application of sulfur (40 mM) improved the total free amino acid contents in all studied maize organs. It was shown by statistically significant Sa × S interaction for leaf and root and non-significant for shoot (Table 3).
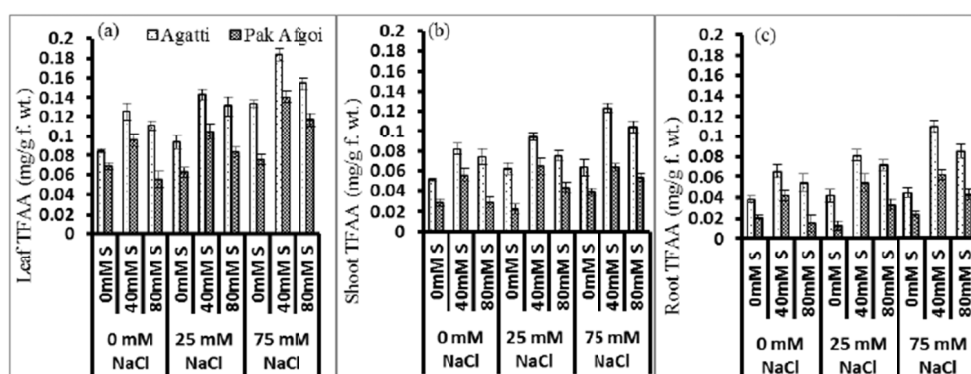

Figure 5. Effect of different levels of sulfur (S) on total free amino acid content in leaf (a) shoot (b) root (c) of different maize (*Zea mays* L.) cultivars under saline conditions

Salt tolerant maize cultivar (Agaitti, 2003) accumulated high total free amino acid contents as compared to salt sensitive variety (Pak Afgoi, 2003). It was evident form significant V × Sa × S interaction (Table 3). The order of accumulation of total free amino acid contents in maize organs was leaf > shoot > root (Figure 5).

Table 3. Mean squares from analysis of variance (ANOVA) of the data for total soluble sugars and total free amino acid contents of maize subjected to different levels of salinity and sulfur

SOV	df	Leaf TFA	Shoot TFA	Root TFA
Variety (V)	1	0.021 ***	0.018 ***	0.013 ***
Salinity (Sa)	2	0.0092 ***	0.0020 ***	0.0023 ***
Sulfur (S)	2	0.0093 ***	0.0058 ***	0.0067 ***
V × Sa	2	1.74e-4 ns	2.42e-4 ns	1.23e-4 ns
V × S	2	1.92e-4 ns	2.30e-4 ns	3.24e-4 ns
Sa × S	4	3.61e-4 *	1.14e-4 ns	3.98e-4 **
V × Sa × S	4	3.49e-4 *	2.62e-4 *	8.98e-5 ns
Error	36	1.20E-04	8.97E-05	1.01E-04

Note. *, **, *** = significant at 0.05, 0.01 and 0.001 levels, respectively. ns = non-significant.

Abbreviation: Exponent (e), TFF = Total Free Amino Acid.

3.2 Inorganic Osmolytes

3.2.1 Sodium (Na$^+$)

Results revealed that salinity increased the sodium (Na$^+$) contents in both studied maize cultivars. It was shown by statistically significant V × Sa interaction for shoot. However, in root and leaf, V × Sa interaction was found non-significant (Table 4). At 75 mM salt level, sodium (Na$^+$) contents very found very high (Figure 6).
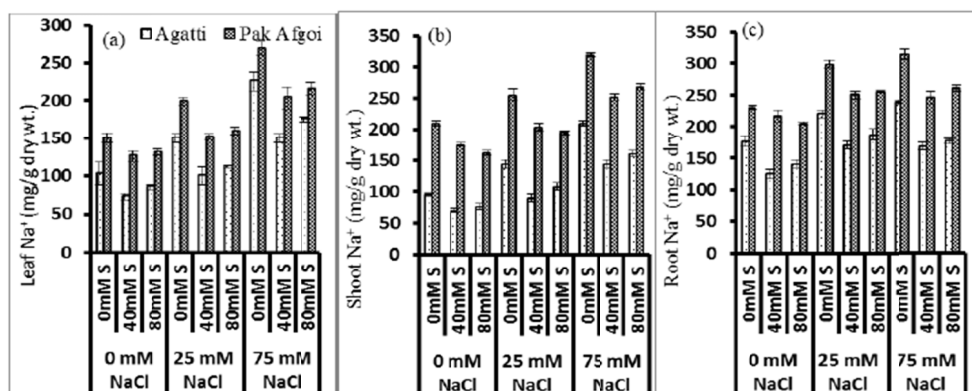

Figure 6. Effect of different levels of sulfur (S) on Na$^+$ content in leaf (a) shoot (b) root (c) of different maize (*Zea mays* L.) cultivars under saline conditions

The application of sulfur (40, 80 mM) significantly lowered the sodium (Na$^+$) contents in all studied maize organs at both studied salt levels (25, 75 mM). It was evident from statistically significant Sa × S interactive effect (Table 4). Leaf had high sodium contents as compared to shoot and root. Moreover, sulfur at 40 mM level lowered the sodium (Na$^+$) contents in Agaitti (2003) as compared to Pak Afgoi (2003) (Figure 6).

Table 4. Mean squares from analysis of variance (ANOVA) of the data for sodium (Na$^+$) and potassium (K$^+$) contents of maize subjected to different levels of salinity and sulfur

SOV	df	Leaf Na$^+$	Shoot Na$^+$	Root Na$^+$	Leaf K$^+$	Shoot K$^+$	Root K$^+$
Variety (V)	1	30706.87 ***	147796.33 ***	75298.84 ***	285871.13 ***	322634.74 ***	29 ***
Salinity (Sa)	2	41258.27 ***	41078.29 ***	15390.76 ***	101395.78 ***	87805.52 ***	58592.55 ***
Sulfur (S)	2	11438.75 ***	13295.002 ***	13069.02 ***	54869.21 ***	68620.39 ***	43139.16 ***
V × Sa	2	19.27 ns	66.51 ns	82.94 ns	554.57 **	3603.46 *	3531.72 *
V × S	2	93.05 ns	438.54 **	224.82 ns	209.68 ns	170.12 ns	56.72 ns
Sa × S	4	776.68 **	551.33 ***	531.58 **	183.66 ns	681.06 ns	657.90 ns
V × Sa × S	4	10.05 ns	108.38 ns	229.44 ns	116.46 ns	382.26 ns	1077.44 ns
Error	36	194.22	78.64	120.17	100.95	844.67	810.17

Note. *, **, *** = significant at 0.05, 0.01 and 0.001 levels, respectively. ns = non-significant.

Abbreviation: Exponent (e), Na$^+$ = Sodium, K$^+$ = Potassium.

3.2.2 Potassium (K$^+$)

Statistical analysis revealed that salinity reduced the potassium (K$^+$) contents in shoot, leaf and root of both maize cultivars (Figure 7). It was evident form statistically significant V × Sa interaction (Table 4). At 40 mM sulfur level, potassium (K$^+$) contents very improved in both varieties (Agaitti, 2003; Pak Afgoi, 2003) (Figure 7). However, both levels of sulfur (40, 80 mM) improved the potassium (K$^+$) contents at both levels of salt treatment (25, 75 mM) in both maize cultivars. Maximum potassium (K$^+$) contents were found in leaf then shoot and root respectively (Figure 7).
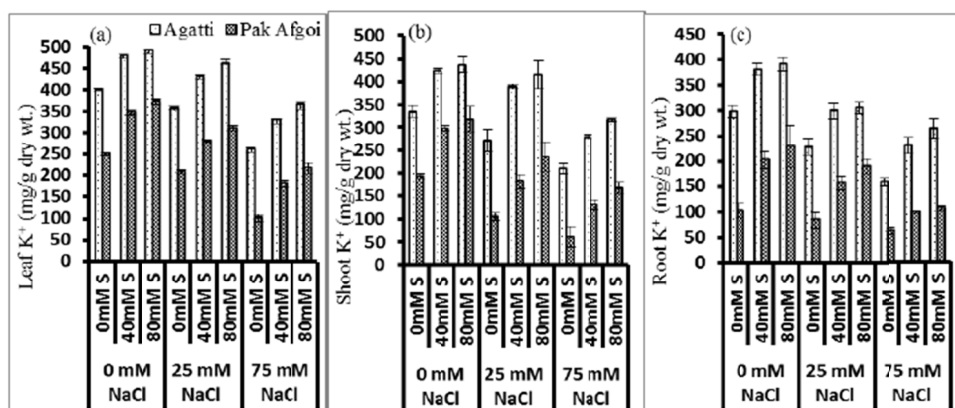
Figure 7. Effect of different levels of sulfur (S) on K^+ content in leaf (a) shoot (b) root (c) of different maize (*Zea mays* L.) cultivars under saline conditions

3.2.3 Calcium (Ca^{2+})

Calcium (Ca^{2+}) contents were reduced by salt application (25, 75 mM) in all studied maize organs (leaf, shoot, root) (Figure 8). It was evident from significant V × Sa interaction for leaf and shoot, however in root V × Sa interaction was found non-significant.
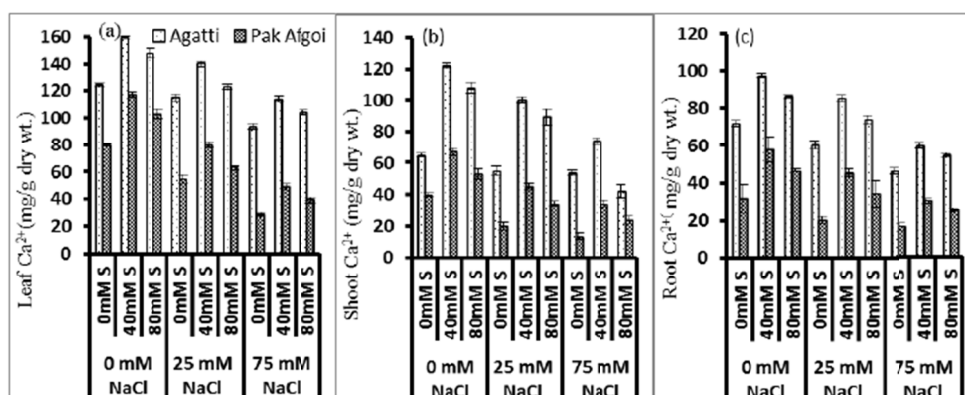

Figure 8. Effect of different levels of sulfur (S) on Ca^{2+} content in leaf (a) shoot (b) root (c) of different maize (*Zea mays* L.) cultivars under saline conditions

The exogenous application of sulfur (40, 80 mM) improved the calcium (Ca^{2+}) contents in both studied maize cultivars. It was shown by statistically significant Sa × S and V × S interaction in leaf and shoot while in root this interaction was found non-significant (Table 5). Also both levels of sulfur (40, 80 mM) lowered the toxic effects of salinity by improving the calcium (Ca^{2+}) contents at higher levels of salinity in salt tolerant (Agaitti, 2003) and salt sensitive (Pak Afgoi, 2003) cultivars (Figure 8). It was revealed from statistically significant V × Sa × S interaction (Table 5).

Table 5. Mean squares from analysis of variance (ANOVA) of the data for calcium (Ca^{2+}) and K^+/Na^+ ratio of maize subjected to different levels of salinity and sulfur

SOV	df	Leaf Ca^{2+}	Shoot Ca^{2+}	Root Ca^{2+}	Leaf K^+/Na^+ ratio	Shoot K^+/Na^+ ratio	Root K^+/Na^+ ratio
Variety (V)	1	42887.33 ***	23856.01 ***	18113.35 ***	55.23 ***	15.99 ***	7.34 ***
Salinity (Sa)	2	11499.46 ***	5691.72 ***	3126.76 ***	31.44 ***	9.17 ***	3.61 ***
Sulfur (S)	2	3336.05 ***	4811.67 ***	2128.28 ***	9.59 ***	3.52 ***	1.46 ***
V × Sa	2	536.49 ***	317.62 ***	148.35 *	4.30 ***	0.42 ***	0.25 ***
V × S	2	1.24 ns	314.35 ***	0.018 ns	1.30 **	0.14 **	0.10 ***
Sa × S	4	145.27 ***	399.34 ***	66.67 ns	0.45 ns	0.11 **	0.065 **
V × Sa × S	4	1.24 ns	295.96 ***	0.018 ns	0.097 ns	0.022 ns	0.018 ns
Error	36	14.78	22.01	31.71	0.19	0.027	0.012

Note. *, **, *** = significant at 0.05, 0.01 and 0.001 levels, respectively. ns = non-significant.

Abbreviation: Exponent (e), Ca^{2+} = Calcium, Na^+ = Sodium, K^+ = Potassium.

3.2.4 K^+/Na^+

Salinity caused the reduction in K^+/Na^+ ratio in salt tolerant and salt sensitive maize cultivars. Maximum reduction in K^+/Na^+ ratio was found at 75 mM salt applied (Figure 9). However, sulfur application significantly improved the K^+/Na^+ ratio in both varieties. It was evident from statistically significant V × S interaction for all studied maize organs (Table 5). Sulfur also developed salt tolerance in maize plants by reducing the toxic effect of salinity. A statistically significant Sa × S interaction in shoot and root, revealed this fact (Table 5). Salt tolerant variety (Agaitti, 2003) responded well to sulfur application by improving the K^+/Na^+ ratio in comparison to salt sensitive variety (Pak Afgoi, 2003) (Figure 9).
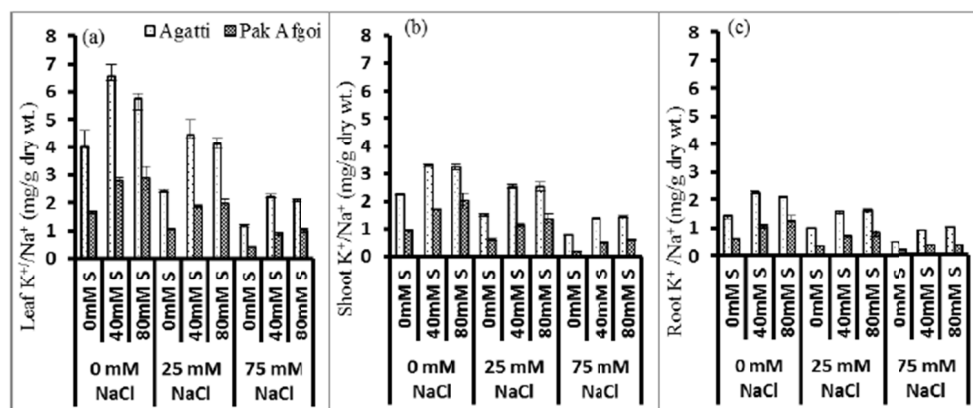

Figure 9. Effect of different levels of sulfur (S) on K^+/Na^+ content in leaf (a) shoot (b) root (c) of different maize (*Zea mays* L.) cultivars under saline conditions

3.2.5 Ca^{2+}/Na^+

Results showed that salt stress (25, 75 mM) reduced the Ca^{2+}/Na^+ ratio in maize plants. It was revealed from statistically significant V × Sa interaction in leaf, shoot and root (Table 6). The application of sulfur not only improved the Ca^{2+}/Na^+ ratio, but also developed salt tolerance in both maize cultivars at all levels of salinity (25, 75 mM). This fact is evident from statistically significant Sa × S interaction, while V × Sa × S interaction was significant only for maize root (Table 6). Agaitti (2003) accumulated high Ca^{2+}/Na^+ ratio as compared to Pak Afgoi 2003 (Figure 10).
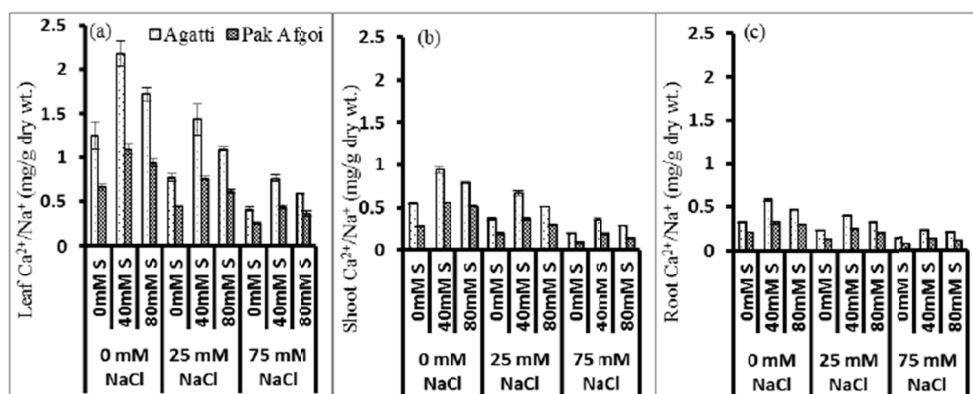
Figure 10. Effect of different levels of sulfur (S) on Ca^{2+}/Na^+ content in leaf (a) shoot (b) root (c) of different maize (*Zea mays* L.) cultivars under saline conditions

Table 6. Mean squares from analysis of variance (ANOVA) of the data for Ca^{2+}/Na^+ ratio and nitrate (NO_3^-) of maize subjected to different levels of salinity and sulfur

SOV	df	Leaf Ca^{2+}/Na^+ ratio	Shoot Ca^{2+}/Na^+ ratio	Root Ca^{2+}/Na^+ ratio	Leaf NO_3^-	Shoot NO_3^-	Root NO_3^-
Variety (V)	1	3.60 ***	0.68 ***	0.24 ***	0.06 ***	0.057 ***	0.05 ***
Salinity (Sa)	2	3.14 ***	0.68 ***	0.22 ***	0.07 ***	0.029 ***	0.05 ***
Sulfur (S)	2	1.02 ***	0.25 ***	0.084 ***	0.05 ***	0.027 ***	0.045 ***
V × Sa	2	0.39 ***	0.03 ***	0.01 ***	1.32e-4 ns	3.88e-4 ns	0.0019 ***
V × S	2	0.13 **	0.012 ***	0.0055 ***	1.32e-4 ns	0.0012 *	2.06e-4 ns
Sa × S	4	0.064 **	0.017 ***	0.0046 ***	0.0045 ***	0.0025 ***	0.0068 ***
V × Sa × S	4	0.011 ns	9.16e-4 ns	0.0011 **	1.32e-4 ns	0.0014 **	2.28e-4 ns
Error	36	0.016	6.87E-04	2.64E-04	3.48E-04	3.05E-04	1.71E-04

Note. *, **, *** = significant at 0.05, 0.01 and 0.001 levels, respectively. ns = non-significant.

Abbreviation: Exponent (e), Ca^{2+} = Calcium, Na^+ = Sodium, NO_3^- = Nitrate.

3.2.6 Nitrate (NO_3^-)

The application of salinity reduced the nitrate (NO_3^-) contents in all studied maize organs (leaf, shoot and root). The maximum reduction in nitrate contents was found at 75 mM salt level (Figure 11).
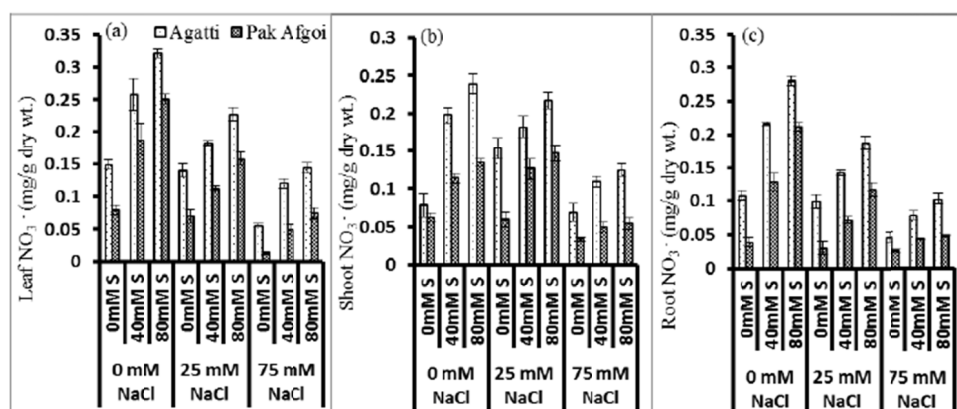

Figure 11. Effect of different levels of sulfur (S) on NO_3^- content in leaf (a) shoot (b) root (c) of different maize (*Zea mays* L.) cultivars under saline conditions

However, both varieties responded differently to salt application. In root, a statistically significant V × Sa interaction was found while in shoot and leaf, V × Sa interaction was non-significant (Table 6).The application of

sulfur (40, 80 mM) improved the nitrate (NO_3^-) contents at all studied salt levels. It was evident from statistically significant Sa × S interactive effect for leaf, root and shoot (Table 6). Moreover, sulfur at 40 mM level improved the salt tolerance in maize plants in both varieties (Agaitti, 2003; Pak Afgoi, 2003). A statistically significant V × Sa × S interaction was found in shoot while in leaf and root this interaction was non-significant (Table 6).

3.2.7 Phosphate (PO_4^{3-})

A marked reduction in phosphate (PO_4^{3-}) contents was found by salt application (Figure 12). However, sulfur application improved the phosphate (PO_4^{3-}) contents in salt tolerant (Agaitti, 2003) and salt sensitive (Pak Afgoi, 2003) maize cultivars. A statistically significant V × S interaction for maize leaf revealed this fact, while in shoot and root V × S interaction was found non-significant (Table 7).
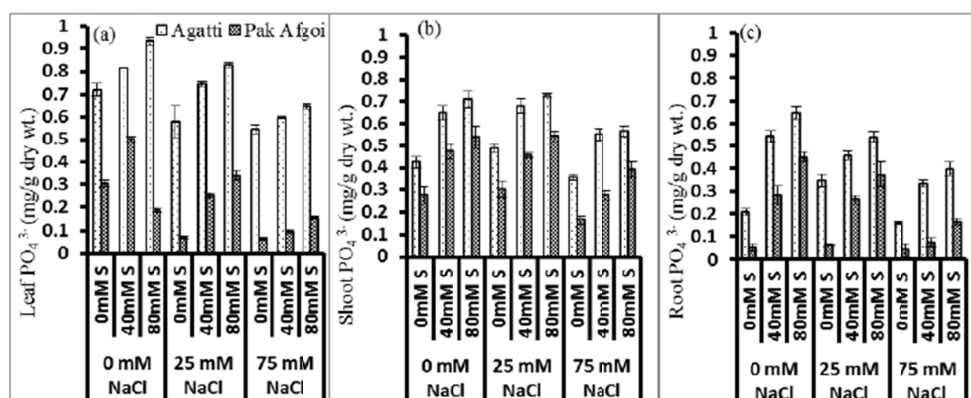

Figure 12. Effect of different levels of sulfur (S) on PO_4^{3-} content in leaf (a) shoot (b) root (c) of different maize (*Zea mays* L.) cultivars under saline conditions

Moreover, sulfur application induced the salt tolerance in maize cultivars. It was shown by statistically significant Sa × S interaction for leaf and root at all studied salt levels (25, 75 mM) while in shoot this interaction was found non-significant (Table 7). In both varieties the application of sulfur improved the phosphate (PO_4^{3-}) contents under salt stress conditions. It was shown by statistically significant V × Sa × S interaction for leaf and root while for shoot, this interaction was non-significant (Table 7). These findings revealed that sulfur application at 80 mM improved the salt tolerance by improving the phosphate (PO_4^{3-}) contents in both maize cultivars (Figure 12).

Table 7. Mean squares from analysis of variance (ANOVA) of the data for phosphate (PO_4^{3-}) and sulfate (SO_4^{2-}) of maize subjected to different levels of salinity and sulfur

SOV	df	Leaf PO_4^{3-}	Shoot PO_4^{3-}	Root PO_4^{3-}	Leaf SO_4^{2-}	Shoot SO_4^{2-}	Root SO_4^{2-}
Variety (V)	1	3.29 ***	0.50 ***	0.58 ***	1340.01 ***	2506.35 ***	663.89 ***
Salinity (Sa)	2	0.23 ***	0.12 ***	0.14 ***	757.22 ***	327.25 ***	411.105 ***
Sulfur (S)	2	0.099 ***	0.28 ***	0.37 ***	1813.53 ***	661.81 ***	950.16 ***
V × Sa	2	3.19e-5 ns	0.0030 ns	1.27e-4 ns	0.018 ns	18.38 *	29.06 **
V × S	2	0.02 ***	0.0036 ns	0.0036 ns	0.018 ns	14.76 ns	80.13 ***
Sa × S	4	0.026 ***	0.0016 ns	0.024 ***	26.63 *	8.79 ns	48.14 ***
V × Sa × S	4	0.026 ***	8.70e-4 ns	0.0077 **	0.018 ns	12.07 ns	3.28 ns
Error	36	0.0013	0.0026	0.0018	7.35	4.85	4.99

Note. *, **, *** = significant at 0.05, 0.01 and 0.001 levels, respectively. ns = non-significant.

Abbreviation: Exponent (e), PO_4^{3-} = Phosphate, SO_4^{2-} = Sulfate.

3.2.8 Sulfate (SO_4^{2-})

Results have shown that sulfate (SO_4^{2-}) contents were decreased by high levels of salinity (75 mM) in both studied maize varieties. It was evident from statistically significant V × Sa interaction for shoot and root, and

non-significant for leaf (Table 7). The application of sulfur improved the sulfate (SO_4^{2-}) contents in both maize varieties, while the maximum improvement in sulfate (SO_4^{2-}) contents was found at 80 M sulfur. It was shown by statistically significant Sa × S interaction for leaf and root (Table 7). Although, sulfur improved the sulfate (SO_4^{2-}) contents in salt tolerant variety (Agaitti, 2003), however, salt sensitive variety (Pak Afgoi, 2003) also improved the sulfate (SO_4^{2-}) contents at all studied salt levels (25, 75 mM). In leaf sulfate (SO_4^{2-}) contents were found vary high as compared to shoot and root (Figure 13).
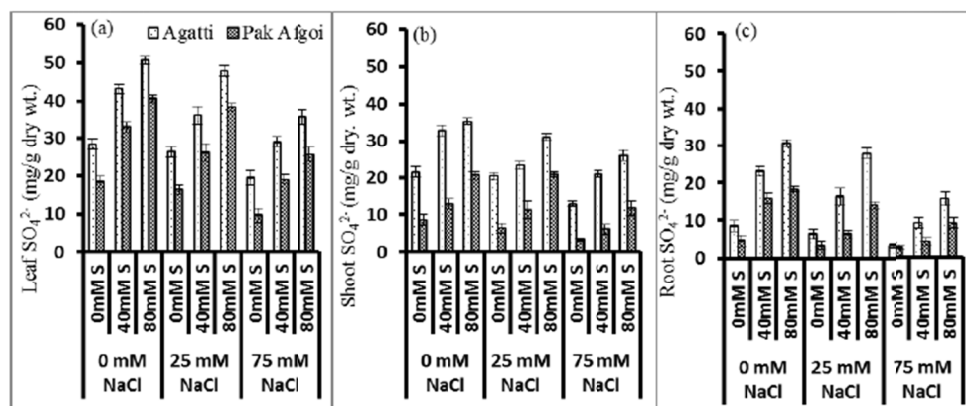

Figure 13. Effect of different levels of sulfur (S) on SO_4^{2-} content in leaf (a) shoot (b) root (c) of different maize (*Zea mays* L.) cultivars under saline conditions

4. Discussion

Osmoprotectants (also called compatible osmolytes) are the organic molecules of small size, have neutral charges and less toxic at elevated concentration (Lang, 2007). Osmolytes not only regulate osmosis but also balance the cell volume by linking to the cytoplasmic organelles, without any disturbance to the usual plant metabolism and fold the proteins to endure the harsh effects of environmental (biotic or abiotic) stresses (Verbruggen & Hermans, 2008). These osmolytes also stabilize the membrane proteins, prevent dehydration, nutrient homeostasis and regulate the osmotic potential inside the plant cell (Burg & Ferraris, 2008).

The current study showed that glycine betaine contents were high in salt stress condition. The findings of this study is supported by previous investigation that confer the accumulation of glycine betaine develops salt tolerance in plants (Sakamoto & Murata, 2002). Glycine betaine accumulation protects the plants from toxic effects of salinity by preventing oxidative stress (Chen & Murata, 2008). Moreover, it plays significant roles in osmotic adjustment, stabilization of embedded proteins, protection of chloroplast and PS II complex and in reducing the reactive oxygen species produced under oxidative stress conditions (Cha-Um & Kirdmanee, 2010). The application of sulfur supported the glycine betaine accumulation in both studied maize cultivars. This may be due to the reason that enzymes containing sulfur promote the biosynthesis of glycine betaine (Rathinasabapathi et al., 1997). Hence, sulfur application develops salt tolerance in plants by increasing the glycine betaine contents in plants.

The results of this study showed that proline concentration was higher in salt tolerant maize cultivar (Agaitti, 2003) in comparison to the salt sensitive maize variety. This finding is supported by previous researches on various crops *i.e.* rice, alfalfa, maize, pigeon pea and potato (Rahnama & Ebrahimzadeh, 2004; Waheed et al., 2006; Shereen et al., 2007; Cha-um & Kirdmanee, 2010). Proline is very much helpful in enduring the adverse conditions of environmental stresses. Proline serves as cytoplasmic osmoticum. Under salt stress condition, high accumulation of proline has been reported in previous studies (Miller et al., 2010). Moreover, it serves as nitrogen reservoir in the periods of restricted growth, hydrate the polymers and scavenge the reactive oxygen species (Kavi Kishor et al., 1995). However, in this study, sulfur application lowered the proline contents in both studied maize varieties (Agaitti, 2003; Pak Afgoi, 2003). This may be due to the reason that excessive amount of proline creates toxic effects in the plants (Jain et al., 2000). Hence, sulfur metabolites regulate the osmolyte concentration for developing salt tolerance in the plants.

The present study showed that salinity increased the total soluble sugars contents to induce salt tolerance in crop plants. It was due to the reason that accumulation of soluble sugars reduces the osmotic potential, water potential, turgidity in plant cell and osmotic adjustment by increasing the storage reserves for the normal functions of

plants under stress conditions (Siringam et al., 2012). Moreover, soluble sugars serve as chelating agent that bound Na^+ with starches and lower the toxic effects of salt on the plants (Xiao et al., 2009). The application of sulfur also increased the soluble sugars contents in both maize cultivars at all levels of treatments. It was supported by the findings of Lunde et al. (2008) who reported the reduction in soluble sugar contents by sulfur deficiency.

In this study, it was found that salinity increased the total soluble protein contents in both studied maize cultivars. It was supported by earlier researches (Chen et al., 2007; Kapoor & Srivastava, 2010). Soluble proteins help to raise the nitrogen level in plants that promotes growth and development under stress conditions. In addition, soluble proteins perform a significant role in osmotic adjustment (Ashraf & Harris, 2004). Sibole et al. (2003) found that by application of salinity (10, 50, 100, 200 mM), the soluble protein contents were increased in the clover plant (*Medicago citrna* L.). The accumulation of soluble protein contents by salt application has been reported in various plants *i.e.* barley, maize, sunflower, rice and mung bean (Khosravinejad et al., 2009; Kapoor & Srivastava, 2010). This study showed that the application of sulfur improved the soluble protein contents in maize plants. It may be due to the reason that sulfur is an important part of amino acids the building blocks of proteins (Gardner et al., 1985). Different metabolites of sulfur (*i.e.* cysteine, thiol) protect the structure of proteins. Hence, sulfur helps in forming the structure and function of proteins in the stress conditions (Malhi & Leach, 2000).

It was found that salt stress enhanced the total free amino acid contents in maize plants. In stress conditions, total free amino acid contents become very high that protects the proteins from degradation (Mansour, 2000). Moreover, this study showed that salt tolerant maize cultivar accumulated high level of total free amino acid in comparison to salt sensitive maize variety. These findings have been supported by previous studies (Ashraf and Tufail, 1995; Ashraf & Fatima, 2004). The application of sulfur improved various amino acid contents in maize plants as sulfur is the constituent of many important amino acids forming various structural and functional proteins in plants (Giovaneli, 1987).

Salt stress causes the disturbance in availability, absorption and transport of nutritional contents in plants (Munns & Tester, 2008). In this study, salinity reduced the beneficial nutrients (K^+, Ca^{2+}, NO_3^-, PO_4^{3-}, SO_4^{2-}, K^+/Na^+, Ca^{2+}/Na^+) in maize plants. It may be due to the reason that salt stress causes the disturbance in external osmotic potential that imbalance the nutrient contents in plants (Murillo-Amador et al., 2002). The imbalance in nutrient contents has been reported in various crops *e.g. Lycopersicon esculentum*, *Spinacia oleracea*, *Physalis peruviana*, as well as in *Zea mays* (Miranda et al., 2010; Collado et al., 2010).

This study revealed that salt stress increased the sodium (Na^+) contents in the maize plants which are in accordance to the findings of Fortmeier et al. (1995). The rise in sodium (Na^+) contents decreased the plant growth in both studied maize cultivars (Agaitti, 2003; Pak Afgoi, 2003). It may be due to the reason that high sodium (Na^+) contents forms ion-pair and precipitates other ions in plant cell (Hu et al., 2005). The reduction in Ca^{2+}, K^+, K^+/Na^+ and Ca^{2+}/Na^+ has been reported in this study. The elevated concentration of sodium (Na^+) changes the root permeability and reduces the uptake of calcium (Ca^{2+}) in plants (Greenway & Munns, 1980). This may be due to the competition in uptake of sodium (Na^+) and calcium (Ca^{2+}) contents and due to reduction in soil water potential affecting root pressure (Sonnevelt et al., 1975). Moreover, high concentration of sodium (Na^+) negatively uptake the potassium (K^+) resulting in reduction in carbon fixation, photosynthetic apparatus and ultimately reduces the photosynthesis in plants (Akram et al., 2010). The results of this study revealed that salt tolerant cultivar (Agaitti, 2003) accumulated low sodium (Na^+) and high potassium (K^+) and calcium (Ca^{2+}) contents in comparison to salt sensitive maize variety (Pak Afgoi, 2003). Therefore, Agaitti (2003) showed high K^+/Na^+ and Ca^{2+}/Na^+ ratio. This may be due to the reason that salt tolerant variety compartmentalizes the sodium (Na^+) in the plants thus transport the potassium and calcium (Munns et al., 2006). Thus, salt tolerant cultivar has high K^+/Na^+ ratio. It was supported by previous studies (Song et al., 2009). In salt tolerant variety the restricted uptake of Na^+ ions maintains plant homeostasis and ultimately overall plant growth. While in salt sensitive variety, plant growth reduced due to disturbance in nutrient homeostasis. These findings are in accordance to previous researches (Eker et al., 2006; Riffat & Ahmad, 2018). Results showed that application of sulfur lowered the Na^+ ions and improved the Ca^{2+}, K^+, K^+/Na^+ and Ca^{2+}/Na^+ in the maize plants. Sulfur helps in maintaining nutrient homeostasis in plants and induces salt tolerance (Singh et al., 2011). Sulfur application increases the Ca^{2+} and K^+ ions and decreases the harmful effects of Na^+ ions in the plants. This results in high K^+/Na^+ and Ca^{2+}/Na^+ ratio that indicate salt tolerance. Thus application of sulfur improves the crop quality and growth and development by maintaining proper nutrient homeostasis in plants under stressful environment (Badr et al., 2002; Prasad et al., 2003).

Results showed that salinity reduced nitrate (NO_3^-) contents in maize plants. It was supported by previous findings of Samra (1985). It may be due to the reason that Na^+ ions cause slow assimilation of nitrate (NO_3^-) contents. Moreover, salt stress shifts the reduction of nitrate from leaf to root (Frechill et al., 2001; Ullrich, 2002), that disturbs the proper availability of nitrate (NO_3^-) to the other parts of plants. The application of sulfur improved the nitrate (NO_3^-) contents in both studied maize varieties. It was in accordance to the previous studies. Reuveny et al. (1980) reported that the deficiency of sulfur causes the reduction in nitrate reductase activity. However, sulfur application improves the nitrogen metabolism and ultimately improves the nitrate contents in stress conditions (Sexton et al., 1993).

Salt stress also reduced the phosphate (PO_4^{3-}) contents in maize plants. Champagnol (1979) reported that salt stress reduced the phosphate (PO_4^{3-}) nutrition in the plants. However, sulfur application at low concentration improved the phosphorous contents in maize plants. These findings are supported by previous researches on various crops i-e. wheat, chickpea and maize, (Islam et al., 2011; Riffat, 2017; Riffat & Ahmad, 2018). Results revealed that salt stress reduced the sulfate (SO_4^{2-}) contents in both studied maize varieties. Riffat & Ahmad (2018) reported that high concentration of salts reduced the sulfate (SO_4^{2-}) contents. While, the sulfur application improved the sulfate (SO_4^{2-}) contents in the maize plants.

5. Conclusions and Recommendations

Salt stress caused changes in the organic and inorganic osmolytes in the plants. The imbalance in nutrient contents disturbs the normal plant metabolism. To overcome the adverse effects of salinity some natural osmoprotectants get accumulated in the maize plants. Among these organic osmolytes, glycine betaine, proline, total soluble sugars, total soluble proteins and total free amino acids has considerable importance. The application of sulfur (40 mM) not only balanced the organic osmolytes contents by lowering the higher accumulation of proline to avoid toxic effects but also induced salt tolerance in maize plants. Among the inorganic osmolytes, salt stress increased the Na^+ contents and lowered the beneficial osmolytes in the maize plants. However, sulfur application at 40 mM proved very effective in improving beneficial osmolytes (K^+, Ca^{2+}, NO_3^-, PO_4^{3-}, SO_4^{2-}, K^+/Na^+ and Ca^{2+}/Na^+) in the plants. Hence, it is recommended that sulfur at 40 mM is very much effective in balancing organic and inorganic osmolytes for improving salt tolerance potential.

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