

# Exogenous Salicylic Acid Mediated Physiological Responses and Improvement in Yield by Modulating Antioxidant Defense System of Wheat under Salinity

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## Abstract

The present study investigated the regulatory roles of exogenous salicylic acid (SA) in physiology, antioxidant defense systems and yield of wheat under different salt stress conditions. The experiment was conducted with two varieties, 'BARI Gom 21' and 'BARI Gom 25' and ten salt stress treatments viz. control (without salt), SA (1 mM salicylic acid), S50 (50 mM NaCl), S50+SA (50 mM NaCl with 1 mM SA), S100 (100 mM NaCl), S100+SA (100 mM NaCl with 1 mM SA), S150 (150 mM NaCl), S150+SA (150 mM NaCl with 1 mM SA), S200 (200 mM NaCl) and S200+SA (200 mM NaCl with 1 mM SA). Leaf relative water content (RWC) and chlorophyll (chl) content reduced due to salt stress. The malondialdehyde (MDA) and H<sub>2</sub>O<sub>2</sub> were increased under the stress condition. The ascorbate (AsA) content, reduced glutathione (GSH) and GSH/GSSG ratio were reduced by salt stresses (50, 100, 150 and 200 mM NaCl, respectively). But the glutathione disulfide (GSSG) amount increased with an increase in the all levels of salinity. The ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and catalase (CAT) activities showed a significant reduction in response to salt stress, but CAT increased only at 100 mM stress condition. The glutathione S-transferase (GST) and glutathione reductase (GR) activity increased significantly with severe salt stress (200 mM NaCl). Also the activity of peroxidase (POD) was decreased with increasing salinity level. At harvest, salt stresses reduced the effective tiller hill<sup>-1</sup>, 1,000 grain weight, grain yield, straw yield, biological yield and harvest index for both of varieties. However, the number of non-effective tiller hill<sup>-1</sup> significantly increased in response of salt stress. Exogenous 1 mM SA application with salt stress improved physiological parameters, yield and reduced oxidative damage in both cultivars, where 'BARI Gom 25' showed better tolerance. SA application could not improve physiological parameters and yield at extreme level of salt stress (200 mM NaCl).

**Keywords:** abiotic stress, antioxidant defense, oxidative stress, phytohormone, reactive oxygen species

## Introduction

Different abiotic stresses like salinity, drought, extreme temperatures, metal toxicity, flooding, UV-B radiation, ozone, etc. hamper plant growth, metabolism and productivity, as crop plants are sessile organisms (Hasanuzzaman *et al.*, 2012a, b; 2013; 2014). Among the abiotic stresses, salt stress is a major environmental threat to agriculture, and its adverse impacts are getting more serious problems in regions where saline water is used for irrigation

(Türkan and Demiral, 2009). More than 30% of the total agricultural production in world comes from around 17% of the cultivable land which is under irrigation and irrigated agriculture (Hillel, 2000). It is estimated that at least 20% of total irrigated lands in the world is salt-affected (Pitman and Läuchli, 2002; Munns and Tester, 2008). In most of the cases, the negative effects of salinity have been attributed to increase in Na<sup>+</sup> and Cl<sup>-</sup> ions in different plants hence these ions produce the critical conditions for plant survival by intercepting different plant mechanisms. Although both Na<sup>+</sup> and Cl<sup>-</sup> are the major ions produce many physiological

disorders in plant, Cl<sup>-</sup> is the most dangerous (Tavakkoli *et al.*, 2010). Salinity at higher levels causes both hyperionic and hyperosmotic stress and can lead to plant demise. The outcome of these effects may cause membrane damage, nutrient imbalance, altered levels of growth regulators, enzymatic inhibition and metabolic dysfunction, including photosynthesis which ultimately leading to plant death (Mahajan and Tuteja, 2005; Hasanuzzaman *et al.*, 2012a). Molecular and biochemical studies of the salt stress responses of plants have demonstrated significant increases in reactive oxygen species (ROS) such as, singlet oxygen (1O<sub>2</sub>), superoxide (O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and hydroxyl radical (OH) (Mittler, 2002; Tanou *et al.*, 2009; Pérez-López *et al.*, 2010). In general, plant cells are adequately equipped to keep ROS within the limits that are generated as a consequence of normal cellular metabolic activities. Under different stress conditions, ROS generation often exceeds the overall cellular antioxidative potential leading to stress-induced adverse effects on plant growth and physiology. A steady state balanced is required to protect plant cells from oxidative damage (Hasanuzzaman *et al.*, 2011). Plants possess an efficient non-enzymatic (AsA, GSH, α-tocopherol, phenolic compounds, alkaloids and non-protein amino acids) and enzymatic (SOD, CAT, APX, MDHAR, DHAR, GR, GPX, GST and POD) antioxidant defense systems which work in concert to control the cascades of uncontrolled oxidation and protect plant cells from oxidative damage by scavenging ROS (Gill and Tuteja, 2010). These antioxidant defense systems are found in almost all cellular compartments, demonstrating the importance of ROS detoxification for cellular survival (Gill and Tuteja, 2010). Therefore, efforts to increase the salt tolerance of crop plants are very important to ensure global food security, as well as for water and land conservation. Now, is the right time to be strategic: first by understanding the reasons – fundamental to complex – for yield reductions so that precise research planning can be brought about to cope with increasing salinity problems. With that view, plant scientists are now searching for ways to make the plants adaptive under saline conditions. Researchers are trying to understand the effects of salt stress on plants so that they can modify the plant's external growing condition as well as change the plant from within by applying different exogenous protectants including trace elements and phytohormones by molecular mechanisms. Salicylic acid (SA) is a common plant-produced phenolic compound and a potential endogenous plant hormone that plays an important role in plant growth and development (Hasanuzzaman *et al.*, 2014). The role of SA is intensively studied in plant responses to biotic stress. In recent years, the involvement of SA in the response to abiotic stresses has come into light. Several studies support a major role of SA in plant adaptation to the changing environment, and induce plant tolerance to various abiotic stresses including elevated NaCl (Stevens *et al.*, 2006; Arfan *et al.*, 2007; Gunes *et al.*, 2007). It is a well observed fact that SA potentially generates a wide array of metabolic responses in plants and also affects plant water relations (Hayat *et al.*, 2010).

## Materials and Methods

### *Plant materials and stress treatments*

Seed of a salt tolerant ('BARI Gom 25') and salt sensitive ('BARI Gom 21') variety of wheat were collected from Bangladesh Agriculture Research Institute, Joydebpur, Gazipur, Bangladesh. A pot experiment was conducted at the experimental shed of the Department of Agronomy, Sher-e-Bangla Agricultural University, Dhaka (90°77' E longitude and 23°77' N latitude) during the period of November 2013 to March 2014. Empty earthen pots with 18 inch depth were used for the experiment. Twelve kilogram sun-dried soils were put in each pot.

The soil and fertilizers were mixed well before placing the soils in the pots. Fertilizers used in the experimental pots were urea, triple super phosphate, muriate of potash and gypsum @ 4.6, 4.1, 2.7 and 1.2 g/pot. One-third of urea and the whole amount of other fertilizers were incorporated with soil at final pot preparation before sowing. Rest of the nitrogen were applied in two equal splits one at 30 days after sowing (DAS) and the other at 60 DAS. Fifteen healthy seeds of each variety were sown in each pot. After germination 9-10 plants were allowed to grow in each pot. There were five salinity levels including control where developed by adding respected amount commercial NaCl salt to the soil/pot as water dissolved solution. The salinity levels were C (control), S50 (50 mM NaCl), S100 (100 mM NaCl), S150 (150 mM NaCl) and S200 (200 mM NaCl). The salinity treatments were applied on 28, 35, 42, 49, 56 and 63 DAS (days after sowing). Salicylic acid (SA) was used as a protectants. The concentration of SA was 1mM and applied as spray solution under non-saline (SA) and saline (S50, S100, S150 and S200) condition. The experiment was laid out in a Randomized Completely Block Design (RCBD) with three replications.

### *Measurement of relative water content (%)*

Three leaves were randomly selected from each pot and cut with scissors. Relative water content (RWC) was measured according to Barrs and Weatherley (1962). Leaf laminae were weighed (Fresh weight, FW) and then immediately floated on distilled water in a petri dish for 4 h in the dark. Turgid weights (TW) were obtained after drying excess surface water with paper towels. Dry weights (DW) were measured after drying at 80 °C for 48 h. Then calculation was done using the following formula:

$$\text{RWC (\%)} = (\text{FW} - \text{DW}) / (\text{TW} - \text{DW}) \times 100.$$

### *Determination of chlorophyll content*

Three leaves were randomly selected from each pot. The top and bottom of each leaf were measured with atLEAF as atLEAF value. Then it was averaged and total chlorophyll content was measured by the conversion of atLEAF value into SPAD units and then total chl content was measured.

### *Measurement of lipid peroxidation*

The level of lipid peroxidation was measured by estimating MDA, a decomposition product of the peroxidized polyunsaturated fatty acid component of the membrane lipid, using thiobarbituric acid (TBA) as the

reactive material following the method of Heath and Packer (1968) with slight modifications. The leaf samples (0.5 g) were homogenized in 3 ml 5% (w/v) trichloroacetic acid (TCA) and the homogenate was centrifuged at 11,500×g for 15 min. One ml supernatant was mixed with 4 ml of TBA reagent (0.5% of TBA in 20% TCA). The reaction mixture was heated at 95 °C for 30 min in a water bath and then quickly cooled in an ice bath and centrifuged at 11,500×g for 15 min. The absorbance of the coloured supernatant was measured at 532 nm and was corrected for non-specific absorbance at 600 nm. The concentration of MDA was calculated by using the extinction coefficient of 155 mM<sup>-1</sup> cm<sup>-1</sup> and expressed as nmol of MDA g<sup>-1</sup> fresh weight.

#### Measurement of H<sub>2</sub>O<sub>2</sub>

H<sub>2</sub>O<sub>2</sub> was assayed according to the method described by Yu *et al.* (2003). H<sub>2</sub>O<sub>2</sub> was extracted by homogenizing 0.5 g of leaf samples with 3 ml of 50 mM K-phosphate buffer pH (6.5) at 4 °C. The homogenate was centrifuged at 11,500×g for 15 min. 3 ml of supernatant was mixed with 1 ml of 0.1% TiCl<sub>4</sub> in 20% H<sub>2</sub>SO<sub>4</sub> (v/v), and the mixture was then centrifuged at 11,500 g for 12 min at room temperature. The optical absorption of the supernatant was measured spectrophotometrically at 410 nm to determine the H<sub>2</sub>O<sub>2</sub> content ( $\epsilon = 0.28 \mu\text{M}^{-1} \text{cm}^{-1}$ ) and expressed as mol g<sup>-1</sup> fresh weight.

#### Extraction and measurement of ascorbate and glutathione

Wheat leaves (0.5 g fresh weight) were homogenized in 3 mL ice-cold acidic extraction buffer (5% metaphosphoric acid containing 1 mM EDTA) using a mortar and pestle. Homogenates were centrifuged at 11,500 ×g for 15 min at 4 °C and the supernatant was collected for analysis of ascorbate and glutathione.

Ascorbate content was determined following the method of Huang *et al.* (2005) with some modifications. The supernatant was neutralized with 0.5 M K-P buffer (pH 7.0). The AsA was assayed spectrophotometrically at 265 nm in 100 mM KP buffer (pH 7.0) with 0.5 unit of ascorbate oxidase (AO). A specific standard curve with AsA was used for quantification.

The glutathione pool was assayed according to previously described methods of Paradiso *et al.* (2008) with modifications utilizing 200 span style of aliquots of supernatant neutralized with 300 span style of 0.5 M K-P buffer (pH 7.0). Based on enzymatic recycling, GSH is oxidized by 5, 5'- dithio- bis (2-nitrobenzoic acid) (DTNB) and reduced by NADPH in the presence of GR, and glutathione content is evaluated by the rate of absorption changes at 412 nm of 2-nitro-5-thiobenzoic acid (NTB) generated from the reduction of DTNB. GSSG was determined after removal of GSH by 2-vinylpyridine derivatization. Standard curves with known concentrations of GSH and GSSG were used. The content of GSH was calculated by subtracting GSSG from total GSH.

#### Determination of protein

The protein concentration of each sample was determined following the method of Bradford (1976) using BSA as a protein standard.

#### Enzyme extraction and assays

Using a pre-cooled mortar and pestle, 0.5 g of leaf tissue was homogenized in 1 ml of 50 mM ice-cold K-phosphate buffer (pH 7.0) containing 100 mM KCl, 1 mM ascorbate, 5 mM β-mercaptoethanol and 10% (w/v) glycerol. The homogenates were centrifuged at 11,500 × g for 15 min and the supernatants were used for determination of enzyme activity. All procedures were performed at a temperature 0-4 °C.

Ascorbate peroxidase (EC: 1.11.1.11) activity was assayed following the method of Nakano and Asada (1981). The reaction buffer solution contained 50 mM K-phosphate buffer (pH 7.0), 0.5 mM AsA, 0.1 mM H<sub>2</sub>O<sub>2</sub>, 0.1 mM EDTA, and enzyme extract in a final volume of 700 μL. The reaction was started by the addition of H<sub>2</sub>O<sub>2</sub> and the activity was measured by observing the decrease in absorbance at 290 nm for 1 min using an extinction coefficient of 2.8 mM<sup>-1</sup> cm<sup>-1</sup>.

Monodehydroascorbate reductase (EC: 1.6.5.4) activity was determined by the method of Hossain *et al.* (1984). The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADPH, 2.5 mM AsA, and 0.5 unit of AO and enzyme solution in a final volume of 700 μL. The reaction was started by the addition of AO. The activity was calculated from the change in ascorbate at 340 nm for 1 min using an extinction coefficient of 6.2 mM<sup>-1</sup> cm<sup>-1</sup>.

Dehydroascorbate reductase (EC: 1.8.5.1) activity was determined by the procedure of Nakano and Asada (1981). The reaction buffer contained 50 mM K-phosphate buffer (pH 7.0), 2.5 mM GSH, and 0.1 mM DHA. The reaction was started by adding the sample solution to the reaction buffer solution. The activity was calculated from the change in absorbance at 265 nm for 1 min using an extinction coefficient of 14 mM<sup>-1</sup> cm<sup>-1</sup>.

Glutathione reductase (EC: 1.6.4.2) activity was measured by the method of Hossain *et al.* (2006). The reaction mixture contained 0.1 M K-phosphate buffer (pH 7.8), 1 mM EDTA, 1 mM GSSG, 0.2 mM NADPH, and enzyme solution in a final volume of 1 ml. The reaction was initiated with GSSG and the decrease in absorbance at 340 nm due to NADPH oxidation was recorded for 1 min. The activity was calculated using an extinction coefficient of 6.2 mM<sup>-1</sup> cm<sup>-1</sup>.

Glutathione S-transferase (EC: 2.5.1.18) activity was determined spectrophotometrically by the method of Hossain *et al.* (2006) with some modifications. The reaction mixture contained 100 mM Tris-HCl buffer (pH 6.5), 1.5 mM GSH, 1mM 1-chloro-2, 4-dinitrobenzene (CDNB) and enzyme solution in a final volume of 700 μL. The enzyme reaction was initiated by the addition of CDNB and the increase in absorbance was measured at 340 nm for 1 min. The activity was calculated using the extinction coefficient of 9.6 mM<sup>-1</sup> cm<sup>-1</sup>.

POD activity was determined by the method of Shannon *et al.* (1966). The reaction mixture contained 2.9 ml of 0.1 M phosphate buffer (pH 7.0), 0.04 ml of 0.1 M H<sub>2</sub>O<sub>2</sub>, 0.04 ml of 0.2% O-dianisidine and 0.02 ml of enzyme extract. The change in absorbance was read at 470 nm for 4 min. One enzyme unit is defined as change in 1 unit of absorbance min<sup>-1</sup>.

Catalase (EC: 1.11.1.6) activity was measured according

to the method of Hossain *et al.* (2006) by monitoring the decrease of absorbance at 240 nm for 1 min caused by the decomposition of H<sub>2</sub>O<sub>2</sub>. The reaction mixture contained 50 mM K-phosphate buffer (pH 7.0), 15 mM H<sub>2</sub>O<sub>2</sub> and enzyme solution in a final volume of 700  $\mu$ L. The reaction was initiated with enzyme extract and the activity was calculated using the extinction coefficient of 39.4 M<sup>-1</sup> cm<sup>-1</sup>.

#### Measurement of yield and yield contributing parameter

Plant height was measured from the soil level to the apex of the leaf or spike in randomly 5 plants of each pot. The total number of tillers hill<sup>-1</sup> was counted from selected samples and grouped in effective and non-effective tillers plant<sup>-1</sup>. Then spike length was recorded from the basal nodes of the rachis to apex of each spike. Grains of 5 randomly selected spike of each replication were counted and then the average number of grains for each spike was determined. One hundred clean sun dried grains were counted from the seed stock obtained from the sample plants and weighed by using an electronic balance. Then it was converted into thousand grain weight. The grains were separated by threshing per plant and then sun dried and weighed. The straw was separated by threshing per plant and weighed. Biological yield was calculated by using the following formula:

Biological yield = Grain yield + straw yield

Finally harvest index was calculated by using the following formula of Gardner *et al.* (1985):

Harvest index (HI) = (Grain Yield)/(Biological Yield) × 100

#### Statistical analysis

The data obtained for different parameters were statistically analyzed following computer based software XLSTAT 2014 (AddinSoft, 2014) and mean separation was done by LSD at 5% level of significance.

## Results

### Relative water content (RWC)

Upon exposure to salt stress, leaf relative water content decreased significantly in both wheat varieties when compared to their controls (Fig. 1A). However, decline in RWC was lower in 'BARI Gom 25' as compared to 'BARI Gom 21'. Addition of SA, in combination with salt stress significantly increased relative water content in both varieties, compared to addition of salt only. But, in case of 200 mM NaCl, SA treatment could not increase relative water content of wheat plant.

### Chlorophyll content

Chlorophyll content is also affected by salinity stress, according to Fig. 1B. In case of 'BARI Gom 21', chlorophyll content decreased 19, 23, 28, and 31% at 50, 100, 150 and 200 mM NaCl, respectively (Fig. 1B). On the contrary, reductions in chl content were 12, 23, 26 and 28% at 50, 100, 150 and 200 mM NaCl, respectively. Though SA treatment significantly increased the chl content under stress condition but it failed to increase chl content under 200 mM NaCl. However, when plant treated with SA only, chl content was not affected significantly in relation to control.

### Lipid peroxidation and H<sub>2</sub>O<sub>2</sub> level

The malonaldehyde (MDA) content (indicator of lipid peroxidation) sharply increased at any level of salt stress in both wheat varieties (Fig. 2A). The highest amount of MDA content was noted in the case of 200 mM NaCl, whereas the lowest amount of MDA content was found when seedling were treated with SA alone in salt sensitive and salt tolerant variety respectively. However, the rate on increment was higher in salt sensitive 'BARI Gom 21' (Fig. 2A). The levels of H<sub>2</sub>O<sub>2</sub> also increased noticeably upon exposure to NaCl. In 'BARI Gom 21', the H<sub>2</sub>O<sub>2</sub> content was increased by 93 and 184% at 150 and 200 mM NaCl,

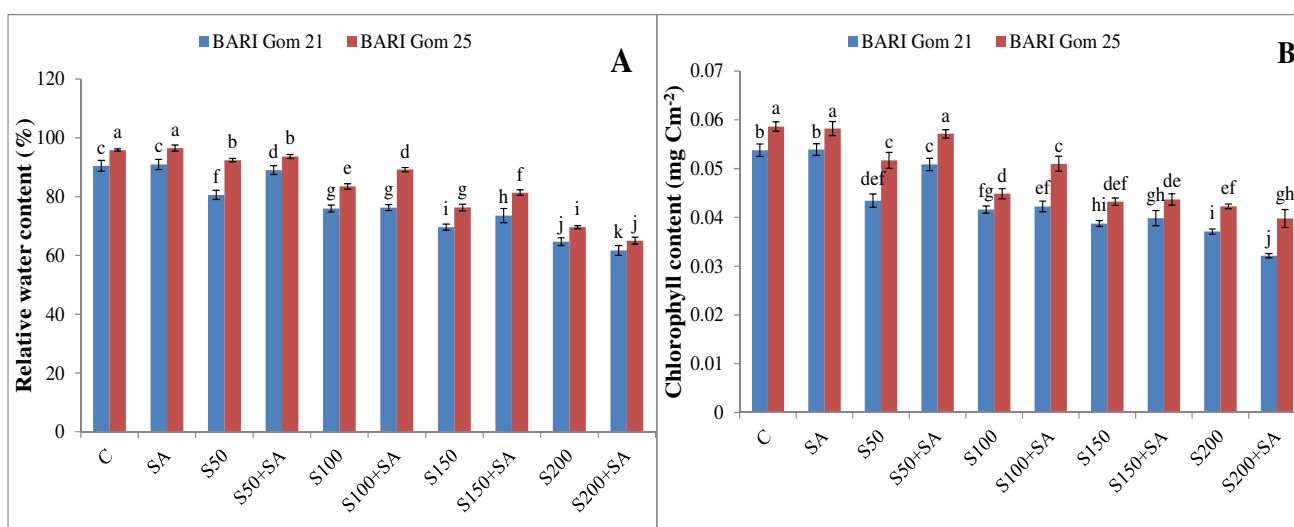


Fig. 1. (A) Leaf relative water content and (B) chlorophyll content in salt sensitive and salt tolerant wheat plants induced by exogenous salicylic acid under salt stress. S50, S100, S150 and S200 indicate 50 mM, 100 mM, 150 mM and 200 mM NaCl, respectively. SA indicates 1 mM salicylic acid spray, respectively. Mean ( $\pm$ SD) was calculated from three replicates for each treatment. Values in a column with different letters are significantly different at  $p \leq 0.05$  applying LSD test

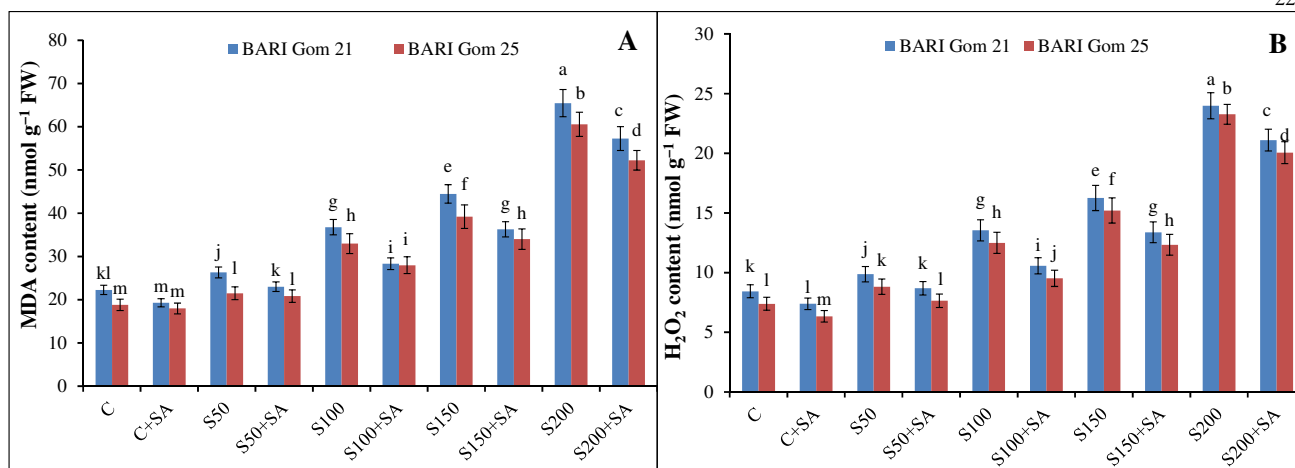


Fig. 2. (A) MDA content and (B)  $H_2O_2$  content in salt sensitive and salt tolerant wheat plants induced by exogenous salicylic acid under salt stress. S50, S100, S150 and S200 indicate 50 mM, 100 mM, 150 mM and 200 mM NaCl, respectively. SA indicates 1 mM salicylic acid spray, respectively. Mean ( $\pm$ SD) was calculated from three replicates for each treatment. Values in a column with different letters are significantly different at  $p \leq 0.05$  applying LSD test

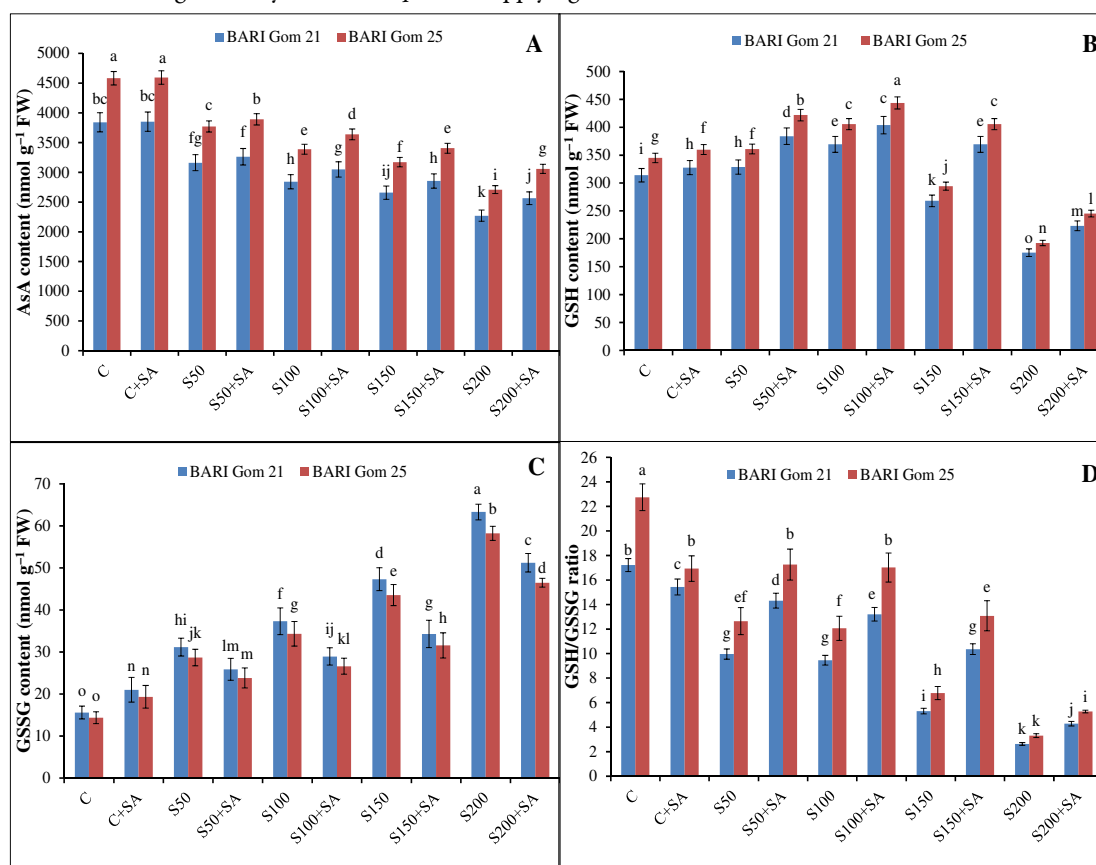


Fig. 3. (A) AsA content, (B) GSH content, (C) GSSG content and (D) GSH/GSSG ratio in salt sensitive and salt tolerant wheat plants induced by exogenous salicylic acid under salt stress. S50, S100, S150 and S200 indicate 50 mM, 100 mM, 150 mM and 200 mM NaCl, respectively. SA indicates 1 mM salicylic acid spray, respectively. Mean ( $\pm$ SD) was calculated from three replicates for each treatment. Values in a column with different letters are significantly different at  $p \leq 0.05$  applying LSD test

while in 'BARI Gom 25' it was increased by 106 and 215%, respectively, compared to control (Fig. 2B). SA could maintain the  $H_2O_2$  content lower in salt-stressed seedlings compared to the seedlings grown without SA supplementation (Fig. 2B). In all cases there was significant difference between 'BARI Gom 21' and 'BARI Gom 25' in respect of  $H_2O_2$  content.

#### Antioxidant defense system

According to (Fig. 3A) gradual decrease in AsA content over control was observed, for both 'BARI Gom 21' and 'BARI Gom 25', as the plant exposed to salt stress. Compared to control AsA content decrease 18, 26, 31 and 41% in case of 'BARI Gom 21' and 'BARI Gom 25' due to 50, 100, 150 and 200 mM NaCl. When seedling treated

with SA, AsA content increases significantly for all cases. AsA content of 'BARI Gom 25' was always higher than that of 'BARI Gom 21'. There was no significant difference between control and SA treated alone seedling AsA content for both varieties. Significant increase in GSH content were observed (5 and 18% by the 50 and 100 mM NaCl stresses respectively) in response to salt stress, compared to the untreated control (Fig. 3B). However, sharp decrease was also observed when plant exposed to 150 and 200 mM NaCl for both genotypes. An increase in GSH content was also observed in SA treated salt-stressed seedlings and, particularly at the 100 mM NaCl, the SA treated seedlings showed a significant increase (9%) in GSH content compared to seedlings of 'BARI Gom 21' and 'BARI Gom 25' subjected to salt stress alone. The highest amount of GSSG content was 63 and 58 nmol g<sup>-1</sup> FW salinity level 200 mM NaCl, whereas the lowest amount of MDA content were 16 and 14 nmol g<sup>-1</sup> FW found when seedling without stress treatment or SA in salt sensitive 'BARI Gom 21' and salt tolerant 'BARI Gom 25' variety respectively (Fig. 3C). In salt sensitive 'BARI Gom 21' the levels were increased by 139 and 305% at 100 and 200 mM NaCl, respectively. Exogenous SA, on the other hand, maintained the GSSG content significantly lower under salt stress compared to the seedlings grown without SA supplementation. The ratio of GSH/GSSG decreased markedly under salt stress in dose dependent manners and it greatly varied with varieties (Fig. 3D). In salt sensitive 'BARI Gom 21', 150 and 200 mM NaCl resulted in 69 and 85% decrease in GSH/GSSG ration, while in salt tolerant 'BARI Gom 25', it decreased by 70 and 85%, respectively, compared to control (Fig. 3D). In all cases there was significant difference between 'BARI Gom 21' and 'BARI

Gom 25' in respect of GSH/GSSG except at 200 mM NaCl.

Imposition of salt stress of 100 mM NaCl significantly increased the APX activity by 30% in salt sensitive 'BARI Gom 21', while in salt tolerant 'BARI Gom 25' it was increased by 31% compared to control. APX activity was decreased by 12% in salt sensitive cultivar and 11% in salt tolerant cultivar (Fig. 4A). Exogenous SA supplementation in salt stressed seedlings maintained higher APX activities, compared to salt stress alone, whereas in salt tolerant 'BARI Gom 25' the activity was always higher than 'BARI Gom 21' (Fig. 4A). In salt sensitive 'BARI Gom 21', the activity decreased by any level of salt stress (24 and 39% lower at 100 and 200 mM NaCl, respectively, compared to the control). Salt tolerant 'BARI Gom 25' showed significant increase in CAT activity under mild stress (50 mM NaCl), whereas a noticeable decrease (23%) was observed at severe stress (200 mM). However, exogenous SA enhanced the CAT activity in salt-treated seedlings (Fig. 5A). Salt stress at any level decreased the MDHAR activity. The highest activity was 43 nmol min<sup>-1</sup> mg<sup>-1</sup> protein found 'BARI Gom 25' treated with SA alone, while the lowest MDHAR activity was 26  $\mu$ mol min<sup>-1</sup> mg<sup>-1</sup> protein found in 'BARI Gom 21' exposed to 200 mM NaCl. Exogenous SA addition under any levels of salt stress significantly increased MDHAR activities irrespective of cultivars (Fig. 4B). Salt stress caused a marked decrease in DHAR activity at any level of stress except when seedling exposed to 200 mM NaCl. In 'BARI Gom 21', due to exogenous SA application DHAR activities were increased by 10 and 21% at 150 and 200 mM NaCl, respectively. In 'BARI Gom 25', exogenous SA supplemented seedlings showed increased DHAR activities by 25% at 150 mM NaCl, however no significant change in

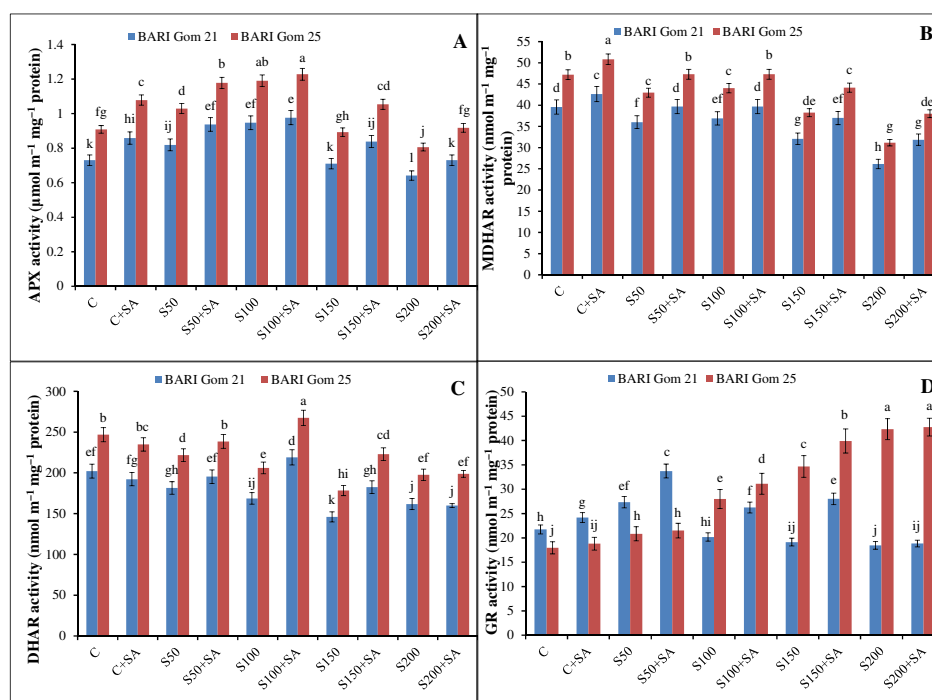


Fig. 4. (A) APX activity, (B) MDHAR activity, (C) DHAR activity and (D) GR activity in salt sensitive and salt tolerant wheat plants induced by exogenous salicylic acid under salt stress. S50, S100, S150 and S200 indicate 50 mM, 100 mM, 150 mM NaCl and 200 mM NaCl, respectively. In Y-axis, m<sup>-1</sup> indicates 'per minute'. SA indicates 1 mM salicylic acid spray, respectively. Mean ( $\pm$ SD) was calculated from three replicates for each treatment. Values in a column with different letters are significantly different at  $p \leq 0.05$  applying LSD test.

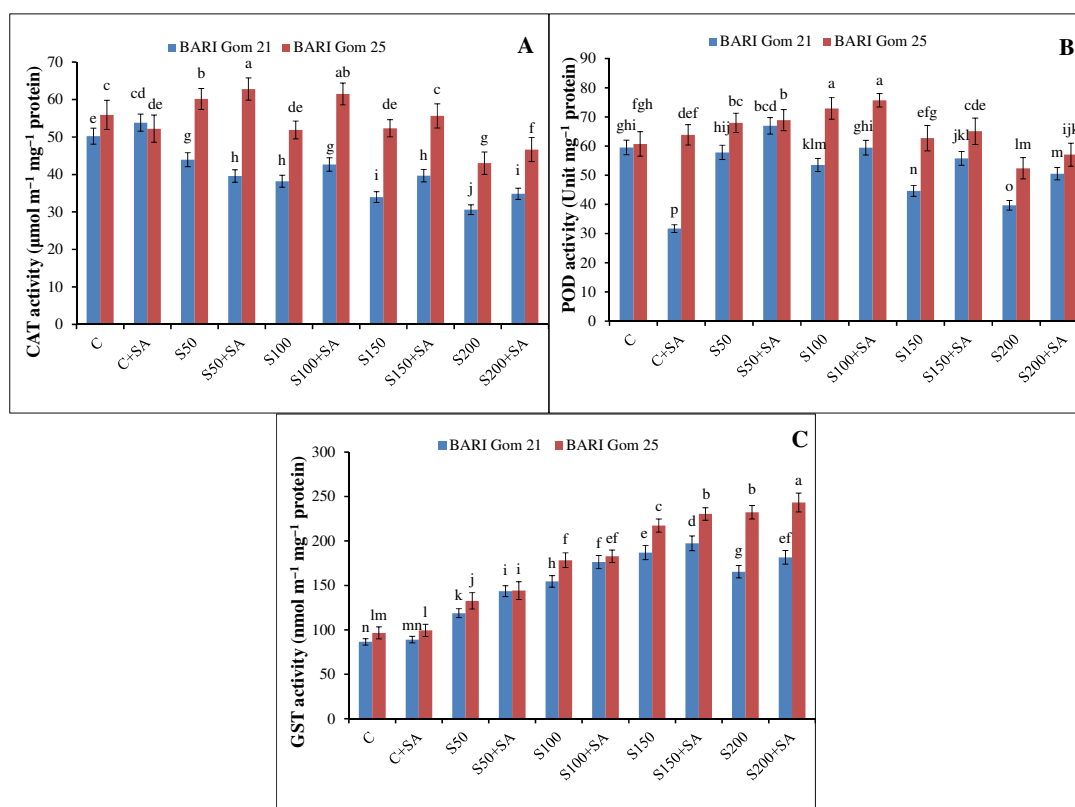


Fig. 5. (A) CAT activity, (B) POD activity and (C) GST activity in salt sensitive and salt tolerant wheat plants induced by exogenous salicylic acid under salt stress. S50, S100, S150 and S200 indicate 50 mM, 100 mM, 150 mM NaCl and 200 mM NaCl, respectively. In Y-axis,  $m^{-1}$  indicates 'per minute'. SA indicates 1 mM salicylic acid spray, respectively. Mean ( $\pm$ SD) was calculated from three replicates for each treatment. Values in a column with different letters are significantly different at  $p \leq 0.05$  applying LSD test

DHAR activity observed due to SA supplementation at 200 mM NaCl, compared to salt stress alone (Fig. 4C). Compared to control, the salt sensitive 'BARI Gom 21' had decreased GR activities of 12% and 15% in exposure to 150 and 200 mM NaCl, respectively (Fig. 4D). In opposition, salt tolerant 'BARI Gom 25' had significantly higher GR activities of 35% and 42% with 150 and 200 mM NaCl, respectively. Nonetheless, exogenous SA enhanced its activity further in both sensitive and tolerant varieties irrespective of salt doses, compared to the activity in the seedlings exposed to salt stress alone (Fig. 4D). Salt stress caused significant decrease in POD activities 25% and 33% in 'BARI Gom 21' whereas 'BARI Gom 25' showed 3% increase and 14% decrease in POD activity at 150 and 200 mM NaCl respectively (Fig. 5B). For 'BARI Gom 25' POD activities ranges from 63 to 76 unit  $mg^{-1}$  protein and in many case showed insignificant difference among different treatment. The activity of GST sharply increased in all wheat seedlings induced by all levels of salt stress although its activity was slightly higher in salt tolerant 'BARI Gom 25' (Fig. 5C). SA increased GST activity significantly. GST activity was higher in all cases except when seedling treated with SA under 50 mM NaCl.

#### Yield contributing characters

Exposure to salt stress resulted in significant decreases in effective tiller number hill<sup>-1</sup>: 22, 29, 44 and 56% for 'BARI Gom 21' and 20, 27, 34 and 53% for 'BARI Gom 25' at 50, 100, 150 and 200 mM NaCl, respectively when compared

to unstressed control plant (Fig. 6A). Addition of exogenous SA combination with salinity stress significantly increased the effective tiller number up to 150 mM NaCl. Furthermore, in case of 200 mM NaCl there had no statistically difference from each other of both varieties. Salinity stress caused increased number of non-effective tiller hill<sup>-1</sup> of both varieties. The highest non-effective tiller was found in 200 mM salt stressed condition of 'BARI Gom 21' which was treated with SA (Fig. 6B). SA supplementation reduced the number of non-effective tiller in the salt stressed condition. Saline treatment reduced the length of spike compared to control and SA treated plant. At 150 mM NaCl, length of spike was decreased 39% in 'BARI Gom 21' and 40% in 'BARI Gom 25' (Fig. 7A). In contrary, exogenous SA supplementation caused increased the length of spike of both varieties. Significantly higher number of spikelet per spike was recorded in controlled condition, only SA treated and SA treated 50 mM NaCl of 'BARI Gom 25'. After all, both of variety gave same result in case of 200 mM NaCl. SA treated 200 mM NaCl gave significantly lower number of spikelet per spike than 200 mM NaCl without treatment for both of variety (Fig. 7B). Marked decreases in 1,000 grain weight were observed in response to salt stress. Anyhow, salt stressed plants treated with SA up to 150 mM NaCl had significantly higher 1,000 grain weight in both varieties compared to plants which were subjected to salt stress without SA (Fig. 7C). Comparing cultivars, under control conditions, 'BARI Gom 25' produced more 1,000 grain weight in comparison

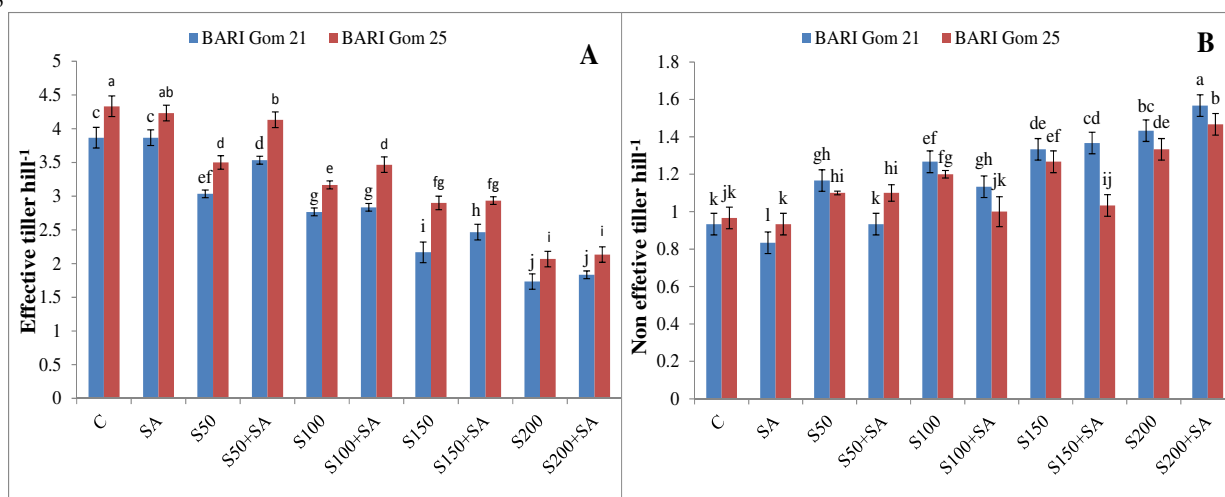


Fig. 6. (A) Effective tiller hill<sup>-1</sup> and (B) Non effective tiller hill<sup>-1</sup> in salt sensitive and salt tolerant wheat plants induced by exogenous salicylic acid under salt stress. S50, S100, S150 and S200 indicate 50 mM, 100 mM, 150 mM and 200 mM NaCl, respectively. SA indicates 1 mM salicylic acid spray, respectively. Mean ( $\pm$ SD) was calculated from three replicates for each treatment. Values in a column with different letters are significantly different at  $p \leq 0.05$  applying LSD test

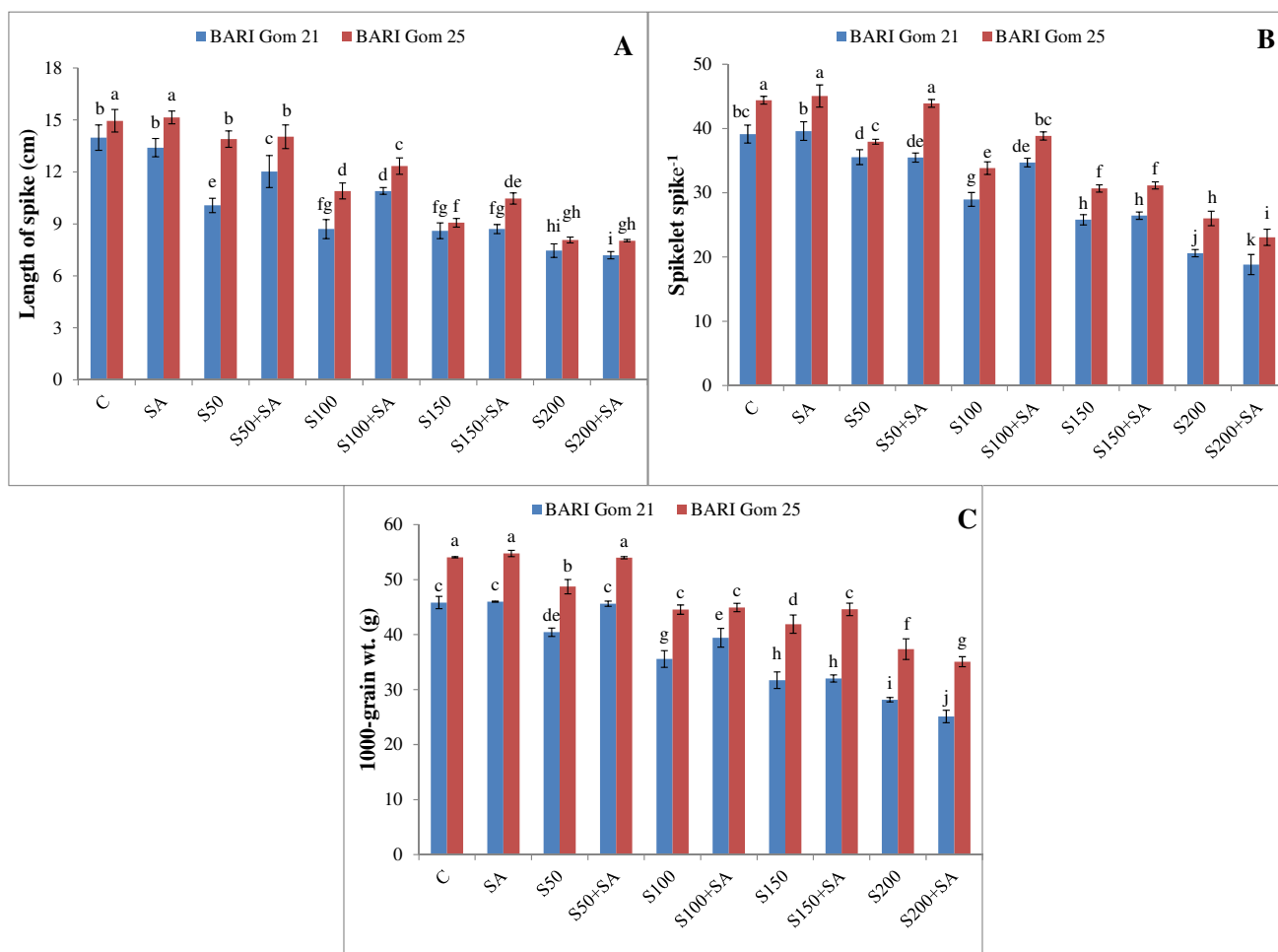


Fig. 7. (A) Length of spike, (B) spikelet spike<sup>-1</sup> and (C) 1,000-grain wt. in salt sensitive and salt tolerant wheat plants induced by exogenous salicylic acid under salt stress. S50, S100, S150 and S200 indicate 50 mM, 100 mM, 150 mM and 200 mM NaCl, respectively. SA indicates 1 mM salicylic acid spray, respectively. Mean ( $\pm$ SD) was calculated from three replicates for each treatment. Values in a column with different letters are significantly different at  $p \leq 0.05$  applying LSD test



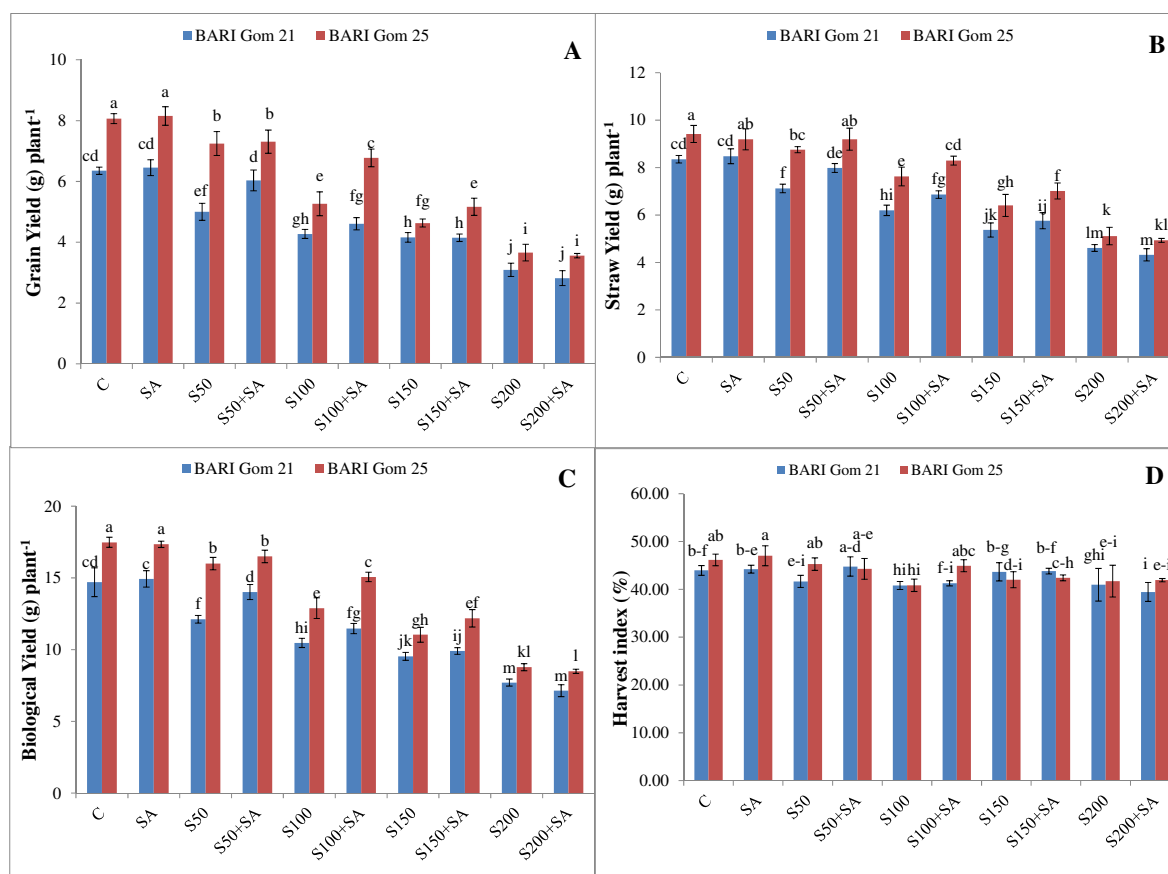


Fig. 8. (A) Grain yield, (B) straw yield, (C) biological yield and (D) harvest index in salt sensitive and salt tolerant wheat plants induced by exogenous salicylic acid under salt stress. S50, S100, S150 and S200 indicate 50 mM, 100 mM, 150 mM and 200 mM NaCl, respectively. SA indicates 1 mM salicylic acid spray, respectively. Mean ( $\pm$ SD) was calculated from three replicates for each treatment. Values in a column with different letters are significantly different at  $p \leq 0.05$  applying LSD test

to 'BARI Gom 21'. However, both wheat cultivars behaved similarly under saline environment.

### Yields

Significant variation was observed for grain yield due to different salinity treatments (Fig. 8A). The highest grain yield was found in control (40.33 g) and only SA treated plant (40.77 g) of 'BARI Gom 25' variety (Fig. 8A). For all that, the lowest grain yield was found in 200 mM NaCl of both varieties which was not increased or decreased after treating with SA. Straw yield was noticeably decreased in both wheat varieties under salt stressed condition. Straw yield was decreased by 11, 32, 42 and 51% for 'BARI Gom 21' and 15, 28, 41 and 50% for 'BARI Gom 25' at 50, 100, 150 and 200 mM NaCl respectively, compared to respective control (Fig. 8B). However, exogenous application with SA mitigated the salt effect up to 150 mM NaCl for both of varieties. The highest amount of biological yield (17.48 and 17.34 g) was observed in untreated control and only SA treated plant of 'BARI Gom 25' and the lowest amount of biological yield (7.70 and 7.15 g) was observed in seedling exposed to 200 mM NaCl and SA treated 200 mM NaCl condition of 'BARI Gom 21' (Fig. 8C). Sharp decreases in harvest index were observed) in response to salt stress, compared to the respective control and untreated control (Fig. 8D). SA treated salt stressed plants had significantly higher HI, compared to plants subjected to salt stress

without SA treatment. However, the level was significantly lower than that of untreated control. The HI of SA treated control plants was similar to that of the untreated control.

### Discussion

Several biochemical and physiological alterations like decrease in water content in plant tissues, decline in photosynthetic pigments and oxidative stress caused by salt stress. A secondary oxidative stress caused by the accumulation of reactive oxygen species (ROS) including singlet oxygen ( $^1O_2$ ), superoxide ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radicals ( $OH\cdot$ ) which generates by salt stress (Ahmad *et al.*, 2011; Hasanuzzaman *et al.*, 2013).

The common phenomenon of plant growth under salt stress is declining RWC and it is considered as the efficient indicator for evaluating plants for tolerance to stress as salt stress causes osmotic stress. In this study, RWC significantly decreased in wheat leaves irrespective to NaCl concentration and the wheat cultivars due to salt stress (Fig. 1A). There was same result found which was reported earlier in decreasing RWC due to salt stress (Katerji *et al.*, 1997; Vysotskaya *et al.*, 2010; Chaparzadeh and Mehrnejad, 2013). However, supplementation of SA with salt stress increased RWC which was due to the increment of water holding capacity in their leaf tissue (Fig. 1A). Foliar spraying with SA increased RWC under salt stressed condition were

also reported for wheat (El-bassiouny and Bekheta, 2005; Mohammadi *et al.*, 2013) and other crops including *Torreyia grandis* (Li *et al.*, 2014), *Brassica napus* (Mohammadreza *et al.*, 2012), strawberry (Karlidag *et al.*, 2009), cucumber (Yildirim *et al.*, 2008), barley (El-Tayeb, 2005), tomato (Tari *et al.*, 2002; Szepesi *et al.*, 2005). The RWC was slightly higher in 'BARI Gom 25' compared to 'BARI Gom 21' which was due to its better salt tolerance ability.

In our experiment, Chl content was reduced due to salt stress for both varieties. However, salt sensitive 'BARI Gom 21' gave slightly higher reduction compared to salt tolerant 'BARI Gom 25' (Fig. 1B). Photosynthesis pigment biosynthesis alters due to salt stress (Maxwel and Jhonson, 2000). Similarly, the adverse effect of salt stress on chl content was observed in many leaves of plant; Kaya *et al.* (2002) in strawberry. However, SA supplementation was mostly turned over this effect (Fig. 1B). These results are in agreement with Li *et al.* (2014) for *Torreyia grandis*, Mohammadi *et al.* (2013) for wheat, Karlidag *et al.* (2009) for strawberry, Yildirim *et al.* (2008) for cucumber, Moharekar *et al.* (2003) for wheat.

The important index of determining the degree of oxidative stress is considered as lipid peroxidation because abiotic stress including salt stress increased MDA content with the extent of oxidative stress (Hasanuzzaman *et al.*, 2011). As oxidation product, MDA formed when ROS such as OH. And  $1O_2$  attack PUFA (Gill and Tuteja, 2010). Higher accumulation of  $H_2O_2$  also causes oxidative stress in plant. In this study, with the increase in salinity level, MDA and  $H_2O_2$  content also increased. Foliar spraying with SA reduced MDA and  $H_2O_2$  content significantly under salt stress condition (Fig. 2A, B). Exogenous SA treated plant have decreased amount of MDA and  $H_2O_2$  were observed by the other researchers (Hasanuzzaman *et al.*, 2014 in *Brassica napus*, Erdal *et al.*, 2011 in wheat, El-bassiouny and Bekheta, 2005 in wheat).

AsA is able to donate electron to many enzymatic and non-enzymatic reactions what makes it an important ROS scavenging molecule. It can protect membrane by scavenging OH and  $O_2^-$  directly regenerating  $\alpha$ -tocopherol from tocopheroxyl radical (Gill and Tuteja, 2010). GSH is another important substance especially for photosynthetic organelles such as chloroplast. AsA and GSH play vital role in the AsA - GSH cycle to enhance stress tolerance under stress condition (Pastori *et al.*, 2003). AsA - GSH cycle composed of APX, MDHAR, DHAR and GR and these enzymes works coordinately to remove ROS such as  $H_2O_2$  (Kadioglu *et al.*, 2011). AsA content strongly related to oxidative stress tolerance and higher AsA content in plants showed better tolerance to oxidative stress (Nahar *et al.*, 2012). Increased AsA or GSH content can effectively reduce ROS produced under stress conditions including salt stress and thus prevents oxidative stress. In the present study, it is examined that the performance of salt tolerance and salt sensitive wheat cultivars against different salinity levels and we also examined how they are protected from salt stress by exogenous SA application. It was observed that under mild salt stress condition AsA level of salt sensitive 'BARI Gom 21' was reduced whereas the AsA level of 'BARI Gom 21' was higher (Fig. 3A). Severe salt stress also reduced the AsA level of salt sensitive and salt tolerant

cultivar. In this study, a slight increase in APX activity was observed in leaves of salt treated seedlings which were supported by Gusman *et al.* (2013), Tari *et al.* (2015). However, SA supplementation could not enhance the activity further under severe salt stress (Fig. 4A). This result is correlated to MDHAR and DHAR activities which regulate the recycling of AsA within the cell. From Fig. 4B and 4C it is clear that when the MDHAR or DHAR activity was reduced in salt sensitive 'BARI Gom 21', then its AsA levels were reduced irrespective of different salt doses. The higher MDHAR and DHAR activities of salt tolerant 'BARI Gom 21' were also related to its AsA levels. In our experiment, the GSH content (Fig. 3B) also increased with increased salinity stress, but decrease under severe stress. Similar results reported by Hasanuzzaman *et al.* (2014) and Alam *et al.* (2013). The increased GSH content might be due to the increase in GR activities as well as higher GSH biosynthesis (Mittova *et al.*, 2004). Under stressful condition GR helps in maintaining the GSH redox state by recycling of GSSG to GSH. It also plays a vital role in maintenance of sulfhydryl ( $-SH$ ) group and acts as a substrate for glutathione S-transferases (Yusuf *et al.*, 2012). However, supplementation with SA under salinity stress showed significant increase of both AsA and GSH (Fig. 3A, B) which indicated a clear role of SA in producing non-enzymatic antioxidant. SA might took part in the regeneration of AsA by up-regulating the related enzymes i.e. MDHAR and DHAR; SA also accelerated efficient recycling of GSH is also ensured by GR activity. In this experiment salt stress could increase the GR activity to a small extent. However, when SA treated seedlings were subjected to salt stress the activity markedly increased which rendered rapid recycling of GSH in line with better synthesis of GSH under salt stress conditions (Fig. 4D). The role of SA in enhancing the activity of GR was reported in many plant studies (He and Zhu, 2008). Earlier, it was reported the correlation between enhanced GR activity and better GSH levels as well as abiotic stress tolerance including salinity (Hasanuzzaman *et al.*, 2011; Hasanuzzaman and Fujita, 2011; Hasanuzzaman and Fujita, 2013). In this study, AsA-GSH cycle actively work in tolerant varieties than that of susceptible varieties supported by Sekmen *et al.* (2007), Aghaei *et al.* (2009), Hasanuzzaman *et al.* (2014).

In our experiment, the GSSG content at severe salinity stress was astonishingly higher (Fig. 3C) than control ones. This increase might be partly attributed to a decrease in the rate of GSH recycling or to an increase in the rate of degradation of GSH (Noctor and Foyer, 1998). However, SA treated salinity-stressed seedlings showed significantly lower GSSG (Fig. 3C). The GSH/GSSG ratio also markedly enhanced by SA application under salt stress condition (Fig. 3D). It has been suggested that the GSH/GSSG ratio, indicative of the cellular redox balance, may be involved in ROS perception (Shao *et al.*, 2005). Similar observations were reported by several researchers (Kadioglu *et al.*, 2011; Hasanuzzaman and Fujita, 2011; Nahar *et al.*, 2012).

Catalase is one of the vital enzymes in scavenging  $H_2O_2$  in plant cells exposed to various abiotic stresses due its higher turnover rate of reaction (Garg and Manchanda, 2009). The role of CAT in scavenging  $H_2O_2$  was observed

in several studies (Hasanuzzaman *et al.*, 2011; Hasanuzzaman and Fujita, 2013). In this study, CAT activity was significantly decreased upon exposure to salt stress in susceptible variety 'BARI Gom 21' and this decrease in CAT activity in 'BARI Gom 21' under salt stress might be due to its inactivation by the accumulated H<sub>2</sub>O<sub>2</sub> induced by water shortage or ineffective enzyme synthesis or change in assembly of enzyme sub-units (Gupta *et al.*, 2009). On the other hand, CAT activity was significantly increased at mild salt stress and decreased under severe salt stress in 'BARI Gom 21' (Fig. 5A) (Gupta *et al.*, 2009; Khan *et al.*, 2009). This trend was supported by earlier reports (Azooz *et al.*, 2009; Lin *et al.*, 2010; Hasanuzzaman *et al.*, 2014). On the contrary, SA-supplemented salt-stressed seedlings showed enhanced activity CAT than those under salt treatment without SA which suggests an unambiguous role of SA in scavenging H<sub>2</sub>O<sub>2</sub> under salt stress. Similar increases in CAT activity after SA supplementation was observed under salt stress by other researchers (Yusuf *et al.*, 2008; Noriega *et al.*, 2012). POD activity increased under salinity stress (Rohman *et al.*, 2015; Li *et al.*, 2014). In this study, POD activity increased at mild stress but decreased at severe stress. In higher plants, H<sub>2</sub>O<sub>2</sub> is scavenged by the ascorbate-glutathione pathway and/or by CAT and non-specific PODs (Scandalios, 2005; Miller *et al.*, 2010). CAT, POD and APX are reported to scavenge H<sub>2</sub>O<sub>2</sub> to water in plant species (Gill and Tujeta, 2010; Miller *et al.*, 2010). The increased activities of POD and GPX under salt stress played important role in H<sub>2</sub>O<sub>2</sub> scavenging (Rohman *et al.*, 2015). SA in salt treatments increased the activities of POD (Fig. 5B) which reduced the H<sub>2</sub>O<sub>2</sub> level and MDA production as well. Li *et al.* (2014) found upregulation of SOD, POD, CAT and APX by application of SA in salt stressed *T. grandis* seedlings. Plant GSTs are also associated with responses to various forms of abiotic stress (Hossain *et al.*, 2006; Dixon *et al.*, 2010) and stress tolerance is often correlated with enhanced activity of GST (Hasanuzzaman *et al.*, 2012a, b). In both wheat varieties of our experiment, GST activity markedly increased under salt stress where comparatively higher activity was observed in salt tolerant 'BARI Gom 21' (Fig. 5C). The hereby results are partially supported by Hoque *et al.* (2008) and Hasanuzzaman *et al.* (2014).

A result of the assimilation of metabolic reactions in plants is yield; consequently, yield can be affected by this metabolic activity at any period of plant growth which is influenced by any factor (Ibrahim and Aldesuquy, 2003). In this study, salt stress reduced yield and yield attributes such as spike length, effective tiller plant-1, non-effective tiller plant-1, number of spikelet spike-1, 1,000 grain weight, grain yield, straw yield, biological yield and harvest index in both wheat cultivars (Fig. 6(A, B), 7(A-C) and 8(A-D)). The reduction of photosynthetic pigments, carbohydrates accumulation (polysaccharides) and nitrogenous compounds (total nitrogen and protein) attributes the reduction of yield under salt stressed condition. Yield and yield components reduction under similar condition have also been reported by the other researchers for different crops (Aldesuquy *et al.*, 2012 for wheat; Sankar *et al.*, 2008 for *Abelmoschus esculentus* and Arfan *et al.*, 2007 for wheat). They clearly indicated that salt tolerant genotypes

showed less reduction in yield plants in respect of susceptible ones. Therefore, under salt stress condition 'BARI Gom 25' had better yield than that of 'BARI Gom 21'. However, SA treated plants under salt stress condition increased in all the studied yield criteria. These findings are in agreement with those reported by Ali and Mahmoud (2013), Arfan *et al.* (2007) and Singh and Usha (2003) on wheat, Gunes *et al.* (2007) on maize and Elwan and El-Hamahmy (2009) on pepper. Arfan *et al.* (2007) reported that, under saline condition grain yield increasing along with increase in 100 grain weight, number of grains and number of spikelets spike-1 with SA application.

## Conclusions

Based on the results of the present experiment, it can be therefore, concluded that exogenous SA spray is an effective way to overcome the adverse effects of osmotic stress on growth, physiology and yield components of wheat. It could be partially attributed to the increase in non-enzymatic and enzymatic antioxidants. In all the cases, 'BARI Gom 25' was a better performer under salt stress compared to salt sensitive 'BARI Gom 21'. All parameters decreased at any level of salt stress. Exceptions were abnormal seedling, non-effective tiller hill-1, MDA content, H<sub>2</sub>O<sub>2</sub>, GSSG content and GST activity which increased in response to salinity.

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