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Salt-Induced Changes in Physio-Biochemical and Antioxidant Defense System in Mustard Genotypes

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Abstract: Salinity stress is a major factor limiting plant growth and productivity of many crops including oilseed. The present study investigated the identification of salt tolerant mustard genotypes and better understanding the mechanism of salinity tolerance. Salt stresses significantly reduced relative water content (RWC), chlorophyll (Chl) content, K^+ and K^+/Na^+ ratio, photosynthetic rate (P_N), transpiration rate (Tr), stomatal conductance (gs), intercellular CO_2 concentration (Ci) and increased the levels of proline (Pro) and lipid peroxidation (MDA) contents, Na^+, superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) in both tolerant and sensitive mustard genotypes. The tolerant genotypes maintained higher Pro and lower MDA content than the salt sensitive genotypes under stress condition. The activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione peroxidase (GPX), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) were increased with increasing salinity in salt tolerant genotypes, BJ-1603, BARI Sarisha-11 and BARI Sarisha-16, but the activities were unchanged in salt sensitive genotype, BARI Sarisha-14. Besides, the increment of ascorbate peroxidase (APX) activity was higher in salt sensitive genotype as compared to tolerant ones. However, the activities of glutathione reductase (GR) and glutathione *S*-transferase (GST) were increased sharply at stress conditions in tolerant genotypes as compared to sensitive genotype. Higher accumulation of Pro along with improved physiological and biochemical parameters as well as reduced oxidative damage by up-regulation of antioxidant defense system are the mechanisms of salt tolerance in selected mustard genotypes, BJ-1603 and BARI Sarisha-16.

Keywords: Mustard; saline sensitivity; photosynthesis; antioxidants; ionic imbalance; oxidative stress

1 Introduction

Salinity is known to adversely affects the production of most crops worldwide [1] because their growth, biomass and yield are significantly suppressed by this abiotic means [2,3]. Plants grow in salt affected soils are exposed to a number of unfavorable conditions such as low soil water potential, nutrient imbalance and higher accumulation of Na^+ and Cl^- or K^+ deficiency in cells. Generally, plants exposed to salinity stress undergo changes of different metabolic processes [4]. The ability of plants to counteract the damaging effect of salt stress can be judged by examining various morpho-physiological and biochemical parameters such as water uptake, inorganic nutrients, hormonal regulation, compatible solutes as well as oxidative defense system [5,6]. Higher concentration of salt in plant cells decrease K^+ ions and increase Na^+ uptake as Na^+ causes K^+ efflux and triggers K^+ leakage from plant cells. However, Na^+ also displaces Ca^{2+} from membranes, which increases intracellular Na^+ under salt stress. Moreover, under higher salt-stress condition, Na^+ content exceeds to K^+, resulting in a higher Na^+/K^+ ratio as well as nutrient imbalance [7]. Besides, lower Na^+/K^+ ratio in plants under saline conditions is an indicator or selection criterion of salt tolerance in crops [8,9].

Lower chlorophyll loss by NaCl stress is an indicator of salt tolerance in crops [10,11]. On the other hand, photosynthesis is also a premier physiological process in plants, and it is affected by salt stress. Reduction in photosynthesis not only affects the opening and closing of stomata but also decreases the assimilation of CO_2. However, photosynthetic capacity under salt stress depends on photosynthesizing tissue, photosynthetic pigments, stomatal and non-stomatal factors which affect the CO_2 assimilation (gas exchange and metabolism) [12]. A numerous studies have been reported that a significant reduction in photosynthesis was found in sunflower [13], *Brassica spp*. [14], pea, radish, and turnip [6,15], and maize [16], and wheat [17].

Salt-induced oxidative stress inhibited the growth of most plants as a result of enhanced generation of reactive oxygen species (ROS) which include superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and singlet oxygen ($^1O_2$) [5]. ROS are formed as byproducts of normal cellular metabolism and necessary for enzymatic reactions when plants are exposed to a lower level of salinity [18]. The over-production of ROS resulting in cellular damage through oxidation of lipids, protein, and nucleic acids [19]. Plants adapt to salt stress through reducing ROS by accumulation of certain protective compatible solutes like proline, glycine betaine, polyols, trehalose, etc. [20]. It is well established that plant tolerance to salinity stress depends on ion homeostasis, osmotic adjustment and efficient and synchronous action of various components of the antioxidant defense system. Plants always try to keep well-developed enzymatic and non-enzymatic antioxidant defense system to overcome the deleterious effects of ROS [21]. The antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione *S*-transferase (GST), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), while non-enzymatic antioxidants includes ascorbate, glutathione, α-tocopherol, carotenoids, and phenolic compounds, non-protein amino acids etc. [21,22]. They act together in scavenging or detoxifying ROS and subsequent protection of plant cells from oxidative damage [19,23]. Although the protective roles of antioxidants have been extensively studied in different plant species the underlying saline tolerant mechanism is not fully understood.

Mustard (*Brassica spp*) is an economically important oilseed crop in Bangladesh. Among mustard *Brassica campestris* contributes 85–90% of the total mustard production. Out of 2.83 million hectares of the coastal areas of Bangladesh about 0.88 million hectares are affected by salinity [24]. However, till now, no saline tolerant mustard variety have been developed, and its tolerance mechanisms still unclear. Therefore, the present investigation was undertaken to search out salt tolerant mustard genotypes and understanding insight into the tolerant mechanism of salt tolerance.

2 Materials and Methods

2.1 Plant Materials and Stress Treatments

The experiment was carried out in the green house and central laboratory of Bangladesh Agricultural Research Institute (BARI), Gazipur, Bangladesh. One hundred and twenty five mustard genotypes were previously screened against 0, 8 and 12 dSm^{-1} levels of salinity. Among them, genotypes BJ-1603 and BARI Sarisha-16 survived whole life in all salinity levels and thus, they were considering as tolerant genotypes. On the other hand, BARI Sarisha-14 was found the most salt sensitive genotype. In this study, we included a previously reported tolerant variety, BARI Sarisha-11 [25] as tolerant materials. Besides, BARI Sarisha-14 was used as salt sensitive genotypes. Before sowing, seeds were surface sterilized with 1% sodium hypochlorite solution for 10 minutes and were vigorously rinsed with distilled water. The plastic pots (35 cm diameters at top and 30 cm at bottom) were filled with sandy loam soil and seeds were sown. Hoagland's nutrient solution was applied on alternate days to each pot. The pot experiment was conducted following completely randomized design (CRD) with three replications under control conditions (relative humidity 60–70%, temperature 25 ± 2°C, light 655 μmole $m^{-1}s^{-1}$ and photoperiod from 12 to 14 h) in green house. After 16–18 days of germination, seedlings were thinned to maintain nine plants each pot. The 0, 8, and 12 dSm^{-1} NaCl were mixed with Hoagland's nutrient solution and were applied to respective pots. The sodium chloride solution was applied step-wise in aliquots of 0 to 12 dSm^{-1} attained. In control treatment, fresh water was added instead of NaCl solution. Salinity levels were measured by direct soil EC meter. After, 26–28 days the exposure to NaCl stress, six plants per replication were harvested and separated into shoots and roots. The samples were then oven-dried at 80°C for one week to record dry masses and the remaining plants were used for recording data in fully expanded leaves for the following variables:

2.1.1 Relative Water Content

Relative water content (RWC) was measured according to the method of [26]. Leaves (third and fourth) were weighed (fresh wt, FW) and then immediately floated on distilled water in a petri-dish to saturate for next 12 h in the dark. Turgid weight (TW) was measured after gently removing excess surface water with paper towel. Dry weight (DW) was measured after oven drying at 80°C for 48 h. The calculation was done using the following formula: RWC (%) = [(FW − DW) × 100]/[TW − DW]

2.1.2 Chlorophyll Content

The chlorophyll (Chl) content was measured according to [27] by homogenizing leaf samples (0.5 g) with 10 ml of acetone (80% v/v) followed by centrifuging at 9,000 × g for 10 min. Absorbance was measured with a UV-vis spectrophotometer at 663 and 645 nm for Chl a and Chl b content, respectively.

2.1.3 Photosynthetic Parameters

Gas exchange parameters were determined using a LiCOR 6400 open system portable infrared gas analyzer (IRGA) (Lincoln, USA). These parameters were determined in noon and cloudless clear days when light intensity was fully expanded leaves (at 9 a.m. to 2.00 p.m.). A young fully expanded leaf (first and third) was selected and used for the data of photosynthetic rate, transpiration rate, sub-stomatal CO_2 concentration and stomatal conductance. Before taking the measurement, the IRGA was calibrated and the zero of the instrument was adjusted approximately every 30 min during the measurement. Leaf was enclosed in a 1 L gas exchange chamber for 60 second. The conditions which were used for the equipment/leaf chamber were as follows: ambient pressure 99.2 kPa, atmospheric CO_2 concentration (Cref) 400 μmol L^{-1}, leaf surface area 6 cm^2, PAR (Qleaf) was maximum up to 900–1000 μmol m^{-2} s^{-1} and the chamber water vapor pressure varied from 4.0 to 5.8 mbar [28].

2.1.4 Inorganic Sodium and Potassium Ions

Sodium (Na^+) and potassium (K^+) ion were determined following [29]. The fully expanded youngest leaf (100 mg) from top was well-ground. Dry ground materials were digested in 8.0 ml concentrated HNO_3. After digestion, the volume of each sample was made up to 10 ml deionized water. The Na^+ and K^+ ions were estimated with a flame emission spectrophotometer (Jenway PFP7).

2.1.5 Proline Content

Proline content in mustard leaves was measured the following protocol of [30]. The amount of proline was estimated by comparison with a standard curve.

2.1.6 Lipid Peroxidation

The level of lipid peroxidation was measured by estimating melondialdehyde (MDA), a decomposition product of the per-oxidized polyunsaturated fatty acid component of the membrane lipid, using 2-thiobarbituric acid (TBA) as the reactive material [31]. The concentration of MDA was calculated by using the extinction coefficient of 155 mM^{-1} cm^{-1} and was expressed as nmole of MDA g^{-1} FW.

2.1.7 Hydrogen Peroxide (H_2O_2) and Superoxide ($O_2^{\bullet-}$) Generation Rate

Hydrogen peroxide and superoxide radical were measured according to the method described by [32]. Hydrogen peroxide and superoxide radical were calculated from a standard curve of $NaNO_2$.

2.1.8 Histochemically Detection of Superoxide and Hydrogen Peroxide

Histochemically detection of superoxide and hydrogen peroxide were visualized by staining of leaves according to the method described in [33] with slight modification. Briefly, the second or third leaves were stained in 0.1% nitrobluetetrazolium (NBT) and 1% of 3,3-diaminobenzidine (DAB) solution for 12 h under dark condition for $O_2^{\bullet-}$ and H_2O_2 detection, respectively. Incubated leaves were then decolorized by immersing in boiling ethanol which was allowed to visualization of blue insoluble formazan (for $O_2^{\bullet-}$) or deep brown polymerization product (for H_2O_2).

2.1.9 Extraction of Soluble Protein

Using a pre-cooled mortar and pestle, 0.5 g of fresh leaves of mustard seedlings were homogenized in 0.0090 g ascorbic acid, 10 ml of 500 mM ice-cold K-P buffer (pH 7.0), 5 ml KCl in 1 M solution, 5 mM 25 µl β-marcaptoethanol and volume up to 50 ml. The homogenate was centrifuged at 11,500 × g for 15 min at 4°C, and the supernatant was used for enzyme assay.

2.1.10 Determination of Protein

The protein concentration in the leaf extracts were determined according to the method of [34] using BSA as a protein standard.

2.1.11 Assay of Enzymatic Activities

Activities of enzymes were assayed spectrophotometrically (Shimadzu UV-1800, Japan). Superoxide dismutase (SOD, EC: 1.15.1.1) activity of whole cell homogenates prepared on ice in 50 mM K-phosphate buffer (pH 7.8, with 1.34 mM diethylenetriaminepenta acetic acid, EDTA) was determined using an indirect competitive inhibition assay [35]. One unit of activity was defined as that amount of protein required to inhibit NBT reduction by 50%. SOD activity was expressed as units as min^{-1} mg^{-1} protein. Peroxidase (POD, EC: 1.11.1.7) activity was estimated according to [36]. Catalase (CAT, EC: 1.11.1.6) activity was measured according to the method of [36] by monitoring the decrease of absorbance at 240 nm for 1 min caused by the decomposition (or degradation) of H_2O_2. The reaction was initiated with enzyme extract and the activity was calculated using the extinction coefficient of 39.4 M^{-1} cm^{-1}. Ascorbate peroxidase (APX, EC: 1.11.1.11) activity was assayed following the method of [37]. The

reaction was started by the addition of H_2O_2, 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^{-1} cm^{-1}. Monodehydroascorbate reductase (MDHAR, EC: 1.6.5.4) activity was determined by the method of [38]. The activity was calculated from the change in ascorbate at 340 nm for 1 min using an extinction coefficient of 6.2 mM^{-1} cm^{-1}. Dehydroascorbate reductase (DHAR, EC: 1.8.5.1) activity was determined by the procedure of [37]. The activity was calculated from the change in absorbance at 265 nm for 1 min using an extinction coefficient of 14 mM^{-1} cm^{-1}. Glutathione peroxidase (GPX, EC: 1.11.1.9) activity was measured as described by [39] using H_2O_2 as a substrate. The reaction was stated by the addition of H_2O_2. The oxidation of NADPH was recorded at 340 nm for 1 min, and the activity was calculated using the extinction coefficient of 6.62 mM^{-1} cm^{-1}. Glutathione reductase (GR, EC: 1.6.4.2) activity was measured by the method of [36] using an extinction coefficient of 6.2 mM^{-1} cm^{-1}. Glutathione *S*-transferase (GST, EC: 2.5.1.18) activity was determined spectrophotometrically by the method of [36].

2.1.12 Statistical Analysis

All data obtained was analyzed by SAS (Version 9.3) program following complete randomized design and the mean differences were compared by Duncan Multiple Range Test (DMRT). Values of mean ± SE were calculated from three replications (n = 3), and different letters on bars within a genotype are significant among the treatments at $p \leq 0.05$.

3 Results

3.1 Growth Attributes

Salt stress significantly decreased shoot and root length, and fresh and dry weight of all mustard genotypes when compared with the control (Fig. 1). At higher salinity stress, more reduction of the values was observed in BARI Sarisha-14 as compared to other genotypes. Data showed that our selected mustard genotypes, BJ-1603, BARI Sarisha-16 as well as BARI Sarisha-11 had higher shoot and root length, fresh and dry biomass than the sensitive genotype BARI Sarisha-14 under saline conditions (Figs. 1A–1D). Importantly, all the parameters were significantly higher in all tolerant genotypes than sensitive genotype at 12 dSm^{-1} salinity.

3.2 Relative Water Content (RWC) and Chlorophyll (Chl) Content

Under salt stress, leaf RWC revealed significant reduction in studied genotypes as compared to the control plants (Fig. 2A). Maximum reduction of RWC was observed in salt sensitive genotype BARI Sarisha-14 while minimum reduction was observed in tolerant genotypes. Decrease in RWC was more pronounced in sensitive genotypes at 12 dSm^{-1} NaCl stress. Like RWC, Chl content was also decreased in stressed seedlings (Fig. 2B). The differences in Chl content among the genotypes become clear at higher salinity level. Data also showed that the Chl content at 12 dSm^{-1} NaCl in sensitive genotype was significantly lower than that in other genotypes.

3.3 Photosynthetic Rate (P_N), Transpiration Rate (Tr), Stomatal Conductance (gs) and Intercellular CO_2 Concentration (Ci)

Salt stress caused a significant reduction in P_N, *Tr*, *gs*, and *Ci* of all mustard genotypes compared to control treatment (Fig. 3). Salinity caused significant decreases in P_N, *Tr* and *gs* in salt sensitive mustard genotype, BARI Sarisha-14 compared to salt tolerant BARI Sarisha-11 variety as well as our selected tolerant genotypes BJ-1603 and BARI Sarisha-16 (Figs. 3A–3C). On the other hand, in BARI Sarisha-11, BJ-1603 and BARI Sarisha-16, the *Ci* was significantly higher than BARI Sarisha-14 (Fig. 3D).

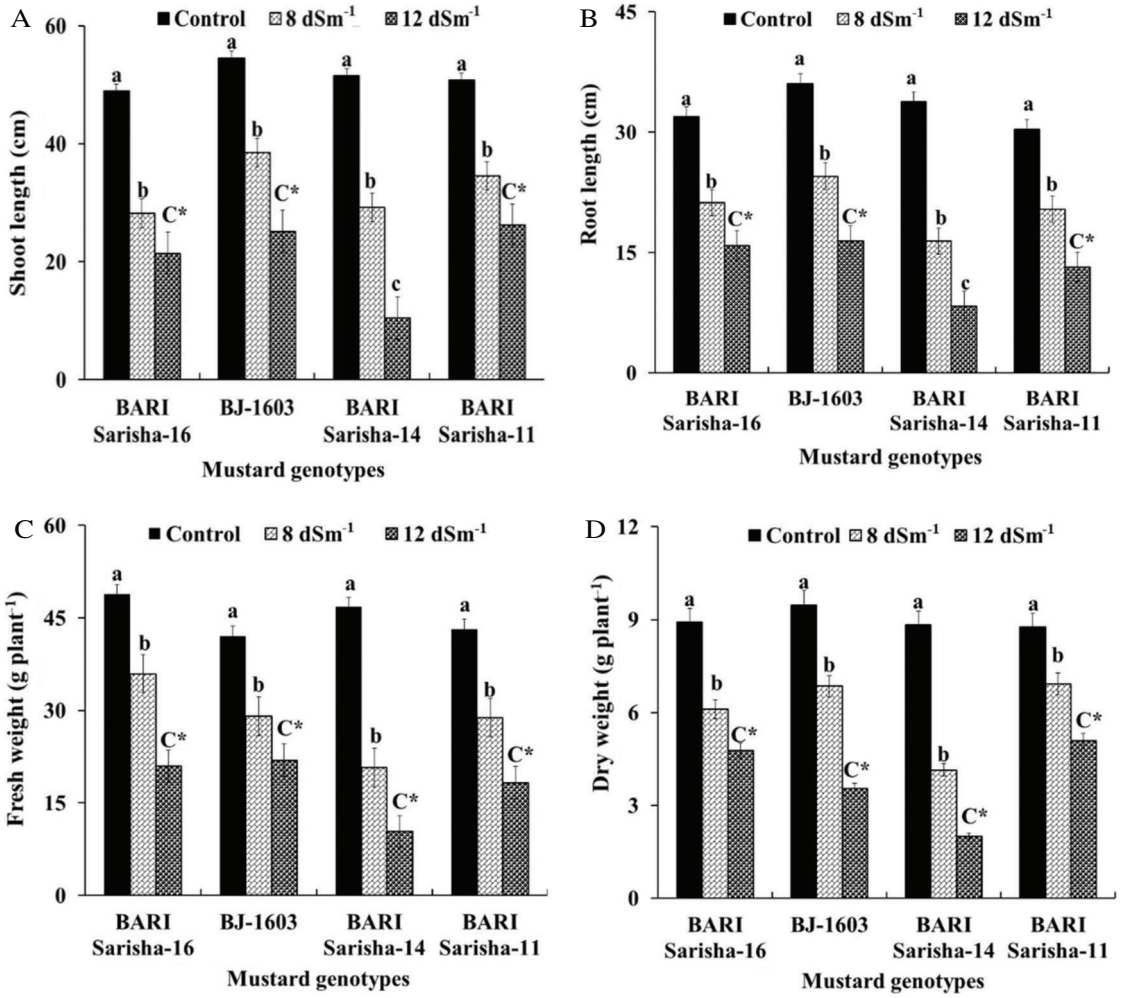

Figure 1: Effect of salinity on shoot length (A), root length (B), fresh weight (C), and dry weight (D) of different mustard genotypes. Data are presented as mean ± SD. Asterisk mark indicated significant different from sensitive genotype

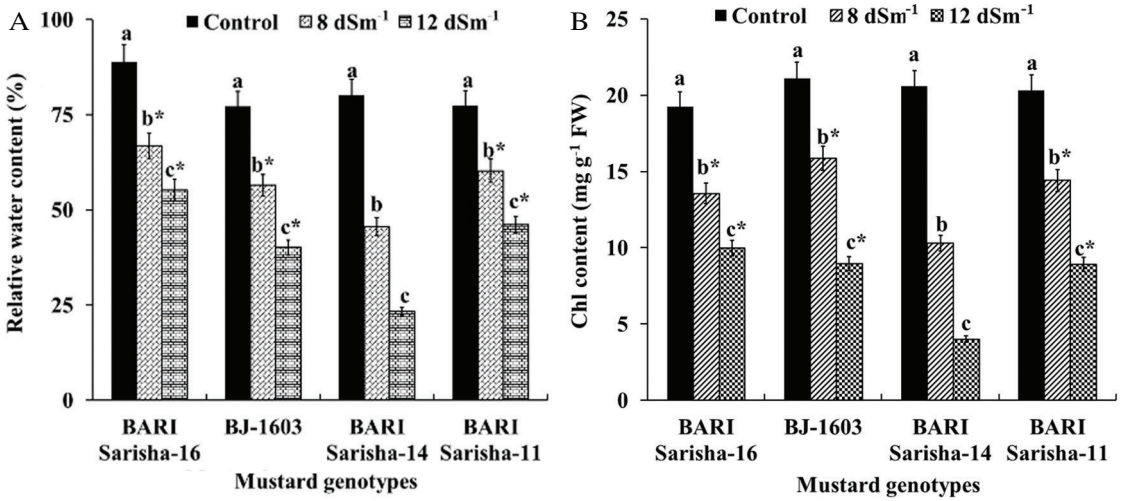

Figure 2: Effect of salinity on leaf relative water content (A) and chlorophyll content (B) of different mustard genotypes. Data are presented as mean ± SD. Asterisk mark indicated significant different from sensitive genotype

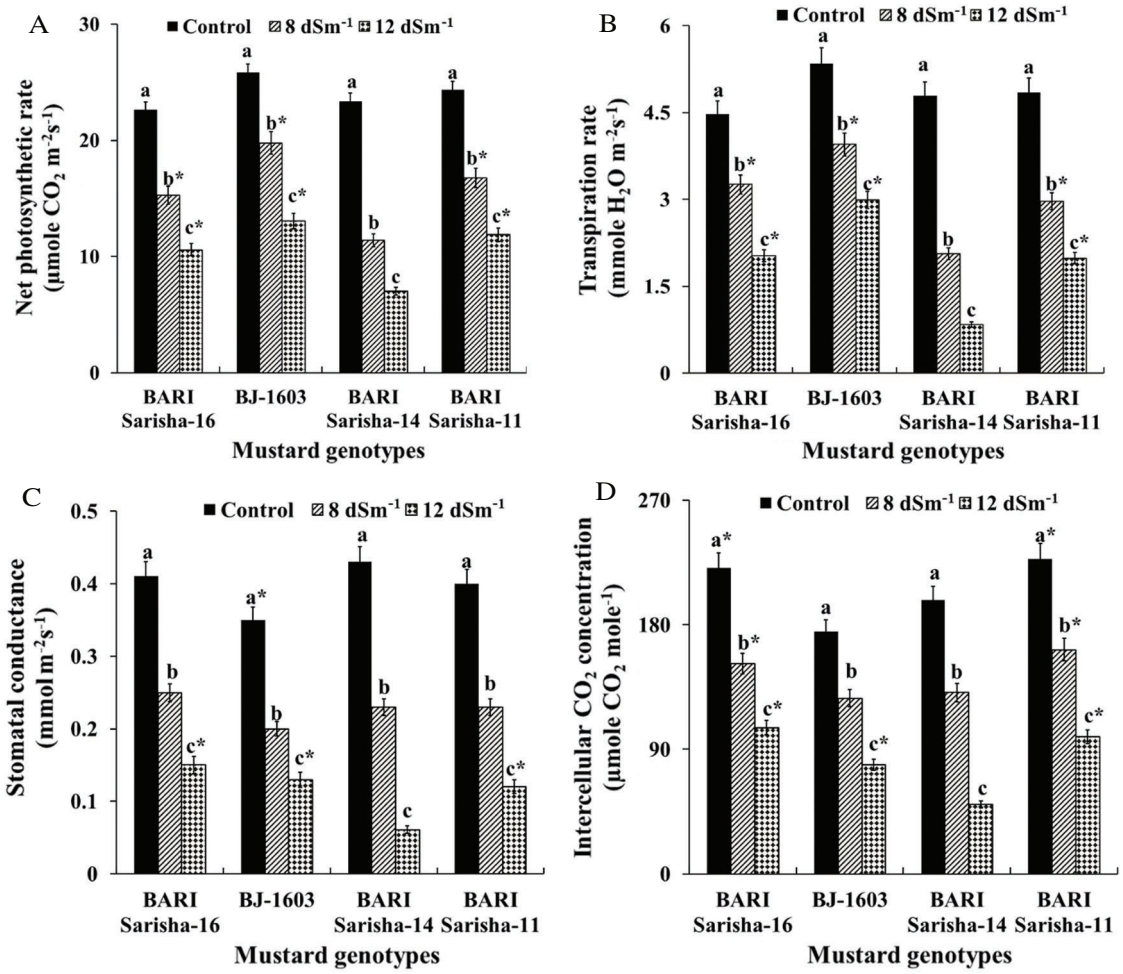

Figure 3: Effect of salinity on photosynthetic rate (A), transpiration rate (B), stomatal conductance (C) and intercellular CO_2 concentration (D) of different mustard genotypes. Asterisk mark indicated significant different from sensitive genotype

3.4 Na^+ and K^+ Concentration and Their Ratio

As compared to control, Na^+ content was significantly increased with increasing salinity in all mustard genotypes (Fig. 4A). The lowest Na^+ concentration was observed in selected genotypes BARI Sarisha-16, BJ-1603 while it was the highest in salt sensitive genotype BARI Sarisha-14 at both salinity levels. The trend of K^+ content was almost reverse to that of Na^+ showing decreasing K^+ concentration in all mustard genotypes with increasing salinity (Fig. 4B). However, this decrease in K^+ was more prominent in BARI Sarisha-14 as compared to salt tolerant BARI Sarisha-11 as well as selected tolerant genotypes BJ-1603 and BARI Sarisha-16. The increasing uptake of Na^+ with increasing salinity ultimately decrease K^+/Na^+ ratio (Fig. 4C). BARI Sarisha-16 and BJ-1603 maintained higher K^+/Na^+ ratio showing better performance under saline condition.

3.5 Endogenous Proline (Pro) and Malondialdehyde (MDA) Content

Salt stress caused significant increase in endogenous Pro content in seedlings of all mustard genotypes. However, the increment was significantly higher at salt tolerant BARI Sarisha-11 as well as the tolerant genotypes (BJ-1603, BARI Sarisha-16) compared to salt sensitive genotype, BARI Sarisha-14 (Fig. 5A).

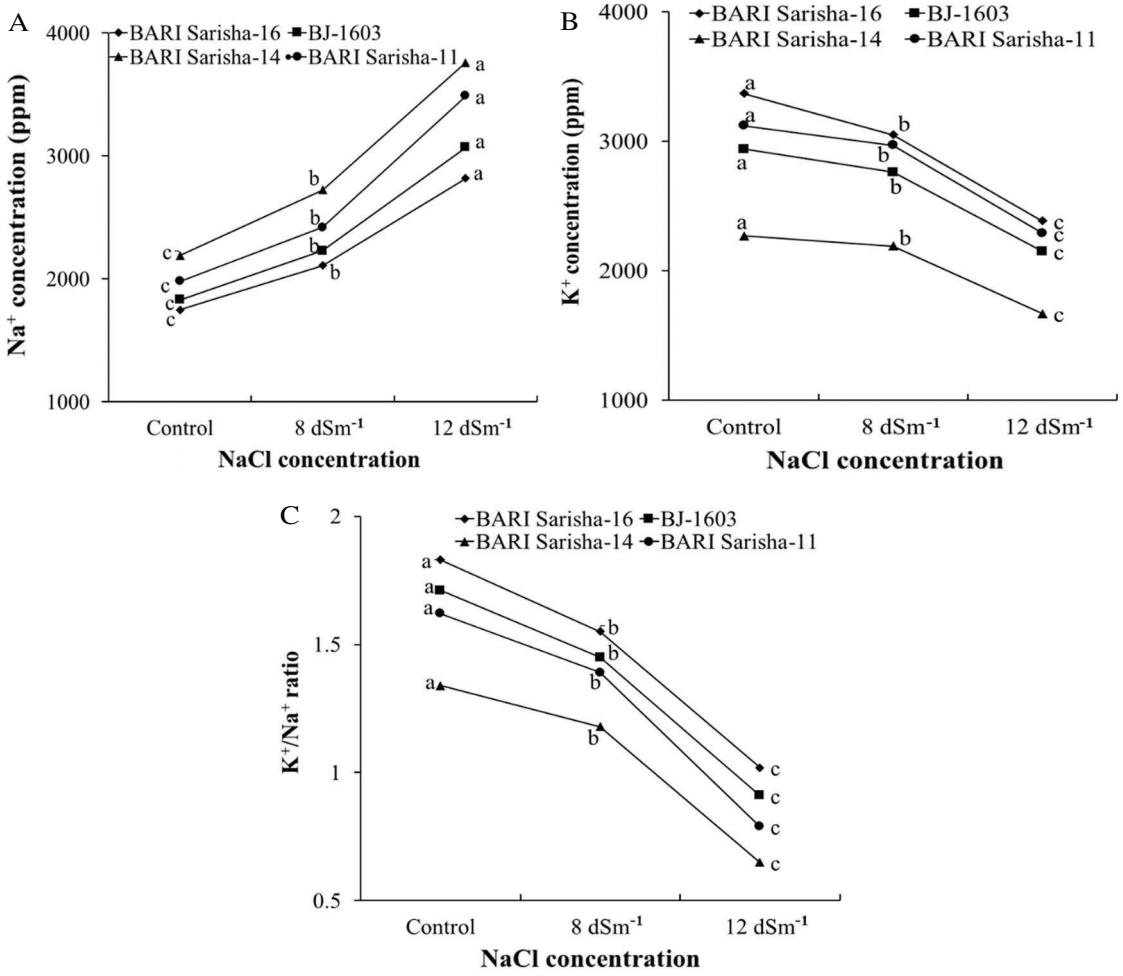

Figure 4: Effect of salinity on Na^+ concentration (A), K^+ concentration (B), and K^+/Na^+ ratio (C) of different mustard genotypes

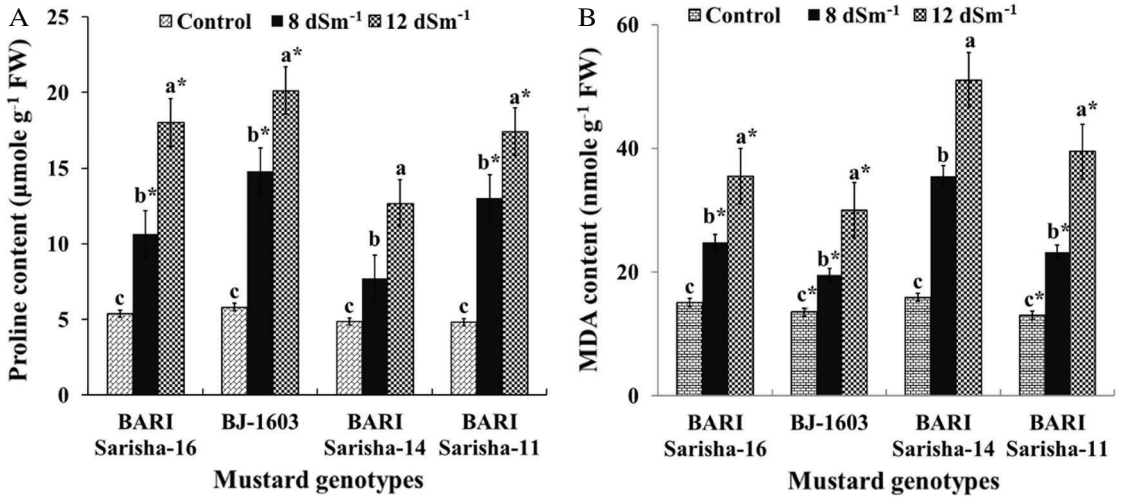

Figure 5: Effect of salinity on proline (A) and lipid peroxidation (MDA) content (B) of different mustard genotypes. Asterisk mark indicated significant different from sensitive genotype

Among the genotypes, Pro content was found the highest in observed highest in BJ-1603 at 8 dS^{-1}m and 12 dS^{-1}m NaCl. On the other hand, the MDA content sharply increased at different level of salt stress in mustard genotypes. However, the increment was significantly higher in salt sensitive genotype, BARI Sarisha-14 as compared to salt tolerant genotypes at 12 dSm^{-1} NaCl stress (Fig. 5B).

3.6 Hydrogen Peroxide (H_2O_2) and Superoxide ($O_2^{\bullet-}$) Generation Rate

The H_2O_2 and $O_2^{\bullet-}$ generation were increased significantly under salt stress as compared to respective control (Figs. 6A and 6B). It was clear that salt sensitive genotype BARI Sarisha-14 showed significantly higher H_2O_2 and $O_2^{\bullet-}$ generation as compared to those in salt tolerant genotypes at 12 dSm^{-1} NaCl (Figs. 6A and 6B).

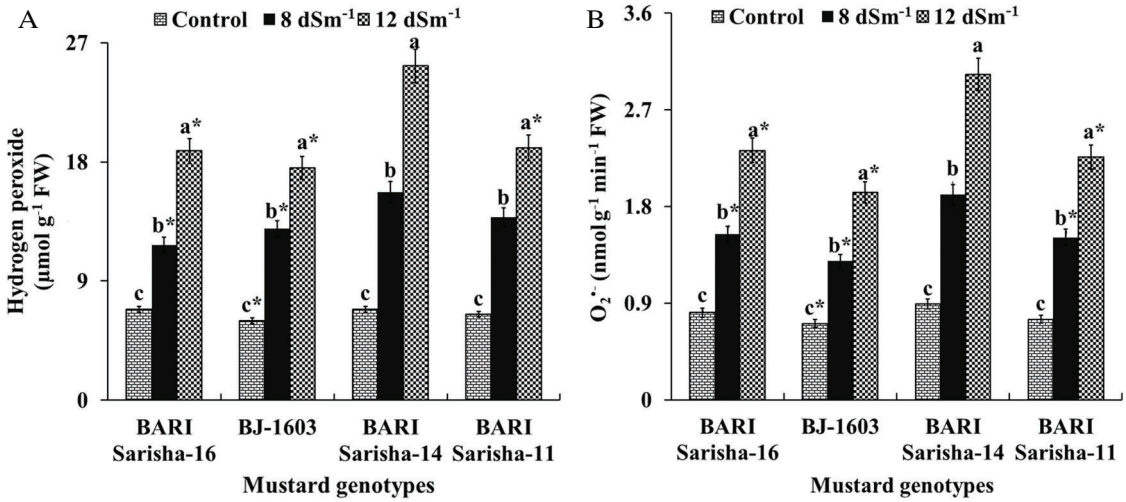

Figure 6: Effect of salinity on hydrogen peroxide (H_2O_2) (A) and superoxide ($O_2^{\bullet-}$) generation (B) of different mustard genotypes. Asterisk mark indicated significant different from sensitive genotype

3.7 Histo-Chemically Detection of Hydrogen Peroxide (H_2O_2) and Superoxide ($O_2^{\bullet-}$) Generation in Mustard Leaves

Histochemical staining detected higher H_2O_2 and $O_2^{\bullet-}$ generation at 12 dSm^{-1} NaCl in tolerant and sensitive genotypes compared to control. Importantly, it was clear that our selected genotypes showed high dark blue insoluble formazan product or spots and deep brown polymerization product or spots localization, in the salt-treated mustard seedling leaves (Figs. 7A and 7B). Contrary, salt sensitive

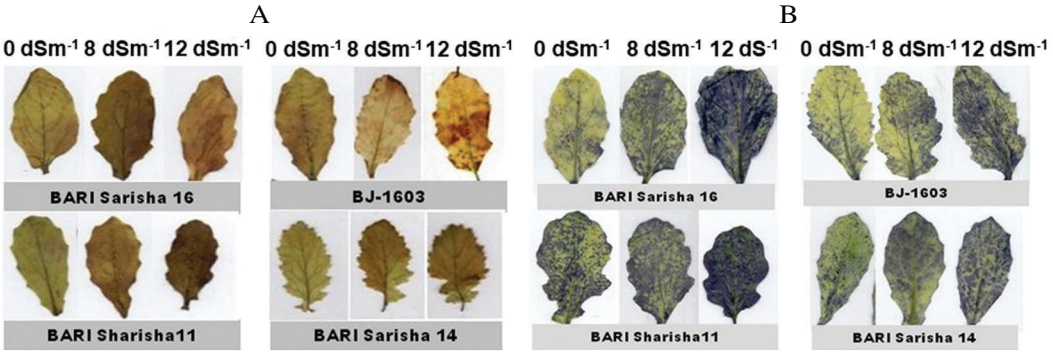

Figure 7: Histo-chemical detection of H_2O_2 (deep brown) (A) and $O_2^{\bullet-}$ generation (bluish) (B) in leaves of mustard seedlings under salt stress

genotype BARI Sarisha-14 showed light dark-blue insoluble formazan product or spots and light deep-brown polymerization product or spots in the salt-treated mustard leaves (Figs. 7A and 7B).

3.8 Activities of Anti-Oxidant Enzymes

3.8.1 Super Oxide Dismutase (SOD), Catalase (CAT), Peroxidase (POD) and Glutathione Peroxidase (GPX)

Under salt stress, the activities of SOD and CAT were unchanged in salt sensitive genotype BARI Sarisha-14 while those were increased in salt tolerant genotypes, BARI Sarisha-11, BJ-1603 and BARI Sarisha-16 (Figs. 8A and 8B). It was clear that salt tolerant genotypes showed significantly higher SOD and CAT activity compared to salt sensitive variety at 12 dSm^{-1} salt level (Figs. 8A and 8B). Besides, salinity increased POD activity significantly in tolerant mustard genotypes (Fig. 8C). Contrary, POD activity was remained unchanged in salt sensitive genotype at 8 and 12 dSm^{-1} salt stresses (Fig. 8C). Similarly, salt stress increased GPX activity significantly in tolerant genotypes, BARI Sarisha-11, BJ-1603, and BARI Sarisha-16 while that activity in salt sensitive genotype BARI Sarisha-14 was unchanged as compared to control (Fig. 8D).

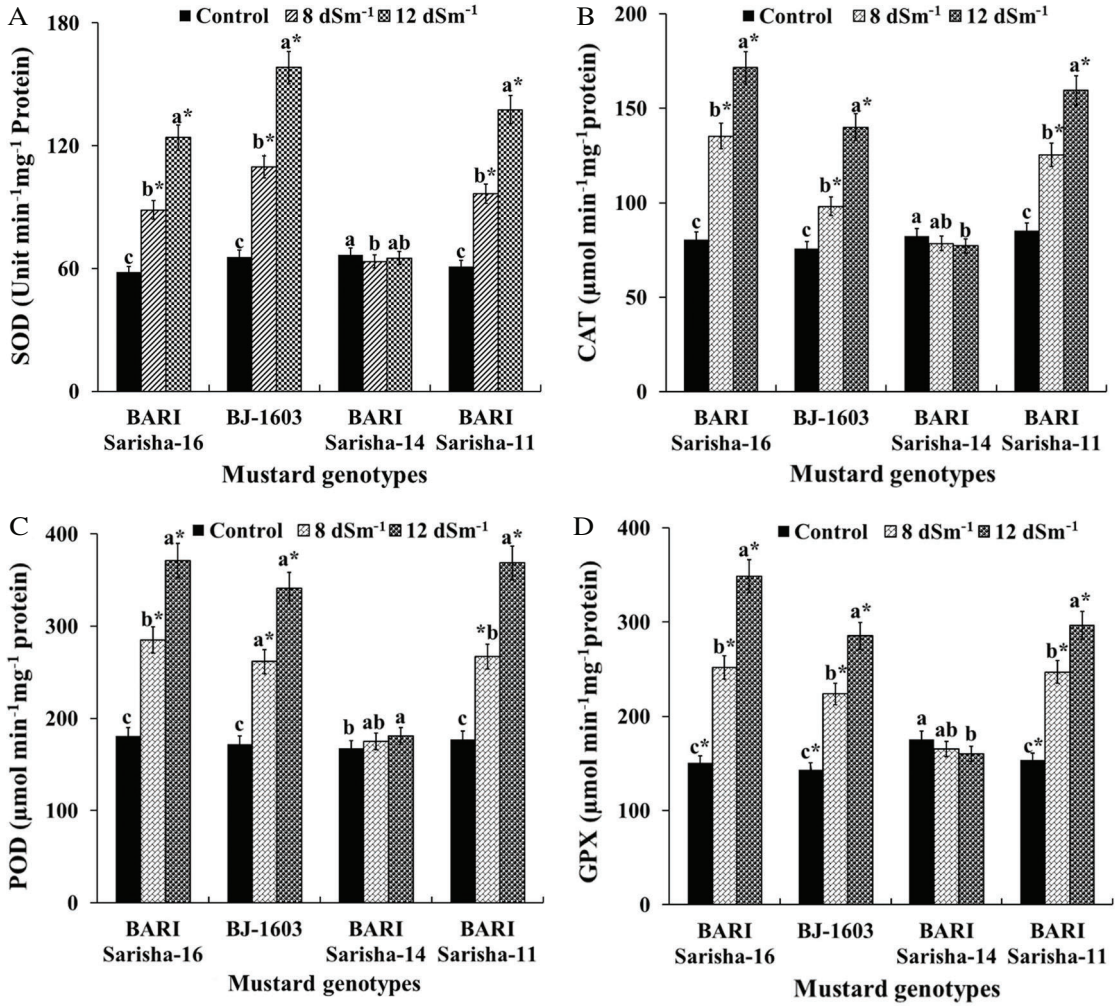

Figure 8: Effect of salinity on specific activity of super oxide dismutase (SOD) (A), catalase (CAT) (B), peroxidase (POD) (C), and glutathione peroxidase (GPX) (D) of different mustard genotypes. Asterisk mark indicated significant different from sensitive genotype

3.8.2 Ascorbate Peroxidase (APX), Monodehydroascorbate Reductase (MDHAR), Dehydroascorbate Reductase (DHAR) and Glutathione Reductase (GR)

Salinity increased APX activity significantly in all mustard genotypes (Fig. 9A). It was remarkable that salt sensitive genotype had higher APX activity in control as well as salt stress condition (9A). As compare to control, salt stress was decreased the MDHAR activity in salt sensitive genotype BARI Sarisha-14 (Fig. 9B). In contrast, the activity increased gradually and significantly in salt tolerant genotypes with increasing salt levels. Similar pattern was also found in DHAR activity in the mustard genotypes (Fig. 9C). The activity of GR increased with increasing salt levels in all the genotypes as compared to control (Fig. 9D). However, the increment was significantly higher in salt tolerant genotypes compared to salt sensitive genotype.

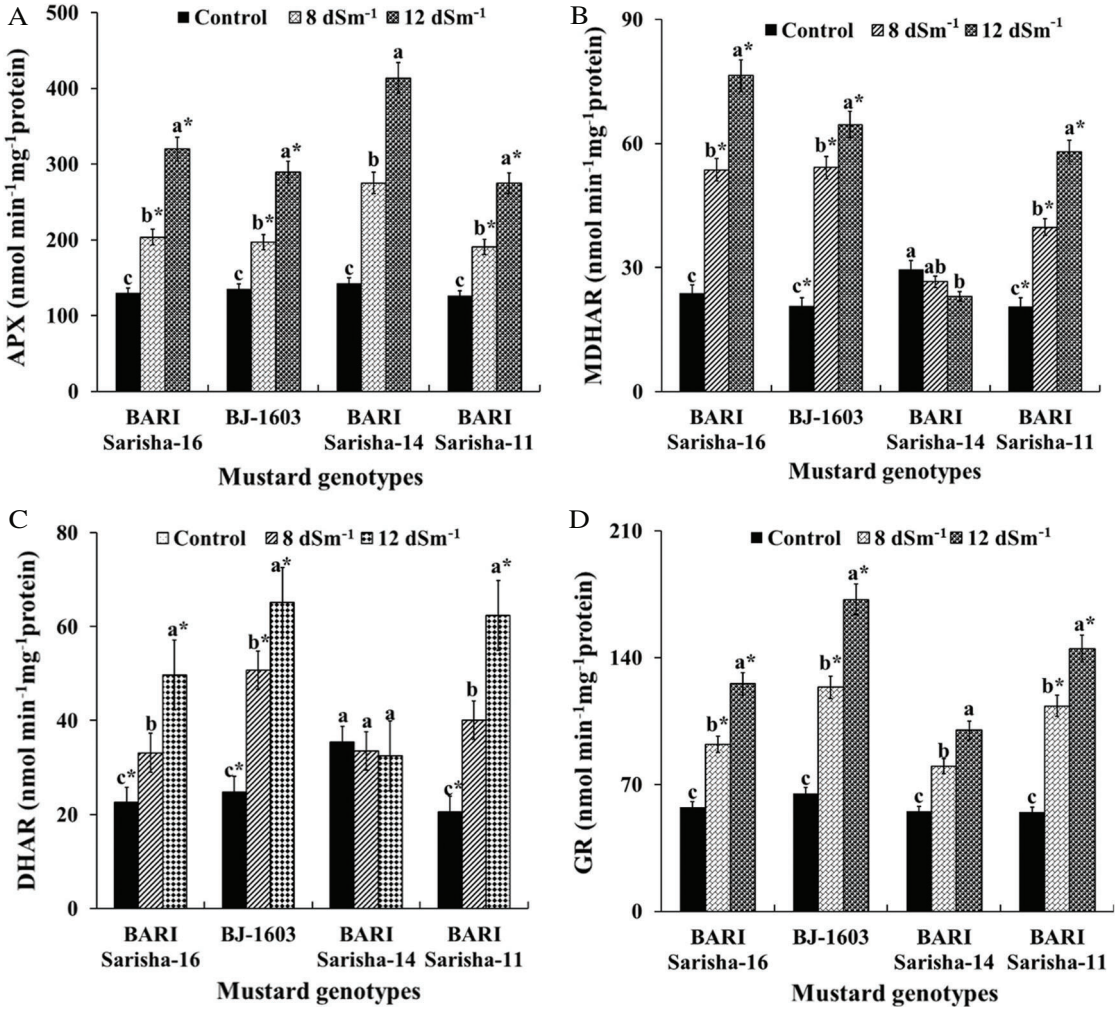

Figure 9: Effect of salinity onspecific activity of ascorbate peroxidase (APX) (A), monodehydroascorbatereductase (MDHAR) (B), dehydroascorbatereductase (DHAR) (C) and glutathione reductase (GR) (D) of different mustard genotypes Asterisk mark indicated significant different from sensitive genotype

3.8.3 Glutathione S-Transferase (GST)

Salinity increased GST activity significantly in all mustard genotypes compared to control (Fig. 10). Importantly, the salt tolerant genotypes, BARI Sarisha-11, BJ-1603, and BARI Sarisha-16 maintained

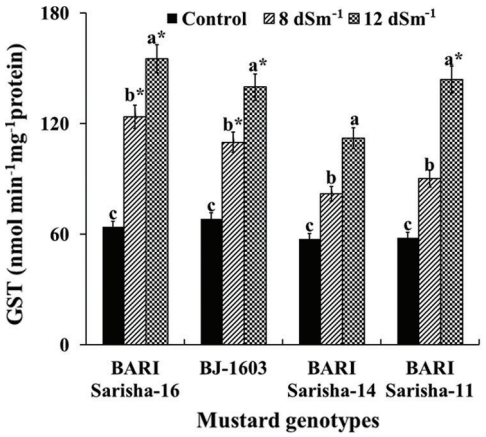

Figure 10: Effect of salinity on activity of glutathione *S*-Transferase (GST) of different mustard genotypes. Asterisk mark indicated significant different from sensitive genotype

significantly higher GST activity than salt sensitive genotype BARI Sarisha-14 at 8 and 12 dSm^{-1} salt stresses (Fig. 10).

4 Discussion

Salinity is one of the major environmental factors limiting crop productivity via several morphological, biochemical and physiological alterations. In this study, salt stress at 12 dSm^{-1} considerably decreased shoot and root length, and fresh and dry weight of all mustard genotypes and the decrease were higher in sensitive genotype BARI Sarisha-14 (Figs. 1A–1D). Salt-induced reduction in different growth attributes are parallel to previous report in different crops such as sunflower [40], maize [41], wheat [42] and okra [43]. Moreover, salt stress causes reduction in cell division as well as cell elongation [44] mainly due to salt-induced perturbate in uptake of nutrients, cytoplasmic enzyme inhibition, turgor loss [44], high accumulation of reactive oxygen species [45] hormonal imbalance [46] which in turn impairs plant growth in terms of yield or biomass production.

Regulation of water balance measured in terms of leaf RWC is considered as an important indicator for evaluating plants for tolerance to salt stress [47]. Moreover, osmotic stress caused by salinity, reduction of RWC is a common phenomenon in plants growth. In this study, salt stress caused significant higher decrease in RWC in leaves of salt sensitive genotype (Fig. 2A). Higher loss of leaf water content lead to malfunction of metabolic process. Similar decrease in RWC due to salt stress was reported earlier in a susceptible rice genotype by [48] and pea [47]. Salinity is known to cause water deficits, which in turn leads to decreased RWC. In addition, soluble salts in the soil hamper the uptake of water and nutrients which induce osmotic effects and ion toxicity [49].

Salinity stress caused higher losses of chlorophyllin leaves of salt sensitive variety BARI Sarisha-14 seedlings compared to tolerant variety BARI Sarisha-11 as well as selected mustard genotypes (BJ-1603, BARI Sarisha-16) (Fig. 2B). The present results are in good agreement previous findings in maize [36] and rice [48]. This reduction of chlorophyll contents under salinity stress could be due to the increased activity of chlorophyllase enzyme [50] or due to the disruption of fine structure of chloroplast and instability of pigment protein complexes by ions. Ion accumulation in leaves also adversely affected chlorophyll concentration [51]. Therefore, the tolerant genotypes maintained higher Chl as compared to salt sensitive genotypes suggesting tolerant genotypes can continue photosyntheic rate to sustain in saline condition.

Saline stress decreased the photosynthesis rate (P_N), stomatal conductance *(gs)*, intercellular CO_2 concentration (*Ci*) and transpiration rate (*Tr*) of all mustard genotypes. However, a stronger reduction was

observed in salt sensitive genotype BARI Sarisha-14 compared to salt tolerant BARI Sarisha-11 as well as selected genotypes BJ-1603 and BARI Sarisha-16 (Figs. 3A–3D) might be due to lower Chl content. Stomatal closure due to salt-induced abscisic acid accumulation is one of the vital factors which cause retardation in vital photosynthetic processes [40]. On the other hand, *Tr* and *gs* are directly involved in photosynthesis, decrease in *Tr* and *gs* results in the decrease in *Ci* and P_N. Higher *gs* in plant are known to increase CO_2 diffusion into leaf, thereby favoring higher P_N. Higher CO_2 assimilation rates could in turn favour a high growth and higher crop yield. Photosynthesis was markedly decreased by salt stress and this was accompanied by decrease *gs* (Figs. 3A–3C). Moreover, photosynthesis has a well-established role in plant growth and dry matter production and salinity tolerance is related to the maintenance of P_N and *gs* [11]. A positive significant relation between P_N and *gs* may suggest that the reductions in P_N were largely associated with stomatal closure, and therefore stomatal effects could be the most important to justify photosynthesis depression. From these observations, it is clear that inter-genotypic variation in the studied mustard genotypes for salt tolerance were only due to genetic variation in photosynthetic rate which could be used as an effective selection criterion for salt tolerance in different crops. Besides, *Tr* and *Ci* rate were also decreased considerably with increasing salt concentration (Figs. 3B and 3D), resulting in reducing biomass production. In this study, our selected tolerant mustard genotypes had better growth compared to salt sensitive genotype under salt stress condition due to higher P_N as well as higher biomass production. This result correlates with earlier findings on different crops okra [43], sunflower [52] and safflower [53].

Under salt stress, the increase in Na^+ ion content and decrease in K^+ ion uptake disturbs ionic imbalance as observed in many plant species [54]. Ion flux regulation is important for ensuring ionic balance where the concentration of essential ions is higher and the toxic ions below the range. In this study, the lowest Na^+ concentration was observed in selected genotypes, BJ-1603, BARI Sarisha-16 and the highest in salt sensitive variety BARI Sarisha-14 at both salinity levels (Fig. 4A). Moreover, higher accumulation of Na^+ and reduction of K^+ ion concentration was observed in salt sensitive genotype BARI Sarisha-14, resulting in a Na^+ and K^+ antagonism or with reversal (Fig. 4B). Importantly, maintenance of adequate K^+ in plant tissues under salt stress depends upon selective cellular K^+ and Na^+ distribution and compartmentation within plant parts [55]. Higher K^+/Na^+ ratio is an indicator of salt tolerance and minimum damage of plants under salt stress condition. On the other hand, lower K^+/Na^+ ratio indicates Na^+ mediated damage of plants. Maintaining of high K^+/Na^+ ratio is essential for the plants to survive under salt stress and the ratio may be used as a possible criterion for selecting salt tolerant genotypes [54]. Our results are in good support of the above data, as well as of the findings of [56].

Higher proline accumulation under salt stress is often suggested as a selection criterion for the stress tolerance of most plant species [20,57]. In our experiment, tolerant BARI Sarisha-11 as well as our selected genotypes, BJ-1603, BARI Sarisha-16 showed enhanced proline accumulation under different salt stress conditions (Fig. 5A). However, tolerant and our selected genotypes showed significantly higher Pro content than salt sensitive genotype BARI Sarisha-14 due to its adaptive features of higher tolerance. Higher proline in tolerant genotypes can function in cellular osmotic adjustment, protection of cellular macromolecules from damage by salts, detoxification of ROS, protection of membrane integrity and stabilization of proteins or enzymes, storage of nitrogen and scavenging of free radicals [20,57]. Thus accumulation of higher proline in the leaves of selected tolerant mustard genotypes might be involved in one or more of above processes and contributed to salinity tolerance. Generally, relatively high accumulation of proline in salt tolerant genotypes has been reported previously in *B. juncea* [57] and pea [58].

The MDA (indicator of lipid peroxidation) is a well-known index for determining the extent of oxidative stress because increased MDA content has been found to be highly correlated with oxidative damages induced by various abiotic stresses including salinity [59]. However, lower level of MDA content in tolerant maize indicated better protection against oxidative damage under saline stress [36]. According to

Labudda [60], the high MDA content in cell is correlated with water deficit stress sensitivity while lower MDA content displays higher antioxidative ability, reflecting higher tolerance to stress. In this study, lower MDA concentration was observed in the tolerant genotypes and significantly higher in salt sensitive genotype under salinity, suggesting lower oxidative damage to sustain under salinity (Fig. 5B). This means that salt sensitive genotype showed more lipid peroxidation than did the tolerant genotypes. Such a pattern of MDA accumulation has already been observed in wheat [61] and okra [43].

Salt stress leads to oxidative stress through an increase in ROS, such as superoxide ($O_2^{\bullet-}$), hydrogen per oxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$) [5]. These ROS are highly reactive and can modify protein, lipid, DNA disorders and pigments to lead cell death [21]. Therefore, they must be kept under non-toxic level. With this view, a genotype with lower level of $O_2^{\bullet-}$ and H_2O_2 have least possibility of oxidative damage. Importantly, salt sensitive BARI Sarisha-14 has more possibility of cell damage due to higher $O_2^{\bullet-}$ and H_2O_2 as well as MDA. Previously, tolerant genotypes under salinity have been reported with lower $O_2^{\bullet-}$ and H_2O_2 as well MDA [36,48]. Therefore, lower ROS in the tolerant genotypes (Figs. 6A and 6B) may be important for cellular survival under salt stress, which was in agreement with several previous reports [62]. On the other hand, histochemical staining showed that salt tolerant genotypes showed high dark blue insoluble formazan product or spots and deep brown polymerization product or spots localization, in the salt-treated mustard seedling leaves (Figs. 7A and 7B). Contrary, salt sensitive genotype BARI Sarisha-14 showed light dark-blue insoluble formazan product or spots and light deep-brown polymerization product or spots in the salt-treated mustard leaves (Figs. 7A and 7B). These results are also supported the results of H_2O_2 production and $O_2^{\bullet-}$ generation rate in Figs. 6A and 6B.

Salt stress-induced overproduction of ROS and sub-sequent elevated activities of antioxidant enzymes have been reported in many plant species. Importantly, the activities of antioxidant enzymes in salt tolerant genotypes are up-regulated under salt stress while in salt sensitive genotypes those are down regulated [63]. In this study, we observed remarkable increases in $O_2^{\bullet-}$ and H_2O_2 contents in mustard seedlings of BARI Sarisha-11 as well as our selected genotypes BJ-1603 and BARI Sarisha-16 (Figs. 8A and 8B). ROS-scavenging enzymes and antioxidant molecules in plants prevents or alleviate the damage from $O_2^{\bullet-}$ and H_2O_2 where $O_2^{\bullet-}$ can be dismutated into H_2O_2 by SOD in chloroplasts, mitochondria, cytoplasm and peroxisome [64]. Moreover, the enhanced activity of SOD minimizes abiotic oxidative stress and has a significant role in the adaptation of a plant to stressed environments [63]. In our experiment salt stress down-regulated the SOD activity in salt sensitive BARI Sarisha 14 while it clearly up-regulated in salt tolerant BARI Sarisha-11, BJ-1603 and BARI Sarisha-16 (Fig. 8A). Higher SOD in the tolerant genotypes can provide first line protection to lessen the oxidative damage.

Catalase is a potential enzyme which has higher turnover rate and is capable to dismutase two molecules of H_2O_2 to water and oxygen and it is considered as an efficient ROS detoxifier [21]. There are plenty of reports on the changes in CAT activity or expression and those supported the notion that it is the most efficient H_2O_2 scavenging enzyme [22]. In our experiment, salt sensitive BARI Sarisha-14 showed decreased in CAT activity at any level of NaCl (Fig. 8B), which might be due to in effective enzyme synthesis or change in assembly of enzyme subunits. On the other hand, in salt tolerant genotypes, BJ-1603 and BARI Sarisha-16 the CAT activity increased under salt stress (Fig. 8B). Therefore, increased activity of CAT played important role in H_2O_2 metabolism in tolerant genotypes. Similar effects were also observed in several recent studies [48,36].

The increased activities of POD under salt stress played important role in H_2O_2 scavenging [36]. H_2O_2 is scavenged by the ascorbate-glutathione pathway by non-specific PODs in higher plants [65]. Increasing POD activity by salinity stress has been reported in various plants [10]. In this study, tolerant genotypes BJ-1603, BARI Sarisha-16 and BARI Sarisha-11 had higher specific POD activity (Fig. 8C), suggesting its role in H_2O_2 metabolism. On the other hand, the salt sensitive genotype had less POD activity.

The GPX is another vital enzyme of antioxidant defense system which has storing affinity for H_2O_2, can efficiently scavenge H_2O_2 and thus provide protection against salt stress [23]. In this study, salt tolerant variety BARI Sarisha-11 as well as our selected genotypes BJ-1603 and BARI Sarisha-16 showed higher activities of GPX as compared to salt sensitive genotype BARI Sarisha-14 which was due to an increased synthesis of the enzymes or an increased activation of constitutive enzyme pools (Fig. 8D). In salt sensitive genotype GPX activity unchanged at different level of salt stresses. This indicates in efficient detoxification of ROS in salt sensitive variety. Differential response of GPX activity in salt sensitive and tolerant varieties was reported in many studies [66–69].

The four enzymes of AsA-GSH cycle, namely, APX, MDHAR, DHAR and GR are vital for antioxidant defense because they are involved in maintaining the AsA and GSH pool [19,23]. In our experiment, APX activity increased in all mustard varieties when exposed to salt stress. APX catalyzes the reduction of H_2O_2 to H_2O by using AsA to MDHAR or DHAR [70]. On the other hand, AsA is maintained in cell with the help of MDHAR using in presence of NADP and DHAR using GSH by converting DHA [71]. Thus higher inductions in APX activity in susceptible genotypes (Fig. 9A) are capable to decompose H_2O_2 to H_2O by using AsA. Since MDHAR and DHAR maintain AsA, the increased activities of MDHAR and DHAR might have important role in AsA recycling in the tolerant genotypes thus they helped in APX mediated H_2O_2 metabolism to confer tolerance in this genotype. Contrary, decreasing activity of MDHAR and DHAR in sensitive genotype might be not able to minimize the H_2O_2 mediated oxidative damage. Decrease of MDHAR and DHAR activity in salt sensitive genotype under salt stress was also reported in earlier studies [67,68]. On the other hand, GR is one of important antioxidant enzymes of AsA-GSH cycle which is important for maintaining GSH pool in plant cells. GR is necessary for accelerating the H_2O_2 scavenging as well as to enhance plant tolerance against oxidative stress [41]. In our experiment, data showed that tolerant genotypes had higher increment in GR activity than the salt sensitive genotype, suggesting their better maintain of GSH under salt stress. Higher activity of GR in stress tolerant plants was also observed in several studies [36].

Plant GSTs are multifunctional family of enzymes which activity reported to increase in tolerant and sensitive genotypes under salt stress. Although, the primary role of these enzymes is to the detoxification of xenobiotics, it also plays a role in antioxidant defense system [63]. Plant GSTs are also associated with responses to various forms of abiotic stress [72] and stress tolerance is often correlated with enhanced activity of GST [63]. In our experiment, GST activity markedly increased in tolerant and sensitive genotypes under salt stress where significantly higher activity was observed in salt tolerant BARI Sarisha-11 and our selected genotypes BJ-1603 and BARI Sarisha-16 (Fig. 10B). Our results are partially supported by the study of [48]. However, the higher GST activity in tolerant genotypes under higher salt stress might also be involved in proper growth of mustard seedlings [73].

5 Conclusion

From the above discussion, it would be concluded that salinity impairs plant growth and biomass production in mustard genotypes due to reduction in RWC and different photosynthetic parameters. The increase in Na^+ concentration, MDA content, H_2O_2 concentration and $O_2^{\bullet-}$ generation are the indicators of oxidative damage in mustard seedlings. From our comparative studies of salt tolerant and salt sensitive genotypes, the selected tolerant mustard genotypes were better performer or maintained better physiological conditions and alleviated oxidative damages by increasing production of proline, enhancing the activities of SOD, CAT, POD, GPX, MDHAR, DHAR, GR and GST along with better stomatal regulation under salt stress. However, studies on their further protective mechanisms and signaling cascades are the future scope of research. Such studies are useful in breeding programmes and agronomic practices for the selection of superior mustard varieties capable of withstanding in stress conditions.

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