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Exogenous ascorbic acid improved tolerance in maize (*Zea mays* L.) by increasing antioxidant activity under salinity stress

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Salinity causes additional manufacture of reactive oxygen species (ROS) in plants, and ascorbate plays important role in maintaining of ROS scavenging antioxidant enzymes. In this study, the role of exogenous ascorbic acid (AsA) was examined on growth, chlorophyll and oxidative stress related enzymatic and non-enzymatic antioxidants in three maize hybrids under NaCl mediated salt stress. In hydroponic culture, AsA was applied at 0.5 and 1.0 mM with and without 12 dSm^{-1} NaCl, each treatment comprised two independent experiments with three replications. After four weeks, plants were harvested for recording growth and biochemical parameters. Root length, shoot length, dry matter accumulation, chlorophyll (*Chl a* and *Chl b*), AsA, reduced glutathione (GSH) and activity of ascorbate peroxidase (APX) were markedly reduced by salt stress, while H_2O_2 and Malondialdehyde (MDA) content were increased significantly. Exogenous application of AsA in saline treatment significantly improved root length, shoot length, dry matter accumulation, chlorophyll, AsA, GSH and APX activity while it decreased the contents of oxidized glutathione (GSSG) significantly in all the hybrids. However, content of dehydroascorbate (DHA) was reduced only in 900M Gold and PS-999. On the other hand, activity of monodehydroascorbatereductase (MDHAR) was increased only in Super gold and 900M gold (by 0.5 mM AsA) while dehydroascorbatereductase (DHAR) activity increased in Super gold only. The results of the present study evidently concluded that exogenous AsA application responded differentially in maize genotypes under salt stress and mitigated the negative effects of salinity.

Key words: Maize, salinity, reactive oxygen species, antioxidant, ascorbic acid.

INTRODUCTION

Salt-stress is one of the most prime hindrances in salt affected area of the world for crop production. At present, nearly 6.5% of whole area of the world and around 20% of the cultured land is affected by salinity (Hakim et al., 2014). Salinity changes various physiological and biochemical characteristics which reduce the plant growth

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and yield production (Munns, 2005). Plant grown at high salt stress in the soil brings about hyperosmolarity, ion disequilibrium, nutrient imbalance and creation of reactive oxygen species (ROS) through molecular damage (Nawaz et al., 2010). The excess gathering of ROS at various abiotic stresses in plants which are extremely reactive and noxious and encourage to damage of carbohydrates, proteins, lipids and DNA which ultimately consequences to oxidative stress (Gill and Tuteja, 2010). Hydrogen peroxide (H_2O_2), produced by salt-stress, is the most stable among all the ROS and reacts with above molecules. Using enzymatic and nonenzymatic antioxidants treatment, these ROS would be managed strategically within a fine influential range (Bose et al., 2013). Moreover, exogenous antioxidant overcomes the low production of growth regulators at stress condition (Ejaz et al., 2012).

The importance and changes in ascorbic acid (AsA) levels in plant cells in response to varying environmental stress conditions are well established (Noctor and Foyer, 1998; Venkatesh and Park, 2014). Functionally, it is an essential metabolite which operates as antioxidant and acts as a significant protagonist of several plant species for salt tolerance (Hameed et al., 2012; Ozgur et al., 2013). Actually, it associates with H_2O_2 metabolism as well as lipid hydroxyl peroxidase and also reacts with various sorts of biotic actions in plant as a patron or receptor in electron transportation system and also as an enzyme co-factor (Conklin, 2001), this approach effectively minimizes the stress impact of salinity and encourage the plant growth. However, exogenous claim of non-enzymatic antioxidant is an imperious suppository for salt sensitive variety.

Several investigations were performed on different plant or crop that exhibited the application of exogenous AsA significantly mitigated salt stress effect and promoted growth and yield (Ejaz et al., 2012; Abou-Leila et al., 2012; Hameed et al., 2015). Nevertheless, few reports are available on the exogenous role of AsA in mitigating salinity-mediated oxidative damage. Therefore, this investigation was carried out to apprehend the role of exogenous AsA application on maize growth and physiological responses to salt stress.

MATERIALS AND METHODS

Plant materials

Three commercial maize hybrids, that is, Super gold, 900M gold and PS-999 were used as experimental materials.

Plant growth condition

The phenotyping was carried out in the green house of Plant Breeding Division, Bangladesh Agricultural Research Institute (BARI), Gazipur-1701 in hydroponic culture, where the temperature was maintained around 28 to 30°C for 14 h, and 22°C for 10 h under light and dark conditions, respectively. The relative humidity and light intensity of the greenhouse room were 50% and 657 µmol $m^{-2}s^{-1}$, respectively. The study was accompanied with completely randomized design (CRD), specifically, four treatments (Control, 12 dSm^{-1} NaCl, 12 dSm^{-1} NaCl +0.5 mM AsA and 12 dSm^{-1} NaCl +1.0 mM AsA) were set and two independent experiments, each containing three replications, were conducted. Seeds were sown under water saturated quartz granule after surface sterilization (with 0.05% $HgCl_2$ for 10 min) and washed three times with deionized water and covered with black polyethylene for 4 days for germination and kept under light for another 6 days. After the exclusion of endosperm, ten days old seedlings were wrapped with sponge tightly and put in the hole of the cover. Each cover has six holes, where six plants were put into for transplanting in hydroponic pot containing half-strength Hoagland solution. The continuous air was supplied to the nutrient solution for ventilation and the nutrient solution was changed every four days. Salt treatments were applied and changed both normal and treated solution every four days interval. During the changing time of solution, the pot was washed with a brush for minimizing the contamination of fungal growth. Culture duration was almost five weeks in the nutrients solution. After five weeks, the seedlings were taken out for measurement of growth parameters and biochemical analysis.

Growth parameter measurement

Root length and shoot length were measured during harvesting whereas total dry matters were determined after keeping the samples in oven at 60°C for seven days.

Biochemical analysis

Measurement of H_2O_2

H_2O_2 was assessed following the method of Yu et al. (2003).

Measurement of lipid peroxidation

The concentrations of lipid peroxidation were estimated by determining MDA, a disintegration product of the peroxided polyunsaturated fatty acid constituent of the membrane lipid with thiobarbituric acid (TBA) as the reactive material succeeding the way of Heath and Packer (1968).

Chlorophyll measurement

Extraction and determination of Chlorophyll (*Chl a* and *Chl b*) were done according to the method of Arnon (1949). *Chl* were computed via following equations and articulated in mg g^{-1} fresh weight (FW).

$$Chl\ a\ (mg\ g^{-1}) = (0.0127) \times (A_{663}) - (0.00269) \times (A_{645})$$
$$Chl\ b\ (mg\ g^{-1}) = (0.0229) \times (A_{645}) - (0.00468) \times (A_{663})$$

Extraction and measurement of ascorbate and glutathione

Ascorbate levels were examined following the process of Huang et al. (2005). The glutathione mere was analyzed using with Yu et al. (2003) methods.

Protein determination

Following to the method of Bradford (1976), the protein levels in the leaf excerpts were measured by BSA as a protein standard.

Enzyme extraction and analyzes

Enzyme extraction was executed affording to the technique as labeled by Hossain et al. (2005). APX (EC: 1.11.1.11) and DHAR (EC: 1.8.5.1) activities were assayed following the technique of Nakano and Asada (1981). MDHAR (EC: 1.6.5.4) activity was computed by the method followed by Hossain et al. (2010).

Statistical analysis

The statistical analysis was completed following complete randomized design (CRD) and the mean differences were compared by Tukey's test using Statistical Tool for Agricultural Research (STAR) Version 2.0.1 for window (IRRI, 2014). Data of mean ± standard error (SE) were recorded from two independent experiments with three replications and P≤0.05 was considered as significant.

RESULTS

Growth parameters

Salt stress significantly decreased all the studied growth parameters (Figure 1A to C). However, application of 0.5 mM AsA in salt treatment markedly increased root length, shoot length and dry matter accumulation in compared to salt stress, though 1.0 mM AsA application showed non-significant effects. Root length reduced near about 50% as compared to control of all maize hybrids under salt stress and presence of 0.5 mM AsA increased root length by 16.69% in Super gold, 17.17% in 900M gold and 19.17% in PS-999 compare to that in stress condition, while slight improvement (3.25% in 900M gold, 2.77% in Super gold and 1.37% in PS-999) was recorded in 1.0 mM AsA application. Salinity reduced shoot length by 30.29% in Super gold, 41.80% in 900M gold and 37.84% in PS-999, while application of 0.5 mM AsA improved 12.19, 14.55 and 11.95% in Super Gold, 900M gold and PS-999, respectively, overstress condition. On the other hand, seedlings treated with 1.0 mM AsA showed relatively lower growth than those treated with 0.5 mM AsA. Dry matter accumulation declined above 50% in all maize genotypes under salt stress, but exogenous AsA (0.5 mM) application increased the accumulation by 28.25, 26.86 and 30.51% in Super gold, 900M gold and PS-999, respectively.

Chl contents

The present study showed that *Chl* contents (*Chl a* and *Chl b*) were decreased significantly by salt stress and exogenous AsA (0.5 mM) application in salt treatment remarkably increased the contents of both *Chl a* and *Chl b* in all genotypes (Table 1). However, 1.0 mM AsA decreased the *Chl* contents compared to those at 0.5 mM AsA.

Salinity decreased *Chl a* content by 23.18% in Super gold, 26.86% in 900M gold and 21.68% in PS-999 while exogenous AsA (0.5 mM) increased the content by 9.55, 15.30 and 16.81% in Super gold, 900M gold and PS-999, respectively, over salt treatment without AsA. In contrast, an increase of *Chl a* and *Chl b* in the application of 1.0 mM AsA was comparatively lower than in the application of 0.5 mM AsA. At the same time, salinity decreased the level of *Chl b* by 29.63% in Super gold, 35.50% in 900M gold and 42.01% in PS-999 whereas 0.50 mM increased the content by 41.59%, 50.44% and 55.50% in Super gold, 900M gold and PS-999, respectively, and 1.0 mM AsA increased the level by 32.66, 27.58 and 37.50%, respectively.

H_2O_2 and Malondialdehyde (MDA) contents

The levels of H_2O_2 and MDA amplified enormously in salt-stressed seedlings, but the exogenous application of AsA reduced the contents in all studied maize hybrids (Figure 2A and B). As compare to normal condition, the concentrations of H_2O_2 under salt-stress were higher by 106.97% in Super gold, 154.47% in 900M gold and 79.65% in PS-999, while the level of MDA increased by 134.21% in Super gold, 156.66% in 900M gold and 95.43% in PS-990. Application of 0.5 Mm AsA reduced the contents of H_2O_2 and MDA more efficiently than in the application of 1.0 mM AsA, although the significant difference was not found between the treatments. As compared to salinity, application of 0.5 mM AsA decreased the content of H_2O_2 by 20.85, 21.73 and 21.29% in Super gold, 900M gold and PS-999, respectively, while it decreased the content MDA by 33.36, 29.12 and 15.05% in Super gold, 900M gold and PS-999, respectively.

Non-enzymatic antioxidant contents

The present study showed that salt stress significantly reduced ascorbate content in the seedlings of maize hybrids compared to respective controls (Figure 3A). Exogenous application of AsA in saline treatment sharply increased the level (Figure 3A). The highest positive effect was observed with 1.0 mM AsA (119.35, 187.67 and 135.62% increment in Super gold, 900M gold and PS-999 respectively) application as compared to o.5 mM AsA (29.82, 81.30 and 116.85% in Super gold, 900M gold and PS-999, respectively) in all the maize hybrids.

Unlike AsA, DHA content increased dramatically under saline stress as compared to control in all genotypes (Figure 3B). On the other hand, DHA content reduced by 22.87% in 900M gold and 16.53% in PS-999 by 0.5 mM AsA while the content reduced by 37.72% in 900M gold and 21.02% in PS-99 by 1.0 mM AsA in compared to salinity treatment alone. In contrast, exogenous AsA increased DHA content in Super gold.

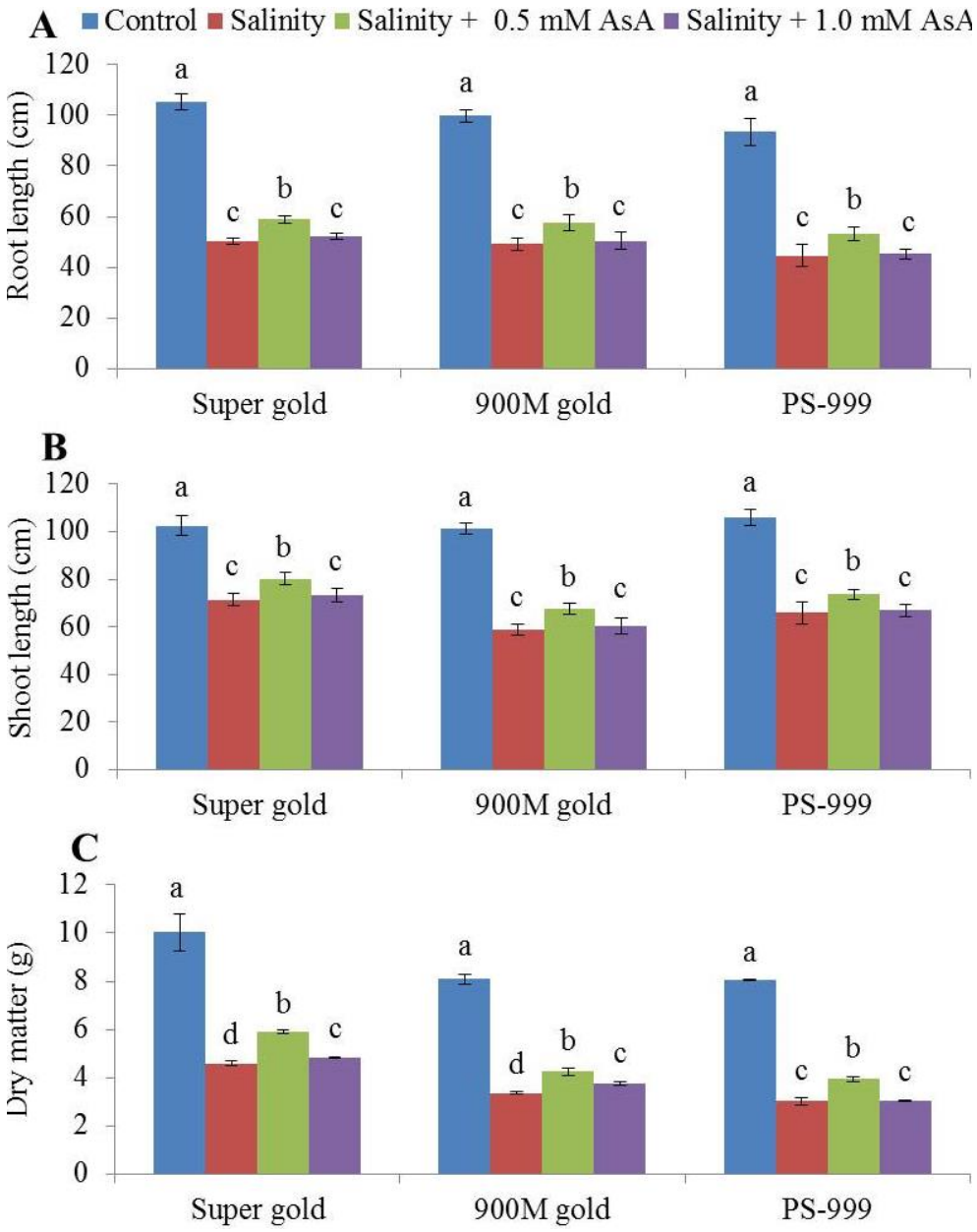

Figure 1. Effect of salinity and AsA on root length (A), shoot length (B) and dry matter accumulation (C) in maze seedlings. Bars denote mean ± SE. Dissimilar letters among different treatments within a variety are significant at P≤0.05%.

Table 1. Impact of AsA on *Chl* contents in leaves of maize hybrids grown under salt stress.

Treatments	*Chl a*			*Chl b*		
	Super gold	**900M gold**	**PS-999**	**Super gold**	**900M gold**	**PS-999**
Control	0.67 ± 0.038^{a}	0.71 ± 0.030^{a}	0.65 ± 0.029^{a}	0.88 ± 0.016^{a}	0.87 ± 0.025^{a}	0.90 ± 0.037^{a}
Saline	0.51 ± 0.033^{c}	0.52 ± 0.035^{c}	0.51 ± 0.049^{c}	0.62 ± 0.025^{d}	0.56 ± 0.039^{d}	0.52 ± 0.038^{d}
Saline + 0.5 mM AsA	0.56 ± 0.022^{b}	0.60 ± 0.050^{b}	0.60 ± 0.016^{b}	0.88 ± 0.036^{b}	0.84 ± 0.038^{b}	0.81 ± 0.030^{b}
Saline + 1.0 mM AsA	0.53 ± 0.026^{c}	0.53 ± 0.055^{c}	0.52 ± 0.019^{c}	0.82 ± 0.026^{c}	0.71 ± 0.058^{c}	0.71 ± 0.027^{c}

Data are mean of two independent experiments with three replicates ± SE. Dissimilar letters among different treatments within a variety are significant at P≤0.05%.

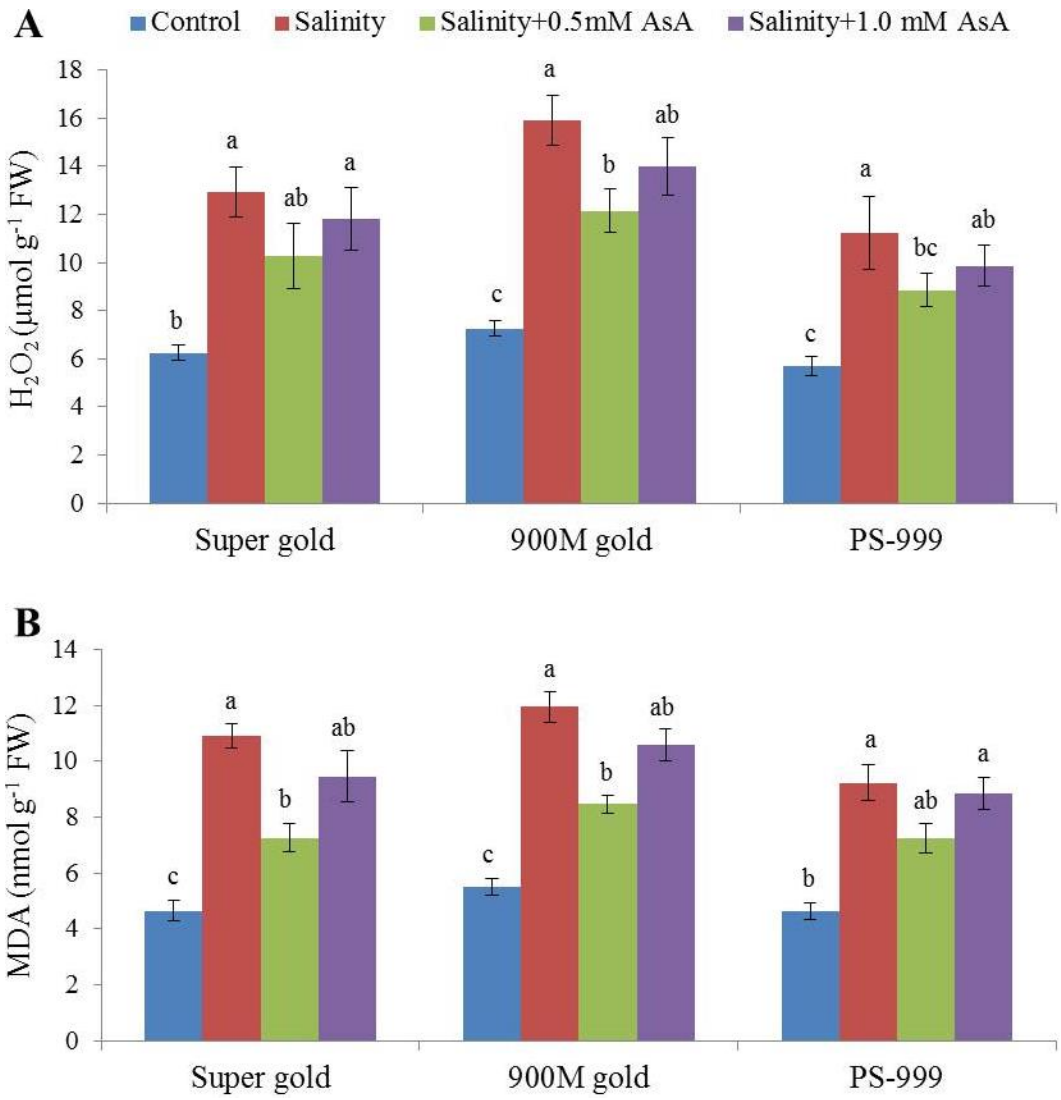

Figure 2. Effect of salinity and exogenous AsA application on H_2O_2 (A) and MDA (B). Bars denote mean ± standard error. Dissimilar letters among different treatments within a variety are significant at P≤0.05%.

Reduced glutathione (GSH) content decreased significantly by salinity in all maize hybrids under salt stress over control (Figure 3C). Due to the application of AsA, GSH improved remarkably in all genotypes. However, 1.0 mM AsA caused the highest increase in GSH by 60.37% in Super gold, 62.62% in 900M gold and 73.16% in PS-999 over the content under salinity.

The content of GSSG increased dramatically under saline conditions in compared to control in all three maize hybrids while exogenous AsA treatments resulted in a decrease in the content of GSSG (Figure 3D). Salt stress increased GSSG content by 305.16% in Super gold, 160.81% in 900M gold and 250.74% in PS-999 whereas, 0.5 mM AsA reduced the content by 33.54, 36.29 and 32.62% in Super gold, 900M gold and PS-999, respectively, and 1.0 mM AsA reduced the content by 39.40, 40.44 and 24.20%, respectively.

Activity of enzymatic antioxidant

Salinity caused the substantial diminution in the activity of APX in all of maize leaves hybrids. Application of AsA in saline solution improved the APX activity in all the genotypes (Figure 4A). Application of 0.5 mM AsA amended more APX activity than 1.0 mM AsA while compare to the stress condition, 0.5 mM AsA promoted the activity by 58.44, 52.29 and 40.63% in Super gold, 900M gold and PS-999, respectively. On the other hand, 1.0 mM AsA increased the activity by 38.61, 39.12 and 21.32% in Super gold, 900M gold and PS-999, respectively.

In the case of MDHAR and DHAR, the activities varied with genotypes in both normal and saline condition (Figure 4B and C). Salinity decreased the activity of MDHAR in Super Gold and 900M gold while it increased

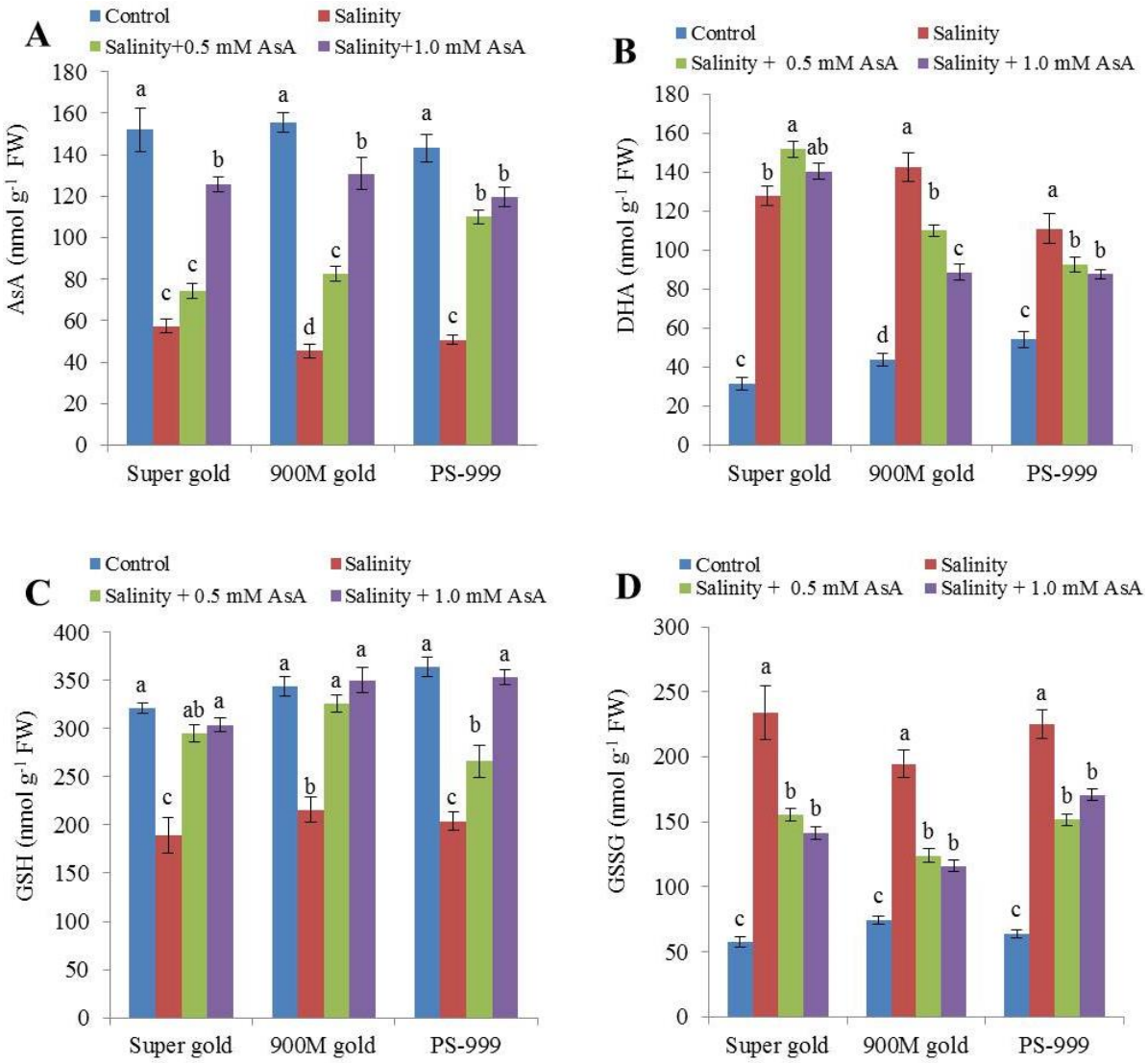

Figure 3. Effect of salinity and exogenous AsA application on endogenous AsA (A), DHA (B), GSH (C) and GSSG (D) contents. Bars denote mean ± standard error. Dissimilar letters among different treatments within a variety are significant at P≤0.05%.

the activity in PS-999. On the other hand, DHAR activity was decreased only in 900M gold. Application of 0.5 mM AsA increased MDHAR activity in Super gold whereas the activity increased with the concentration of AsA when compared to salt treatment. On the other hand, DHAR activity decreased in AsA treated seedlings of 900M gold and PS-999, but increased in seedlings of Super gold.

DISCUSSION

The results attained in the present investigation noticeably exhibited that the three maize genotypes revealed a remarkable decrease in root length, shoot length and dry matter accumulation under salinity (Figure 1A to C). However, exogenous AsA (0.5 mM) treatment significantly improved the all studied growth parameters (Figure 1), signifying useful role of AsA in maize under salinity. It is well recognized that Na^+ is a toxic element which hinders the different metabolic activities at higher concentration (Gul et al., 2015). Higher gathering of Na^+ and Cl^- ions in the cytoplasm due to noxiousness, the creation of ROS under salt-stress, nutritional imbalance and desiccation of the tissues via the low water potential are the four salt stress factors that affect the plant responses which caused by the intrusion of salty ions through crucial nutrients in fixation and translocation progressions (Arab and Ehsanpour, 2006). Moreover, during salt stress, osmotic and ionic effects decrease the plant growth (Munns, 2002; Ghoulam et al., 2002). Root

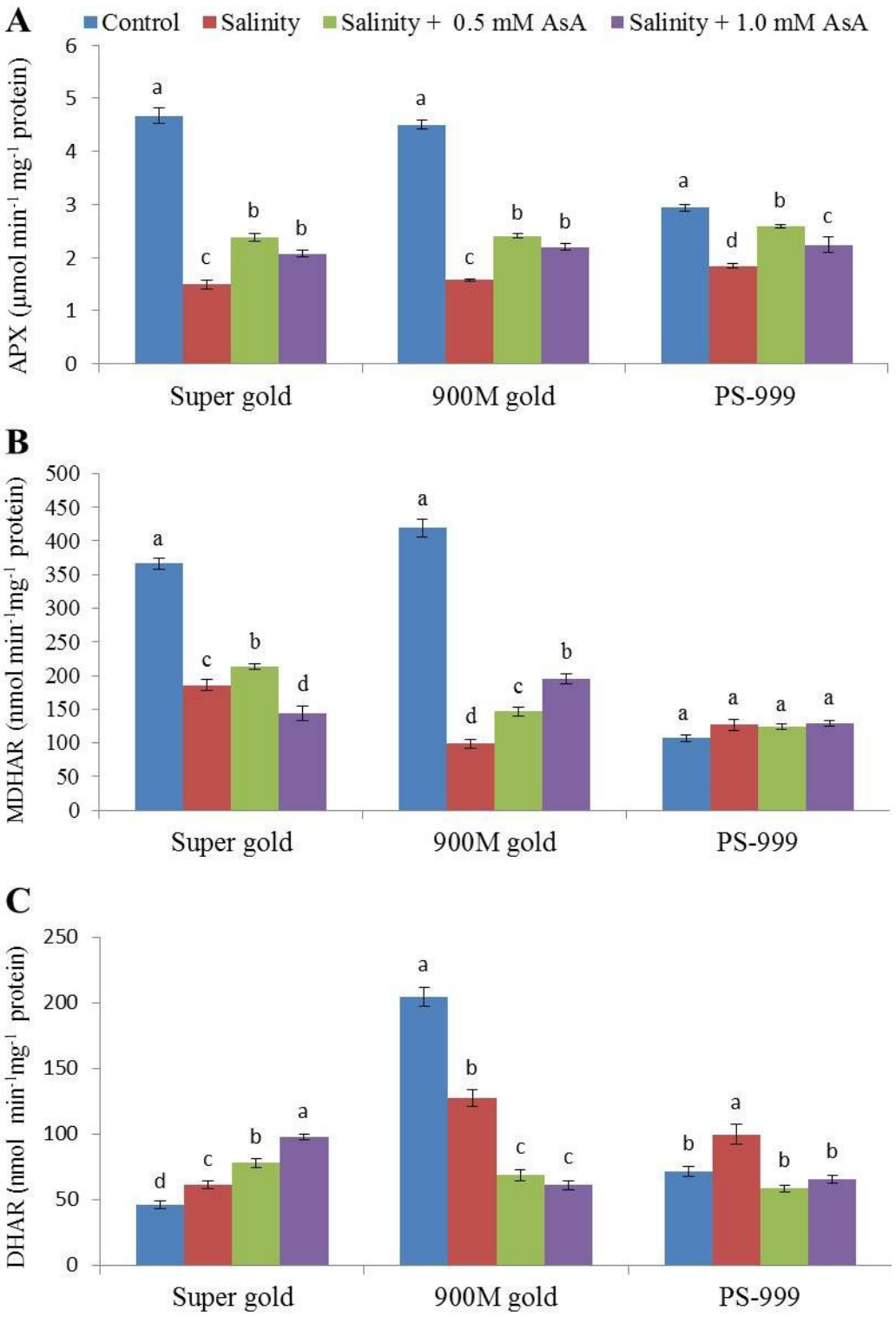

Figure 4. Effect of salinity and application of AsA on APX (A), MDHAR (B) and DHAR activity (C). Bars denote mean ± standard error. Dissimilar letters of different treatments for same variety on bars represent their comparative significance at P≤0.05%.

growth is sensitive to high salt concentrations solution (Akram et al., 2010) and roots are sharply reduced by salinity (Cramer et al., 1988; Ashraf et al., 2005). With increasing salinity, reduction in plant height is the

distinctive influence of the toxic ions accumulation in cells which rigorously disturb cell division and expansion (Munns, 1993). Biomass accumulation was meticulously affected due to increasing salinity thus; fresh and dry weights were decreased (Akram et al., 2010). Similar result was shown from the previous experiment (Majeed et al., 2014; Hoque et al., 2015). Promotion of plant growth as a result of AsA treatment has also been described in several plant species (Tuna et al., 2013 in maize; Gul et al., 2015 in Guar; Ejaz et al., 2012 in sugarcane; Alhasnawi et al., 2015 in rice).

In the present observation, salt stress sharply reduced the photosynthetic pigment (*Chl a* and *Chl b*) whereas the exogenous treatment of AsA appreciably delayed the loss of chlorophyll contents of all maize hybrids (Table 1). The reduction in leaf *Chl* content probably decline due to biosynthesis or amplified degradation of chlorophyll at salt stress (Gul et al., 2015). The most probable reason for chlorophyll degradation is a production of proteolytic enzymes such as chlorophyllase (Zhao et al., 2007) and substantial accumulation of H_2O_2 (Tuna et al., 2013). Similar finding was investigated by Tuna et al. (2013). Therefore, our investigation revealed that exogenous AsA application significantly improved *Chl* content of maize hybrids (Table 1) which indicated that AsA protects photosynthetic pigment apparatus. This result was supported by the previous experiment of different crops (Beltagi, 2008 in chickpea; Khan et al., 2010 in Mustard; Rafique et al., 2011 in Pumpkin).

Salt stress is extensively described to enhance production of H_2O_2, which can diminish essential cell components (Gill and Tuteja, 2010). Our observation also showed that salinity increased the H_2O_2 levels while both concentrations of exogenous AsA application in salt stress reduced the H_2O_2 concentration of all hybrids (Figure 2A). Similar finding were documented by Dolatabadian and Jouneghani (2009) and Ebrahimian and Bybordi (2012). On the other hand, malondialdehyde (MDA) is the end-product of lipid peroxidation. Under salt stress, the level of lipid peroxidation is used as a pointer of free radical damage to cell membranes. Thus, MDA has been commonly designated as assortment to measure the salt stress injury (Katsuhara et al., 2005; Jaleel et al., 2007). In the present study, application of AsA in maize seedlings decreased the salt induced MDA content (Figure 2B) suggesting that exogenous application of AsA is useful in reducing the cell damage.

Ascorbate plays manifold role related to plant growth, such as in cell division, cell wall expansion and other developmental progressions through molecular mechanisms (Asada, 1999; Pignocchi and Foyer, 2003). In our observation, salt stress significantly reduced AsA content which is improved due to AsA application in salt treatment (Figure 3A). Turan and Tripathy (2012) reported that salt stress reduced AsA contents in rice seedling and Sairam et al. (2005) also noted that AsA content in *Triticum aestivum* decreased in saline soil. In contrast, Yildiztugay et al. (2013) in *Sphaerophysa kotschyana* plant and Eltelib et al. (2012) in transgenic tobacco plant observed that endogenous AsA contents amplified at the saline condition. Augmented AsA concentrations in AsA treated plant could be as a result of its better biosynthesis and recovering through the Asada-Halliwell-Foyer pathway. Franceschi and Tarlyn (2002) documented that treated leaf cells improved 2 to 3 times of AsA in compares to the untreated sink cells/tissues of a plant. Endogenous AsA levels and Asada-Halliwell-Foyer pathway enzymes significantly increased due to the application of exogenous AsA in *Limonium stocksii* seedlings (Hameed et al., 2015) which support our present investigation.

In ascorbate-glutathione cycle, APX, DHAR and MDHAR are vital enzymes intricate in sustaining the AsA (Noctor and Foyer, 1998). In this cycle, APX reduced H_2O_2 to H_2O using ascorbate as the definite electron benefactor (Smirnoff, 2000). AsA is oxidized to DHA under oxidative stress (Noctor and Foyer, 1998). In our observation, DHA content significantly increased under saline condition over control and exogenous AsA application reduced the DHA stuffing (Figure 3B). Alternatively, salt-stress meaningfully decreased the APX activity of all maize hybrids in our observation and exogenous AsA positively improved APX activity (Figure 4A). Neto et al. (2006) noted that salinity reduces the activity of APX in roots organs of salt sensitive maize and upgraded the activity in salt endurance genotype. This result suggested that response APX fluctuates in different genotypes. MDHAR and DHAR are two crucial enzymes which are equally essential in regulating and regeneration of AsA level (Wang et al., 2010). In our study, MDHAR and DHAR activity showed fairly different response in different treatment and genotypes (Figure 4B and C). Sharp reduction was detected in MDHAR activity in Super gold and 900M gold under salt stress and PS-999 exposed significant increase over control. Super gold and 900M gold also displayed better response to AsA treatments. On the other hand, PS-999 almost did not show the response to AsA treatments. Rohman et al. (2016) demonstrated that MDHAR activity increased in tolerant maize inbreds while decreased in sensitive inbreds under salinity. However, AsA treatment increased the activity of MDHAR which assisted in a renewal of AsA in maize and detoxify the excesses H_2O_2 level.The result of the study agreed with observations of Ebrahimian and Bybordi (2012). In case of DHAR activity, Super gold exposed the increase of activity under salt stress and further promoted due to AsA treatment (Figure 4C). In contrast, 900M gold and PS-999 showed significant demotion in DHAR activity due to AsA application which may be responsible for lower regeneration of AsA and/or used other metabolic activities. Hossain et al. (2013) found their study that non-significance variation appearance in MDHAR activity and the noticeable increase in DHAR activity under saline condition.

The redox state maintained by GSH acts as a defensive role in salt tolerance (Shalata et al., 2001). Furthermore, the central action of GSH is to renew of ascorbate via reduction of DHA through DHAR activity in the antioxidant protection system (Noctor and Foyer, 1998). In our study, exogenous AsA noticeably increased GSH contents under salt stress (Figure 3C). Due to availability of exogenous AsA, GSH might not be used in AsA maintenance via DHAR activity. However, this higher GSH might be used by glutathione peroxidase (GPX, an important H_2O_2 decomposer). Nevertheless, amplified activity of DHAR in Super gold can use GSH. In the present study, GSSG contents meaningfully increased of all maize hybrids under salt stress and further decreased in the presence of AsA treatment (Figure 3D). The increased contents of GSSG under salt-stress in plant perhaps recognized to the reaction of GSH with oxyradicals formed by oxidative stress or diminished GR activity (Aravind and Prasad, 2005). Therefore, AsA application reduced the oxidation GSH. These findings were supported with earlier reports which exposed the intensification of the GSSG contents in salt treated plant (Rohman et al., 2016). However, APX conferred tolerance to all maize genotypes. The variation of MADAR and DHAR activity suggested that AsA mediated tolerance under salinity varies with maize genotypes.

Conclusion

In conclusion, it is clear that application of AsA as non-enzymatic antioxidant alleviated the adverse effect of salinity in maize hybrids and improved all growth and some biochemical attributes by regulating the antioxidative protection mechanisms. Therefore, AsA can performance efficiently in plants as medication when applied at the appropriate concentration during stress environment.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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