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Saline sensitivity leads to oxidative stress and increases the antioxidants in presence of proline and betaine in maize (*Zea mays* L.) inbred

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Abstract

The oxidative stress, antioxidant and glyoxalase systems in two differently saline sensitive maize inbreds (i.e., CZ-27; tolerant and CZ-37; susceptible) in presence of proline and betaine were studied for better understanding of salinity tolerance mechanism. Five days old seedlings were imposed to 16 dSm^{-1} salinity for 10 days. Water content, chlorophyll (Chl), reactive oxygen species (ROS), lipid peroxidation, methylglyoxal (MG), lipoxigenase (LOX) activity, enzymatic and non-enzymatic antioxidants and glyoxalases were investigated in fully expanded leaves. Salinity caused higher reduction in leaf water and chlorophyll content as well as increased in levels of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), melondialdehyde (MDA), LOX and MG in both inbreds. However, the levels were higher in CZ-37 as compared to CZ-27. Proline and betaine treatments in salinity made significantly delay in loss of leaf water and breakdown of chlorophyll. The salinity caused more oxidation of reduced glutathione (GSH) and ascorbic acid (ASA) as well as inhibited the synthesis of cysteine in CZ-37. The levels of glutathione- and ascorbate-redox suggested that use of both proline and betaine helped better maintenance of GSH and ASA under salinity in CZ-37 compared to CZ-27. Under salinity, the activities of superoxide dismutase (SOD) and peroxidase (POD) increased in both inbreds, but the magnitude was higher in CZ-37 than in CZ-27. On the other hand, the activities of catalase (CAT), ascorbate peroxidase (APX) and glyoxalase-II (Gly-II) decreased in both inbreds, but the levels were higher in CZ-27. Though the activities of glutathione peroxidase (GPX) and monodehydroascorbate reductase (MDHAR) decreased in CZ-37, the activity of dehydroascorbate reductase (DHAR) increased. The application of proline and betaine increased the activities of SOD, POD, APX, GPX, glutathione reductase (GR), DHAR, glutathione *S*-transferase (GST) and Gly-II in both inbreds, however, the increments were higher for SOD, POD, DHAR, GST and Gly-II in CZ-37. The higher enzymatic and non-enzymatic antioxidants and glyoxalases under salinity in presence of proline and betaine in CZ-37 suggested better cellular protection from excess accumulation of ROS, MG and other toxic metabolites.

Keywords: Salinity, oxidative stress, maize, antioxidants, proline and betaine.
Abbreviations: Chl_Chlorophyll; ROS_Reactive oxygen species; MDA_Melondialdehyde; MG_Methylglyoxal; LOX_Lipoxygenase; SOD_Superoxide dismutase; POD_Peroxidase; CAT_Catalase; GPX_Glutathione peroxidase; GST_Glutathione *S*_transferase; APX_Ascorbate peroxidase; AO_Ascorbate oxidae; MDHAR_Monodehydroascorbate reductase; DHAR_Dehydroascorbate reductase; GR_Glutathione reductase; ASA_Ascorbic acid; DHA_Dehydroascorbate; GSH_Reduced glutathione; GSSG_Oxidized glutathione; Gly-I_Glyoxalase-I; Gly-II_Glyoxalase-II; CDNB_1-Chloro-2,4-dinitrobenzene; SLG_S-D-lactoyl glutathione RWC_ Relative water content; FW_Fresh weight; DW_Dry weight; TW_Turgid weight; DTNB_5,5′-dithio-bis (2-nitrobenzoicacid); NTB_ 2-nitro-5-thiobenzoicacid; NBT_Nitro blue tetrazolium; BSA_Albumin from bovine serum; dSm^{-1}_Salinity measuring unit (decisimen per meter); CRD_Complete randomized design.

Introduction

Salinity is one of the most important abiotic stress limiting growth and productivity of crops. High exogenous salt concentrations cause ionic imbalance in the cells resulting in ion toxicity and osmotic stress (Demiral and Türkan, 2005; Mandhania et al., 2006). Osmotic stress induced by salinity causes oxidative stress in plant and thus produces ROS such as superoxide radicals $O_2^{\bullet-}$, singlet oxygen ($^1O_2$), hydroxyl radicals ($OH^{\bullet}$) and H_2O_2 (Misra and Gupta, 2006; Hasegawa et al., 2000; Apel and Hirt, 2004) and MG (Yadav et al., 2005a, b). In plant cells, ROS are highly reactive and toxic which can lead to cell death by damaging proteins, lipids, DNA and carbohydrates (Noctor and Foyer, 1998; Apel and Hirt, 2004). On the other hand, MG is a potential cytotoxic that can react with and modify other molecules including DNA and proteins (Yadav et al., 2005a). Therefore, ROS are highly toxic and must be detoxified by cellular responses to survive and grow (Gratäo et al., 2005). However, the abiotic stress tolerance mechanism is not clear and needs integrated approaches of biochemical, physiological and molecular intervention.

Proline and betaine are the most common compatible solutes which contribute to osmotic adjustment and stabilization and protection of membranes, proteins and enzymes from damaging effects of salinity (McNeil et al., 1999; Ashraf and Foolad, 2007). In addition, they can scavenge free radicals and ROS (Hasegawa et al., 2000; Hong et al., 2000; Okuma et al., 2000, 2004; Chen and Dickman, 2005). Exogenous proline and betaine also improve salt tolerance by enhancing stress-protective proteins (Apel and Hirt, 2004; Molla et al., 2014) and reducing

oxidation of lipid membranes (Demiral and Türkan, 2004; Okuma et al., 2004). To protect cells from ROS-induced cellular injury, plants have evolved a complex antioxidant system that plays a significant role in ROS signaling in plants (Noctor and Foyer, 1998; Noctor et al., 2012). Efficient scavenging of ROS produced during various environmental stresses including salinity requires the action of several non-enzymatic as well as enzymatic antioxidants present in the tissue (Choudhury et al., 2013). The antioxidant defense of plants includes SOD, CAT, GPX, GST, APX, MDHAR, DHAR, and GR along with non-enzymatic components such as; ASA and GSH (Hasanuzzaman et al., 2014). On the other hand, cytotoxic MG is detoxified and GSH homeostasis is maintained via glyoxalase system (Yadav et al., 2005a) which consists of two enzymes: Gly-I and Gly-II. It was reported that the coordinated induction or regulation both of the antioxidant and glyoxalase pathway enzymes are necessary to obtain substantial tolerance in plant against oxidative stress (Singla-Pareek et al., 2008; Saxena et al., 2011). A good number of research group reported the role of glyoxalase systems in plant responses to salt stress (Noctor and Foyer, 1998; Shalata et al., 2001; Mittova et al., 2003a, b; Singla-Pareek et al., 2008; Yadav et al., 2005a, b; Hoque et al., 2007a, b, 2008; Saxena et al., 2011). Although the protective roles of antioxidants have been extensively studied in different plant species, the underlying saline tolerant mechanism is not fully understood. Similarly, the accumulation of proline and betaine under stress and their protective role in cell has been reported in several plants while the role of exogenous proline and betaine has not been widely studied considering the whole antioxidant system. In this study, ROS, their related cytotoxic metabolites, MG, enzymatic and non-enzymatic antioxidants and glyoxalase system along with water content, chlorophyll, proline and cysteine were studied in fully expanded leaves of two maize inbreds (one saline tolerant and another susceptible) in presence of exogenous proline and betaine for better understanding the saline tolerance mechanism.

Results

Leaf relative water content

Under salinity stress, leaf relative water content (RWC) decreased in both inbreds while 17% decrease was found in tolerant inbred CZ-27 and 44% in susceptible inbred CZ-37 over control (Fig. 1). The application of 15 mM of proline with saline maintained the RWC considerably higher by 15% and 50% in CZ-27 and CZ-37, respectively, over salinity. Correspondingly, application of 15 mM of betaine with saline helped to maintain higher RWC level by 13% and 47% in CZ-27 and CZ-37, respectively.

Chlorophyll content

Chlorophyll (Chl) contents of maize leaves were decreased significantly under salinity and the magnitude of loss was higher in CZ-37 (Table 1). Salinity decreased Chl*a* contents by 31% in CZ-27 and 57% in CZ-37. Salinity also decreased the level of Chl*b* by 28% and 62% in CZ-27 and CZ-37, respectively. Application of proline with saline increased Chl*a* by 33% and 46% in CZ-27 and CZ-37, respectively, while application of betaine with saline increased the Chl*a* by 27% and 51% in CZ-27 and CZ-37, respectively. Correspondingly, application of proline with saline increased Chl*b* by 18% in CZ-27 and 63% in CZ-37, whereas increases of Chl*b* by betaine were 14% in CZ-27 and 54% in CZ-37 (Table 1).

Proline and cysteine contents

Sharp increase of proline in seedlings of both inbreds under salinity was observed while the content was 21% higher in CZ-27 (Fig. 2A). As compared to CZ-27, higher accumulation of proline was observed in CZ-37 under both proline and betaine treated salinity treatments. Notably, proline treatment in salinity increased the proline content by 46% in leaves of CZ-37. As an important peptide of GSH, cysteine was also estimated, where saline stress decreased significantly the content of cysteine in both inbreds compared to respective control. However, the content was 38% lower in CZ-37. Application of proline increased the cysteine content in saline treated seedlings by 12% and 40% in CZ-27 and CZ-37, respectively, over salinity (Fig. 2B). Similarly, application of betaine increased the cysteine content by 14% in CZ-27 and 53% in CZ-37.

$O_2^{\bullet-}$ generation and H_2O_2 content

Salinity stress significantly increased the formation rate of $O_2^{\bullet-}$ and the content of H_2O_2 in both inbreds. Under salinity, the contents of $O_2^{\bullet-}$ and H_2O_2 were 34% and 21% higher in CZ-37 compared to those in CZ-27 (Fig. 3A, B). Application of proline in salinity reduced the contents of $O_2^{\bullet-}$ and H_2O_2 by 14% and 20%, respectively, in CZ-27 while betaine reduced the contents of $O_2^{\bullet-}$ and H_2O_2 by 11% and 22%, respectively, in CZ-37.

Lipid peroxidation and LOX activity

Salinity stress significantly increased lipid peroxidation (as MDA) and LOX activity in both inbreds (Fig. 4A, B). Under salinity stress, MDA and of LOX activity were 36% and 31% higher in CZ-37 as compared to those in CZ-27. Both proline and betaine treatments in salinity reduced the MDA and LOX activity in both inbreds, but the levels were still higher in CZ-37. (Fig. 4A, B).

Activities of antioxidant enzymes

Under saline stress, the activities of SOD and POD increased over control in both inbreds, and the activities were further induced by proline and betaine (Fig. 5A, B). On the other hand, CAT activity decreased under salinity and remained almost similar in proline and betaine treated seedlings of both inbreds (Fig. 5C). However, saline stress decreased the activity of APX by 27% and 40% in CZ-27 and CZ-37, respectively, compared to control (Fig. 5D). Use of proline in salinity increased the APX activity by 22% and 23% in CZ-27 and CZ-37, respectively, over salinity, whereas use of betaine in salinity increased the activity by 45% and 47% in CZ-27 and CZ-37, respectively. Saline stress increased the GPX activity in CZ-27 while it decreased the activity in CZ-37 (Fig. 6A). Application of proline and betaine in salinity increased GPX activity considerably higher by 48% and 44%, respectively, in CZ-27 over salinity (Fig. 6A). Salinity increased GR activity in both inbreds, but the level was 11% higher in CZ-27 (Fig. 6B). Notably, use of proline in salinity increased the activity significantly in both inbreds (Fig. 6B).
As compared to control, significant changes in the activity of MDHAR were not found in the seedlings of CZ-27 under salinity with or without proline and betaine (Fig. 7A). On the other hand, salinity decreased the activity in CZ-37, and

Table 1. Contents of Chl in leaves of maize seedlings in presence or absence of proline (Pro) and betaine (Bet) at 10 day under salinity stress. Values represent the mean ± SE from three independent experiments. Values with the same letters within a column are not significantly different at P≤0.05.

Treatments	Chl*a*		Chl*b*	
	CZ-27	CZ-37	CZ-27	CZ-37
Control	1.31±0.21^{a}	1.40±0.11^{a}	2.03±0.14^{a}	1.99±0.002^{a}
Saline	0.91±0.01^{b}	0.61±0.05^{c}	1.47±0.12^{b}	0.76±0.05^{c}
Saline+Proline	1.21±0.12^{ab}	0.89±0.08^{bc}	1.74±0.14^{ab}	1.24±0.09^{b}
Saline+Betaine	1.16±0.09^{b}	0.92±0.04^{b}	1.68±0.17^{ab}	1.17±0.11^{b}

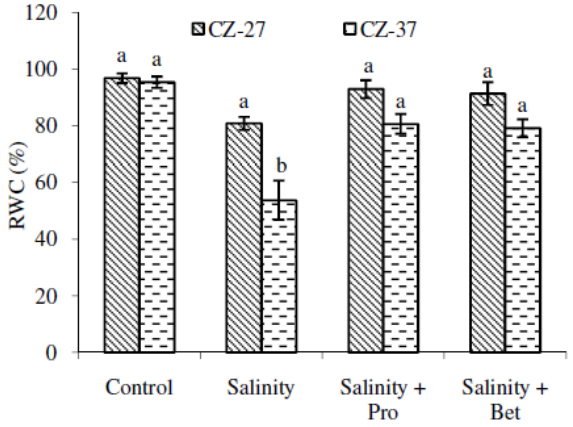

Fig 1. Relative water content (RWC) in leaves of maize seedlings maintained by proline (Pro) and betaine (Bet) at 10 day under salinity stress. Values represent the mean ± SE from three independent experiments. Bars with the same letters are not significantly different at P≤0.05.

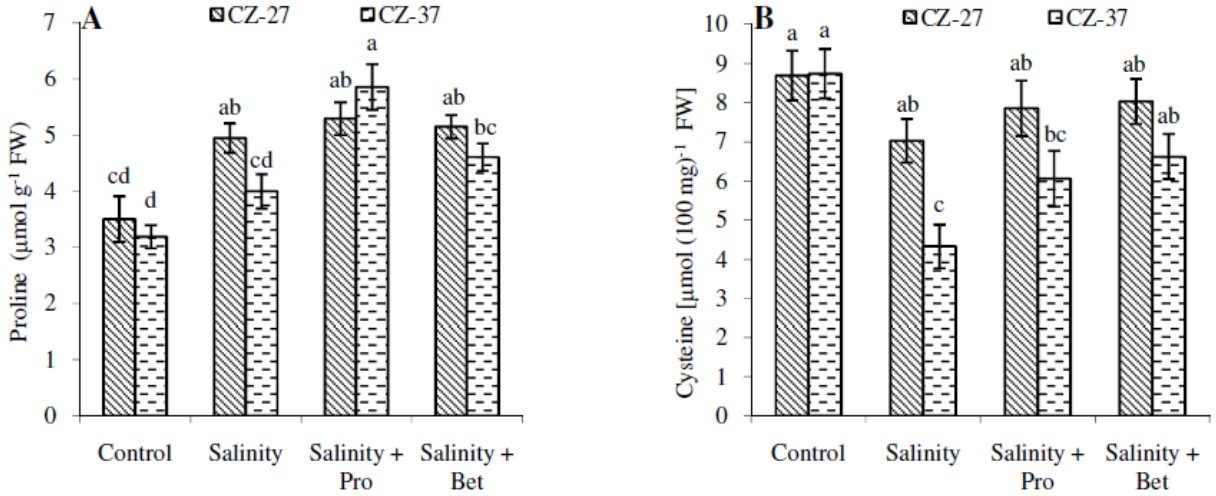

Fig 2. Contents of proline (A) and cysteine (B) in leaves of maize seedlings in presence or absence of proline (Pro) and betaine (Bet) at 10 day under salinity stress. Values represent the mean ± SE from three independent experiments. Bars with the same letters are not significantly different at P≤0.05.

addition of proline and betaine in salinity increased the activity by 43% and 31% in CZ-27 and CZ-37, respectively (Fig. 7A). In case of DHAR, salinity increased the activity in both inbreds. However, activity was 37% higher in CZ-27 as compared to CZ-37 (Fig. 7B). In presence of proline and betaine, the activity increased more in both inbreds. Remarkable increase was also observed in GST activity in maize seedlings of both inbreds under salinity stress in presence and absence of proline and betaine (Fig. 8). However, as compared to CZ-27, the activity of GST was higher in CZ-37 under control and stress condition.

Glutathione and ascorbate levels

Saline stress caused significant decreases in the content of GSH (22% in CZ-27 and 50% in CZ-37) and ASA (31% in CZ-27 and 55% in CZ-37) (Fig. 9A and 10A). In contrast, salinity increased GSSG and DHA contents significantly and altered the glutathione- and ascorbate-redox state (Fig. 9B, C and 10B, C). However, the oxidation of GSH and ASA was higher in CZ-37. Both proline and betaine in salinity increased the GSH and ASA contents. At the same time, they decreased the GSSG and DHA contents resulting in improved GSH- and ASA-redox (12% in CZ-27 and 42% in

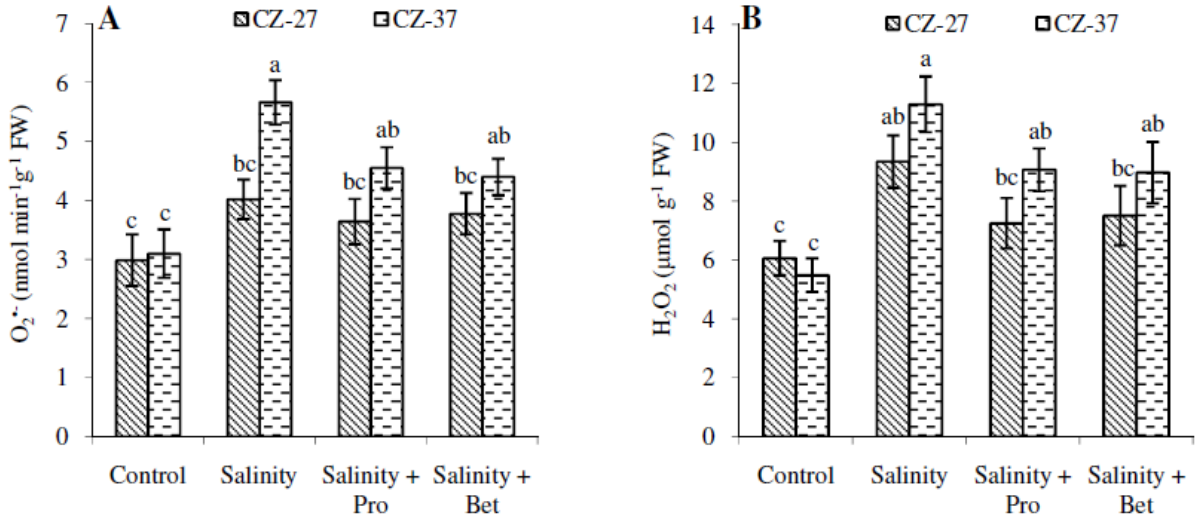

Fig 3. 'Generation rate of $O_2^{\bullet-}$ ion (A) and contents of H_2O_2 (B) in leaves of maize seedlings in presence or absence of proline (Pro) and betaine (Bet) at 10 day under salinity stress. Values represent the mean ± SE from three independent experiments. Bars with the same letters are not significantly different at P≤0.05.

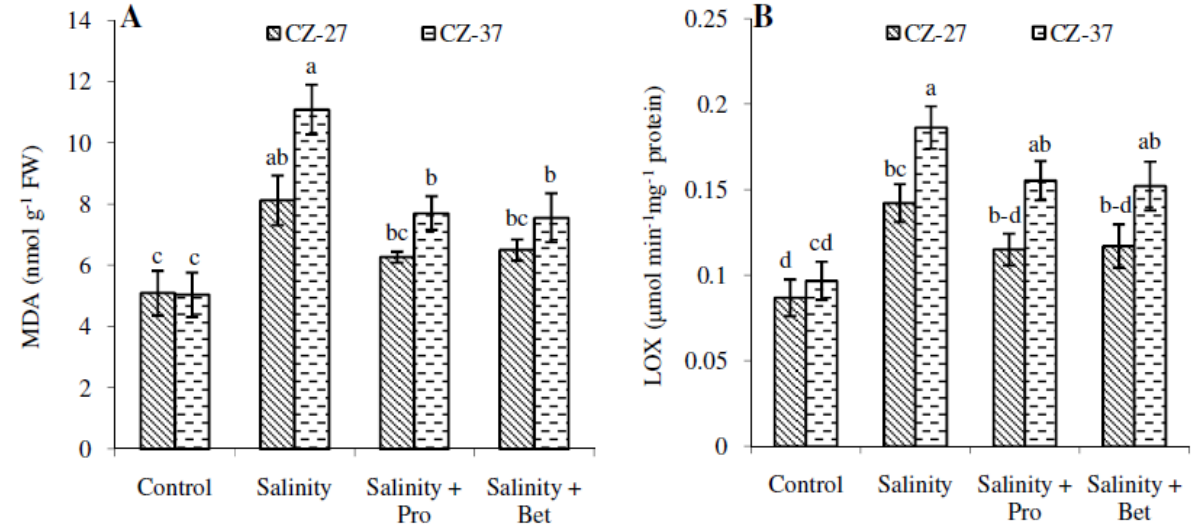

Fig 4. Contents of MDA (A) and activity of LOX (B) in leaves of maize seedlings in presence or absence of proline (Pro) and betaine (Bet) at 10 day under salinity stress. Values represent the mean ± SE from three independent experiments. Bars with the same letters are not significantly different at P≤0.05.

CZ-37 by proline and 10% in CZ-27 and 46% in CZ-37 by betaine) (Fig. 9C and 10C).

Methylglyoxal detoxification

Significance increase was observed in the content of MG in leaves of both inbreds under salinity stress, whereas the MG contents were 2.41 and 2.36 times higher in CZ-27 and CZ-37, respectively, over control (Fig. 11A). Proline decreased the MG content by 27% in CZ-27 and 29% in CZ-37 while betaine decreased the content by 27% in CZ-27 and 25% in CZ-37 when compared with those in salinity (Fig. 12A).
Under salinity, Gly-I activity increased by 64% and 48% in CZ-27 and CZ-37, respectively, over control (Fig. 11B). Though proline increased the activity in salinity stressed seedlings of both inbreds, there was no effect of betaine on Gly-I activity. On the other hand, salinity decreased by 22% and 31% in CZ-27 and CZ-37, respectively, over control (Fig. 11C). Application of proline increased Gly-II activity by 29% and 37% in CZ-27 and CZ-37, respectively, while application of betaine increased the activity by 18% and 19% in CZ-27 and CZ-37, respectively (Fig. 11C).

Discussion

Salinity is one of the major environmental factors limiting crop productivity which causes several biochemical and physiological alterations. The deleterious effects of salinity on plant growth and photosynthesis are also associated with oxidative damage resulting from the imbalance between productions of ROS and antioxidant defense (Manchanda and Garg, 2008; Foyer et al., 1994; Foyer and Noctor, 2005). To protect themselves from the toxic ROS, plant cells and its organelles like chloroplast, mitochondria and peroxisomes employ antioxidant defense systems. A great deal of research has established that the induction of the cellular antioxidant machinery is important for protection against various stresses (Gill and Tujeta, 2010; Noctor et al., 2012). However, in over production of ROS, these defense systems are required to be upregulated more than their normal limit (Gill and Tujeta,

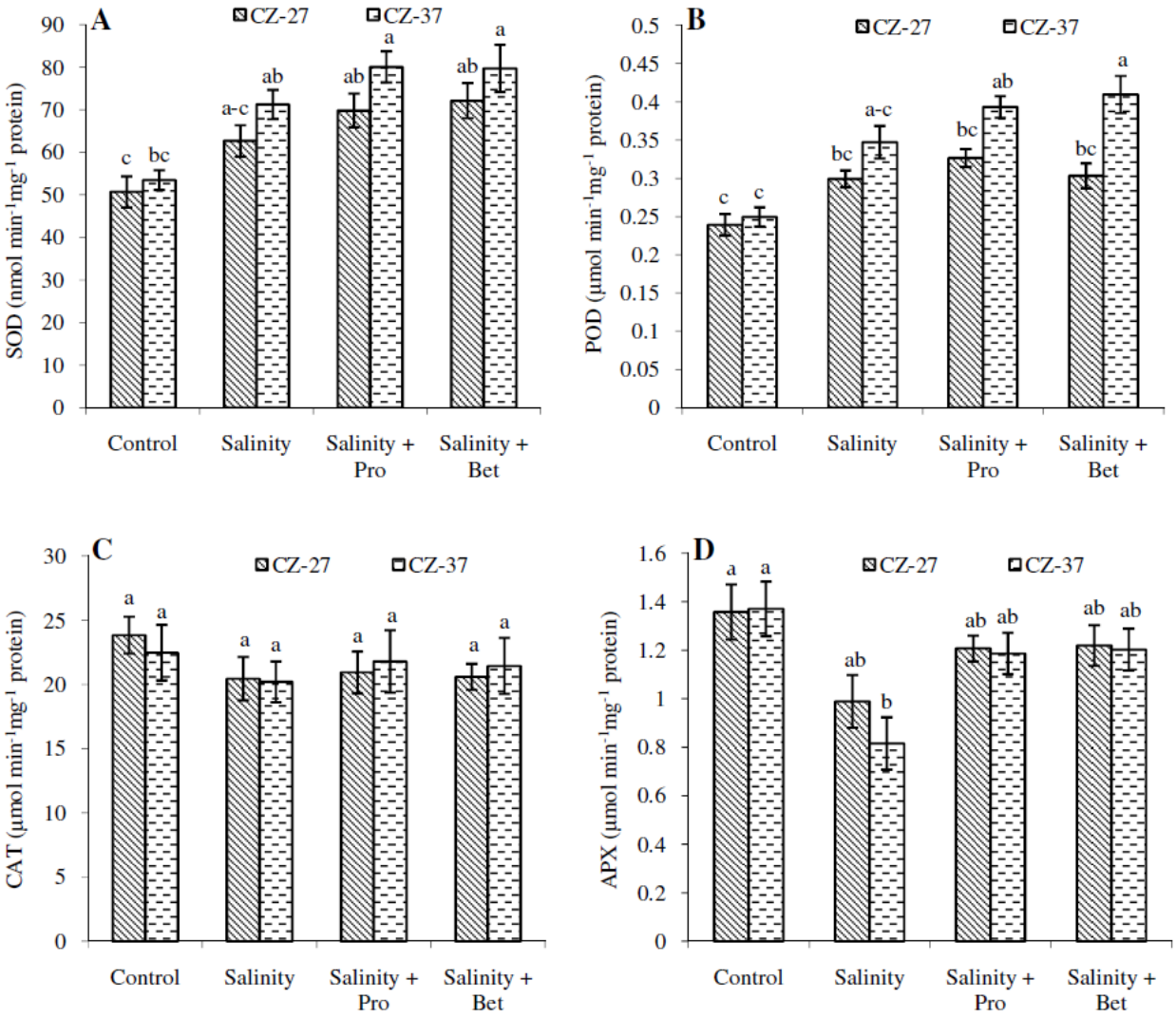

Fig 5. Activities of SOD (A), POD (B), CAT (C) and APX (D) in leaves of maize seedlings in presence or absence of proline (Pro) and betaine (Bet) at 10 day under salinity stress. Values represent the mean ± SE from three independent experiments. Bars with the same letters are not significantly different at P≤0.05.

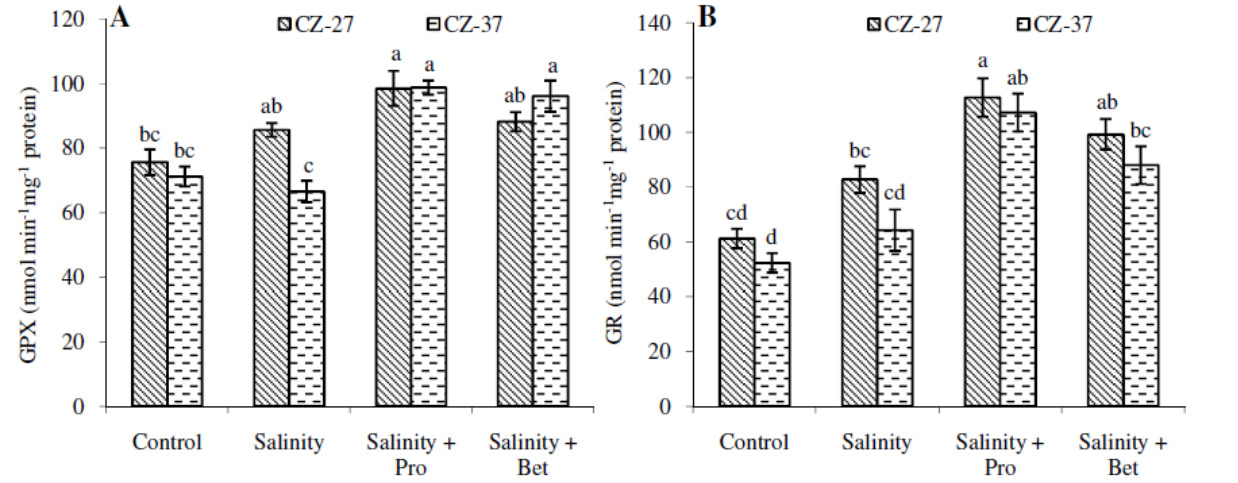

Fig 6. Activities of GPX (A) and GR (B) in leaves of maize seedlings in presence or absence of proline (Pro) and betaine (Bet) at 10 day under salinity stress. Values represent the mean ± SE from three independent experiments. Bars with the same letters are not significantly different at P≤0.05.

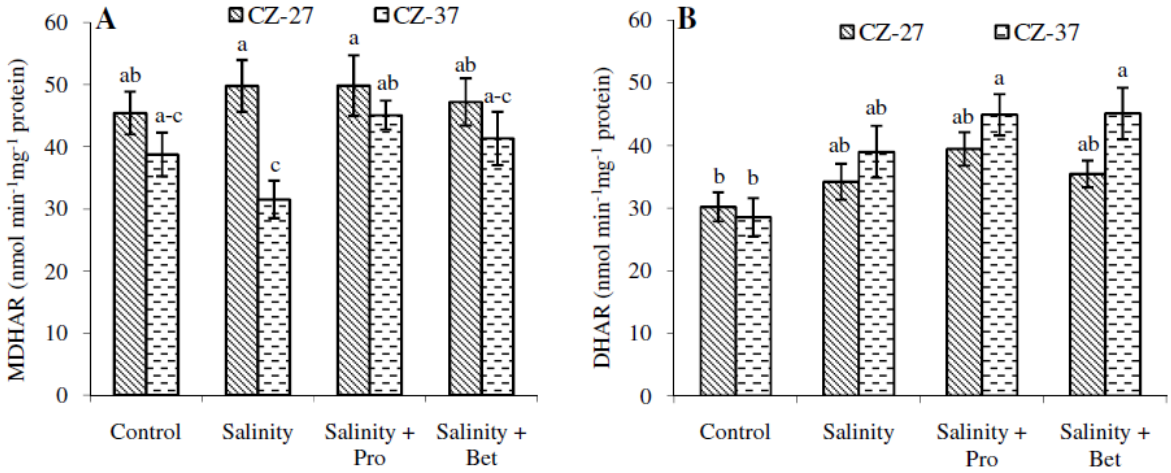

Fig 7. Activities of MDHAR (A) and DHAR (B) in leaves of maize seedlings in presence or absence of proline (Pro) and betaine (Bet) at 10 day under salinity stress. Values represent the mean ± SE from three independent experiments. Bars with the same letters are not significantly different at P≤0.05.

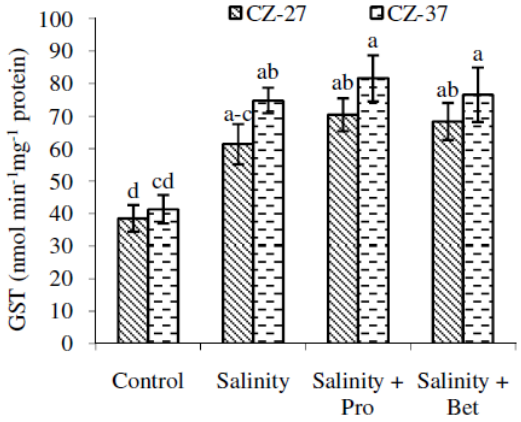

Fig 8. Activities of GST in leaves of maize seedlings in presence or absence of proline (Pro) and betaine (Bet) at 10 day under salinity stress. Values represent the mean ± SE from three independent experiments. Bars with the same letters are not significantly different at P≤0.05.

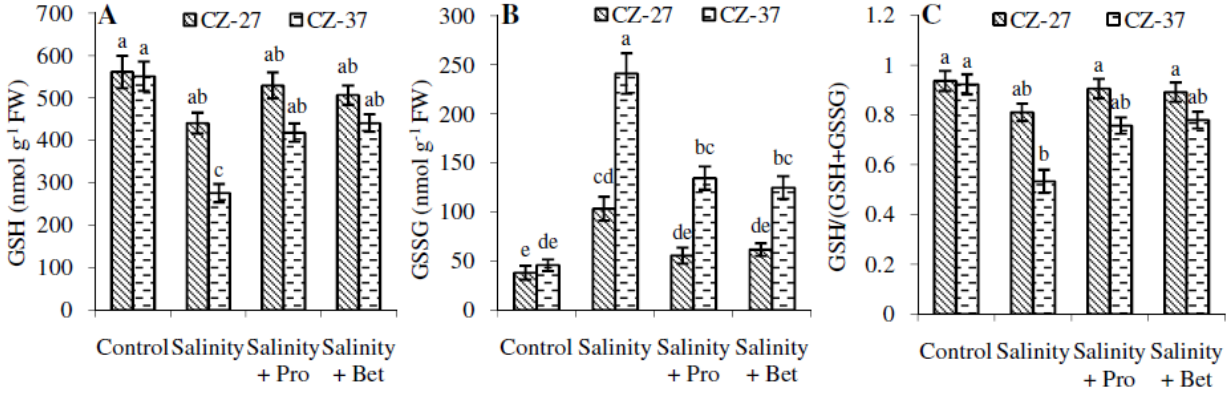

Fig 9. Contents of GSH (A), GSSG (B) and Glutathione redox state [GSH/(GSH+GSSG) ratio] (C) in leaves of maize seedlings maintained by proline (Pro) and betaine (Bet) at 10 day under salinity stress. Values represent the mean ± SE from three independent experiments. Bars with the same letters are not significantly different at P≤0.05.

2010). Proline and betaine are most important organic compatible solutes which have been reported to protect the plants from salt-induced damages through osmoprotection and antioxidant defense as well (Ashraf and Foolad, 2007; Hoque et al., 2008; Nounjan et al., 2012; Molla et al., 2014). In osmotic stress caused by salinity, reduction of RWC is a common phenomenon in plants growth and hence, RWC is considered as an important indicator for evaluating plants for tolerance to salt stress. In our study, salt stress caused significant decrease in RWC in leaves of susceptible inbred, and proline and betaine maintained significantly higher RWC (Fig. 1). Similar decrease in RWC due to salt stress was reported earlier in a susceptible rice genotype by Hasanuzzaman et al. (2014). Salinity stress caused higher losses of Chl*a* and Chl*b* in leaves of CZ-37 seedlings compared to those in CZ-27 (Table 1), and the present results support to previous findings in maize (Cha-um and Kirdmanee, 2009) and rice (Hasanuzzaman et al., 2014). However, application of proline and betaine in salt treated seedlings showed enhanced RWC which was due to the retention in water in their tissue (Table 1). This reduction of chlorophyll contents under salinity stress could be due to the increased activity of chlorophyllase enzyme or due to the disruption of fine structure of chloroplast and instability of pigment protein complexes by ions. These results are in agreement with Sakr et al. (2012). In CZ-27, the chlorophyll contents were higher which might be due to better tolerance to salinity in this inbred. However, significant difference was not found between proline and betaine treated seedlings.

Proline accumulation under stress condition is often suggested as a selection criterion for the stress tolerance of most plant species (Ashraf and Foolad, 2007; Hayat et al., 2012). In our experiment, tolerant inbred CZ-27 showed enhanced proline accumulation under saline stress (Fig. 2A). It has been shown that exogenous application of proline and betaine mitigates the adverse effect of salinity by detoxifying the ions and protects the plant cells by maintaining osmotic balance (Ashraf and Foolad, 2007; Hoque et al., 2007a, b; Okuma et al., 2000, 2004; Nawaz and Ashraf, 2010; Sakr et al., 2012; Hasanuzzaman et al., 2014). In the present study, the seedlings received proline and betaine provided significant evidence for assessing salt tolerance at seedling stage in maize. However, higher accumulation of proline in susceptible inbred under proline treatment suggested its physiological roles under saline stress. It was also observed that cysteine (a component of tripeptide GSH) content was strongly inhibited by salinity stress in CZ-37 (Fig. 2B), which might affect the GSH synthesis. Application of proline and betaine in saline reduced the inhibition of cysteine synthesis in maize seedlings. ROS generation is a common phenomenon in crop under salinity (Hernández et al., 2000; Huang et al., 2005; Noctor et al., 2012). In this study, we observed remarkable increases in $O_2^{\bullet-}$ and H_2O_2 contents in seedlings of CZ-37 (Fig. 3A, B). ROS-scavenging enzymes and antioxidant molecules in plants prevent 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 peroxisomes (Bowler et al., 1992). In our case, exogenous proline and betaine increased SOD activity in both inbreds which correlated negatively with $O_2^{\bullet-}$ generation. Therefore, an increase in SOD activity can be induced by the addition of proline and betaine, and this will enhance the dismutation of $O_2^{\bullet-}$ in salinity stressed maize seedlings. However, the higher induction of SOD in CZ-37 resulted in dismuting of higher $O_2^{\bullet-}$ generation in presence or absence of proline and betaine (Fig. 3A). MDA is regarded as a marker for evaluation of lipid peroxidation or damage to plasmalemma and organelle membranes that increases with environmental stresses including salinity (Garg and Manchanda, 2009). On the other hand, H_2O_2, produced through action of SOD, is a toxic compound, which is injurious to the cell, and excessive accumulation of H_2O_2 is one of the indicators of oxidative stress (Apel and Hirt, 2004). In present study, both MDA and H_2O_2 increased under salinity stress (Fig. 3B, 4A), which was in agreement with several previous reports (Azooz et al., 2009; Saha et al., 2010; Weisany et al., 2012). On the contrary, saline treated seedlings with proline and betaine maintained lower H_2O_2 and MDA contents (Fig. 3B, 4A), which was due to their higher antioxidant defense system. Exogenous proline- and betaine-induced upregulation of antioxidant defense and concomitant decrease in MDA and H_2O_2 contents was observed in many plant species (Nounjan et al., 2012; Yan et al., 2011). On the other hand, LOX catalyzes the peroxidation of polyunsaturated fatty acids to their corresponding hydroperoxides (Doderer et al., 1992). In this study, LOX activity was sharply increased in salt treated seedlings of CZ-37 as compared to CZ-27 (Fig. 4B). Therefore, the increased LOX activity was assumed as reasons for increased lipid peroxidation in CZ-37 (Demiral and Türkan, 2004; Azooz et al., 2009; Sánchez-Rodríguez et al., 2012). The exogenous proline and betaine reduced the activity of LOX and provided protective role under saline stress and it supported the findings of Sánchez-Rodríguez et al. (2012) and Hasanuzzaman et al. (2014). In higher plants, H_2O_2 is scavenged by the ascorbate-glutathione pathway and/or by CAT and non-specific PODs (Asada, 1994; Scandalios, 1994; Miller et al., 2010). CAT, POD, GPX and APX scavenge H_2O_2 to water and have been reported in plant species (Miller et al. 2010, Gill and Tujeta, 2010). CAT, as compared to APX, GPX and POD, with low affinity towards H_2O_2 but with a high processing rate (Scandalios, 2005), may become the principal enzymatic H_2O_2 scavenger in plants under salinity stress, where the cellular H_2O_2 level becomes several fold higher than found in plants grown under normal conditions (Nor'aini et al., 1997; Cheeseman, 2006). This is essentially because, unlike other H_2O_2 scavenging enzymes (APX, GPX and POD), enzymatic reaction of CAT is not saturated with increasing concentrations of the peroxide and is independent of other cellular reductants for instituting its activity (Scandalios, 2005). However, a large body of literature reports suggest that as compared to unstressed plants the CAT activity is significantly down regulated in salinity stressed plants (Lee et al., 2001; Shim et al., 2003; Cavlcanti et al., 2004; Demiral and Türkan, 2004), suggesting that the enzyme may not serve as the major scavenger of H_2O_2 under salinity offence to plants (Cavlcanti et al., 2004). In this study, the CAT activity was not found to increase in both inbreds (Fig. 5C) which might be due to ineffective enzyme synthesis or change in assembly of enzyme subunits (Gupta et al., 2009). Like CAT, APX activity also decreased under salt stress (Fig. 5D). On the other hand, the increased activities of POD in both inbreds and GPX activity in CZ-27 under salt stress played important role in H_2O_2 scavenging (Fig. 5B, 6A). Therefore, the tolerance in CZ-27 under salinity might also be due to higher activities of APX and GPX under salinity. Proline and betaine in salt treatments increased the activities of POD, APX and GPX in both inbreds (Fig.5C, D, 6A) which reduced the H_2O_2 level and MDA production as well (Fig. 3B, 4B). In ascorbate-glutathione cycle, ASA and GSH have vital roles in development of plant stress tolerance to adverse environmental conditions (Nakano and Asada, 1987; Apel and Hirt, 2004). In our study, the reduced content of ASA

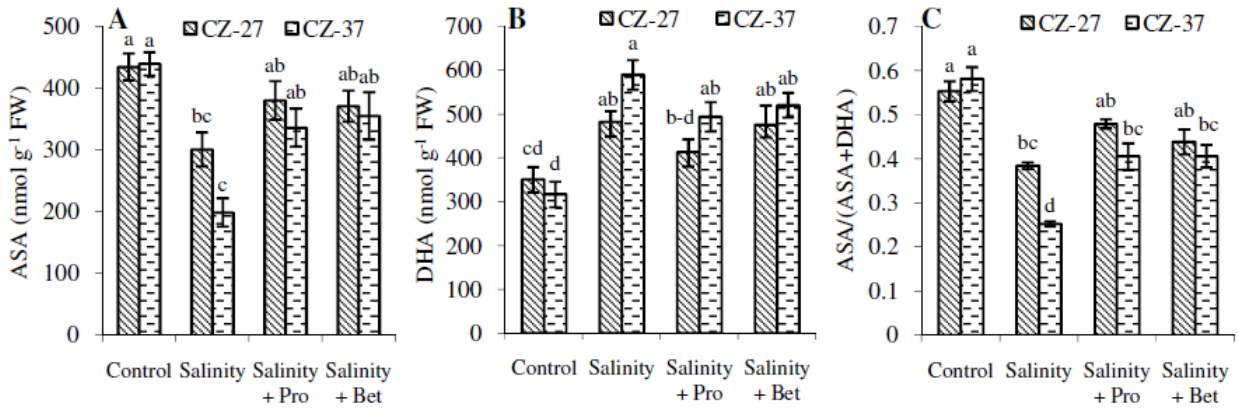

Fig 10. Contents of ASA (A), DHA (B) and Ascorbate redox state [ASC/(ASC+DHA) ratio] (C) in leaves of maize seedlings maintained by proline (Pro) and betaine (Bet) at 10 day under salinity stress. Values represent the mean ± SE from three independent experiments. Bars with the same letters are not significantly different at P≤0.05.

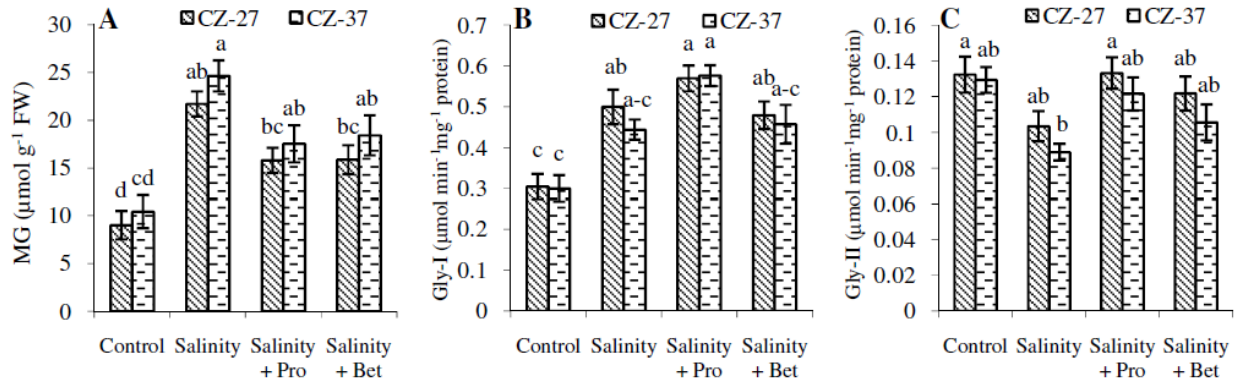

Fig 11. Contents of MG (A) and activities of Gly-I (B) and Gly-II (C) in leaves of maize seedlings in presence or absence of proline (Pro) and betaine (Bet) at 10 day under salinity stress. Values represent the mean ± SE from three independent experiments. Bars with the same letters are not significantly different at P≤0.05.

Fig 12. Comparative tolerance to salinity of two maize inbreds; CZ-27, tolerant and CZ-37, susceptible, in presence or absence of proline (Pro) and betaine (Bet). Five day seedlings were imposed to 16 dSm^{-1} salinity for 10 days.

and ascorbate redox suggested more oxidation of ASA under saline stress (Fig. 10A, C). In ascorbate-glutathione cycle, APX, MDHAR and DHAR are important enzymes involved in maintaining the ASA. APX uses two molecules of ascorbate to reduce H_2O_2 to water, with the concomitant generation of two molecules of monodehydroascorbate (MDHA) that disproportionates to ascorbate and DHA (Noctor and Foyer, 1998). The higher contents of DHA in CZ-37 might be resulted from more oxidation of ASA (Fig. 10B). On the other hand, the decreased amount of APX activity under salinity stress might be due to inactivation, and exogenous proline and betaine enhanced the activity which indicated the H_2O_2 scavenging role of proline and betaine (Fig. 5D). This result was in agreement with other findings (Patade et al., 2014; Nounjan et al., 2012). MDHAR and DHAR are two important enzymes related to the regeneration of ASA which are equally important in regulating ASA level and its redox state under oxidative stress condition (Wang et al., 2010). In our study, under saline stress, the activity of MDHAR increased in CZ-27 while decreased in CZ-37 (Fig. 7A). The increased MDHAR activity which was supposed to increase ASA in CZ-27 might be used in H_2O_2 decomposition. Exogenous proline and betaine enhanced the DHAR activity in CZ-37 which helped in regeneration of ASA in CZ-37. The central role of GSH in the antioxidant defense system is due to its ability to regenerate ascorbate through reduction of DHA via DHAR activity (Noctor and Foyer, 1998). GSH also plays a vital role in the antioxidant defense system as well as glyoxalase system by acting as a substrate or cofactor for some enzymes. Furthermore, GSH plays a protective role in salt tolerance by the maintenance of the redox state (Shalata et al., 2001). The increased level of GSH pool is generally regarded as a protective response against oxidative stress. GR is an important enzyme which is important for maintaining high ratio of GSH in plant cells, also necessary for accelerating the H_2O_2 scavenging (Apel and Hirt, 2004). In this study, increased GR activities under salinity with or without proline and betaine treatments were higher in CZ-27 suggesting better maintenance of GSH and GSH-redox in this inbred (Fig. 6B, 9A, C). GPX uses GSH for decomposing H_2O_2 and converts to GSSG while GST uses GSH in detoxification and leaf senescence (Noctor et al., 2012). In this study, the higher GST activity in CZ-37 (Fig. 8) might be involved in leaf senescence. At the same time, high GPX activity suggested better tolerance of CZ-27 by decomposition of H_2O_2. Importantly, the reduced cysteine content in CZ-37 under salinity (Fig. 2B) might also be important reason of lower GSH. Therefore, the higher induction of SOD, POD, MDHAR, DHAR, GPX, GR and GST activities in proline and betaine treated seedling in CZ-37 could help in increase tolerance through decomposition of H_2O_2, maintaining in ASA and GSH pool and detoxification and other physiological roles. Our results corroborated with findings of Patade et al. (2014) and Hasanuzzaman et al. (2014). The glyoxalase system consists of two enzymes (Gly-I and Gly-II) acts to convert the potential cytotoxic MG to non-toxic hydroxyacids such as lactate. Gly-I uses GSH to convert MG to S-D-lactoyl glutathione (SLG), while the hydrolytic reaction catalyzed by Gly-II liberates the lactic acid and free GSH (Noctor et al., 2012). In several plant species, upregulation or overexpression of these enzymes increases tolerance to abiotic stresses (Singla-Pareek et al., 2008; Saxena et al., 2011). The present investigation demonstrated that salt stress caused significant accumulation of MG in both inbred (Fig. 11A). Contrary, though the activity of Gly-I increased, Gly-II decreased under salinity in both inbreds. Therefore, it could be speculated that the high MG might be transferred to SLG, but lower Gly-II hampered the MG detoxification as well as GSH recycling. Proline and betaine increased the activities of both Gly-I and Gly-II along with lower contents of MG (Fig. 11B, C) suggested the evidence for their protective role in glyoxalase system for conferring saline stress tolerance (Hoque et al., 2008).

Materials and Methods

Plant materials and stress treatments

Five days old seedlings of a tolerant maize inbred CZ-27 and a susceptible inbred CZ-37 grown in plastic pot under green house condition were subjected to impose 16 dSm^{-1} salinity induced by NaCl and observed for 10 days (Fig. 13). Fifteen millimolar of proline or betaine was applied with saline water. After arising salinity to 16 dSm^{-1}, the level was maintained by adding saline solution of higher or lower concentration. Hyponex (Japan) was used as nutrition. Control treatments were also maintained under same condition. Salinity levels were measured by an EC meter (HI 993300). Data were taken on different parameters in fully expanded leaves of 10 day stressed seedlings.

Relative water content

To measure RWC, fresh weight (FW), turgid weight (TW) and dry weight (DW) of leaves were recorded. The RWC was calculated by the following formula: RWC (%) = (FW-DW) ×100/(TW-DW).

Chlorophyll contents

For Chl determination, five hundred milligram of fresh leaf material was homogenized in 10 ml of 80% acetone. The absorbance aliquot was read at 645, 663 and 470 nm with a spectrophotometer (UV-1800, Shimadzu, Japan) against 80% acetone as blank. Chl contents were calculated using the formula of Arnon (1949) and were expressed in mg g^{-1} fresh weight (FW).

Extraction and measurement of ascorbate and glutathione

Maize leaves (0.5 g fresh weight) were homogenized in 3 ml ice-cold acidic extraction buffer containing 5% meta-phosphoric acid containing 1 mM EDTA. Homogenates were centrifuged at 11,500×g for 15 min at 4°C, and the supernatant was used for analysis of ascorbate and glutathione spectrophotometrically (UV-1800, Shimadzu, Japan). Ascorbate content was determined following the method of Huang et al. (2005). The supernatant was neutralized using 0.5 M potassium-phosphate (K-P) buffer (pH 7.0). The reduced ascorbate was assayed at 265 nm in 100 mM K-P buffer (pH 5.6) with 0.5 unit of ascorbate oxidase (AO). A specific standard curve of ASA was used for quantification. The glutathione pool was assayed according to Yu et al. (2003), utilizing 0.4 ml of aliquots of supernatant neutralized with 0.6 ml of 0.5 M potassium-phosphate (K-P) buffer (pH 7.0). Based on enzymatic recycling, glutathione was oxidized by 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) and reduced by NADPH in the presence of GR, and total glutathione content was evaluated by the rate of absorption changes at 412 nm of 2-nitro-5-thiobenzoic acid (NTB) generated from the reduction of DTNB. Oxidized glutathione (GSSG) was determined after removal of GSH by 2-vinylpyridine derivatization. GSH was found by deduction of

GSSG from total glutathione. Specific standard curves of GSH and GSSG were used.

Extraction of soluble protein for assay activity

Maize leaf (0.5g) was homogenized in 1 ml of 50 mM ice-cold K-P buffer (pH 7.0) by mortar and pestle 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 10 min and the supernatants were used for determination of enzyme activities. All procedures were performed below 4 °C.

Assay of enzymatic activities

SOD (EC 1.15.1.1): SOD activity of whole cell homogenates prepared on ice in 50 mM K-P buffer (p^H 7.8, with 1.34 mM diethylenetriaminepentaacetic acid) was determined using an indirect competitive inhibition assay (Spitz, and Oberley, 1989). This assay is based on the competition between SOD and an indicator molecule NBT for superoxide production from xanthine and xanthine oxidase. One unit of activity was defined as that amount of protein required to inhibit NBT reduction by 50%. POD (EC 1.11.1.7): POD activity was estimated according to Hemeda and Klein (1990). The reaction mixture contained 25 mM K-P buffer (pH 7.0), 0.05% guaiacol, 10 mM H_2O_2 and enzyme. Activity was determined by the increase in absorbance at 470 nm due to guaiacol oxidation for 1 min using extinction coefficient of 26.6 mM^{-1} cm^{-1}. APX (EC: 1.11.1.11): APX activity was assayed following the method of Nakano and Asada (1981). The reaction buffer solution contained 50 mM K-P buffer (pH 7.0), 0.5 mM ASA, 0.1 mM H_2O_2, 0.1 mM EDTA, and enzyme extract in a final volume of 0.7 ml. 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}. MDHAR (EC: 1.6.5.4): MDHAR activity was determined by the method of Hossain et al. (2010). 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 0.7 ml. 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^{-1} cm^{-1}. DHAR (EC: 1.8.5.1): DHAR activity was determined by the procedure of Nakano and Asada (1981). The reaction buffer contained 50 mM K-P 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 extinction coefficient of 14 mM^{-1} cm^{-1}. GR (EC: 1.6.4.2): GR activity was measured by the method of Hossain et al. (2010). The reaction mixture contained 0.1 M K-P 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^{-1} cm^{-1}**.** GST (EC: 2.5.1.18): GST activity was determined by the method of Rohman et al. (2010). The reaction mixture contained 100 mM Tris-HCl buffer (pH 6.5), 1.5 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), and enzyme solution in a final volume of 0.7 ml. 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^{-1} cm^{-1}. GPX (EC: 1.11.1.9): GPX activity was measured as described by Hasanuzzaman et al. (2014) using H_2O_2 as a substrate. The reaction mixture consisted of 100 mM Na-phosphate buffer (pH 7.5), 1 mM EDTA, 1 mM NaN_3, 0.12 mM NADPH, 2 mM GSH, 1 unit GR, 0.6 mM H_2O_2, and 20 µl of sample solution. The reaction was started 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}. CAT, EC: 1.11.1.6): CAT activity was measured according to the method of Hossain et al. (2010) by monitoring the decrease of absorbance at 240 nm for 1 min caused by the decomposition of H_2O_2. The reaction mixture contained 50 mM K-P buffer (pH 7.0), 15 mM H_2O_2, and enzyme solution in a final volume of 0.7 ml. The reaction was initiated with enzyme extract, and the activity was calculated using the extinction coefficient of 39.4 M^{-1} cm^{-1}. Gly-I (EC: 4.4.1.5): Gly-I assay was carried out according to Yadav et al. (2005a). Briefly, the assay mixture contained 100 mM K-P buffer (pH 7.0), 15 mM magnesium sulfate, 1.7 mM reduced glutathione, and 3.5 mM MG in a final volume of 0.7 ml. The reaction was started by the addition of MG, and the increase in absorbance was recorded at 240 nm for 1 min. The activity was calculated using the extinction coefficient of 3.37 mM^{-1} cm^{-1}. Gly-II (EC: 3.1.2.6): Gly-II activity was determined according to the method of Principato et al. (1987) by monitoring the formation of GSH at 412 nm for 1 min. The reaction mixture contained 100 mM Tris-HCl buffer (pH 7.2), 0.2 mM DTNB, and 1 mM SLG in a final volume of 1 ml. The reaction was started by the addition of SLG, and the activity was calculated using the extinction coefficient of 13.6 mM^{-1} cm^{-1}. LOX (EC: 1.13.11.12): LOX activity was measured following Doderer et al. (1992) with modification. The substrate solution was prepared by adding 35µl linoleic acid to 5 ml distilled water containing 50 µl Tween-20. The solution was kept at pH 9.0 by adding 0.2 M NaOH until all the linoleic acid was dissolved and the pH remained stable. After adjusting the pH to 6.5 by adding 0.2 M HCI, 0.1 M phosphate buffer (pH 6.5) was added to a total vol. of 100 ml. The substrate solution was flushed with and kept under a nitrogen atmosphere. LOX activity was determined spectrophotometrically by adding 10 µl of sample to 590 µl substrate solution. The increase in absorbance at 234 nm was measured for 1min at 25°C. The activity was expressed as µ mol hydroperoxide formed min^{-1} mg^{-1} protein using a molar extinction coefficient of 25,000 M^{-1} cm^{-1}.

Measurement of the $O_2^{\bullet-}$ generation rate and H_2O_2

$O_2^{\bullet-}$was determined according to the method of Elstner and Heupel (1976). The $O_2^{\bullet-}$ concentration was calculated from a standard curve of $NaNO_2$. H_2O_2 was assayed according to the method described by Yu et al. (2003). The H_2O_2 content was determined by using extinction co-efficient of 0.28 μM^{-1} cm^{-1} and expressed as micromoles per gram FW.

Measurement of lipid peroxidation

The level of lipid peroxidation was measured by estimating melondialdehyde (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). The concentration of MDA was calculated by using the extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nanomole of MDA per gram FW.

Determination of proline and cysteine

Proline was measured according to Bates et al. (1973) based on proline's reaction with ninhydrin. Cysteine was estimated as described in Gaitonde (1967).

Measurement of MG

About 0.3 g leaf tissue was extracted in 3 ml of 5% perchloric acid and centrifuged at 4°C at 11,000×*g* for 10 min. The supernatant was decolorized by adding charcoal (10 mg/ml) and centrifuged at 11,000×*g* for 10 min. The supernatant was neutralized by saturated solution of potassium carbonate at room temperature and centrifuged again at 11,000×*g* for 10 min. Neutralized supernatant was used for MG estimation following the method of Wild et al. (2012). The formation of the product N-α-acetyl-*S*-(1-hydroxy-2-oxo-prop-1-yl) cysteine was recorded at a wave length of 288 nm. Data was calculated with standard curve of MG solutions in sodium dihydrogen phosphate.

Determination of protein

The protein concentration in the leaf extracts was determined according to the method of Bradford (1976) using BSA as a protein standard.

Data analysis

Data were analyzed by SAS (Version 9.3) program following complete randomized design (CRD) and the mean differences were compared by Duncan's Multiple Range Test. Values of mean ± SE were calculated from three independent experiments with three replications and $P \leq 0.05$ was considered to be significant.

Conclusion

Considering the above results, under saline stress, the accumulation of ROS, MDA and MG were higher in susceptible genotypes CZ-37. The ASA and GSG pool were also found to be more oxidized in this inbred. Both proline and betaine played significant role in physiology and ROS and MG detoxification through enhancing the antioxidative (enzymatic and non-enzymatic) and detoxification systems. However, under salinity, both proline and betaine showed better role in increasing ASA and GSH and many of the ROS scavenging enzymes in CZ-37, probably to protect cellular damage from higher ROS and toxic metabolites. In this study, we found a contrast feature of enzymatic and non-enzymatic responses in two differently saline sensitive inbreds maize and the information will help in understanding the underlying saline tolerance mechanism of the crop.

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Conflict of interests

The authors have declared that no conflicts of interests exist.

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