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RESEARCH ARTICLE

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# Coordinate induction of antioxidant defense and glyoxalase system by exogenous proline and glycinebetaine is correlated with salt tolerance in mung bean

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**Abstract** The purpose of this study was to assess the synergistic effects of exogenously applied proline and glycinebetaine (betaine) in antioxidant defense and methylglyoxal (MG) detoxification system in mung bean seedlings subjected to salt stress (200 $mmol \cdot L^{-1}$ NaCl, 48 h). Seven-day-old mung bean seedlings were exposed to salt stress after pre-treatment with proline or betaine. Salt stress caused a sharp increase in reduced glutathione (GSH) and oxidized glutathione (GSSG) content in leaves, while the GSH/GSSG ratio and ascorbate (AsA) content decreased significantly. The glutathione reductase (GR), glutathione peroxidase (GPX), glutathione S-transferase (GST) and glyoxalase II (Gly II) activities were increased in response to salt stress, while the monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), catalase (CAT) and glyoxalase I (Gly I) activities sharply decreased with an associated increase in hydrogen peroxide ($H_2O_2$) and lipid peroxidation level (MDA). Proline or betaine pre-treatment had little influence on non-enzymatic and enzymatic components as compared to those of the untreated control. However, proline or betaine pre-treated salt-stressed seedlings showed an increase in AsA, GSH content, GSH/GSSG ratio and maintained higher activities of APX, DHAR, GR, GST, GPX, CAT, Gly I and Gly II involved in ROS and MG detoxification system as compared to those of the untreated control and mostly also salt-stressed plants with a simultaneous decrease in GSSG content, $H_2O_2$ and MDA level. These results together with our previous results suggest that coordinate induction of antioxidant defense and glyoxalase system by proline and betaine rendered the plants tolerant to salinity-induced oxidative stress in a synergistic fashion.

**Keywords** salt stress, reactive oxygen species, antioxidant defense, methylglyoxal detoxification system, glycinebetaine, proline, mung bean

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## 1 Introduction

Induction of stress tolerance in plants has been a major focus of research over many decades because reduction in the productivity of agricultural and horticultural crops in response to various environmental challenges is a major economic loss. Therefore, the development of methods to induce stress tolerance in plants against adverse conditions is vital and necessary. High salinity, most commonly mediated by NaCl, is one of the major abiotic stresses globally. Increased salinization of arable land is expected to have devastating effects, resulting in 30% land loss within the next 25 y and up to 50% by the middle of the 21st century (Wang et al., 2003). Although a wide range of genetic adaptations to saline conditions has been observed and a number of significant physiologic responses have been associated with tolerance, the underlying mechanisms of salt tolerance in plants are not fully understood (Zhu, 2002). Attempts to improve yield under stress conditions by plant breeding have been largely unsuccessful, primarily due to the multigenic origin of the adaptive responses. Therefore, understanding the biochemical and molecular responses to salt stress is essential for a holistic perception of plant resistance mechanisms under salt stress

conditions.

High salt concentration in the soil or in the irrigation water can have a devastating effect on plant metabolism, disrupting cellular homeostasis and uncoupling major physiologic processes. A direct result of salt-induced cellular changes is an enhanced accumulation of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide radical ($O_2\cdot^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($\cdot OH$) (Khan and Panda, 2008; Pérez-López et al., 2009; Tanou et al., 2009). In addition to ROS, methylglyoxal (MG) was found to accumulate in excessive amounts in plants in response to various stresses including salt stress (Yadav et al., 2005a, 2005b; Singla-Pareek et al., 2006; Hossain et al., 2009). ROS and MG are highly toxic to plant cell, and in absence of any protective mechanism, they can react with proteins, lipids, DNA and inactivate antioxidant defense system (Yadav et al., 2005b; El-Shabrawi et al., 2010). Plants have a battery of enzymatic and non-enzymatic scavenging pathways or detoxification systems which function as an extremely efficient cooperative system to counter the deleterious effects of ROS and MG (Fig. 1) (Apel and Hirt, 2004; Yadav et al., 2005a, 2005b; Hossain et al., 2009, 2010). These antioxidants include the enzymes, superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), and water-soluble compounds, such as AsA and GSH. The ascorbate–glutathione (AsA-GSH) cycle involves APX, MDHAR, DHAR, GR, AsA and GSH in a series of cyclic reactions to detoxify $H_2O_2$ and regenerate AsA and GSH. Both GSH and AsA also serve as chemical scavengers of reactive oxygen in non-enzymatic reactions (Noctor and Foyer, 1998; Asada, 1999). Recently, it is well known that GPXs are ubiquitously occurring enzymes in plant cells that use GSH to reduce $H_2O_2$, organic and lipid hydroperoxides. Importantly, GST catalyzes the detoxification of lipid peroxides and xenobiotics by conjugating them with GSH (Noctor et al., 2002) and helps them in sequestration into vacuole of a plant cell. In plants, MG is detoxified mainly via the glyoxalase system, comprising two enzymes, glyoxalase I (Gly I) and glyoxalase II (Gly II). Gly I catalyzes the formation of *S*-D-lactoylglutathione (SLG) from the hemithioacetal formed non-enzymatically from MG and GSH, while Gly II catalyzes the hydrolysis of SLG to regenerate GSH and liberate D-lactate (Thornalley, 1990). In addition, CAT eliminates $H_2O_2$ by breaking it down to $H_2O$ and $O_2$ and does not require any reducing equivalent (Mallick and Mohn, 2000). The results of numerous studies suggest that the alleviation of oxidative damage and increased resistance to salt stress is often correlated with a more efficient antioxidative and glyoxalase system (Mittova et al., 2003a, 2003b; Sekmen et al., 2007; Aghaei et al., 2009; El-Shabrawi et al., 2010; Hossain and Fujita, 2010).

The typical first response of all plants to salt stress is osmotic adjustment. Compatible solutes, such as proline and betaine, are known to accumulate under conditions of environmental stresses and play a pivotal role in the process of osmotic adjustment in higher plants. Salt stress upregulates the enzymes involved in proline and betaine biosyntheses in several plant species (Hare et al., 1998; Sumithra et al., 2006), and elevated levels of proline and betaine accumulated in plant cells correlate with enhanced stress tolerance (Munns, 2005; Desingh and Kanagaraj, 2007; Ahmad et al., 2010a). Apart from their osmoprotective roles, both proline and betaine contribute to scavenging free radicals and buffering cellular redox potential and stabilize many functional units such as the complex II electron transport, membranes and proteins under stressful conditions (McNeil et al., 2001; Molinari et al., 2004). Higher antioxidative enzyme activities in stress

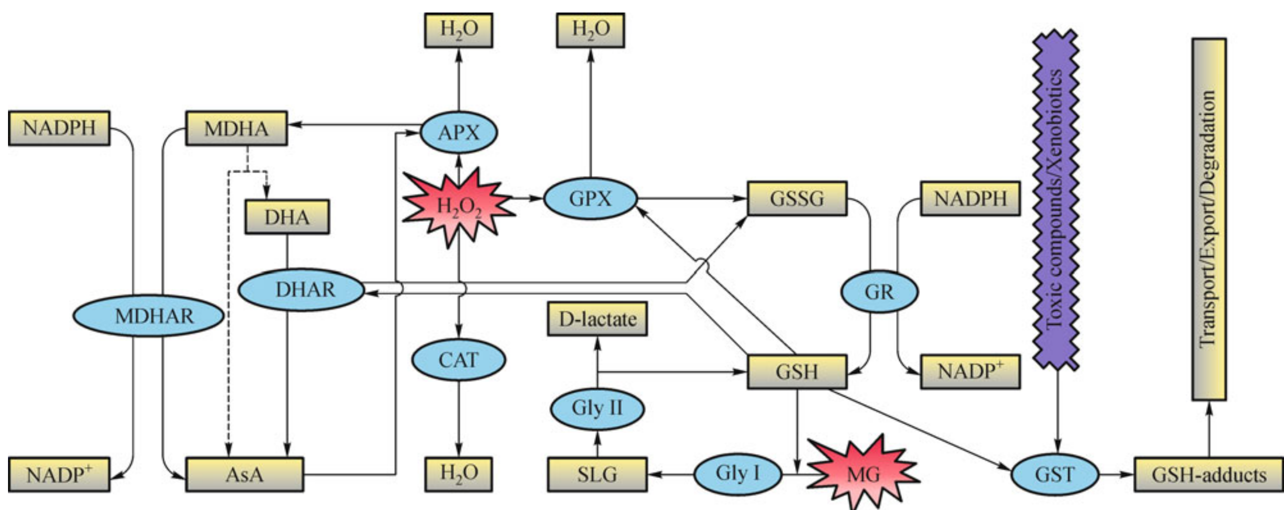


**Fig. 1** Schematic illustration of possible metabolic interaction of antioxidant defense and MG detoxification systems in plant cells
Note: Abbreviations are defined in the text.

conditions either by in vivo proline or betaine synthesis or by exogenous application have also been shown to improve salt stress tolerance (Hoque et al., 2007; Raza et al., 2007; Huang et al., 2009; Ahmad et al., 2010b; Meloni and Martinez, 2010). To our knowledge, there is currently no information available on the possible beneficial effects of exogenous proline or betaine in AsA-GSH cycle in mung bean seedlings grown under salinity conditions.

In our recent study, we showed that exogenous betaine and proline enhanced the tolerance to cadmium (Cd) stress in mung bean seedlings by enhancing antioxidant defense and MG detoxification system (Hossain et al., 2010). In addition, we earlier demonstrated that MG level, Gly I and II activities and Gly I protein expression were increased in response to various abiotic stresses including salt stress (Hossain et al., 2009; Hossain and Fujita, 2009). Therefore, to further explore the biochemical and physiologic mechanisms of betaine or proline mediated plant stress tolerance, in this study, we investigated the possible regulatory role of exogenous proline or betaine on antioxidant defense and MG detoxification system in mung bean seedlings subjected to short-term salt stress. We hypothesized that coordinate induction antioxidant and glyoxalase system components offered by exogenous proline and betaine might contribute to better protection against NaCl-induced oxidative damage.

## 2 Materials and methods

### 2.1 Plant materials and stress treatments

Mung bean (*Vigna radiata* cv. Binamoog-1) seeds of uniform size were selected and surface-sterilized with 70% ethanol followed by washing several times with distilled water. The seeds were then soaked with distilled water for 20 min and sown in Petri plates (9 cm) lined with 4 layers of filter paper moistened with 15 mL of distilled water for germination under controlled conditions (light, 100 μmol photon $\cdot m^{-2} \cdot s^{-1}$; temp, 25±2°C; RH, 65%–70%) for three days. Germinated seedlings were then grown in Petri plates that contained 1000-fold diluted Hyponex solution (Type: 5-10-5, Hyponex, Japan). Six-day-old mung bean seedlings of approximately equal sizes were employed to proline or betaine pre-treatment. For betaine and proline pre-treatment, root portions of the seedlings were immersed with 5 $mmol \cdot L^{-1}$ proline or 5 $mmol \cdot L^{-1}$ betaine in Hyponex solution for 24 h. Proline or betaine treated and non-treated seedlings were then subjected to 200 $mmol \cdot L^{-1}$ NaCl stress in Hyponex solution and grown under the above conditions for 48 h. Untreated control and proline or betaine pre-treated non-stressed seedlings were grown in Hyponex solution only.

### 2.2 Extraction and analysis of ascorbate and glutathione

Mung bean leaves (0.5 g fresh weight) were homogenized in 1.5-mL ice-cold acidic extraction buffer (6% metaphosphoric acid containing 1 $mmol \cdot L^{-1}$ EDTA) using a mortar and pestle. Homogenates were centrifuged at 11500 × *g* for 15 min at 4°C, and the supernatant was collected for analysis of ascorbate and glutathione.

Ascorbate content was determined following the method of Huang et al. (2005) with some modifications. The supernatant was neutralized with 0.5 $mol \cdot L^{-1}$ K-phosphate buffer (pH 7.0). The reduced ascorbate was assayed spectrophotometrically at 265 nm in 100 $mmol \cdot L^{-1}$ K-phosphate buffer (pH 5.6) with 0.5 unit of ascorbate oxidase (AO). A specific standard curve with AsA was used for quantification. The glutathione pool was assayed according to previously described methods (Yu et al., 2002) with modifications (Paradiso et al., 2008) utilizing 0.4-mL aliquots of supernatant neutralized with 0.6 mL of 0.5 $mol \cdot L^{-1}$ K-phosphate buffer (pH 7.5). 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 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. GSSG was determined after removal of GSH by 2-vinylpyridine derivatization. Standard curves were generated with reduced and oxidized glutathione.

### 2.3 Enzyme extraction and assays

Using a pre-cooled mortar and pestle, 0.5 g of leaf tissue was homogenized in 1 mL of 50 $mmol \cdot L^{-1}$ ice-cold K-phosphate buffer (pH 7.0) containing 100 $mmol \cdot L^{-1}$ KCl, 1 $mmol \cdot L^{-1}$ ascorbate, 5 $mmol \cdot L^{-1}$ $\beta$-mercaptoethanol and 10% (w/v) glycerol. The homogenates were centrifuged at 11500 × *g* for 10 min, and the supernatants were used for determination of enzyme activity. All procedures were performed at 0–4°C.

APX (EC: 1.11.1.11) activity was assayed following the method of Nakano and Asada (1981). The reaction buffer solution contained 50 $mmol \cdot L^{-1}$ K-phosphate buffer (pH 7.0), 0.5 $mmol \cdot L^{-1}$ AsA, 0.1 $mmol \cdot L^{-1}$ $H_2O_2$, 0.1 $mmol \cdot L^{-1}$ 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 $mM^{-1} \cdot cm^{-1}$.

MDHAR (EC: 1.6.5.4) activity was determined by the method of Hossain et al. (1984). The reaction mixture contained 50 $mmol \cdot L^{-1}$ Tris-HCl buffer (pH 7.5), 0.2 $mmol \cdot L^{-1}$ NADPH, 2.5 $mmol \cdot L^{-1}$ AsA, 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 absorbance at 340 nm for 1 min using an extinction coefficient of 6.2 $mM^{-1}cm^{-1}$.

DHAR (EC: 1.8.5.1) activity was determined by the procedure of Nakano and Asada (1981). The reaction buffer contained 50 $mmol \cdot L^{-1}$ K-phosphate buffer (pH

7.0), 2.5 mmol·$L^{-1}$ GSH, and 0.1 mmol·$L^{-1}$ 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) activity was measured by the method of Hossain and Fujita (2010). The reaction mixture contained 0.1 mol·$L^{-1}$ K-phosphate buffer (pH 7.8), 1 mmol·$L^{-1}$ EDTA, 1 mmol·$L^{-1}$ GSSG, 0.2 mmol·$L^{-1}$ 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 $mM^{-1}$·$cm^{-1}$.

GPX (EC: 1.11.1.9) activity was measured as described by Hossain and Fujita (2010) using $H_2O_2$ as a substrate. The reaction mixture consisted of 100 mmol·$L^{-1}$ Na-phosphate buffer (pH 7.5), 1 mmol·$L^{-1}$ EDTA, 1 mmol·$L^{-1}$ $NaN_3$, 0.12 mmol·$L^{-1}$ NADPH, 2 mmol·$L^{-1}$ GSH, 1 unit GR, 0.6 mmol·$L^{-1}$ $H_2O_2$ 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 $mM^{-1}$·$cm^{-1}$.

GST (EC: 2.5.1.18) activity was determined spectrophotometrically by the method of Hossain and Fujita (2010). The reaction mixture contained 100 mmol·$L^{-1}$ Tris-HCl buffer (pH 6.5), 1.5 mmol·$L^{-1}$ GSH, 1 mmol·$L^{-1}$ 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 $mM^{-1}$·$cm^{-1}$.

CAT (EC: 1.11.1.6) activity was measured according to the method of Hossain et al. (2009) 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 mmol·$L^{-1}$ K-phosphate buffer (pH 7.0), 15 mmol·$L^{-1}$ $H_2O_2$ 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) assay was carried out according to the method of Hossain and Fujita (2009). Briefly, the assay mixture contained 100 mmol·$L^{-1}$ K-phosphate buffer (pH 7.0), 15 mmol·$L^{-1}$ magnesium sulfate, 1.7 mmol·$L^{-1}$ GSH and 3.5 mmol·$L^{-1}$ 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) activity was determined according to the method of Hossain and Fujita (2009) by monitoring the formation of GSH at 412 nm for 1 min. The reaction mixture contained 100 mmol·$L^{-1}$ Tris-HCl buffer (pH 7.2), 0.2 mmol·$L^{-1}$ DTNB and 1 mmol·$L^{-1}$ *S*-D- lactoylglutathione (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 $mM^{-1}$·$cm^{-1}$.

### 2.4 Lipid peroxidation

The level of lipid peroxidation was measured in leaf tissue by estimating MDA, a decomposition product of the peroxidized polyunsaturated fatty acid component of the membrane lipid, using thiobarbituric acid (TBA) as the reactive material following the method of Heath and Packer (1968) with slight modifications (Hossain and Fujita, 2010). The concentration of MDA was calculated by using the extinction coefficient of 155 $mM^{-1}cm^{-1}$ and expressed as nmol MDA·$g^{-1}$ fresh weight.

### 2.5 Measurement of $H_2O_2$

$H_2O_2$ was assayed according to the method described by Hossain and Fujita (2010). $H_2O_2$ was extracted by homogenizing 0.5 g leaf tissue with 3 mL of 50 mmol·$L^{-1}$ K-phosphate buffer pH (6.5) at 4°C. The homogenate was centrifuged at 11500 × *g* for 15 min. A 3-mL sample of supernatant was mixed with 1 mL 0.1% $TiCl_4$ in 20% $H_2SO_4$ (v/v), and the mixture was then centrifuged at 11500 × g for 15 min at room temperature. The optical absorption of the supernatant was measured spectrophotometrically at 410 nm to determine the $H_2O_2$ content ($\epsilon$ = 0.28 $\mu M^{-1}$·$cm^{-1}$) and expressed as μmol·$g^{-1}$ fresh weight.

### 2.6 Determination of protein

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

### 2.7 Statistical analysis

All data obtained were subjected to one-way analysis of variance (ANOVA), and the mean differences were compared by a least significant difference (LSD) test using MSTAT-C. Differences at $P < 0.05$ were considered significant.

## 3 Results

### 3.1 Cellular ascorbate and glutathione contents

A significant decrease (43%) in AsA content was observed in response to salt stress as compared to the untreated control (Fig. 2). However, proline- and betaine-pretreated salt-stressed seedlings showed 24% and 13% higher in

AsA content as compared to the seedlings subjected to salt stress without pre-treatment. AsA content in proline or betaine pre-treated non-stress seedlings was similar to untreated control.

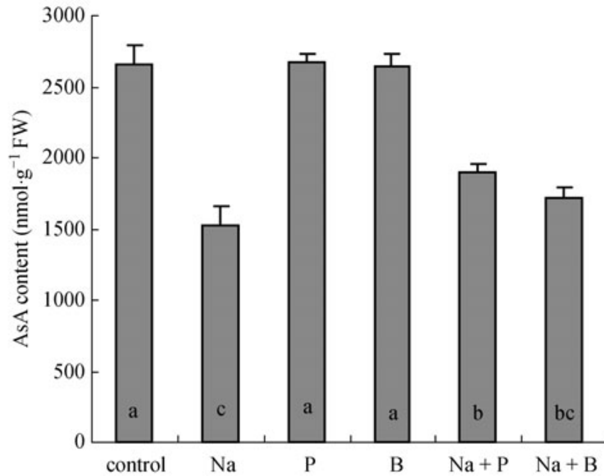


**Fig. 2** Reduced ascorbate (AsA) contents in mung bean seedlings induced by proline and betaine under salt stress conditions

Note: Na, P, B, Na + P and Na + B indicates 200 $mmol \cdot L^{-1}$ NaCl, 5 $mmol \cdot L^{-1}$ proline, 5 $mmol \cdot L^{-1}$ betaine, 200 $mmol \cdot L^{-1}$ NaCl + 5 $mmol \cdot L^{-1}$ proline and 200 $mmol \cdot L^{-1}$ NaCl + 5 $mmol \cdot L^{-1}$ betaine treatments, respectively. Mean (±SD) is calculated from three independent experiments. Bars with different letters are significantly different at $P < 0.05$.

Salt stress caused a significant increase in GSH content (118%) as compared to the untreated control (Fig. 3A). Proline- and betaine-pretreated salt-stressed seedlings showed 161% and 145% increase in GSH content as compared with the untreated control. Proline- and betaine-pretreated salt-stressed showed a significant increase in GSH content as compared with seedlings treated with salt alone.

A profound increase in GSSG content (188%) was observed in response to salt stress as compared to the untreated control (Fig. 3B). Proline- and betaine-pretreated salt-stressed seedlings showed 88% and 72% increase in GSSG content as compared with the untreated control. Proline- and betaine-pretreated salt-stressed seedlings showed 34% and 40% decrease in GSSG content as compared to the seedlings treated with salt alone.

A significant decrease (24%) in GSSH/GSSG ratio was observed in response to salt stress as compared to the untreated control (Fig. 3C). Proline- and betaine-pretreated salt-stressed seedlings showed 39% and 42% increase in GSH/GSSG ratio as compared with the untreated control.

3.2 Antioxidant enzymes

APX activity remains unchanged in response to salt stress

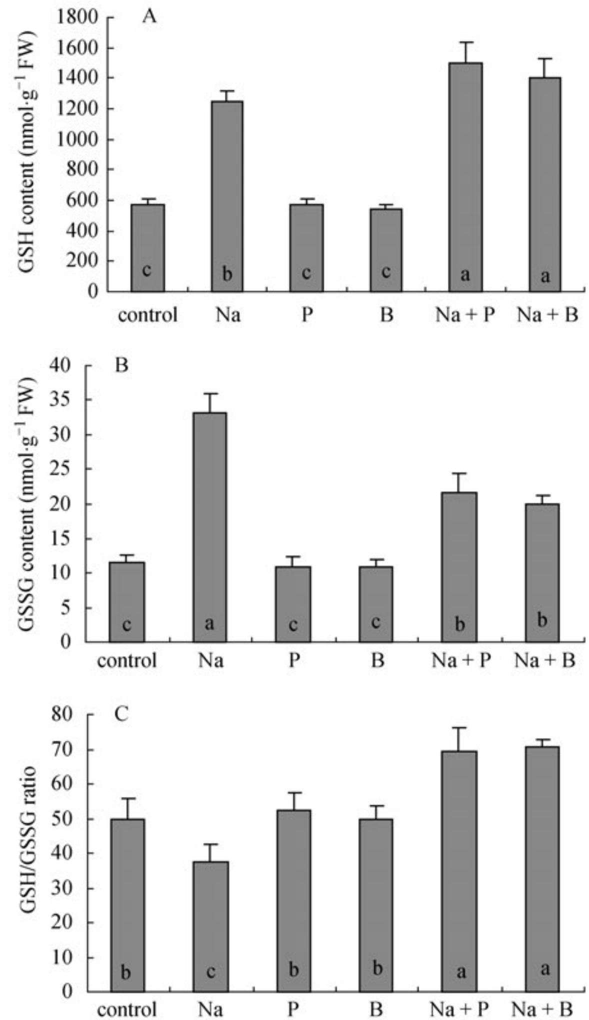


**Fig. 3** Glutathione accumulation in mung bean seedlings induced by proline and betaine under salt stress conditions

Note: A is reduced glutathione (GSH), B is oxidized glutathione (GSSG) and C is GSH/GSSG ratio. Na, P, B, Na + P and Na + B indicate 200 $mmol \cdot L^{-1}$ NaCl, 5 $mmol \cdot L^{-1}$ proline, 5 $mmol \cdot L^{-1}$ betaine, 200 $mmol \cdot L^{-1}$ NaCl + 5 $mmol \cdot L^{-1}$ proline and 200 $mmol \cdot L^{-1}$ NaCl + 5 $mmol \cdot L^{-1}$ betaine treatments, respectively. Mean (±SD) is calculated from three independent experiments. Bars with different letters are significantly different at $P < 0.05$.

(Fig. 4A). Proline- and betaine pre-treated salt-stressed seedlings showed 13% and 6% increase in APX activity as compared with the untreated control. Proline and betaine pre-treated salt-stressed seedlings showed 20% and 12% increase in APX activity as compared to the seedlings subjected to salt stress without pre-treatment.

MDHAR activity decreased significantly (39%) in response to salt stress as compared to the untreated control (Fig. 4B). Proline- and betaine-pretreated salt-stressed seedlings showed a slight increase in MDHAR activity as compared to the seedlings treated with salt alone; however, the difference was insignificant.

A significant decrease (21%) in DHAR activity was

observed in response to salt stress as compared with the untreated control (Fig. 4C). Proline- and betaine-pretreated salt-stressed seedlings had significantly higher DHAR activity (37% and 29% by proline and betaine, respectively) as compared to the seedlings subjected to salt stress without pre-treatment.

A slight increase in GR activity was observed in response to salt as compared with the untreated control (Fig. 4D). Proline- and betaine-pretreated salt-stressed seedlings had significantly higher GR activity (20% and 29% by proline and betaine, respectively) as compared to the seedlings subjected to salt stress without pre-treatment.

GST activity was increased significantly (70%) in response to salt stress as compared with the untreated control (Fig. 4E). Proline- and betaine-pretreated salt-stressed seedlings showed 87% and 103% increase in GST activity as compared with the untreated control. However, betaine-pretreated salt-stressed seedlings had significantly higher (19%) GST activity as compared to the seedlings treated with salt alone.

GPX activity was increased by 24% as compared with the untreated control in response to salt stress (Fig. 4F). Proline- and betaine-pretreated salt-stressed seedlings showed 51% and 33% increase in GPX activity as compared to the untreated control. However, proline-pretreated seedlings showed a significant increase (21%) as compared to the seedlings treated with salt alone.

A significant decrease in CAT activity (59%) was observed as compared with the untreated control (Fig. 4G) in response to salt stress. However, both proline- and betaine-pretreated salt-stressed seedlings had significantly higher CAT activity (26% and 42% by proline and betaine, respectively) as compared to the seedlings treated with salt alone.

### 3.3 Glyoxalase pathway enzymes

A significant decrease (27%) in Gly I activity was observed in response to salt stress (Fig. 5A) as compared to the untreated control. However, proline- and betaine-pretreated salt-stressed seedlings had significantly higher Gly I activity (26% and 17% by proline and betaine, respectively) as compared to the seedlings treated with salt alone.

A significant increase (24%) in Gly II activity was observed in response to salt stress as compared with untreated control (Fig. 5B). Proline- and betaine-pretreated salt-stressed seedlings showed 41% and 33% increase in Gly II activity as compared with untreated control. Proline-pretreated salt-stressed seedlings had significantly higher Gly II activity as compared to the seedlings treated with salt alone.

### 3.4 $H_2O_2$ and lipid peroxidation (MDA)

Salt stress resulted in a significant increase (79%) in $H_2O_2$ content as compared with the untreated control (Fig. 6A). Proline- and betaine-pretreated salt-stressed seedlings showed 48% and 43% increase in $H_2O_2$ content as compared with the untreated control; however, the levels were significantly lower than those of the seedlings subjected to salt stress without pre-treatment.

Lipid peroxidation (measured in terms of MDA) showed a significant increase (44%) in response to salt stress (Fig. 6B). Proline- and betaine-pretreated salt-stressed seedlings showed 17% and 25% increase in MDA content as compared with the untreated control; however, the MDA levels were significantly lower (19% and 13% by proline and betaine, respectively) than those of the seedlings subjected to salt stress alone.

## 4 Discussion

Plants tolerate abiotic stresses, including salinity-induced oxidative stress, by modulating multiple genes and coordinating the action of various genes from different pathways. Recently, the fundamental role of antioxidant pathway and glyoxalase pathway in plant stress tolerance has come to light (Yadav et al., 2005a, 2005b; Hoque et al., 2008; Hossain et al., 2009; Hossain and Fujita, 2009; El-Shabrawi et al., 2010; Hossain and Fujita, 2010; Hossain et al., 2010). It is now apparent that the functions of compatible solutes such as proline and betaine are not as straightforward as initially believed. Recent studies showed that proline or betaine fulfils a plethora of functions in imparting stress tolerance of plants. However, there are very few reports on the regulatory role of exogenous proline or betaine in antioxidant and glyoxalase pathway (Fig. 1) that acts additively and coordinately during salt tolerance. In this study, we evaluated the synergistic effects of exogenous proline and betaine in improving salt tolerance by investigating the metabolites such as AsA, GSH and $H_2O_2$, and the activity of their associate enzymes involves in antioxidant defense and glyoxalase system.

In plant cells, AsA is involved in the protection of a wide range of cellular compartments against oxidative attacks due to its ultra crucial ability to function as a donor of electrons in a broad range of enzymatic and non-enzymatic reactions (Noctor and Foyer, 1998). AsA possesses the ability to directly scavenge ROS and assists the detoxification form $H_2O_2$ through its reduction via the AsA-GSH cycle. Higher levels of endogenous AsA in plants are necessary to offset oxidative stress in addition to regulating other plant metabolic processes (Smirnoff, 2000). Exogenous application of AsA in wheat and chickpea improves salt tolerance by improving photosynthetic capacity of plants against salt-induced oxidative stress, maintaining ion homeostasis, inducing protein stability and reducing lipid peroxidation (Shalata and Neumann, 2001; Athar et al., 2008). Additionally, exogenous application of

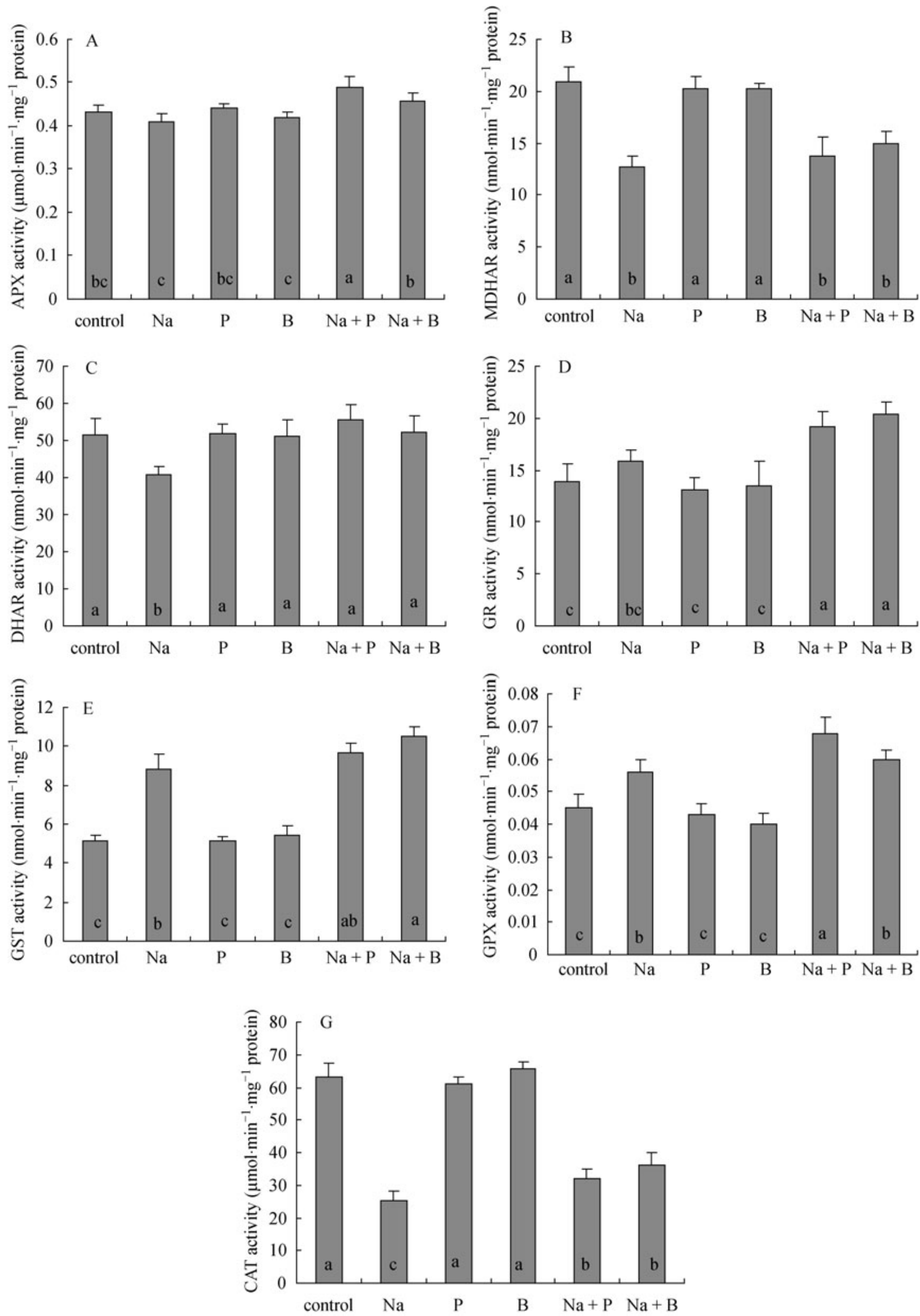


**Fig. 4** Activities of APX (A), MDHAR (B), DHAR (C), GR (D), GPX (E), GST (F) and CAT (G) in mung bean seedlings induced by proline and betaine under salt stress conditions

Note: Na, P, B, Na + P and Na + B indicates 200 mmol·$L^{-1}$ NaCl, 5 mmol·$L^{-1}$ proline, 5 mmol·$L^{-1}$ betaine, 200 mmol·$L^{-1}$ NaCl + 5 mmol·$L^{-1}$ proline and 200 mmol·$L^{-1}$ NaCl + 5 mmol·$L^{-1}$ betaine treatments, respectively. Mean (±SD) is calculated from three independent experiments. Bars with different letters are significantly different at $P < 0.05$.

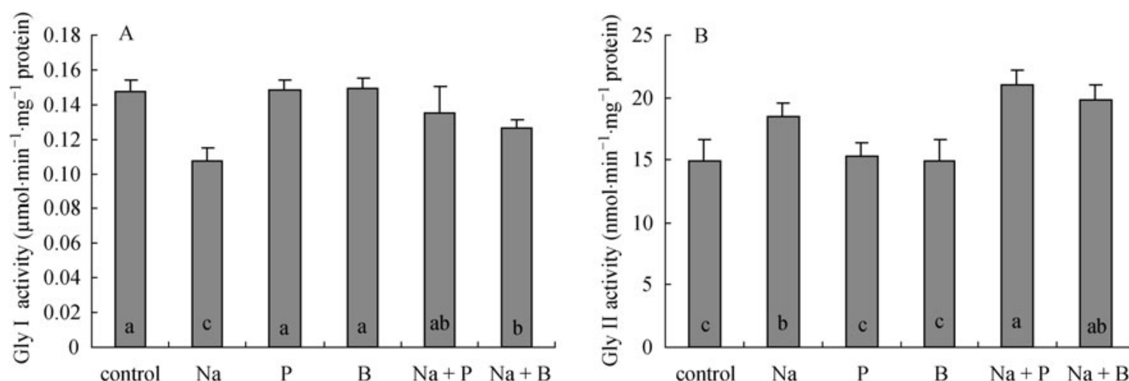


**Fig. 5** Activities of Gly I (A) and Gly II (B) in mung bean seedlings induced by proline and betaine under salt stress conditions
Note: Na, P, B, Na + P and Na + B indicate 200 mmol·$L^{-1}$ NaCl, 5 mmol·$L^{-1}$ proline, 5 mmol·$L^{-1}$ betaine, 200 mmol·$L^{-1}$ NaCl + 5 mmol·$L^{-1}$ proline and 200 mmol·$L^{-1}$ NaCl + 5 mmol·$L^{-1}$ betaine treatments, respectively. Mean (±SD) is calculated from three independent experiments. Bars with different letters are significantly different at $P < 0.05$.

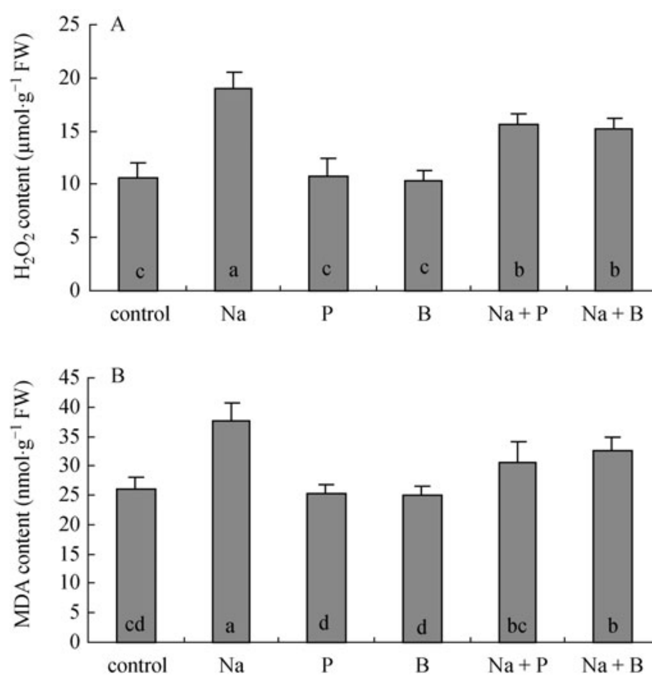


**Fig. 6** Changes in $H_2O_2$ concentration (A) and lipid peroxidation (represented by MDA) (B) level in mung bean seedlings induced by proline and betaine under salt stress conditions
Note: Na, P, B, Na + P and Na + B indicates 200 mmol·$L^{-1}$ NaCl, 5 mmol·$L^{-1}$ proline, 5 mmol·$L^{-1}$ betaine, 200 mmol·$L^{-1}$ NaCl + 5 mmol·$L^{-1}$ proline and 200 mmol·$L^{-1}$ NaCl + 5 mmol·$L^{-1}$ betaine treatments, respectively. Mean (±SD) is calculated from three independent experiments. Bars with different letters are significantly different at $P < 0.05$.

AsA influences many enzyme activities and minimizes the damage caused by oxidative process through synergic function with other antioxidants (Asada, 1999). Results obtained in our study revealed that salt stress significantly decreased AsA content (Fig. 2), although APX activity remained unchanged. Sharp decrease in AsA content is probably due to direct scavenging of ROS, since AsA is believed to be the first line of defense against oxidative stress. Decrease in AsA content in salt-sensitive cultivars and AsA deficient mutant was also reported (Hernandez et al., 2000; Shalata et al., 2001; Mittova et al., 2003a; Huang et al., 2005). The biosynthetic capacity of AsA was impaired under severe stress conditions because the AsA pool was generally determined by both of its rates of regeneration and synthesis (Song et al., 2005). It was also reported that regeneration of AsA under salinity was insufficient or that AsA synthesis was lower than AsA catabolism (Shalata et al., 2001). Importantly, proline- or

betaine-pretreated salt-stressed seedlings maintained higher AsA content as compared with the seedlings subjected to salt stress without pre-treatment indicating their role in AsA regeneration. However, we cannot exclude the influence of proline or betaine on biosynthetic capacity of AsA under salt stress conditions.

Like AsA, GSH is one of the major non-protein thiols and crucial metabolites found abundantly in all cell compartments of plants. The chemical reactivity and high water solubility of the thiol group of GSH make it particularly suitable to serve a broad range of biochemical functions to protect plants against oxidative stress. Its pivotal role as an antioxidant derives from the fact that GSH participates in the AsA-GSH cycle as the reducing agent of DHAR, while it also possesses the ability to protect the integrity of the cellular plasma membrane by maintaining α-tocopherol and zeaxanthine in the reduced state as well as protecting proteins from denaturation caused by oxidation of protein thiol groups (Paradiso et al., 2008). In addition, GSH exerts its antioxidant ability through the direct ROS scavenging as well as by acting as the substrate of GPX, GST and Gly I enzymes that participate in removal of ROS, MG and endogenous toxic compounds. The property of GSH is of great biologic importance since it allows fine-tuning of the cellular redox environment under normal conditions and upon onset of stress. GSH level is increased under stressful conditions, and such an increase gives the ability to cells to counteract the oxidation of GSH and provokes alteration of gene expression of different pathways directly or through interplay with regulatory protein and/or transcription factors and orchestrated activation of genes encoding enzymes related to GSH metabolism (May and Leaver 1993; Secenji et al., 2010). In our study, salt stress caused a sharp increase in GSH content (Fig. 3A). Increases in GSH content in response to salt stress was also reported in mung bean seedlings (Sumithra et al., 2006; Hossain and Fujita, 2010). This can be explained based on the increase in GR activity as well as a higher rate of GSH biosynthesis (Mittova et al., 2003b). Conversion of DHA to AsA by DHAR requires extensive utilization of GSH (De Gara et al., 2000). However, in our experiment under salt stress, the significant increases in GSSG content were observed, although DHAR activity decreased (Fig. 3B and 4C). The formation of GSSG in salt-treated seedlings might be due to the reaction of GSH with oxyradicals generated due to oxidative stress or due to enhancement of GPX and GST activities that decompose $H_2O_2$ and organic hydroperoxide or insufficient increase of GR activity (Shalata et al., 2001; Aravind and Prasad, 2005; Hossain and Fujita, 2010). However, both proline- and betaine-pretreated salt-stressed seedlings maintained a higher GSH and GSH/GSSG ratio as compared to the untreated control and seedlings subjected to salt stress without pre-treatment (Fig. 3A, C). Therefore, both proline and betaine play a crucial role in maintaining higher GSH level either through efficient functioning of GR or by modulating higher GSH synthesis (Kocsy et al., 2005; Xu et al., 2009; Hossain et al., 2010; Hossain and Fujita, 2010). However, GSH accumulation is not the only factor for stress tolerance (Yu et al., 2002).

APX is a primary enzyme of the AsA-GSH cycle that suppresses the accumulation of $H_2O_2$ in most cellular compartments by catalyzing the conversion of $H_2O_2$ to water. APX has a high affinity for $H_2O_2$ than CAT and POD, and it may have a more crucial role in the management of ROS stress or may be responsible for the fine modulation of ROS signaling. In the present study, upon imposition of salt stress, APX activity remained at a control level (Fig. 4A). Our result was consistent with the results of Mittova et al. (2003a). However, proline- and betaine-pretreated salt-stressed seedlings had higher APX activities. Confirming our results, Demiral and Türkan (2004), Hoque et al. (2007) and Ben Ahmed et al. (2010) also observed proline- and betaine-induced APX activity under salt stress conditions. These results suggest that both exogenous proline and betaine could contribute to detoxifying $H_2O_2$ by enhancing APX activity under salt stress and a strict control of $H_2O_2$ level for intracellular signaling. In vivo glycinebetaine synthesizing transgenic tobacco plants under salt stress conditions also showed a higher APX activity (Ahmad et al., 2010b).

The activities of MDHAR and DHAR are crucial to regenerate AsA level and its redox state under oxidative stress condition (Eltayeb et al., 2006, 2007; Wang et al., 2010). Salt stress caused a sharp decrease in MDHAR and DHAR activities which was accompanied by a sharp decrease of AsA content (Fig. 2 and 4B, C). Theses results suggested that the turnover rate of ascorbate oxidation and reduction via AsA-GSH cycle was not efficient under salt stress conditions. Decrease in MDHAR and DHAR activities was also reported in salt-sensitive tomato and pea (Hernández et al., 2000; Shalata et al., 2001; Mittova et al., 2003a). However, proline- and betaine-pretreated salt-stressed seedlings under severe salt stress showed a significant increase in DHAR activity (Fig. 4C) accompanied by an increased level of AsA as compared to the seedlings treated with salt alone (Fig. 2) which indicates proline and betaine might play an important role in AsA recycling. Similar increase in DHAR and MDHAR activity by exogenous and proline and betaine was also reported (Hoque et al., 2007; Hossain et al., 2010).

GR plays a crucial role against oxidative stress by maintaining the glutathione in its reduced state, which is necessary for regeneration of ascorbate into its reduced form, thus ensuring smooth operation of the AsA-GSH cycle. In most of the biologic functions, GSH is oxidized to GSSG, which should be converted back to GSH in plant cells to perform normal physiologic functions. Hence, rapid recycling of GSH is more essential than synthesis of GSH under salt stress conditions. GR is responsible for recycling of GSSG to GSH and controls three important redox couples. The increase of GR activity increased the

ratio of $NADP^+$/NADPH and ensured availability of $NADP^+$ to accept electrons from the photosynthetic electron transport chain, thus reducing the formation of $O_2\cdot^-$ which reduced the facilitation of metal-catalyzed formation of the ·OH through the Haber-Weiss reaction (Sudhakar et al. 2001). It was observed that stress-tolerant plants tended to have high activities of GR (Mittova et al., 2003b; Sekmen et al., 2007; Aghaei et al., 2009). Additionally, overexpression of GR increased antioxidant activity and improved tolerance to oxidative stress (Noctor et al., 1998; Potters et al., 2004). Our results showed a slight increase in GR activity under salt stress conditions. But the increase in GR activity is not high enough to protect the overaccumulation of GSSG which was attributed to significant decrease in the GSH/GSSG ratio. However, proline and betaine pre-treated salt-stressed seedlings had higher GR activity (Fig. 4D). These results agree well with those of Demiral and Türkan (2004), Kumar and Yadav (2009) and Hoque et al. (2007) who found that exogenous betaine and proline increased the GR activity under salinity. Increased GR activity by proline and betaine under salt stress conditions contributes to the maintenance of higher GSH/GSSG ratio and GSH level, which is used by DHAR and other GSH-dependent enzymes involved in the antioxidant defense and glyoxalase systems (Hossain and Fujita, 2010; Hossain et al., 2010).

CAT is one of the principal $H_2O_2$ scavenging enzymes which can directly dismutase $H_2O_2$ and is indispensable for ROS detoxification during stress. Increased CAT activity is supposed to be an adaptive trait possibly helping to overcome the damage to tissue metabolism by reducing toxic levels of $H_2O_2$ (Sekmen et al., 2007). Our results indicated a sharp decline in CAT activity (Fig. 4G) which suggests that CAT appears not to be an effective scavenger of $H_2O_2$ in *Vigna radiata*, and the increase in $H_2O_2$ is possibly due to the decreased activity of CAT (Parida and Jha, 2010). A decline in CAT activity due to salt stress was also reported in different crop species (Sekmen et al., 2007; Aghaei et al., 2009; Hossain et al., 2010; Lin et al., 2010) which agrees with our results. Importantly, proline- or betaine-pretreated salt-stressed seedlings maintained a higher CAT activity as compared to the seedlings subjected to salt stress without pre-treatment. This is in full accordance with the recent reports (Khedr et al., 2003; Demiral and Türkan, 2004; Hoque et al., 2007; Ben Ahmed et al., 2010; Newaz and Ashraf, 2010) which showed that the activity of CAT increased in the presence of exogenous proline or betaine. Similar protective effects of proline or betaine were also observed in our previous studies with mung bean under Cd-stress. In vivo glycinebetaine synthesizing transgenic tobacco plants under salt stress conditions also showed higher CAT activity (Ahmad et al., 2010b).

GSTs are well-characterized detoxification enzymes with potential antioxidant properties involved in plant stress tolerance (Fujita and Hossain, 2003). GSTs are best known for the detoxification of xenobiotics, but they can also act as antioxidants by tagging oxidative degradation products for removal. GSTs act by catalyzing the conjugation of GSH with electrophilic, often hydrophobic toxic compounds to form derivates that can be secreted from the cells, sequestered in the vacuole, or catabolized (Dalton et al., 2009). In addition to GST, GPX catalyzes the GSH-dependent reduction of $H_2O_2$ and organic peroxides, including lipid peroxides, to water or alcohols, thus helping to protect the plant cells against the oxidative damage (Eshdat et al., 1997). The upregulation of GST protein in shoot tissues of *A. lagopoides* subjected to high level of salt stress showed that this detoxifying enzyme had a major role in the tolerance of high levels of salt stress (Sobhanian et al., 2010). The major function of GPXs in plants appears to be the scavenging of phospholipid hydroperoxides and thereby the protection of cell membranes from peroxidative damage (Gueta-Dahan et al., 1997). Overexpression of GST or GPX gene induces salt and drought tolerance in tobacco and *Arabidopsis* (Yoshimura et al., 2004; Ji et al., 2010). Our results showed a sharp increase in GST and GPX activity under salt stress (Fig. 4E, F). Increase in GST and GPX activities in response to salt stress was also reported in different plant species (Mittova et al., 2003b; Halusková et al., 2009). Therefore, it is possible that in the case of strong stress conditions, where basal antioxidant mechanisms are exhausted, the more effective responses are activated including GST/GPX or GPX. In the present investigation, the increases of GST and GPX were not high enough to protect cells from salt-induced oxidative damage. However, the fact that exogenous proline and betaine increased GST and GPX activities and suppressed the production of $H_2O_2$ and MDA level indicates that both of them are able to reduce salt-induced oxidative damage by increasing GST and GPX activities. Similar induction of GST and GPX activity by exogenous proline and betaine was also reported (Hoque et al., 2008; Kumar and Yadav, 2009; Hossain and Fujita, 2010).

The glyoxalase pathway is a cyclic metabolic pathway which removes toxic 2-oxoaldehydes such as MG by converting them to the corresponding non-toxic 2-hydroxycarbozxylic acid like D-lectate. The toxic compound MG builds up under salt stress conditions, and its detoxification might be a strategy for conferring tolerance against various abiotic stresses (Hoque et al., 2008; Hossain et al., 2009; Hossain and Fujita, 2009; Kumar and Yadav, 2009; El-Shabrawi et al., 2010; Hossain and Fujita, 2010; Hossain et al., 2010). A high level of MG accumulation is toxic to cells, since it inhibits cell proliferation (Ray et al., 1994) and results in a number of adverse effects such as increasing the degradation of proteins and inactivating the antioxidant defense system (Martins et al., 2001; Wu and Juurlink, 2002). Apart from MG, pathway intermediate SLG (substrate for Gly II) has

also been found to be cytotoxic at high concentrations in that it inhibits DNA synthesis (Thornalley, 1996). Besides detoxification of MG, the glyoxalase system could also play a role in providing tolerance under stress by recycling GSH that would be "trapped" spontaneously by MG to form hemithioacetal, thereby maintaining glutathione homeostasis (Creighton et al., 1988). Higher Gly I and Gly II activities might protect plants against MG that is formed during abiotic stresses (Veena et al., 1999; Jain et al., 2002; Saxena et al., 2005; Hossain et al., 2009; Hossain and Fujita, 2009). The engineering of glyoxalase pathway enzymes in tobacco, *Brassica* and *Arabidopsis* inhibits an increase in MG level under salt and heavy metal stress and confers tolerance by increasing the GSH-based detoxification system and decreasing lipid peroxidation (Veena et al., 1999; Singla-Pareek et al., 2003, 2006, 2008; Yadav et al., 2005a, 2005b). In the present study, we observed a significant decrease in Gly I activity while the Gly II activity increased (Fig. 5A, B). Decrease in Gly I activity in response to salt stress was also reported (El-Shabrawi et al., 2010), which might be due to inactivation or proteolytic degradation of enzymes. In contrast, an increase in Gly II activity in response to salt stress was corroborated well with our previous study (Hossain et al., 2009; Hossain and Fujita, 2009, 2010). In the light of the results obtained in the present study and supporting evidences from literature, we infer that the detoxification of MG via the glyoxalase system is not sufficient under salt stress conditions. In contrast, betaine and proline pretreatment could partially alleviate the salinity-induced oxidative damage by maintaining higher Gly I and II activities, suggesting that both of them were able to enhance GSH regeneration via the glyoxalase system. As GSH is recycled by the glyoxalase system, it was assumed that an increased level of MG was detoxified efficiently in the proline- or betaine-pretreated seedlings, thus creating the possibility of the upregulation of GSH and GSH/GSSG ratio via the glyoxalase system under salt stress in addition with the AsA-GSH pathway.

ROS is able to initiate a chain reaction on polyunsaturated fatty acids that lead to the lipid peroxidation, and the peroxidation of lipid membrane represented by MDA is both a reflection and measure of stress-induced damage at the cellular level. $H_2O_2$ at low concentrations is suggested to be involved in the signaling of many processes; however, excessive accumulation of $H_2O_2$ can lead to oxidative stress. It is thus the interest of plant to regulate the levels of $H_2O_2$ under salt stress rather than to remove it completely. In the current experiment, salt stress significantly increased MDA and $H_2O_2$ level (Fig. 6A, B). These results corroborate previous reports where salinity causes sharp increase in $H_2O_2$ and MDA levels in mungbean seedlings (Sumithra et al., 2006; Hossain and Fujita, 2010; Saha et al., 2010). An increase in $H_2O_2$ and MDA contents upon salt stress has also been reported in different plant species (Pérez-López et al., 2009; Khan et al., 2010), and the control of the levels of $H_2O_2$ and MDA is thought to be a mechanism by which plants tolerate the stress (Sekmen et al., 2007). A sharp increase in the level of $H_2O_2$ and MDA in response to salt stress was due to insufficient antioxidant defense and MG detoxification system. However, proline or betaine pretreatment significantly decreased the MDA and $H_2O_2$ level as compared to the seedlings subjected to salt stress without pre-treatment. In addition, relatively lower $H_2O_2$ levels, as observed during salt stress in proline- and betaine-pretreated seedlings, might play a secondary role in stress signaling network by inducing defense pathways (Dat et al., 2000).

This study provided an insight into the role of exogenous proline and betaine in regulating biochemical response to NaCl toxicity. Our results indicated that exogenous application of betaine or proline was an effective way to improve (short-term) salt tolerance. The enhanced tolerance could partially attribute to the increased non-enzymatic antioxidant and enzymatic activities of antioxidant and glyoxalase systems. Based on the above results, together with our previous results (Hossain and Fujita, 2010; Hossain et al., 2010) and available results found in the literature (Hoque et al., 2008; Kumar and Yadav, 2009; El-Shabrawi et al., 2010), we, therefore, concluded that antioxidant defense system and glyoxalase system are co-regulated/correlated in controlling ROS and MG levels and the damage caused by them under stressful conditions. Nevertheless, more research is needed to answer the following questions: What is the effective concentration of proline and betaine over a range of mung bean cultivars grown under salt stress conditions and what is the proper stage of plant developmental for exogenous betaine or proline application? Additionally, further studies are required to elucidate the molecular mechanism and signaling pathways underlying the role of proline and betaine in salinity tolerance of plants.

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