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Roles of exogenous glutathione in antioxidant defense system and methylglyoxal detoxification during salt stress in mung bean

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Abstract

The protective roles of glutathione (GSH) applied on salt stress-affected mung bean (*Vigna radiata* L.) seedlings were studied. The salt stress (200 mM NaCl) significantly increased the malondialdehyde (MDA), methylglyoxal (MG), H_2O_2, and proline (Pro) content, $O_2^{\bullet-}$ generation rate, and lipoxygenase (LOX) activity and reduced the leaf relative water content (RWC) and chlorophyll (Chl) content. The salt stress also significantly reduced the ascorbate (AsA) content, increased the endogenous GSH and glutathione disulfide (GSSG) content, and reduced the GSH/GSSG ratio. The activities of mono-dehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and catalase (CAT) decreased; and the activities of ascorbate peroxidase (APX), glutathione reductase (GR), superoxide dismutase (SOD), glutathione *S*-transferase (GST), and glutathione peroxidase (GPX) increased under the salt stress. The activities of glyoxalase I (Gly I) and glyoxalase II (Gly II) decreased under the salt stress (except the Gly II activity at 48 h). Mung bean seedlings which were treated with NaCl together with GSH showed an improved AsA and GSH content, GSH/GSSG ratio, higher activities of APX (only at 24 h), MDHAR, DHAR, GR, SOD (only at 24 h), CAT, GPX (only at 48 h), GST (only at 24 h), Gly I and Gly II under the salt stress compared with those treated with NaCl alone. The improved antioxidant and glyoxalase systems by GSH application decreased the MDA, H_2O_2, and MG content, $O_2^{\bullet-}$ generation rate, as well as increased the leaf RWC and Chl content. Thus, exogenous GSH improved the response of the mung bean seedlings to the salt stress.

Additional key words: abiotic stress tolerance, ascorbate-glutathione cycle, glyoxalase enzymes, osmotic stress, reactive oxygen species.

Introduction

Salinity is one of the most severe constraints for agriculture worldwide, especially in arid and semi-arid regions. Salt stress adversely affects physiology and biochemistry of plants primarily by creating osmotic stress, ionic imbalance, and toxicity (Hajlaoui *et al.* 2009). Reactive oxygen species (ROS), which include superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and singlet oxygen ($^1O_2$), are generated in response to salt stress. ROS can cause peroxidation of lipids and oxidation of amino acids. Protein carbonylation

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Abbreviations: AO - ascorbate oxidase; APX - ascorbate peroxidase; AsA – ascorbate; BSA - bovine serum albumin; CAT - catalase; CDNB - 1-chloro-2,4-dinitrobenzene; Chl - chlorophyll; DHA - dehydroascorbate; DHAR - dehydroascorbate reductase; DTNB - 5,5´-dithio-bis-(2-nitrobenzoic) acid; EDTA - ethylenediaminetetraacetic acid; Gly - glyoxalase; GR - glutathione reductase; GSH - reduced glutathione; GSSG - oxidized glutathione; GPX - glutathione peroxidase; GST - glutathione *S*-transferase; LOX - lipoxygenase; MDA - malondialdehyde; MDHA - monodehydroascorbate; MDHAR - monodehydroascorbate reductase; MG - methylglyoxal; NADPH - nicotinamide adenosine dinucleotide phosphate; NTB - 2-nitro-5-thiobenzoic acid; PEG - polyethylene glycol; Pro - proline; ROS - reactive oxygen species; RWC - relative water content; SLG - *S*-D-lactoyl-glutathione; SOD - superoxide dismutase; TBA - thiobarbituric acid; TCA - trichloroacetic acid.
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is a widely used marker of protein oxidation (Job *et al.* 2005, Moller *et al.* 2007) which may occur due to direct oxidation of amino acid side chains (Shringarpure and Davies 2002). ROS can also cause oxidation of nucleic acids, substantial cellular damage, and even programmed cell death (Gill and Tuteja 2010, Hasanuzzaman *et al.* 2012, 2013). Methylglyoxal (MG), a toxic compound that develops through the glycolysis pathway or through some other biochemical reactions, can be over-produced many fold under different abiotic stresses including salt stress (Yadav *et al.* 2005). Methylglyoxal can also cause oxidative damage to a cell and its ultrastructural components, and can even cause DNA damage and mutation (Wang *et al.* 2009). As both ROS and MG are responsible for damaging plant cells under salt stress, elimination of these toxic compounds is considered a strategy for developing salt stress tolerance. In plants, ROS are scavenged by the antioxidant defense system and MG is detoxified by the glyoxalase system. Therefore, an upregulation of these systems under stress conditions is considered vital in enhancing stress tolerance. The antioxidant defense system includes non-enzymatic antioxidants like ascorbate (AsA), glutathione (GSH), carotenoids, flavanones, and anthocyanins. The enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), mono-dehydro-ascorbate reductase (MDHAR), dehydro-ascorbate reductase (DHAR), glutathione reductase (GR), glutathione peroxidase (GPX), and glutathione *S*-transferase (GST) (Hasanuzzaman *et al.* 2012). The glyoxalase enzymes, glyoxalase I (Gly I) and glyoxalase II (Gly II), comprise the glyoxalase system which detoxifies MG (Yadav *et al.* 2005, 2008).

Glutathione (GSH, γ-glutamylcysteinylglycine) is a tripeptide, a major source of non-protein thiols in most plant cells, and it is widely distributed in different cellular organelles (Bergmann and Rennenberg 1993). GSH can be taken up by roots, loaded into the xylem, and transported into leaves *via* the transpiration stream. GSH can be synthesized within plants and be transported *via* the phloem (Rennenberg *et al.* 1979, Schneider *et al.* 1994, Herschbach *et al.* 2000). The content of GSH in different cells and tissues is the result of equilibrium between its synthesis, degradation, exploitation, and transportation. The exchange and transport of GSH between the cytosol, apoplast, and phloem requires specific plasma membrane transport systems driven by ATP hydrolysis. It imports oxidised glutathione (GSSG) and glutathione conjugates (GS-X) rather than GSH (Foyer *et al.* 2001 and references therein). Identifying the plasma-membrane transport system and a potential for multidrug-resistance-associated proteins (MRPs) have opened the door to assess the transport of GSH or GS-X compounds into the vacuole and apoplast in regulating GSH homeostasis, and a further detailed study is needed to explore their roles. GSH plays an important role in responding to environmental stresses. It directly acts as antioxidant maintaining homeostasis among different components of the antioxidant system. It has roles in detoxifying xenobiotics (Marrs 1996) and chelating heavy metals (Grill *et al.* 1989). In the glyoxalase system, MG is detoxified by the activities of the Gly I and Gly II enzymes where GSH is utilized as substrate (Yadav *et al.* 2008). Some other important physiological functions of GSH are regulating inter-organ sulfur allocation (Lappartient and Touraine 1996), maintaining cellular redox regulation, stress signal transduction, and gene expression (Baier and Dietz 1997).

There has been some research concerning the role of exogenous GSH (Kattab 2007, Chen *et al.* 2012, Nahar *et al*. 2015a,b). However, GSH-induced modulation of the antioxidant defense and MG detoxification systems has not been studied intensively yet. Therefore, the present study investigates the protective role of applied GSH on salt stress-affected mung bean seedlings with a special consideration of the antioxidant and glyoxalase systems.

Materials and methods

Mung bean (*Vigna radiata* cv. Binamoog-1) seeds were sown in Petri dishes with six layers of filter paper moistened with 10 cm^3 of distilled water. The Petri dishes were placed in the dark for 48 h for germination. Water was sprayed according to need. Germinated seedlings were transferred and grown in a growth chamber under an irradiance of 350 μmol(photon) m^{-2} s^{-1}, a temperature of 25 ± 2 °C, and a relative humidity of 65 - 70 %. One Petri dish contained 30 seedlings and was considered as one set of seedlings. *Hyponex* (Tokyo, Japan) solution was diluted 10 000-fold and applied as nutrient solution. Salt stress (200 mM NaCl), 1 mM GSH, and NaCl + GSH were applied to the nutrient solution of 6-d-old seedlings. The control plants were grown in the *Hyponex* solution only. The experiment was conducted using a completely randomized design with three replications. Data were taken after 24 and 48 h.

Leaf relative water content (RWC) was measured according to Barrs and Weatherley (1962). Fresh mass (FM), mass of fully water saturated leaves (SM), and dry mass (DM) were determined, and leaf RWC was calculated using the following formula:

$$RWC\ [\%] = [(FM - DM)/(SM - DM)] \times 100.$$

For measurement of chlorophyll (Chl) content, leaf tissue (0.5 g) was homogenized with 10 cm^3 (v/v) of 80 % acetone in a pre-chilled mortar and pestle and then centrifuged at 5 000 *g*. The absorbance of the supernatant was measured with a UV-visible spectrophotometer (Shimazdu, Japan) at 663 and 645 nm; the Chl content was calculated according to Arnon (1949).

Proline (Pro) was appraised according to Bates *et al.* (1973). Leaves were homogenized in 3 % (m/v) sulfosalicylic acid and centrifuged at 11 500 *g* for 12 min. The supernatant (1 cm^3) was placed in a centrifuge tube

with 1 cm^3 of acid ninhydrin, 1 cm^3 of glacial acetic acid, and 1 cm^3 of 6 M phosphoric acid and mixed thoroughly. After incubating the mixture at 100 °C for 1 h and cooling to 4 °C, the absorbance was read at 520 nm.

Lipid peroxidation was estimated by measuring the content of malondialdehyde (MDA, a decomposition product of peroxidized polyunsaturated fatty acids) following the method of Heath and Packer (1968) with slight modifications (Hasanuzzaman *et al.* 2011a). Leaf samples (0.5 g) were homogenized in 3 cm^3 of 5 % (m/v) trichloroacetic acid (TCA) and centrifuged at 11 500 *g* for 10 min. The supernatant (1 cm^3) was mixed with 4 cm^3 of a thiobarbituric acid (TBA) reagent [0.5 % (m/v) of TBA in 20 % TCA]. The reaction mixture was boiled at 95 °C for 30 min in a water bath and then quickly cooled in ice. The absorbance of the supernatant was measured at 532 nm. The content of MDA was calculated using a coefficient of absorbance (ε) of 155 mM^{-1} cm^{-1}.

H_2O_2 content was assayed according to Yu *et al.* (2003). Leaves were extracted in a potassium phosphate buffer (pH 6.5), centrifuged at 11 500 *g*, then treated with a mixture of $TiCl_4$ (0.075 %, m/v) in 20 % (v/v) H_2SO_4, and the absorbance of mixture was measured at 410 nm.

The rate of $O_2^{\bullet-}$ generation was determined following Yang *et al.* (2010) with some modifications. Fresh leaves (0.3 g) were homogenized in 3 cm^3 of a65 mM potassium phosphate buffer (pH 7.8) and centrifuged at 5 000 *g* for 10 min. The supernatant was mixed with the extraction buffer and 10 mM hydroxylamine hydrochloride (at the ratio of 10.7:9.1:1) and incubated at 25 °C for 20 min. The solution was then mixed with 17 mM sulfanilamide and 7 mM naphthylamine (at the ratio of 1:1:1) and incubated again at 25 °C for 20 min. The absorbance was measured at 530 nm. The rate of $O_2^{\bullet-}$ generation was calculated using a standard curve of $NaNO_2$ (Elstner and Heupel 1976).

For determination of methylglyoxal (MG) content, leaves (0.5 g) were homogenized in 5 % (m/v) perchloric acid and centrifuged at 11 000 *g* and 4 ºC for 10 min. The supernatant was decolorized by adding charcoal and neutralized using a saturated solution of potassium carbonate at room temperature. The neutralized supernatant was used to estimate MG by adding sodium dihydrogen phosphate and *N*-acetyl-L-cysteine (at the ratio of 25:24:1) to a final volume of 1 cm^3. Formation of the product *N*-*α*-acetyl-*S*-(1-hydroxy-2-oxo-prop-1-yl) cysteine was recorded after 10 min at 288 nm according to Wild *et al.* (2012). The MG content within the plant sample was calculated using a standard curve of known concentrations of MG.

For determination of AsA and GSH content, leaves (0.5 g) were homogenized in 5 % (m/v) meta-phosphoric acid containing 1 mM Na_2EDTA, centrifuged at 11 500 *g* and 4 °C for 15 min, and the supernatant was collected to analyze AsA and GSH. The AsA content was determined following the method of Huang *et al.* (2005) with some modifications as described by Hasanuzzaman *et al.* (2011a). The supernatant was neutralized with 0.5 M K-phosphate buffer (pH 7.0). The reduced ascorbate was assayed spectrophotometrically at 265 nm in 100 mM 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 GSH and GSSG pool was assayed according to previously described methods (Yu *et al.* 2003) with modifications as described by Paradiso *et al.* (2008) and Hasanuzzaman *et al.* (2011a) utilizing 0.4 cm^3 of aliquots of supernatant neutralized with 0.6 cm^3 of 0.5 M K-phosphate buffer (pH 7.5). Based on enzymatic recycling, glutathione is oxidized by 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB) and reduced by NADPH in the presence of GR, and glutathione content is evaluated by the rate of absorption changes at 412 nm of 2-nitro-5-thiobenzoic acid (NTB) generated from the reduction of DTNB. GSSG was determined after removal of GSH by 2-vinylpyridine derivatization. Standard curves with known concentrations of GSH and GSSG were used. The protein content of each sample was determined following the method of Bradford (1976) using bovine serum albumine (BSA) as standard.

For enzyme extraction, leaf tissue (0.5 g) was homogenized in 1 cm^3 of a 50 mM ice-cold potassium phosphate buffer (pH 7.0) containing 100 mM KCl, 1 mM AsA, 5 mM β-mercaptoethanol, and 10 % (v/v) glycerol. The homogenate were centrifuged at 11 500 *g* for 15 min and the supernatant was used to determine enzyme activity. All procedures were performed at 0 - 4 °C.

The APX (EC: 1.11.1.11) activity was measured according to Nakano and Asada (1981) in a mixture of a 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM AsA, 0.1 mM H_2O_2, 0.1 mM EDTA, the enzyme extract (a final volume of 700 mm^3). The reaction was started by adding H_2O_2. The absorbance was measured at 290 nm for 1 min and the activity was determined using ε = 2.8 mM^{-1} cm^{-1}.

The MDHAR (EC: 1.6.5.4) activity was assayed according to Hossain *et al.* (1984) in a reaction mixture containing a 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADPH, 2.5 mM AsA, 0.5 units of ascorbate oxidase (AO), and the enzyme extract to a final volume of 700 mm^3. The reaction was started by adding AO. The absorbance was taken at 340 nm; the activity was calculated from a change in the absorbance after 1 min (ε = 6.2 mM^{-1} cm^{-1}).

The DHAR (EC: 1.8.5.1) activity was measured according to Nakano and Asada (1981) in a solution containing a 50 mM potassium phosphate buffer (pH 7.0), 2.5 mM GSH, 0.1 mM EDTA, and the solution of dehydro-ascorbic acid (the solution was prepared by dissolving 0.0010 g dehydroascorbic acid in 4.2 cm^3 of distilled water). The activity was calculated from a change in the absorbance at 265 nm after 1 min (ε = 14 mM^{-1} cm^{-1}).

The GR (EC: 1.6.4.2) activity was measured according to Hasanuzzaman *et al.* (2011b). An assay buffer was prepared by adding 20 mm^3 of 1 mM EDTA to 2 cm^3 of a 0.5 M potassium phosphate buffer, pH 7.0, to give its final concentration of 0.1 M. The GSSG was prepared by adding 0.00306 g of GSSG to 2 cm^3 of a

0.5 M potassium phosphate buffer. The NADPH (1 mM) was prepared by adding 36 mm^3 of NADPH from a stock to 2 cm^3 of a 0.5 M potassium phosphate buffer. The assay procedure was started by mixing distilled water, the buffer solution, the enzyme sample, and the NADPH solution (a final volume of 1 cm^3). The reaction was initiated with GSSG. A decrease in the absorbance at 340 nm was recorded for 1 min, and the activity was calculated ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$).

The SOD (EC 1.15.1.1) activity was assayed using a xanthine-xanthine oxidase system (El-Shabrawi *et al.* 2010). A potassium phosphate buffer (50 mM), 2.24 mM nitroblue tetrazoliam chloride (NBT), catalase (0.1 units), xanthine oxidase (0.1 units), xanthine (2.36 mM), and the enzyme extract were in a reaction mixture of a final volume 1 cm^3. Catalase was added to avoid H_2O_2-mediated inactivation of CuZn-SOD. A change in the absorbance was read at 560 nm. The SOD activity was expressed as units (the amount of the enzyme needed to inhibit NTB reduction by 50 %).

The CAT (EC: 1.11.1.6) activity was assayed according to Hasanuzzaman *et al.* (2011a) by mixing distilled water, a 50 mM potassium phosphate buffer (pH 7.0), the enzyme extract, and H_2O_2 (15 mM) to a final volume of 620 mm^3. A decrease in the absorbance (by decomposition of H_2O_2) at 240 nm was recorded for 1 min. The reaction was initiated with the enzyme extract; the activity was calculated using $\varepsilon = 39.4\ M^{-1}\ cm^{-1}$.

The GST (EC: 2.5.1.18) activity was assayed according to Hossain *et al.* (2006) in a reaction mixture containing a 100 mM Tris-HCl buffer (pH 6.5), 1.5 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), and the enzyme extract (a final volume of 700 mm^3). The reaction was initiated by CDNB addition; the increase in the absorbance was measured at 340 nm for 1 min. The activity was calculated using $\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$.

The GPX (EC: 1.11.1.9) activity was measured as described by Elia *et al.* (2003) with a slight modification as described by Hasanuzzaman *et al*. (2011a). A reaction mixture consisted of a 100 mM potassium phosphate buffer (pH 7.0), 1 mM Na_2EDTA, 1 mM NaN_3, 0.12 mM NADPH, 2 mM GSH, 1 unit of GR, 0.6 mM H_2O_2 (as substrate), and 20 mm^3 of the enzyme extract. The oxidation of NADPH was recorded at 340 nm for 1 min. The activity was calculated using $\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$.

Glyoxalase I (EC: 4.4.1.5) was measured according to Hasanuzzaman *et al.* (2011a): GSH (100 mM) and MG (35 mM) solutions were prepared. An assay buffer was prepared by combining a 0.1 M sodium phosphate buffer and 16 mM $MgSO_4$. The Gly I activity was assayed by adding the GSH solution, the assay buffer, distilled water, and the enzyme extract to a cuvette of a spectrophotometer (a final volume of 700 mm^3). The reaction was started by adding MG; an increase in the absorbance was recorded at 240 nm for 1 min. The activity was calculated using $\varepsilon = 3.37\ mM^{-1}\ cm^{-1}$.

Glyoxalase II (EC: 3.1.2.6) was determined as described by Principato *et al.* (1987): A buffer was prepared by adding a 0.5 M Tris-HCl buffer (pH 7.2), distilled water, and DTNB. An S-D-lactoylglutathione (SLG) solution was prepared by adding 0.002 g of SLG to 1.04 cm^3 of distilled water and mixing. The assay procedure was started by adding the buffer, distilled water, the enzyme extract, and SLG as substrate (to a final volume of 1 cm^3). The formation of GSH at 412 nm was monitored for 1 min. The activity was calculated using $\varepsilon = 13.6\ mM^{-1}\ cm^{-1}$.

The LOX (EC 1.13.11.12) activity was estimated according to the method of Doderer *et al.* (1992) by monitoring the increase in absorbance at 234 nm using linoleic acid as substrate. A 17.5 mm^3 of linoleic acid (a final concentration of 8 mM) was added to 2.5 cm^3 of distilled water containing 0.025 cm^3 of *Tween-20* and was stired for 30 min. Phosphate buffer (pH 6.5, 0.25 mM) was added to the solution to make a total volume of 50 cm^3. Enzyme extract (10 mm^3) was mixed with 590 mm^3 of substrate solution for spectrophotometric determination of absorbance. The activity was calculated using $\varepsilon = 25\ mM^{-1}\ cm^{-1}$).

All data obtained were subjected to analysis of variance (*ANOVA*) and mean differences were compared by the Duncan's multiple range test (DMRT) using the *XLSTAT v. 2015.1.01* software (*Addinsoft*, Paris, France). Differences at $P \leq 0.05$ were considered significant.

Results

The salt stress resulted in severe oxidative damage to the mung bean seedlings. The lipid peroxidation (the MDA content) increased by 86 and 90 %, the H_2O_2 content increased by 48 and 80 %, the $O_2^{\bullet-}$ generation rate increased by 75 and 86 %, and the LOX activity increased by 30 and 36 % after 24 and 48 h, respectively, compared with the control seedlings. However, the addition of GSH significantly eliminated the NaCl-induced oxidative stress as indicated by a reduced MDA and H_2O_2 content, $O_2^{\bullet-}$ generation rate, and LOX activity compared with the seedlings treated only with NaCl (Fig. 1*A-D*). The salt stress also significantly increased the Pro content (by 172 and 407 % after 24 and 48 h, respectively). Adding GSH to the NaCl-treated seedlings significantly reduced the Pro content (Table 1).

Exposure of mung bean seedlings to NaCl significantly reduced the leaf RWC (by 22 and 30 % after 24 and 48 h, respectively), compared with the control. However, when GSH was added, the RWC less decreased compared with the seedlings treated with NaCl alone (Table 1). The salt stress also decreased the Chl *a* content by 15 and 18 % and the Chl *b* content by 29 % (both after 24 and 48 h). The GSH application to the NaCl-treated seedlings improved the Chl *b* and total Chl content, but it

Table 1. The leaf relative water content (RWC), and the content of proline (Pro), chlorophyll [Chl *a*, Chl *b*, and Chl (*a*+*b*)] in mung bean seedlings as affected by 1 mM GSH and 200 mM NaCl. Means ± SE, $n = 3$. Different letters mark significantly different means at $P \leq 0.05$ (DMRT).

Parameter	Duration	Control	GSH	NaCl	NaCl+GSH
RWC [%]	24 h	90.96 ± 3.33 a	90.66 ± 2.51 a	70.78 ± 4.32 c	82.73 ± 2.78 b
	48 h	90.02 ± 2.81 a	91.69 ± .49 a	62.50 ± 4.92 d	76.52 ± 3.83 bc
Proline [μmol g^{-1}(f.m.)]	24 h	2.87 ± 0.38 cd	3.70 ± 0.30 cd	7.81 ± 0.47 b	4.95 ± 0.68 c
	48 h	2.40 ± 0.10 d	2.63 ± 0.33 d	12.16 ± 1.14 a	9.49 ± 0.87 b
Chl *a* [mg g^{-1}(f.m.)]	24 h	1.35 ± 0.04 a	1.37 ± 0.06 a	1.15 ± 0.11 bc	1.25 ± 0.17 ab
	48 h	1.31 ± 0.02 a	1.35 ± 0.03 a	1.07 ± 0.04 c	1.15 ± 0.07 bc
Chl *b* [mg g^{-1}(f.m.)]	24 h	1.10 ± 0.06 b	1.27 ± 0.05 a	0.78 ± 0.07 d	1.01 ± 0.07 bc
	48 h	1.10 ± 0.09 b	1.27 ± 0.07 a	0.79 ± 0.04 d	0.95 ± 0.02 c
Chl (*a*+*b*) [mg g^{-1}(f.m.)]	24 h	2.44 ± 0.02 b	2.64 ± 0.10 a	1.93 ± 0.14 e	2.26 ± 0.11 c
	48 h	2.41 ± 0.08 bc	2.62 ± 0.07 a	1.86 ± 0.05 e	2.09 ± 0.10 d

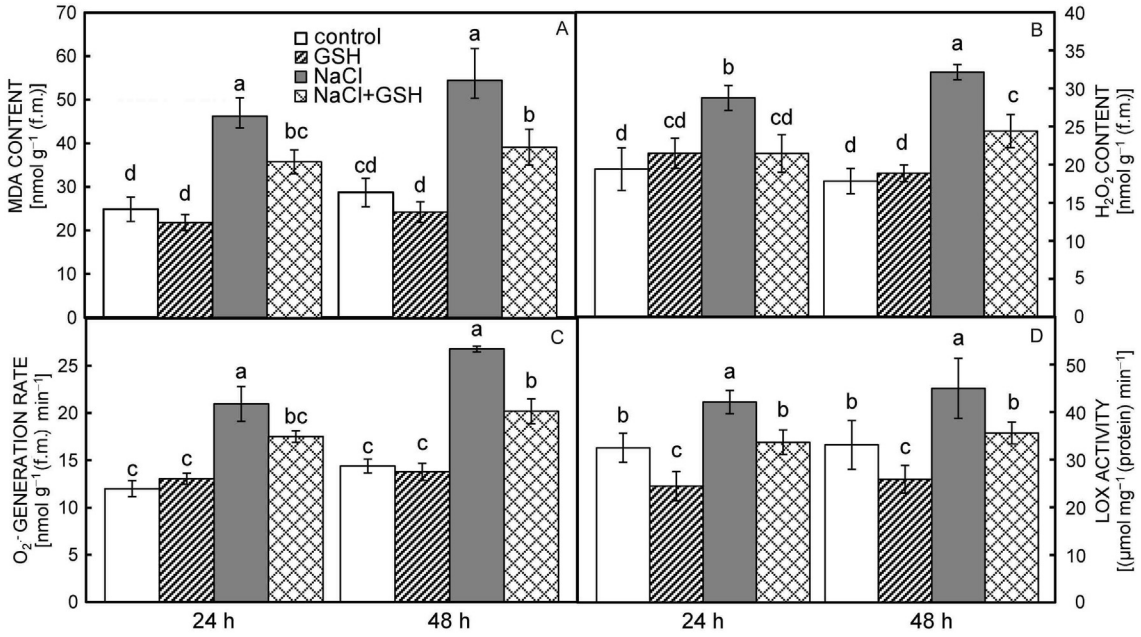

Fig. 1. The content of MDA (*A*) and H_2O_2 (*B*), the superoxide generation rate (*C*) and LOX activity (*D*) in mung bean seedlings as affected by 1 mM GSH and 200 mM NaCl. Means ± SE, $n = 3$. Different letters mark significantly different means at $P \leq 0.05$ (DMRT).

did not improve the Chl *a* content (Table 1).

The salt stress significantly decreased the AsA content of the mung bean seedlings compared with the non-stressed control seedlings. Exogenous GSH recovered the AsA content; it was 31 and 36 % higher in the GSH + NaCl treated seedlings compared with the seedlings treated with NaCl only (Fig. 2*A*).

A significant increase in an endogenous GSH content was evident under the salt stress at 24 h, but after 48 h of the stress, the GSH content increased only slightly compared with the control seedlings. The exogenous GSH together with the salt stress maintained the GSH content as high as under the salt-stress only (Fig. 2*B*). The content of GSSG also increased markedly under the salt stress. In contrast to the GSH content, the NaCl + GSH treatment significantly reduced the GSSG content (by 23 and 34 % after 24 and 48 h, respectively, compared with the NaCl treatment only; Fig. 2*C*). The GSH/GSSG ratio was slightly reduced under the NaCl stress compared with the control. However, compared to the salt stress alone, the exogenous GSH significantly increased the GSH/GSSG ratio after 24 h and slightly after 48 h (Fig. 2*D*).

The SOD activity increased under the salt stress by 16 and 22 % after 24 and 48 h, respectively. An increase in the SOD activity was observed under GSH + NaCl more after 24 h (Fig. 3*A*). The CAT activity was reduced markedly by 28 and 44 % under the NaCl stress after 24 and 48 h, respectively, compared with the non-stressed seedlings. The addition of GSH increased the CAT

activity compared with the salt-only stress (Fig. 3*B*). The enzymes of the AsA-GSH cycle (APX, MDHAR, DHAR, and GR) showed differential responses to NaCl and GSH. The salt stress for 24 h increased the activity of APX by 31 % compared with the control, whereas the 48-h stress did not significantly increase its activity. The exogenous GSH enhanced the APX activity only after 24 h of the salt stress (Fig. 3*C*). The NaCl treatment reduced the MDHAR activity by 31 % (at both 24 and 48 h) compared with the non-stressed control seedlings, and the addition of GSH led to a less reduction (Fig. 3*D*). The activity of DHAR also decreased under the salinity (by 18 and 28 % after 24 and 48 h, respectively) and was restored by adding GSH (Fig. 3*E*). The activity of GR in the seedlings increased under the salt stress at either duration, compared with the control, and adding GSH further upregulated the GR activity by 15 and 17 % at 24 and 48 h, respectively, compared with NaCl only (Fig. 3*F*). The GST activity increased significantly in the NaCl affected seedlings compared with the control. The GST activity further increased with GSH application to the salt-stressed seedlings at 24 h (Fig. 3*G*). The salt stress slightly increased the GPX activity after 24 h, and after 48 h, its activity did not change compared with the control. The exogenous GSH did not change the GPX activity in the NaCl-stressed seedlings after 24 h, but after 48 h, the GPX activity significantly increased after adding GSH (Fig. 3*H*).

The activities of Gly I and Gly II were reduced at 24 and 48 h of the salt stress compared with the control, and their activities were restored by GSH supplementation (Table 2). The salt stress greatly increased the MG content by 74 and 102 % after 24 and 48 h, respectively, compared with the control seedlings. The GSH addition reduced the MG content significantly compared with the seedlings stressed with NaCl alone (Table 2).

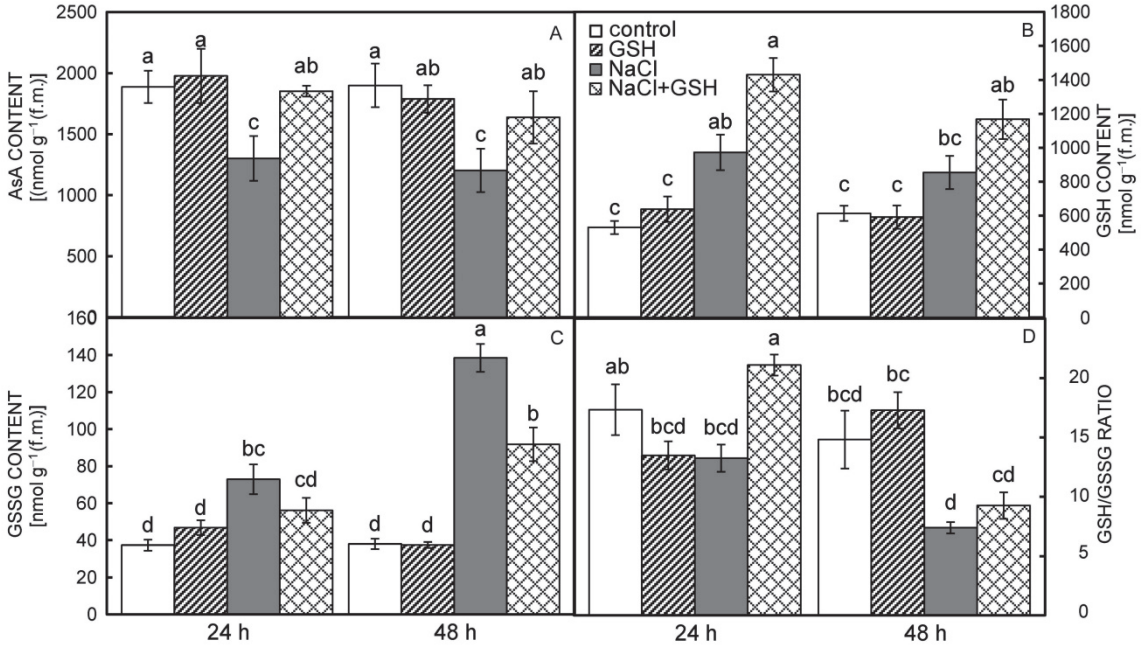

Fig. 2. The content of AsA (*A*), GSH (*B*), and GSSG (*C*), and GSH/GSSG ratio (*D*) in mung bean seedlings as affected by 1 mM GSH and 200 mM NaCl. Means ± SE, $n = 3$. Different letters mark significantly different means at $P \leq 0.05$ (DMRT).

Table 2. Activities of Gly I and Gly II and the methylglyoxal content in mung bean seedlings as affected by 1 mM GSH and 200 mM NaCl. Means ± SE, $n = 3$. Different letters mark significantly different means at $P \leq 0.05$ (DMRT).

Parameter	Duration	Control	GSH	NaCl	NaCl+GSH
Gly I	24 h	0.19 ± 0.005 b	0.23 ± 0.017 ab	0.14 ± 0.0163 c	0.24 ± 0.024 a
[mg^{-1}(protein) min^{-1}]	48 h	0.20 ± 0.010 ab	0.20 ± 0.018 a	0.17 ± 0.015 c	0.24 ± 0.039 a
Gly II	24 h	11.78 ± 2.050 bc	13.43 ± 1.530 abc	9.92 ± 0.747 c	15.21 ± 1.990 ab
[µmol mg^{-1}(protein) min^{-1}]	48 h	15.09 ± 0.980 abc	13.21 ± 1.879 abc	11.62 ± 1.060 bc	15.99 ± 2.260 a
MG content	24 h	17.75 ± 1.025 c	21.66 ± 1.480 bc	30.89 ± 3.664 a	25.22 ± 0.614 bc
[µmol g^{-1}(f.m.)]	48 h	18.29 ± 0.490 c	24.69 ± 1.075 bc	36.89 ± 3.590 a	28.57 ± 1.160 bc

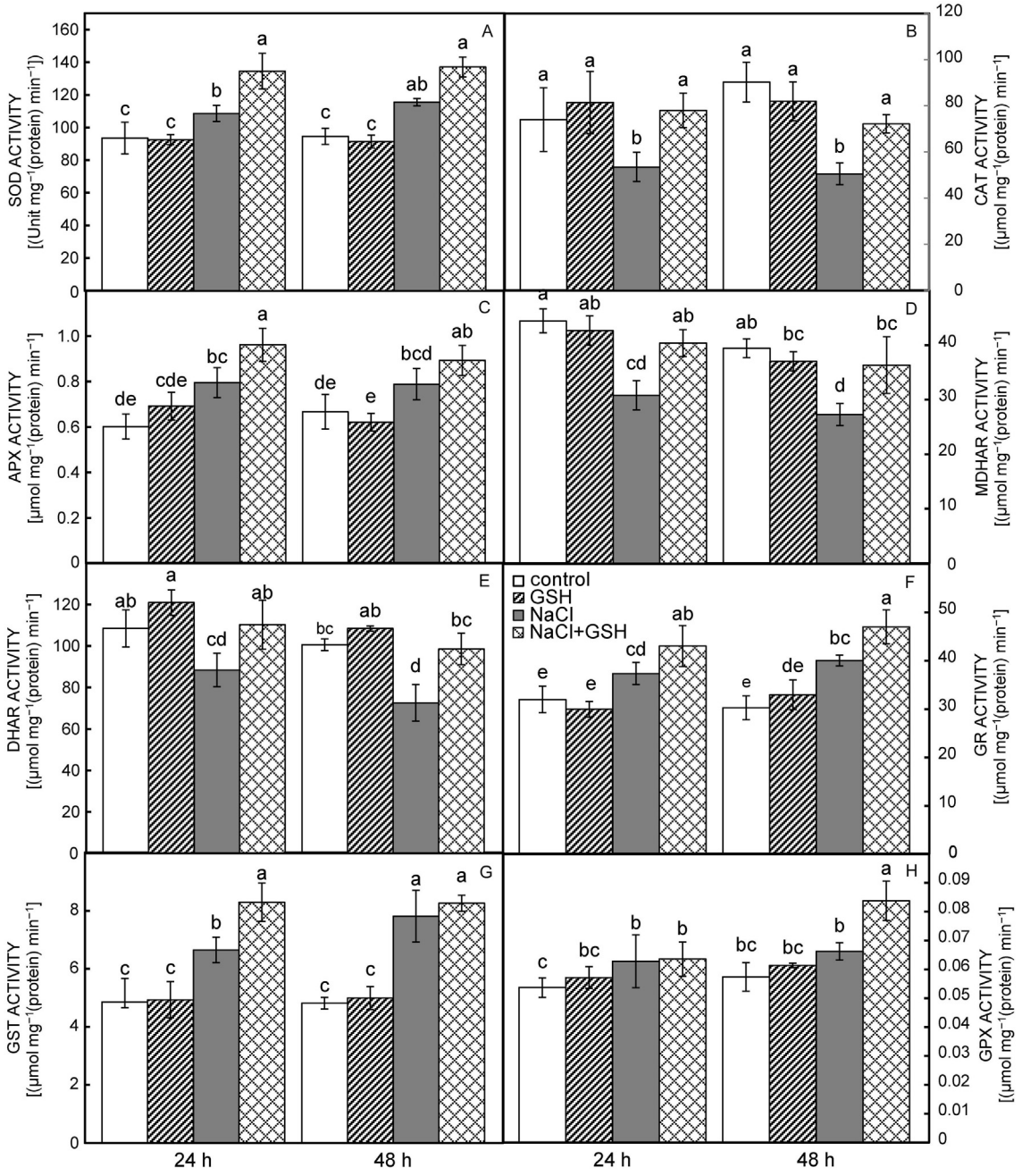

Fig. 3. Activities of SOD (*A*), CAT (*B*), APX (*C*), MDHAR (*D*), DHAR (*E*), GR (*F*), GST (*G*), and GPX (*H*) in mung bean seedlings as affected by 1 mM GSH and 200 mM NaCl. Means ± SE, $n = 3$. Different letters mark significantly different means at $P \leq 0.05$ (DMRT).

Discussion

Salinity aggravates several physiological disorders within plants. One of the most common effects of salt stress is increased production of ROS which are rapidly scavenged by plant antioxidative mechanisms, but a severe salt stress often impairs the antioxidant system and causes oxidative stress (Foyer and Noctor 2003). Methylglyoxal, which can be detoxified by the Gly system, is also overproduced under salt stress and is also responsible for oxidative damage (Yadav *et al.* 2005, 2008). Thus, the antioxidant system together with the Gly system are important in plants under salinity. Improved protection mechanisms are considered as indicators of enhanced salt tolerance (Aghaleh *et al.* 2014, Xu *et al.* 2014). Hossain and Fujita (2010) and Hossain *et al.*

(2011) reported that a coordinated induction of the antioxidant defense and Gly systems can confer salt stress tolerance to mung bean plants.

Many studies have shown that GSH is a vital component in both the antioxidant system and Gly system with multiple roles for eliminating ROS and MG, and GSH also improves plant growth and physiological adaptation under stress conditions (Foyer and Noctor 2003, Srivalli and Khanna-Chopra 2008, Yadav *et al.* 2008). Considering the vital roles of GSH, this study was based on a hypothesis that exogenous GSH might enhance salt stress tolerance in mung bean seedlings by improving the antioxidant system, glyoxalase system, and physiological processes. That is why this study examined the effects of the exogenous GSH on different non-enzymatic components, antioxidant enzymes, and oxidative stress: the effects of the exogenous GSH on components of the Gly system and MG content, and the effects of the exogenous GSH on some physiological parameters under salt stress conditions.

In the present study, the salt stress caused oxidative stress in the mung bean seedlings, which was apparent from the elevated MDA and H_2O_2 content and the increased $O_2^{\bullet-}$ generation rate (Fig. 1*A-C*). These kinds of salt-aggravated oxidative damage are similar to previous findings (Hasanuzzaman *et al.* 2011a). Decreases in oxidative stress by adding GSH to the salt media was evident in the reduction of oxidative stress indicators, and GSH was more effective for alleviation of the 24-h NaCl stress compared with the 48-h stress (Fig. 1*A-C*). Singlet oxygen and superoxide anions are generated when LOX catalyzes the oxidation of fatty acids (Lynch and Thompson 1984). Thus, a higher LOX activity results in oxidative stress by generating ROS (Molassiotis *et al.* 2006). An increased LOX activity is one of the reasons for an increased lipid peroxidation under stress conditions (Aziz and Larher 1998). An extreme increase in the LOX activity was observed under the salt stress in this experiment (Fig. 1*D*), and the stress also increased lipid peroxidation (Fig. 1*D*). A less increase in the LOX activity and the MDA content were observed after adding GSH. A relationship between a reduced LOX activity and a reduced MDA content under salt stress was also observed by Roychoudhury *et al.* (2011).

A decrease in leaf RWC indicates a limited water availability for cell extension (Katerji *et al.* 1997). Leaf wilting symptoms were observed under the salinity because of the reduction in the leaf RWC (Table 1). Under salt stress, restricted transpiration contributes to a limited flow of water from the soil to the plant (Steudle 2000). The RWC was partially restored by the GSH supplementation in the NaCl-treated seedlings (Table 1).

Proline is an important osmolyte and indicator of stress, it reestablishes an osmotic balance under stress conditions including salinity (Kattab 2007). Proline is also a ROS scavenger and a source of nitrogen and carbon required for stress recovery (Aziz *et al.* 1998, Khedr *et al.* 2003). In this study, Pro increased with the salt stress at both the durations (Table 1), and adding GSH to the NaCl solution resulted in a reduced Pro content especially after 24 h of the stress (Table 1).

Salinity impairs photosynthesis in various ways, one of which is destruction of photosynthetic pigments or hindrance of their production (Sudhir and Murthy 2004). The salt stress significantly decreased the Chl *a*, Chl *b*, and total Chl content in the present study (Table 1), which is similar to previous results (*e.g.*, Kattab 2007). The GSH addition to the NaCl solution prevented the reduction of the Chl content. Kattab (2007) also reported that an exogenous GSH in salt-stressed *Brassica napus* plants ameliorates a reduction in photosynthetic pigments. The probable reasons might be prevention or retardation of Chl degradation resulting from oxidative stress.

Ascorbate, having the ability to donate electrons in a number of enzymatic and non-enzymatic reactions, acts as powerful ROS scavenger. Ascorbate either directly scavenges ROS or aids in the detoxification of H_2O_2 through the AsA-GSH cycle. Generally, plants with a high AsA content show improved tolerance to oxidative stress (Foyer and Noctor 2003). The salt stress significantly reduced the AsA content (Fig. 2*A*) because of excess ROS production or by impaired biosynthesis or regeneration of AsA under the severe stress conditions (Song *et al.* 2005). In contrast to this, the increase in the AsA content was observed (Fig. 2*A*) under the exogenous GSH application to the salt-stressed seedlings. The exogenous GSH helped to enhance the MDHAR and DHAR activities (Fig. 3*D,E*), which could improve the AsA content in this experiment. A similar increase in AsA content by the exogenous GSH was observed in *Brassica napus* (Kattab 2007).

The endogenous GSH has multiple functions in relation to the antioxidant defense system. Glutathione has a protective role in enhancing salt tolerance by reactions with ROS, and it helps in AsA regeneration (Foyer and Noctor 2003). These roles of GSH are considered beneficial to plants under salt stress (Huang *et al.* 2005). The high content of endogenous GSH after GSH addition was documented (Fig. 2*B*; Kattab 2007). In contrast, the presence of an oxidized form of GSH, GSSG, is a sign of stress. In the current experiment, the salinity caused a significant increase in the GSSG content (Fig. 2*C*). A similar increase in GSSG content was reported in rapeseed seedlings under salt stress (Hasanuzzaman *et al.* 2011b). However, after the GSH addition, GSSG less increased, similarly as observed Sumithra *et al.* (2006) in chickpea. The higher GSH/GSSG ratio shows better salt and other stress tolerance capacities (Hasanuzzaman *et al.* 2011a,b, Alam *et al.* 2014), and the decreased GSH/GSSG ratio was significantly restored after the GSH application (Fig. 2*D* and Hasanuzzaman *et al.* 2011b). It was observed that the GR activity increased under the salt stress and especially after the addition of GSH (Fig. 3*F*). Glutathione reductase helps to recycle GSH, which affects the GSH pool and the GSH/GSSG ratio of plants to some extent. The GSH content of plants is also regulated by GSH

biosynthetic pathway enzymes.

The frontline defence against ROS is provided by SOD which removes $O_2^{\bullet-}$ (Hasanuzzaman *et al.* 2012). The SOD activity increased significantly in the salt stress-affected mung bean seedlings (Fig. 3*A*), and a similar increase in SOD activity under a salinity stress was previously demonstrated by other researches (Mishra *et al.* 2013, Aghaleh *et al.* 2014). A further increase in the SOD activity was observed after the GSH supplementation but only after 24 h (Fig. 3*A*). Liu *et al.* (2009) observed a higher endogenous GSH content and SOD activity in cucumber under chilling stress. Catalase directly dismutates H_2O_2 into H_2O and O_2 (Garg and Manchanda 2009). The activity of CAT in the mung bean seedlings decreased under the salt stress (Fig. 3*B*), and a reduced activity may have a role in increasing H_2O_2 production. This result is in agreement with earlier findings (Hasanuzzaman *et al.* 2011b). In contrast, the CAT activity was similar to the control after the addition of GSH to the NaCl solution (Fig. 3*B*). A similar result was also observed previously (Kattab 2007).

In the AsA-GSH cycle, the first step is scavenging H_2O_2 by APX (Nakano and Asada 1981). A significant increase in the APX activity under the salinity was the characteristic result of this study. The GSH application further increased the APX activity under the salinity (Fig. 3*C*) similarly as observed by Kattab (2007) and Hasanuzzaman *et al.* (2011b). The enzymes MDHAR and DHAR play a vital role in AsA regeneration. Monodehydroascorbate dehydrogenase is present in peroxisomes and scavenges H_2O_2 (Del Río *et al.* 2002). Salt stress decreases MDHAR and DHAR activities (Hasanuzzaman *et al.* 2011b), which is similar to the results of the present study (Fig. 3*D,E*). However, the GSH application recovered both the DHAR and MDHAR activities. As was mentioned above, GR is another important enzyme in the AsA-GSH system and helps to maintain the GSH pool by catalyzing the NADPH-dependent reduction of the disulphide bond of GSSG (Gill and Tuteja 2010, Gill *et al.* 2013). In this study, the GR activity increased significantly under the salinity compared with the control (Fig. 3*F*). Its activity was further enhanced by the GSH application.

Plant GST is a large family of enzymes playing roles in hormone homeostasis, vacuolar sequestration of anthocyanin, tyrosine metabolism, and hydroxyperoxide detoxification (Dixon *et al.* 2010). The GST activity of the NaCl-treated mung bean seedlings increased markedly, which is supported by recent findings of Naliwajski and Skłodowska (2014) who observed a salt induced increase in GST activity in cucumber. The GSH addition caused a further increase in the GST activity in the salt affected plants (Fig. 3*G*), which might improve the plant status under the salt stress. Glutathione peroxidase reduces H_2O_2 by using GSH and protects plant cells from oxidative damage (Gill and Tuteja 2010). A salinity-induced increase in GPX activity has been documented in previous studies (Kattab 2007, Hasanuzzaman *et al.* 2011b, Naliwajski and Skłodowska 2014). Similarly in this study, the GPX activity increased significantly at 24 h of the salt stress. Its activity also increased after the GSH addition but only after 48 h (Fig. 3*H*). An exogenous GSH application improved GPX activity in salt-stressed *Brassica napus* as reported by Kattab (2007).

Under abiotic stresses, MG production increases significantly and can either act directly as potential toxic agent affecting various plant processes or deplete a GSH pool. The Gly system, comprising the Gly I and Gly II enzymes, effectively eliminates MG by utilizing GSH as substrate and subsequently reduces oxidative stress. Thus, a higher content of GSH is also considered beneficial in reducing oxidative stress induced by MG (Yadav *et al.* 2008). The MG content also increased under salt stress (Yadav *et al.* 2005, El-Shabrawi *et al.* 2010), which corroborates with our findings (Table 2). Improving plant tolerance by improving the Gly system is often achieved by using exogenous protectants (El-Shabrawi *et al.* 2010) and exogenous GSH as protectant has been shown effective in the present study as it reduced the MG content by improving the Gly I and Gly II activities (Table 2) and increasing the endogenous GSH content. Other reports also express similar findings where exogenous GSH application in salt-stressed *Nicotiana tabacum* plants significantly reduces an MG content (Yadav *et al.* 2005). Similarly, an exogenous GSH application in high temperature-affected mung bean seedlings reduces an MG content significantly by improving Gly I and Gly II activities and increasing an endogenous GSH content (Nahar *et al*. 2015a).

Conclusion

The regulatory roles of the exogenously-applied GSH in the antioxidant and Gly systems and its effect on other physiological parameters were investigated in the mung bean seedlings (*Vigna radiata* cv. Binamoog-1) subjected to the salt stress for the different durations. The salt stress significantly reduced the leaf RWC, Chl content, Pro content, generated severe oxidative stress, and markedly raised the MG content. The severity of damage increased with the increase in stress duration. The salt stress also modulated the non-enzymatic and enzymatic antioxidants, and components of the MG detoxification system. However, the exogenous GSH was able to modulate and upregulate the antioxidant components and the Gly enzymes. Reduced glutathione was shown to be efficient in improving physiological adaptation of the mung bean seedlings under the salt stress.

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