Plant Biotechnol Rep (2011) 5:353–365
DOI 10.1007/s11816-011-0189-9

ORIGINAL ARTICLE

Nitric oxide modulates antioxidant defense and the methylglyoxal detoxification system and reduces salinity-induced damage of wheat seedlings

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Received: 4 July 2010 / Accepted: 22 June 2011 / Published online: 19 July 2011
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Abstract The present study investigates the possible regulatory role of exogenous nitric oxide (NO) in antioxidant defense and methylglyoxal (MG) detoxification systems of wheat seedlings exposed to salt stress (150 and 300 mM NaCl, 4 days). Seedlings were pre-treated for 24 h with 1 mM sodium nitroprusside, a NO donor, and then subjected to salt stress. The ascorbate (AsA) content decreased significantly with increased salt stress. The amount of reduced glutathione (GSH) and glutathione disulfide (GSSG) and the GSH/GSSG ratio increased with an increase in the level of salt stress. The glutathione *S*-transferase (GST) activity increased significantly with severe salt stress (300 mM). The ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), catalase (CAT) and glutathione peroxidase (GPX) activities did not show significant changes in response to salt stress. The glutathione reductase (GR), glyoxalase I (Gly I), and glyoxalase II (Gly II) activities decreased upon the imposition of salt stress, especially at 300 mM NaCl, with a concomitant increase in the H_2O_2 and lipid peroxidation levels. Exogenous NO pretreatment of the seedlings had little influence on the non-enzymatic and enzymatic components compared to the seedlings of the untreated control. Further investigation revealed that NO pre-treatment had a synergistic effect; that is, the pre-treatment increased the AsA and GSH content and the GSH/GSSG ratio, as well as the activities of MDHAR, DHAR, GR, GST, GPX, Gly I, and Gly II in most of the seedlings subjected to salt stress. These results suggest that the exogenous application of NO rendered the plants more tolerant to salinity-induced oxidative damage by enhancing their antioxidant defense and MG detoxification systems.

Keywords Antioxidant defense · Ascorbate–glutathione cycle · Glyoxalase system · Salt stress · Reactive oxygen species · Sodium nitroprusside

Abbreviations

AO	Ascorbate oxidase
APX	Ascorbate peroxidase
AsA	Ascorbic acid
CAT	Catalase
CDNB	1-Chloro-2, 4-dinitrobenzene
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
DTNB	5,5′-Dithio-bis (2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
Gly I	Glyoxalase I
Gly II	Glyoxalase II
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GPX	Glutathione peroxidase
GST	Glutathione *S*-transferase

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MDA Malondialdehyde
MDHA Monodehydroascorbate
MDHAR Monodehydroascorbate reductase
MG Methylglyoxal
NO Nitric oxide
NTB 2-Nitro-5-thiobenzoic acid
ROS Reactive oxygen species
SLG *S*-D-lactoylglutathione
SNP Sodium nitroprusside
TBA Thiobarbituric acid
TCA Trichloroacetic acid

Introduction

Salt stress is a major environmental threat to agriculture, and its adverse impacts are getting more serious problems in regions where saline water is used for irrigation (Türkan and Demiral 2009). Therefore, efforts to increase the salt tolerance of crop plants are very important to ensure global food security, as well as for water and land conservation. A high salt concentration in the soil or in irrigation water can have a devastating effect on plant metabolism; that is, it can result in the disruption of cellular homeostasis and uncoupling of major physiological and biochemical processes. Plants can respond and adapt to salt stress by altering their cellular metabolism and invoking various defense mechanisms (Ghosh et al. 2011). The survival of plants under this stressful condition depends on their abilities to perceive the stimulus, generate and transmit a signal, and initiate various physiological and biochemical changes (Tanou et al. 2009a; El-Shabrawi et al. 2010). Molecular and biochemical studies of the salt stress responses of plants have demonstrated significant increases in reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH·) (Mittler 2002; Tanou et al. 2009a; Pérez-López et al. 2010). The ROS accumulation during stress greatly depends on the balance between ROS production and ROS scavenging (Mittler et al. 2004), which in turn depends on changes in the growth conditions, including the severity and duration of the stress, as well as the ability of the tissue to rapidly acclimate to the energy imbalance (Miller et al. 2010). A strict control of ROS levels is essential to prevent their toxicity and ensure an accurate execution of their signaling function (Mittler et al. 2004). Plants have well-developed enzymatic and non-enzymatic antioxidant defense systems to counter the deleterious effects of ROS. The antioxidant defenses of plants include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione *S*-transferase (GST), and the four enzymes of the ascorbate–glutathione (AsA–GSH) cycle: ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR), along with non-enzymatic components such as ascorbate (AsA) and glutathione (GSH). Apart from its function to detoxify ROS and thereby combat the potentially harmful effect of environmental stress, the AsA–GSH cycle is also included in redox sensing and signaling. Under a stress condition, the redox signals could interfere with signaling networks complementary to the antioxidant system and could regulate defense gene expression (Kuźniak and Sklodowska 2005).

Recent studies in plants have demonstrated that the amount of methylglyoxal (MG) increases considerably in response to various abiotic stresses, including salinity (Hossain et al. 2010). In plants, MG is detoxified mainly via the glyoxalase system, which comprises two enzymes: glyoxalase I (Gly I) and glyoxalase II (Gly II). Gly I converts MG to *S*-D-lactoylglutathione (SLG) by utilizing GSH, whereas Gly II converts SLG to D-lactic acid, and, during this reaction, GSH is recycled back. The presence and characterization of both Gly I and Gly II have been reported in many plants, and the genes encoding these enzymes have been cloned and found to be regulated under various environmental conditions (Veena et al. 1999; Yadav et al. 2005a, b; Singla-Pareek et al. 2008; Hossain and Fujita 2009). Additionally, MG functions as a signal initiator for the activation of stress-activated protein kinases (Takatsume et al. 2006). Extensive research findings support the idea that coordinated inductions or regulations of the antioxidant and glyoxalase pathway enzymes are necessary to obtain substantial tolerance against oxidative stress (Fig. 1; Hoque et al. 2007; Hossain et al. 2010, 2011; El-Shabrawi et al. 2010). The overexpression of the glyoxalase pathway enzymes in transgenic tobacco and rice plants has been found to lower the levels of ROS and MG under stress conditions by maintaining GSH homeostasis and increasing antioxidant enzyme activities (Yadav et al. 2005b; Singla-Pareek et al. 2006).

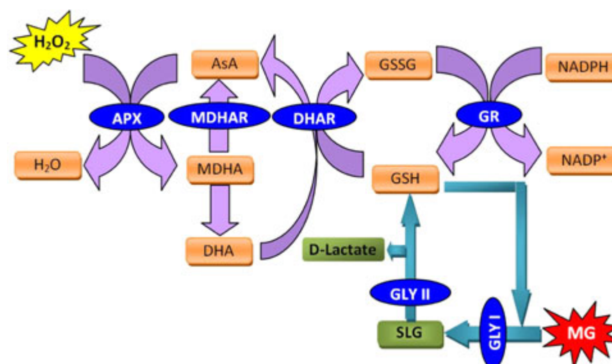

Fig. 1 Ascorbate–glutathione (AsA–GSH) cycle and glyoxalase system in plants involved in ROS and MG detoxification. Abbreviations are described in the text

Nitric oxide (NO) is a highly reactive, membrane-permeable free radical that has recently emerged as an important signaling molecule and antioxidant. NO triggers many kinds of redox-regulated (defense-related) gene expressions, directly or indirectly, to establish plant stress tolerance (Polverari et al. 2003; Sung and Hong 2010). The application of an NO donor, sodium nitroprusside (SNP), confers tolerance to various abiotic stresses in plants by enhancing their antioxidant defense system (Neill et al. 2002; Tian and Lei 2006; Sheokand et al. 2008; Zheng et al. 2009; Singh et al. 2009; Xu et al. 2010). Several lines of study have shown that the protective effect of NO against abiotic stress is closely related to the NO-mediated reduction of ROS in plants (Beligni and Lamattina 1999; Wang and Yang 2005; Hasanuzzaman et al. 2010). Shi et al. (2007) observed that application of NO donor reduced the salinity-induced damage while the effects were reverted by addition of NO scavenger. In addition to a direct ROS scavenging activity and the modulation of lipid peroxidation by lipoxygenase inhibition, NO may also protect plant cells against oxidative processes by stimulating GSH synthesis. It has been indicated that the GSH biosynthesis is stimulated in response to NO in plant and animal cells (Moellering et al. 1998; Kim et al. 2004; Innocenti et al. 2007). The regulation of GSH synthesis by NO raises the question of the physiological roles that may be sustained by such a modulation. Recent studies have shown that GSH plays an important role in regulating the MG level and enhances the oxidative stress tolerance of plants (Yadav et al. 2005a, b; Hossain et al. 2010; El-Shabrawi et al. 2010; Hasanuzzaman and Fujita 2011). With regard to recent reports indicating the importance of the glyoxalase pathway in plant stress tolerance, we speculated that the stimulation of GSH synthesis by NO may provide a regulatory role in MG detoxification by influencing glyoxalase pathway enzymes, as the first enzyme (Gly I) of this pathway uses GSH as a cofactor during MG detoxification (Yadav et al. 2005a). Considering the above viewpoints, in the present study, we investigated the regulatory role of exogenous NO in the antioxidant defense and MG detoxification system of wheat seedlings subjected to salt stress. To the best of our knowledge, this is the first study on the regulatory effects of exogenous NO on the glyoxalase system in plants under salinity stress.

Materials and methods

Plant materials and stress treatments

Wheat (*Triticum aestivum* cv. Pradip) seeds of uniform size were selected and surface-sterilized with 70% ethanol followed by washing several times with sterile distilled water. The seeds were then soaked with distilled water for 10 min and sown in Petri plates (9 cm) lined with 6 layers of filter paper moistened with 10 ml of distilled water for germination under controlled conditions (light 100 µmol photon m^{-2} s^{-1}, temp 25 ± 2°C, RH 65–70%) for 3 days. Germinated seedlings were then grown in Petri dishes that contained 10,000-fold diluted Hyponex solution (Hyponex, Japan). Six-day-old wheat seedlings of approximately equal sizes were employed to NO pre-treatment. For NO pre-treatment root portion of the seedlings were immersed with Hyponex solution that contained 1 mM sodium nitroprusside (SNP; Wako, Japan), a NO donor, for 24 h. SNP-treated and non-treated seedlings were then subjected to two different levels of salt stress (150 and 300 mM NaCl) in Hyponex solution and grown under the above conditions for 4 days. Control plants were grown in Hyponex solution only. The experiment was repeated three times under the same conditions.

Extraction and analysis of ascorbate and glutathione

Wheat leaves (0.5 g fresh weight) were homogenized in 3 ml ice-cold acidic extraction buffer (5% meta-phosphoric acid containing 1 mM EDTA) using a mortar and pestle. Homogenates were centrifuged at 11,500*g* for 12 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 M K-phosphate buffer (pH 7.0). The reduced ascorbate was assayed spectrophotometrically at 265 nm in 100 mM K-phosphate buffer (pH 7.0) 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. 2003) with modifications (Paradiso et al. 2008) utilizing 0.4 ml of aliquots of supernatant neutralized with 0.6 ml of 0.5 M K-phosphate buffer (pH 7.0). 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.

Determination of protein

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

Enzyme extraction and assays

Using a pre-cooled mortar and pestle, 0.5 g of leaf tissue was homogenized in 1 ml of 50 mM ice-cold K-phosphate buffer (pH 7.0) 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 15 min and the supernatants were used for determination of enzyme activity. All procedures were performed at a temperature 0–4°C.

Ascorbate peroxidase (EC: 1.11.1.11) activity was assayed following the method of Nakano and Asada (1981). The reaction buffer solution contained 50 mM K-phosphate 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 700 µl. The reaction was started by the addition of H_2O_2 and the activity was measured by observing the decrease in absorbance at 290 nm for 1 min using an extinction coefficient of 2.8 mM^{-1} cm^{-1}.

Monodehydroascorbate reductase (EC: 1.6.5.4) activity was determined by the method of Hossain et al. (1984). The reaction mixture contained 50 mM Tris–HCl buffer (pH 7.5), 0.2 mM NADPH, 2.5 mM AsA, 0.5 unit of AO and enzyme solution in a final volume of 700 µl. 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}.

Dehydroascorbate reductase (EC: 1.8.5.1) activity was determined by the procedure of Nakano and Asada (1981). The reaction buffer contained 50 mM K-phosphate 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 an extinction coefficient of 14 mM^{-1} cm^{-1}.

Glutathione reductase (EC: 1.6.4.2) activity was measured by the method of Hossain et al. (2010). The reaction mixture contained 0.1 M K-phosphate 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}.

Glutathione *S*-transferase (EC: 2.5.1.18) activity was determined spectrophotometrically by the method of Hossain et al. (2006) with some modifications. 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 700 µl. 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}.

Glutathione peroxidase (EC: 1.11.1.9) activity was measured as described by Elia et al. (2003) 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}.

Catalase (EC: 1.11.1.6) 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-phosphate buffer (pH 7.0), 15 mM H_2O_2 and enzyme solution in a final volume of 700 µl. The reaction was initiated with enzyme extract and the activity was calculated using the extinction coefficient of 39.4 M^{-1} cm^{-1}.

Glyoxalase I (EC: 4.4.1.5) assay was carried out according to Hossain et al. (2009). Briefly, the assay mixture contained 100 mM K-phosphate buffer (pH 7.0), 15 mM magnesium sulphate, 1.7 mM GSH and 3.5 mM MG in a final volume of 700 µl. 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}.

Glyoxalase II (EC: 3.1.2.6) 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 *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}.

Measurement of H_2O_2

H_2O_2 was assayed according to the method described by Yu et al. (2003). H_2O_2 was extracted by homogenizing 0.5 g of leaf samples with 3 ml of 50 mM K-phosphate buffer pH (6.5) at 4°C. The homogenate was centrifuged at 11,500*g* for 15 min. Three ml of supernatant was mixed with 1 ml of 0.1% $TiCl_4$ in 20% H_2SO_4 (v/v), and the mixture was then centrifuged at 11,500*g* for 12 min at room temperature. The optical absorption of the supernatant was measured spectrophotometrically at 410 nm to determine the H_2O_2 content ($\epsilon = 0.28$ μM^{-1} cm^{-1}) and expressed as µmol g^{-1} fresh weight.

Measurement of lipid peroxidation

The level of lipid peroxidation was measured by estimating MDA, a decomposition product of the peroxidized

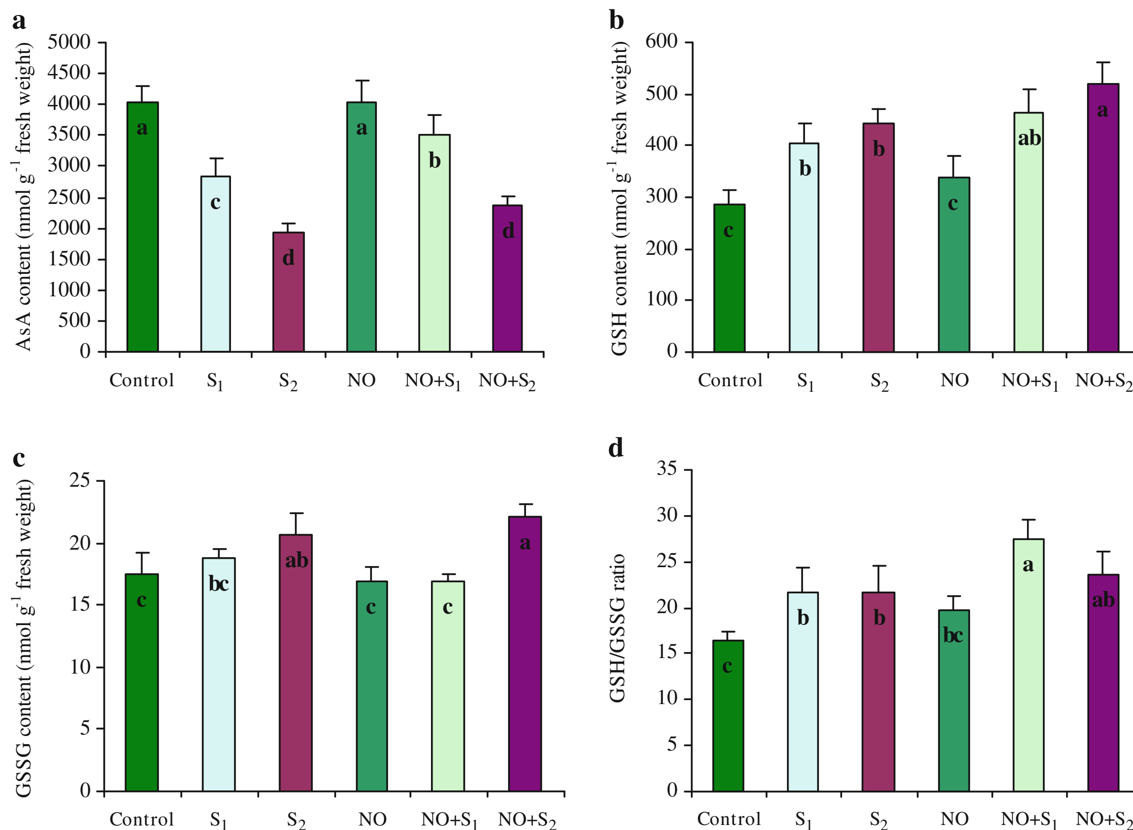

Fig. 2 **a** Reduced ascorbate (AsA), **b** reduced glutathione (GSH), **c** oxidized glutathione (GSSG), and **d** GSH/GSSG ratio in wheat seedlings induced by nitric oxide under salt stress conditions. S_1, S_2, *NO*, $NO + S_1$ and $NO + S_2$ indicates 150 mM NaCl, 300 mM NaCl, SNP, 150 mM NaCl + SNP and 300 mM NaCl + SNP treatments, respectively. Mean (±SD) was calculated from three replicates for each treatment. *Bars* with different *letters* are significantly different at $P < 0.05$, applying a LSD test

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. The leaf samples (0.5 g) were homogenized in 3 ml 5% (w/v) trichloroacetic acid (TCA) and the homogenate was centrifuged at 11,500*g* for 10 min. One ml supernatant was mixed with 4 ml of TBA reagent (0.5% of TBA in 20% TCA). The reaction mixture was heated at 95°C for 30 min in a water bath and then quickly cooled in an ice bath and centrifuged at 11,500*g* for 15 min. The absorbance of the colored supernatant was measured at 532 nm and was corrected for non-specific absorbance at 600 nm. The concentration of MDA was calculated by using the extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol of MDA g^{-1} fresh weight.

Statistical analysis

All data obtained were subjected to analysis of variance (ANOVA) and the mean differences were compared by a least significant difference (LSD) test using MSTAT-C software (MSTAT-C 1990). Differences at $P < 0.05$ were considered significant.

Results

AsA and GSH contents

Sharp decreases in AsA content were observed (30 and 52% by the 150 and 300 mM NaCl stresses, respectively) in response to salt stress, compared to the untreated control (Fig. 2a). NO pre-treated salt-stressed seedlings had significantly higher AsA content at 150 mM NaCl stress, compared to seedlings subjected to salt stress without pre-treatment. However, the level was significantly lower than that of the untreated control. The AsA level of NO pre-treated control seedlings was similar to that of the untreated control.

Significant increases in GSH content were observed (42 and 55% by the 150 and 300 mM NaCl stresses, respectively) in response to salt stress, compared to the untreated control (Fig. 2b). An increase in GSH content was also observed in NO pre-treated salt-stressed seedlings and, particularly at the 300 mM stress, the NO pre-treated seedlings showed a significant increase (17%) in GSH content compared to seedlings subjected to salt stress alone.

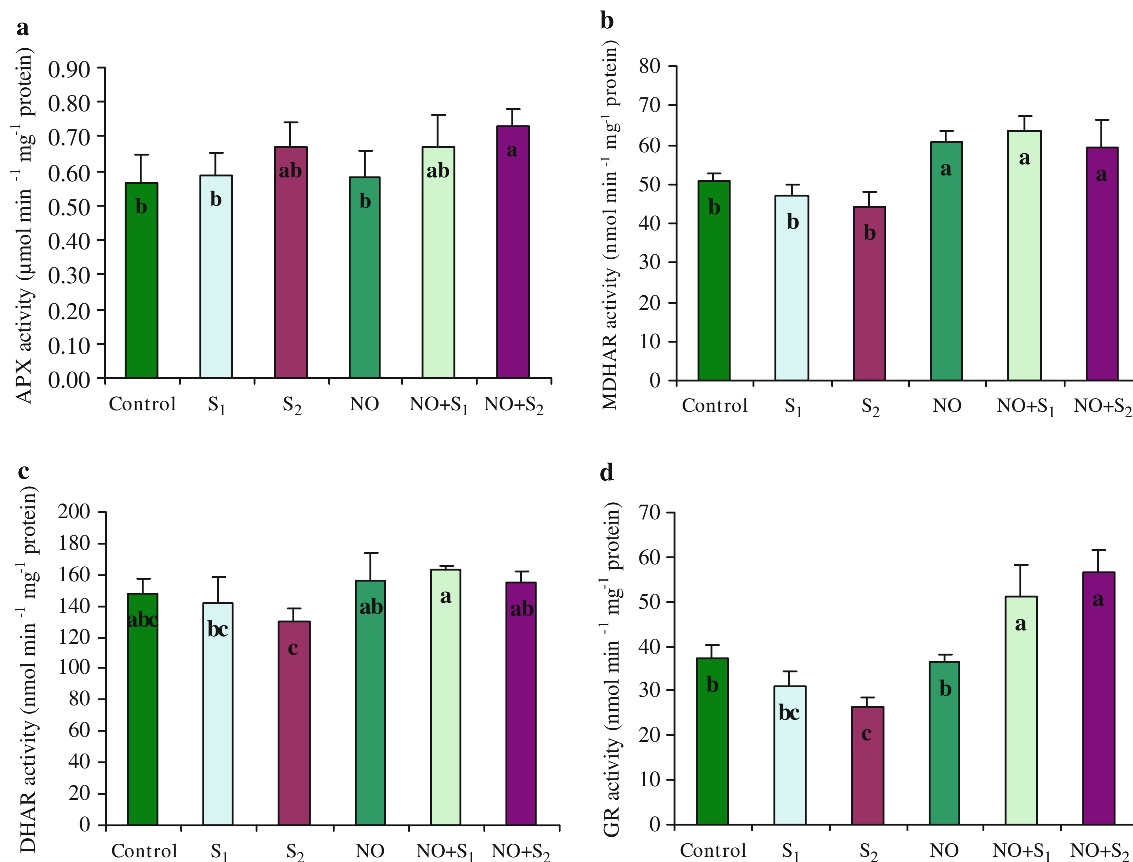

Fig. 3 Activities of APX (**a**), MDHAR (**b**), DHAR (**c**), and GR (**d**) in wheat seedlings induced by nitric oxide under salt stress conditions. S_1, S_2, *NO*, $NO + S_1$ and $NO + S_2$ indicates 150 mM NaCl, 300 mM NaCl, SNP, 150 mM NaCl + SNP and 300 mM NaCl + SNP treatments, respectively. Mean (±SD) was calculated from three replicates for each treatment. *Bars* with different *letters* are significantly different at $P < 0.05$, applying a LSD test

No significant differences were observed in the GSSG content in response to the 150 mM NaCl, whereas at the 300 mM NaCl stress, a significant increase (18%) in GSSG content was observed compared to control. However, NO pre-treated seedlings did not show statistically significant changes in GSSG content compared to the seedlings subjected to salt stress alone (Fig. 2c).

A significant increase in the GSH/GSSG ratio was observed in response to salt stress (32% by both the 150 and 300 mM NaCl stresses) (Fig. 2d). At the 150 mM NaCl stress, the NO pre-treated seedlings showed a significant (26%) increase in the GSH/GSSG ratio compared to those treated with salt alone.

ROS scavenging enzymes

A slight increase in APX activity was observed at the 300 mM NaCl stress, compared to the untreated control (Fig. 3a) with no statistical significance. Similarly, NO pre-treated salt-stressed seedlings did not show any significant increase in APX activity compared to the seedlings subjected to salt stress without pre-treatment.

A slight decrease in MDHAR activity was observed in response to salt stress (Fig. 3b) with no statistical significance. The NO pre-treated control and salt-stressed seedlings had significantly higher MDHAR activities than the untreated control and the seedlings subjected to salt stress without pre-treatment.

A little decrease in DHAR activity was observed under NaCl stress, compared to the untreated control (Fig. 3c) with no statistical significance. The NO pre-treated salt-stressed seedlings had significantly higher DHAR activities (20 and 15% by the 150 and 300 mM NaCl stresses, respectively), compared to the seedlings subjected to salt stress without pre-treatment.

A slight decrease in GR activity was observed at the 150 mM NaCl stress, whereas a significant decrease (29%) was observed at the 300 mM NaCl stress (Fig. 3d), compared to the untreated control. The NO pre-treated salt-stressed seedlings showed significant increases in GR

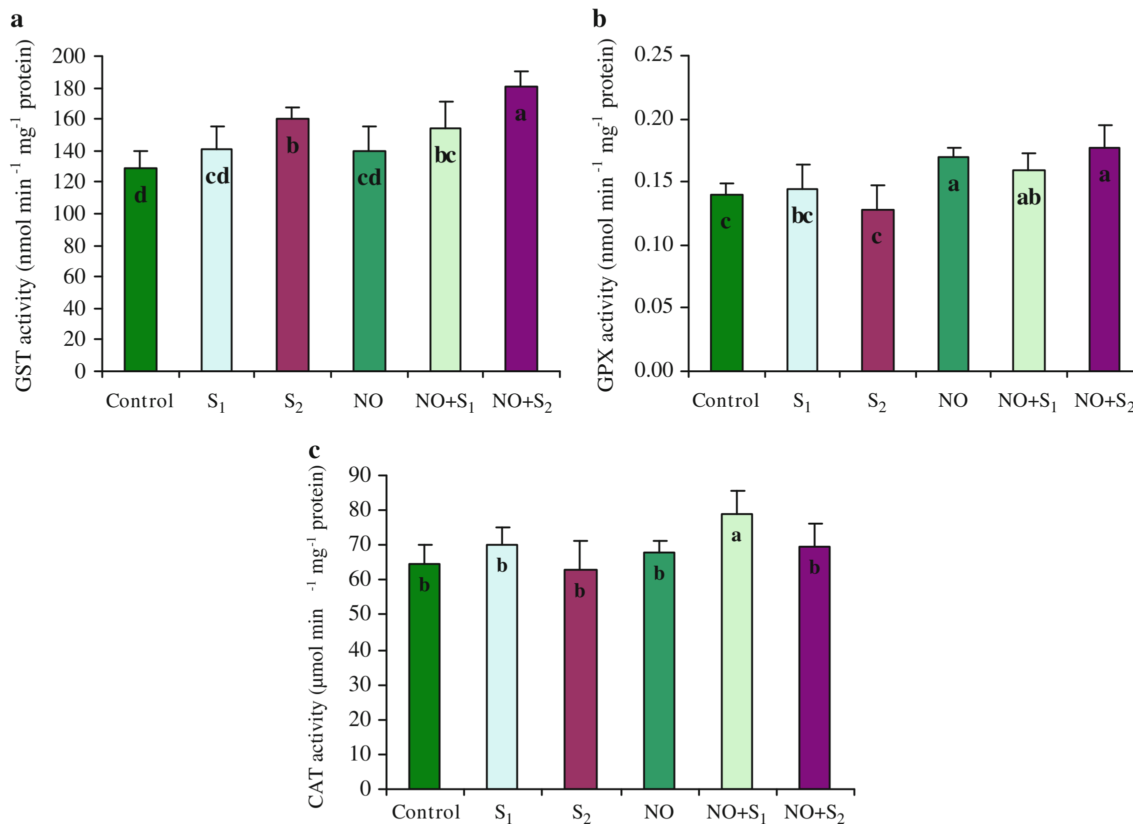

Fig. 4 Activities of GST (**a**), GPX (**b**), and CAT (**c**) in wheat seedlings induced by nitric oxide under salt stress conditions. S_1, S_2, *NO*, $NO + S_1$ and $NO + S_2$ indicates 150 mM NaCl, 300 mM NaCl, SNP, 150 mM NaCl + SNP and 300 mM NaCl + SNP treatments, respectively. Mean (±SD) was calculated from three replicates for each treatment. *Bars* with different *letters* are significantly different at $P < 0.05$, applying a LSD test

activity (37 and 53% by the 150 and 300 mM NaCl stresses, respectively), compared to the seedlings subjected to salt stress.

The GST activity was slightly increased at the 150 mM NaCl stress with no statistical significance, whereas a significant increase (25%) was observed at the 300 mM NaCl stress, compared to the untreated control (Fig. 4a). At the 300 mM NaCl stress, the NO pre-treated seedlings maintained significantly higher GST activity than the seedlings subjected to salt stress without pre-treatment.

A slight decrease in GPX activity was observed at 300 mM NaCl stress (Fig. 4b) compared to the untreated control with no statistical significance. The NO pre-treated salt-stressed seedlings had higher GPX activity and, particularly, a significant increase was observed at the 300 mM NaCl stress, compared to the seedlings subjected to salt stress without pre-treatment. The NO pre-treated non-stressed seedlings had significantly higher GPX activity than the untreated control.

The activity of CAT remained almost unchanged in response to salt stress (Fig. 4c). The NO pre-treated salt-stressed seedlings had significantly higher CAT activity at the 150 mM NaCl stress, compared to the seedlings subjected to salt stress without pretreatment. The CAT activity of the NO pre-treated control seedlings was similar to that of the untreated control.

Activities of glyoxalase enzymes

Significant decreases in Gly I activity were observed (12 and 26% by the 150 and 300 mM NaCl stresses, respectively) in response to salt stress (Fig. 5a), compared to the untreated control. The NO pre-treated salt-stressed seedlings had significantly higher Gly I activities (26 and 25% by the 150 and 300 mM NaCl stresses, respectively), compared to the seedlings subjected to salt alone.

A slight decrease in Gly II activity was observed at the 150 mM NaCl stress (Fig. 5b) with no statistical significance, whereas a significant (13%) decrease was observed at the 300 mM NaCl stress, compared to the untreated control. At the 300 mM NaCl stress, the NO pre-treated salt-stressed seedlings had significantly higher Gly II activity, compared to the seedlings subjected to salt stress alone.

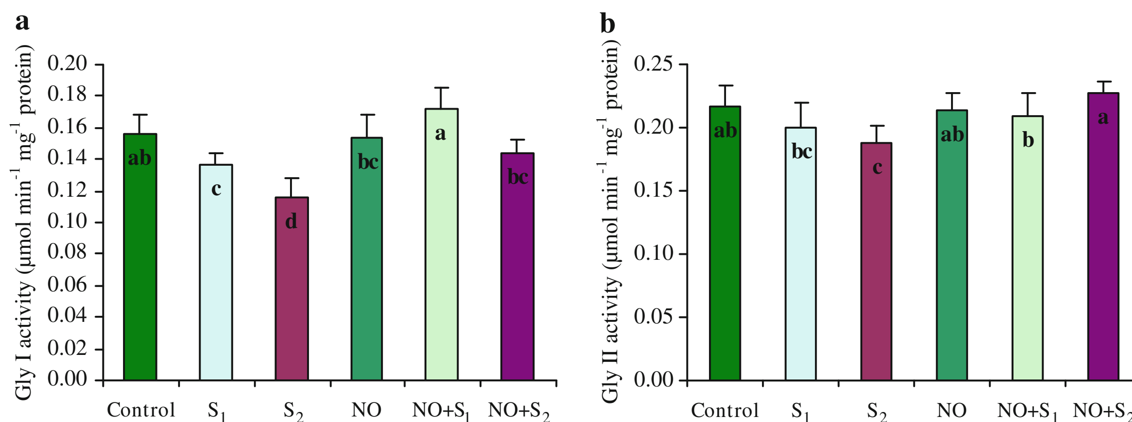

Fig. 5 Activities of Gly I (**a**), and Gly II (**b**) in wheat seedlings induced by nitric oxide under salt stress conditions. S_1, S_2, *NO*, $NO + S_1$ and $NO + S_2$ indicates 150 mM NaCl, 300 mM NaCl, SNP, 150 mM NaCl + SNP and 300 mM NaCl + SNP treatmenst, respectively. Mean (±SD) was calculated from three replicates for each treatment. *Bars* with different *letters* are significantly different at $P < 0.05$, applying a LSD test

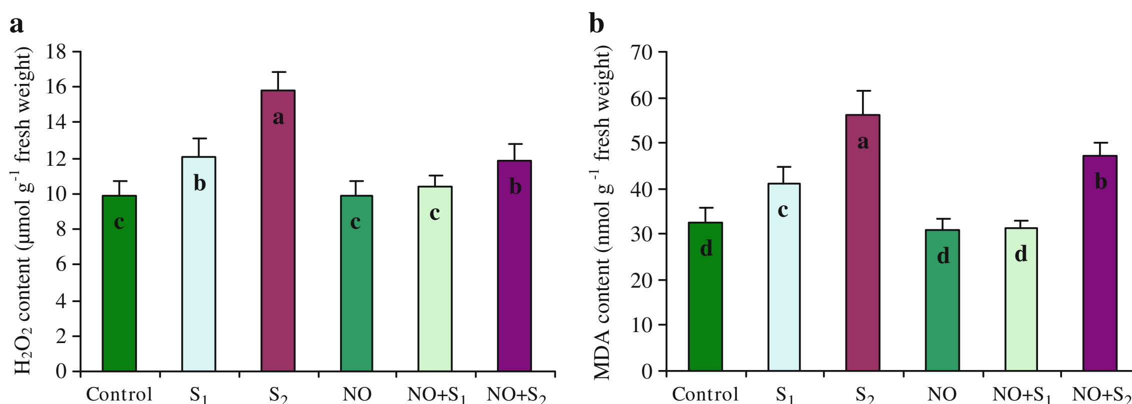

Fig. 6 H_2O_2 (**a**), and lipid peroxidation (represented by MDA) level (**b**) in wheat seedlings induced by nitric oxide under salt stress conditions. S_1, S_2, *NO*, $NO + S_1$ and $NO + S_2$ indicates 150 mM NaCl, 300 mM NaCl, SNP, 150 mM NaCl + SNP and 300 mM NaCl + SNP treatments, respectively. Mean (±SD) was calculated from three replicates for each treatment. *Bars* with different *letters* are significantly different at $P < 0.05$, applying a LSD test

H_2O_2 and lipid peroxidation

A significant increase in the H_2O_2 level was observed in wheat leaves in response to salt stress (Fig. 6a) and, particularly, a profound increase (60%) was observed at the 300 mM NaCl stress, compared to the untreated control. The NO pre-treated salt-stressed seedlings maintained significantly lower levels of H_2O_2 content (21 and 16% at the 150 and 300 mM NaCl stresses, respectively), compared to the seedlings subjected to salt stress without pre-treatment.

The lipid peroxidation levels in leaf tissues, measured as the MDA content, are represented in Fig. 6b. A significant increase in MDA content was observed at the 150 mM NaCl stress (27%). However, a sharp increase (73%) was observed at the 300 mM NaCl stress, compared to the untreated control. The NO pre-treated salt-stressed seedlings showed a significant decrease in MDA content (24 and 16% at the 150 and 300 mM NaCl stress, respectively), compared to the seedlings subjected to salt alone.

Phenotypic observations also showed that the NO pre-treated salt-stressed seedlings had better visual symptoms (less chlorosis/yellowing), compared to the seedlings without pre-treatment (Fig. 7).

Discussion

This study provided an insight into the role of NO in regulating the biochemical response of wheat seedlings to salt stress. Our results demonstrated that NO pre-treatment in wheat seedlings enhanced the protection against salt-induced oxidative damage. These observations were mainly attributed to the protective effect of exogenously

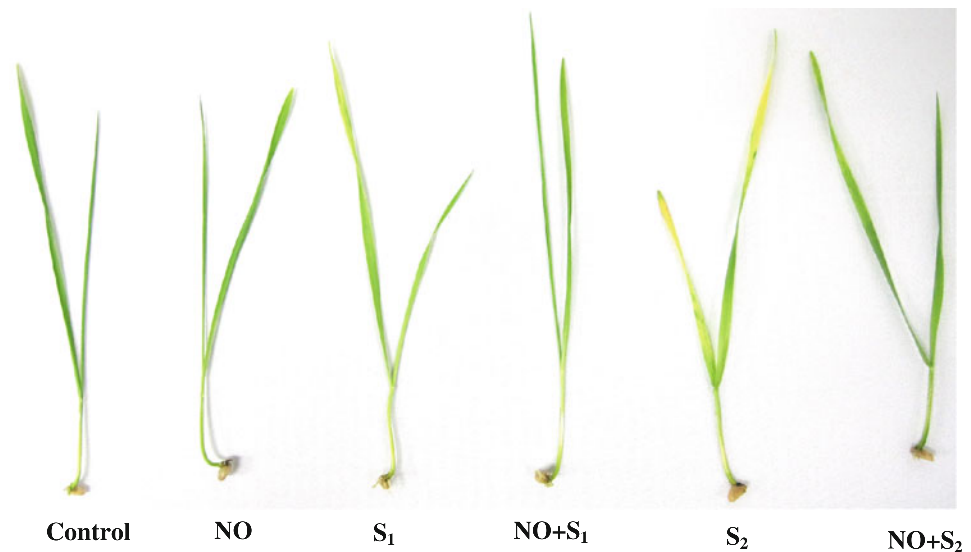

Fig. 7 Phenological appearance of wheat leaves induced by nitric oxide under salt stress conditions. S_1, S_2, *NO*, $NO + S_1$ and $NO + S_2$ indicates 150 mM NaCl, 300 mM NaCl, SNP, 150 mM NaCl + SNP and 300 mM NaCl + SNP treatments, respectively

applied NO, because the components of the antioxidant defense and glyoxalase systems were upregulated in the presence of a NO donor, which allowed the plants to cope better with salinity stress. Several recent studies have demonstrated the diverse functions of NO, in accordance with the pleiotropic role of this molecule in plant physiology (Neill et al. 2002; Zhang et al. 2007; Tanou et al. 2009b).

To counter the deleterious effects of ROS, plants are equipped with an array of non-enzymatic scavengers and antioxidant enzymes acting in concert to alleviate cellular damage under oxidative stress conditions. AsA is one of the most abundant non-enzymatic antioxidant, serving as a major contributor to the cellular redox state and protecting plants against oxidative damage (Smirnoff 2000). It is the substrate of APX which is a critical component of the AsA–GSH cycle for H_2O_2 detoxification (Nakano and Asada 1981; Dalton et al. 1986). In our present study, AsA content significantly decreased with an increased level of salt stress which corroborated other reports (Mittova et al. 2003a; Huang et al. 2005; Hasanuzzaman et al. 2011). The regeneration of AsA under salinity is insufficient or AsA synthesis is lower than AsA catabolism (Shalata et al. 2001; Amor et al. 2006). Importantly, under the mild salt stress (150 mM), the NO pre-treated seedlings maintained significantly higher AsA content than the control and the seedlings treated with salt alone, which indicates that NO might play a role in AsA regeneration. Like AsA, glutathione (GSH) plays a pivotal role in preventing cell against oxidative damage by equilibrating redox status. GSH can participate not only in scavenging H_2O_2 through the AsA–GSH cycle but also in direct reactions with other ROS (May et al. 1998). The increased level of the GSH pool is generally regarded as a protective response against oxidative stress. In this study, salt stress (150 and 300 mM NaCl) caused a significant increase in GSH content (Fig. 2b), which was consistent with previous studies (Hu et al. 2009; Xu et al. 2009). However, the NO pre-treated salt-stressed (300 mM) seedlings showed a significant increase in the level of GSH compared to seedlings subjected to salt stress alone because GSH synthesis is enhanced by NO treatment (Moellering et al. 1998; Innocenti et al. 2007). The increased level of GSSG in salt-treated seedlings may be attributed to an increase in DHAR activity, which utilizes GSH as an electron donor. However, in our experiment under severe salt stress, a significant increase in GSSG content was observed, even though the DHAR activity remained unchanged (Fig. 3c). The formation of GSSG in salt-treated seedlings might be due to the reaction of GSH with oxyradicals generated by oxidative stress or decreased GR activity (Shalata et al. 2001; Aravind and Prasad 2005).

Ascorbate peroxidase catalyzes the reduction of H_2O_2 into H_2O (Zhang et al. 2008) and modulates the concentration of H_2O_2 to a level sufficient for second messenger activity. In our experiments, the APX activity slightly increased upon the imposition of salt stress, although it was statistically insignificant (Fig. 3a). Similar trends were also observed by Zhu et al. (2004) and Mittova et al. (2004). The NO pre-treated salt-stressed seedlings did not show significant increase in APX activity compared to the seedlings treated with salt alone. These results suggest that exogenous NO could not contribute very well in the detoxification of H_2O_2 by enhancing APX activity under salt stress. This result is in agreement with Sheokand et al. (2008). MDHAR and DHAR are two important enzymes in regulating the AsA level and its redox state under stress (Eltayeb et al. 2006, 2007; Wang et al. 2010). The results obtained in this study showed a slight decrease in the MDHAR and DHAR activities under salt stress, which were accompanied by a decreased level of AsA (Figs. 2a

and 3b, c), indicating insufficient regeneration of AsA from MDHA and DHA under severe salt stress (Hernandez et al. 2000; Shalata et al. 2001). However, the NO pre-treated seedlings under salt stress showed significant increases in their MDHAR and DHAR activities (Fig. 3b, c), accompanied by an increased level of AsA, compared to the seedlings treated with salt alone (Fig. 2a). This result indicates that NO must have a role in AsA recycling under moderate salt stress (150 mM). The AsA levels found in the present study also support this, and our results are in agreement with the results of Shi et al. (2007). GR is responsible for recycling GSSG to GSH and controls the redox couples in plant cells. It has been observed that stress-tolerant plants tend to have high GR activities (Mittova et al. 2003b; Sekmen et al. 2007). In our study, a slight decrease in GR activity was observed at the 150 mM NaCl stress, whereas a significant decrease was observed at the 300 mM NaCl stress. These results agree well with those of Bandeoglu et al. (2004), who observed that the GR activity decreased under severe salt stress conditions. In contrast, the NO pre-treated salt-stressed seedlings showed sharp increases in GR activity, which were significantly higher than those of the seedlings treated with salt alone (Fig. 3d). Increases in the GR activity of NO-treated seedlings have also been reported (Laspina et al. 2005; Sang et al. 2008; Xu et al. 2010). The increased GR activity by NO under salt stress maintained a higher GSH/GSSG ratio and GSH level (Hoque et al. 2007; Hossain and Fujita 2010; Hossain et al. 2010, 2011).

Glutathione *S*-transferases constitute a family of multifunctional enzymes which have been shown to confer tolerance to abiotic stress (Fujita and Hossain 2003; Hossain et al. 2011). GPX is considered as an important ROS scavenger because of its broader substrate specificities and stronger affinity for H_2O_2 compared to CAT (Brigelius-Flohé and Flohé 2003). Salt stress led to an increase in GST activity, and a robust increase was observed under severe salt stress (Fig. 4a). Increased GST and GPX activities in response to drought and salinity stress have also been reported (Halušková et al. 2009; Hasanuzzaman and Fujita 2011; Hasanuzzaman et al. 2011). Meanwhile, additional increases in GPX and GST activities by NO might provide a protective role against NaCl-induced oxidative damage which was consistent with a previous report (Laspina et al. 2005). CAT is a key antioxidant enzyme which decomposes H_2O_2 to H_2O. In our experiment, we did not find any significant change in CAT activity in response to salt stress. This result was corroborated with the findings of de Azevedo Neto et al. (2006) and Da Costa et al. (2005). In contrast, at 150 mM NaCl stress, the NO pre-treated seedlings showed a significant increase in CAT activity. Similar to our result, significant increases in CAT activity by NO treatment were also reported in different crops under various abiotic stresses (Jin et al. 2010; Xu et al. 2010). However, at 300 mM NaCl, the NO pre-treatment had little influence on CAT activity.

Efficient MG detoxification has been used as a potential biochemical marker for plant stress tolerance. At a high concentration of MG, GSH may be trapped as *S*-2-hydroxyacylglutathione, resulting in GSH depletion (Kalapos et al. 1992). Higher Gly I and Gly II activities might protect plants against the MG that is formed during abiotic stresses (Veena et al. 1999; Jain et al. 2002; Hossain et al. 2009, 2010; Hossain and Fujita 2010; Hasanuzzaman et al. 2011). Our study showed that, upon the imposition of salt stress, Gly I and Gly II activities decreased. These results agree well with recent reports (Hoque et al. 2007; El-Shabrawi et al. 2010). These decreases in Gly I and Gly II activities suggest that the detoxification of MG via the glyoxalase system is not sufficient. In contrast, the NO pre-treated seedlings maintained higher Gly I and Gly II activities, thus creating the possibility of the up-regulation of the GSH level and GSH/GSSG ratio via the glyoxalase system (Fig. 2b, d).

Finally, the present study suggests that salinity-induced oxidative damage in wheat seedlings, as indicated by the higher levels of H_2O_2 and MDA (Fig. 6a, b), is probably due to the inhibition or insufficient induction of the antioxidant defense and MG detoxification systems. In contrast, the NO pre-treated seedlings maintained significantly lower H_2O_2 and MDA levels, compared to the seedlings treated with salt alone. Similar protective effects of NO under salt stress have been reported by other researchers (Li et al. 2008; Sheokand et al. 2008; Zheng et al. 2009). The biochemical results of our experiments were well correlated with the phenotypic appearance of the seedlings (Fig. 7). However, the complete elucidation of the regulatory role of endogenous NO, as well as its detailed signaling mechanism, would be helpful in improving our understanding of NO-mediated salt stress tolerance.

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