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Modulation of antioxidant enzyme activities and metabolites ratios by nitric oxide in short-term salt stressed soybean root nodules

I. Egbichi, M. Keyster, A. Jacobs, A. Klein and N. Ludidi

Abstract

Several abiotic factors cause molecular damage to plants either directly or through the accumulation of reactive oxygen species such as hydrogen peroxide (H_2O_2). We investigated if application of nitric oxide (NO) donor 2,2′-(hydroxynitrosohydrazono) bis-ethanimine (DETA/NO) could reduce the toxic effect resulting from short-term salt stress. Salt treatment (150 mM NaCl) alone and in combination with 10 μM DETA/NO or 10 μM DETA were given to matured soybean root nodules for 24 h. Salt stress resulted in high H_2O_2 level and lipid peroxidation while application of DETA/NO effectively reduced H_2O_2 level and prevented lipid peroxidation in the soybean root nodules. NO treatment increased the activities of ascorbate peroxidase and dehydroascorbate reductase under salt stress. Whereas short-term salt stress reduced AsA/DHAsA and GSH/GSSG ratios, application of the NO donor resulted in an increase of the reduced form of the antioxidant metabolites thus increasing the AsA/DHAsA and GSH/GSSG ratios. Our data suggests a protective role of NO against salt stress.

1. Introduction

Abiotic stresses such as extreme temperatures, drought, salinity and chemical toxicity have been associated with pronounced decline in crop yield worldwide (Boyer, 1982; Bray et al., 2000). Amongst these stress factors, salinity is considered as one of the major factors that hinder plant growth and productivity (Pitman and LaEuchli, 2002; Majeed et al., 2010). One of the effects of salinity is oxidative damage at the cellular level and is due to increased production of reactive oxygen species (ROS) such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO•) in plant cells (Mittler, 2002). Accumulation of ROS causes cellular damage through oxidation of lipids, proteins and nucleic acids (Foyer and Noctor, 2000). There are several lines of evidence associating high salinity with changes in lipid metabolism. Amongst the various biomolecules, polyunsaturated fatty acids (PUFA) are the most susceptible targets to oxidative attacks mediated by ROS (Spiteller, 2003). Reaction of ROS with the PUFA leads to peroxidation which subsequently leads to degradation of biological membranes,

rapid desiccation and cell death (Skrzyn and Krupa, 2006). Amongst the different ROS, H_2O_2 is regarded as the most stable and at low concentration functions as a signaling molecule (Desikan et al., 2004) but at high concentration, it becomes toxic and leads to programmed cell death, hence it is crucial for plants to regulate H_2O_2 intracellular concentrations (Quan et al., 2008).

Nitric oxide (NO) is well recognized as an important signaling molecule in plants and is involved in several physiological processes such as promotion of seed germination or reduction of seed dormancy (Beligni and Lamattina, 2000; Bethke et al., 2006, 2007; Libourel et al., 2006), regulation of plant development and senescence (Leshem et al., 1998; Guo and Crawford, 2005; Mishina et al., 2007) and suppression of floral transition (He et al., 2004). There are other reports suggesting the role of NO in regulating the expression of genes involved in nodule development and nodule functioning in Medicago truncatula (Ferrarini et al., 2008). More importantly, there are several studies showing an increase of NO production under unfavorable environmental conditions, hence suggesting the role of NO in mediating responses to abiotic stresses such as heat (Leshem and Haramaty, 1996), drought, ultraviolet radiation (Mackerness et al., 2001), extreme temperature (Neill et al., 2003; Zhao et al., 2009) and heavy metals (Kopyra and Gwozdz, 2003).

Plants are fully equipped with an array of antioxidant defenses aimed at protecting them from the oxidative effects exerted by ROS. Amongst the anti-oxidant enzymes, ascorbate peroxidase (APX.EC1.11.1.11) is crucial in regulating the level of H_2O_2 in plants and utilizes ascorbate (AsA) as its specific electron donor to reduce H_2O_2 into H_2O with the concomitant generation of monodehydroascorbate (MDHA) (Dalton et al., 1986, 1993; Iturbe et al., 2001) and oxidized form of AsA-dehydroascorbate (DHAsA) (Zhang and Kirkham, 1996). The antioxidant metabolite AsA plays an essential role in the removal of H_2O_2 by either reacting directly with H_2O_2 or via a reaction catalyzed by APX. It is regenerated from MDHA and DHAsA in a reaction catalyzed by NAD(P) H-dependent monodehydroascorbate reductase (MDHAR, E.C. 1.6.5.4) and dehydroascorbate reductase (DHAR, E.C. 1.8.5.1). Glutathione GSH is crucial in the regeneration of AsA and as such also functions in the regulation of H_2O_2 concentration and control of redox state in plants (Kocsy et al., 2000, 2001).

There are studies (Meneguzzo et al., 1999; Sairam et al., 2005) correlating, amongst other antioxidant enzymes, the rate and extent of increase of APX enzymatic activity under salt stress with plant tolerance to salinity. Furthermore, application of the NO donor 2,2′- (hydroxynitrosohydrazono) bis-ethanimine (DETA/NO) resulted in an increase in NO content and enzymatic activity of three soybean APX isoforms in a dose-dependent manner in a study by Keyster et al. (2011). We thus investigated the degree of salt stress on nodulated

soybean (Glycine max L. Merr. cv. PAN 626) root exposed to high salt concentrations for a period of 24 h and the mechanism through which NO could reduce the toxic effects of salt stress.

2. Materials and method

2.1. Growth and treatment of plants

Plant growth and treatments were done by modifying a method previously described by Leach et al. (2010). Soybean (G. max L. Merr. cv. PAN 626) seeds were surface-sterilized in 0.35% sodium hypochlorite for 10 min, followed by five washes with sterile distilled water. The seeds were imbibed in sterile distilled water for 1 h and inoculated with Bradyrhizobium japonicum supplied as the commercial peat-based HiStick 2 Soybean Inoculant (Becker Underwood Ltd.). Seeds were sown in filtered silica sand that had been pre-soaked in distilled water, in 15 cm diameter plastic pots (one plant per pot) and the sand was kept moist by watering only with distilled water during germination.

The germinated seedlings were grown on a 25/19 °C day/night temperature cycle under a 16/8 hour light/dark cycle, at a photosynthetic photon flux density of 300 μmol photons·m^{-2}·s^{-1} during the day phase, in a completely randomized design. Once the plants reached the VC stage (when unifoliolate leaves are fully expanded), they were supplied with nitrogen free nutrient solution at a three day interval until they reached the V3 stage (third trifoliolate).

Plants of the same phenological stage and similar height were selected for all experiments. The treatment was performed once the plants were at the V3 stage. The plants were chosen randomly and divided into six groups. The first group, treated with nitrogen-free nutrient solution only, served as the untreated control. The second group was treated with nitrogen-free nutrient solution containing 10 μM DETA/NO. The third group was treated with nitrogen-free nutrient solution containing 10 μM DETA, serving as a negative control. The fourth, fifth and sixth groups were treated with nitrogen free nutrient solution containing 150 mM NaCl, 150 mM NaCl plus 10 μM DETA and 150 mM NaCl plus 10 μM DETA/NO respectively.

2.2. Protein extraction from nodule tissue

Extracts were obtained from soybean root nodules by grinding the nodule tissue into a fine powder in liquid nitrogen and homogenizing 500 mg of the tissue with either 1 ml of homogenizing buffer [40 mM K_2HPO_4, pH 7.4, 1 mM ethylene diamine tetra-acetic acid (EDTA), 5% (w/v) poly vinyl pyrrolidone (PVP) molecular weight = 40,000] for the determination of APX enzymatic activity, antioxidant metabolites and estimation of DHAR activity or 10% trichloroacetic acid (TCA) for H_2O_2 content and lipid peroxidation. The resulting homogenates were centrifuged at 12,000 ×g for 20 min at 4 °C and the supernatants were used for biochemical assays.

2.3. Measurement of H_2O_2 content

H_2O_2 content was determined in the nodule extracts by modifying a method previously described by Velikova et al. (2000). G. max nodule tissue (100 mg) was ground to a fine powder in liquid nitrogen and homogenized in 400 µl of cold 6% (w/v) TCA. The extracts were centrifuged at 12,000 ×g for 30 min at 4 °C and 50 µl of the supernatant was used to initiate the reaction in a mixture (total volume of 200 µl) containing 5 mM K_2HPO_4, pH 5.0 and 0.5 M KI. The reaction was incubated at 25 °C for 20 min and absorbance readings were recorded at 390 nm. H_2O_2 content was calculated using a standard curve based on the absorbance ($A_{390\ nm}$) of H_2O_2 standards.

2.4. Lipid peroxidation

Lipid peroxidation was determined in soybean root nodules by measuring malondialdehyde (MDA) formation, using the thiobarbituric acid (TBA) method as previously described by Buege and Aust (1978). Plant tissue (100 mg) was ground into a fine powder in liquid nitrogen. The tissue was homogenized in 400 µl of cold 5% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 ×g for 30 min at 4 °C. Aliquots (100 µl) of the supernatant were mixed with 400 µl of 0.5% TBA (prepared in 20% TCA). The mixture was incubated at 95 °C for 30 min and the reaction was stopped by placing the mixture on ice for 5 min. The mixture was further centrifuged at 12,000 ×g for 5 min at 4 °C. The absorbance of the supernatant was measured at 532 nm and 600 nm. After subtracting the non-specific absorbance ($A_{600\ nm}$), the MDA concentration was determined by its extinction coefficient of 155 $mM^{-1}\ cm^{-1}$and expressed as nmol g^{-1} of fresh weight.

2.5. Determination of APX enzymatic activity

Plant APX activities were measured in nodule extracts by modifying a method previously described by Asada (1984). The nodule extracts which were supplemented with ascorbate to a final concentration of 2 mM, were added to the assay buffer containing 50 mM K_2HPO_4, pH 7.0, 0.1 mM EDTA and 50 mM ascorbate. The reaction was initiated by adding 1.2 mM H_2O_2 in a final reaction volume of 200 µl and APX activity was calculated based on the change in absorbance at 290 nm using the extinction co-efficient of 2.8 $mM^{-1}\ cm^{-1}$.

For the determination of the response of G. max APX isoforms to exogenously applied NO under salinity stress, electrophoretic APX separation was carried out as previously described by Mittler and Zilinskas (1993) and non-denaturing polyacrylamide gel electrophoresis was performed at 4 °C in 7.5% polyacrylamide mini gels. Prior to loading extracts containing 50 µg of protein into the wells, gels were equilibrated with running buffer containing 2 mM ascorbate for 30 min at 4

°C. After the electrophoresis, gels were incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 20 min and then transferred to solutions containing 50 mM potassium phosphate buffer (pH 7.8), 4 mM ascorbate and 2 mM H_2O_2 for 20 min. The gels were washed in the buffer for a minute and submerged in a solution of 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM N,N,N′, N′-tetra methyl ethylene di-amine and 2.5 mM nitroblue tetrazolium for 10–20 min with gentle agitation in the presence of light. The gel images were captured and analyzed by densitometry using AlphaEase FC imaging software (Alpha Innotech Corporation).

2.6. **Antioxidant metabolite assay**

AsA and DHAsA were determined in soybean root nodules by modifying a method previously described by Law et al. (1983). The following solutions were freshly prepared accordingly: 0.1 M standard solutions of AsA and DHAsA dissolved in 6% (w/v) TCA, 10 mM dithiothreitol (DTT) dissolved in 0.2 M phosphate buffer (pH 7.4), 0.5% (w/v) N-ethylmaleimide (NEM) and 3% (w/v) $FeCl_3$. The reaction was carried out in a 96 well-plate and to measure the total ascorbate, the reaction mixture consisted of 10 µl of plant extract, 10 µl of 10 mM DTT, 10 µl of 0.5% NEM and 20 µl of 0.2 M phosphate buffer (pH 7.4). For AsA content, the reaction mixture contained 10 µl of plant extract, 30 µl of 0.2 M phosphate buffer (pH 7.4) and 10 µl distilled H_2O. The rest of the steps were similar for both estimations. The following were added accordingly to each well of the plates; 50 µl of 10% TCA, 40 µl of 42% H_3PO_4 and 40 µl of 4% 2,2′-dipyrydyl amimade in 70% (v/v) ethanol. The total reaction mixture was made up to 200 µl by the addition of 20 µl of 3% iron (III) chloride ($FeCl_3$). The solution was mixed and the plate was incubated at 42 °C for 15 min. The absorbance was recorded at 525 nm and DHAsA was calculated as the difference between total ascorbate and AsA.

Total (GSH + GSSG) and oxidized (GSSG) glutathione were determined in soybean root nodule extracts by modifying a method previously described by Griffith (1980). Nodule extracts (1 ml) were neutralized with 360 µl of 1 M triethanolamine and 40 µl of 2-vinyl pyridine was added for the measurement of GSSG to obtain GSH. The mixture was allowed to stand at room temp for 1 h. The rest of the steps were similar for both estimations of total and oxidized glutathione. The following were added accordingly to each well of the plates; 190 µl of reaction mixture consisting of 125 mM phosphate buffer (pH 7.5) containing 6.3 mM EDTA, 0.5 units of glutathione reductase, 0.3 mM NADPH, 6 mM 5-(3-carboxy-4-nitrophenyl)disulfanyl-2-nitrobenzoic acid (DTNB) and 10 µl of each of the above treated soybean root nodule extracts. Change in absorbance at 412 nm was recorded for 4 min. A reference curve was prepared with GSSG.

2.7. Determination of DHAR enzymatic activity

DHAR activity was measured in soybean root nodule extracts by modifying a method previously described by De Tullio et al. (1998). 10 µl of the plant extracts (50 µg of protein) were diluted in 40 µl distilled H_2O and added into each well of a 96 well plate containing 80 µl solution containing 2 mM GSH and 40 µl of 500 mM phosphate buffer (pH 7.0). For the blank solution, 80 µl of distilled H_2O was added in place of GSH. The reaction was initiated by the addition of 30 µl of 6.6 mM DHAsA. The assay measured the formation of AsA at 265 nm ($\epsilon = 14\ mM^{-1}\ cm^{-1}$). Changes in absorbance at 265 nm were followed for a minute. The rate of enzymatic DHAsA reduction was corrected by subtracting the values obtained in the absence of substrate GSH.

2.8. Determination of protein concentration

Protein concentrations for all assays were measured in the extracts as instructed for the RC DC Protein Assay Kit 11 (Bio-Rad Laboratories).

2.9. Statistical analysis

One-way analysis of variance (ANOVA) test was used for all data to evaluate statistical validity of the results and means were compared using Tukey–Kramer test at 5% level of significance, using GraphPad
Prism 5.03 software.

3. Result

3.1. H_2O_2 content in soybean root nodules

The level of H_2O_2 did not show any significant difference in the DETA (negative control) treated plants when compared with untreated controls (Fig. 1). Application of DETA/NO resulted in approximately 25% decrease in H_2O_2 levels when compared to the untreated control. H_2O_2 content was increased by approximately 15% in response to 150 mM NaCl when compared to untreated controls. However there was no significant difference in the level of H_2O_2 content between 150 mM NaCl and 150 mM NaCl combined with DETA. Application of 10 µM DETA/ NO in combination with 150 mM NaCl in soybean root nodule resulted in approximately 12% reduction of H_2O_2 content.

3.2 Changes in lipid peroxidation

Salt stress induced oxidative damage to membrane lipids, as revealed by the amount of malondialdehyde produced in salt-treated nodules. There was no marked difference in the lipid peroxidation level between the untreated control and DETA-treated soybean root nodules (Fig. 2). Soybean root nodules treated with 10 µM DETA/NO exhibited low levels of lipid peroxidation. Lipid peroxidation was reduced by approximately 24% in response to 10 µM DETA/NO when compared to untreated controls. The injury caused by salt to cellular membranes due to lipid peroxidation as reflected by the accumulation of the

MDA levels was significantly increased by the addition of 150 mM NaCl. Nodules treated with 150 mM NaCl showed a 20% increase in lipid peroxidation when compared to the untreated control. A similar trend was also observed in soybean root nodules treated with 10 μM DETA in combination with 150 mM NaCl. Interestingly, exogenous application of 10 μM DETA/NO combined with 150 mM NaCl resulted in almost complete amelioration of the toxic effect of salt stress on lipid peroxidation. As shown in Fig. 2, there was no marked significant difference on the level of lipid peroxidation in the 10 μM DETA/ NO combined with 150 mM NaCl treated soybean root nodules when compared to the untreated control.

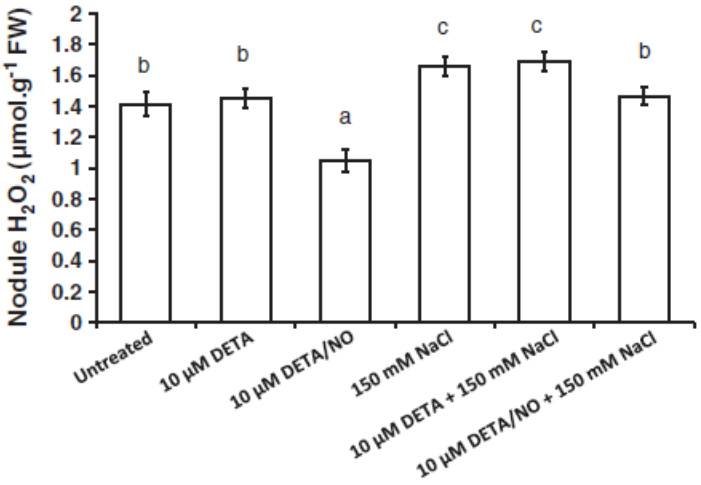

Fig. 1. Effect of exogenously applied NO (10 μM DETA/NO) and salt stress (150 mM NaCl) on soybean root nodule H_2O_2 content. Error bars represent the mean (±SE; n = 3) from data that are representative of three independent experiments. Values sharing a common letter are not significantly different at $p < 0.05$.

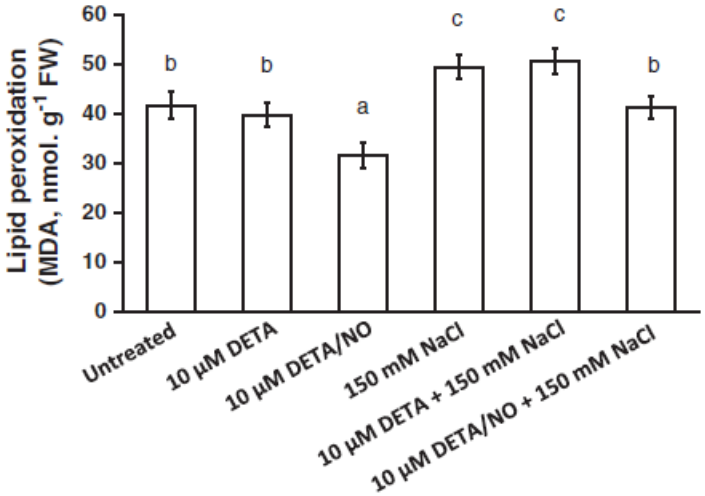

Fig. 2. Effect of 150 mM NaCl and exogenously applied NO (10 μM DETA/NO) or DETA (10 μM) on lipid peroxidation in soybean root nodules. Error bars represent the mean (±SE; n = 3) from data that are representative of three independent experiments. Values sharing a common letter are not significantly different at $p < 0.05$.

3.3 Effect of DETA/NO on total APX enzymatic activity in salt-treated soybean root nodules

Given that there was a marked decrease in the level of H_2O_2 and lipid peroxidation in 10 μM DETA/NO treated plants as well as those treated with 10 μM DETA/NO combined with 150 mM NaCl, we analyzed the effect of exogenous application of DETA/NO on total APX enzymatic activity in salt-treated soybean root nodules. There was no marked difference in the total APX enzyme activity between the untreated control and DETA-treated soybean root nodules (Fig. 3). However APX activities showed an increase in response to both salinity stress and the NO donor. The NO-induced increase in APX activity was 2.8-fold higher in response to 10 μM DETA/NO than the controls, whereas the APX activity was approximately 65% higher in response to 150 mM NaCl when compared to the untreated controls. There was also no significant difference in the level of APX activity in response to 150 mM NaCl when compared to 10 μ M DETA + 150 mM NaCl. Interestingly, treatment of soybean root nodules with 10 μM DETA/NO + 150 mM NaCl resulted in the highest up-regulation of total APX enzymatic activity in root nodules. The increase in APX activity observed was 3.5-fold higher than the untreated control.

3.4 Effect of exogenously applied NO on three APX isoforms in NaCl treated soybean root nodules

Since the total APX activity was differentially upregulated in response to various treatments we further investigated the response of individual APX isoforms to NO in NaCl-stressed soybean root nodules using in-gel APX enzymatic assays coupled with pixel intensity analyses. Soybean root nodules treated with 150 mM NaCl or its combination with DETA; NO donor (10 μM DETA/NO) or its combination with 150 mM NaCl showed higher increase in APX activity in an ascending order (Fig. 4). This is visually evident from the varying intensities of the isoforms of the APXs in the native gel. However, further observation suggests that APX activity bands from the untreated and DETA treated samples have similar intensity.

Densitometry analyses of activity gels (Fig. 5A) showed that the enzymatic activity of GmAPX 1 was up-regulated by approximately 34% in response to 10 μM DETA/NO when compared to GmAPX 1 enzymatic activity of untreated root nodule tissue. Also, pixel intensities obtained indicate an up-regulation of enzyme activity by approximately 27% in response to 150 mM NaCl when compared to GmAPX 1 enzymatic activity of untreated root nodule tissue. A similar level of enzyme activity was observed in response to treatment with the combination of 10 μM DETA and 150 mM NaCl. Interestingly, pixel intensities obtained from the combined treatment of 150 mM NaCl and 10 μM DETA/NO had the highest value.

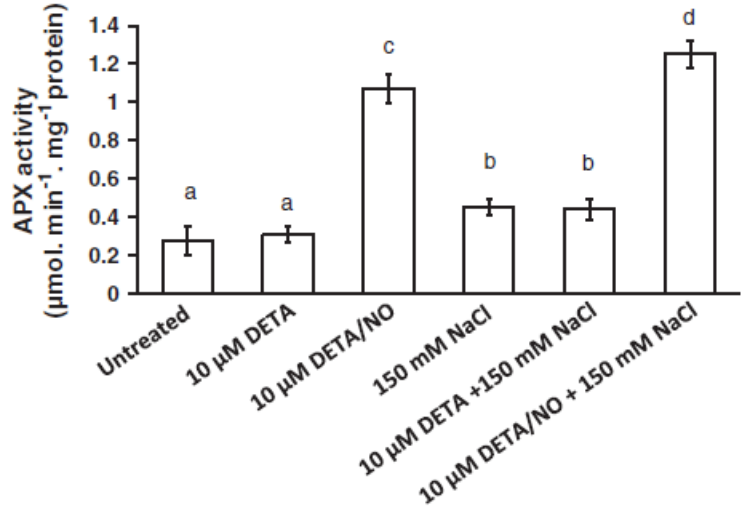

Fig. 3. Effect of exogenously applied NO (10 µM DETA/NO or 10 µM DETA) and salt (150 mM or 10 µM DETA + 150 mM NaCl) on APX activity in soybean root nodules. Error bars represent the mean (±SE; n = 3) from data that are representative of three independent experiments. Values sharing a common letter are not significantly different at $p < 0.05$.

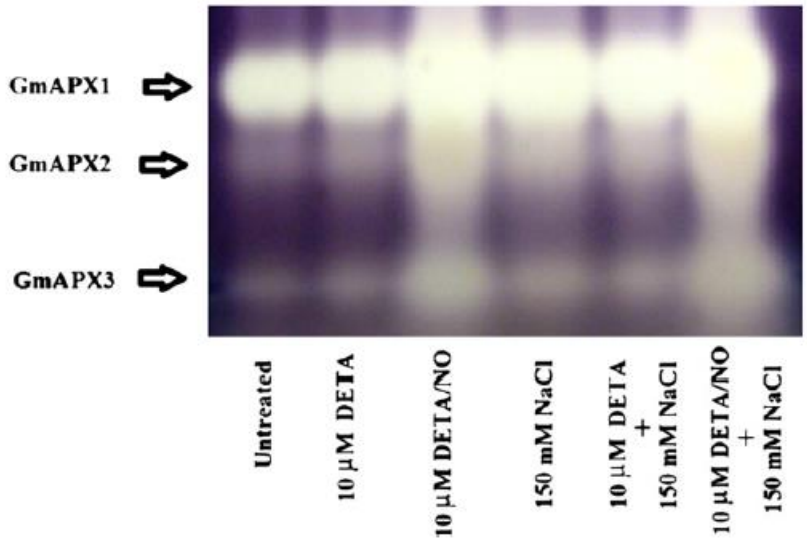

Fig. 4. Effect of NO and NaCl on APX activity of *Glycine max* roots. Lanes 1–6: Untreated, 10 µM DETA, 10 µM DETA/NO, 150 mM NaCl, 10 µM DETA + 150 mM NaCl and 10 µM DETA/NO + 150 mM NaCl respectively. The three isoforms are referred to as GmAPX 1, GmAPX 2 and GmAPX 3 on the basis of their migration on the native PAGE gel.

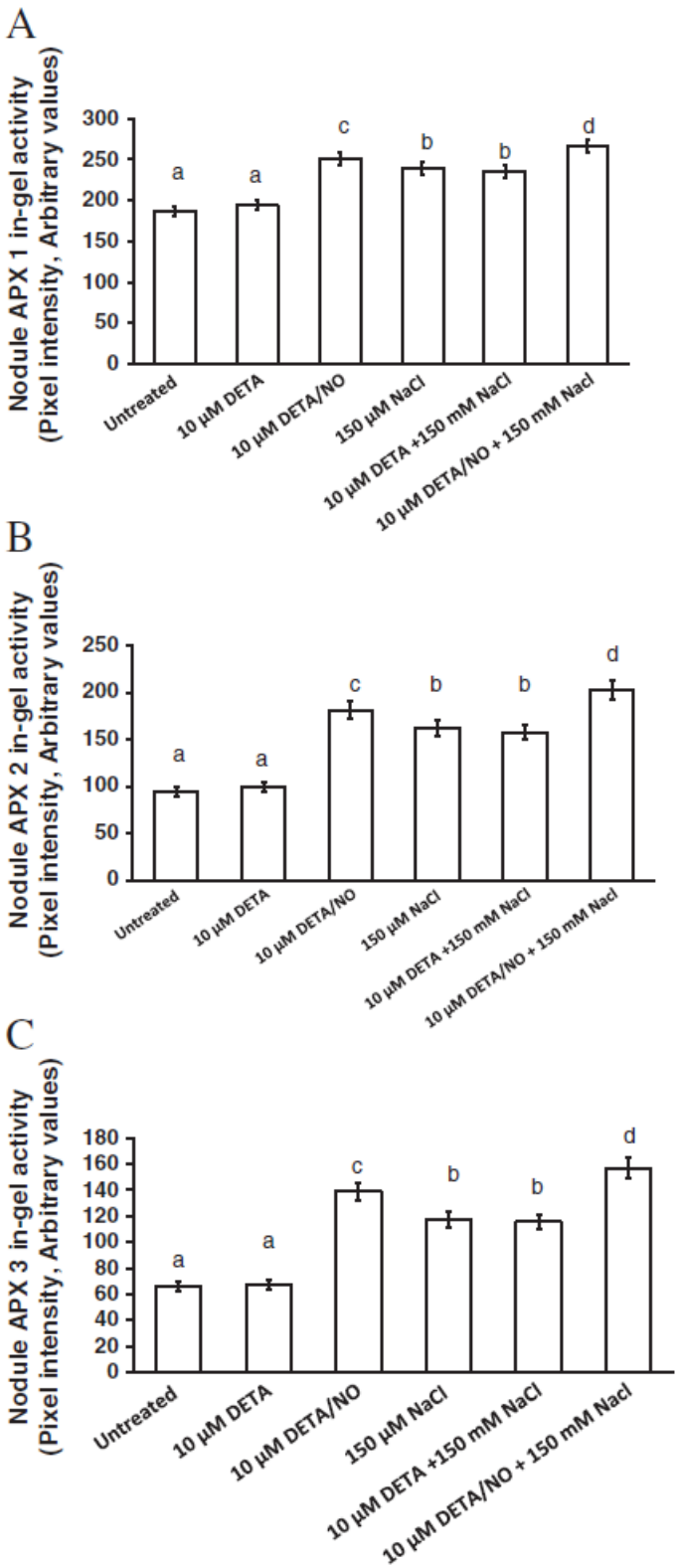

Fig. 5. Pixel intensities signifying the level of enzymatic activity of nodule GmAPX 2 isoform, derived from analysis of the intensity of the bands. Response of (A) GmAPX 1 (B) GmAPX 2 (C) GmAPX 3 to treatment with 10 µM DETA, 10 µM DETA/NO, 150 mM NaCl, 10 µM DETA + 150 mM NaCl or 10 µM DETA/NO + 150 mM NaCl. Error bars represent the means (±SE; n = 3) of three independent experiments. Values sharing a common letter are not significantly different at $p < 0.05$.

The enzymatic activity of GmAPX 1 was up-regulated by approximately 42% in response to 10 μM DETA/NO + 150 mM NaCl when compared with GmAPX 1 enzymatic activity of soybean untreated sample.

The enzymatic activity of GmAPX 2 was up-regulated by approximately 92% in response to 10 μM DETA/NO when compared to the activity of GmAPX 2 in the untreated sample (Fig. 5B). The activity of GmAPX 2 in response to salt treatment or a combination of NaCl with DETA, which was also similar for these two treatments, was less than those of the plants treated with the NO donor. The enzyme was up regulated by approximately 67% in response to the salt treatment, however the enzyme activity was more induced in the combined treatment of NaCl and NO donor, with an up-regulation of approximately 115% in response to the 10 μM DETA/NO + 150 mM NaCl treatment.

Densitometry analysis of the GmAPX 3 (Fig. 5C) also shows a similar trend of enzyme induction by the various treatments as observed for GmAPX 2 (Fig. 5B). Whereas there was no significant difference for GmAPX 3 in response to the DETA treatment when compared with the untreated, GmAPX 3 was up-regulated by approximately 110% in response to 10 μM DETA/NO, approximately 77% in response to 150 mM NaCl or its combination with 10 μM DETA and induction of this isoform increased by 137% in response to 10 μM DETA/NO + 150 mM NaCl when compared to GmAPX 3 activity of the untreated root nodule tissue.

3.5 Levels of AsA and DHAsA

In view to the fact that the metabolism of H_2O_2 involving APX utilizes AsA as its electron donor, cellular levels of AsA and DHAsA were investigated in the variously treated soybean root nodules. There was no significant difference in AsA or DHAsA content of DETA treated soybean nodules when compared with the untreated samples (Fig. 6A and B). Whereas there was a pronounced increase in AsA content in DETA/NO-treated soybean root nodules when compared to the untreated control and the corresponding increase in the level of its oxidized form (DHAsA) was moderate when compared to the untreated control. AsA was increased by approximately 43% and DHAsA by 20% in DETA/NO-treated soybean root nodules when compared to the level of the antioxidant metabolites in untreated levels. Treatment of soybean root nodules with 150 mM NaCl (either alone or in combination with 10 μM DETA) resulted in a slight increase of AsA and a higher increase in DHAsA. The salt-treated soybean root nodules had an increase of 20% in their AsA content whereas the increase in DHAsA was approximately 67% when compared to their respective untreated controls. However supplementing the salt treatment with an NO donor (10 μM DETA/NO) restored AsA content considerably although not to the levels of the untreated control and a similar restoration of DHAsA was observed for this treatment.

Whereas there was no significant difference on the antioxidant ratio between the untreated and DETA-treated soybean root nodules, a decrease (26%) in the AsA/DHAsA ratio was observed on the salt-treated soybean root nodules when compared with the untreated samples (Fig. 6C). However, DETA/NO treatments had a positive effect towards increasing (19%) the ascorbate ratio.

3.6 Levels of GSH and GSSG

Since GSH is a crucial antioxidant that participates in the AsA–GSH cycle for the scavenging of H_2O_2, the effect of exogenously applied NO, as DETA/NO, on GSH content in salinity-induced oxidative stress in soybean root nodules was investigated. Whereas there was no significant difference in the levels of GSH and GSSG in the 10 μM DETA-treated when compared to untreated values, a marked increase of GSH and a corresponding marginal increase in GSSG in response to 10 μM DETA/ NO were observed (Fig. 7A and B). GSH level showed approximately 63% increase in response to 10 μM DETA/NO whereas a 23% increase in the level of GSSG was observed in response to this treatment. On the other hand, a moderate increase of GSH with a high increase in GSSG levels was observed in response to 150 mM NaCl. This suggests an induction of oxidative stress by salt. GSH levels showed a 20% increase whereas the GSSG levels showed approximately 63% increase in nodules exposed to 150 mM NaCl. This trend was similar in the treatment where 150 mM NaCl was combined with 10 μM DETA. However the combination of 10 μM DETA/NO with 150 mM NaCl was effective in increasing GSH level by 50% when compared to the untreated plants but there was also an increase of GSSG by 52%.

Application of the NO donor was effective in increasing the GSH/ GSSG ratio by 35% when compared to the untreated controls (Fig. 7C). The salt treatment resulted to a decrease of the GSH/GSSG ratio by approximately 26% when compared to the untreated samples. However, supplementation of 10 μM DETA/NO to 150 mM NaCl was able to restore the shift of GSH from an oxidized state to the reduced state hence maintaining the GSH/GSSG ratio at a level similar to the level of nodules from untreated plants.

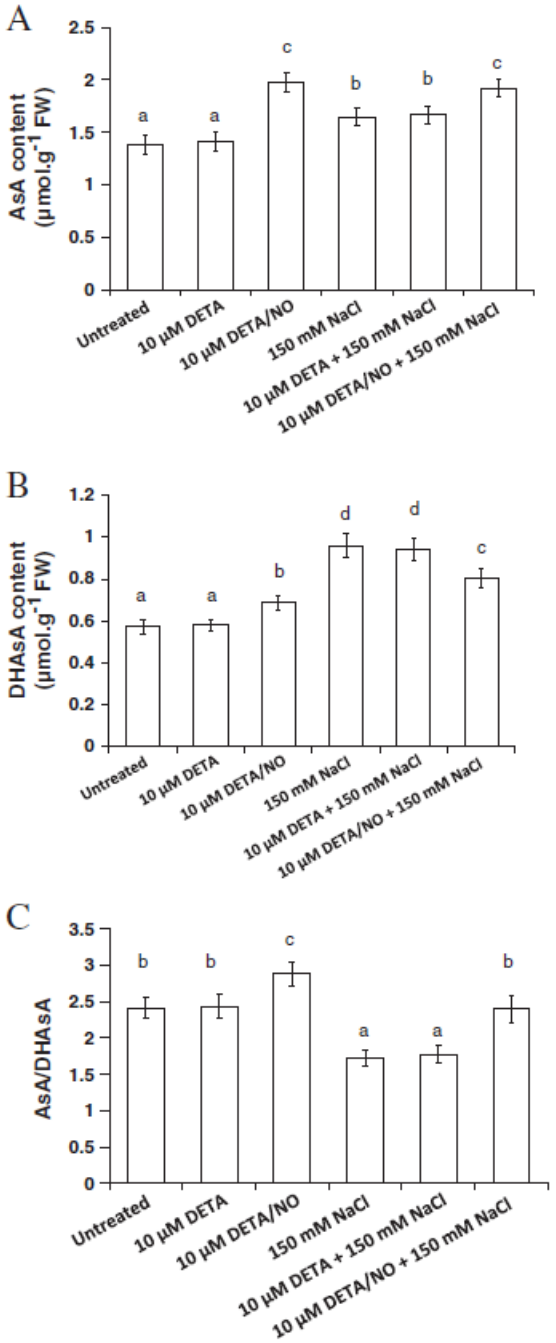

Fig. 6. Effect of NaCl and DETA/NO treatments on ascorbate content (A), DHAsA content (B) and ascorbate redox ratio (C) in soybean root nodules. Treatments: Untreated, 10 µM DETA, 10 µM DETA/NO, 150 mM NaCl, 10 µM DETA + 150 mM NaCl and 10 µM DETA/NO + 150 mM NaCl. The data are mean values ± SE ($n = 3$). Values sharing a common letter are not significantly different at $p < 0.05$.

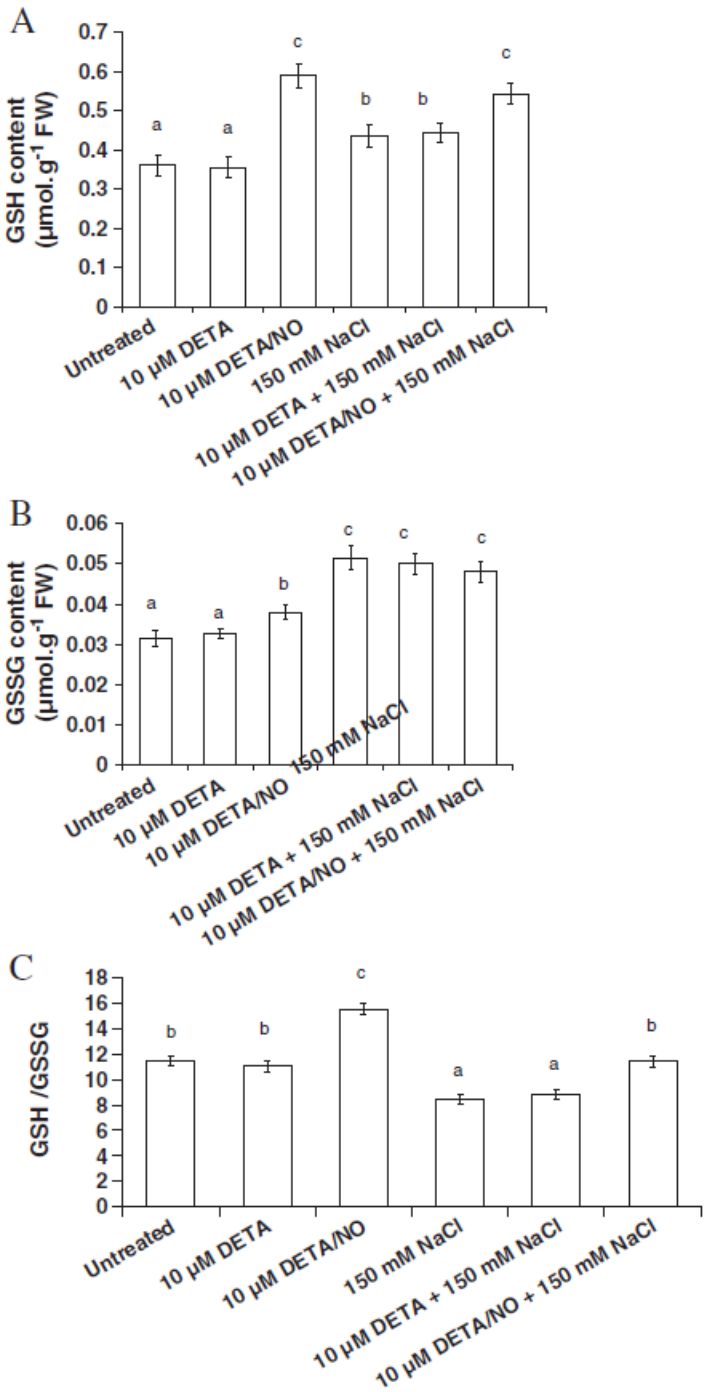

Fig. 7. Effect of NaCl and DETA/NO treatments on glutathione content (A), GSSG content (B) and glutathione redox ratio (C) in soybean root nodules. Treatments: Untreated, 10 µM DETA, 10 µM DETA/NO, 150 mM NaCl, 10 µM DETA + 150 mM NaCl and 10 µM DETA/NO + 150 mM NaCl. The data are mean values ± SE (n = 3). Values sharing a common letter are not significantly different at $p < 0.05$.

3.7. Effect of NO donor and salt on soybean root nodule DHAR activity

DHAR catalyzes the reduction of DHAsA to AsA by utilizing reduced GSH. Since the product of this reaction is required by APX as an electron donor for scavenging of H_2O_2, further investigation was carried out to determine the effect of NO on DHAR

activity in salinity-induced stress in soybean root nodules. When compared with untreated control plants, treatment with 10 μM DETA exhibited no significant difference in DHAR activity (Fig. 8). However treatment of soybean root nodules with the NO donor resulted to an increased (64%) DHAR activity. Although an increase in DHAR activity was observed in response to both 150 mM NaCl and 150 mM NaCl combined with 10 μM DETA, the increase was less pronounced (18% increase of DHAR activity) in these treatments when compared with the increase caused by the NO donor. However, supplementation of the salt treatment with NO (150 mM NaCl + 10 μM DETA/NO treatment) resulted in elevation of DHAR activity by 40% when compared to the enzymatic activity of untreated soybean nodules.

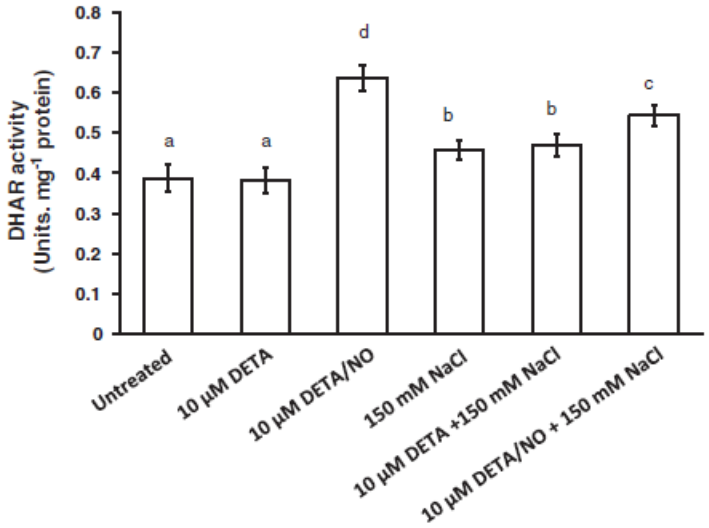

Fig. 8. Effect of exogenously applied NO (10 μM DETA) and salt (150 mM NaCl) on DHAR activity in soybean root nodules. Error bars represent the mean (±SE; n = 3) from data that are representative of three independent experiments. Values sharing a common letter are not significantly different at $p < 0.05$.

4. Discussion

We have shown that application of 150 mM NaCl, either alone or combined with 10 μM DETA, to the soybean root nodules caused lipid peroxidation. This effect of salt stress on lipid peroxidation has previously been described (Shi et al., 2007; Li et al., 2008). However the data obtained from this study suggests that application of DETA/NO can protect plants from the effect of salt induced-membrane damage. This is evident not only from the ability of NO released from DETA/NO to maintain cellular membrane integrity as evidenced by the low level of lipid peroxidation of the soybean root nodules but also by reducing the level of lipid peroxidation when supplemented on salt-treated plants. A similar protective effect of NO on membrane injury has been reported under salt stress (Zhao et al., 2004). This protective role of NO against salt-induced membrane lipid peroxidation could be attributed to its ability to scavenge reactive intermediates (Kopyra and Gwozdz, 2003). Furthermore, some evidence suggests that the reaction

of NO with lipid peroxyl radicals reduces lipid peroxidation (Beligni and Lamattina, 1999; Van Breusegem et al., 2001).

Apart from extensive induction of lipid peroxidation, salinity stress is also associated with increased production of H_2O_2 (Sudhakar et al., 2001). The accumulation of H_2O_2 during salinity stress can arise as a result of the imbalance in the rate of production and removal of ROS. As such, H_2O_2 content is often used as a marker that indicates the extent of oxidative stress in plant cells. We showed that samples treated with salt either alone or salt in combination with DETA recorded the highest level of H_2O_2 content. The result obtained from this study is similar, with reference to increased H_2O_2 content, to those observed in response to salt stress in various other studies using plant species such as rice (Uchida et al., 2002), barley (Li et al., 2008) and cucumber (Shi et al., 2007) and were also associated with oxidative stress. Application of NO decreased the H_2O_2 content and reversed the salinity-induced stress. This effect of NO on H_2O_2 has been reported in a similar study (Shi et al., 2007) thus suggesting that NO is a crucial molecule involved in tolerance to salt stress.

Tolerance to oxidative stress is mostly associated with increased antioxidant activity. This suggests that the underlying mechanism for the protective role of NO against oxidative damage could depend on its role as a signaling molecule involved in the activation of plant antioxidant enzymes. In our study, the total activities of APX and DHAR under salt stress were increased when compared to untreated controls, a mechanism often regarded as a defense response against salt stress (Mittova et al., 2004). The marginal increase of APX enzymatic activity in response to salt treatment or the combination of salt with the DETA, and the corresponding increased lipid peroxidation and H_2O_2 content in soybean root nodules in these treatments point towards limited ability of the plant to induce its antioxidant system sufficiently to improve salt tolerance. Exogenously applied NO promoted APX and DHAR activities and this was maintained in treatments where NO was added in combination with salt, which was associated with efficient removal of H_2O_2 from the cell. These findings are in agreement with other studies which reported an NO-induced increase in APX and DHAR under salt stress in cucumber roots (Shi et al., 2007) and APX induction under salt stress in barley leaves (Li et al., 2008). The inference made from the total APX activity can also be applied on the native gel staining of APX activity where NO increased the pixel intensities of the three APX isoforms, clearly showing NO-enhanced APX activity. Furthermore, analysis of the in-gel enzymatic activities also indicates that the effect of NO on the three APX isoforms was more pronounced when combined with 150 mM NaCl. It is evident that NO functions as an antioxidant and the results obtained from this study are supported by data in other literature (Lamattina et al., 2003; Laspina et al., 2005).

For effective conversion of H_2O_2 to H_2O, APX requires two molecules of AsA and also depends on DHAR for the reduction of DHAsA to AsA — a reaction that requires GSH as the reducing substrate (Foyer and Halliwell, 1976; Nakano and Asada, 1987; Mehlhorn et al., 1996). These antioxidants are important redox molecules that participate in the AsA/GSH cycle and their redox status directly affects the activity of APX. In fact, the ratios of these antioxidant metabolites are mostly used as markers of redox status.

The results presented in this work show that 150 mM NaCl directly affects the total AsA level, increasing the oxidation of AsA to DHAsA and this resulted in decreased AsA/DHAsA ratio. Although the DETA treatment had no significant effect on the AsA/DHAsA ratio, application of 10 μM DETA/NO increased the ratio in the absence of NaCl and effectively alleviated the effect of NaCl by maintaining the AsA/DHAsA ratios to a level similar to those of the untreated plants. The fact that NO maintained the AsA/DHAsA ratio in the salt treatment is likely through increased DHAR activity and subsequent effective AsA regeneration. This is evident from the increased DHAR activity in response to supplementation of NO to salt-treated samples. The data obtained from NaCl alone or in combination with DETA, which lacks the NO moiety, shows a slight increase of DHAR activity, which is linked to the decreased AsA/ DHAsA ratio. This study suggests that salinity-induced stress restricts the level of induction of DHAR activity while allowing for consumption of AsA by the elevated APX activity and the resulting effect is a poor AsA/DHAsA ratio.

The GSH content increased slightly under salt treatment (either alone or combined with DETA) whereas there was highly elevated GSSG content in these treatments. It is possible that the accumulation of GSSG in 150 mM NaCl could be due to the reaction of GSH with ROS generated by the high salt level. Hence a decrease in the GSH/GSSG ratio was observed. Whereas DETA/NO treatment increased the ratios much above those in the untreated controls, NO was able to increase the GSH levels under salt stress in order to compensate for the high GSSG. As a result, the GSH/GSSG ratio was restored by exogenously applied NO in the salt treatment back to the level similar to the untreated control. It is possible that NO modulated GSH content in soybean root nodules by regulating its biosynthesis or via enhancement of glutathione reductase activity (Meister, 1995).

In conclusion, a higher level of AsA and GSH pool or their recovery under salt stress respectively is consistent with the high activities of APX and DHAR enzymes in NO treatments (either alone or in combination with NaCl). This further demonstrates the function of NO in promoting H_2O_2 scavenging through APX activity.

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