Exogenous Nitric Oxide (as Sodium Nitroprusside) Ameliorates Polyethylene Glycol-Induced Osmotic Stress in Hydroponically Grown Maize Roots

Evren Yildiztugay · Ceyda Ozfidan-Konakci · Mustafa Kucukoduk

Abstract The present study was designed to examine whether exogenous sodium nitroprusside (SNP) supplementation has any ameliorating action against PEG-induced osmotic stress in *Zea mays* cv. FRB-73 roots. Twenty percent or 40 % polyethylene glycol (PEG6000; −0.5 MPa and −1.76 MPa, respectively) treatment alone or in combination with 150 and 300 μM SNP was applied to hydroponically grown maize roots for 72 h. Although only catalase (CAT) activity increased when maize roots were exposed to PEG-induced osmotic stress, induction of this antioxidant enzyme was inadequate to detoxify the extreme levels of reactive oxygen species, as evidenced by growth, water content, superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$) scavenging activity, and TBARS content. However, supplementation of PEG-exposed specimens with SNP significantly alleviated stress-induced damage through effective water management and enhancement of antioxidant defense markers including the enzymatic/non-enzymatic systems. Exogenously applied SNP under stress resulted in the up-regulation of glutathione peroxidase (GPX), glutathione S-transferase (GST), ascorbate peroxidase (APX), glutathione reductase (GR), total ascorbate, and glutathione contents involved in ascorbate–glutathione cycle. On the other hand, growth rate, osmotic potential, CAT, APX, GR, and GPX increased in maize roots exposed to both concentrations of SNP alone, but activities of monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase decreased. Based on the above results, an exogenous supply of both 150 and 300 μM SNP to maize roots was protective for PEG-induced toxicity. The present study provides new insights into the mechanisms of SNP (NO donor) amelioration of PEG-induced osmotic stress damages in hydroponically grown maize roots.

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Introduction

Drought stress is one of the most serious environmental problems with well characterized limiting effects on plant growth and productivity. Plants exposed to drought stress tend to exhibit stomatal closure, which prevents water loss, but decreases CO_2 influx. This consequently leads to a decrease in carbon fixation and in $NADP^+$ reduction in photosynthesis. As a result of this, electrons are shuttled to an alternative electron acceptor, oxygen, producing toxic reactive oxygen species (ROS) (Gill and Tuteja 2010). ROS including the superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\bullet}$) can directly damage important vital molecules of plants such as proteins, amino acids, or nucleic acids (Mittler and others 2004). Survival under stress conditions depends on the plant's capacity to scavenge these extreme ROS levels. Stress-induced ROS accumulation is counteracted by enzymatic/non-enzymatic antioxidant systems that include

a variety of scavengers, such as superoxide dismutase (SOD), catalase (CAT), peroxidases (POX), glutathione peroxidase (GPX), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), ascorbate, and glutathione contents (Shi and others 2007). Also, osmotic regulators such as proline (Pro) are helpful against the consequences of oxidative damage.

Nitric oxide (NO) is a small diffusible signaling molecule participating in a wide variety of physiological processes including germination, flowering, fruit ripening, and organ senescence (Crawford and Guo 2005). NO signaling is usually related to its cross-talk with ROS. For example, the defense response in plants results from the simultaneous and balanced production of NO and $O_2^{\bullet-}$ or H_2O_2 (Wu and Wu 2008). Moreover, NO is believed to be involved in activation of antioxidant enzymes by ROS accumulation in that NO has been demonstrated to enhance the expression of genes encoding antioxidative enzymes (Kopyra and Gwóźdz 2004). Such evidence may suggest a role for NO in the up-regulation of antioxidant isozymes by ROS accumulation, although the mechanism involved is ambiguous. On the other hand, NO promotes normal growth and development of plants at lower concentrations under stress (Beligni and Lamattina 2001). For example, Zhang and others (2004) reported that NO enhanced salt tolerance by increasing K^+ accumulation and decreasing Na^+ accumulation in maize roots and leaves. Sodium nitroprusside (SNP), a NO donor, has been found to have similar protective effects on NaCl-induced impairment of PSII in rice seedlings (Uchida and others 2002). In addition to having cytoprotectant properties, NO may also have a role as a cytotoxin in plants (Beligni and Lamattina 2001). NO clearly perturbs normal metabolism of plants when applied at high doses (Yamasaki 2000). For instance, high levels of NO are associated with potential impairment of photosynthetic electron transport, inhibition of shoot and root development, DNA damage, and cell death (Gould and others 2003). These results indicate that NO mediates either negative or positive effects depending upon concentrations and plant species. Therefore, there is still a debate about protective or toxic effects of SNP in plants under stress.

Most previous studies have focused on shoot and leaf material and cell cultures, but roots have been much less investigated with respect to the effects of NO under stress. Although there are a number of studies using SNP inhibitors, information about ameliorative effects of exogenous SNP on plant roots exposed to stress could not be found in these studies. Therefore, understanding of these physiological and biochemical changes in which osmotic stress triggers peroxidation or antioxidant defense-related responses and an amelioration mechanism induced by NO

in roots is of critical importance. Some chemicals such as polyethylene glycol (PEG) cause osmotically-induced stress when added to soil or growth media of plants. Considering the pivotal role of SNP on tolerance mechanisms in stressed plants, the present work was undertaken to investigate the efficacy of SNP in alleviating PEG-induced alterations. For this, a general relationship was established among the regulation of the antioxidant defense system, osmo-protectant accumulation, and the induced tolerance with exogenously applied SNP in maize plants grown under different intensities of PEG-induced osmotic stress. This work may increase our understanding of the mechanisms of NO amelioration of osmotic-stress damage in maize roots.

Materials and Methods

Plant Material and Experimental Design

Seeds of *Zea mays* cv. FRB-73 were provided from Bahri Dagdas International Agricultural Research Institute, Konya, Turkey. Seeds were treated with 5 % hypochloric acid for 10 min, rinsed five times with sterile distilled water, and then allowed to germinate on double-layer filter paper wetted with distilled water. Germinated *Z. mays* seedlings were transferred to half strength Hoagland solution and were grown under controlled conditions (16/8 h light/dark regime at 23 °C, 70 % relative humidity and 350 μmol m^{-2} s^{-1} photosynthetic photon flux density). The seedlings were grown for 21 days in this solution and subsequently transferred to fresh Hoagland solution containing various concentrations of polyethylene glycol (20 % −0.5 MPa osmotic potential- and 40 % PEG6000 −1.76 MPa osmotic potential-) with or without the NO donor sodium nitroprusside (SNP, 150 and 300 μM) treatments for 72 h. Plants were harvested after 72 h of treatment and then stored at −86 °C until further analyses.

Determination of Growth Rate, Relative Water Content and Osmotic Potential

Six plants were used for the control group and for each treatment group. After the samples were dried, dry weights (DW) were measured. RGR values were calculated according to the following formula by Hunt and others (2002):

$$RGR = [\ln(DW_2) - \ln(DW_1)]/(t_2 - t_1),$$

where DW_1 dry weight (g) at t_1; DW_2 dry weight (g) at t_2, t_1 initial harvest, and t_2 final harvest.

After the stress exposure period, six roots were weighed and their fresh weights (FW) were recorded. The samples were kept in ultrapure water for 8 h and then the turgid

roots (TW) were measured again. After oven drying at 75 °C for 72 h, DW was reported. Root RWC was calculated by the formula given by Smart and Bingham ([1974]):

$$RWC\ (\%) = [(FW - DW)/(TW - DW)] \times 100$$

Roots were homogenized by a glass rod. After centrifugation (12,000×*g* for 10 min), the extraction was directly used for Ψ_π determination. Root osmolarity (c) was measured by a Vapro Vapor pressure Osmometer 5600 and converted from mosmoles kg^{-1} to MPa using the formula as follows: Ψ_π (MPa) $= -c$ (mosmoles kg^{-1}) $\times 2.58 \times 10^{-3}$ according to the Van't Hoff equation.

Determination of Proline Content

Determination of proline (Pro) content was done according to the procedure defined by Bates and others ([1973]). The roots of maize were treated with 2-hydroxy-5-sulfobenzoic acid, and the extract was centrifuged at 5,000×*g* for 5 min. The supernatant including acid ninhydrin and glacial acetic acid was heated at 95 °C. After stopping the reaction, toluene was added to the solution and the absorbance was reported at 520 nm.

Determination of ROS Accumulation

$O_2^{\bullet-}$ levels were estimated according to Xu and others ([2006]), with some modifications. One milliliter of 1 mM hydroxylammonium chloride was added to 0.5 ml of the supernatant and incubated for 1 h at 25 °C. The addition of 1 ml 4-aminobenzenesulfonic acid (17 mM) and 1 ml α-naphthylamine (7 mM) for 20 min at 25 °C altered the color, and the specific absorption at 530 nm was determined. Sodium nitrite was used as a standard solution to calculate the $O_2^{\bullet-}$ levels.

Determination of H_2O_2 content was performed according to Liu and others ([2010]). Roots were homogenized in cold acetone and centrifuged at 3,000×*g* at 4 °C for 10 min. The supernatant was mixed with titanium reagent (prepared in concentrated hydrochloric acid containing 20 % (v/v) titanium tetrachloride), and then ammonium hydroxide was added to precipitate the titanium-peroxide complex. The reaction mixture was centrifuged at 16,000×*g* at 4 °C for 10 min, and the pellet was washed with cold acetone. The pellet was dissolved in 1 M H_2SO_4. The absorbance of the solution was measured at 410 nm. H_2O_2 concentrations were calculated using a standard curve prepared with known concentrations of H_2O_2.

$OH^\bullet$ scavenging activity was determined according to Chung and others ([1997]), with minor changes. Competition between deoxyribose and the sample for $OH^\bullet$ generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system was measured to determine the $OH^\bullet$ scavenging activity. The reaction mixture contained 20 mM Na–phosphate buffer (pH 7.0), 10 mM 2-deoxyribose, 10 mM $FeSO_4$, 10 mM EDTA, 10 mM H_2O_2, and sample. The mixture was incubated at 37 °C for 2 h. A mixture of 0.75 ml of 2.8 % (w/v) trichloroacetic acid and 0.75 ml of 1 % (w/v) thiobarbituric acid in 50 mM NaOH was boiled for 20 min. After the mixture cooled, absorbance was measured at 520 nm against a blank solution. The $OH^\bullet$ scavenging activity was calculated using the following formula:

$$OH^\bullet scavenging\ activity\ (\%) = [(A_0 - A_1)/A_0] \times 100,$$

where A_0 is the absorbance of the blank and A_1 is the absorbance of the sample.

Enzyme Extraction and Determination of Enzyme and/or Isozyme Composition

For protein and enzyme extractions, 0.5 g of each sample was ground to a fine powder using liquid nitrogen and then homogenized in 50 mM Tris–HCl (pH 7.8) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.2 % (w/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 2 mM dithiothreitol (DTT). For APX activity determination, 5 mM ascorbate (AsA) was added to the homogenization buffer, and 2 % (w/v) polyvinylpyrrolidone was used instead of DTT. Samples were centrifuged at 14,000×*g* for 30 min, and supernatants were used for the determination of protein content and enzyme activities. The total soluble protein content of the enzyme extracts was determined (Bradford [1976]) using bovine serum albumin as a standard. All spectrophotometric analyses were conducted on a Shimadzu spectrophotometer (UV 1800).

Samples containing equal amounts of protein (85 μg) were subjected to non-denaturing polyacrylamide gel electrophoresis (PAGE) as described by Laemmli ([1970]) with minor modifications. SOD activity was detected by photochemical staining using riboflavin and NBT (Beauchamp and Fridovich [1971]). The units of activity for each SOD isozyme were calculated by running a SOD standard from bovine liver (Sigma Chemical Co., St. Louis, MO, USA). The different types of SOD were discriminated by incubating gels with different types of SOD inhibitors before staining: Mn-SOD activity was resistant to both inhibitor treatments, and Cu/Zn-SOD activity was sensitive to 2 mM KCN. Cu/Zn-SOD and Fe-SOD activities were inhibited by 3 mM H_2O_2 (Vitória and others [2001]). The total SOD (EC 1.15.1.1) activity assay was based on the method of Beauchamp and Fridovich ([1971]), which uses spectrophotometric analysis at 560 nm to measure the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT). One unit of specific enzyme activity

was defined as the quantity of SOD required to produce a 50 % inhibition of NBT reduction.

NADPH oxidase (NOX) isozymes were identified by the NBT reduction method as described by Sagi and Fluhr (2006). Non-denaturing PAGE was performed in 7.5 % (w/v) polyacrylamide mini gels, and 40 µg protein sample was loaded per lane. Gels were stained in 50 mM Tris–HCl buffer (pH 7.4), 0.2 mM NBT, 0.1 mM $MgCl_2$, and 1 mM $CaCl_2$, in the dark for 20 min. After 0.2 mM NADPH·Na$_4$ was added, the appearance of blue formazan bands was observed. Total NOX (EC 1.6.3.1) activity was measured according to Jiang and Zhang (2002). The assay medium contained 50 mM Tris–HCl buffer (pH 7.5), 0.5 mM 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, disodium salt (XTT), 100 µM NADPH·Na$_4$, and 20 µg protein sample. After the addition of NADPH, XTT reduction was followed at 470 nm. The corrections for background production were determined in the presence of 50 units of SOD. Activity was calculated using the extinction coefficient, 2.16×10^4 M^{-1} cm^{-1}. One unit of NOX was defined as 1 nmol XTT oxidized min^{-1} ml^{-1}.

After electrophoresis of samples containing 40 µg protein, CAT isozymes were detected according to Woodbury and others (1971). The electrophoretic separation was performed on non-denaturing polyacrylamide mini slab gels (8×10 cm^2) under constant current (30 mA). The gels were incubated in 0.01 % (v/v) H_2O_2 for 15 min and were incubated for 20 min in staining solution containing 1 % (w/v) $FeCl_3$ and 1 % (w/v) $K_3Fe(CN_6)$. Total CAT (EC 1.11.1.6) activity was estimated according to the method of Bergmeyer (1970), which measured the initial rate of H_2O_2 disappearance at 240 nm. The decrease in absorption was followed for 3 min, and one unit of CAT was defined as 1 mmol H_2O_2 decomposed min^{-1} ml^{-1}.

POX isozymes were detected according to Seevers and others (1971). Electrophoretic separation of samples containing 40 µg protein was performed on non-denaturing polyacrylamide mini slab gels (8×10 cm^2) under constant current (30 mA). The gels were incubated for 30 min at 25 °C in 200 mM sodium acetate buffer (pH 5.0) containing 1.3 mM benzidine and 3 % (v/v) H_2O_2. The gels were stored in 7 % (v/v) acetic acid until the assay was carried out. Total POX (EC 1.11.1.7) activity was based on the method described by Herzog and Fahimi (1973). The increase in the absorbance at 465 nm was followed for 3 min. One unit of POX activity was defined as mmol H_2O_2 decomposed min^{-1} ml^{-1}.

Electrophoretic APX separation was performed according to Mittler and Zilinskas (1993). Non-denaturing PAGE was carried out in 7.5 % (w/v) polyacrylamide mini slab gels (8×10 cm^2) supported by 10 % (v/v) glycerol. Before the samples (40 µg protein) were loaded, gels were equilibrated with running buffer containing 2 mM AsA for

30 min. Subsequently, electrophoresis gels were incubated in 50 mM K–phosphate buffer (pH 7.0) containing 2 mM AsA for 20 min and then were transferred to solutions containing 50 mM K–phosphate buffer (pH 7.8), 4 mM AsA, and 2 mM H_2O_2 for 20 min. The gels were washed in the buffer for 1 min and submerged in a solution of 50 mM K–phosphate buffer (pH 7.8) containing 28 mM N,N,N',N'-tetramethylethylenediamine and 2.5 mM NBT for 10–20 min with gentle agitation in the presence of light. Total APX (EC 1.11.1.11) activity was measured according to Nakano and Asada (1981). The assay depends on the decrease in absorbance at 290 nm. The concentration of oxidized AsA was calculated by using a 2.8 mM^{-1} cm^{-1} extinction coefficient. One unit of APX was defined as 1 mmol AsA oxidized min^{-1} ml^{-1}.

GR isozymes were detected using 7.5 % native PAGE according to Hou and others (2004). After electrophoresis of the samples containing 40 µg protein, GR isozymes were detected by incubating the gels in a solution containing 10 mM Tris–HCl (pH 7.9), 4 mM GSSG, 1.5 mM NADPH·Na$_4$, and 2 mM 5,5'-dithiobis (2-nitro-benzoic acid) (DTNB) for 20 min. After a brief rinse with 50 mM Tris–HCl buffer (pH 7.9), GR activity was negatively stained by 1.2 mM MTT (Thiazolyl blue tetrazolium bromide), 1 mM 2,6-dichloroindophenol, and 1.6 mM N-methylphenazonium methyl sulfate for 5–10 min at room temperature. Total GR (EC 1.6.4.2) activity was measured according to Foyer and Halliwell (1976). Activity was calculated using the extinction coefficient of NADPH (6.2 mM^{-1} cm^{-1}). One unit of GR was defined as 1 mmol GSSG reduced min^{-1} ml^{-1}.

Monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) activity was assayed by the method of Miyake and Asada (1992). The reaction mixture contained 50 mM of Hepes–KOH (pH 7.6), 1 mM NADPH, 2.5 mM AsA and 2.5 U AsA oxidase (EC 1.10.3.3), and enzyme extract. The MDHAR activity was measured by decrease in absorbance as the amount of enzyme that oxidizes 1 mM NADPH per minute at 340 nm. A molar extinction coefficient of 6.2 mM^{-1}-cm^{-1} was used for the calculation of enzyme activity.

Dehydroascorbate reductase (DHAR; EC 1.8.5.1) activity was measured according to Dalton and others (1986). DHAR activity was measured by increase in absorbance at 265 nm due to ascorbate formation. A molar extinction coefficient of 14.6 mM^{-1} cm^{-1} was used for the calculation of enzyme activity.

Glutathione-s-transferase (GST; EC 2.5.1.18) activity was assayed according to Habig and Jacoby (1981). The reaction mixture consisted of 0.2 M K–phosphate buffer (pH 7.0), 0.1 M 1-chloro,2,4-dinitrobenzene (CDNB), and distilled water. The reaction was started by the addition of enzyme extract and the increase in absorbance was measured at 340 nm for 1 min. The activity was calculated

using the extinction coefficient of the conjugate ($9.6 \, mM^{-1} \, cm^{-1}$).

Glutathione peroxidase (GPX; EC 1.11.1.9) activity was measured as described by Elia and others (2003) with minor modification. The reaction mixture consisted of 100 mM K–phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM NaN_3, 0.12 mM NADPH, 2 mM GSH, 1 unit GR enzyme, 0.6 mM H_2O_2, and 20 µl of sample solution. The oxidation of NADPH was recorded at 340 nm for 1 min, and the activity was calculated using the extinction coefficient of $6.62 \, mM^{-1} \, cm^{-1}$.

Content of total ascorbate (tAsA) was estimated according to Mukherjee and Choudhuri (1983) with a slight modification. Fresh root tissue was extracted with 6 % trichloroacetic acid. Two milliliters of the extract was mixed with 2 % dinitrophenylhydrazine followed by the addition of one drop 10 % thiourea. The mixture was boiled for 20 min in a water bath, and after cooling to room temperature, 80 % (v/v) H_2SO_4 was added to the mixture in an ice bath. The absorbance was recorded at 530 nm. The glutathione pool (tGlut; reduced and oxidized glutathione) was assayed according to Paradiso and others (2008), utilizing aliquots of supernatant neutralized with 0.5 M K–phosphate buffer (pH 7.0). Based on enzymatic recycling, glutathione is oxidized by DTNB and reduced by NADPH in the presence of GR, and glutathione content is evaluated by the rate of absorption changes at 412 nm. Oxidized glutathione (GSSG) was determined after removal of GSH by 2-vinylpyridine derivatization. Standard curves with known concentrations of GSH and GSSG were used for the quantification.

Gels stained for SOD, CAT, POX, APX, GR, and NOX activities were photographed with the Gel Doc XR+ System and then analyzed with Image Lab software v4.0.1 (Bio-Rad, California, USA). Known standard amounts of enzymes (0.5 units of SOD and CAT, and 0.2 units of POX) were loaded onto gels. The units of isozyme activity for each group were calculated by comparison with the standard and are given as a graphic below each gel photo. For each isozyme set/group, the average values (shown with the same symbol) were not significantly different at $p > 0.05$ using Tukey's post-test.

Determination of Lipid Peroxidation Levels

The level of lipid peroxidation was determined by thiobarbituric acid reactive substances (TBARS) according to Madhava Rao and Sresty (2000). TBARS concentration was calculated from the absorbance at 532 nm, and measurements were corrected for nonspecific turbidity by subtracting the absorbance at 600 nm. The concentration of TBARS was calculated using an extinction coefficient of $155 \, mM^{-1} \, cm^{-1}$.

Statistical Analysis

The experiments were repeated thrice independently and each data point was the mean of six replicates. All data obtained were subjected to a one-way analysis of variance (ANOVA). Statistical analysis of the values was performed by using SPSS 20.0. Tukey's post-test was used to compare the treatment groups. Comparisons with $p < 0.05$ were considered significantly different. In all the figures, the error bars represent standard errors of the means.

Results

Effects of SNP on Growth Rate, Relative Water Content and Osmotic Potential

With increasing PEG supply from 20 to 40 %, the root growth rate significantly decreased (Table 1). Reduction in RGR reached maximum levels at high-PEG concentration (68 %). However, addition of 150 or 300 µM SNP markedly decreased the symptoms of PEG toxicity on growth of maize roots. On the other hand, both applied SNP treatments increased RGR under normal conditions.

RWC gradually decreased with increasing concentration of stressor (Table 1). A 12 % decline in RWC was observed at 20 % PEG, which further decreased to 18–40 % PEG treatment. The higher SNP treatment was the most effective on RWC with the 40 % PEG treatment, resulting in 19 % amelioration. Moreover, both SNP treatments alone had no effect on RWC.

PEG-induced osmotic stress caused a significant decrease in Ψ_π of roots as compared to controls (Table 1). This trend was more pronounced for 40 % PEG stress. Ψ_π increased with addition of SNP under stress in comparison to the PEG treatments alone. For example, Ψ_π increased from −0.74 MPa (after 40 % PEG treatment) to −0.59 MPa (after 40 % PEG plus 300 µM SNP treatment) in maize roots for 72 h. On the other hand, under non-stress conditions, exogenously applied SNP increased Ψ_π, especially pronounced in 300 µM SNP-treated maize plants.

Effects of SNP on Pro Content

As shown in Table 1, Pro content increased after PEG-stress treatment, reaching maximal levels (227 %) at 40 % PEG. The enhancements in Pro content of maize roots following 150 µM SNP treatment were 9 and 4 % after 72 h of 20 and 40 % PEG stress compared to PEG alone, respectively. On the other hand, the 300 µM SNP treatment under 40 % PEG stress did not create any remarkable effect on Pro content. Similarly, 150 or 300 µM SNP alone

Table 1 Effect of exogenous 150 and 300 μM SNP treatment on growth rate (RGR; mg mg^{-1} days^{-1}), root water content (RWC; %), osmotic potential (Ψ_π; MPa), and proline content (Pro; μmol g^{-1} FW) in *Zea mays* exposed to 20 and 40 % PEG-6000 for 72 h. The letters were used to denote the level of significance of the difference among the treatment groups

	RGR	RWC	Ψ_π	Proline
Control	0.155 ± 0.045[b]	89.8 ± 5.18[c]	−0.56 ± 0.012[c]	2.11 ± 0.45[a]
SNP1	0.164 ± 0.042[c]	90.6 ± 3.16[c]	−0.43 ± 0.017[d]	2.15 ± 0.42[a]
SNP2	0.176 ± 0.05[d]	88.5 ± 0.74[c]	−0.48 ± 0.044[d]	2.38 ± 0.42[a]
PEG1	0.055 ± 0.01[a]	79.4 ± 2.56[b]	−0.67 ± 0.016[b]	5.63 ± 0.36[c]
PEG1+SNP1	0.183 ± 0.041[e]	87.9 ± 4.27[c]	−0.56 ± 0.067[c]	6.11 ± 0.41[d]
PEG1+SNP2	0.201 ± 0.03[f]	88 ± 5.05[c]	−0.58 ± 0.041[c]	5.32 ± 0.3[b]
PEG2	0.049 ± 0.01[a]	73.3 ± 1.88[a]	−0.74 ± 0.031[a]	6.89 ± 0.87[e]
PEG2+SNP1	0.183 ± 0.018[e]	86.3 ± 2.89[c]	−0.55 ± 0.038[c]	7.16 ± 0.18[f]
PEG2+SNP2	0.184 ± 0.03[e]	87.1 ± 2.67[c]	−0.59 ± 0.037[c]	6.74 ± 0.03[e]
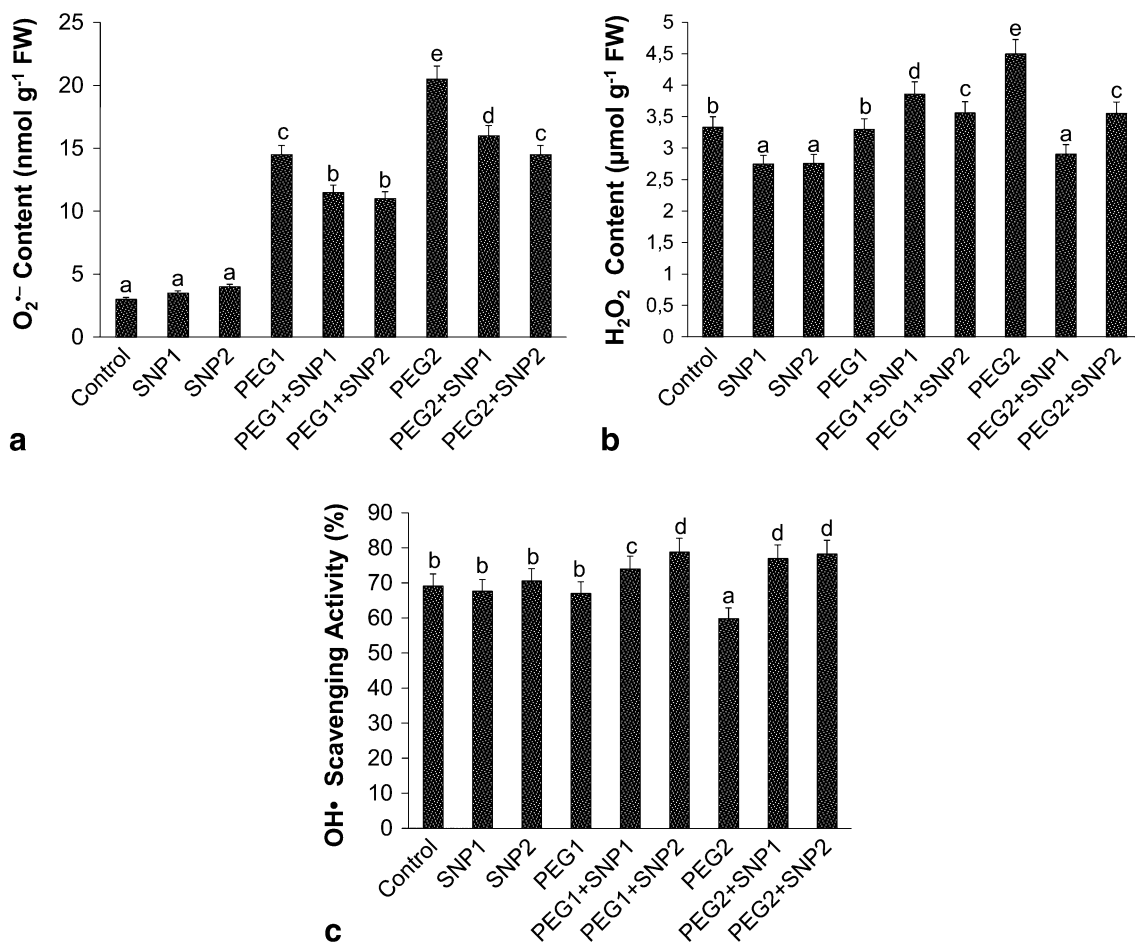
Fig. 1 Effect of exogenous 150 and 300 μM SNP treatment on O$_2^{\bullet-}$ content (**a**), H$_2$O$_2$ content (**b**), and scavenging activity of OH$^\bullet$ (**c**) in *Zea mays* exposed to 20 and 40 % PEG-6000 for 72 h

did not change the Pro content of maize plants during the experimental period.

Effects of SNP on ROS Accumulation

As shown in Fig. 1a, PEG caused a significant increase in O$_2^{\bullet-}$ content in roots. The highest O$_2^{\bullet-}$ content was observed at 40 % PEG stress, which was sevenfold higher than that of the control. However, upon addition of SNP together with PEG, a significant decrease in O$_2^{\bullet-}$ content was detected compared with PEG treatment alone. One hundred fifty or 300 μM SNP treatments alone did not create any remarkable effect on O$_2^{\bullet-}$ content. After 72 h of 20 % PEG stress, H$_2$O$_2$ content in maize roots was close to
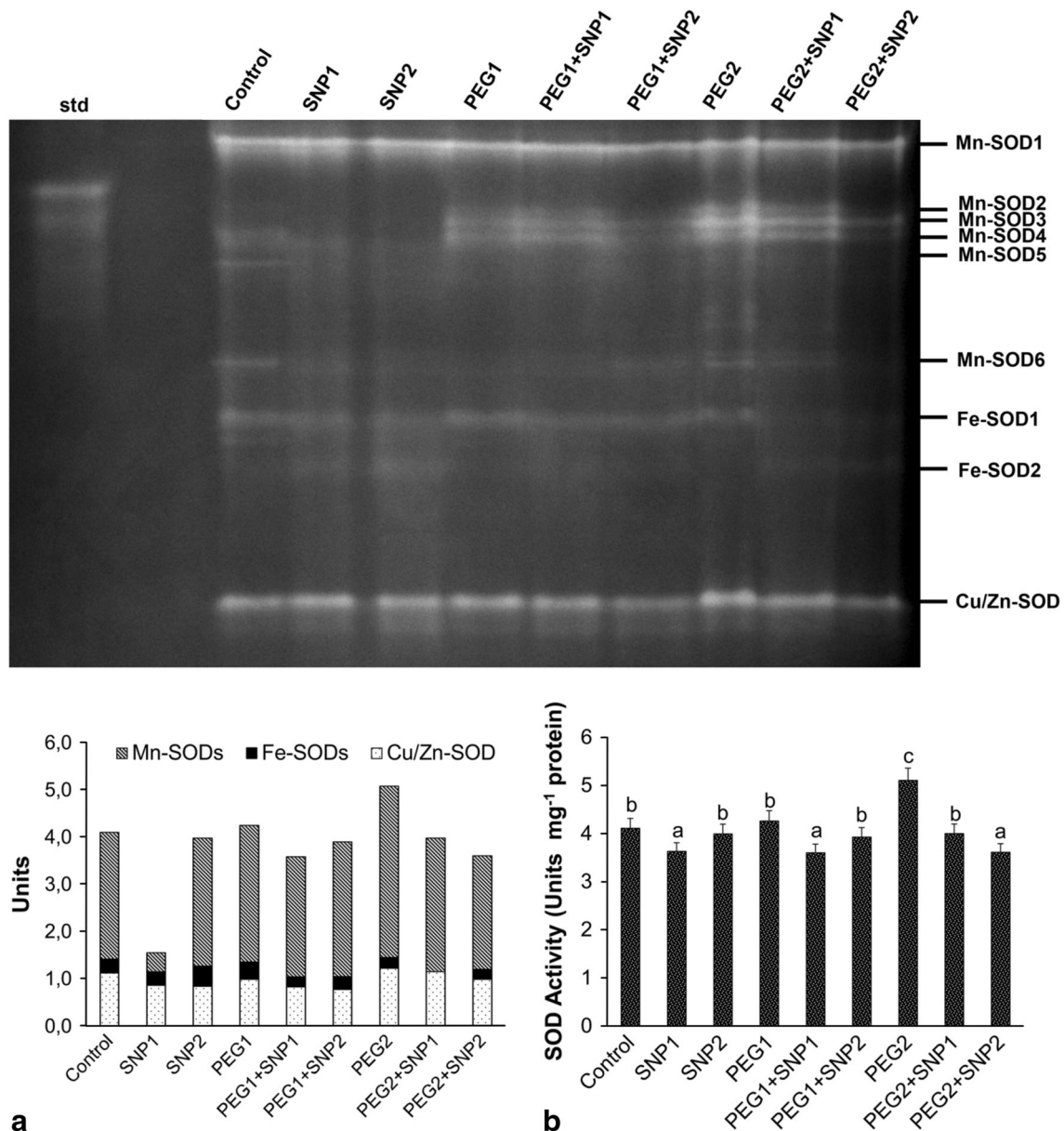
Fig. 2 Effect of exogenous 150 and 300 μM SNP treatment on contribution of different types of SOD isozymes (**a**) and total SOD activity (**b**) in *Zea mays* exposed to 20 and 40 % PEG-6000 for 72 h

control levels, whereas H_2O_2 content showed a rapid increase by 35 % in 40 % PEG-treated maize roots (Fig. 1b). In contrast, except for PEG (20 %) and 150 or 300 μM SNP treatment, H_2O_2 content decreased in the 40 % PEG+SNP group as compared to PEG treatment alone. The maximum decrease in H_2O_2 content (35 %) was observed when plants were subjected to 40 % PEG stress in combination with 150 μM SNP. Similar results were also observed in SNP-treated maize roots alone. The $OH^\bullet$ scavenging activity decreased only with 40 % PEG-induced osmotic stress (Fig. 1c). However, exogenously applied SNP with increasing PEG concentrations caused an increase in $OH^\bullet$ scavenging activity. The degree of these increases was higher in SNP+40 % PEG-treated maize plants than SNP+20 % PEG-treated plants. The $OH^\bullet$ scavenging activity was not significantly affected by SNP concentrations alone.

Effects of SNP on Enzymatic/Non-Enzymatic Compounds

Using different specific inhibitors, nine SOD isozymes were detected in extracts of root soluble proteins: six Mn-SODs, two Fe-SODs, and one Cu/Zn-SOD (Fig. 2). The

Fig. 3 Effect of exogenous 150 and 300 μM SNP treatment on Native-PAGE separation of NOX isozymes (**a**) and total NOX activity (**b**) in *Zea mays* exposed to 20 and 40 % PEG-6000 for 72 h
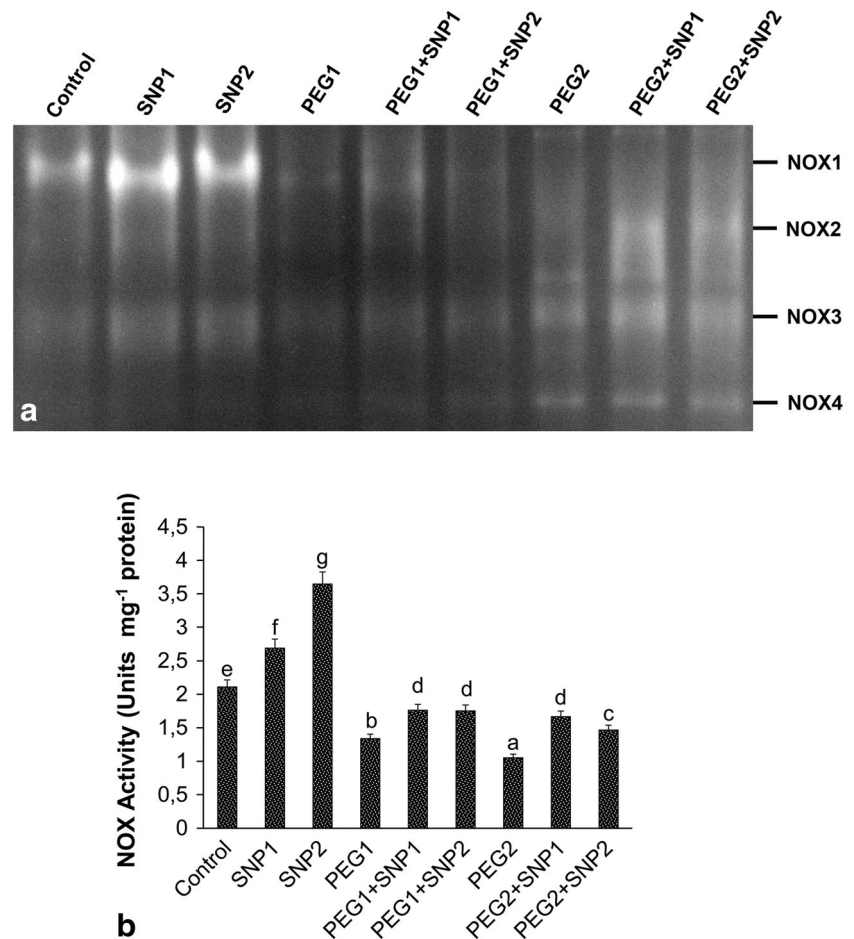

increase in SOD activity was only in 40 % PEG-treated roots and seemed to be mainly due to the activity of Mn-SOD isozymes in maize plants after 72 h (Fig. 2a). The activity of Mn-SODs accounted for approximately 29 % of the total SOD activity at 40 % PEG stress treatment for 72 h. However, except for 20 % PEG+300 μM SNP, the addition of SNP to PEG treatment solution resulted in a significant decrease in both activities of total SOD and SOD isozymes (Cu/Zn-SOD) compared to PEG treatment alone. On the other hand, a decline in total SOD activity was observed when maize roots were treated with 150 μM SNP (Fig. 2b).

As shown in Fig. 3, a significant decrease in NOX activity was observed in PEG-treated roots. For instance, NOX activity (NOX1 and NOX3 isozymes) of roots exposed to 40 % PEG decreased from 2.11 to 1.05 U mg^{-1} protein, 50 % of the control group level (Fig. 3a, b). Compared with PEG treatment alone, maize roots treated with PEG+SNP had more the NOX activity. Similarly, NOX activity in roots increased with SNP treatment alone and reached the maximum levels (73 %) at the high-SNP concentration.

In native PAGE, gel analyses revealed four CAT isozymes: CAT1-4 (Fig. 4a). Total CAT activity increased significantly even when PEG stress was rather severe. This data was supported by native PAGE gels (Fig. 4a), which revealed more intense CAT1, CAT2, and CAT3 isozymes at both PEG stress treatments, over control groups. Moreover, except 40 % PEG+300 μM SNP, exogenous SNP treatment under stress (SNP+PEG) maintained the induction in CAT activity compared with PEG stress alone. Total CAT activity was 81 and 101 % in 150 and 300 μM SNP-treated alone plants at 72 h, respectively (Fig. 4b). Three POX isozymes were presented in the roots of maize plants (Fig. 4c). In comparison with non-stress conditions, the total activity of POX decreased and was close to control levels in 20 and 40 % PEG-treated plants, respectively (Fig. 4d). Both SNP+PEG and SNP treatments alone caused a decline in POX activity in regards to intensities of POX1.

After native PAGE analysis, only two APX isozymes, denominated APX1 and APX2, were markedly detected in roots (Fig. 5a). When maize roots were exposed to PEG stress, both APX and GR activities remained almost
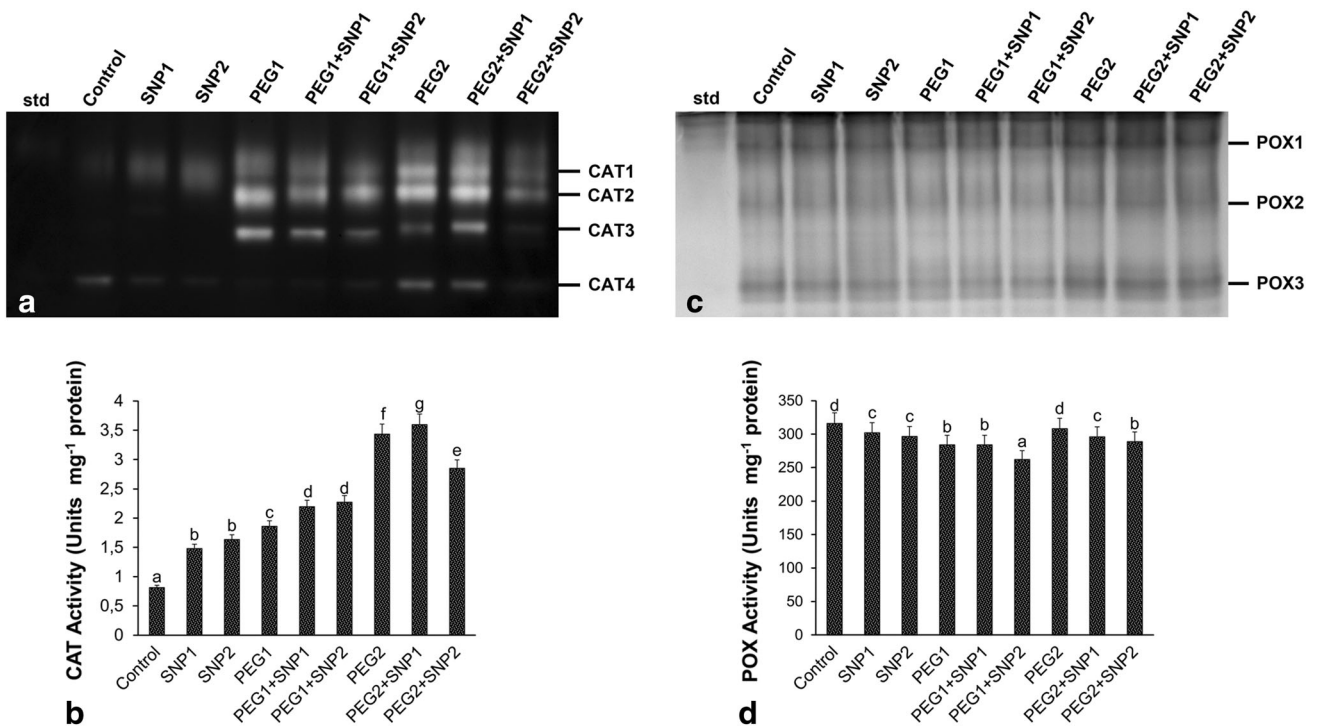
Fig. 4 Effect of exogenous 150 and 300 µM SNP treatment on Native-PAGE separation of CAT isozymes (**a**), total CAT activity (**b**), Native-PAGE separation of POX isozymes (**c**), and total POX activity (**d**) in *Zea mays* exposed to 20 and 40 % PEG-6000 for 72 h
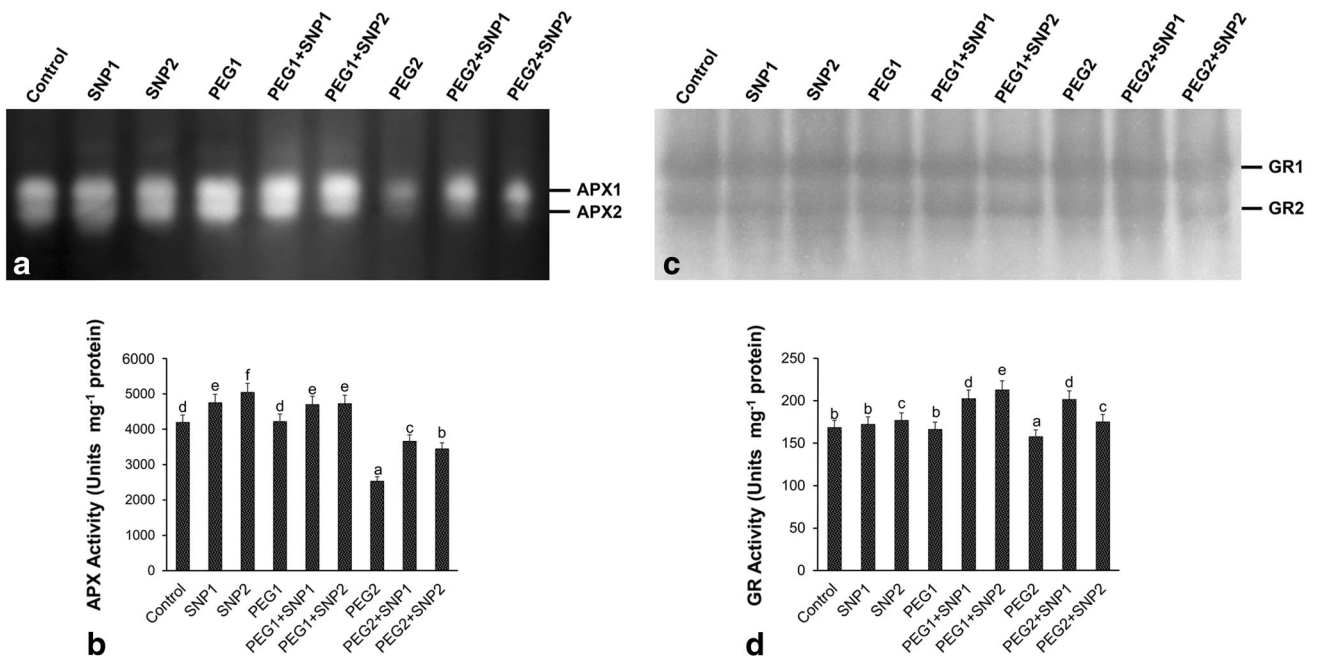
Fig. 5 Effect of exogenous 150 and 300 µM SNP treatment on Native-PAGE separation of APX isozymes (**a**), total APX activity (**b**), Native-PAGE separation of GR isozymes (**c**), and total GR activity (**d**) in *Zea mays* exposed to 20 and 40 % PEG-6000 for 72 h

unchanged at low-PEG concentration, but decreased at high-PEG treatment (Fig. 5b–d). This reduction in APX (APX1 isozyme) and GR (GR1 isozyme) activity was 40

and 6 %, respectively, in the highest PEG treatment (Fig. 5a–c). However, as compared to the group treated with PEG alone, this decline induced by PEG stress was

Table 2 Effect of exogenous 150 and 300 μM SNP treatment on monodehydroascorbate reductase (MDHAR; μmol mg^{-1} protein), dehydroascorbate reductase (DHAR; μmol mg^{-1} protein), glutathione S-transferase (GST; μmol g^{-1} protein), and glutathione peroxidase (GPX; μmol g^{-1} protein) in *Zea mays* exposed to 20 % and 40 % PEG-6000 for 72 h. The letters were used to denote the level of significance of the difference among the treatment groups

	MDHAR	DHAR	GST	GPX
Control	0.724 ± 0.06 [g]	37.14 ± 0.53[e]	0.904 ± 0.06[d]	7.2 ± 0.33[a]
SNP1	0.499 ± 0.09[d]	26.36 ± 0.71[d]	0.856 ± 0.05[c]	8.53 ± 0.76[c]
SNP2	0.484 ± 0.06[c]	24.36 ± 0.89[c]	0.852 ± 0.04[c]	9.02 ± 0.24[e]
PEG1	0.609 ± 0.04[f]	24.34 ± 1.16[c]	0.691 ± 0.03[a]	7.06 ± 0.33[a]
PEG1+SNP1	0.422 ± 0.04[b]	22.27 ± 0.94[b]	0.766 ± 0.09[b]	8.99 ± 0.33[d]
PEG1+SNP2	0.46 ± 0.03[c]	23.44 ± 0.55[c]	0.779 ± 0.04[b]	8.98 ± 0.14[d]
PEG2	0.599 ± 0.04[e]	23.02 ± 1.88[c]	1.292 ± 0.01[e]	6.57 ± 0.22[a]
PEG2+SNP1	0.475 ± 0.01[c]	20.82 ± 2.12[a]	1.676 ± 0.01 [g]	8.07 ± 0.78[b]
PEG2+SNP2	0.362 ± 0.04[a]	22.11 ± 1.16[b]	1.401 ± 0.04[f]	8.17 ± 0.24[b]

alleviated by exogenously applied SNP treatment. Moreover, SNP under optimal conditions caused a significant increase in APX and GR activities throughout the experiment.

Results presented in Table 2 show that both MDHAR and DHAR activities in all PEG treatments decreased during the experiment. For example, MDHAR and DHAR activity decreased upon exposure to the high-PEG concentration, where the reduction was 17 and 38 % as compared to control, respectively. Similarly, the SNP supplemented PEG-stressed plants maintained significantly lower activity of these enzymes, as compared to the roots subjected to stress without SNP. Also, drastic decreases in MDHAR and DHAR activities were observed in response to SNP treatment alone. GST and GPX activities in maize treated with osmotic stress are shown in Table 2. Although GST activity of maize roots showed the most up-regulation, 43 %, in response to 40 % PEG stress, the GPX activity remained unchanged at 72 h of stress. SNP supplementation combined with PEG stress significantly enhanced both GST and GPX activities compared to stress alone. With SNP treatment alone, GST activity slightly decreased, whereas GPX activity increased.

In maize roots treated with low- and high-PEG concentrations, tAsA content decreased 14 and 40 %, respectively, compared to the controls, whereas SNP+PEG treatments increased tAsA content in comparison to PEG stress alone (Table 3). Although tAsA content decreased in 150 μM SNP-treated plants alone, this content increased at 300 μM SNP treatment alone in comparison to control levels. tGlut content was gradually increased by increasing PEG concentrations (Table 3). Also, a significant decrease in the GSH:GSSG ratio was observed in response to 20 and 40 % PEG stress, which was 37 and 6 % lower at 72 h when compared to control, respectively. The increase of tGlut was prevented by

Table 3 Effect of exogenous 150 and 300 μM SNP treatment on total ascorbate (tAsA; nmol g^{-1} FW), total glutathione (tGlut; nmol g^{-1} FW), and GSH/GSSG ratio in *Zea mays* exposed to 20 and 40 % PEG-6000 for 72 h. The letters were used to denote the level of significance of the difference among the treatment groups

	tAsA	tGlut	GSH/GSSG
Control	846.58 ± 2.9[e]	235.67 ± 2.63[a]	4.08 ± 0.91[c]
SNP1	773.07 ± 10.4[d]	245.88 ± 5.54[b]	3.56 ± 0.71[b]
SNP2	857.51 ± 9.32[f]	246.31 ± 1.97[b]	4.12 ± 0.72[c]
PEG1	725.1 ± 3.36[c]	373.97 ± 6.43[c]	2.59 ± 0.46[a]
PEG1+SNP1	845.13 ± 3.91[e]	570.72 ± 9.12[h]	5.86 ± 0.49[e]
PEG1+SNP2	877.54 ± 6.93[g]	451.19 ± 7.56[e]	4.35 ± 0.52[d]
PEG2	509.71 ± 9.85[a]	438.29 ± 7.21[d]	3.84 ± 0.34[b]
PEG2+SNP1	768.92 ± 11.3[d]	520.93 ± 8.72[g]	6.04 ± 0.35[f]
PEG2+SNP2	608.08 ± 2.82[b]	514.75 ± 3.23[f]	4.19 ± 0.59[c]
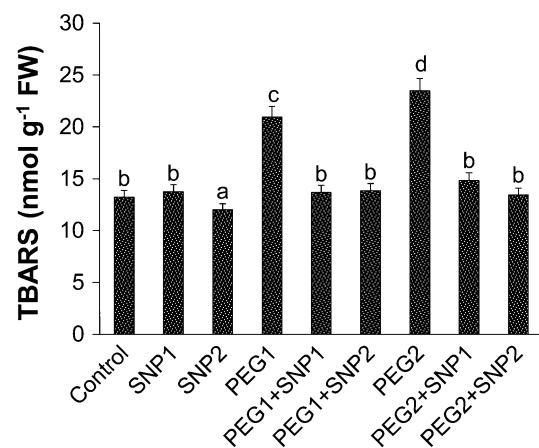
Fig. 6 Effect of exogenous 150 and 300 μM SNP treatment on lipid peroxidation (TBARS content) in *Zea mays* exposed to 20 and 40 % PEG-6000 for 72 h

exogenously applied SNP under the osmotic stress. Also, tGlut content was strongly induced in maize roots treated with both SNP treatments alone.

Effects of SNP on Lipid Peroxidation

PEG-induced osmotic stress adversely affected the membrane integrity of maize roots. Maize plants treated with 20 and 40 % PEG for 72 h showed a 58 and 78 % increase in TBARS content, respectively (Fig. 6). However, amelioration against PEG-induced membrane injury was provided by exogenous application of two SNP concentrations (150 or 300 μM) throughout the experiment. In other words, both concentrations of SNP were effective in bringing down the lipid peroxidation levels by 34–43 %. Moreover, 150 μM SNP treatment alone did not change TBARS levels, whereas it decreased in 300 μM SNP-treated plants.

Discussion

Exposure to different osmotic potential causes visible toxicity with symptoms such as reduction of plant growth (Silva and others 2010). Compared with controls, the growth rate of maize seedlings under PEG stress was markedly lower. Exogenously applied 150 or 300 μM SNP in plants exposed to osmotic stress exhibited a significant promotion of growth rate as compared with the PEG-treated plants. These significant differences in growth of maize roots were also found when SNP was administered in the absence of PEG. It was previously reported that treatment with SNP alone resulted in much more growth induction in Catharanthus roseus (Xu and others 2005), as observed in our study. Such an induction might be due to (i) root tip elongation induced by NO-releasing compounds (Gouvea and others 1997) and/or (ii) induction of the auxin-signaling pathway by SNP (Pagnussat and others 2002). Like growth inhibition, in maize plants, PEG-induced osmotic stress also caused a decrease in RWC, consistent with previous observations of Merewitz and others (2011). However, supplementation of SNP efficiently reduced the toxic symptoms of PEG-induced decreases in water content. This result was consistent with a previous study in which SNP-treated wheat seedlings exposed to water stress tended to retain more water content (Garcia-Mata and Lamattina 2001). The increase in water retention induced by exogenously applied SNP in stress-treated plants might be linked to (i) stomatal closure, (ii) a reduced transpiration rate, or (iii) promoted adventitious root development. PEG stress maximally decreased root Ψ_π in 40 % PEG-treated maize plants. The lower Ψ_π of maize is likely connected with its lower RWC under stress conditions. Similarly, Lefèvre and others (2009)

demonstrated that the presence of PEG stress highly affected Ψ_π of Oryza sativa. In this study, after 72 h of stress exposure, Ψ_π increased in SNP-treated roots compared to PEG stress alone; this may be explained by a re-absorption of water and an increase in the root RWC. The ability of 150 μM SNP to maintain a high RWC in stressed-maize roots might be attributed to its contribution to osmotic adjustment by increase in Pro levels. Our results showed that Pro content in roots treated with 300 μM SNP under PEG stress decreased or had no significant changes, but it was greater in 150 μM SNP plus PEG-treated maize plants than that in PEG treatment alone. These results seem to indicate that Pro accumulation had no role in the osmotic adjustment production in 300 μM SNP-treated Z. mays under stress. These observations may be attributed to the role of SNP as a potent mediator in stress-induced physiological responses and water relations of maize plants.

Although ROS initiate several oxidatively destructive processes, they also trigger various signaling pathways. NO signaling and its cross-talk with ROS could interfere with the signal transduction pathways of the defense mechanism against stress (Zaninotto and others 2006). Osmotic stress is reported to enhance the total activity and overexpression of the isoenzymatic pattern of antioxidant enzymes for scavenging of toxic ROS levels in plants (Mittler and others Mittler and others 2004). Results of the present study also revealed that 40 % PEG treatment induced ROS accumulation as indicated by the increased $O_2^{\bullet-}$ and H_2O_2 content. Although SOD activity, responsible for the elimination of $O_2^{\bullet-}$ in cells, did not change in 20 % PEG-treated roots, it showed a remarkable increase after 40 % PEG. Therefore, H_2O_2 content increased only at high-PEG concentrations due to promotion of the conversion of $O_2^{\bullet-}$ to H_2O_2, by SOD activity. On the other hand, the remaining H_2O_2 content in the low-PEG concentration resulted from the reduced activities of Fe-SODs and Cu/Zn-SOD isozymes. Also, high-NOX activity in plants under stress conditions suggests that NOX is involved in the induction of oxidative stress through enhanced generation of $O_2^{\bullet-}$ (Jones and others 2007). However, in our study, NOX did not contribute to $O_2^{\bullet-}$ production in stress-treated plants. Although plants with exogenous SNP treatment under stress did not show an increase in SOD activity, a reduced amount of $O_2^{\bullet-}$ was observed in maize roots with SNP+PEG treatment, which is an important step in protecting cells. Contrary to this result, positive effects of NO on SOD activity under salt stress conditions have been reported (Li and others 2008). Suppression of SOD activation may be explained by NO itself being a direct scavenger of $O_2^{\bullet-}$ (Bavita and others 2012). After SNP treatment alone, the $O_2^{\bullet-}$ content did not change in maize roots, which was consistent with results observed by Kopyra and Gwóźdź (2003). Our results indicated that 40 %

PEG-induced accumulation of H_2O_2 in maize roots may be, at least in part, due to PEG-enhanced SOD activity. Only CAT activity (especially CAT1, CAT2, and CAT4 isozymes) was increased to remove the high level of H_2O_2 content in 40 % PEG-treated plants. Besides, NO may effectively reduce the level of ROS generated by stress (Xiong and others 2010). SNP supplemention of maize roots grown under 20 % PEG stress prevented increased CAT activity. The increased CAT activities in NO-supplemented plants under various forms of stress have been reported earlier (Jin and others 2010). Also, APX and GR could play a role in scavenging of H_2O_2 toxicity in maize plants exposed to both SNP supplementations under both PEG stresses, which indicates a protective role for exogenous NO in scavenging H_2O_2 in maize under stress. It seems that these results lead to alleviation of the oxidative damage as indicated by the lowered H_2O_2 levels. The present results are in agreement with Tian and Lei (2006) and Yang and others (2008), who found that NO increased the activity of CAT, APX, and GR under osmotic stress. Furthermore, in our study, NO treatments had no obvious effect on POX activity, just as reported by Sheokand and others (2010) in chickpea plants. On the other hand, Kopyra and Gwóźdź (2003) reported that CAT and POX activities in *Lupinus luteus* were markedly increased and decreased, respectively, after exposure to SNP treatment alone as supported by our results.

As well as enzymatic antioxidants, non-enzymatic antioxidants (ascorbic acid and glutathione) have a role in mechanisms of ROS detoxification in plants (Mittler and others 2004). The oxidized form of AsA produced by the action of APX is regenerated via the glutathione–ascorbate cycle involving MDHAR and DHAR and GSSG reduction by GR using the reducing power of NADPH (Khatun and others 2008). APX reduction requires AsA as a substrate and DHAR conversion back to AsA needs glutathione as substrate, so both glutathione and ascorbate are important. As MDHAR and DHAR are equally important in regulating the level of AsA and its redox state under oxidative stress (Eltayeb and others 2007), the decreases in these enzyme activities were reflected by decreased AsA content under stress conditions. In our study, the decreased tAsA content was accompanied by a decline in MDHAR and DHAR activities in maize roots exposed to PEG treatment. Despite in our experiments, APX increased greatly with SNP treatment under stress, which would indicate an elevated use of tAsA to detoxify H_2O_2 through APX activity, tAsA content also greatly increased.

Another important H_2O_2 scavenger that associates with GSH is GPX, which may also play an important role for the reduction of lipid peroxides and other hydroperoxides by GSH. Increasing of GST with GPX activity provides protection against oxidative stress (Roxas and others 2000).

However, in our study, GST and GPX activities decreased or remained similar to control levels in low-PEG concentration treated-maize roots, respectively. The induction of GST was only observed at 40 % PEG. This observation agrees with Kuzniak and Sklodowska (2005) who suggested that the enhanced level of GST activity resulted from tomato plants exposed to salt stress. However, higher levels of both GST and GPX persisted with exogenously applied SNP treatments in PEG-treated maize roots. These results suggested that GST/GPX over-expression provides increased glutathione-dependent peroxidase scavenging and alterations in ascorbate and glutathione metabolism, leading to reduced oxidative damage (Sairam and Tyagi 2004). The induction of GST and GPX in the SNP+PEG group was consistent with results of tAsA, tGlut, and GSH:GSSG ratio. Li and others (1998) suggest that NO could be playing a role in the elevation of GSH levels, either by increasing the biosynthesis rate of this metabolite or increased supply of cysteine.

H_2O_2 can also lead to the production of $OH^\bullet$ and is very reactive on important biomolecules or membranes in plants (Karuppanapandian and others 2011). In this study, as suggested by results in lipid peroxidation in root membranes of maize plants, the $OH^\bullet$ scavenging activity decreased at high-PEG concentration. However, both SNP concentrations under stress conditions improved the capacity of $OH^\bullet$ scavenging. Therefore, the enhanced scavenging ability of $OH^\bullet$ inhibited ROS accumulation in maize roots, and thus plants were protected from lipid peroxidation of membrane systems and oxidative damages. On the other hand, no change in the scavenging of this radical was observed with SNP treatment alone.

An enhanced level of lipid peroxidation under osmotic stress indicated oxidative damage in maize roots, meaning that lipid peroxidation might be a consequence of toxic ROS generation. In this study, the reported changes in the TBARS content were consistent with previous results observed by Zhao and others (2009). NO reportedly protects membrane integrity by inhibition of lipid peroxidation, either through scavenging of lipid peroxyl radicals or by inhibiting peroxidation enzymes. The supplementation of SNP-treatment solution with PEG caused a substantive reduction in TBARS accumulation induced by stress. The results were consistent with Jin and others (2010), including a protective role of SNP by reacting with lipid radicals and stopping the propagation of lipid oxidation. This may be due to (i) the antioxidant activity of SNP itself against induced-ROS under stress conditions, (ii) the signaling effect of this SNP on the gene expression and the antioxidant enzyme activity, or (iii) maintaining higher ATP-ase activity (Shi and Others 2007).

In conclusion, PEG-induced osmotic stress causes oxidative damage in maize roots through excessive generation of

ROS, and this effect is greater in 40 % PEG-treated plants. Although only the increase of CAT activity is responsible for the important protective effect when maize roots are exposed to PEG stress, induction of this antioxidant enzyme is inadequate to detoxify high levels of ROS as evidenced by growth, water content, OH$^\bullet$ scavenging activity, and level of lipid peroxidation, which suggests that maize roots are not able to prevent damage caused by oxidative stress. However, exogenously applied SNP could improve the dehydration tolerance against stress through effective water management and osmoregulation as well as enhancing the antioxidant enzyme/non-enzyme systems. Despite a decline in SOD and POX activities, the principal ROS scavenging enzymes in SNP treated-maize roots under PEG stress are GPX, GST, and APX, GR, MDHAR, DHAR involved in ascorbate–glutathione cycle, total ascorbate and glutathione which help to decrease the oxidative damage to biomolecules. It is possible that these antioxidant enzymes/non-enzymes can be activated and cooperate with one another to scavenge ROS and alleviate oxidative injury imposed by PEG in maize roots. The present study provides new insights into the roles and interactions of SNP, ROS, and antioxidants in hydroponically grown maize roots under PEG-induced osmotic stress.

Conflict of interest: The authors declare that they have no conflict of interest.

References

Bates L, Waldrenn R, Teare I (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39:205–207

Bavita A, Shashi B, Navtej SB (2012) Nitric oxide alleviates oxidative damage induced by high temperature stress in wheat. Indian J Exp Biol 50:372–378

Beauchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44:276–287

Beligni MV, Lamattina L (2001) Nitric oxide in plants: the history is just beginning. Plant Cell Environ 24:267–278

Bergmeyer N (1970) Methoden der enzymatischen analyse, vol 1. Akademie Verlag, Berlin

Bradford MM (1976) A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of the protein-dye binding. Anal Biochem 72:248–254

Chung SK, Osawa T, Kawakishi S (1997) Hydroxyl radical-scavenging effects of spices and scavengers from brown mustard (*Brassica nigra*). Biosci Biotech Bioch 61:118–123

Crawford NM, Guo FQ (2005) New insights into nitric oxide metabolism and functions. Trends Plant Sci 259:1360–1385

Dalton DA, Russell SA, Hanus FJ, Pascoe GA, Evans HJ (1986) Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soybean root nodules. P Natl Acad Sci USA 83:3811–3815

Elia AC, Galarini R, Taticchi MI, Dorr AJM, Mantilacci L (2003) Antioxidant responses and bioaccumulation in Ictalurus melas under mercury exposure. Ecotox Environ Safe 55:162–167

Eltayeb AL, Kawano N, Badawi GH, Kaminaka H, Sanekata T, Shibahar T, Inanaga S, Tanaka K (2007) Overexpression of monodehydroascorbate reductase in transgenic tobacco confers enhanced tolerance to ozone, salt and polyethylene glycol stresses. Planta 225:1255–1264

Foyer CH, Halliwell B (1976) The presence of glutathione and glutathione reductase in chloroplast: a proposed role in ascorbic acid metabolism. Planta 133:21–25

Garcia-Mata C, Lamattina L (2001) Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress. Plant Physiol 126:1196–1204

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Bioch 48:909–930

Gould KS, Lamotte O, Klinguer A, Pugin A, Wendehenne D (2003) Nitric oxide production in tobacco leaf cells: a generalized stress response? Plant, Cell Environ 26:1851–1862

Gouvea CMCP, Souza JF, Magalhaes ACN, Martins IS (1997) NO-releasing substances that induce growth elongation in maize root segments. Plant Growth Regul 21:183–187

Habig WH, Jacoby WB (1981) Assays for differentiation of glutathione S-transferase. Method Enzymol 77:398–405

Herzog V, Fahimi H (1973) Determination of the activity of peroxidase. Anal Biochem 55:554–562

Hou D, Fujii M, Terahara N, Yoshimoto M (2004) Molecular mechanisms behind the chemo preventive effects of anthocyanidins. J Biomed Biotechnol 5:321–325

Hunt R, Causton DR, Shipley B, Askew AP (2002) A modern tool for classical plant growth analysis. Ann Bot 90:485–488

Jiang M, Zhang J (2002) Involvement of plasma membrane NADPH oxidase in abscicic acid and water stress-induced antioxidant defense in leaves of maize seedlings. Planta 215:1022–1030

Jin JW, Xu YF, Huang YF (2010) Protective effect of nitric oxide against arsenic-induced oxidative damage in tall fescue leaves. Afr J Biotechnol 9:1619–1627

Jones MA, Raymond MJ, Yang Z, Smirnoff N (2007) NADPH oxidase-dependent reactive oxygen species formation required for root hair growth depends on ROP GTPase. J Exp Bot 58:1261–1270

Karuppanapandian T, Wang HW, Prabakaran N, Jeyalakshmi K, Kwon M, Manoharan K, Kim W (2011) 2,4-Dichlorophenoxy-acetic acid-induced leaf senescence in mung bean (*Vigna radiata* L. Wilczek) and senescence inhibition by co-treatment with silver nanoparticles. Plant Physiol Bioch 49:168–177

Khatun S, Ali MB, Hahn EJ, Paek KY (2008) Copper toxicity in *Withania somnifera*: growth and antioxidant enzymes responses of in vitro grown plants. Environ Exp Bot 64:279–285

Kopyra M, Gwóźdź EA (2003) Nitric oxide stimulates seed germination and counteracts the inhibitory effect of heavy metals and salinity on root growth of *Lupinus luteus*. Plant Physiol Bioch 41:1011–1017

Kopyra M, Gwóźdź EA (2004) The role of nitric oxide in plant growth regulation and responses to abiotic stresses. Acta Physiol Plant 26:459–472

Kuzniak E, Sklodowska M (2005) Compartment-specific role of the ascorbate-glutathione cycle in the response to tomato leaf cells to Botrytis cinerea infection. J Exp Bot 413:921–933

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lefévre I, Marchal G, Correal E, Zanuzzi A, Lutts S (2009) Variation in response to heavy metals during vegetative growth in Dorycnium pentaphyllum Scop. Plant Growth Regul 59:1–11

Li L, Lee TK, Meier PJ, Ballatori N (1998) Identification of glutathione as a driving force and leukotriene C4 as a substrate for oatp1, the hepatic sinusoidal organic solute transporter. J Biol Chem 273:16184–16191

Li QY, Niu HB, Yin J, Wang MB, Shao HB, Deng DZ, Chen XX, Ren JP, Li YC (2008) Protective role of exogenous nitric oxide against oxidative-stress induced by salt stress in barley (Hordeum vulgare). Colloid Surf B 65:220–225

Liu ZJ, Guo YK, Bai JG (2010) Exogenous hydrogen peroxide changes antioxidant enzyme activity and protects ultrastructure in leaves of two cucumber ecotypes under osmotic stress. J Plant Growth Regul 29:171–183

Madhava Rao KV, Sresty TVS (2000) Antioxidative parameters in the seedlings of pigeon pea [Cajanus cajan (L.) Millspaugh] in response to Zn and Ni stresses. Plant Sci 157:113–128

Merewitz E, Gianfagna T, Huang B (2011) Protein accumulation in leaves and roots associated with improved drought tolerance in creeping bentgrass expressing an ipt gene for cytokinin synthesis. J Exp Bot 62(15):5311–5333

Mittler R, Zilinskas BA (1993) Detection of ascorbate peroxidase activity in native gels by inhibition of the ascorbate dependent reduction of nitroblue tetrazolium. Anal Biochem 212:540–546

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. Trends Plant Sci 9:490–498

Miyake C, Asada K (1992) Thylakoid-bound ascorbate peroxidase in spinach chloroplasts and photoreduction of its primary oxidation product monodehydroascorbate radicals in thylakoids. Plant Cell Physiol 33:541–553

Mukherjee SP, Choudhuri MA (1983) Implications of water stress-induced changes in the levels of endogenous ascorbic acid and hydrogen peroxide in Vigna seedlings. Physiol Plant 58:166–170

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22:867–880

Pagnussat GC, Simontacchi M, Puntarulo S, Lamattina L (2002) Nitric oxide is required for root organogenesis. Plant Physiol 129:954–956

Paradiso A, Berardino R, Pinto MC, Toppi LS, Storelli MM, Tommasi F, Gara L (2008) Increase in ascorbate–glutathione metabolism as local and precocious systemic responses induced by cadmium in durum wheat plants. Plant Cell Physiol 49:362–374

Roxas VP, Lodhi SA, Garrett DK, Mahan JR, Allen RD (2000) Stress tolerance in transgenic tobacco seedlings that overexpress glutathione S-transferase/glutathione peroxidase. Plant Cell Physiol 41:1229–1234

Sagi M, Fluhr R (2006) Production of reactive oxygen species by plant NADPH oxidases. Plant Physiol 141(2):336–340

Sairam RK, Tyagi A (2004) Physiology and molecular biology of salinity stress tolerance in plants. Curr Sci 86:407–421

Seevers FM, Daly JM, Catedral FF (1971) The role of peroxidase isozymes in resistance to wheat stem rust. Plant Physiol 48:353–360

Sheokand S, Bhankar V, Sawhney V (2010) Ameliorative effect of exogenous nitric oxide on oxidative metabolism in NaCl treated Chickpea plants. Braz J Plant Physiol 22(2):81–90

Shi Q, Ding F, Wang X, Wei M (2007) Exogenous nitric oxide protect cucumber roots against oxidative stress induced by salt stress. Plant Physiol Biochem 45:542–550

Silva EN, Ferreira-Silva SL, Fontenele AV, Ribeiro RV, Viégas RA, Silveira JAG (2010) Photosynthetic changes and protective mechanisms against oxidative damage subjected to isolated and combined drought and heat stresses in Jatropha curcas plants. J Plant Physiol 167:1157–1164

Smart RE, Bingham GE (1974) Rapid estimates of relative water content. Plant Physiol 53:258–260

Tian X, Lei Y (2006) Nitric oxide treatment alleviates drought stress in wheat seedlings. Biol Plantarum 50(4):775–778

Uchida A, Jagendorf AT, Hibino T, Takabe T (2002) Effects of hydrogen peroxide and nitric oxide on both salt and heat tolerance in rice. Plant Sci 163:515–523

Vitória AP, Lea PJ, Azevedo RA (2001) Antioxidant enzymes responses to cadmium in radish tissues. Phytochemistry 57:701–710

Woodbury W, Spencer AK, Stahman MA (1971) An improved procedure for using ferricyanide for detecting catalase isozymes. Anal Biochem 44:301–305

Wu SJ, Wu JY (2008) Extracellular ATP-induced NO production and its dependence on membrane Ca^{2+} flux in Salvia miltiorrhiza hairy roots. J Exp Bot 59:4007–4016

Xiong J, Fu G, Tao L, Zhu C (2010) Roles of nitric oxide in alleviating heavy metal toxicity in plants. Arch Biochem Biophys 497:13–20

Xu MJ, Dong JF, Zhu MY (2005) Effect of nitric oxide on catharanthine production and growth of Catharanthus roseus suspension cells. Biotechnol Bioeng 89:367–371

Xu S, Li J, Zhang X, Wei H, Cui L (2006) Effects of heat acclimation pretreatment on changes of membrane lipid peroxidation, antioxidant metabolites, and ultrastructure of chloroplasts in two cool-season turfgrass species under heat stress. Environ Exp Bot 56:274–285

Yamasaki H (2000) Nitrite-dependent nitric oxide production pathway: implications for involvement of active nitrogen species in photoinhibition in vivo. Philos T Roy Soc B 355:1477–1488

Yang Y, Han C, Liu Q, Lin B, Wang J (2008) Effect of drought and low light on growth and enzymatic antioxidant system of Picea asperata seedlings. Acta Physiol Plant 30:433–440

Zaninotto F, La Camera S, Polverari A, Delledonne M (2006) Cross talk between reactive nitrogen and oxygen species during the hypersensitive disease resistance response. Plant Physiol 141:379–383

Zhang YY, Liu J, Liu YL (2004) Nitric oxide alleviates growth inhibition of maize seedlings under salt stress (in Chinese). J Plant Physiol Mol Biol 30:455–459

Zhao HX, Li Y, Duan BL, Korpelainen H, Li CY (2009) Sex-related adaptive responses of Populus cathayana to photoperiod transitions. Plant Cell Environ 32:1401–1411