

Effects of Various Chemical Agents for Alleviation of Drought Stress in Rice Plants (*Oryza sativa* L.)

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Abstract

Drought stress is one of the main causes for crop yield reduction in the majority of agricultural regions of the world. The effects of sodium nitroprusside (SNP; nitric oxide donor) treatment on induced drought stress were investigated. Drought stress induced by polyethylene glycol (PEG) with different concentrations 5%, 10%, 15% and 20% and previous four concentrations of both poly ethylene glycol and 100 μ M of SNP on rice (*Oryza sativa*) culture were used. The results showed that, while drought conditions increased after four weeks of administration, the stress signals increased markedly via H_2O_2 and MDA as a response to elevated oxidative damage promoted by lipid peroxidation under elevated free radical formation. The decrease in water potential also increased the contents of AsA and GSH as a strongly antioxidant defense compound against induced oxidative damage, total soluble sugars, total amino acids, total phenols and PAL activity. All PEG and nitric oxide treatments significantly increased the total soluble phenols contents and PAL activity induced under oxidative stress condition and SNP signaling action controlled the oxidative damage. In addition, the increase in the activity of various antioxidant defense enzymes SOD, APX, GR and CAT represent the protective activity to counteract the oxidative injury promoted by drought conditions.

Keywords: drought, nitric oxide, oxidative stress, antioxidant, lipid peroxidation, rice

Abbreviations: AsA-ascorbic acid; APX-ascorbate peroxidase; DasA- dehydroascorbic acid; dwt- dry weight; EDTA- ethylenediamine-N-N-N-N-tetraacetic acid; FAA- free amino acids; FW- fresh weight; GSH- glutathione; GSSG- oxidised glutathione; GR- glutathione reductase; mBBr- monobromobimane; ROS- reactive oxygen species; CAT- catalase; MDA- malondialdehyde; NBT- nitroblue tetrazolium; PAL- phenylalanine ammonia lyase; PEG- polyethylene glycol; POD- peroxidase; SNP- sodium nitroprusside; and SOD- superoxide dismutase

Introduction

Drought stress induces several physiological, biochemical and molecular responses in several crop plants, which would help them to adapt to such limiting environmental conditions (Bajaj *et al.*, 1999; Arora *et al.*, 2002). It inhibits the photosynthesis of plants, causes changes of chlorophyll contents and components and damage to the photosynthetic apparatus (Escuredo *et al.*, 1998). It also inhibits the photochemical activities and decreases the activities of enzymes in the Calvin cycle (Monakhova and Chernyadev, 2002). The alteration of antioxidant metabolisms is one of the fundamental metabolic processes that may influence the drought tolerance of perennial grasses (Da Costa and Huang, 2007). A common effect of drought stress is the disturbance between the generation and quenching of reactive oxygen species (ROS) (Smirnov, 1998).

Reactive oxygen species (ROS) are the byproducts of many degenerative reactions in crop plants, which will affect the regular metabolism by damaging the cellular components (Foyer and Noctor, 2002). Extensive study on oxidative stress has demonstrated that exposure of plants

to adverse environmental conditions induces the overproduction of ROS such as superoxide radical ($O_2^{\cdot-}$), (H_2O_2) and hydroxyl radical (OH^{\cdot}) in plant cells (Wise and Naylor, 1987).

As a consequence, plants' evolved cellular adaptive responses like up-regulation of oxidative stress protectors and accumulation of protective solutes (Horling *et al.*, 2003). Antioxidant defense enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POD), glutathione reductase (GR) and monodehydroascorbate reductase (MDAR) are the systems designed to minimize the concentrations of superoxide and hydrogen peroxide. Superoxide-dismutase (SOD) catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. (H_2O_2) is eliminated by catalase and peroxidases, which include both enzymic and non-enzymic H_2O_2 degradation (Peltzer *et al.*, 2002). The antioxidants such as ascorbate (AsA) and glutathione (GSH) are involved in scavenging ROS primarily by the Halliwell-Asada pathway, which scavenges H_2O_2 , while MDAR and GR are involved in the regeneration of ascorbate (Horemans *et al.*, 2000).

Drought is also known to affect the metabolism of soluble carbohydrates, a group of compounds that may act as compatible solutes, as well as antioxidants. These compounds usually increase as a result of water deficit (Smirnov, 1993; Smirnov and Cumbes, 1989). Another group of compounds, which may be affected by water deficit, are free amino acids. Proline and total free amino acids are often increased in water-stressed leaves (Pinheiro *et al.*, 2004; Van Heerden and Krüger, 2002). Accumulation of protective solutes like proline and soluble sugar in the leaf is a unique plant response to environmental stresses, specifically to drought stress (Sakamoto and Murata, 2002). Proline acts as an osmoprotectant (Yoshida *et al.*, 1997; Heuer and Nadler, 1998) and soluble sugar also acts as an osmoprotectant (Kameli and Losel, 1995; Rekika *et al.*, 1998; Shao *et al.*, 2005a) during stress. Proline may also act as antioxidant (Smirnov and Cumbes, 1989; Reddy *et al.*, 2004b).

The activation of PAL activity is a common response of plant cells to biotic and abiotic stresses and may also function as antioxidants because of their free-radical trapping properties (Haslam, 1998). Studies with several different species of plants have shown PAL is activated by many environmental factors, which is consistent to the increase in PAL activity in rice at our experiment.

Nitric oxide (NO) is a uniquely volatile molecule in plants (Velikova *et al.*, 2008). It is a signaling molecule that has been implicated in the activation of plant defenses. Nitric oxide is a bioactive free radical which plays important roles in many physiological processes in plants, such as growth, development, senescence and adaptive responses to multiple stresses (Zhao *et al.*, 2004; Graziano and Lamattina, 2005). Under ROS-related toxicity NO may act as a chain breaker and thus limit the oxidative damage. Recently, a function of nitric oxide in the protection of plants against oxidative stress under various adverse conditions was reported (Beligni *et al.*, 2002; Shi *et al.*, 2005). Many previous studies have reported presence of NO in the plant kingdom and its involvement in growth, development and defense responses (Beligni and Lamattina, 1999). Tu *et al.* (2003) found that 0.1 mM SNP delayed the senescence of wheat leaves by inhibition of the degradation of chlorophyll and soluble proteins, especially Rubisco.

The aim of the present study was to investigate the effects of sodium nitroprusside (SNP; nitric oxide donor) treatment of antioxidant mechanisms induced by developing drought stress in rice plants mediated by polyethylene glycol (PEG) and to evaluate the changes of selected biochemical parameters known as protective substances at different intensities of drought stress.

Materials and methods

Plant materials and cell culture

The plant cell line for the study was initiated from the callus induced from the young stems of rice (*Oryza sativa*)

plants (cultivar). The callus was initiated in a MS-Medium supplemented with 4.0 mg/l of Naphthaleneacetic acid (NAA), 4.0 mg/l of kinetin, 20 g/l sucrose and 8 g/l agar. The callus line had been in culture for 5 months by the time of this study. The medium was adjusted to pH 5.8 and then sterilized at 121°C for 20 min, incubated in dark at 25°C. The Subculture occurred every 4 weeks.

Experimental design and culture treatment

Chemicals used in the experiment were mainly obtained from Sigma and other chemical companies. For the experiment of external addition, 8 treatments were used. Polyethylene glycol in different concentrations 5%, 10%, 15% and 20% as source of drought stress; and various combinations of both polyethylene glycol 5%, 10%, 15% and 20% and 100 µM of Sodium nitroprusside (SNP as Nitric Oxide donors) and negative control without PEG or SNP) on rice culture and monitoring total soluble amino acids, total soluble carbohydrates, total Ascorbic acids and oxidative burst response of GSH content, MDA accumulation, hydrogen peroxide content, PAL activity and total soluble phenols contents and the role of various antioxidant defense enzymes activity against oxidative damage during the defense process (ascorbic peroxidase, SOD, glutathione reductase and catalase activity) of rice callus, which was pre-treated with NO donors Sodium nitroprusside SNP (100µM) treatment. Against the control, all parameters and enzymes activities have been monitored after four weeks of administration.

This NO donor and its dosage used in the experiments were chosen based on previous studies (Doke, 1983). They were all pre-dissolved in distilled water of the final concentrations in the culture and sterilized by filtration, then added to the culture medium. All the following responses were measured in the solid cultures. All treatments were performed in triplicate and the results were represented by their mean ± standard error (S.E.)

After treatments, callus cells were harvested at time intervals, washed twice with 100 ml water on a porous-glass funnel with filter paper (Whatman No.1) then frozen in liquid nitrogen and stored in the deep freezer for further investigations and analysis.

Preparation of enzyme extracts

Samples of 0.25g was homogenized in 5 ml of 50 mM phosphate buffer pH 7.0 containing 1.0 N NaCl, 1% PVP (Sigma) M.W. 40,000, 1 mM ascorbate (Sigma) at 4°C. After centrifugation at 15,000 × g for 15 min the supernatant was collected.

Assay of protein content

Soluble proteins were measured by the Bio-Rad micro assay modification of the Bradford (1976) procedure using crystalline bovine serum albumin as a reference.

*Determination of oxidative burst**Lipid peroxidation (MDA contents)*

Thiobarbituric acid reaction (TBA) as described by Heath and Packer (1968). Fresh mass (200 mg) from culture was homogenized in 2 ml of 0.1% (w/v) trichloroacetic acid (TCA), followed by centrifugation at $12,000 \times g$ for 20 min. The supernatant (1 ml) obtained was mixed with an equal volume of TCA (10%) containing 0.5% (w/v) TBA or no TBA as the blank and heated at 95°C for 30 min and then cooled in ice. The reaction product was centrifuged at $12,000 \times g$ for 15 min and the supernatant absorbance was measured at 400, 532 and 600 nm. The MDA equivalent was derived from the absorbance according to Hodges *et al.* (1999).

Assay of hydrogen peroxide concentration

Hydrogen peroxide was measured by the method described by (Capaldi and Taylor, 1983) with a slight modification. The ground callus in 5% TCA (2.5 ml per 0.5 g callus) with 50 mg active charcoal at 0°C , and centrifuged for 10 min at $15,000 \times g$. Supernatant was collected, neutralized with 4 N KOH to pH 3.6 and used for H_2O_2 assay. The reaction mixture contained 200 μl of leaf extract, 100 μl of 3.4 mM 3-methylbenzothiazoline hydrazone (MBTH). The reaction was initiated by adding 500 μl of horseradish peroxidase solution (90 U per 100 ml) in 0.2 M sodium acetate (pH 3.6). Two minutes later 1400 μl of 1 N HCl was added. Absorbance was read at 630 nm after 15 min.

Determination of total glutathione

The level of total acid-soluble SH compound (glutathione GSH) was determined with Ellman's reagent according to (De Vos *et al.*, 1992). Samples of 0.5 g were homogenized in 6% m-phosphoric acid (pH 2.8) containing 1 mM EDTA. The buffer was mixed with 630 μl of 0.5 M K_2HPO_4 and 25 μl of mM 5, 5'-dithiobis (2-nitrobenzoic acid) (final pH 7). The absorbance at 412 nm was read after 2 min. The GSH concentration was determined from a standard curve.

Ascorbic acid determination

Levels of AsA followed the procedure described by Singh *et al.* (2006) with few modifications. Briefly, fresh leaf sample of a known weight (1 g) was extracted with 3 ml of 5% (w/v) trichloroacetic acid (TCA) and centrifuged at $18,000 \times g$ for 15 min. AsA was determined in a reaction mixture consisting of 0.2 ml of supernatant, 0.5 ml of 150 mM phosphate buffer (pH 7.4, containing 5 mM EDTA) and 0.2 ml of deionized water. Colour was developed in reaction mixtures with the addition of 0.4 ml of 10% (w/v) TCA, 0.4 ml of 44% (v/v) phosphoric acid, 0.4 ml of α,α -dipyridyl in 70% (v/v) ethanol and 0.2 ml of 3% (w/v) FeCl_3 . The reaction mixtures were incubated at 40°C for 40 min. and the absorbance was read at 532 nm.

*Determination of antioxidant defense enzymes activity**Assay of SOD activity*

The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of NBT using the method of (Beauchamp and Fridovich, 1971). The 3 ml reaction mixture contained 50 mM phosphate buffer pH 7.8, 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 1.0 mM EDTA and 20 μl enzyme extract. Riboflavin was added last and the reaction was initiated by placing the tubes 30 cm below 15 W fluorescent lamps. The reaction was started by switching on the light and was allowed to run for 10 min. Switching off the light stopped the reaction and the tubes were covered with black cloth. Non-illuminated tubes served as control. The absorbance at 560 nm was read. One unit of SOD is the amount of extracts that gives 50% inhibition the rate of NBT reduction.

Assay of ascorbate peroxidase (APX) activity

Ascorbate peroxidase activity was estimated according to the method of Nakano and Asada (1981). Enzyme activity was determined by the decrease in absorbance of ascorbate at 290 nm. The reaction mixture consisted of enzymatic extract, 50 mM sodium phosphate buffer (cold), pH 7, 0.5 mM ascorbate, 0.5 mM H_2O_2 and 0.1 mM EDTA, in a 0.3 ml final volume. The reaction started after the hydrogen peroxide addition. The molar extinction coefficient $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ was used to calculate ascorbate peroxidase activity. Enzyme activity was expressed as unit's mg-1 protein. One unit of enzyme was the amount necessary to decompose 1 μmol of substrate per minute at 25°C .

Assay of glutathione reductase (GR) activity

The activity of GR was determined based on the decrease in absorbance at 340 nm due to the oxidation of NADPH to NADP according to the method of Foyer and Halliwell (1976), with minor modifications. The reaction mixture (3 mL) consisted of 50 mM Tris-HCl (pH 7.6), 5 mM MgCl_2 , 0.5 mM GSSG, 0.2 mM NADPH and 0.1 mL enzyme extract. The reaction was started by the addition of GSSG and the NADPH oxidation rate was monitored at 340 nm for 3 min. Enzyme activity was determined using the molar extinction coefficient for NADPH ($6.2 \text{ mM}^{-1} \text{ cm}^{-1}$).

Assay of catalase activity

Catalase activity was determined by consumption of H_2O_2 using the method of (Dhindsa *et al.*, 1981). The reaction mixture contained 50 mM potassium phosphate buffer pH 7.0, 15 mM H_2O_2 and enzyme extract. The consumption of H_2O_2 was spectrophotometrically monitored at 240 nm ($\epsilon = 45.2 \text{ M}^{-1} \text{ cm}^{-1}$). The enzyme activity was expressed in $\mu\text{M H}_2\text{O}_2 \text{ min}^{-1}$.

Evaluation of elicitation induces secondary products-based defense compounds against drought stress.

Assay of phenylalanine ammonia lyase (PAL)

Phenylalanine ammonia lyase activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm as described by (Dickerson *et al.*, 1984). A known weight of callus was homogenized in 5 ml of 0.1 M sodium borate buffer, pH 7.0 containing 0.1 g of insoluble polyvinyl pyrrolidone (PVP). The homogenate was centrifuged at 15000 g and for 20 min. The supernatant was used as the enzyme source for assay. Samples containing 0.4 ml of enzyme extract were incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 30°C. The reaction was arrested by adding 0.5 ml of 1 M trichloroacetic acid and incubated at 37°C for 5 min. The blank contains 0.4 ml of crude enzyme extract and 2.7 ml of 0.1 M borate buffer (pH 8.8) and absorbance was measured at 290 nm and used an extinction coefficient of 9630 per min cm⁷¹ for trans-cinnamic acid in 0.1 M borate buffer (pH 8.8). The absorbance 9630 is equal to 1 mol/1 min or the absorbance is 0.963, the product formed is 100 nmol/ml/min. The enzyme activity was expressed on the fresh weight basis of the amount of trans-cinnamic acid.

Assay of phenol

The phenolic assay was conducted as per the method of (Zieslin and Ben-zaken, 1993). The samples were homogenized at the rate of 0.1 g per 1 ml of 80% methanol and the methanolic extract was kept in a water bath at 70°C for 15 min with frequent agitation. One ml of methanolic extract was added to 5 ml of distilled water and 250 ml of Folin-Ciocalteu reagent (1 N) was added and the solution was kept at 25°C for 30 min. Finally, 1 ml of saturated solution of Na₂CO₃ and 1 ml of distilled water were added and the reaction mixture was incubated for 1 h at 25°C. After the blue color development, the absorbance was recorded at 725 nm. The contents of total soluble phenols were calculated according to a standard curve obtained from a Folin-Ciocalteu reaction with a catechol solution. The phenol content was expressed as phenol equivalents in mg/g fresh weight of callus tissues.

Assay of total amino acids

Total amino acids were determined according to the method of Rosein (1957) using 0.5 g samples hydrolyzed in 6 N HCl for 24 hr at 105°C. 1 ml of the extract, 0.5 ml of acetate buffer and 0.5 ml of ninhydrin solution were added then heated in a water bath at 100°C for 15 minutes. Immediately after removal from the water bath 3 ml of 50% isopropyl alcohol-water diluents was added to the mixture which was then shaken vigorously. After cooling to room temperature the samples were read on a spectrophotometer at 570 nm. Leucine in 0.1 M, pH 5.0 citrate buffer was used as the standard.

Assay of total soluble sugars

Total soluble sugars were determined in ethanol extract of plant tissue by the phenol-Sulphoric acid method as described by (Dubois *et al.*, 1956). Two grams of the fresh ground samples were accurately weighted then extracted by boiling in 80% neutral aqueous ethanol for 6 hours. The extract was filtered through whatman filter paper No. 1. After filtration, the clear solution was made up to a known volume with ethanol solution. An aliquot ethanol extract (10 ml) was transferred to clean dry beaker and heated to dryness in water bath. The residue was then dissolved in water and quantitatively transferred to 25 ml volumetric flask and made up to the mark with distilled water. 1 ml of water extract, 1 ml of the phenol solution (5%) and 5 ml of sulfuric acid (96%) were added. The O.D was measured at 490 nm using Unicam spectrophotometer against blank, which prepared as the test except that the distilled water was added instead of the sample. Graphic plot of the O.D values against various standard solutions of different concentrations of glucose was used as a standard curve.

Statistical analyses

All determinations done in triplicate. Statistical analyses were performed using SPSS (version 10) program. Mean and standard error were descriptive measures of quantitative data using the analysis of variance test (ANOVA) for independent samples. P-values <0.05 were considered significant.

Results and discussion

Drought stress and (Total amino acids, soluble carbohydrates and ascorbic acid)

Soluble sugar as important osmoregulation matter can decrease osmotic potential and improve stability of soluble protein. As shown in Tab. 1, the increase in drought stress and SNP treatments reflected a highly significant increase in the total amino acids, total soluble sugars and total ascorbic acids. Total soluble sugar content increased rapidly and reached to its maximum at higher treatments of PEG (20%) also in both PEG (20 %) and SNP from 12.76 mg/g¹FW in negative control to 23.44 mg/g¹FW in PEG (20%) and 23.54 mg/g¹FW in PEG (20%) + SNP which increased about two folds. Soluble sugar, in addition to their storage functions, was considered to have an important role in controlling cellular metabolism. The highest significant increase in the total amino acids and total ascorbic acids have been observed with the PEG (20 %) + SNP treatment, which reflect the induction effect of SNP addition on the gene expression of somatically functional molecules. The present results indicated that total amino acids and soluble sugar are significant contributors to metabolism under stress as mentioned before by (Harding *et al.*, 2003; Shao *et al.*, 2005 a, b and c). Thus, sucrose seems to play a key role in the integration of plant growth and seems to be part of a wider mechanism for balancing

Tab. 1. Effects of drought stress by polyethylene glycol and sodium nitroprusside treatments on the contents of total soluble carbohydrates, total amino acids and ascorbic acid concentrations (mg/g⁻¹ FW) in rice plants

Treatments	Total soluble sugars (mg/g ⁻¹ FW)	Total amino acids (mg/g ⁻¹ FW)	Total ascorbic acids (mg/g ⁻¹ FW)
Negative control	12.76 ± 0.96 ^d	2.01 ± 0.19 ^h	7.61 ± 0.48 ^f
PEG (5%)	13.87 ± 0.89 ^d	3.03 ± 0.16 ^e	11.43 ± 0.97 ^e
PEG (10%)	17.64 ± 1.23 ^c	3.54 ± 0.28 ^e	13.21 ± 0.99 ^{de}
PEG (15%)	21.44 ± 1.74 ^{ab}	4.39 ± 0.26 ^d	14.34 ± 1.18 ^{cd}
PEG (20%)	23.44 ± 1.78 ^a	4.76 ± 0.29 ^c	15.98 ± 1.38 ^{bc}
PEG (5%)+SNP	16.54 ± 1.58 ^c	3.21 ± 0.23 ^f	12.25 ± 0.98 ^e
PEG (10%)+SNP	20.12 ± 1.79 ^b	4.25 ± 0.32 ^d	14.57 ± 1.38 ^{cd}
PEG (15%)+SNP	22.12 ± 1.59 ^{ab}	4.98 ± 0.31 ^b	16.84 ± 1.45 ^b
PEG (20%)+SNP	23.54 ± 1.49 ^a	5.64 ± 0.39 ^a	21.95 ± 1.98 ^a
LSD	1.715	0.173	1.618

Values are means ± standard error (SE) and the different letters in the same row indicated significant difference at P < 0.05

carbon acquisition and allocation within and between organs (Farrar and *et al.*, 2000; Foyer *et al.*, 2003; Shao *et al.*, 2005c). When plants were subjected to stress and the stimulation of sugar accumulation was proportional to osmotic adjustment as shown in Tab. 1. These observations seem to indicate that sugar accumulation has some role in the osmotic adjustment production. It has been suggested that under water stress soluble sugars can function in two ways, which are difficult to separate: as osmotic agents and as osmoprotectors (Bohnert *et al.*, 1995). As osmoprotectors, sugars stabilize proteins and membranes, most likely substituting the water in the formation of hydrogen bonds with polypeptide polar residues (Crowe *et al.*, 1992) and phospholipid phosphate groups (Strauss and Hauser, 1986).

Also, the drought conditions caused a marked increase in the total amino acids after the high dose of PEG and (PEG and SNP) by more than 2.37 and 2.8 folds respectively, above negative control (Tab. 1). The presence of amino acids especially proline may play a role in the protection from desiccation and from the harmful effects derived from solute accumulation. Leigh *et al.* (1981) observed that proline is predominantly confined to the cytoplasm, which could mean that proline affects osmotic adjustment in certain organelles. However, proline can act as an osmoprotector of cytosolic enzymes and cellular structures (Csonka, 1989). Proline accumulation may help the plant to survive for short periods of drought and recover from stress. Besides, high proline concentration measured in sludge-treated nodules could also contribute to a protective role as scavenger of ROS (Koca *et al.*, 2007; Türkan and Demiral, 2009). All these factors could result in improved adaptation ability and growth of plants under drought conditions.

Our results are in agreement with (Van Heerden and Krüger, 2002; Pinheiro *et al.*, 2004) who found that the increase in total amino acid is usually correlated to adaptation with water deficit.

Among the non-enzymatic antioxidants, ascorbate is found to be one of the best characterized compounds, required for many key metabolic functions in plant cells (Smirnoff and Wheeler, 2000). Ascorbate acts as an antioxidant, protecting cells against oxidative stress. AA has the capacity to eliminate different ROS including singlet oxygen, superoxide and hydroxyl radicals (Foyer, 2001). Ascorbic acid (AsA) was the major water-soluble antioxidant in plant leaves. Our results are in agreement with Smirnoff and Pallanca (1996) and Reddy *et al.* (2004a), who found that, marked increase of foliar ascorbic acid under drought indicates active stress adaptation in apple trees and mulberry. In our present study, the contents of ascorbate (Tab. 1) increased 2.1 and 2.9 folds in callus rice after high dose of drought stress and NO treatments.

Drought stress, SNP, lipid peroxidation, H₂O₂ content and GSH content

These data reflected that there was a significant increase in the various oxidative indicators. Lipid peroxidation, H₂O₂ and total glutathione contents under various PEG drought stress treatments were compared with the control negative group. The results show that the addition of SNP to various PEG treatments significantly reduced the oxidative stress induced by drought conditions (Tab. 2). This may be due to the antioxidant activity of the SNP itself against reactive oxidant molecules initiated under stress condition, or it could be attributed to the signaling effect of this SNP on the gene expression and the enzyme activity level of various anti oxidant defense enzymes. And also may be due to the anti oxidant defense molecules, such as

Tab. 2. Effects of drought stress by polyethylene glycol (PEG) and SNP treatments on the contents of lipid peroxidation (MDA), H₂O₂ and GSH in rice plants

Treatments	MDA (nmol/g FW)	H ₂ O ₂ (μmol/g FW)	GSH (μmol/g FW)
Negative control	0.89 ± 0.06 ^c	80.86 ± 5.76 ⁱ	3.67 ± 0.27 ^e
PEG (5%)	5.75 ± 0.23 ^d	243.54 ± 13.76 ^f	5.87 ± 0.36 ^d
PEG (10%)	8.43 ± 0.37 ^b	264.98 ± 18.32 ^c	6.19 ± 0.43 ^d
PEG (15%)	9.43 ± 0.49 ^b	327.86 ± 15.76 ^c	7.98 ± 0.65 ^c
PEG (20%)	11.98 ± 0.78 ^a	387.90 ± 23.46 ^a	8.54 ± 0.75 ^c
PEG (5%)+SNP	4.85 ± 0.36 ^d	189.24 ± 12.14 ^h	7.24 ± 0.39 ^{cd}
PEG (10%)+SNP	6.27 ± 0.58 ^{cd}	212.95 ± 17.56 ^g	8.32 ± 0.59 ^c
PEG (15%)+SNP	7.93 ± 0.62 ^{bc}	289.67 ± 22.11 ^d	9.87 ± 0.78 ^b
PEG (20%)+SNP	8.00 ± 0.73 ^{bc}	349.47 ± 23.71 ^b	12.64 ± 0.99 ^a
LSD	1.514	16.183	1.184

Values are means ± standard error (SE) and the different letters in the same row indicated significant difference at P < 0.05

total soluble phenolic compounds. The lipid peroxidation product as MDA content of the treated plants by PEG was significantly higher than that of the control plants, beginning at (5-20 % PEG; PEG and SNP) treatments (Tab. 2). The levels of MDA content reached the highest values in the treatment of PEG (20%); PEG20% and SNP treatments. The levels of MDA in stressed plants were greater than that of control plants, about 13 and 9 times, respectively.

The observed changes in the MDA content were consistent with previous results observed by Fu and Huang (2001) and Bai *et al.* (2006). These authors stated that, an enhanced level of lipid peroxidation of grasses and maize under drought stress indicated oxidative damage to plants, it means lipid peroxidation may be a consequence of generation of reactive oxygen species ($\text{OH}\cdot$, O_2^- , H_2O_2). Moreover, H_2O_2 content results showed a marked increase in all PEG treatments compared to untreated plants (Tab. 2), this increment reached its maximum at the highest PEG +SNP treatment and recorded 23%. Our results are in agreement with Gong *et al.* (2005) who found marked increase in H_2O_2 content in wheat plants as a result of drought stress.

Antioxidant content of glutathione (GSH) in rice treated with drought stress was shown in Tab. 2. Glutathione was higher in all treated plants under drought stress. The increased of GSH content was gradually increased by increasing of the time of PEG treatments. However, GSH may play a protective role in scavenging of singlet oxygen, peroxides and hydroxyl radicals and is involved in recycling reduced of ascorbic acid (AsA) in the ascorbate-glutathione pathway in chloroplasts (Foyer, 1993). The increase of GSH may be driven by an enhancement of H_2O_2 formation in the drought. The treated callus with 100 μM SNP had the lowest content of MDA and H_2O_2 and the highest antioxidant content of GSH and ascorbate. As it is now commonly accepted NO as a second messenger in

plants, it is supposed that low concentration of NO might be a signal molecule to induce/stabilize the expression of many antioxidant compounds (Gong *et al.*, 2005).

Drought stress, antioxidant enzymes activities and total phenols content

The drought-stressed treatments had double times increase in APX and GR activities at the maximum treatment PEG (20%) which reached 25.65 Unit/mg protein and 6.96 $\mu\text{mol}/\text{mg}$ protein/min respectively, in comparison to untreated cultures which recorded 12.61 Unit/mg protein and 2.88 $\mu\text{mol}/\text{mg}$ protein/min respectively, and three times over the treatment with NO. While SOD and CAT activity was approximately 1.5 and 2 times higher than the untreated cells at the highest treatment of PEG. Therefore the treatment of NO after PEG (20%) showed significant increase by 1.7 and 3.7 times higher than control plants. These activities change is a strong hint that the drought treatment actually led to oxidative stress. While the treatment with a low concentration of NO might be improve the activity of antioxidative enzymes including SOD, APX, GR and CAT.

Catalases and peroxidases (CAT and POD) play an essential role in scavenging for H_2O_2 toxicity. The combined action of CAT and SOD converts the toxic superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) to water and molecular oxygen (O_2), thus averting the cellular damage under unfavorable conditions like drought stress (Noctor *et al.*, 2000; Reddy *et al.*, 2000; Chaitanya *et al.*, 2002). The activity of GR which was relatively high in drought-stressed plants might be able to increase the ratio of $\text{NADP}^+/\text{NADPH}$, thereby ensuring availability of NADP^+ to accept electrons from photosynthetic electron transport chain and to facilitate the regeneration of oxidized ascorbate (Noctor *et al.*, 2002). Higher antioxidant system activity was also observed in the drought-tolerant cultivars of wheat, Lascano *et al.* (2001), coffee, Lima, *et*

Tab. 3. Effects of drought stress by means of polyethylene glycol (PEG) and SNP treatments on the activities of superoxide dismutase (SOD), Ascorbate peroxidase (APX), glutathione reductase (GR) and catalase (CAT) in rice plants

Treatments	SOD activity (Unit/mg protein)	APX activity (Unit/mg protein)	GR activity ($\mu\text{mol}/\text{mg}$ protein/min)	CAT activity (n mol/mg protein/min)
Negative control	170.2 \pm 10.65 ^c	12.61 \pm 1.01 ^f	2.88 \pm 0.18 ⁱ	19.26 \pm 1.18 ^f
PEG (5%)	173.4 \pm 12.43 ^c	13.65 \pm 1.34 ^f	4.28 \pm 0.24 ^h	21.35 \pm 1.67 ^f
PEG (10%)	183.7 \pm 14.54 ^{dc}	20.12 \pm 1.87 ^c	5.64 \pm 0.47 ^g	27.84 \pm 1.99 ^{ef}
PEG (15%)	211.3 \pm 17.83 ^{cd}	22.95 \pm 2.01 ^d	5.98 \pm 0.39 ^f	41.37 \pm 2.37 ^d
PEG (20%)	256.5 \pm 18.65 ^b	25.65 \pm 2.33 ^c	6.96 \pm 0.52 ^d	58.97 \pm 3.23 ^b
PEG (5%) +SNP	190.5 \pm 15.38 ^{dc}	18.54 \pm 1.46 ^c	6.43 \pm 0.52 ^c	24.95 \pm 1.96 ^f
PEG(10%)+SNP	210.5 \pm 17.67 ^{cd}	25.43 \pm 2.91 ^c	7.94 \pm 0.49 ^c	33.96 \pm 2.37 ^c
PEG(15%)+SNP	230.9 \pm 18.56 ^c	31.37 \pm 2.74 ^b	8.43 \pm 0.67 ^b	50.59 \pm 3.87 ^c
PEG(20%)+SNP	300.5 \pm 22.69 ^a	42.56 \pm 3.02 ^a	9.03 \pm 0.49 ^a	71.29 \pm 4.07 ^a
LSD	24.232	1.651	0.225	6.66

Values are means \pm standard error (SE) and the different letters in the same row indicated significant difference at $P < 0.05$

Tab. 4. Effects of drought stress by means of polyethylene glycol (PEG) and SNP treatments on activity, % relative activity of phenylalanine ammonia lyase (PAL) and total phenols, % relative content in rice plants

Treatments	PAL activity (Unit/mg protein)	% Relative activity	Total phenols (mg/g FW)	% Relative content
Negative control	6.78 ± 0.37 ^h	100	1.06 ± 0.09 ^f	100
PEG (5%)	12.54 ± 1.07 ^g	184.96	2.62 ± 0.12 ^e	247.16
PEG (10%)	19.63 ± 1.15 ^{ef}	289.53	3.86 ± 0.16 ^d	364.15
PEG (15%)	23.63 ± 1.34 ^e	348.53	4.76 ± 0.31 ^c	432.07
PEG (20%)	43.87 ± 2.67 ^c	647.05	5.57 ± 0.49 ^b	525.47
PEG (5%) +SNP	17.54 ± 1.27 ^f	258.70	3.87 ± 0.20 ^d	356.09
PEG (10%) +SNP	32.58 ± 2.02 ^d	480.53	5.32 ± 0.37 ^b	501.89
PEG (15%) +SNP	51.64 ± 3.62 ^b	761.65	5.58 ± 0.41 ^b	526.42
PEG (20%) +SNP	74.95 ± 4.03 ^a	1105.45	6.65 ± 0.59 ^a	627.36
LSD	4.752		0.357	

Values are means ± standard error (SE) and the different letters in the same row indicated significant difference at $P < 0.05$

al. (2002), rice Guo *et al.* (2006) and caper, Ozkur *et al.* (2009) compared to their susceptible cultivars. Furthermore, high antioxidants capacity under salt stress or iron deficiency could prevent cell damage and correlate with plant tolerance to salt stress (El-Beltagi *et al.*, 2008; Salama *et al.*, 2009).

The enhanced scavenging ability for H_2O_2 in tolerant cultivars inhibited the accumulation of ROS and thus protected the plants from lipid peroxidation of membrane systems and oxidative damages under drought stress.

As it is now commonly accepted NO as is second messenger in plants, and thus it is supposed that a low concentration of NO might be a signal molecule to induce/stabilize the expression of many antioxidative enzymes (Frank *et al.*, 2000). Zhang *et al.*, (2003) found in rice leaves that NO increased the activity of SOD, GR, CAT and APX under osmotic stress. The protective effect of NO may also be related to its ability to react with some ROS, such as O_2^- , making NO act as a chain breaker and show its proposed antioxidant properties (Conner and Grisham, 1996). Moreover it has been reported that NO can react with lipid alcoxyl ($LO\cdot$) and peroxy ($LOO\cdot$) radicals, leading to the expectation that NO could stop the propagation of radical-mediated lipid oxidation in a direct fashion (Lamotte *et al.*, 2004). Thus NO may help plants to survive stressful conditions through its action as signaling molecule to activate antioxidative enzymes and reaction with active oxygen and lipid radicals directly.

In this experiment, the increased activity of CAT, SOD, GR and APX by NO may be due to the fact that NO-mediation improved the resistance of rice cells against oxidative burst. The present results are in agreement with Wang *et al.* (2005); Tian and Lei (2006) and Yang *et al.* (2008) who found that, NO increase the activities of SOD, CAT, APX, GR and PAL under drought stress to counteract oxidative injury.

Effect of NO administration on the activity of phenylalanine ammonia lyase (PAL) and Total phenols, percentage (%) in drought-stressed rice callus are represented in

Tab. 4. The increase in the percentage (%) of PAL activity in rice cells under 5,10,15 and 20% PEG and SNP were 258.70, 480.53, 761.65 and 1105.45 % respectively, of the control values (100 %). The data revealed that, the high increase in PAL activity ($Unit\ mg\ protein^{-1}$) which was triggered by both NO administration and drought stress conditions. In addition, there was a positive correlation between NO, PEG % and PAL activity. In accordance, (Goodman *et al.*, 1967) found that, the increase in APX and PAL activity might have frequently enhanced the phenol content in challenging inoculated plant cells. Our results are in agreement with Rizhsky *et al.* (2002) and Tian and Lei (2006) who found that, drought stress by PEG treatments caused an increase in PAL activity in tobacco and wheat plants.

At PEG (20%) and $100\mu M$ SNP treatment, the relative percentage (%) content of total phenols was increased by 627.35% of the control 100% in rice stressed-plant. These data in Tab. 4 show that there was a high accumulation of total soluble phenols under drought stress. On the other hand, NO treatment significantly increased the total soluble phenols contents.

These data were in accordance with Goodman *et al.* (1967), who found that, Multifold increase of phenols after challenging with elicitation in the present study may be due to the excess production of H_2O_2 in elicited plant cells through increased respiration (Farkas and Kiraly, 1962) or due to the activation of hexose-monophosphate pathway, acetate pathway and release of bound phenols by hydrolytic enzymes.

Conclusions

In conclusion, PEG induced drought stress could cause oxidative damage to rice growth through excessive generation of ROS and proper concentrations of exogenous NO could improve the dehydration tolerance through enhancing the antioxidant systems. Our results provide some evi-

dences to the important functions of NO in the plantkingdom, which need to undergo further research.

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