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Effects of Exogenous Nitric Oxide on Photosynthesis, Antioxidant Capacity and Proline Accumulation in Wheat Seedlings Subjected to Osmotic Stress

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Abstract: Nitric oxide (NO) is a active molecule involved in mediation of various biotic and abiotic stress-induced physiological responses in plants. In the present study, using SNP (sodium nitroprusside) as NO donor and cPTIO [2-(4-carboxypheny-4,4,5,5-tetramethylimidazoline-1-1oxyl-3-oxide, potassium salt] as specific NO scavenger, we examined the ability of exogenous NO to alleviate oxidative damage, accelerate proline accumulation and enhance photosynthesis in leaves of wheat seedlings subjected to osmotic stress. Wheat seedlings were exposed to Hoagland solution containing 15% polyethylene glycol (PEG), or 15% PEG plus 0.3mmol L^{-1} SNP or 15% PEG plus 0.3 mmol L^{-1} SNP and 0.5 mmol L^{-1} cPTIO for 24 h. The results showed that osmotic stress induced decrease in superoxide dismutase (SOD) and catalase (CAT) activity and overproduction of O^{-}_{2} in wheat leaves, which in turn caused exacerbation of lipid peroxidation and depression of photosynthesis. Application of NO donor SNP retarded decrease in SOD and CAT activity, increase in O^{-}_{2} production and hence inhibited lipid peroxidation. As a result, F_m/F_o, F_v/F_m, |ÕPS2 and Pn in leaves of wheat seedlings subjected to osmotic stress were increased. Meanwhile, proline accumulation was accelerated and higher relative water content (RWC), Ψ_w and lower leaf water loss (LWL) were maintained by the application of SNP under osmotic stress. However, such effects of SNP were reversed by the addition of NO scavenger cPTIO. It was indicated that effects of NO on preventing wheat leaves from osmotic stress-induced damage might be specific.

Key words: Wheat Nitric oxide · Osmotic stress · Reactive oxygen species · Osmotic adjustment · Photosynthesis

INTRODUCTION

Drought is the most important environmental factor limiting crop productivity in many regions of the world. At the whole-plant level, the effect of water stress is usually perceived as a decrease in photosynthesis and growth. At the molecular level, the negative effect is associated with oxidative damage to plant cell produced by osmotic stress, due to imbalance between production of Reactive Oxygen Species (ROS) and antioxidant defenses [1, 2]. Accordingly, higher capacity to detoxify reactive oxygen species contributed to increasing drought tolerance of plants [3, 4]. During osmotic stress caused by water deficit, another plant strategy that may confer stress tolerance is the rapid accumulation of compatible osmolytes such as proline and glycine-betain [5, 6].

Nitrite oxide (NO) is a lipophilic molecule that diffuses through membranes. Although first described as a signal molecule in animals, accumulating evidence shows that NO is an important signal molecule involved in plant response to biotic and abiotic stresses [7-9]. It has been shown that some phytohormones play roles in regulating plant growth and response to stresses by inducing NO formation. Abscisic acid (ABA) triggered NO production, which in turn led to the stimulation of antioxidant enzyme activities [10]. Polyamines (Pas) induced NO biosynthesis in specific tissues in Arabidopsis seedlings, especially in the elongation zone of root tips and primary leaves [11]. Some researchers applied exogenous NO directly to plants to elucidate the role of NO in plant growth and stress tolerance. The results showed that application of exogenous NO confers resistance to salt [9], heavy metals [12], chilling [13] and ultraviolet-B radiation

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stresses [14]. Although it has been shown that exogenous application of NO donors can enhance adaptive plant responses against drought stress through inducing stomatal closure [8], the mechanism of drought tolerance induced by NO is not clear till now. Additionally, NO is itself a reactive nitrogen species and its effects on different types of cells have proved to be either protective or toxic, depending on its concentration and on the situation. In systems where toxicity is incurred predominantly from ROS, NO may act as a chain breaker and thus limit damage [15].

In this study, using SNP (sodium nitroprusside) as NO donor and cPTIO [2-(4-carboxypheny)-4,4,5,5-tetramethylimidazoline-1-1oxyl-3-oxide, potassium salt] as specific NO scavenger, we examined the ability of exogenous NO to alleviate oxidative damage, accelerate proline accumulation and enhance photosynthesis in leaves of wheat seedlings subjected to osmotic stress. The aim was to provide experimental basis for understanding the mechanism of drought tolerance induced by NO.

MATERIALS AND METHODS

Plants and Treatments: Wheat (*Triticum aestivum* L. var Yunong949) seeds were sterilized with 15% (v/v) H_2O_2 and soaked in distilled water for 12 h at room temperature after fully washing. The seeds were incubated at 25±1°C till germination and then transferred to trays containing sterilized sand. Trays were kept at 25±1°C, with a Photosynthetically Active Radiation (PAR) of 300 μmol m^{-2} s^{-1} and 12 h photoperiod. Seedlings were watered with Hoagland solution. After 8 days, uniform seedlings were selected and exposed to various treatment solutions: (1) Hoagland solution (Control); (2) Hoagland solution + 15% PEG6000 (T1) ; (3) Hoagland solution + 15% PEG6000 + 0.3 mmol L^{-1} SNP (T2); (4) Hoagland solution + 15% PEG6000 + 0.3 mmol L^{-1} SNP + 0.5 mmol L^{-1} cPTIO (T3). Both SNP and cPTIO were bought from Sigma co (USA). Other growing conditions were the same. After 24 h of treatment, the first expanded leaf from top was sampled to determine the relevant physiological characteristics of treated plants.

Assay of Superoxide Disnutase and Catalase Activity: Leaf tissues were extracted in 50 mM sodium phosphate buffer (pH 7.0) and centrifuged at 16000 g for 10 min at 4°C. The supernatant was used for assays of Superoxide Disnutase (SOD) and Catalase (CAT) activity. SOD activity was determined by the method of Beauchamp and Fridovich [16] with some modifications. The supernatant was incubated in an assay medium containing 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 mM NBT(nitroblue tetrazolium), 2 μM riboflavin and 100 nM EDTA. Changes in absorbance were monitored at 470 nm. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT reduction under light. Catalase activity was determined spectrophotometrically by recording the decrease in absorbance of H_2O_2 (extinction coefficient 0.0394 mM^{-1} cm^{-1}) within 1 min at 240 nm according to the method of Aebi [17]. The 3 ml reaction solution contained 15 mM H_2O_2, 50 mM phosphate buffer (pH 7.0) and 50μl of enzyme extract. The reaction was initiated by adding enzyme extract.

Measurement of Superoxide Anion and Lipid Peroxidation: The rate of superoxide anion (O^{-}_{2}) production was determined by the method of Elstner and Heupel [18] with some modifications [19]. Lipid peroxidation was estimated by malondialdehyde (MDA) following the method of Larkindale and Knight [20] with modifications [19].

Determination of Proline Concentration: Proline was extracted and determined by the method of Bates *et al*. [21]. Leaf segments were homogenized with 3% sulfosalicylic acid and the homogenate was centrifuged at 3000 g for 10 min. After acetic acid and acid ninhydrin were added, the supernatant was boiled for 1 h and then absorbance of the supernatant at 520 nm was determined. Proline concentration was calculated with a standard curve and expressed as μmol g^{-1} fresh mass.

Measurement of Water Status and Leaf Water Loss: Relative water content (RWC) of the leaf was calculated by the formula: RWC = (FW-DW)/(TW-DW)×100, where FW = fresh weight, TW =leaf weight after rehydration for 24 h at 4°C in the dark and DW = leaf dry weight, after dried in the oven at 80°C for 48 h. Water potential (Ψw) was measured with a HR-33T dew point micro-voltmeter (Wescor Inc., Logan, UT, USA) after equilibration in the chamber for 2 h. The measurement of leaf water loss (LWL) was based on Xing *et al*. [22]. After their fresh weight (W1) was recorded when cut from seedlings, the leaves were left to evaporate under room temperature for 3 h and re-weighed (W2). LWL was calculated by the formula: (W1-W2)/W1 ×100.

Determination of Net Photosynthetic O_2-evolving Rate and Chlorophyll Fluorescence Parameters: Net photosynthetic O_2-evolving rate (Pn) in the middle parts of leaves was determined with a Chlorolab-2 oxygen electrode unit (Hansatech Company, UK). Temperature in leaf chamber was regulated to 28°C and photon density (PFD) was 800 μmol m^{-2} s^{-1}. Chlorophyll fluorescence was measured at room temperature (26~28°C) with the FM-2 fluorescence monitor (Hansatech, UK). Before measurement, the leaf samples were kept in darkness for 15 min. Actinic light of 400 μmol m^{-2} s^{-1} and saturating pulse light of 8000 μmol m^{-2} s^{-1} were used. Chlorophyll fluorescence parameters were calculated by the method of Schreiber *et al*. [23] and Genty *et al*. [24].

Statistical Analysis: All the experiments were performed in triplicate except chlorophyll fluorescence parameters among which 10 leaves were measured per treatment. Data in the figures and tables are mean values±s.d (standard deviation). Differences between treatments were tested by Duncan test after ANOVA (analysis of variance).

RESULTS

Effects of Exogenous NO Donor and Scavenger on SOD and CAT activity: The results showed that, compared with control, osmotic stress (T1) resulted in a significant decrease in SOD (Fig.1) and CAT (Fig.2) activity. The reduction in SOD and CAT activity caused by PEG was distinctly retarded by the addition of 0.3 mmol L^{-1} SNP (T2). If 0.5 mmol L^{-1} cPTIO was added to the culturing solution simultaneously (T3), effect of SNP was reversed and SOD and CAT activity decreased nearly to T1.

Table 1: Effect of exogenous NO on Relative Water Content (RWC) and leaf water loss (LSL) of wheat leaves. Control: seedlings grown in Hoagland solution; T1: seedlings grown in mixture of Hoagland solution and 15% PEG6000; T2: seedlings grown in mixture of Hoagland solution, 15% PEG6000 and 0.3 mmol L^{-1} SNP; T3: seedlings grown in mixture of Hoagland solution, 15% PEG6000, 0.3 mmol L^{-1} SNP and 0.5mmol L^{-1} cPTIO. Different letters in the same column indicated significant differences at <0.05 level

Treatment	RWC(%)	Ψw (MPa)	LWL(%)
Control	94.1±1.62 a	-0.71±0.03 a	16.3±1.31a
T1	74.8±1.17 c	-1.49±0.04 c	11.6±1.24b
T2	88.3±1.44 b	-1.02±0.02 b	8.7±1.19c
T3	76.3±1.55 c	-1.37±0.04 c	10.5±1.22b

Table 2: Effect of exogenous NO donor and scavenger on chlorophyll fluorescence parameters of wheat leaves under osmotic stress. Control: seedlings grown in Hoagland solution; T1: seedlings grown in mixture of Hoagland solution and 15% PEG6000; T2: seedlings grown in mixture of Hoagland solution, 15% PEG6000 and 0.3 mmol L^{-1} SNP; T3: seedlings grown in mixture of Hoagland solution, 15% PEG6000, 0.3 mmol L^{-1} SNP and 0.5mmol L^{-1} cPTIO

Treatment	F_m/F_o	F_v/F_m	PS
CK	3.643±0.038	0.776±0.074	0.144±0.015
T1	1.486±0.041	0.415±0.053	0.113±0.012
T2	2.597±0.039	0.648±0.049	0.129±0.016
T3	1.612±0.027	0.476±0.061	0.117±0.014

Effects of Exogenous NO Donor and Scavenger on O^-_2 Production and Lipid Peroxidation: As shown in Fig.3 and Fig.4, osmotic stress caused an increase in O^-_2 production rate and MDA content (T1), which implicated that oxidative damage was induced by osmotic stress. The application of 0.3 mmol L^{-1} SNP suppressed the rise of O^-_2 production rate and MDA content under osmotic stress. The effect of SNP was largely depressed by simultaneous addition of cPTIO.

Effects of Exogenous NO Donor and Scavenger on Proline Accumulation, Water Status and LWL: Results in Fig. 5 demonstrated that osmotic stress induced accumulation of proline in wheat leaves and the proline accumulation under osmotic stress was markedly accelerated by exogenous NO donor SNP. However, when NO scavenger cPTIO was added, the effect of SNP was almost eliminated and proline concentration in wheat leaves decreased to the level of T1.

In accord with change in proline concentration, osmotic stress resulted in an obvious decrease in water potential (Ψw) and Relative Water Content (RWC) of wheat leaves. Meanwhile, water-losing rate in detached leaves (LWL) was significantly increased. Higher Ψw and RWC were maintained and leaf water loss in wheat leaves was retarded by the application of SNP. But these effects of SNP were nearly removed by the addition of cPTIO (Table 1).

Effects of Exogenous NO and NO Scavenger on Chlorophyll Fluorescence Parameters and Pn: As shown in Table 2 and Fig. 6, Fm/F_o (electron transfer through PS2), F_v/F_m (primary photochemical efficiency of PS2),φPS2 (quantum yield of PS2) and Pn in leaves of wheat seedlings decreased remarkably when subjected to osmotic stress. The decreases in F_m/F_o, F_v/F_m, φS2 and Pn

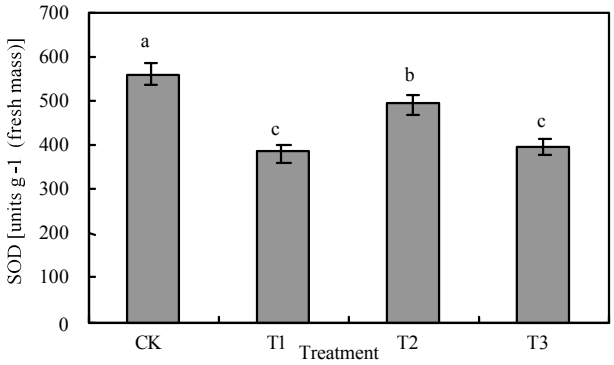

Fig. 1: Effect of exogenous NO donor and scavenger on activity of Superoxide Dismutase (SOD) in wheat leaves under osmotic stress. Control: seedlings grown in Hoagland solution; T1: Seedlings grown in mixture of Hoagland solution and 15% PEG6000; T2: Seedlings grown in mixture of Hoagland solution, 15% PEG6000 and 0.3 mmol L^{-1} SNP; T3: seedlings grown in mixture of Hoagland solution, 15% PEG6000, 0.3 mmol L^{-1} SNP and 0.5mmol L^{-1} cPTIO. Bars indicated standard deviations. Different letters indicated significant differences at <0.05 level

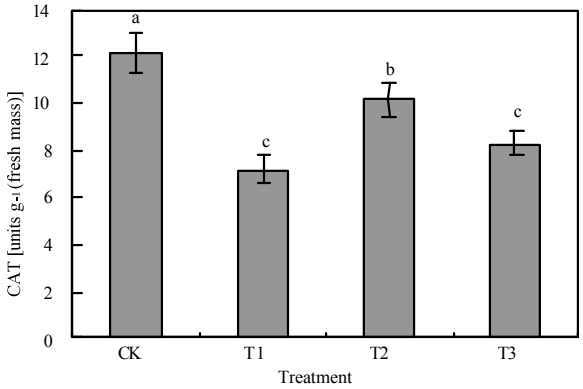

Fig. 2: Effect of exogenous NO donor and scavenger on activity of catalase (CAT) in wheat leaves under osmotic stress. Control: seedlings grown in Hoagland solution; T1: Seedlings grown in mixture of Hoagland solution and 15% PEG6000; T2: Seedlings grown in mixture of Hoagland solution, 15% PEG6000 and 0.3 mmol L^{-1} SNP; T3: Seedlings grown in mixture of Hoagland solution, 15% PEG6000, 0.3 mmol L^{-1} SNP and 0.5mmol L^{1} cPTIO. Bars indicated standard deviations. Different letters indicated significant differences at <0.05 level

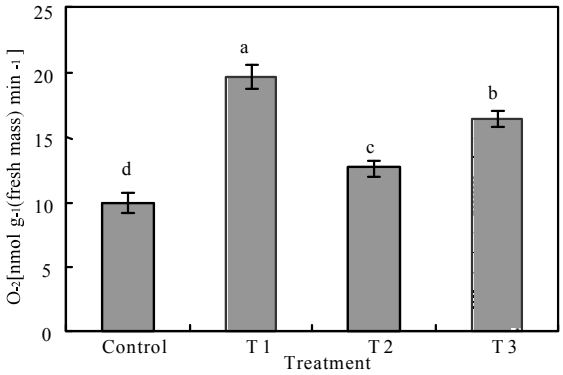

Fig. 3: Effect of exogenous NO donor and scavenger on production rate of O_2^- in wheat leaves under osmotic stress. Control: seedlings grown in Hoagland solution; T1: Seedlings grown in mixture of Hoagland solution and 15% PEG6000; T2: Seedlings grown in mixture of Hoagland solution, 15% PEG6000 and 0.3 mmol L^{-1} SNP; T3: seedlings grown in mixture of Hoagland solution, 15% PEG6000, 0.3 mmol L^{-1} SNP and 0.5mmol L^{-1} cPTIO. Bars indicated standard deviations. Different letters indicated significant differences at <0.05 level

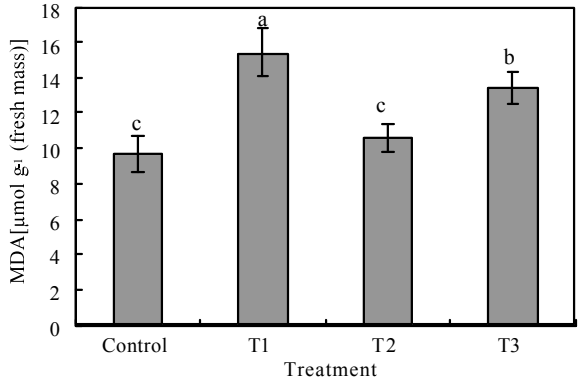

Fig. 4: Effect of exogenous NO donor and scavenger on concentration of malondialdehyde (MDA) in wheat leaves under osmotic stress. Control: seedlings grown in Hoagland solution; T1: Seedlings grown in mixture of Hoagland solution and 15% PEG6000; T2: Seedlings grown in mixture of Hoagland solution, 15% PEG6000 and 0.3 mmol L^{-1} SNP; T3: Seedlings grown in mixture of Hoagland solution, 15% PEG6000, 0.3 mmol L^{-1} SNP and 0.5mmol L^{-1} cPTIO. Bars indicated standard deviations. Different letters indicated significant differences at <0.05 level

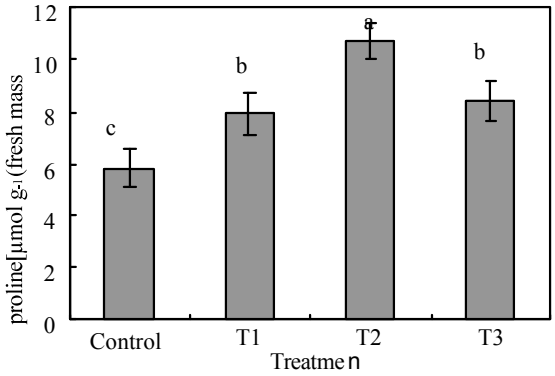

Fig. 5: Effect of exogenous NO donor and scavenger on proline concentration in wheat leaves under osmotic stress. Control: seedlings grown in Hoagland solution; T1: Seedlings grown in mixture of Hoagland solution and 15% PEG6000; T2: Seedlings grown in mixture of Hoagland solution, 15% PEG6000 and 0.3 mmol L^{-1} SNP; T3: Seedlings grown in mixture of Hoagland solution, 15% PEG6000, 0.3 mmol L^{-1} SNP and 0.5mmol L^{-1} cPTIO. Bars indicated standard deviations. Different letters indicated significant differences at <0.05 level

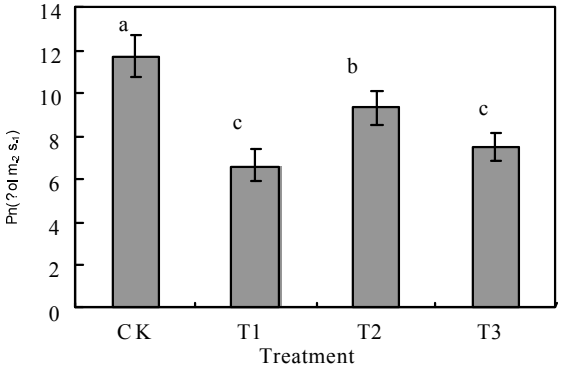

Fig. 6: Influence of exogenous NO donor and scavenger on net photosynthetic O_2-evolving rate (Pn) of wheat leaves under osmotic stress. Control: seedlings grown in Hoagland solution; T1: Seedlings grown in mixture of Hoagland solution and 15% PEG6000; T2: seedlings grown in mixture of Hoagland solution, 15% PEG6000 and 0.3 mmol L^{-1} SNP; T3: Seedlings grown in mixture of Hoagland solution, 15% PEG6000, 0.3 mmol L^{-1} SNP and 0.5mmol L^{-1} cPTIO. Bars indicated standard deviations. Different letters indicated significant differences at <0.05 level

induced by osmotic stress were greatly moderated by the use of exogenous NO donor SNP. Nevertheless, the effects of SNP on photosynthesis were counteracted by NO scavenger cPTIO.

DISCUSSION

Reactive chemical intermediates derived from various substances have been invoked as causative agents in many toxicological mechanisms. Reactive oxygen species (ROS) are the important ones in biological systems, due to their abundance and interconvertibility [25]. As an important member of ROS, O^{-}_{2} has been shown to directly react with proteins containing Fe-S clusters, heme groups or S-S bonds and oxidize them [26]. In this sense, O^{-}_{2} is devastating to electron transfer in photosynthesis. Furthermore, Rubisco, a key enzyme in carbon assimilation in the stroma of plant chloroplasts is very sensitive to oxidative stress. Oxidative stress causes cross-linking of large subunits of the enzyme by S-S [27]. Other targets of ROS are biological membranes. In plants, O^{-}_{2}-mediated cell death symptoms are presumably due to induced lipid peroxidation and subsequent membrane damage [28]. In accord with many other studies, the results from this work indicated that osmotic stress induced decreases in SOD and CAT activity and overproduction of O^{-}_{2} in wheat leaves, which in turn caused exacerbation of lipid peroxidation and depression of photosynthesis. Application of NO donor SNP suppressed decline in SOD and CAT activity and increase in O^{-}_{2} production under osmotic stress (Fig.1-3). Therefore, lipid peroxidation was evidently inhibited (Fig. 4). As a result, Fm/Fo, Fv/Fm, φPS2 and Pn in leaves of wheat seedlings subjected to osmotic stress was increased (Table 2, Fig. 6). However, such effects of SNP were counteracted by the addition of NO scavenger cPTIO.

In many plants, free proline accumulates in response to the imposition of a wide range of biotic and abiotic stresses. As a compatible molecule in cell, proline possesses the ability to mediate osmotic adjustment, stabilize subcellular structures and scavenge free radicals. Besides, proline accumulation may reduce stress-induced cellular acidification or prime oxidative respiration to provide energy for recovery. Moreover, high level of proline synthesis during stress may maintain $NAD(P)^{+}$/NAD(P)H ratios at values compatible with metabolism under normal conditions [29]. The result in Fig.5 showed that osmotic stress led to an increase in proline concentration in wheat leaves and much more proline accumulated when NO donor SNP was used. Consequently, higher RWC, Ψw and lower LWL were maintained by the application of SNP (Table 1). The effects of SNP on proline accumulation and water status in wheat leaves were reversed by NO scavenger cPTIO.

In conclusion, we obtained experimental evidence indicating that exogenous NO is involved in alleviation of osmotic stress-induced oxidative damage and stimulation of proline accumulation in wheat leaves under osmotic stress. Owing to the ability of NO to reduce oxidative damage and stimulate proline accumulation, higher RWC, Ψw and lower LWL were maintained and photosynthesis was improved in leaves of wheat seedlings subjected to osmotic stress. The effects of NO appear to be specific because the NO scavenger cPTIO could reverse the effects of SNP. In order to get an insight into the function of NO in alleviating damage caused by osmotic stress, further research is needed. For example, endogenous NO concentration and activity of NO synthetic enzyme should be determined.

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