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Full Length Research Paper

Protective roles of nitric oxide on antioxidant systems in tall fescue leaves under high-light stress

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Nitric oxide (NO) is an important molecule involved in many physiological processes. In this study, the effect of NO on oxidative damage caused by high levels of light was investigated in tall fescue leaves. Tall fescue was developed in relative low light intensity (100 µmol m^{-2} s^{-1}) for 21 days and then transferred to high light (500 µmol m^{-2} s^{-1}). Tall fescue leaves was supplied with NO donor, sodium nitroprusside (SNP), before high-light treatment to determine the physiological mechanisms of NO on tall fescue tolerance to high-light stress. Treatment of tall fescue leaves with 100 µM SNP before high-light stress resulted in alleviated light-induced electrolyte leakage, malondialdehyde and carbonyl contents in tall fescue leaves. The levels of H_2O_2 and superoxide radical ($O_2^{\bullet-}$) were reduced as well. Moreover, the activities of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) increased in tall fescue in presence of SNP under high-light stress. This pattern was reversed by application of NO scavenger, 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) before light treatment. Pronounced increases of NO production were found in tall fescue leaves after exposure to high-light stress. The results suggested that high-light stress elevated NO level and that NO might act as a signalling molecule to enhance antioxidant enzyme activities, further protecting against injuries caused by high-light stress.

Key words: Antioxidant, high-light stress, nitric oxide, tall fescue.

INTRODUCTION

Light is essential for plant growth and development, but when plants are subjected to excessive light, active oxygen generation is increased (Asada, 2006), often resulting in photo-oxidative damages; thus light can also be one of the most deleterious environmental factor. Singlet oxygen (O_2^1), superoxide radicals ($O_2^{\cdot-}$) hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$) are collectively called reactive oxygen species (ROS). The effects of these ROS can cause the oxidation of lipids, proteins and enzymes necessary for the proper functioning of the chloroplast and the cell as a whole. To avoid ROS induced cellular injury, plants employ various antioxidative enzymes such as superoxide dismutase (SOD: EC 1.15.1.1), catalase (CAT: EC 1.11.1.6) and ascorbate peroxidase (APX: EC 1.11.1.6) and low molecular weight antioxidants such as ascorbate (ASC), glutathione (GSH) and α-tocopherol (Mittler, 2002; Apel and Hirt, 2004) in the scavenging of these radicals.

Nitric oxide (NO) is an important signaling molecule involved in many plant physiological processes (Lamotte et al., 2005; Crawford, 2006; Corpas et al., 2008; Zhang et al., 2009). NO generates dual effects on plant, which is determined by its concentration, action site and physiological conditions of cells (Beligni and Lamattina, 1999). On one hand, NO participates in plant growth and signal transduction of acclimatization through the direct effect of effective molecular reaction or indirect effect of changing potential difference of cellular redox. On the other hand, higher concentration of NO can react with $O_2^{\cdot-}$ to produce many exogenous peroxynitrite, which could form peroxynitrous acid after protonation and damage structure and function of bio-macromolecule (Beligni and Lamattina, 2000; Martinez et al., 2000; Wendehenne et al., 2001; Neill

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et al., 2003). Several studies have shown that NO is involve in the regulation of plant responses to various environmental stresses. Increasing exogenous NO content under abiotic stresses may alleviate injury and enable plant cells to survive better (Song et al., 2006; Shi et al., 2007; Sun et al., 2007; Vital et al., 2008; Zhao et al., 2008). However, excessive NO released in plants and sustained high NO concentration might be cytotoxic (Arasimowicz and Floryszak-Wieczorek, 2007).

Tall fescue (*Festuca arundinacea*) is a widely used cold-season turfgrass species in China. Tall fescue is used as understorey turfgrass species and has leaves that are adapted to low light (Burner, 2003). As a result, it is often propagated under low light levels and can suffer damage if transferred to full sunlight. For this reason, it is important to obtain new light-tolerant cultivars. In a preliminary experiment, different varieties of tall fescue were found to exhibit distinct photoacclimation. Arid3 was not photobleached under high-light stress and this result suggested that Arid3 was light-tolerant turfgrass. High-light stress leads to enhanced ROS production. ROS-scavenging enzymes activities were reported to increase under high-light stress to mitigate oxidative damage (Burritt and Mackenzie, 2003; Ali et al., 2005a; Jiang et al., 2005). There have been no reports in which the influence of light levels on the production of ROS and antioxidant metabolism in tall fescue had been considered. On the other hand, NO was able to increase antioxidant enzymes activities under abiotic stresses to alleviate oxidative damage (Arasimowicz and Floryszak-Wieczorek, 2007), but there is little information on how NO is involved in the regulation of plant responses to light stress. Therefore, the objectives of this study were to determine whether antioxidant metabolism play in the acclimation of tall fescue following transfer of plants grown in the low light, to high light; and to elucidate the role of NO (applied exogenous NO or depleted endogenous NO) in alleviating light-induced oxidative damage in leaves of tall fescue.

MATERIALS AND METHODS

Plant materials and treatments

Seeds of tall fescue (*F. arundinacea* cv. Arid3) were obtained from Beijing Clover Seed and Turf CO., Ltd., China. Seeds were surface sterilized in 0.1% (w/v) sodium hypochlorite, rinsed several times in distilled water and germinated on moist filter paper at room temperature for 7 d. Seedlings were selected and placed into 5 L black plastic containers containing 4 L of solution. Each plastic container contained six plants. Seedlings cultured hydroponically in a continuously aerated nutrient solution containing 4 mM $Ca(NO_3)_2$, 4 mM KNO_3, 1 mM KH_2PO_4, 2 mM $MgSO_4$, 46 µM H_3BO_3, 10 µM $MnSO_4$, 50 µM Fe-EDTA, 1.0 µM $ZnSO_4$, 0.05 µM H_2MoO_4, 0.95 µM $CuSO_4$. The nutrient solution pH was adjusted close to 6.5 by adding H_2SO_4 or KOH. Nutrient solution was re-newed once a week. The plants were grown in a plant incubator at a day/night temperature 25/20°C, a relative humidity of 70%, a day/night regime of 14/10 h and a photosynthetic photo flux density (PPFD) at the height of the plants of 100 µmol m^{-2} s^{-1}. Light was provided by a fluorescent lamp.

The leaves of plants that were maintained in relative low light (L, 100 µmol m^{-2} s^{-1} PPFD) and high light (H, 500 µmol m^{-2} s^{-1} PPFD) for the duration of the experiment were referred to as low-light-leaves and high-light-leaves, respectively, while those that developed fully in the low light and were then transferred to high light were referred to as transferred-leaves. Stress treatments were carried out after 21 d of pre-culture. Plants developed fully in the low light (100 µmol m^{-2} s^{-1} PPFD) and were transferred to high light (500 µmol m^{-2} s^{-1} PPFD). Sodium nitroprusside (SNP; Sigma, USA) was used as NO donor. The potas-sium salt of 2-(4-carboxyphenyl)-4,4,5,5-tetramethy-limidazoline-1-oxyl-3-oxide (PTIO; Sigma, USA) was used as NO scavenger. 100 µM SNP and 200 µM PTIO were applied to tall fescue seedlings through the roots incubated (Laspina et al., 2005; Sun et al., 2007) in 4 L of nutrient solution (regenerated once a day) with high-light treatments. The 21-day-old seedlings were incubated in the solutions for 5 and 10 d at a day/night temperature 25/20°C, a relative humidity of 70%, day/night regime of 14/10 h. After 5 and 10 d of treatment, plants were harvested and frozen in liquid nitrogen and then stored at -80°C for further analysis.

Membrane permeability measurement

Membrane permeability (MP) was determined by the modified method of Song et al. (2006). The fresh leaves (0.5 g) were washed in deionized water and placed in petri dishes with 5 ml of deionized water at 25°C for 2 h. After the incubation, the conductivity was measured (C_1). Then, the samples were boiled for 20 min and conduc-tivity was read again (C_2). Electrolyte leakage was expressed as a percentage of the total conductivity after boiling (MP % = $C_1/C_2 \times 100$).

Analysis of lipid peroxidation

The level of lipid peroxidation was expressed as the amount of malondialdehyde (MDA) produced with a slight modification of the thiobarbituric acid method described by Buege and Aust (1978). Leaves (0.5 g) were homo-genized with a mortar and pestle in 10% trichloroacetic acid and then the homogenate was centrifuged at 4000 × g for 30 min. A 2 ml aliquot of supernatant was mixed with 2 ml of 10% trichloroacetic acid containing 0.5% thiobarbituric acid. The mixture was heated at 100°C for 30 min. The absorbance of the supernatant was mea-sured at 532 nm, with a reading at 600 nm subtracted from it to account for non-specific turbidity.

Analysis of oxidative damage to protein

Leaves (1.0) was homogenized in 50 mM potassium pho-sphate buffer (pH 7.0) containing 3 mM EDTA and 1 mM PMSF and leupeptin and centrifuged at 10000 × g for 15 min at 4°C. Contaminating nucleic acids were removed by treatment with streptomycin sulfate and the oxidative damage to proteins was estimated as the protein carbonyl content, as determined by reaction with 2,4-dinitrophenylhydrazine (Reznick and Packer, 1994).

Determination of hydrogen peroxide and superoxide radical

Hydrogen peroxide content was measured according to Veljovic-Jovanovic et al. (2002). Leaves (0.5 g) were ground in liquid nitrogen and the powder was extracted in 2 ml 1 M $HClO_4$ in the presence of 5% PVP. The homo-genate was centrifuged at 12000 × g for 10 min and the supernatant was neutralized with 5 M K_2CO_3

(pH 5.6) in the presence of 0.1 ml of 0.3 M phosphate buffer (pH 5.6).The solution was centrifuged at 12000 × g for 1 min and the sample was incubated for 10 min with 1 U ascorbate oxidase to oxidize ascorbate prior to assay. The reaction mixture contained 0.1 M phosphate buffer (pH 6.5), 3.3 mM 3-(dimethylamino) benzoic acid, 0.07 mM 3-methy-2-benzothiazoline hydrazone, 0.3 U POX and 200 µl supernatant. Changes in absorbance at 590 nm were monitored at 25℃. Superoxide radical pro-duction rate was determined by the modified method of Elstner and Heupel (1976). Leaves (1.0 g) were homo-genized in 3 ml of 50 mM potassium phosphate buffer (pH 7.8) and centrifuged at 12000 × g for 20 min. The incubation mixture contained 1 ml of supernatant, 1 ml of 50 mM potassium phosphate buffer (pH 7.8) and 1 ml of 1 mM hydroxyl aminonium chloride and the mixture was incubated in 25℃ for 20 min. The mixture was sub-sequently incubated with 2 ml of 17 mM sulphanilic acid and 2 ml of 7 mM α-naphthyl amine at 25℃ for 20 min. The final solution was mixed with an equal volume of ethyl ether and the absorbance of the pink phase was read at 530 nm. The production rate of superoxide radical was calculated based on a standard curve.

Antioxidant enzyme activity

Leaves (1.0 g) were homogenized with a mortar and pestle at 4℃ in 5 ml 50 mM phosphate buffer (pH 7.0) containing 1mM EDTA, 1% PVP. The homogenate was centrifuged at 12000 × g for 30 min at 4℃ and the supernatant was collected for enzyme assays.

The activity of SOD was measured by nitroblue tetrazolium (NBT) method of Beauchamp and Fridovich (1971). One unit of SOD was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm. The activity of CAT was determined by following the consumption of H_2O_2 at 240 nm (E = 39.4 mM^{-1} cm^{-1}) by the method of Aebi (1984). The activity of APX was measured according to Nakano and Asada (1981) by monitoring the rate of ascorbate oxidation at 290 nm (E = 2.8 mM^{-1} cm^{-1}). Enzyme activities were expressed on the basis of per unit protein weight. Protein content in the supernatant was deter-mined using bovine serum albumin (BSA) as a standard (Bradford, 1976).

NO content determination

NO content determination was performed according to Murphy and Noack (1994) with some modifications. Fresh Leaves (0.5 g) were incubated with 100 units of catalase and 100 units of superoxide dismutase for 5 min to remove endogenous ROS before addition of 5 ml oxyhaemoglobin (5 mM). After 2 min incubation, NO concentrations were estimated by following the con-version of oxyhaemoglobin to methaemoglobin spectro-photometrically at 577 and 591 nm.

Statistical analysis

Each experiment was repeated at least three times. Values were expressed as means ± SD. Statistical analy-ses were performed by analysis of variance (ANOVA). Means were separated using Duncan's multiple range test at 5% level of significance.

RESULTS

Effect of NO on membrane permeability (MP)

Membrane permeability (MP) in low-light-leaves was not significantly different from those found in high-light-leaves. Transfer to high light caused a significant rapidly increased MP (at P <0.05). Five days following transfer, MP remarkably increased by 35.5% and then showed little decrease after 10 days treatment (Figure 1A). Treatment of plant leaves with NO donor, SNP before high-light stress resulted in significant decrease of MP in transferred-leaves (at P < 0.05), especially 10 d after treatment. NO scavenger, PTIO were utilized to further clarify the role of SNP. The results showed that addition of PTIO enhanced MP to similar levels in transferred-leaves under high-light stress, indicating that PTIO scavenged endogenous NO.

Effect of NO on lipid peroxidation

As observed for membrane permeability, the levels of lipid hyperoxides in low-light-leaves was not significantly different from those found in high-light-leaves, but increased rapidly when plants were transferred to high light (at P < 0.05). High-light stress significantly increased MDA content in transferred-leaves both 5 and 10 d after treatment (Figure 1B). Supplementation with NO donor, SNP before high-light stress remarkably (at P < 0.05) reduced MDA content in transferred-leaves (at P < 0.05). However, when endogenous NO was removed, MDA content rose evidently, indicating that severe lipid peroxidation was caused.

NO prevents protein oxidative damage

Carbonyl content in low-light-leaves was not significantly different from that of high-light-leaves of tall fescue (Figure 1C). Carbonyl content of transferred-leaves increased following transfer to high light (at P < 0.05); with levels remaining significantly elevated for 5 d and then declined to normal levels after 10 d treatment. Application of exogenous NO dramatically prevented protein oxidative damage in transferred-leaves under high-light stress (at P < 0.05), especially 10 d after treatment.

Effect of NO on H_2O_2 and $O_2^{\bullet-}$ production

Compared to low-light-leaves, high-light stress caused significant accumulations (at P<0.05) of H_2O_2 and $O_2^{\cdot-}$ production in transferred-leaves (Figure 2). Application of NO significantly reduced the accumulation of H_2O_2 and $O_2^{\cdot-}$ (at P < 0.05). Addition of PTIO remarkably increased H_2O_2 and $O_2^{\cdot-}$ levels in transferred-leaves of tall fescue under high-light stress (Figure 2).

Antioxidant enzyme activity

High-light stress had different effects on SOD, CAT and

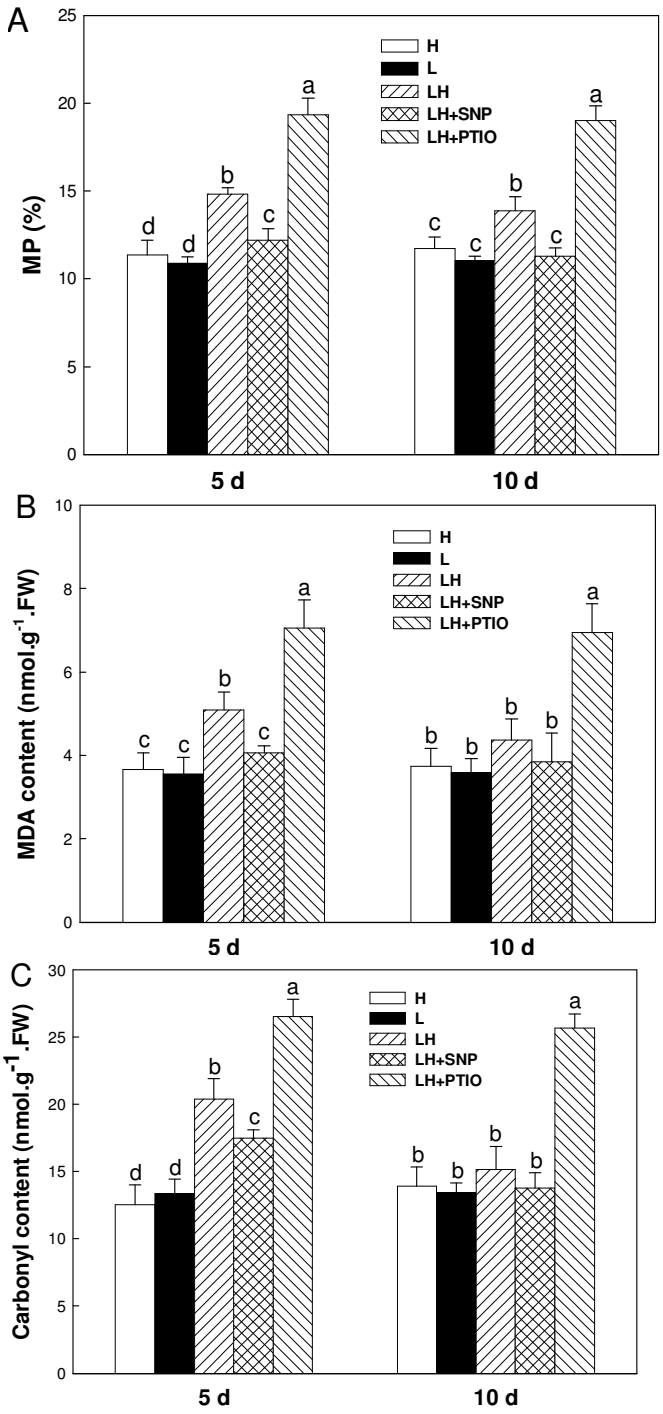

Figure 1. Effect of NO on Membrane permeability (A), MDA content (B) Carbonyl content (C) in tall fescue leaves under high-light stress. Different treatments represents: H, L, LH, LH + SNP (100 μM) and LH+PTIO (200 μM). Values are means ± SD (n = 3). Bars with different letters are significantly different at the 5% level.

APX activity both on the 5 and 10 days after treatment in transferred-leaves (Figure 3A). On the 10th day after treatment, high-light stress greatly induced SOD, CAT

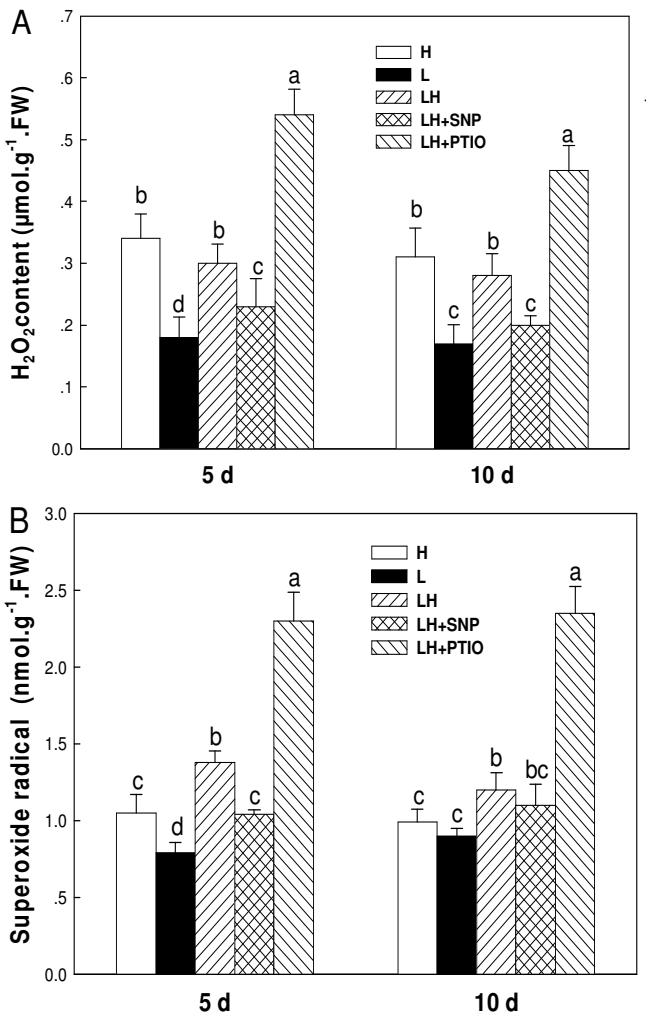

Figure 2. Effect of NO on levels of H_2O_2 (A) and superoxide radical (B) in tall fescue leaves under high-light stress. Values are means ± SD (n = 3). Bars with different letters are significantly different at the 5% level.

and APX activity (at $P < 0.05$). Under high-light stress, application of NO kept SOD and CAT with a relative high activity on the 5th day of treatment but slightly decreased APX activity on the 10th day treatment. Under normal condition, high-light-leaves had higher activities of antioxidant enzymes than low-light-leaves.

NO production

To further reveal the relationship between NO accumulation and high-light stress, NO production was measured. Transfer to high-light caused significantly increased endogenous NO production both 5 and 10 d after treatment. NO production increased by 199.6 and 179.2% in transferred-leaves, respectively. Application of NO scavenger PTIO merely reduced NO content (Figure 4).

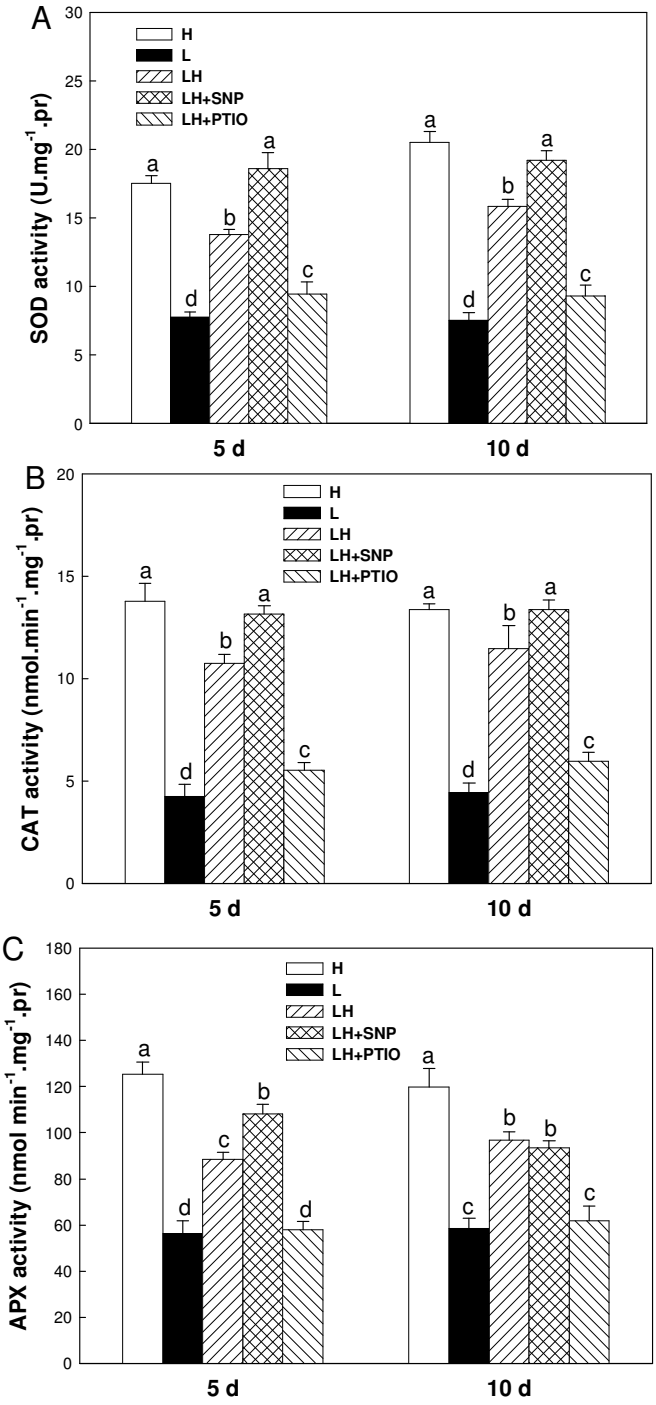

Figure 3. Effect of NO on the activities of antioxidant enzymes of SOD (A), CAT (B) and APX (C) in tall fescue leaves under high-light stress. Values are means ± SD (n = 3). Bars with different letters are significantly different at the 5% level.

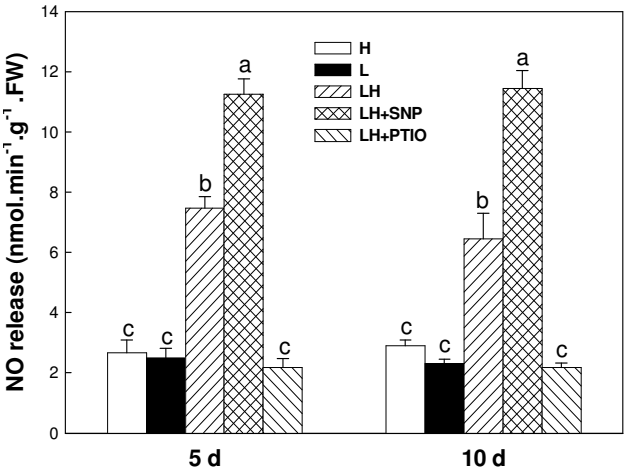

Figure 4. NO production in tall fescue leaves under high-light stress. Values are means ± SD (n = 3). Bars with different letters are significantly different at the 5% level.

DISCUSSION

Light is the major environmental constraint to growth and reproduction of understory turfgrass species. High light intensities might have contributed to a degradation of chlorophyll contents, whereas low light intensities likely prevented breakdown of chlorophyll in leaves of tall fescue (Wherley et al., 2005). Thus, anatomical and physiological adaptations limited photosynthetic capacity and the ability to respond to increased irradiance and CO_2 of tall fescue grown continuously in low light intensities (Allard et al., 1991). Recent research indicated that the value of Fv/Fm has been used to understand photosynthesis affected by light intensities. The lower Fv/Fm in plants was due to photo-inhibition under high-light stress and turfgrasses grown under low light might suffer photo-inhibition when they were removed to high-light stress (jiang et al., 2005).

Photo-inhibition occurs when plants are exposed to a photosynthetic photon flux density (PPFD) higher than that required for the rate of CO_2 fixation, which further led to increased ROS generation (Asada, 2006). Many studies have reported that transfer from low light to high light caused enhanced H_2O_2 accumulation in plant leaves (Ali et al., 2005; Burritt and Mackenzie, 2003). The result in the present study showed that the levels of H_2O_2 and $O_2^{\cdot-}$ increased in tall fescue transferred-leaves (Figure 2).The increased SOD activity may account for the increased accumulation of $O_2^{\cdot-}$ in transferred-leaves (Figure 3A). Over production of ROS caused the oxidation of membrane lipids, proteins and enzymes necessary for the proper functioning of the chloroplasts and cells as a whole (Mittler, 2002). The increase in membrane permeability, MDA and carbonyl content under high-light stress indicated that high light induced oxidative damage on membrane lipid and proteins (Figure 1).

Previous studies have indicated that enhancements in the activities of activated oxygen scavenging enzymes generally accompany exposure to high-light stress (Foyer et al., 1997). For example, it has been reported that ROS-scavenging enzymes activities increased under highlight

stress in pumpkin, periwinkle (Logan et al., 1998a), begonias (Burritt and Mackenzie, 2003), seashore paspalum, bermudagrass (jiang et al., 2005) and sevenson flower leaves (Liu et al., 2006). The result of this study indicated that tall fescue transferred-leaves exhibitted increases in the activities of antioxidant enzymes (SOD, CAT and APX) both 5 and 10 d after highlight treatment (Figure 3). The difference indicated that the influence of high-light stress on the antioxidant enzymes are very complex and related to the plant treatment time, light intensity, plant tissues, plant species and genotype. SOD is a major scavenger of $O_2^{\cdot-}$, catalyzing the dismutation of superoxide to H_2O_2 and O_2. CAT and APX are important H_2O_2 detoxifying enzymes. Increases in the activities of all antioxidant enzymes suggested that generation of ROS induced increased activities of antioxidant enzymes located in different cellular compartments (Logan et al., 1998b). Pronounced increase in antioxidant enzymes activities and the relatively low level of ROS in transferred-leaves indicated that tall fescue alleviated oxidative injuries through raising antioxidant enzymes activities to scavenge newly-produced ROS.

In many studies, it was found that NO could counteract oxidative damage and had protective effect against various stressful conditions (Song et al., 2006; Arasimowicz and Floryszak-Wieczorek, 2007; Shi et al., 2007; Sun et al., 2007; Tian et al., 2007; Vital et al., 2008; Zhao et al., 2008; Zhang et al., 2009). This study provided evidence that 100 µM SNP treatments was able to protect transferred-leaves of tall fescue from MDA increase by delaying the accumulation of H_2O_2 and $O_2^{\cdot-}$. Addition of exogenous NO with SNP regulated the ROS levels in transferred-leaves of tall fescue relating to the activation of SOD, CAT and APX, prevented lipid peroxidation and proteins damage, whereas a reversed pattern was found with the supplementation of NO savenger PTIO. Protective effect of NO might be related to two possibilities. On one hand, low concentration of NO induced the expression or activation of antioxidant enzymes, which was proven in plants (Laspina et al., 2005; Shi et al., 2007; Zhang et al., 2009). Recent research also proved that NO can induce APX and CAT antioxidant genes in *Arabidopsis* suspension cells (Huang et al., 2002). On the other hand, NO can detoxify ROS, such as reacting with $O_2^{\cdot-}$ and generating peroxynitrite ($ONOO^-$). In the physiological pH range, $ONOO^-$ is an unstable product. However, $ONOO^-$ can be protonated and decomposed to a nitrate anion and a proton, or it can react with hydrogen peroxide to yield a nitrite anion and oxygen (Martinez et al., 2000; Wendehenne et al., 2001). Thus, NO might provide protection against oxidative stress. Transferred-leaves of tall fescue suffered severe peroxidative damage when endogenous NO was removed by PTIO, which further proved that NO plays an important role in photoprotection.

In conclusion, ROS metabolism is clearly important for tall fescue during acclimatization to high-light stress. The acquisition of tolerance to high-light stress tall fescue transferred-leaves, are owed to the significantly increased antioxidant enzymes activities. As a bioactive antioxidant, NO protects tall fescue leaves against light-induced oxidative damage by reacting with ROS directly or inducing activities of ROS-scavenging enzymes.

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