Exogenously Applied Nitric Oxide Enhances the Drought Tolerance in Hulless Barley

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Abstract: Drought stress is a severe threat to high altitude hulless barley production, which causes oxidative damage, disturbs water relations and photosynthesis, while exogenously applied nitric oxide (NO) has the potential to alleviate these effects. In the present study, the role of NO in improving drought tolerance of hulless barley was evaluated. At the three leaf stage, sodium nitroprusside (SNP), a NO donor, was applied at 50, 100 and 150 μmol l⁻¹ under drought stress, the controls, were kept at full field water capacity without NO treatment. The results showed that drought stress seriously reduced the hulless barley growth and physiological attributes, but NO application alleviated the stress effects. Drought tolerance in hulless barley was strongly related to the maintenance of water content and enhanced capacity of antioxidants, improved stability of cellular membranes and enhanced photosynthetic capacity, plausibly by signaling action of NO. Among the NO treatments, 100 μmol l⁻¹ SNP was the most effective.

Key words: Drought stress, Hulless barley, Leaf growth, Nitric Oxide, Physiological.

Materials and Methods

Seeds of hulless barley named Zangqing25 were used as experimental material. Selected healthy seeds were surface sterilized with 0.1% HgCl₂ solution for 5 min and thoroughly rinsed with tap water, then, seeds were pre-soaked in Hoagland nutrient solution at 25°C for 24 h.
vernalization. After a week, seeds were sown in plastic pots (25 cm in diameter and 18 cm in height) filled with silicon in a phytotron with photosynthetically active photon flux density of 100 μmol m⁻² s⁻¹, day temperature 28°C, 70–80% relative humidity (RH) and photoperiod of 13 h. The plants were well-watered (100% field capacity) up to three leaf stage, all plants were subjected to drought stress and exogenous NO application except the control plants which were kept well watered (CK). Experimental design was completely randomized with four replications. Drought stress (DS) was induced by maintaining the soil moisture at 30% of field capacity by curtailing the water supply. For exogenous NO application, SNP50 (S50), SNP100 (S100) or SNP150 (S150) μmol l⁻¹ was applied every two days under DS.

All the observations were made 1 week after the start of DS treatments. The seedlings were tested for vigour after carefully removing from the soil. Seedling fresh weight was determined immediately after harvest, and dry weight was determined after drying at 75°C to constant weight. Relative water contents (RWC), was calculated following the method of Jeon et al. (2006).

Hydrogen peroxide (H₂O₂) content in leaf tissues was estimated using titanium reagent following the method of Teranishi et al.,(1974). Sample preparation and hydrogen peroxide estimation were performed as described previously (Prasad, 1997). Lipid peroxidation was measured in terms of malondialdehyde (MDA) content, following the method of Heath and Packer (1968). To determine membrane permeability, we measured leaf electrolyte leakage following the protocol of Blum and Ebercon (1981). Free proline (Pro) was estimated following the method of Bates et al. (1973) and superoxide dismutas (SOD) activity following the method of McCord and Fridovich (1969). Catalase (CAT) activity was measured following the modified method of Luck (1974).

The net photosynthetic rate (Pn), stomatal conductance (gs) and transpiration rate (E) in the penultimate expanded leaves were measured using a portable infrared gas analyzer-based photosynthesis system (LI-6400, Li Cor, Inc., Lincoln, NE, USA). Instantaneous water-use efficiency (IWUE) was calculated as the ratio of net photosynthetic rate to transpiration rate (Condon et al., 2002). Data were recorded at 0900 to 1100 am. During data collection, relative air humidity was about 75%, the leaf temperature 28°C and the ambient CO₂ concentration 380 μmol mol⁻¹.

Chlorophyll fluorescence and leaf gas exchange were measured simultaneously. Chlorophyll fluorescence was measured using a PAM-2100 fluorometer (Heinz Walz, Effeltrich, Germany). Initial fluorescence (Fo) and maximal fluorescence were measured after adaptation to darkness for 30 min. The intensity of saturation pulses used to determine the maximal fluorescence emission in the presence (Fm’) or absence (Fm) of quenching was 4000 μmol (photon) m⁻² s⁻¹, 0.8 s. The “actinic light” was set at 1200 μmol (photon) m⁻² s⁻¹. Steady-state fluorescence (Fs), basic fluorescence after light induction (Fo’), and the variable fluorescence in both dark (Fv = Fm – Fo) and light (ΔF=Fm’–Fs) were also determined. Several photochemical variables were calculated based on the chlorophyll fluorescence parameters measured: maximal PSII photochemical efficiency (Fv/Fm), effective quantum yield of PSII (ΦPSII = Δ F/Fm’), apparent electron transport rate (ETR = Δ F/Fm’×PPFD × 0.5 × 0.84) and photochemical fluorescence quenching [qP = (Fm’–Fs)/ (Fm’– Fo’)] (Maxwell and Johnson, 2000). For ETR estimation, 0.5 was used as the fraction of excitation energy distributed to PSII, and 0.84 as the fraction of light absorption.

The data and figures were subjected to statistical analysis by analysis of variance using SAS software package (SAS version8.0 for Windows, SAS Inc., IL, USA). Least significant difference test was applied to compare the mean values.

Results

Drought stress significantly reduced seedling plant height, seeding fresh and dry weight and relative water content (Fig. 1a, b, c, d); however, NO treatment improved the plant growth under drought stress. Amongst NO treatments, SNP with 100 μmol NO (S100) was the most effective (Fig. 1a, b, c, d).

Leaf H₂O₂ content, MDA content and membrane permeability were lowest in well-watered conditions as shown in Fig. 1 (e, f, g), and were significantly increased by drought stress. However, NO application significantly decreased the leaf H₂O₂ content, MDA content and membrane permeability under drought stress. Minimum leaf H₂O₂ content, MDA content and membrane permeability were observed in S100 treatment, followed by S150 and S50. Likewise, minimum proline content was observed in hulless barley raised under well-watered conditions; while upon exposure to drought stress, proline contents increased. Besides, NO application further increased the free proline contents of leaf (Fig. 1h). Although SOD activity was decreased by drought stress, NO application significantly improved this attribute (Fig. 1i). SOD was higher in S100 than in other treatments. Maximum CAT contents were observed in well-watered hulless barley seedlings, which were decreased significantly upon exposure to drought; nevertheless, NO application improved the CAT activity under stressful conditions, S100 being the most effective (Fig. 1j).

Pn, gs and E were maximum under well-watered conditions, while drought stress significantly reduced these parameters, application of NO significantly improved the Pn but decreased gs and E under drought stress (Fig. 2a, b, c). Furthermore, IWUE was minimum in well-watered conditions, while drought stress significantly reduced seedling plant height, seeding fresh and dry weight and relative water content (Fig. 1a, b, c, d); however, NO treatment improved the plant growth under drought stress. Amongst NO treatments, SNP with 100 μmol NO (S100) was the most effective (Fig. 1a, b, c, d). Leaf H₂O₂ content, MDA content and membrane permeability were lowest in well-watered conditions as shown in Fig. 1 (e, f, g), and were significantly increased by drought stress. However, NO application significantly decreased the leaf H₂O₂ content, MDA content and membrane permeability under drought stress. Minimum leaf H₂O₂ content, MDA content and membrane permeability were observed in S100 treatment, followed by S150 and S50. Likewise, minimum proline content was observed in hulless barley raised under well-watered conditions; while upon exposure to drought stress, proline contents increased. Besides, NO application further increased the free proline contents of leaf (Fig. 1h). Although SOD activity was decreased by drought stress, NO application significantly improved this attribute (Fig. 1i). SOD was higher in S100 than in other treatments. Maximum CAT contents were observed in well-watered hulless barley seedlings, which were decreased significantly upon exposure to drought; nevertheless, NO application improved the CAT activity under stressful conditions, S100 being the most effective (Fig. 1j).

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treatment, and drought-stress increased IWUE, NO treatments also improved IWUE under drought stress (Fig. 2d). Maximum Fv/Fm, ΦPSII, ETR and qP were observed in well-watered plants, and drought-stress significantly decreased these parameters, while NO treatment improved the chlorophyll fluorescence parameters under drought stress. SNP with 100 μmol l⁻¹ SNP showed the greatest effect among the treatments (Fig. 2e, f, g, h).

**Discussion**

In this work, drought stress significantly decreased the growth and physiological attributes, while exogenous application of NO alleviated the adversaries of drought stress (Fig. 1 and Fig. 2). 100 μmol l⁻¹ SNP was more effective in inducing drought tolerance than other treatments (Fig. 1a, e, d). In addition, relative water content of hulless barley was inhibited by drought stress, but NO application substantially improved RWC (Fig. 1b), indicating that NO application substantially improved leaf water status.

Several studies have shown that exogenous NO ameliorates the oxidative stress induced by a range of abiotic conditions such as the presence of heavy metal ions, salinity, high temperatures, the presence of H₂O₂,
dehydration, UV irradiation, and the presence of paraquat (Farooq et al., 2009a). In our study, oxidative stress was also proven to be generated as a consequence of drought in plants by increasing the leaf H$_2$O$_2$, MDA contents and membrane permeability (García-Mata and Lamattina, 2001). However, NO application alleviated drought effects, as revealed by substantially reduced membrane permeability, electrolyte leakage and lowered leaf H$_2$O$_2$ and MDA contents (Fig. 1c, f, g). Enhanced electrolyte leakage and MDA contents are important manifestations of oxidative damage to the biological membranes (Farooq et al., 2008). Such changes quite often arise because of the generation of reactive oxygen species (ROS), mainly H$_2$O$_2$, which is a relatively long-lived molecule (Apel and Hirt, 2004). The ROS react with proteins, lipids and DNA and impairing the normal cellular functions (Foyer and Fletcher, 2001). This further indicates that NO can improve cell wall elasticity, act on the phospholipids bilayer, improve the fluidness of the membrane, and ultimately leads to improved plant growth (Leshem and Hamaraty, 1996).

Leaf free proline is not only an osmoprotectant, but it might also eliminate ROS and improve the antioxidant ability, stabilize the structure of biological macromolecules, decrease cell acidity and relieve the toxicity of NH$_4^+$ (Hou and Tang, 1999). Our study demonstrated that proline contents increased under drought stress and NO application further increased the leaf free proline contents (Fig. 1h), indicating that the accumulation of compatible osmolytes is related to osmoregulation under drought, which enables additional water to be taken up from the environment, thus offsetting the immediate effect of water...
shortages on the cells (Farooq et al., 2009a). In addition, ROD in plants is scavenged by a variety of antioxidant enzymes and/or lipid- and water-soluble molecules. Of these, antioxidant enzymes are the most effective against oxidative damage (Foyer and Fletcher, 2001). In present study, SOD activity and CAT activity were decreased by drought stress, and NO application significantly improved this attribute (Fig. 1i, j). This was mainly due to NO may enhance the antioxidant capacity of cells by increasing the activities of antioxidant enzymes such as SOD activity, which converts superoxide to H$_2$O$_2$, and catalase and ascorbate peroxidase, both of which remove H$_2$O$_2$ (Zhang et al., 2007).

Drought stress decreased Pn, gs and E (transpiration rate) and increased IWUE in this study, while NO application increased Pn and IWUE, and reduced gs and E (Fig. 2a, b, c, d). Furthermore, Chen et al. (2014) reported that NO contributed to the greening of etiolated barley seedling through increasing chlorophyll content, enhancing Pn and photochemical quantum yield of PSII, as well as promoting the development of thylakoid membranes. NO application increased Pn under drought stress which might be associated with the contents of photosynthetic pigments increased by NO treatment (Cao et al., 2011). Decreased gs indicated that NO application induces stomatal closure that may result from an increase in intracellular Ca$^{2+}$ concentrations in the guard cell. Increase of Ca$^{2+}$ concentration precedes stomatal closure (García-Mata and Lamattina, 2001; Neill et al., 2008). Our experiments also showed greater reduction in stomatal conductance under water deficits resulted in slightly increased IWUE and NO treatment further enhanced it (Fig. 1j). This was mainly due to the rapid adjustment of water loss through transpiration and absorption of CO$_2$ through stomatal regulation (Wu et al., 2008). In addition, under drought stress, we found reduction in Fv/Fm (Fig. 2e) which indicated damage to an important portion of the PSII reaction center (Zhang et al., 2010). $\Phi$PSII and ETR decreased mainly because water deficit decreased the efficiency of excitation energy capture of open PSII reaction centers (Fig. 2f, g) (Roháček and Barták, 1999). The decrease in $\Phi$P suggests that water deficit might harm the PSII reaction centers and promote closure of PS reaction centers (Fig. 2h) (Roháček and Barták, 1999), while NO treatment, especially, 100 $\mu$mol l$^{-1}$ SNP could alleviate the damage by preventing the inhibition of photochemical activity, improving the “opening” of the PSII reaction centers, promoting light photosynthetic electron transport, and thereby enhancing photosynthetic electron transport capacity.

In summary, our study showed that NO treatment significantly improved the drought tolerance. Maintenance of the growth, and water status, improved antioxidant activities, reduced ROS injuries, improved cellular membranes stability and, photosynthesis were taken as the principal indicators of hulless barley under drought stress.

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* In Chinese with English abstract.