Ecotoxicology and Environmental Safety 74 (2011) 67–73

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Nitric oxide supplementation alleviates ammonium toxicity in the submerged macrophyte *Hydrilla verticillata* (L.f.) Royle

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ARTICLE INFO

Article history:
Received 6 May 2010
Received in revised form
2 July 2010
Accepted 3 July 2010
Available online 23 July 2010

Keywords:
Nitric oxide
Ammonium
Submerged plant
Hydrilla verticillata
Oxidative damage
Malondialdehyde
Ascorbic acid
Sodium nitroprusside
Glutathione S-transferase
Stress

ABSTRACT

The likely protective effects of nitric oxide (NO) against ammonium toxicity were investigated in the submerged macrophyte *Hydrilla verticillata*. The plants were subjected to ammonium stress (3 mM ammonium chloride) in the presence of sodium nitroprusside (SNP, 10 μM), an NO donor. Treatment with SNP significantly increased the NO content and partially reversed the ammonium-induced negative effects, including membrane damage and the decrease in levels of chlorophyll, malondialdehyde, glutathione and ascorbic acid. Further, SNP application increased the catalytic activities of ascorbate peroxidase, superoxide dismutase, guaiacol peroxidase, catalase and glutathione S-transferase, but decreased that of NADH-oxidase. Histochemical staining showed that SNP application caused a significant decrease in the levels of superoxides and hydrogen peroxide. In contrast, application of other breakdown products of SNP (10 μM sodium ferrocyanide, 10 μM sodium nitrite and 10 μM sodium nitrate) failed to show any protective effect. The results suggest that the increased intracellular NO, resulting from SNP application, improved the antioxidant capacity of *H. verticillata* plants in coping with ammonium-induced oxidative stress.

1. Introduction

Ammonium is one of the most common ionic forms of inorganic nitrogen in aquatic ecosystems (Camargo and Alonso, 2006). It is an important source of nitrogen for plants and is produced intracellularly in various biological processes including photorespiration, amino acid metabolism and hydrolysis of nitrogen-carrying and -storage molecules (Masclaux-Daubresse et al., 2006). However, high levels of ammonium are detrimental to plant cells. An excess of ammonium can disturb nutrient uptake and hormone balance, reduce content of soluble carbohydrates, increase the free amino acid level, and have negative effects on chlorophyll metabolism and photosynthesis (Britto and Kronzucker, 2002; Wang et al., 2008). Ammonium stress also induces the generation of excessive reactive oxygen species (ROS), including superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) (Wang et al., 2010).

ROS are the byproducts of aerobic metabolism in an organism, and excess ROS often damage lipids, proteins and even DNA. Hence, antioxidant systems are important to control the levels of ROS (Mittler, 2002). These include antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and guaiacol peroxidase (POD) and antioxidant compounds such as glutathione, ascorbic acid and carotene. SOD acts as the first-line of defense against ROS by catalyzing O_2^- into H_2O_2. The latter can be further decomposed into harmless byproducts O_2 and H_2O (Hegedus et al., 2001) non-enzymatically or with the help of H_2O_2-scavenging enzymes such as CAT, APX and POD. A rapidly activated and efficient antioxidant response is critical for plants to cope with oxidative stress.

Nitric oxide (NO) is a highly diffusible gas and is synthesized by nitric oxide synthases (NOS) in mammalian cells (Chung et al., 2001). In plants, NO may be produced from NO_2^- by nitrate reductase with the participation of NAD(P)H (Kaiser et al., 2002) and/or be released non-enzymatically from NO_2^- in the presence of a reductant, such as ascorbate (Weitzberg and Lundberg, 1998). NOS-like activity has been widely detected in plants. Notably, inhibitors of mammalian NOS can also inhibit the NO generation in plant cells (Zhao et al., 2004). However, no NOS gene (protein) has been definitively identified in plants to date (Wilson et al., 2008). Increasing evidences show that NO plays important roles in many key physiological processes including plant growth, germination, cell death, mitochondrial functions, root organogenesis and floral regulation (Beligni et al., 2002; Neill et al., 2003;

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doi:10.1016/j.ecoenv.2010.07.005

He et al., 2004; Wilson et al., 2008). Interestingly, application of the sodium nitroprusside (SNP) an NO donor, can enhance the ability of plants in combating various stresses such as pathogen infection, salt, drought, heavy metals, UV-B-radiation and heat (Neill et al., 2003; Zhao et al., 2008). One of the possible roles for NO in stress management in plants is its ability to detoxify ROS directly or indirectly (Shi et al., 2005; Beligni et al., 2002). However, the effects of NO on submerged plants remain unclear.

The decline of submerged plants in water columns containing excess ammonium is of concern (Cao et al., 2007). Ammonium-induced oxidative stress in aquatic plants has been reported by our group among others (Nimptsch and Pflugmacher, 2007; Wang et al., 2008; Wang and Greger, 2004). In the present study, the effects of NO provided in the form of exogenous application of SNP, were investigated in *H. verticillata* (L.f.) Royle plants under ammonium stress. The resultant changes in the levels of total chlorophyll, ROS, malondialdehyde (MDA) and relative electrolyte leakage (REL%), as well as the catalytic activities of several antioxidant enzymes were evaluated.

2. Materials and methods

2.1. Plant cultural conditions and treatment

Plants of *H. verticillata* were purchased from an aquaculture company in Hangzhou, Zhejiang Province, China and were cultured in a primary culture medium as described previously (Wang et al., 2008). The primary culture medium was supplemented with trace elements and the macro-nutrition elements (1%) adapted from Hoagland's solution, including 0.05 mM $Ca(NO_3)_2$, 0.007 mM KH_2PO_4, 0.2 mM $MgSO_4$ and 0.7 mM K_2SO_4.

For acclimation, plants (7 cm of the tip portion) were cultured in primary culture solution in 70 L plastic containers for at least two weeks under laboratory conditions. Throughout the acclimation and treatment periods, plants were cultured with a photoperiod of 12:12 (light/dark) at a temperature of 24 ± 0.1 °C and an irradiance of 120 μE m^2 s^{-1} under laboratory conditions. SNP (Sigma Chemical Co., USA) solution, used as an exogenous NO donor, was prepared fresh. SNP metabolite (SM) solution, also freshly prepared, consisted of 10 μM sodium ferrocyanide, 10 μM sodium nitrite and 10 μM sodium nitrate. After the period of acclimatization, plants were cultured in the following 4 solutions: primary culture solution (control), or primary culture solution containing either 3 mM NH_4Cl (N), or 3 mM NH_4Cl+10 μM SNP metabolites (N+SM) or 3 mM NH_4Cl+10 μM SNP (N+S).

Ammonium stock solution was prepared fresh as ammonium chloride, according to Nimptsch and Pflugmacher (2007) and diluted to the desired concentration. The ammonium concentrations were determined as described previously (Wang et al., 2010). To verify the amount of ammonium supplied in the medium, for each test solution, samples were taken in triplicate at the beginning and end of the treatment period (12 h and 4 d). Samples were diluted appropriately and immediately applied to AQ2+Discrete Analyzer (Seal analytical, West Sussex, UK) for estimation of total ammonium. Requisite amount of the ammonium was added to the medium accordingly to obtain the final desired concentration.

Plants (5 cm of the tip portion) were harvested after treatments for 12 or 48 h. The harvested material was rinsed with distilled water, blotted, and immediately frozen in liquid nitrogen until further use for biochemical analyses. Experiments were carried out in triplicate. Each replicate included at least 40 plants.

2.2. Determination of photosynthetic pigments

To extract photosynthetic pigments, 0.2 g (M) powdered plant sample was resuspended in 7 mL of 95% ethanol for 24 h in dark, at 4 °C. After centrifugation at 10,000*g* for 10 min, absorbance of the supernatant was measured at 649 and 665 nm. The chlorophyll ($a+b$) concentrations were calculated as described by Vernon (1960) according to the following formula:

Total chlorophyll (mg/g)=[6.45($A665$)+17.72($A649$)]V/M, where, A_{665}=absorbance at 665 nm, A_{649}=absorbance at 649 nm and V=volume of ethanol extract in mL.

2.3. Lipid peroxidation (MDA) and the electrical conductivity

Malondialdehyde was determined in plant leaves according to the method described previously (Wang et al., 2010). Briefly, 0.5 g of powdered sample was homogenized with 5 mL of 1% trichloroacetic acid (TCA) and then centrifuged at 15,000*g* for 10 min. An aliquot of the supernatant (0.5 mL) was mixed with 2 mL of 20% TCA containing 0.5% thiobarbituric acid TBA. After heating at 96 °C for 30 min, the mixture was cooled to 0 °C and then centrifuged at 10, 000*g* for 10 min at 4 °C. The amount of MDA was calculated from the difference in absorbance at 532 and 600 nm using an extinction coefficient of 155 mM^{-1} cm^{-1}.

To determine electrical conductivity, 0.5 g of each plant leaf was washed with double distilled water and subsequently resuspended in 25 mL of deionized water. The conductivity in the bathing solution was determined (C_1) after the incubation at 25 °C for 3 h. Then the conductivity (C_2) was read again in the bathing solution at 25 °C after the samples were heated at 80 °C for 2 h. Percent relative electrolyte leakage (REL%) was expressed as a percentage of the total conductivity by using the formula (Zhao et al., 2004): $REL\% = (C_1/C_2)100$.

2.4. Histochemical detection of O_2^- and H_2O_2

The level of O_2^- was determined according to the method of Doke (1983) that relies on formation of a dark blue precipitate by the reaction of nitro blue tetrazolium (NBT) with superoxide. For H_2O_2 determination, freshly harvested plants were stained in 1% 3,3-dimethoxybenzidine (DAB, pH, 3.8; Sigma Chemical CO., USA) for 7 h under light at 25 °C (Thordal-Christensen et al., 1997). A deep brown polymerization product can be observed with DAB in the presence of H_2O_2. After staining with NBT or DAB, samples were washed with distilled water, boiled in 95% ethanol for 10 min, twice.

2.5. Assay of antioxidant enzymes

For assaying catalytic activities of the various enzymes, 1 g of powdered sample was homogenized in 5 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 2 mM $MgCl_2$ and 1% polyvinylpyrrolidone. For the APX assay, ascorbic acid (AsA), 1 mM was included in the same reaction as above. The homogenate was centrifuged at 12,000*g* for 20 min at 4 °C, and the supernatant was used for the enzyme assays.

NADH oxidase activity was determined following the method described by Ishida et al. (1987). One unit of NADH-oxidase activity was defined as the amount required to hydrolyze 1 μmol *p*-coumaric acid min^{-1} g FW^{-1}. CAT activity was determined by the method described by Aebi (1974). One unit of CAT activity was defined as the amount required to breakdown 1 μmol of H_2O_2 min^{-1} g FW^{-1}. Activity of ascorbate peroxidase (APX) was determined according to the method described by Nakano and Asada (1981). One unit of APX activity was defined as the amount required to oxidize 1 μmol ascorbic acid-oxidized min^{-1} g FW^{-1}.

SOD activity was measured by assaying the inhibition of the photochemical reduction of NBT based on the method of Beauchamp and Fridovich (1971). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT at 560 nm. The guaiacol peroxidase (POD) activity was determined according to the method of Upadhyaya et al. (1985). One unit of POD activity was defined as the amount required to convert 1 μmol guaiacol min^{-1} g FW^{-1}. Activity of glutathione *S*-transferase (GST) was assayed based on the method of Mannervik and Guthenberg (1981). One unit of GST activity was defined as the amount required to convert glutathione 1 μmol min^{-1} g^{-1} FW.

2.6. Determination of ascorbic acid, glutathione and NO

Powdered sample, 1 g, was homogenized in 5 mL of 5% meta-phosphoric acid (w/v). The homogenate was centrifuged at 12,000*g* for 20 min at 4 °C and the supernatant was used for the AsA and GSH assays. The ascorbate assay solution contained 0.4 ml of the sample solution, 0.4 ml of 5% (w/v) TCA and 0.4 ml of absolute ethanol. The color was developed by adding various reagents in the following sequence: 0.2 ml of 0.4% (v/v) H_3PO_4, 0.4 ml of 0.5% (w/v) bathophenanthroline-ethanol and 0.2 ml of 0.03% (w/v) $FeCl_3$. After incubation at 4 °C for 60 min, absorbance of the mixture was recorded at 534 nm (Wang and Greger, 2004). The content of glutathione was determined by the method of Guri (1983) using reduced glutathione (GSH) as a standard. NO content was determined spectrophotometrically by measuring the conversion of oxyhemoglobin to methemoglobin (Zhao et al., 2004).

2.7. Statistical analyses

Each result shown in the figs. is the mean of at least four replicate treatments. Values are mean ± S.E. ($n=4$). All the data were analyzed by ANOVA and Tukey's test, $P < 0.05$.