Ecotoxicology and Environmental Safety 113 (2015) 95–102

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Interaction of nitric oxide and reactive oxygen species and associated regulation of root growth in wheat seedlings under zinc stress

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ARTICLE INFO

Article history:
Received 2 July 2014
Received in revised form
23 November 2014
Accepted 25 November 2014
Available online 6 December 2014

Keywords:
Hydrogen peroxide
Nitric oxide
Root growth
Wheat
Zinc

ABSTRACT

The inhibition of root growth was investigated in wheat seedlings exposed to 3 mM zinc (Zn). Zn treatment with or without 250 μM 2-phenyl-4,4,5,5,-tetrame-thylimidazoline-3-oxide-1-oxyl (PTIO) or 10 μM diphenylene iodonium (DPI) significantly inhibited growth, increased malondialdehyde content and lowered cell viability in roots. The most prominent changes of these three parameters at Zn+DPI treatment could be partly blocked by high PTIO concentration (1 mM). The production of nitric oxide (NO) and hydrogen peroxide (H_2O_2) influenced each other under different treatments, with the highest NO level and the highest H_2O_2 accumulation in Zn+DPI-treated roots. Compared with Zn-stressed roots, catalase, soluble peroxidase (POD), ascorbate peroxidase and superoxide dismutase decreased in Zn+DPI-treated roots, suggesting that ROS generation from plasma membrane (PM) NADPH oxidase was associated with the regulation of antioxidant enzyme activities. Additionally, Zn-treated roots exhibited significant decreases in cell wall-bound POD, diamine oxidase and polyamine oxidase activities. Our results suggested that Zn-induced effects on root growth resulted from NO interaction with H_2O_2 and that Zn+DPI-induced strongest inhibition could be explained by the highest increase in the endogenous NO content and the reduction of extracellular ROS production.

1. Introduction

Nitric oxide (NO) is a stress factor or a signal molecule involved in a variety of plant processes including root growth and plant responses to abiotic stresses (Beligni et al., 2002; De Michele et al., 2009; Böhm et al., 2010). NO may also act as an antioxidant and quench reactive oxygen species (ROS) generated under oxidative stress (Beligni et al., 2002; Zheng et al., 2010). Moreover, in some cases, the effects of NO result from its interaction with ROS. For example, NO worked in oligosaccharide-induced hydrogen peroxide (H_2O_2) production and interacted with ROS to regulate oligosaccharide-induced artemisinin biosynthesis in *Artemisia annua* hairy roots (Zheng et al., 2010). Efficient activation of hypersensitive cell death also required a balance between NO and ROS production in soybean suspension cultures (Delledonne et al., 2001). Recently, we observed that NO stimulated lead-treated seed germination and seedling shoot growth in wheat, indicating the protective role of exogenous NO against lead toxicity (Yang et al., 2010). The study of Xu et al. (2010) showed that NO was associated with the long-term Zn tolerance in *Solanum nigrum*. Although the formation of NO has been well documented in the experiments of different plant systems, the role of endogenous NO during plant responses to different heavy metals seems to be much more puzzling (Arasimowicz–Jelonek et al., 2011).

The rapidly increasing generation of hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) and superoxide anion ($O_2^{\cdot -}$) is one of plant responses to heavy metal stresses. H_2O_2 acts as a secondary messenger that controls such different plant processes as growth and development, and stress responses (Gechev et al., 2006). Furthermore, ROS have the potential to cause oxidative damage to proteins, nucleic acids and other macromolecules, which can severely endanger cell health and viability (Halliwell and Gutteridge, 1999). A previous study demonstrated that ROS production was associated with phytotoxicity due to heavy metal stresses (Posmyk et al., 2009).

As one of the micronutrients essential for normal growth and development of plants, zinc (Zn) becomes toxic to plants if it is

Abbreviations: ASA, ascorbate acid; Zn, zinc; DAF-FM DA, 3-Amino, 4-amino-methyl-2',7'-difluorescein diacetate; DPI, diphenylene iodonium; DW, dry weight; EDTA, ethylenediaminetetraacetic acid; HNO_3, nitric acid; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; NBT, nitrobluetetrazolium; NO, nitric oxide; NOS, nitric oxide synthase; $^{\cdot}OH$, hydroxyl radical; $O_2^{\cdot -}$, superoxide anion; PBS, phosphate buffered solution; PM, plasma membrane; POD, peroxidase; PTIO, 2-phenyl-4,4,5,5-tetrame-thylimidazoline-3-oxide-1-oxyl; PVP, polyvinylpyrrolidone; ROS, reactive oxygen species; TCA, trichloroacetic acid

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http://dx.doi.org/10.1016/j.ecoenv.2014.11.030
0147-6513/

excessive, which may retard plant growth and even lower agricultural products (Cherif et al., 2010; Todeschini et al., 2011). Wheat (*Tritium aestivum* L.) is one of the most important crops in China and many other countries in the world. More recently, we found that zinc treatment resulted in such phytotoxicity as growth inhibition in wheat seedlings and that the effects rose with the increasing concentration (Li et al., 2012a). DPI is a widely used selective NADPH oxidase inhibitor (Auh and Murphy, 1995) and PTIO is used as the scavenger of NO (Arasimowicz–Jelonek et al., 2012). In the present study, the relationship between the root growth and the H_2O_2 and NO generation was further investigated in wheat seedlings under 3 mM Zn treatment in the presence or absence of PTIO or DPI.

2. Material and methods

2.1. Seedling growth

Wheat (*Triticum aestivum*, cv Xihan 3) seeds were purchased from Gansu Agricultrual Academy. The seeds were surface-sterilized with 0.1% (w/v) $HgCl_2$ for 10 min and germinated in the dark at 25 ± 1.5 °C. Uniformly germinated seeds were transferred into Petri dishes and treated with 1/4 Hoagland solution containing 0 and 3 mM $ZnSO_4$ in the presence or absence of 2-phenyl-4,4,5,5-tetrame-thylimidazoline-3-oxide-1-oxyl (PTIO) or diphenylene iodonium (DPI) at 25 ± 2.5 °C under a light irradiance of 300 μ M m^{-2} s^{-1} (12 h light: 12 h dark cycles). Root length was measured 6 days later.

2.2. Zinc content analyses

Roots and leaves were prepared for measuring Zn level according to Achary et al. (2008) with some modifications. Plant material was thoroughly washed with deionized water and dried to constant weight at 80 °C. The dry sample was dissolved in a solution containing 12 ml concentrated nitric acid (HNO_3), 4 ml hydrofluoric acid and 4 ml H_2O_2 (30%), and was digested in a closed microwave digestion system (Multiwave 3000, Anton paar, Austria) for 2.5 h. The chilled sample was transferred into polytetrafluoroethylene beaker, and then evaporated to dryness. The ash residue was dissolved in 1% HNO_3, and total Zn content was measured with flame Atomic Absorption Spectrophotometry (WFX210, China).

2.3. Fluorescence detection of nitric oxide

Endogenous NO level was detected by use of diaminofluorescein diacetate (DAF-FM DA, Sigma),a specific NO fluorescent probe. Wheat roots were incubated with 10 μM DAF-FM DA at 37 °C for 20 min. The extra DAF-FM DA was washed and removed with NaH_2PO_4/Na_2HPO_4 buffer (PBS, pH 7. 4). A Leica MPS60 fluorescent microscope equipped with a red fluorescent protein filter (excitation 450–490 nm, emission 500–530 nm) was used to get fluorescent images.

2.4. Determination of Hydrogen peroxide and superoxide anion levels

The measurement of H_2O_2 level was performed with the method of Sergiev et al. (1997). Plant roots were ground with 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 12,000*g* for 20 min. The supernatant was mixed with 10 mM PBS buffer (pH 7.0) and 1 M KI, and the absorbance of the solution was measured at 390 nm. H_2O_2 concentrations were calculated by use of a standard curve prepared with known H_2O_2 concentrations.

$O_2^{\cdot -}$ generation was determined according to Achary et al. (2012). To develop color resulting from reduction of NBT, wheat roots were immersed in the reaction mixture consisting of 50 mM Tris–HCl buffer (pH 6.4), 0.2 mM nitrobluetetrazolium (NBT), 0.2 mM NADH and 250 mM sucrose, and vacuum-infiltrated for 10–15 min and illuminated at 200 mM m^{-2} s^{-1} for 24 h. The absorbance of blue monoformazan formed in the reaction mixture was measured at 530 nm with an extinction coefficient of ($\varepsilon = 12.8$ mM^{-1} cm^{-1}), and the $O_2^{\cdot -}$ content was expressed as μ M g^{-1} fresh weight (FW).

2.5. Lipid peroxidation determination

Lipid peroxidation was measured based on the method of Zhou (2001). Wheat roots (0.5 g) were homogenized in 0.25% (w/v) thiobarbituric acid, heated at 98 °C for 30 min, and then centrifuged at 10,000*g* for 10 min. The absorbance of the supernatant was measured at 450, 532 and 600 nm, respectively. The concentration of malondialdehyde (MDA) was calculated with an extinction coefficient ($\varepsilon = 155$ mM^{-1} cm^{-1}) and expressed as μ M g^{-1} FW.

2.6. Cell viability analysis

The loss of cell viability was assayed by Evans blue staining (Zanardo et al., 2009). Fresh roots (0.5 g) were incubated in 0.25% (w/v) Evans blue solution for 15 min, washed to remove the excessive and unbound dye. After root tips were soaked in 12 M N'N-dimethylformamide for 50 min at room temperature, the absorbance of released Evans blue was measured at 600 nm.

2.7. Nitric oxide synthase activity measurement

Nitric oxide synthase (NOS) activity was assayed according to the method described by Murphy and Noack (1994) with some modifications. Wheat roots (1 g) together with 1% (w/v) polyvinylpolypyrrolidone (PVP) were ground in liquid N_2 and then resuspended in 5 ml extraction buffer (50 mM Tris–HCl (pH 7.4), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, 1 μM leupeptin, 1 μM pepstatin, 320 mM sucrose and 1 mM phenylmethylsulfonyl fluoride). After centrifugation at 10,000*g* at 4 °C for 30 min, the supernatant was used to analyze NOS activity. 100 U catalase (CAT) and 100 U superoxide dismutase (SOD) were used to remove endogenous ROS for 5 min, and then 5 ml oxyhaemoglobin (5 mM) was added. NOS activity was analyzed by the hemoglobin assay at 401 nm and 421 nm with an extinction coefficient of 77 mM^{-1} cm^{-1}.

2.8. Measurement of antioxidant enzyme activities

Plant material (1 g) was ground with 1 ml of PBS buffer (50 mM, pH 7.8) containing 0.1 mM EDTA and 1% (w/v) PVP. After it was centrifuged at 15,000*g* for 30 min, the supernatant was collected to determine antioxidant enzyme activities. SOD activity was estimated based on the method of Dhindsa and Matowe (1981). The reaction mixture was made up of 50 mM PBS (pH 7.6), 13 mM methionine, 75 μM NBT, 0.1 mM EDTA-Na_2 and an appropriate amount of enzyme extraction, and the reaction was started by the addition of 2 μM lactochrome. After the reaction was illuminated at 25 °C for 10 min with a non-illumination surface as a reference, the absorbance was recorded at 560 nm. The complete reaction medium without enzyme incubated in the dark was used as dark control. One unit (U) of enzyme activity was defined as the quantity of SOD required to produce a 50% inhibition of NBT reduction.

A modification of the method of Aebi (1974) was used to assay CAT activity. The enzyme extraction was added to 50 mM PBS