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Zinc induced phytotoxicity mechanism involved in root growth of *Triticum aestivum* L.

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ABSTRACT

This study investigated the inhibition mechanism of root growth in wheat seedlings when exposed to different zinc (Zn) concentrations. All applied Zn concentration did not affect seed germination, but reduced root length; in contrast, only Zn at 3 mM inhibited significantly the growth of shoot. The loss of cell viability and the significant increases of lignification as well as the increases of hydrogen peroxide (H_2O_2), superoxide radical (O_2^-) and malondialdehyde levels were observed in the root tissue exposed to Zn treatment. And also, Zn stress led to the inhibition of cell-wall bound peroxidase. Moreover, NADPH oxidase inhibitor diphenylene iodonium could block greatly the elevation of O_2^- generation in Zn-treated roots. Therefore, the increased H_2O_2 generation was dependent on the extracellular O_2^- production derived from plasma membrane NADPH oxidase. In addition, the loss of cell viability and the significant increases of lignification in response to the highest Zn concentration may be associated with the remarkable reduction of root growth in wheat seedlings.

1. Introduction

Zinc (Zn), as a component of over 300 enzymes (Gupta et al., 2011), is one of the micronutrients essential for normal growth and development of plants. It is acquired for numerous physiological processes including respiration, photosynthesis and the biosynthesis of plant hormones (Broadley et al., 2007). Moreover, the application of Zn can reverse the phytotoxicity of heavy metals including copper and cadmium (Cd) and be beneficial for the plant's survival (Aravind and Prasad, 2003; Upadhyaya and Panda, 2010). Metal contamination is a widespread phenomenon around the world and mainly originates from increasing industrial and urban activities. For example, high levels of Zn in soils are found in the areas where Zn has been mined or refined. Excessive Zn concentration in soil becomes toxic to plants, which may result in leaf chlorosis, nutrient imbalances and photosynthesis inhibition, thus retarding plant growth and even reducing agricultural products (Kabata-Pendias and Pendias, 1992; Cherif et al., 2010; Todeschini et al., 2011).

Root growth is characterized by high metabolic activity, thus the roots are considered quite susceptible to the stress induced by metals (Finger-Teixeira et al., 2010). It has been indicated that Zn toxicity depends on plant species and growth stage, and that growth inhibition and biomass reduction are the general responses of plants to Zn excess. For instance, in the salt-marsh shrub *Halimione portulacoides*, Zn concentrations greater than 70 mM in the nutrient solution negatively affected plant growth (Cambrollé et al., 2011). Similarly, root growth of *Lolium perenne* was reduced at all levels of Zn excess (≤ 20 mM) in the nutrient solution (Monnet et al., 2001). However, being an essential micronutrient, Zn promoted the growth of *Brassica juncea* seedlings when presented at lower concentrations (Prasad et al., 1999). Additionally, in bean seedlings the root length reduced in Zn-deficient while it exhibited good growth in Zn-sufficient plants (Michael and Krishnaswamy, 2011).

Root length is a parameter which affects plant ability to uptake compounds from its surroundings. Some studies have showed that one reason for the inhibition of the growth of plant seedlings in the presence of metals may be due to the effect of heavy metals on mineral nutrient uptake (Israr et al., 2011; Wang et al., 2012). The inhibitory effect of heavy metals on root growth was also accompanied by increased activity of superoxide dismutase (Kopyra and Gwózdz, 2003). Even though Zn action on the root growth has been clearly established in different plants, the mechanisms by which Zn causes plant injury are not yet fully understood. Moreover, there is no information on the connection between H_2O_2 and lignification levels in plants under Zn stress. Therefore, the aim of this study was to investigate the

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mechanisms of inhibitory effects on root growth due to Zn stress in wheat seedlings, to evaluate the relation between H_2O_2 generation and lignification.

2. Material and method

2.1. Seed germination and seedling growth

Wheat (*Triticum aestivum*, cv Xihan 3) seeds were purchased from Gansu Agricultrual Academy. The seeds were surface-sterilized with 0.1 percent $HgCl_2$ for 10 min and germinated in the dark at 25 ± 1.5 °C. All germination tests were conducted in Petri dishes containing 5 mL of 1/4 Hoagland supplied with 0, 0.5, 1 and 3 mM $ZnSO_4$ (measured concentrations 1.94, 34.4, 66.9 and 197 mg L^{-1} 75 Zn in the solution, respectively), and three replicates of 50 seeds were used for each treatment. The final percentage germination was recorded four days after incubation. Seed germination was defined as a root length of 1.0 mm or more. In addition, after one day of incubation, uniformly germinated seeds were transferred into Petri dishes and treated with 1/4 Hoagland containing 0, 0.5, 1 and 3 mM $ZnSO_4$ at 25 ± 2.5 °C under a light irradiance of 300 μmol m^{-2} s^{-1} (12 h light:12 h dark cycles). Root lengths and shoot lengths were measured after six days.

2.2. Analysis of root cell death

Cell death in root tips was analyzed using propidium iodide (PI). Roots were stained with 10 μg mL^{-1} PI for 10 min. A laser scanning confocal microscope equipped with a red fluorescent protein filter (excitation 535 nm, emission 615 nm) was used for fluorescent images.

2.3. Cell viability analysis

Wheat roots treated with different Zn concentrations were used to determine the loss of cell viability by Evans blue staining (Zanardo et al., 2009). Fresh roots were incubated for 15 min with 30 mL of 0.25 percent Evans blue solution, washed for 30 min to remove excess and unbound dye. After excised root tips (3 cm) were soaked in 3 mL of N, N-dimethylformamide for 50 min at room temperature, the absorbance of released Evans blue was measured at 600 nm, using deionized water as a blank. The loss of cell viability was expressed as absorbance at 600 nm of treated roots in relation to untreated roots.

2.4. Root lignification analysis

Roots were fixed in FAA fixative solution containing 90 mL of 50 percent ethanol, 5 mL of glacial acetic acid and 5 mL of formalin. The roots were dehydrated with ethanol from low concentration to high concentration and conserved in 70 percent ethanol, then transparent with dimethylbenzene, dipped in wax, embedded with wax, sectioned and bonded, dewaxing. After staining with Safranin–Fast Green staining, the obtained sections were dehydrated and transparent to anatomical observation. Micrographs were photographed with a light microscope equipped with a Nikon Coolpix digital camera.

2.5. Estimation and visualization of O_2^- in roots

Superoxide radical (O_2^-) generation was determined by the method of Achary et al. (2012). Hundred and fifty milligram roots were immersed in 6 mL of the reaction mixture containing 50 mM Tris–HCl buffer (pH 6.4), 0.2 mM nitrobluetetrazolium (NBT), 0.2 mM NADH and 250 mM sucrose, with or without 10 μM diphenyleneiodonium chloride (DPI, a specific inhibitor of NADPH-oxidase); vacuum-infiltrated for 10–15 min and illuminated at 200 mmol m^{-2} s^{-1} for 24 h to develop color as a result of reduction of NBT. The absorbance of blue monoformazan formed in the reaction mixture was measured at 530 nm, and the O_2^- content was expressed as μM g^{-1} FW.

2.6. Fluorescence detection of hydrogen peroxide

The generation of H_2O_2 in roots was determined according to the method of Pei et al. (2000). Roots were labeled with 50 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) for 30 min at 37 °C, then washed in Tris–HCl (pH 7.2) buffer to remove excess dye. A Leica MPS60 fluorescent microscope equipped with a red fluorescent protein filter (excitation 450–490 nm, emission 500–530 nm) was used for fluorescent images.

2.7. Measurement of cell wall-POD activity

Cell walls were prepared by homogenizing roots in 50 mM phosphate buffer (PBS, pH 5.8) as described by Lee and Lin (1995). The homogenate was centrifuged at 1000*g* for 10 min and washed at least two times with 50 mM PBS buffer (pH 5.8), the pellets were incubated in 1 M NaCl for 2 h for extracting cell wall-bound POD. After centrifuged at 1000*g* for 10 min, the supernatant containing the ionically bound cell wall-POD enzyme was collected. Enzyme activity was determined according to the procedures of dos Santos et al. (2008). The reaction mixture contained 25 mM PBS (pH 6.8), 2.58 mM guaiacol, 10 mM H_2O_2 and the enzyme extract. The change of the absorbance at 470 nm within 2 min was recorded for calculating POD activity. One unit (U) of POD activity was defined as an absorbance change of 0.01 unit min^{-1}.

2.8. MDA content detection

Malondialdehyde (MDA) was determined according to the method of Zhou (2001) with some modifications. Wheat roots (0.5 g) were homogenized in 5 mL 0.25 percent thiobarbituric acid, then heated at 98 °C for 30 min, quickly cooled on ice and then centrifuged at 10,000*g* for 10 min, the absorbance of the supernatant was measured at 450, 532, 600 nm. The MDA level was expressed as μmol g^{-1} FW.

2.9. Data analysis

Each experiment was replicated at least three times. The data were expressed as means ± standard error (SE). Statistical comparisons were carried out using SPSS 13.0 software with One-Way ANOVA, and significant differences were indicated by different letters ($p < 0.05$).

3. Results

3.1. Seed germination and seedlings growth

As shown in Table 1, the addition of Zn did not affect seed germination of wheat. Zn stress reduced root length and the effects increased with increasing concentration. In contrast, shoot growth is less affected than root growth. No significant difference in shoot length was observed in wheat seedlings exposed to 0.5 and 1 mM Zn; only at 3 mM, Zn inhibited significantly the growth of shoot, as compared to the control (Table 1).

3.2. Cell death and cell viability

As shown in Fig. 1, there was no detectable fluorescence of PI in the nuclei of root tip cells exposed to different Zn concentrations. In addition, the loss of cell viability was evaluated by Evans blue staining in roots after treatment with different Zn concentrations (Fig. 2). Compared to the control, the uptake of Evans blue in root cells decreased under 0.5 mM Zn treatment, but increased significantly under 3 mM Zn treatment; in contrast, 1 mM Zn concentration had no effect on the viability of root tip cells in wheat seedlings.

3.3. Root lignification level

The extent of root tissue lignification in response to Zn excess was determined by staining with Safranin–Fast Green double staining. As shown in Fig. 3, the average level of lignification in

Table 1
Effects of zinc on seed germination and seedling growth in wheat.

$ZnSO_4$ concentration (mM)	Germination percentage (percent)	Root length (cm)	Shoot length (cm)
Control	83.33 ± 0.06a	9.99 ± 0.10d	14.97 ± 0.05b
0.5	82.67 ± 0.07a	6.25 ± 0.08c	14.25 ± 0.01b
1	88.00 ± 0.07a	4.02 ± 0.12b	13.79 ± 1.00b
3	86.00 ± 0.02a	1.13 ± 0. 01a	9.53 ± 0.33a

Notes: Values are expressed as means ± standard error (SE), different small letters in each column indicate significant difference at 0.05 levels.