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Comparative antioxidative responses and proline metabolism in two wheat cultivars under short term lead stress

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ABSTRACT

This study investigated antioxidative response and proline metabolism in two wheat cultivars (*Tritium aestivum* Xihan 2 (Xihan) and Ningchun 4 (Ningchun)) under $Pb(NO_3)_2$ stress. The constitutive H_2O_2 scavenging enzyme activities and proline content in the leaves of drought-tolerant Xihan are higher than those in drought-sensitive Ningchun. Higher Pb concentration reduced chlorophyll content in both cultivars, but Ningchun was more sensitive to lead toxicity than Xihan. The higher activities of antioxidant enzyme and the significant proline accumulation provide protection for two wheat cultivars against lead toxicity, resulting in unaltered MDA content. Analysis of enzyme activities showed that the accumulation of proline induced by lead stress is due to the increases of OAT and GK activities in Xihan seedlings, and to the increase of GK activity in Ningchun leaves. Therefore, the different mechanism of proline metabolism was associated with increased proline levels in two wheat cultivars when exposed to lead stress.

1. Introduction

Contamination of agricultural soils by heavy metal represents a serious environmental problem (Vaculík et al., 2009). Most of the heavy metals are known as growth inhibitors to cause phytotoxicity and decrease the yield and quality of agricultural crops (Kopyra and Gwóźdź, 2003; Atici et al., 2005; Faheed, 2005), even threat human beings by entering the food chain. It has been reported that inhibited biosynthesis of chlorophyll is a frequent symptom of metal toxicity (Van Assche and Clijsters, 1990; Sandalio et al., 2001). Heavy metal toxicity may also be exerted by the fact that heavy metals induce secondary oxidative stress by importing the formation of harmful reactive oxygen species (ROS) (Posmyk et al., 2009). Increased ROS generation must be regulated to avoid unwanted phytotoxicity and oxidative damage (Halliwell and Gutteridge, 1990). Antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) constitute an important protective mechanism to minimize oxidative damage in plant response to environmental stress. Among those, SOD is usually considered the first line of defense against oxidative stress (Wang et al., 2008); CAT, POD and APX consist of main elimination H_2O_2 systems in cells (Mittler, 2002). Stress condition may induce increased antioxidant enzyme activity, which can provide plant protection against ROS damage. Malecka et al. (2001) found increased activities of CAT and SOD in the cytosol, mitochondrial and peroxisomal fractions isolated from root cells of *Pisum sativum* grown under lead treatment (0.5 or 1 mM). Moreover, the correlation between antioxidant capacity and lead tolerance has been demonstrated in a large number of plants, such as *Lupinus luteus* (Kopyra and Gwóźdź, 2003), *Sesbania* (Ruley et al., 2004), *Macrotyloma uniflorum* (Lam.) Verdc. and *Cicer arietinum* L. (Reddy et al., 2005).

In order to survive plants have also evolved non-enzymatic defense mechanisms against oxidative stress. Proline has been demonstrated to scavenge hydroxyl radicals and singlet oxygen, thus providing protection against ROS-induced cell damage (Matysik et al., 2002). Increased proline content induced by heavy metal stress has also been reported in some higher plants (Chen et al., 2001; Zengin and Munzuroglu, 2005; Kováč et al., 2009; Radic et al., 2010). It has been demonstrated that proline is synthesized via two different pathways from either glutamate or ornithine/ariginine (Delauney and Verma, 1993; Kavi-Kishor et al., 2005). The rate-limiting step in glutamate pathway is the r-glutamyl kinase (GK) activity of the P5CS enzyme. Ornithine-δ-aminotransferase (OAT) is a key enzyme that catalyzes the first step in the ornithine pathway (Yang et al., 2009). In addition, proline degradation catalyzed by proline dehydrogenase (PDH) is involved in the accumulation of proline (Hare et al., 1999; Lee and Liu, 1999). Even though much supporting evidence on the effects of heavy metal on proline accumulation is available (Costa and Morel, 1994; Bassi and Sharma, 1993; Kováč et al., 2009), the mechanisms of proline metabolism in response to heavy metal condition have been poorly investigated to date. Studying the effect of lead stress on enzyme activities involved in proline biosynthesis and degradation could

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provide valuable information on physiological significance of its accumulation.

Wheat (*T. aestivum* L.) is one of the most important agricultural crops in China and other countries in the world. Xihan 2 (Xihan) and Ningchun 4 (Ningchun), differing in drought tolerance, are two wheat species widely grown in the north-west of China. The aim of this study was to evaluate phytotoxicity, antioxidative responses and proline metabolism in these two wheat cultivars exposed to short term lead stress. Additionally, malondialdehyde (MDA) content was investigated as an indicator of oxidative stress and lipid peroxidation.

2. Materials and methods

2.1. Plant material and growth condition

In this study, two wheat cultivars (*T. aestivum* cv. Xihan 2 and Ningchun 4, purchased from Gansu Agricultural University and Gansu Agricultural Academy, respectively) were used. Xihan 4 and Ningchun 2 are drought-tolerant and drought-sensitive, respectively. Seeds were surface-sterilized with 0.1% $HgCl_2$ for 10 min, then soaked in water for 24 h and germinated in the dark at 25 ± 1.5 °C for 24 h. The germinated seeds were planted in pots containing quartz sands, and irrigated with distilled water. After growing for 6–7 days at 25 ± 1.5 °C under a light irradiance of 300 μmol m^{-2} s^{-1} (12 h-photo period), wheat seedlings were treated with different concentrations of $Pb(NO_3)_2$ for 3 days. For the control experiment, seedlings were cultured in the absence of heavy metal.

2.2. Lead content determination

The stems and leaves of Wheat seedlings were prepared for Pb measurements according to Wu et al. (2002) with some modifications. Plant material was thoroughly washed with deionized water and dried at 80 °C. The dry sample was dissolved in 10 mL of HNO_3 and total lead content was measured with an Inductive Coupled Plasma Emission Spectrometer (ICP, LABTAM8410).

2.3. Soluble protein content measurement

The whole procedures were carried out at 4 °C. One gram of plant material was ground with 1 mL of chilled NaH_2PO_4/Na_2HPO_4 buffer (PBS, 50 mM, pH 7.8) containing 0.1 mM ethylenediamine tetraacetic acid (EDTA) and 1% polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 12,000 rpm for 30 min, and the supernatant (enzyme extraction) was collected for protein assay and the determination of SOD, POD and CAT activities. The amount of proteins was estimated according to the method proposed by Bradford (1976) and the standard curve was made using bovine serum albumin.

2.4. Chlorophyll content measurement

Chlorophyll was extracted from wheat leaves with 80% acetone. Chlorophyll content was determined spectrophotometrically at 663 and 646 nm according to Lichtenthaler (1987).

2.5. Antioxidant enzyme activity measurement

SOD activity was estimated based on the method described by Dhindsa and Matowe (1981). 50 μL of the enzyme extraction was added to 3 mL of the reaction mixture consisting of 50 mM PBS (pH 7.6), 13 mM methionine, 75 μM nitroblue tetrazolium (NBT) and 0.1 mM EDTA-Na_2. The reaction was started by the addition of 2 μM lactochrome. After illumination for 10 min at 25 °C using a non-illumination surface as 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 enzyme activity (U) was defined as the quantity of SOD required to produce a 50% inhibition of reduction of NBT and the results were given as units of SOD activity per milligram of protein (U mg^{-1} protein).

A modification of the method of Aebi (1974) was used to assay CAT activity. Briefly, 100 μL of the enzyme extraction was added to 3 mL 50 mM PBS buffer (pH 7.0). After 5 min incubation at 25 °C, the reaction was started by the addition of 6 μM H_2O_2 and the absorbance changes were recorded at 240 nm for 2 min. An absorbance change of 0.1 unit min^{-1} was defined as 1 unit of CAT activity, and CAT activity was expressed as U mg^{-1} protein.

POD activity was measured following a modification of the method of Rao et al. (1996). 5 μL of the enzyme extraction was mixed with 3 mL of the reaction mixture containing 50 mM PBS (pH 7.0) and 20 mM guaiacol. After pre-incubation at 25 °C for 5 min, 6 μM H_2O_2 was added to initiate the reaction. Changes of the absorbance at 470 nm within 2 min were recorded for calculating POD activity. One unit of POD activity was defined as an absorbance change of 0.01 unit min^{-1} and POD activity was expressed as U mg^{-1} protein.

0.5 g of plant material was ground with 1 mL of chilled 50 mM PBS buffer (pH 7.0) containing 1 mM EDTA-Na_2 and 1 mM ascorbate (ASA). After centrifugation for 20 min at 10,000 rpm, the supernatant (enzyme extraction) was collected for the measurement of APX activity. APX activity assay was performed as described by Nakano and Asada (1981) with some modifications. The assay was carried out in a reaction mixture consisting of 50 mM PBS (pH 7.0), 0.5 mM ASA, 3 μM H_2O_2 and 100 μL of the enzyme extraction. The changes in the absorbance at 290 nm were recorded at 25 °C for 1 min after the addition of H_2O_2. One unit of APX activity was defined as an absorbance change of 0.1 unit min^{-1} and APX activity was expressed as U mg^{-1} protein.

2.6. Proline content determination

Proline analysis was performed according to Bates et al. (1973) with some modifications. Wheat leaves (0.5 g) were immediately homogenized in 2 mL of 3% sulfosalicylic acid solution, then heated at 98 °C for 10 min. After centrifugation at 12,000 rpm for 15 min, 0.25 mL supernatant was added to 3.75 mL of the reaction medium containing 0.56% sulfosalicylic acid, 0.25% glacial acetic acid and 1.25% ninhydrin solution. The mixture was kept at 95 °C for 60 min, and then the reaction was stopped quickly by an ice bath. Toluene (4 mL) was added to the mixture, the organic phase was extracted and monitored at 520 nm by a spectrophotometer.

2.7. Proline metabolism enzyme activity analysis

About 1 g of plant material was immediately homogenized in 100 mM potassium phosphate buffer (pH 7.9) containing 1 mM EDTA, 15% glycerol and 10 mM 2-mercaptoethanol. The homogenate was centrifuged at 12,000 rpm for 15 min at 4 °C and the supernatant was collected for OAT activity measurement. OAT activity was assayed with ninhydrin according to Kim et al. (1994). 1 mL of the reaction mixture consisted of 50 mM Tris–HCl (pH 8.0), 50 mM L-ornithine, 5 mM β-ketoglutarate, 0.05 mM pyridoxal phosphate and the appropriate amount of crude enzyme extract was incubated at 37 °C for 20 min. After the addition of 0.3 mL of 3 N perchloric acid and 0.2 mL of 2% ninhydrin, the reaction was stopped by boiling for 5 min. The precipitate was collected by centrifugation (11,000 rpm, 30 min, 4 °C) and completely dissolved with 1.5 mL of ethanol, and then the absorbance was recorded at 510 nm. The absorbance of 0.01 at 510 nm was defined as one unit (U) of OAT activity, and the specific enzyme activity was expressed as U mg^{-1} protein.

About 2 g of wheat leaves was ground in 2 mL TD buffer containing 50 mM Tris–HCl buffer (pH 7.0), 1 mM dithiothreitol and 10% glycerol. After centrifugation at 13,000 rpm for 20 min, the supernatant was collected and precipitated by adding solid ammonium sulfate (40% saturation). Then, the soluble fraction obtained by centrifugation (13,000 rpm for 20 min) was saturated with dry ammonium sulfate to a concentration of 80%. After centrifugation at 12,000 rpm for 15 min at 4 °C, the pellet was collected and completely dissolved with 1 mL TD buffer. The crude enzyme solution was obtained after a 24 h dialysis against TD buffer at 4 °C. Glutamyl kinase activity was assayed by the method of Smith et al. (1984) with some modifications. A total volume of 1 mL assay mixture containing 50 mM glutamate, 10 mM ATP, 20 mM $MgCl_2$, 100 mM oxammonium hydrochloride, 50 mM Tris–HCl buffer (pH 7.0) and an appropriate amount of enzyme was incubated at 37 °C for 30 min and then the reaction was stopped by adding 1 mL stop solution (5.5% $FeCl_3$, 2.0% $HClO_4$, 2 M HCl). The precipitate was removed by centrifugation, and the absorbance of the supernatant at 535 nm was recorded against a blank identical to the one mentioned above but lacking ATP. The changes of absorbance of 0.01 h^{-1} at 535 nm were defined as one unit (U) of GK activity, and the specific enzyme activity was expressed as U mg^{-1} protein.

Fresh wheat leaves (0.5 g) were homogenized in the ice-cold extraction buffer (100 mM sodium phosphate, 1 mM cysteine, 0.1 mM EDTA, pH 8.0). After centrifugation at 12,000 rpm for 10 min at 4 °C, the supernatant was used as crude enzyme preparation for measurement of PDH activity. PDH activity was measured as described by Rena and Splittstoesser (1975) with a slight modification. Briefly, the crude extraction was incubated in the reaction buffer (100 mM Na_2CO_3–$NaHCO_3$, 10 mM nicotinamide adenine dinucleotide (NAD), 20 mM L-proline, pH 10.3) at 32 °C for 5 min, and then PDH dependent NAD reduction was monitored at 340 nm for 4 min. One unit (U) of PDH activity was defined as an absorbance change of 0.001 min^{-1} and the specific enzyme activity was expressed as U mg^{-1} protein.

2.8. Lipid peroxidation analysis

Lipid peroxidation was measured according to the method of Zhou (2001) with some modifications. Wheat leaves (0.5 g) were immediately homogenized in 5 mL 0.25% thiobarbituric acid, then heated at 98 °C for 30 min, quickly cooled on ice and then centrifuged at 10,000 rpm for 10 min, the absorbance of the supernatant was measured at 450, 532 and 600 nm, respectively.