Cadmium-Induced Phytotoxicity in Tomato Seedlings Due to the Accumulation of H_2O_2 That Results from the Reduced Activities of H_2O_2 Detoxifying Enzymes

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ABSTRACT : Tomato (*Lycopersicon esculentum*) seedlings exposed to various concentrations of $CdCl_2$ (0-100 μM) in the nutrient solution for up to 9 days were analyzed with the seedling growth, H_2O_2 production, glutathione levels and activity changes of enzymes related to H_2O_2 removal. The growth of seedlings was inhibited with over 50 μM Cd, whereas the levels of H_2O_2 and glutathione were enhanced with Cd exposure level and time. Meanwhile, Cd exposure increased the activities of catalase (CAT) and glutathione reductase (GR) but decreased the activities of dehydroascorbate acid reductase (DHAR) and ascorbate peroxidase (APX) in both leaves and roots. These results suggest that the altered activities of antioxidant enzymes particularly involved in the H_2O_2 removal and the subsequent H_2O_2 accumulation could induce the Cd-induced phytotoxicity.

Key words : Cadmium, Hydrogen peroxide, *Lycopersicon esculentum*

INTRODUCTION

Cadmium (Cd) is one of the most toxic metals in the present environment, and is easily taken up by roots and translocated to different plant parts (Baker *et al*. 1994). The high Cd accumulation generally causes growth inhibition and even plant death due to the reduction of enzyme activity (Schutzendubel *et al*. 2001), photosynthesis (Clijsters and Van Assche 1985), respiration (Kessler and Brand 1995), transpiration (Barcelo and Poschenrieder 1990) and nutrient uptake (Sanita di Toppi and Gabbrielli 1999).

Although how Cd acts at the cellular level and how plants respond to the metal are not understood clearly, an increasing body of evidence suggests that the metal-induced phytotoxicity can be attributed, at least in part, to oxidative damage (Dixit *et al*. 2001, Hegedus *et al*. 2001). Oxidative stress, arising from an imbalance in the regeneration and removal of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), is a challenge faced by all aerobic organisms. In parallel to metal-induced growth inhibition alterations in both the activities of antioxidant enzymes and the levels of antioxidants (Schtzendubel *et al*. 2001) as well as enhanced lipid peroxidation have been observed in plants (Chaoui *et al*. 1997, Weckx and Clijsters 1997, Cho and Park 2000).

Glutathione-ascorbate cycle (Foyer *et al*. 1994) is essential in removing H_2O_2, and the activities of ascorbate peroxidase (APX), glutathione reductase (GR) and dehydroascorbate reductase (DHAR) are crucial in the operation of the cycle (Asada 1999). Adaptation of the plant cells to metal stress involves chelation, compartmentation or exclusion of metal ions (Mehra and Tripathi 2000). Availability of the glutathione in cytosol may be important since glutathione itself has a relatively high affinity for binding Cd (Perrin and Watt 1971) and can be used to synthesize phytochelatins (PC), which bind metals in the cytosol and sequester them in the vacuole (Grill *et al*. 1985, Rauser 1995). However, since high PC synthesis leaded to hypersensitivity to Cd stress (Lee *et al*. 2003) and some plants or cell lines resistant to Cd are not active in PC synthesis (Ernst *et al*. 2000, Ebbs *et al*. 2002, Kupper *et al*. 2004), the role of PC in Cd resistance is not clearly defined. It is possible that inefficient removal of H_2O_2 by the altered operation of glutathione-ascorbate cycle due to glutathione depletion might induce phytotoxicity. In fact, in *Silene cucubalus*, copper caused oxidative stress by depletion of the glutathione due to PC synthesis (De Vos *et al*. 1991). It is still unknown whether the Cd ion directly induces ROS including H_2O_2. Since Cd ion is unable to catalyze Fenton-Haber-Weiss reactions, which generate ROS, how Cd-exposure induce ROS and oxidative stress is also an interesting subject to investigate.

For understanding and finally further enhancing the mechanisms of tolerance, it is crucial to know how the metal-induced phytotoxicity can be induced. The goal of this work was to understand some of the mechanisms involved in Cd-induced phytotoxicity. H_2O_2 production and alterations of the activities of antioxidant enzymes particularly involved in H_2O_2 removal were investigated to

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determine if the Cd-induced phytotoxicity could be due to the accumulation of H_2O_2. The resistant plants capable of detoxifying or storing Cd could be developed based on the understanding of phytotoxicity and might be used in the phytoremediation of Cd-contaminated environment.

MATERIALS AND METHODS

Plant material and growth conditions

Seedlings of tomato (*Lycopersic esculentum*, Mill cv. Seokwang) were germinated in pots containing perlite:vermiculite (1:1) mixture in a controlled environment chamber at 25℃ with 12 h of light (250 µM $m^{-2}s^{-1}$) and 70~80% humidity. Two week-old seedlings were transferred to aerated plastic pots containing half-strength Hoagland's solution (Hoagland and Arnon 1938) with 0, 10, 50 and 100µM Cd (as $CdCl_2$). The solution was changed every 3 d. Plants collected from each treatment after 3, 6 and 9 days were dried for 48 h at 70℃ and weighed for Cd determination. For measurements of H_2O_2 and glutathione levels and enzyme activities, fresh samples were weighed and used.

Cd content

Leaves were washed twice in deionized water, and the roots of intact plants were washed with ice-cold 5 mM $CaCl_2$ solution for 10 min to displace extracellular Cd (Rauser 1987). The plant material was dried for 48 h at 70℃, weighed and ground into fine powder before wet ashing in HNO_3:$HClO_4$ (3:1) solution. Cd was determined directly by atomic absorption spectrophotometry (Varian 200AA equipped with SIPS, Australia) using an air-acetylene flame and Cd hollow-cathode lamp.

Analyses of H_2O_2 and total glutathione

Five hundred milligrams of fresh tissues were ground with liquid nitrogen and suspended in 1.5 ml of 100 mM potassium phosphate buffer, pH 6.8, and the H_2O_2 contents in tissues were measured according to the method of Karabal *et al*. (2003).

For the determination of glutathione, 100 mg of samples were powdered in liquid nitrogen, mixed with 1 mL of 5% (w/v) sulphosalicylic acid and centrifuged (10 min, 4℃, 12,000 g), and the supernatant was analyzed according to the method of Fadzilla *et al*. (1997).

Enzyme assays

All samples (0.2~0.5 g fresh weight) were ground to a fine powder with mortar and pestle under liquid nitrogen. The proteins were extracted with a cold 50 mM KH_2PO_4/K_2HP_4 (pH 7.0) buffer containing 10 gs l^{-1}PVP, 0.2 mM EDTA, 0.1% (w/v) ascorbic acid (only for APX measurement) and 10 ml l^{-1} Triton X-100. After the homogenate was centrifuged at 12000×g for 20 min at 4℃, the supernatant was used for immediate determination of protein content and enzymatic assay. Protein was determined according to the method of Bradford (1976) with BSA as a standard. CAT activity was assayed based on the decrease in absorbance at 240 nm due to degradation of H_2O_2 (= 39.4 mM^{-1} cm^{-1}) (Chance and Maehly 1955). The GR assay using 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) is based on the method of Smith *et al*. (1988). The APX activity was assayed as described by Rao *et al*. (1995). The DHAR activity was assayed by monitoring the increase in absorbance at 265 nm due to ascorbate formation (= 14 mM^{-1} cm^{-1}) (Nakano and Asada 1981) using a reaction mixture described by Prasad *et al*. (1999). All spectrophotometric analyses were conducted on a Bio-20 spectrophotometer (Perkin Elmer, USA).

Statistical analysis

The results are the means ± SE of five independent replicates. The analyses of variance were computed on statistically significant differences determined based on the appropriate *F*-tests. The mean differences were compared utilizing Duncan's multiple range tests.

RESULTS AND DISCUSSION

Effect of Cd on seedling growth and Cd accumulation

Table 1 shows the development of roots and shoots of seedlings and Cd contents accumulated in leaves and roots treated for 9 d in a nutrient solution containing various concentrations of $CdCl_2$. Lengths of both shoot and root of seedlings were significantly reduced with over 50 µM Cd. Both shoot and root lengths were reduced by nearly 25% and 65%, respectively, with 100 µM Cd,

Table 1. Seedling growth and Cd content in leaves and roots exposed to various concentrations of Cd for up to 9 days. Values are means±SE of three independent replicates. Values in a column followed by the same letter are not significantly different at the 0.05 levels according to Duncan's multiple range tests

Cd (µM)	Shoot length (mm/plant)	Root length (mm/plant)	Cd accumulation (µg/g dry weight)	
			Leaf	Root
0	6.10±0.40a	10.15±0.69a	0.06±0.05	0.08±0.06
10	5.42±0.36a	9.60±1.30a	16.69±1.08	165.44±5.86
50	5.08±0.36b	5.81±0.49b	83.93±2.60	322.83±30.92
100	4.55±0.27b	3.58±0.34c	142.67±7.14	441.97±5.09

so that root growth was more sensitive to Cd exposure than shoot growth.

The seedlings accumulated substantial amounts of Cd in the leaves and roots, and the accumulation increased concurrently with the treatments applied. The Cd content in leaves for 9 d was approximately 32% of that in roots. Therefore, roots accumulated much higher Cd than leaves did.

The consistent increase of Cd accumulation in leaves with prolonged treatments indicated that high Cd content in the root was not a limiting factor for the translocation of absorbed Cd during Cd exposure period investigated. The high Cd retention in roots might be due to cross-linking of Cd to carboxyl groups of the cell wall (Barcelo and Poschenrieder 1990, Boominathan and Doran 2003) and /or to an interaction with thiol residues of PCs (Leita *et al*. 1993).

H_2O_2 and glutathione

The effect of Cd on H_2O_2 production is presented for leaves and roots (Fig. 1). Subjecting tomato seedlings to up to 50 μM Cd for 6 d significantly increased the level of H_2O_2 in comparison with control plants, and H_2O_2 content in roots was much higher than in leaves.

Although the mechanism of Cd-induced H_2O_2 formation is not known at present, heavy metals are known to be involved in many ways in the production of ROS including H_2O_2 (Halliwell and Gutterrifge 1984). The H_2O_2 accumulation caused by Cd-exposure may occur in a manner similar to that in plants stressed (Prasad *et al*. 1994), and it is conceivable to suppose that a decrease of enzymic and non-enzymic free radical scavengers caused by heavy metals (De Vos *et al*. 1993) may contribute to the shift in the balance of free-radical metabolism towards H_2O_2 accumulation. Although the dismutation of superoxide radicals produce H_2O_2 in the initial process of ROS removal, the enhanced production of H_2O_2 on metal exposure might not be entirely induced by the increased production of superoxide radical (O_2^-) and activity of SOD since O_2^- production was not high in other plants exposed to metals (Cakmak and Horst 1991). Therefore, the activities of the enzymes involved in H_2O_2 removal might contribute to the accumulation of H_2O_2 particularly in metal environment. Although Cd is not an active transition metal as Cu is (De Vos *et al*. 1993) and then it may not directly generate toxic oxygen species (Ouariti *et al*. 1997), Cd enhance lipoxygenase activity (Somashekaraiah *et al*. 1992), and the products of the lipoxygenase reaction, mainly peroxy, alkoxy and hydroxyl radicals, are themselves reactive and result in further membrane lipid deterioration leading to membrane permeability and subsequent growth inhibition (De Vos *et al*. 1991). The enhnanced production of H_2O_2 could contribute to the suppression of seedling growth.

The Cd-induced progressive changes of total glutathione in leaves and roots were measured (Fig. 2). Total glutathione content increased significantly only in leaves with Cd exposure levels. No significant change with Cd exposure levels was observed in roots.

Glutathione plays a crucial role in controlling and maintaining the intracellular redox state (Noctor and Foyer 1998). The size of the glutathione pool changes in response to various environmental factors including temperature (Wise and Naylor 1987), light (Noctor *et al*. 1996) and heavy metals (Scheller *et al*. 1987, Ruegsegger and Brunold 1992, Schneider and Bergmann 1995). Glutathione synthesis can occur in the chloroplast and the cytosol (Noctor *et al*. 1998a, 1998b) and respond to the availability of amino acid substrates, particularly Cys (Buwalda *et al*. 1990, Xiang *et al*. 2001).

Glutathione changes have also been observed in a number of other plant species (Scheller *et al*. 1987, Vogeli-Lange and Wagner 1996), and the enhanced glutathione levels could be related to the demand of PCs for detoxification of Cd (Vogeli-Lange and Wagner 1996). Therefore, the maintenance of glutathione levels in plants is crucial in metal tolerance. Meanwhile, copper tolerance of *Silene*

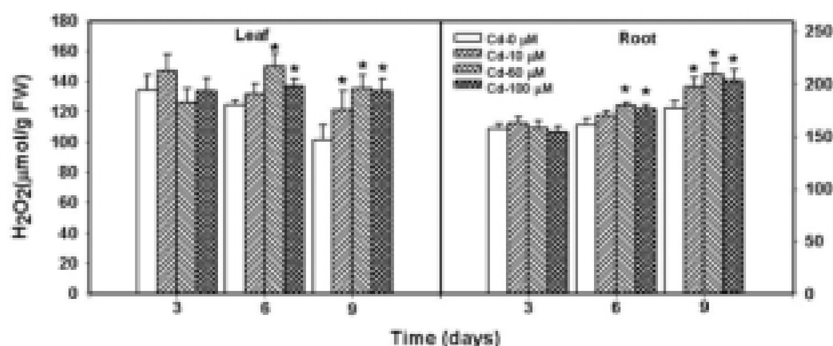

Fig. 1. H_2O_2 content in leaves and roots of tomato seedlings exposed to Cd for 9 days. Values are means±SE of three independent replicates. Significant at the 0.05 levels compared with non-treated control according to *F*-test. n-treated control according to *F*-test.

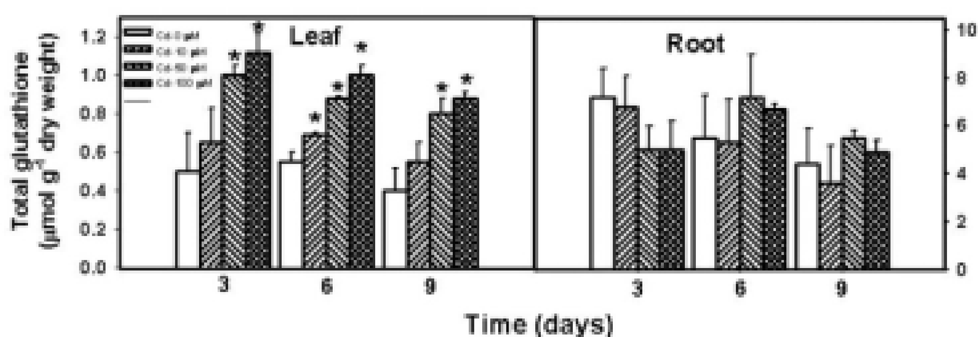

Fig. 2. Concentration of total glutathione in leaves and roots of tomato seedlings exposed to Cd for 9 days. Values are means±SE of three independent replicates. Significant at the 0.05 levels compared with non-treated control according to *F*-test. n-treated control according to *F*-test.

vulgaris was related to their ability to prevent glutathione depletion than to protective functions of PCs (De Vos *et al*. 1992) and Indian mustard plants having 1.5- to 2.5-fold increase in glutathione showed some increase in metal resistance (Zhu *et al*. 1999). These results indicated that there are some other roles of glutathione besides usage for synthesis of PCs in metal stress. Although the roles of glutathione in metal tolerance are not clear and depend on plant species, metals and exposure levels, it was assumed to be important in metal tolerance, functioning as both an antioxidant against ROS including hydrogen peroxide (Alscher 1989) and a chelating agent for metal detoxification. Therefore, glutathione could be crucial in H_2O_2 removal and H_2O_2 level might be increased by the inefficient operation of glutathione-ascorbate cycle, a major H_2O_2 scavenging system in plants.

The no-increase of glutathione in roots might be due to the active synthesis of PCs, which contributed to the high Cd accumulation. Meanwhile, the higher level of H_2O_2 and more suppression of growth in roots might indicate that glutathione levels in roots were not enough to remove H_2O_2.

Enzyme activities

Ten micromoles of Cd were enough to induce CAT activity in leaves and roots on all days measured although no significant increase of the enzyme activity with high Cd levels on day-9 was observed in roots (Fig. 3).

CAT activity might contribute to the no-increase of H_2O_2 on day-3 (Fig. 1). However, further increase of CAT activity over day-6 was not enough to decrease H_2O_2 production, and CAT might not be a major enzyme of H_2O_2 removal during later Cd exposure period. In beans, CAT activity was enough for the efficient defense mechanism against Cu-induced oxidative stress (Weckx and Clijsters 1996).

The activities of enzymes involved in the ascorbate-glutathione pathway in seedlings exposed to Cd were also analyzed (Fig. 4). APX activity is responsible for the degradation of H_2O_2 and production of dehydroascorbate (DASA) from reduced ascorbate (ASA) and DHAR is responsible for the reduction of DASA into ASA. In this pathway, GR is responsible for the reduction of oxidized glutathione (GSSG) into reduced glutathione (GSH) that is required

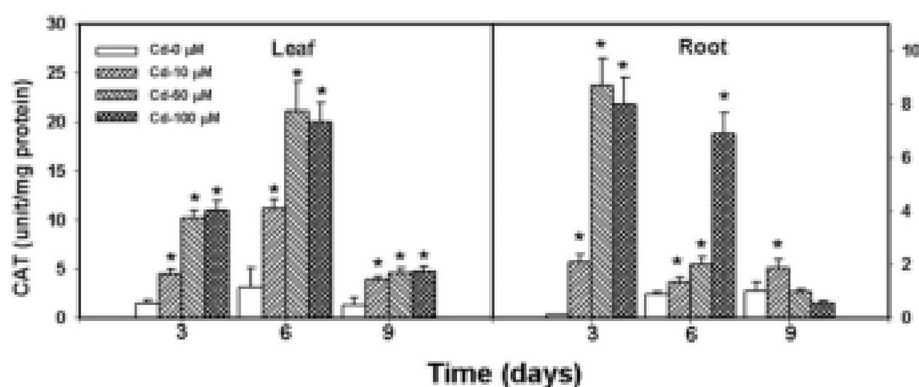

Fig. 3. Activity of CAT in leaves and roots of tomato seedlings exposed to Cd for 9 days. Values are means±SE of three independent replicates. Significant at the 0.05 levels compared with non-treated control according to *F*-test.

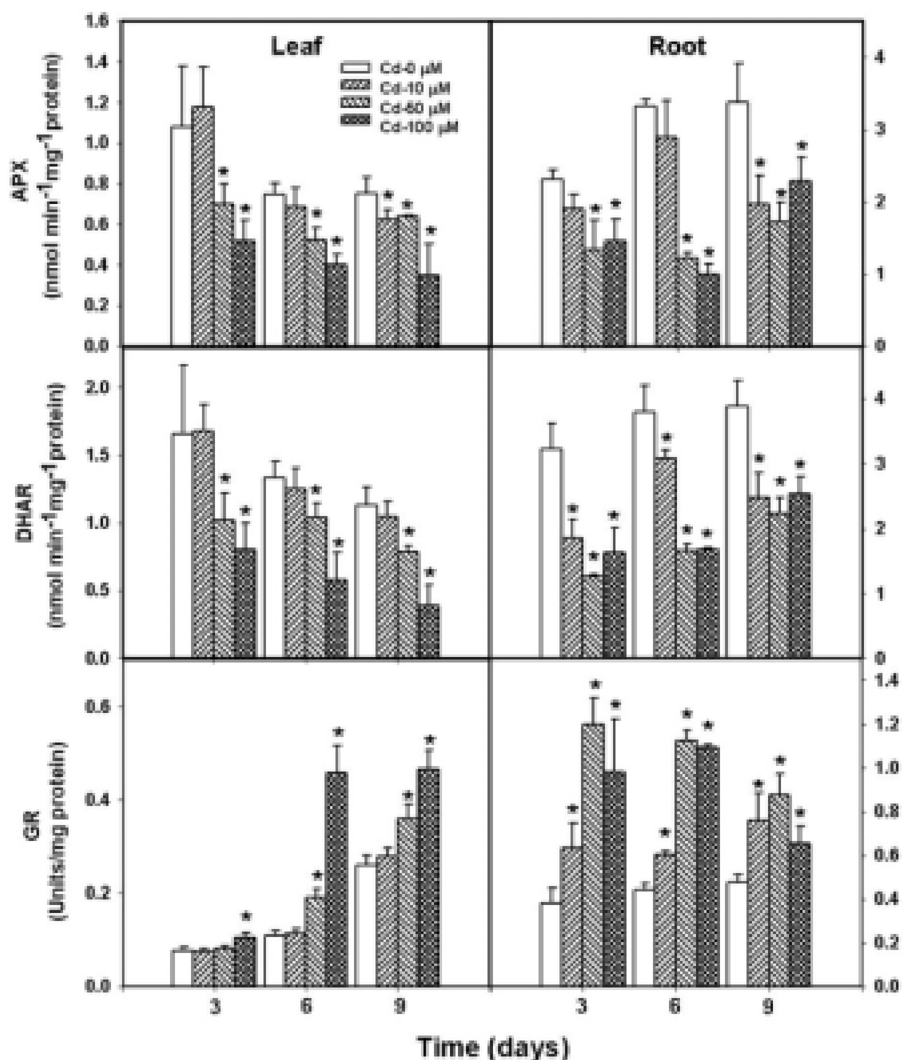

Fig. 4. Activities of antioxidative enzymes involved in glutathione-ascorbate cycle in leaves and roots of tomato seedlings exposed to Cd for 9 days. Values are means±SE of three independent replicates. Significant at the 0.05 levels compared with non-treated control according to *F*-test.

for the activity of DHAR.

The activities of both APX and DHAR in leaves and roots decreased significantly with Cd exposure. Meanwhile, the activity of GR increased significantly with over 50 μM Cd in leaves and with over 10 μM Cd in roots, showing more sensitive and higher activity in roots than in leaves.

The observed increases in GR activity in both leaves and roots appeared to be due to the levels of glutathione and the demand of PC synthesis. GR in plastid and cytosol is a highly specific enzyme that utilizes NADPH to reduce GSSG to 2 molecules of GSH (Schaedle 1977) and can maintain a high level of GSH, and by this means plays an important role in the regulation of cell metabolism (Williams 1976). Over-expression of GR in the plastids showed enhanced Cd tolerance in terms of chlorosis and chlorophyll fluorescence parameters, and the lower Cd stress in the transgenic plants could be from the result of increased glutathione synthesis and subsequent increase of thiols for metal binding in roots and reduced Cd translocation in shoots (Pilon-Smits *et al*. 2000). However, the results obtained in the present experiment showed that glutathione should not be used only for the metal detoxification because of H_2O_2 accumulation.

Since DHAR depends on GSH as an electron donor, it is possible that the decreases in the enzyme activity in both in roots and leaves exposed to Cd could be a response to the relative oxidation of the glutathione pool and other metabolites particularly after 6-d exposure. The low DHAR activity could decrease the activities of other enzymes such as APX in the glutathione-dependent H_2O_2 quenching pathway and then H_2O_2 accumulation occur.

The relationship between H_2O_2 and antioxidant enzyme system in Cd stress has not been explained clearly. In Scots Pine roots (Schutzendubel *et al*. 2001), Cd exposure caused H_2O_2 accumulation in tissues but the accumulation was not parallel to the changes of the systems involved in H_2O_2 removal. In barley seedlings (Hegedus *et al*. 2001), the role of APX instead of CAT was critical in oxidative stress. In bean (Chaoui *et al*. 1997), oxidative stress was mainly induced by the decrease of CAT and glutathione-ascorbate cycle could be activated in leaves. The increased activity of CAT (Fig. 3) in spite of the high H_2O_2 content (Fig. 1) showed that the alteration of glutathione-ascorbate cycle could be the primary reason of H_2O_2 accumulation. The high activities of APX and GR in response to Cd exposure could be associated with Cd tolerance in *Phragmites australis* (Iannelli *et al*. 2002).

Results obtained in this study suggest that H_2O_2 accumulation is a major reason of phytotoxicity of Cd on tomato seedlings, and the H_2O_2 accumulation can be resulted from the inefficient quenching by the altered activities of antioxidant enzymes particularly involved in glutathione-ascorbate pathway.

The decreased hydrogen peroxide scavenging capacity provided by reduced activities of antioxidant enzymes related to ascorbate-glutathione pathway could be a critical factor to induce Cd-induced phytotoxicity. However, further researches are required for the analyses of redox status of glutathione and ascorbate and the developmental changes of PCs to confirm the role of glutathione-ascorbate pathway in Cd-induced phytotoxicity.

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