African Journal of Biotechnology Vol. 10(15), pp. 2936-2943, 11 April, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1230
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Cadmium accumulation and antioxidative defenses in leaves of *Triticum aestivum* L. and *Zea mays* L.

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Accepted 23 February, 2011

Corn (*Zea Mays L.*) and wheat (*Triticum aestivum L.*) seedlings were grown in four cadmium (Cd) concentration levels (0 - 1 mg/l) in a hydroponic system to analyze the antioxidant enzyme system, Cd concentration in the shoots and roots of plants, proline contents, growth responses and chlorophyll contents in the leaves of corn and wheat. The results showed that there was a significant increase in malondialdehyde (MDA) concentration from 0 to 1 mg/l, and peroxidase (POD) and catalase (CAT) activities in the corn and wheat subjected to 0 – 1 mg/l of Cd. However, there was a significant decrease in the superoxide dismutase (SOD) activities in the corn and wheat subjected to 0 - 1 mg/l of Cd. This indicated that Cd stress induced an oxidative stress response in corn and wheat seedlings, characterized by an accumulation of MDA, decrease in the activities of SOD and increase in the activities of POD and CAT. Root and leaf Cd concentrations of corn and wheat increased with their exposure to the Cd level,s and the highest Cd concentration occurred in roots, followed by the leaves. An increase in proline in the leaves of corn and wheat seedlings exposed to Cd occurred as well as a decrease in chlorophyll (CHL) contents and shoots and roots biomass. Thus, Cd levels negatively affected the corn and wheat seedlings growth.

Key words: Cadmium, corn (*Zea mays* L.), wheat (*Triticum aestivum* L.).

INTRODUCTION

With the development of modern industry and agriculture, Cadmium (Cd) has become one of the most harmful and widespread pollutants in agricultural soils, and soil-plant-environment system mainly due to anthropogenic activeties, such as industrial emission, the application of Cd-containing sewage sludge, phosphate fertilizers and municipal waste disposal (Wu et al., 2005, Lima et al., 2006). Although not essential for plant growth, Cd is readily taken up by roots and translocated into leaves in many plant species (Zhou and Qiu, 2005). Moreover, Cd uptake and accumulation in plants poses a serious health issue to humans through the food chain (Shah and Dubey, 1998). Thus, the Cd pollution is of growing concern because it has turned into a potential agricultural, environmental and health issue worldwide.

Cd is easily translocated from plant roots to above ground tissues (Zhou and Qiu, 2005), and potentially threatens human health. Cd in plants interferes with physiological processes: decrease carbon assimilation (Krupa and Baszynski, 1995), induce stomatal closure and disturb plant water status (Perfus-Barbeoch et al., 2002), inhibit chlorophyll synthesis (Chien et al., 2001), damage root tips, reduce nutrient uptake, impair photosynthesis, inhibit plant growth (Das et al., 1997) and generate oxidative stress (Pietrini et al., 2003).

Cd toxicity causes oxidative stress, which can take place possibly by generating reactive oxygen species (ROS) such as superoxide radicals (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) (Romero-Puertas et al., 2002). These oxygen species cause lipid peroxidation, which is reflected by increased malondialdehyde (MDA) concentration (Chaoui et al., 1997). To scavenge ROS and avoid oxidative damage, plants possess the antioxidative enzymes superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), glutathione peroxidase, ascorbate peroxidase and glutathione reductase, as well as non enzyme antioxidants such as ascorbic acid and glutathione (Kanazawa et al., 2000). Superoxide anion radicals produced in different cell compartments are rapidly converted into H_2O_2 in a reaction catalyzed by SOD (Noctor and Foyer, 1998). Catalases are synthesized in a tissue specific and age dependent manner and scavenge H_2O_2 generated during the

photorespiration and β-oxidation of fatty acids (Lin and Kao, 2000). As a very important enzyme for plant respiration, POD can participate in lignin biosynthesis and convert H_2O_2 to H_2O (Asada, 1994). Regulation of antioxidative enzymes can provide plants with an additional protective ability against oxidative stress (Sun et al., 2007). Therefore, it is necessary to determine the change in Cd-induced oxidant stress and antioxidant enzymatic system in order to ascertain plants resistance mechanisms to Cd stress.

Proline accumulation is not only regarded as an indicator of environmental stress but also considered as an important protective role against heavy metal stress (Alia-Saradhi, 1991; Sharma et al., 1998). Cd stress leads to protein degradation through amino acid metabolism resulting in decreased plant growth (Dinakar et al., 2008). Proline levels increase with Cd stress and osmotic adjustment is associated with increase in proline (Rai and Raizada, 1988). The free proline has been found to chelate Cd ion in plants and form a non toxic Cd-proline complex (Sharma et al., 1998). The cumulative capacity of free proline is a manifestation of the self-protection ability of plants exposed to different metal stresses (Sun et al., 2007).

The crop gains significance due to its high commercial and nutrient value and in addition it is also used as fodder especially in some farmland of Shenyang, China. In certain areas of this region, it is noticed that the Cd levels are substantially high which could hamper the plant growth and yield (Sun et al., 2006). Therefore the objective of this study were to examine the effect of Cd on the alternation of physiological reactions, including changes in shoot and root growth, Cd contents in shoot and root, antioxidative enzymes (SOD, POD, and CAT), lipid peroxidation, and free proline contents occurring in leaves of corn and wheat seedlings after different concentrations of Cd exposure. Also, the possible mechanisms of the tolerance of corn and wheat seedlings to Cd stress were discussed.

MATERIALS AND METHODS

Plant growth and solution culture

The seeds were sterilized in 75% ethanol for 10 min and washed several times with sterile distilled water. The seeds were dipped in sterile distilled water and shaken at 144 r/min on an orbital shaker in sterile distilled water for 8 h. Then the seeds were germinated on filter paper moistened with distilled water in a thermostat for two days, the temperature and moisture were kept at 28°C and 60%, respectively. After one week incubation, seedlings with similar biomass were transferred to hydroponic culture under sterile conditions. Plants were grown under a controlled-environment growth chamber with a 16 h light period (light intensity of 350 µmol $m^{-2}s^{-1}$), a 25/15°C light/dark temperature regime, and 60% relative humidity. Plants were harvested after five weeks growth.

Seedlings of the plants were placed through a perforation in a plastic platform in a 450 ml plastic jar containing 400 ml solution, so that the root were immersed in liquid medium and the shoot was above the platform. The nutrition solution used was Hoagland-Arnon solution (Hoagland and Arnon, 1938), which comprised 3 mM KNO_3, 0.5 mM $NH_4H_2PO_4$, 2 mM $Ca(NO_3)_2$, 1 mM $MgSO_4.7H_2O$, 4.5 µM $MnCl_2.4H_2O$, 23 µM H_3BO_3, 0.4 µM $ZnSO_4.7H_2O$, 0.15 µM $CuSO_4.5H_2O$, 0.05 µM $H_2MoO_4.H_2O$ and 22 µM EDTA-Fe. Sterility checks were conducted in the prepared cultures simultaneously. The heavy metal salts (reagent grade) used in this study was $CdCl_2{\cdot}2.5H_2O$ (analytical purity). The salts were separately diluted in deionized water and added to hydroponic culture, respectively. Treatments were prepared at different concentrations of Cd; 0 (control), 0.01, 0.1 and 1 mg/l. All solutions were adjusted to pH 6.0 - 6.2.

The plants were grown in quarter-strength Hoagland's solution in the first week, and then cultures were changed to half-strength Hoagland's solution in the second week. During the next three weeks, the cultures were changed to full strength Hoagland's solution. After the seedlings had grown for five weeks, the seedlings were exposed for one week to the different Cd concentrations. Plants were arranged in a completely randomized design. Cultures were aerated with an aquarium air pump every 2 - 3 days, replaced with fresh solution every 3 - 4 days and supplied deionized water to maintain 400 ml in all treatments.

Plant harvest and Cd analysis

During harvest, the roots and aerials were rinsed with distilled water and shoots and roots were separated. The plant samples were oven dried at 70°C for 48 h to a constant weight, after which dry weight of shoots and roots were determined by electronic balance. After milling, 200 ± 5 mg dried plant tissue were weighed into a 30 ml porcelain crucible. The plant tissues were ashed at 500°C for 5 h in a muffle furnace and cooled down. A mixture of 5 ml HNO_3 (65%), 2 ml H_2O_2 (30%) and 2 ml purified water (Milli-Q reagent grade water) were added at room temperature. Subsequently, the sample volume was adjusted to 20 ml with deionized water and analyzed for cadmium by flame atomic absorption spectroscopy (Spectra AA220, Varian).

Assays of enzyme activity and lipid peroxidation

The 0.5 g fresh weight of leaves was homogenized in a pre-chilled mortar under ice-cold conditions in 5.0 ml 50mM cold Na-phosphate buffer (pH 7.8), with 0.1mM EDTA and 1% (w/v) poly-vinylpyrrolidone (PVP). After centrifuging at 13,000 × g for 30 min at 4°C, the supernatant was used for further analyses. The level of lipid peroxidation was determined in terms of 2-thiobarbituric acid (TBA) reactive metabolite, chiefly MDA. MDA was measured as described by Liu et al. (2004) and expressed as µmol g^{-1} fresh weight. The activity of SOD was measured as described by Krivosheeva et al. (1996). The activity of POD and CAT was determined using guaiacol and H_2O_2 substrates, respectively, as described previously (Wu and von Tiedemann, 2002; Pinhero et al., 1997).

Determination of chlorophyll and free proline

The content of chlorophyll was determined in 80% acetone extract of 0.1 g leaf (Hegedüs et al., 2001) and expressed as mg g^{-1} fresh weight. For the analysis of free proline, 0.5 g fresh weight of leaves and roots was homogenized with 5 ml of 3% sulfosalisylic acid, and the homogenate was cooled after heating for 10 min at 100°C. After centrifugation at 3000 rpm for 10 min, the content of free proline in the supernatant was measured using Ninhydrin reagent at 520 nm (Zhang et al., 1990) and expressed as µg g^{-1} fresh weight.

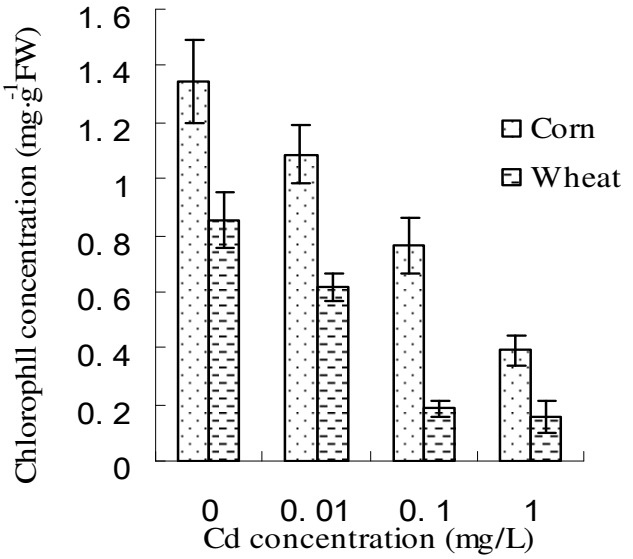

Figure 1. Effect of Cd concentrations on chlorophyll (CHL) content in leaves of corn and wheat after 7 days exposure.

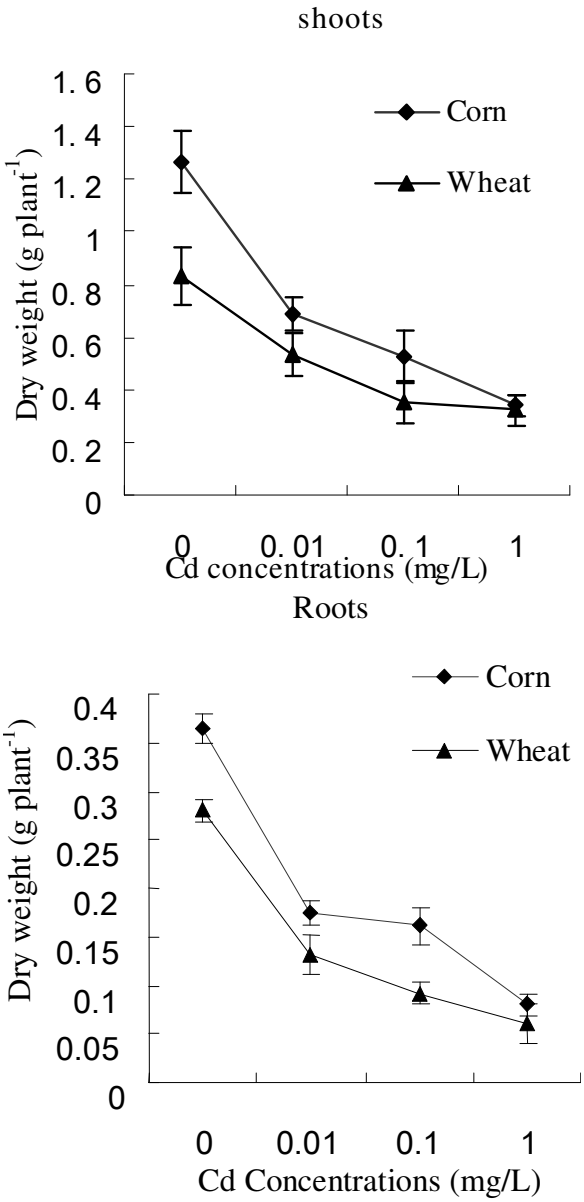

Figure 2. Effect of Cd concentrations on the biomass of shoots and roots of corn and wheat after 7 days exposure.

Statistical analysis

Controls and treatments were performed in triplicate. Data were tested for statistical significance using the software of SPSS13.0 for Windows, followed by the least significant difference (LSD) test for comparison of individual means. The difference was considered significant at the $P < 0.05$ and $P < 0.01$ levels.

RESULTS

Plant growth and Cd bioaccumulation

Chlorophyll concentrations in the youngest leaves of corn and wheat were drastically decreased with increasing Cd concentrations (Figure 1). Compared to the control, the chlorophyll concentrations in the youngest leaves of corn decreased (19.2, 43.3 and 71%), and the chlorophyll concentrations in the youngest leaves of wheat decreased (28.25, 78.17 and 81.77%), respectively with increasing Cd concentrations.

Shoot and root growth of corn and wheat were both restricted and it was strongly reduced with increasing Cd concentrations (Figure 2). The magnitude of inhibition was generally similar for both organs. Shoot and root biomasses of corn and wheat decreased with the increase in Cd concentrations in the solutions. The shoot biomasses of corn and wheat were significantly decreesed by Cd treatments at an average of 45.65 to 73.18% and an average of 36.18 to 61.3% compared with the control. The root biomasses of corn and wheat were significantly decreased by Cd treatments at an average of 52.05 to 78.08% and an average of 52.86 to 78.57% compared with the control.

The Cd accumulation in the shoots and roots of corn and wheat grown at different Cd concentrations are shown in Figure 3. The Cd content in the shoots of corn and wheat increased with the increase in Cd concentrations in the solution ($P < 0.05$), peaked and reached a maximum of 0.32 and 0.35 μmol g^{-1}DW at 1mg l^{-1} Cd, respectively. The Cd content in the roots of corn and wheat also increased with the increase in Cd concentrations in the solution ($P < 0.05$), peaked and reached a maximum of 1.25 and 1.60 μmol g^{-1} DW at 1 mgl^{-1} Cd respectively. In relation to Cd uptake, concentration in roots of corn and wheat was generally, by three or four times, higher than that in shoots particularly at the higher Cd concentrations tested, with very low translocation of

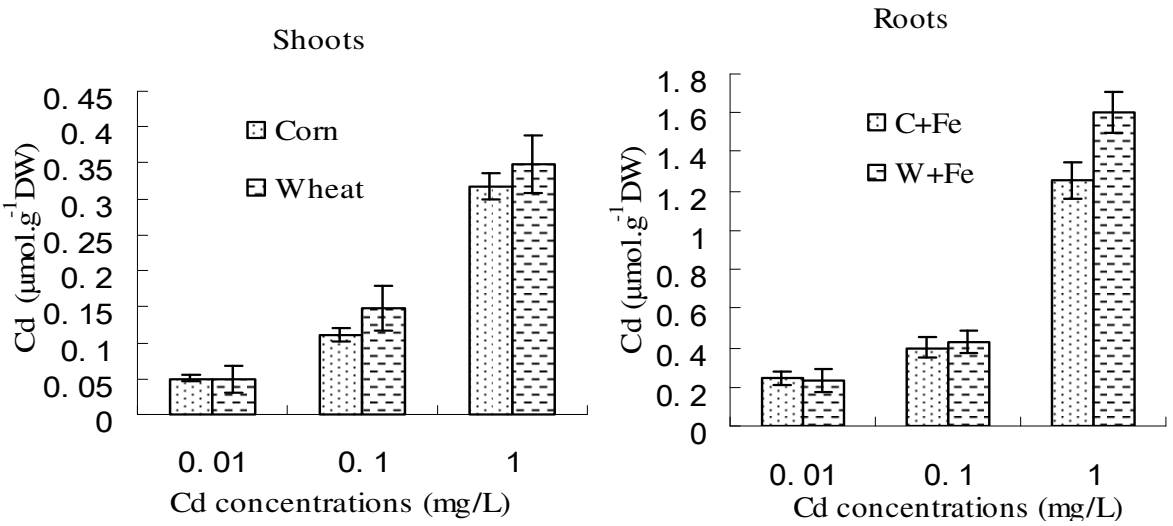

Figure 3. Effect of Cd stress on Cd accumulation in shoots and roots of corn and wheat after 7 days exposure.

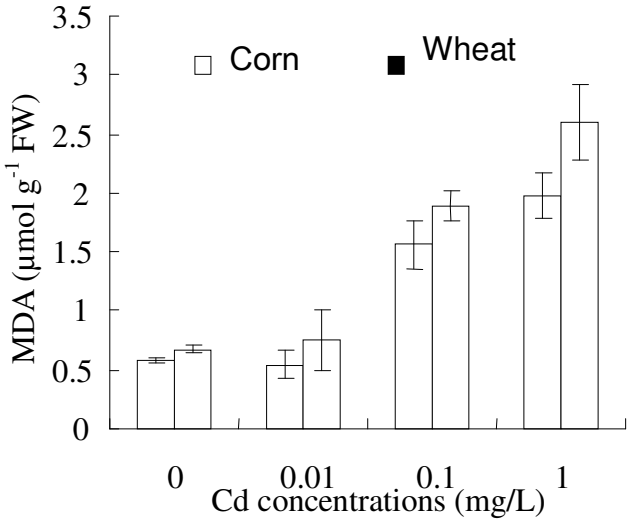

Figure 4. Effect of Cd concentrations on malondialdehyde (MDA) content in leaves of corn and wheat after 7 days exposure.

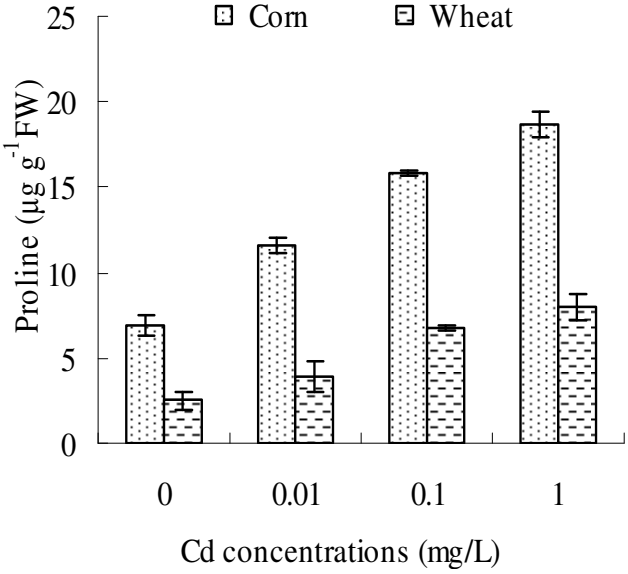

Figure 5. Effect of Cd concentrations on the proline content in the leaves of corn and wheat after 7 days exposure.

Cd to shoots.

Antioxidative responses and oxidative stress

MDA formation is considered as the general indicator of lipid peroxidation (Wang and Zhou, 2006). Exposure to Cd resulted in an accumulation of lipid peroxidation products in corn and wheat leaves (Figure 4). The MDA levels of corn and wheat tended to increase with increasing Cd concentrations, whereas there were no significant ($P > 0.05$) differences in the MDA level in leaves of corn and wheat between Cd 0.01 mg/l and the control. However, when compared with the control, the MDA levels of wheat and corn increased markedly only when the Cd levels in the solution were 0.1 mg/l and 1 mg/l, respectively ($P < 0.05$). The MDA levels of corn and wheat increased by 167.58 and 180.83% in comparison with the control at 0.01 mg/l Cd exposure and by 238.76 and 286.33% at 1mg/l.

As shown in Figure 5, the activity of SOD in the leaves of wheat and corn was significant decreased with an increase in the Cd concentration ($P < 0.05$). Compared to the control plants, the activity of SOD in wheat leaves decreased by 35.68, 61.06 and 74.07%, respectively with increasing Cd concentrations and the activity of SOD in corn leaves decreased by 21.43, 46.94 and 65.36%,

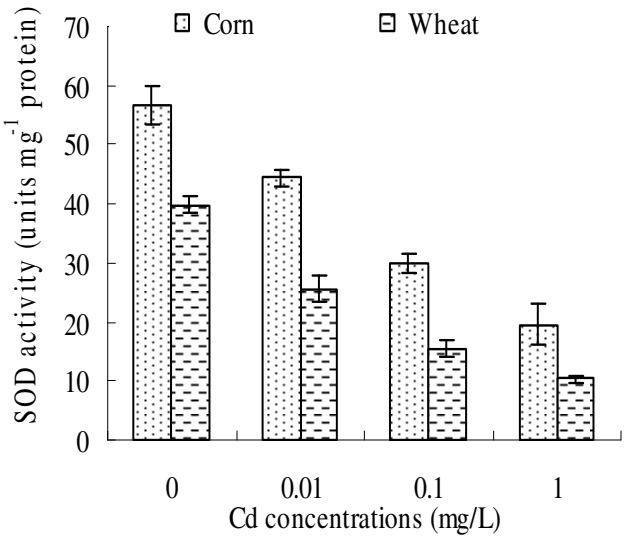

Figure 6. Effect of Cd concentrations on the activity of superoxide dismutase (SOD) in the leaves of corn and wheat after 7 days exposure.

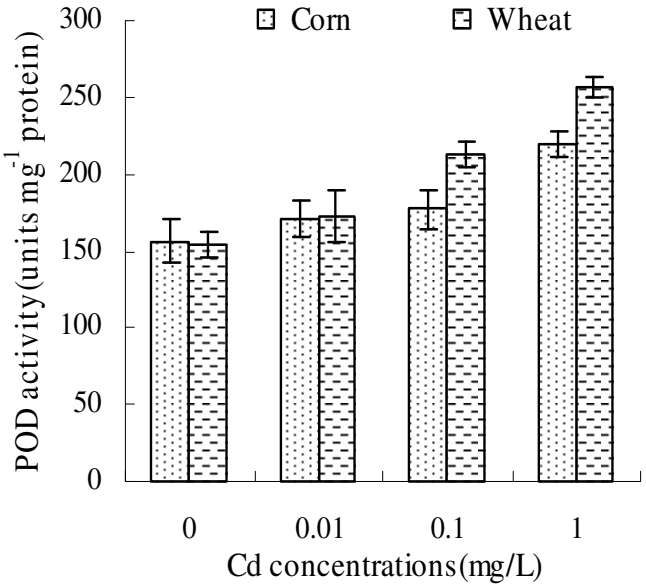

Figure 7. Effect of Cd concentrations on the activity of peroxidase (POD) in the leaves of corn and wheat after 7 days exposure.

respectively with increasing Cd concentrations. Changes of SOD activity were significant with increasing Cd concentrations in the solutions for wheat and corn. The SOD activities of wheat and corn reached the minimum at the level of 1 mg/l of Cd and all the changes were significant when compared with the control ($P < 0.05$).

The POD activity of wheat and corn decreased gradually with the different treatments of Cd, but the changes were not always significant ($P < 0.05$) (Figure 6). Compared with the control, the activity of POD in wheat leaves decreased by 17.09, 32.66 and 39.68%, respecttively with increasing Cd concentrations and the activity of POD in corn leaves decreased by 19.31, 22.21 and 28.81%, respectively with increasing Cd concentrations.

Changes in the activity of CAT in wheat and corn are shown in Figure 7. The activity of CAT in wheat and corn leaves was significantly increased with an increase in Cd concentration ($P < 0.05$). In contrast, the CAT activity in leaves of wheat and corn was significantly enhanced by Cd treatments at an average of 38.09 – 91.81% and an average of 83.15 – 261.09% respectively compared with the control. On the whole, the application of Cd increased the CAT activity in leaves of corn and wheat.

Accumulation of free proline

The stress indicator amino acid proline content increased with the increase in the concentration of cadmium. Compared to the control plants, the content of proline in wheat leaves increased by 59.2, 172 and 220%, respecttively and the content of proline in corn leaves increased by 65.71, 125.71 and 167.14%, respectively with increasing Cd concentrations ($P < 0.05$) (Figure 8).

DISCUSSION

It is well known that Cd inhibits plant growth. This is consistent with the results presented in this study (Figure 2). A concentration of 50 mM Cd resulted in more than 50% growth inhibition in poplar shoots after 48 h (Schützendübel et al., 2002). Furthermore, small and similar retardation of shoot and root growth for corn and wheat seedlings at higher concentrations (0.1 and 1mg/l) of Cd were found in the study. All these results indicated that corn and wheat seedlings were inhibited by the different Cd concentrations. Cd distribution in plant tissues indicate a change in chemical form resulting from complexation with plant-produced ligands. Most of Cd transportation into plants was chelated by chemical compounds such as phytochelatins and organic acids. Therefore, in plants which are not hyperaccumulators of Cd, roots have been shown previously to be the major site of phytochelatin synthesis and hence Cd accumulation (Di Cagno et al., 1999; Zhu et al., 1999). It also was shown that Cd was accumulated in the roots of corn and wheat seedlings to a much higher concentration than in the leaves after Cd application (Figure 3). Similar results have been found in soybean in which about 98% of the accumulated Cd was retained by roots (Bechern and Hofner, 1994).

Cd causes many morphological, physiological and biochemical changes in growing plants. Decrease in the chlorophyll is the primary bioindicator of Cd phytotoxicity. In this study, the total chlorophyll of corn and wheat were decreased with increase in Cd concentrations. The inhibition of chlorophyll content in *Phragmites australis* (Pietrini

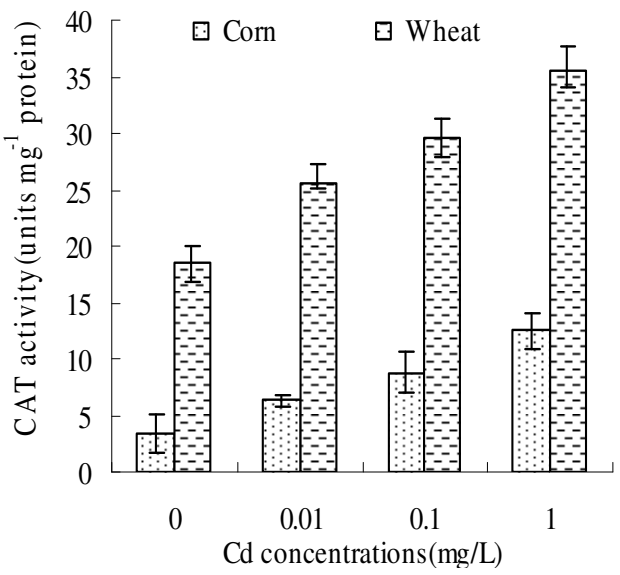

Figure 8. Effect of Cd concentrations on the activity of catalases (CAT) in the leaves of corn and wheat after 7 days exposure.

et al., 2003) and *Vigna mungo* (Singh et al., 2008) leaves exposed to Cd stress were also reported. Chien et al. (2001) observed that higher levels of Cd decreased the chlorophyll content of the leaves of rice plant. Cd can alter both chlorophyll biosynthesis by inhibition of protochlorophyllide reductase and the photosynthetic electron transport by inhibition of the water-splitting enzyme located at the oxidizing site of photosystem II (Baycu et al., 2003). It has been suggested that Cd interferes with chlorophyll biosynthesis and the reduction of chlorophyll content could in turn lead to a decrease in shoot length and biomass at least in part (Orcutt and Nilsen, 2000). Cd-induced growth inhibition in pea which indicated that photosynthesis was affected by the presence of Cd (Sandalio et al., 2001). Lannelli et al. (2002) also demonstrated that long-term exposure to Cd stress induces antioxidant responses in all plant organs, causing direct or indirect formation of reactive oxygen species that interfere with the redox status and significantly reduce chlorophyll content. Additional studies have revealed that Cd antagonizes the uptake and transport of essential elements such as Cu and Zn and irreversibly replaces them in enzyme reactions needed in RNA, DNA, and protein metabolism (Orcutt and Nilsen, 2000).

In the study, corn and wheat seedlings exposed to 0.01mg/l Cd showed no significant ($P > 0.05$) differences in the MDA level in leaves compared to the control plants. However, at higher Cd levels, a significant increase in MDA concentration was observed in leaves of corn and wheat seedlings. A variety of abiotic stresses including heavy metals may cause molecular damage to plant cells either directly or indirectly through the formation of AOS (Greger et al., 1995; Lin and Kao, 2000). Enhancement of O_2^- can produce the hydroperoxyl radical ($^{\cdot}OH$, H_2O_2), which in turn convert fatty acids to toxic lipid peroxides, destroying biological membranes.

Measurement of MDA level is routinely used as an index of lipid peroxidation under stress conditions. Increased MDA in 0.1 or 1 mg/l Cd treatments in leaves of corn and wheat seedlings suggests that higher Cd levels stimulate lipid peroxidation, resulting in irreversible damage to tissue development and function.

Accumulations of Cd in corn and wheat seedlings were accompanied by concomitant induction in the levels of antioxidants (proline) and activities of antioxidative enzymes such as SOD, POD, and CAT. In this study, a significant increase in proline level was observed with increasing Cd treatments (Figure 5). Proline can play an important protective role against heavy metal stress. Free proline accumulation under heavy metal exposure seems to be widespread among plants (Costa and Morel, 1994). The cumulative capacity of free proline is a manifestation of the self-protective ability of plants exposed to different metal (Sun et al., 2007). Dinakar et al. (2008) also reported that Cd treated plant tissues showed a significant increase in proline as compared to the control. Balestrasse et al. (2005) reported that proline levels increased in the roots of soybean plants with Cd stress. It has been suggested that proline accumulation in plants under Cd stress is due to the decrease of the plant water potential and the functional significance of this accumulation could be related to the water balance (Schat et al., 1997). It has been demonstrated that free proline could chelate with Cd ion in plants and form a nontoxic Cd-proline complex (Sharma et al., 1998). Data from literatures indicated that the important role of proline in response of plants to heavy metal toxicity may also be related to its antioxidative properties (Matysik et al., 2002; Siripornadulsil et al., 2002), its function as metal chelator (Sharma et al., 1998) and its ability to protect enzymes from inhibition (Maheshwari and Dubey, 2007).

Antioxidant enzymes and certain metabolites play an important role in adaptation and ultimate survival of plants during periods of stress (Dinakar et al., 2008). Foyer et al., (1994) reported that activities of antioxidative enzymes were inducible by oxidative stress, which reflected a general strategy required to overcome stress.

SOD is one of the stress-resistant enzymes and can catalyze the disproportionation of two O_2^- radicals to H_2O_2 and O_2. H_2O_2 is also toxic to plant cells and CAT could eliminate H_2O_2 by breaking it down directly to form water and oxygen. Therefore, the combination of SOD and CAT plays an important role in the resistance of a plant to environmental stress (Sun et al., 2007). In this study, exposure of corn and wheat to Cd resulted in a decline in SOD activity. The decline in SOD activity in leaves of corn and wheat after Cd exposure especially at Cd 1 mg/l treatment indicated that the scavenging function of SOD was impaired with severe Cd stress. Simultaneously, the increased CAT activity was observed in the leaves of

corn and wheat with increasing Cd concentration. In addition, compare to the CAT, Cd treatments inhibited SOD activity in the leaves of corn and wheat. It was suggested that Cd-induced reduction of SOD could be responsible for an inactivation of the enzyme by H_2O_2 produced in different compartments, where SOD catalyses the disproportionation of superoxide radicals (Vitoria et al., 2001).

POD can catalyze $H_2O_2^-$ dependent oxidation of substrates and can thus take part in improving the mechanical protection in plant tissues (Dong et al., 2006). Corn and wheat were able to maintain high POD activity for detoxifying active oxygen under higher Cd treatments, compared with the control plants (Figure 7). The enhancement of POD activity could have resulted from either ionic microenvironment or tissue specific gene expression in plants. Moreover, POD participating in lignin biosynthesis could build up a physical barrier against toxic heavy metals (Hegedüs et al., 2001; Dong et al., 2006). Therefore, it has been suggested that the Cd treatments inhibited SOD activity in the leaves of corn and wheat, in contrast to POD and CAT. These results also suggested that the enhanced POD and CAT activeties may induce the removal of H_2O_2 when SOD in the leaves of corn and wheat is inactivated, which fits well to the earlier work by Lee and Shin (2003). These results suggest that Cd induced physiological and biochemical changes in plants. The activity of antioxidative enzymes could serve as important components of antioxidative defense mechanism against oxidative injury.

Conclusion

Taken together, the results suggest that the accumulation of Cd in the roots and leaves of corn and wheat seedlings promoted the production of AOS, which was demonstrated by lipid peroxidation and the variation of the activity of the antioxidative enzymes, and that these enzymes can cooperate between each other, especially between POD and CAT, in the detoxification of AOS. In addition, application of Cd significantly increased the level of the free proline in both corn and wheat leaves. The free proline accumulation in corn and wheat leaves played an important role in Cd tolerance.

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