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Toxic Effect of Cadmium as Affected by Humic Acid on Lettuce (*Lactuca sativa* L.) and Antioxidant Changes

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ABSTRACT: We studied the binding of Cd to humic acid. The influence of a Cd-humic complex on enzyme activities (superoxide dismutases, peroxidases), availability of Cd and biomass compared with the behavior of no treatment (control). It was examined through a hydroponics system in lettuce. Treatments were: control (no additions), humic acid (0, 100 and 1000 mg L^{-1}), and Cd (0, 2 and 4 mg L^{-1}). Enzyme activities increased significantly with increasing rates of cadmium. A significant increase ($P<0.05$) in cadmium content of leaves result in a considerable decrease in the biomass ($P<0.05$). Based on these results, it can be concluded that the humic acid applied in the media led to a decrease in the metal adsorption with plant. This material can be used to reduce the availability and mobility of heavy metals in the soils, too.

Keywords: Superoxide Dismutase (SOD), Peroxides (POD), Heavy Metal, Cadmium.

INTRODUCTION

Leachability and plant uptake of cadmium (Cd), and other heavy metals depend on the mobility and availability of the metals in the soil, which in turn are affected by contents of solid and dissolved organic matter. Excellent studies and reviews on the ability of Humic Acid (HA) to affect plant micronutrient uptake due to their ability to complex metals under different environmental conditions have been published (Chen, 1996; Chen and Aviad, 1990). Likewise, a number of studies carried out using nutrient solutions or inert substrates have shown plant capacity to prevent micronutrients absorption from metal–humic complexes. In this way, Pinton *et al.* (1999) and Chen *et al.* (1999) have pointed out the participation of Fe-HA in Fe uptake mechanisms in cucumber, melon and bentgrass. Similar results had been obtained by Miravé *et al.* (1987). The application of HA or metal–HA complexes led to a significant decrease in Fe plant uptake (Garcia-Mina *et al.*, 2004). Lobartini and Orioli (1988) used other plant species, as well as by Mirave and Orioli (1989) and Barnard *et al.* (1992) in relation to Zn plant uptake from Zn–HA complexes, and by others in relation to Cu plant uptake from Cu–HA complexes (Ennis, 1962; Ennis and Brogan, 1961; Gupta, 1986; Gupta and Häni, 1980). These results were in line with those reported by other authors, in which the important role of metal–HA complex diffusion in soil in relation to metal bioavailability was also well documented (Kumar and Prasad, 1988; Bar-Ness and Chen, 1991; Pandeya *et al.*, 1998).

Studies on the effects of these humic substances on plant growth, showed improved effects on growth independent of nutrition (Chen and Aviad, 1990; Dursun *et al.*, 1999; Aydin *et al.*, 1999; Dursun *et al.*, 2002). For instance, humic substances increased dry matter yields of corn and oat seedlings (Lee and Bartlett, 1976; Albuzio *et al.*, 1994) and numbers and lengths of tobacco roots (Mylonas and Mccants, 1980). The typical growth response curves that have been reported as a result of treating plants with humic substances, showed progressively increased growth with increasing concentrations of humic substances (Chen and Aviad, 1990).

Cadmium (Cd), a heavy metal contaminant of agricultural soils enters the environment mainly from industrial processes and phosphate fertilizer application (Wagner, 1993). Cd is readily taken up by plants and quickly accumulates to toxic levels in the food chain. Compared with other heavy metal contaminants, Cd is more toxic than all the others. It has therefore become a serious problem of increasing international concern, and a number of strategies have been proposed for effective management of Cd contaminated agricultural soils. It has been found the order of toxicity to wheat and lettuce on acid soils to be Cd > Ni > Cu > Zn. McKenna and Chaney (1977) stressed that it was not possible to rely on the onset of visible symptoms of Cd toxicity to act as a warning because some crops have accumulated excessive amounts of Cd, which could be hazardous to health without the plants showing symptoms of stress. Kabata-Pendias and Pendias (1992) showed that Cd in potato stalks and barley grain had a steep linear relationship with Cd levels in the soil, whereas spinach leaves showed a high relationship with soil Cd levels. The same result has been shown by Alloway (1990). He found total Cd to be closely correlated with Cd content in the edible portions of cabbage, carrot, lettuce and radish grown on 50 different soils polluted from various sources.

This Cd toxicity may be ascribed to the deterioration of many physiological processes, such as the reduction in the number of chloroplasts (Sandalio *et al.*, 2001), inhibition of chlorophyll synthesis and photosynthesis and changes of the antioxidant status (Somashekaraiah *et al.*, 1992; Pankovic *et al.*, 2000). Inhibition of seed germination, reduction of growth specially roots growth, disturbances in mineral nutrition and carbohydrate metabolism (Moya *et al.*, 1993). Plant species and cultivars differ widely in their ability to absorb, accumulate and tolerate heavy metals. Davis and Calton-Smith (1980) showed that lettuce, spinach, celery and cabbage tended to accumulate relatively high concentrations of Cd. Many studies have found lettuce to be the greatest Cd accumulator among food crops. However, in lettuce, Cd contents were highest in the leaves (MacLean, 1976).

The reduction of biomass by Cd toxicity could be the direct consequences of the inhibition of chlorophyll synthesis (Padmaja *et al.*, 1990) and photosynthesis. Study on the effect of heavy metals on chlorophyll content showed that Cd, in particular, inhibited chlorophyll biosynthesis and decreased total chlorophyll content (Stobart *et al.*, 1985; Padjmaja *et al.*, 1990).

Fortunately plants possess antioxidant enzymes and antioxidant substances that protect them against damaging effects of Reactive Oxygen Species (ROS). The production of ROS is increased by stress conditions (Van Camp *et al.*, 1990; Bowler *et al.*, 1990). One of the primary effects of these molecular species and their products in cells is the peroxidation of membranes (Perl-Treves *et al.*, 1991; Scandialos 1993). Enzymatic antioxidant system, which is one of the protective mechanisms involves Superoxide Dismutase (SOD) located in various cell compartments that catalyze the disproportionate of two O^{2-} radicals to H_2O_2 and O_2. H_2O_2 is eliminated by various antioxidant enzymes such as peroxidase (POD), which converts it to water (Santandrea *et al.*, 2000). These antioxidant enzymes are important in preventing the oxidative stress in plants since their activity is generally increased in plants when exposed to stress conditions and this enhanced activity is related to increased stress tolerance (Allen, 1995; Fecht-Christoffers *et al.*, 2003; Santandrea *et al.*, 2000). Humic acid would be expected to reduce cadmium's bioavailability, and indeed most experiments run in the presence of humic acid show such a decrease (Morel, 1983; Campbell, 1995). So in the present study, we investigated the effect of humic acid on Cd uptake by lettuce and its affect on enzymes activity and biomass.

MATERIALS AND METHODS

Humic substance were extracted from humified forest soil sampled from Mount Jinyun, Chongqing, China, using the classical fractionation procedure for humic substances including extraction, acidification, separation, and purification. [10–21] The separated Humic Acid (HA) were re-dissolved in deionized water by dropwise addition of NaOH solution, then adjusted to around pH 6.0 with dilute HNO_3 and NaOH. The final HA solution have organic carbon concentration of 3.01 mg mL^{-1} and 9.34 $mgmL^{-1}$, respectively.

Culture Conditions and Treatment

Seedlings were produced in a mixture of vermiculate and perlite (2/1 V/V). The plants were transplanted in peat/perlite (1/1 V/V) culture substrate. When the plants reached the 4 to 5 leaves-stage, they were transplanted to the greenhouse with 25°C temperature. Cadmium was added into the Hogland nutrient solution as Cd Cl_2 to obtain two Cd levels; 0 (control, Cd-1), 2 mg L^{-1} (Cd-2) and 4 mg L^{-1} (Cd-3). The pH of the nutrient solution was adjusted to 6 with NaOH or HCl. The experiment was arranged as a Completely Randomized (CRD) design with 7 replicates.

Sampling and Measurement

The second fully expanded leaves were selected for enzymatic analysis. After washing thoroughly with deionised water, they were homogenized with a mortar and pestle under chilled conditions in 50 mM phosphate buffer (Zhang, 1992). The homogenate was centrifuged at 12000 rpm for 10 min at 4°C and the supernatants were used for enzyme assays.

Enzymes Assay

Superoxide Dismutase (SOD) activity was assayed according to Beauchamp and Fridovich (1971) with some modification: the samples (0.5 g) were homogenized in 5 mL extraction buffer consisting of 50 mM phosphate (pH 7.8), 0.1% (w/v) BSA, 0.1% (w/v) ascorbate, 0.05% (w/v) mercaptoethanol. The assay mixture in 3 mL contained 50 m*M* phosphate buffer (pH 7.8), 9.9 mM L-methionine, 57 μM NBT, 0.025% (w/v) Triton X-100, and 0.0044 (w/v) riboflavin. The photo-reduction of NBT (formation of purple formazan) was measured at 560 nm. One unit of SOD activity was defined as that being present in the volume of extracts that caused inhibition of the photo-reduction of NBT by 50%.

Peroxides activity was measured using the method of Chandlee and Scandalios (1984) with some modification. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 6.1), 1% guaiacol (w/v), 0.4% H_2O_2 (v/v) and enzyme extract. The absorbance increase due to oxidation of guaiacol at 470 nm was measured. Enzyme activity was calculated as μM of guaiacol oxidized min^{-1} (g fresh weight)$^{-1}$ at 25 ± 2°C.

Determination of Biomass

At the end of the experiments, plants were harvested and washed in tap water. Leaves were excised from roots by using a steel blade. Then oven dried at 70°C to constant weight. Fresh and Dry weights of leaves and roots were measured.

Determination of Element Content

Cadmium and nutrients content in leaves were determined in dried material (105°C, 48 h). If plant dry matter exceeded 0.5 g, the sample was milled and 0.3 g taken for analysis. The samples were dissolved in HNO_3 (65%) according to the microwave technique. For element analysis, inductively coupled plasma emission (ICP-AES) was used.

Statistical Analysis

All data were subjected to one-way ANOVA and means were compared by Duncan Comparisons with P values of <0.05 being considered significantly different for the culture conditions and treatments.

RESULTS

Effect of Cadmium on Plant Biomass and Cadmium Uptake

A significant difference was observed in dry weight Cd treated plants. Dry weight of lettuce leaves decreased significantly with addition of Cd to the nutrient solution (Figure 1). However, we did not observe significant differences in dry root weight. The negative correlation between weight of leaves and Cd levels was observed ($R^2 = 0.99$, $Y = -0.0588X + 6.77$). The dry root weight and Cd levels had the same trend with $R^2 = 0.98$. Plant Cd concentrations and leaves fresh and dry is shown in Table 1. The uptake of Cd increased as its concentration in the growth medium increased. The leaves had taken up 0.7 and 54.8 ppb Cd in 0 and 4 mg L^{-1} external cadmium concentrations respectively.

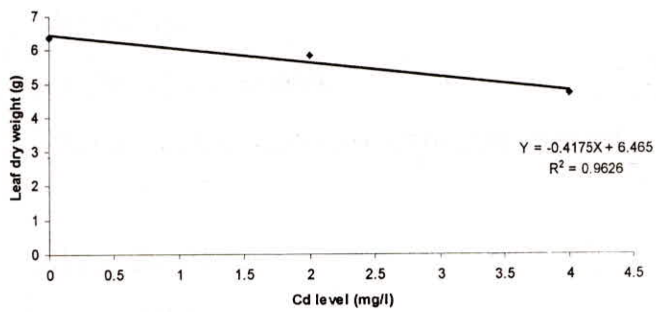

Fig. 1: The correlation between different Cd levels in nutrient solution and dry weight in lettuce leaves plants

Table 1: The effect of cadmium concentration in nutrient solution on the activity of Superoxide Dismutases (SOD), peroxidases (POD), fresh weight and cadmium content in lettuce leaves

Cd Level (mg L^{-1})	*SOD Activity (U g^{-1} W)*	*POD Activity (U g^{-1} W)*	*Fresh Weight of Leaves (g)*	*Cd Content in Leaves (ppb)*
0	156.56 c	5.58 b	96.38 a	0.7 c
2	259.9 b	6.03 b	78.01 b	35.31 b
4	324.07a	8.59 a	55.28 c	54.8 a

†Within each column values followed by the same letter are not significantly different at the 5% level of DMRT

Comparison of the total amount of Cd in the plant and total plant biomass (fresh weight) of all treatments revealed high negative relation with $R^2 = 0.92$. Fresh leaf weight in lettuce decreased significantly with the addition of Cd to the nutrient solution. It was 18% and 42% lower than control in 2 and 4 mg L^{-1} nutrient Cd concentration. The uptake of Cd had high impact on the growth of the plants, from which it is inferred that the amount of Cd in the growth medium was, as expected, toxic.

Effects of Cadmium on SOD and POD Activity

SOD and POD activity in lettuce leaves increased significantly with the addition of Cd to the nutrient solution (Table 1). On average SOD and POD activity in Cd-treated plants increased by 86% and 25% respectively in leaves, compared with the control (non-Cd). SOD activity increased sharply by 259.9 and 324.07 U g^{-1} W while POD activity increased by 6.03 and 8.59 in 2 mg L^{-1}, and 4 mg L^{-1} respectively.

Effete of Humic Acid Plant Biomass and Cadmium Uptake

The effect of humic acid on lettuce biomass and cadmium uptake was shown in Table 2. Significant differences in fresh weight were observed. The fresh weight increased 27% and 13% with 100 and 1000 mg L^{-1} humic acid compared with the control (non-HA) respectively. The leaf Cd concentration when humic acid was used in nutrient solution is very low and not significantly different in humic acid concentrations.

Effete of Humic Acid SOD and POD Activity

SOD and POD activity in lettuce leaves did not change significantly with the addition of HA to the nutrient solution (Table 2). However their activity was lower than cadmium treated plants.

Interaction of Cadmium and Humic Acid on Leaf Cadmium Content and SOD and POD Activity

Humic acid has been very successful in lowering anti-oxidant activity of the measured Enzymes (Figures 4 and 2). The highest activity of POD and SOD was encouraged with 2 and 4 mg L^{-1} cadmium in nutrient solution. Humic acid at 100 mg L^{-1} concentration is more effective on adjustified SOD activity than 1000 mg L^{-1} but no clear differences was observed for POD activity.

Table 2: The effect of humic acid concentration on the activity of superoxide dismutases (SOD), peroxidases (POD), fresh weight and cadmium content in lettuce leaves

Humic Acid Level (mg L^{-1})	*SOD Activity (U g^{-1} W)*	*POD Activity (U g^{-1} W)*	*Leaf Fresh Weight (g)*	*Leaf Cd Content (ppb)*
0	156.56 a	5.58 a	96.38 b	0.7 a
100	137.53 ab	4.10 a	122.39 a	0.59 a
1000	182.03 a	4.04 a	109.23 a	0.95 a

†Within each column values followed by the same letter are not significantly different at the 5% level of DMRT

Humic acid at 100 mg L^{-1} concentration decreased the leaf cadmium content of Cd treated plants (2 and 4 mg L^{-1} nutrient solution) from 35.31 and 54.80 ppb to 21.46 and 32.26 ppb respectively. Higher concentration of HA (1000 mg L^{-1}) did not decreased leaf Cd content of Cd treated plants significantly. Therefore HA shows a good potential for preventing Cd accumulation in lettuce leaves.

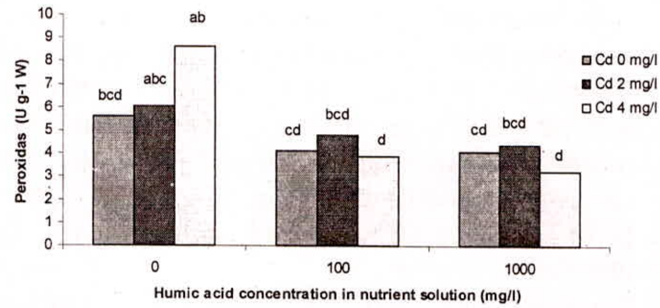

Fig. 2: Interaction between humic acid and cadmium on the activity of peroxidase in lettuce

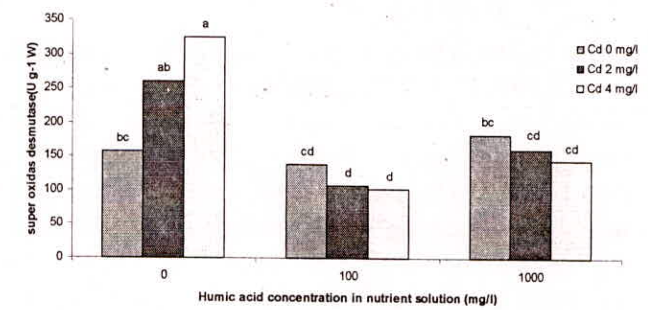

Fig. 3: Interaction between humic acid and cadmium on the activity of superoxide dismutase in lettuce

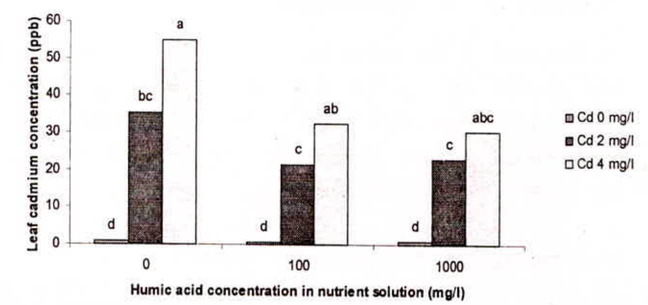

Fig. 4: Intraction between humic acid and cadmium on cadmium content in lettuce

DISCUSSIONS

The results showed that the lettuce Cd uptake was regulated by the Cd concentration in the substrate, since the amount of Cd in the plants increased as the concentration in the nutrient solution increased. Eriksson (1990) found that Cd uptake in wheat was depended on the amount of plant-available Cd in the soil, and was related to the concentration of exchangeable Cd. In lettuce we observed high correlations between the average Cd uptake by leaves and the Cd concentration in the nutrient solution $R^2 = 0.97$. The highest amounts of Cd in the leaves at 4 mg L^{-1} external Cd concentration, which was 54% more than 2 mg L^{-1}. An assumption was made that most of the dissolved Cd was present as Cd^{2+} and could be taken up by the plant. These results are in agreement with those of Andersson (1985).

The nature of Reactive Oxygen Species (ROS) makes them potentially harmful to all cellular components. Fortunately, all plants have efficient ROS scavenging systems. However, during periods of stress situations, the scavenging system may become saturated by the increased rate of radical production (Goering, 1994). Therefore, investigation of responses against different level of stress conditions on the same plant species may be important for understanding and improving defense strategies via the antioxidant enzyme activities. Cd injury to plants is mainly a consequence of altered oxidant levels, thus causing the occurrence of oxidative stress due to accumulation of Active Oxygen Species (AOS), including superoxide radical (O^{-2}), hydroxyl radical (OH^{-1}) and hydrogen peroxide (H_2O_2) (Mazhoudi *et al.*, 1997). The enzymes, Superoxide dismutase and peroxidase are more sensitive than other ones (Albertina Xavier da Rosa Corre^aa *et al.*, 2006) Superoxide dismutase is most commonly used to demonstrate the status of antioxidant capacity (Bowler *et al.*, 1994) and higher increased activity of SOD was observed than POD. The results of the current study indicated that SOD activity significantly increased correlation when plants were exposed to Cd. Dixit *et al.* (2001) reported that SOD activity showed a marked increase in leaves of the Cd treated plant, too. The response of SOD activity to Cd or other stresses is reported to be dependent on stress intensity, species and age of the plants exposed to stress (Shah *et al.*, 2001). The results of this study showed that the oxidative stress was caused by Cd uptake by the plant. Enzymes such as superoxide dismutase and peroxidase play an important role in the cellular defense strategy against oxidative stress caused by toxic heavy metal concentrations which is also supported with other results (Van Assche and Clijsters, 1990; Karataglis *et al.*, 1991; Weckx and Clijsters., 1996).

Humic acid reduced the availability of heavy metals in the soil, thereby minimizing the uptake by plants Through the formation of relatively insoluble complexes, humic acid and its inhibitory influence on the mobility of heavy metals in soil and uptake of the metals by lettuce was reported before (Wang, D.Y., *et al.*, 1997). It is known to form moderately strong complexes with humic acid. Several qualitative studies on aquatic organisms have shown that the presence of humic substances reduces Cd uptake. For example, Sedlacek *et al.* (1983) reported that Cd uptake by the green alga (*Selenastrum capricornutum)* and its toxicity were reduced in the presence of humic substances, and they attributed this decrease in bioavailability to the complexation of Cd in solution by the humic substances. Pempkowiak and Kosakowska (1998) also noted decreased Cd uptake by *Chlorella vulgaris* in the presence of marine humic substances uptake. Recently, Bunluesin *et al.* showed Cd uptake by plant tissue, toxicity symptoms and accumulation, were reduced by addition of HA (Bunluesin *et al.*, 2007). In this research Cd and HA interaction showed that HA has impressive effect on lettuce fresh and weight. As the other reports showed that humic acid can stimulate shoot and root growth, dry and fresh weight and improved resistance to environmental stress in plants (Delfine *et al.*, 2005; Hartwigsen and Evans., 2000), Because of its bonding ability by plants to Cd and preventing its absorption Studies on the positive effects of humic substances on plant growth have resulted in improved effects on growth independent of nutrition (Chen and Aviad, 1990; Dursun *et al.*, 1999; Aydin *et al.*, 1999; Dursun *et al.*, 2002). For instance, in controlled experiments, humic substances increased dry matter and yield of corn and oat seedlings (Lee and Bartlett, 1976; Albuzio *et al.*, 1994) and numbers and lengths of tobacco roots (Mylonas and Mccants, 1980). The typical growth response curves that have been reported as a result of treating plants with humic substances, show progressively increased growth with increasing concentrations of humic substances, but there was usually a decrease in growth at higher concentrations of these materials (Chen and Aviad, 1990). But the result of Mesutu (2005) is conversed with our result, may be because of different HA concentration. He used low HA concentration around 50 mg L^{-1}. Consequently, it can be inferred that the more concentration of HA has the better effect on weight.

Taken together, the results of this study showed that changes in antioxidative enzyme activity were a more sensitive indicator of effects of Cd exposure than classical (height and biomass) endpoints. However, since the accumulation of cadmium in plants does not result in obvious morphological characteristics, its hazardous nature may not be apparent. There is therefore a need for further studies to explore ways of phytoremidiation of polluted soils or developing plant species that are resistance to Cd toxicity. In conclusion, humic acid decreased lettuce cadmium content and uptake of cadmium.

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