Bull. Environ. Contam. Toxicol. (2003) 70:1036–1044

DOI: 10.1007/s00128-003-0087-0

Bulletin of Environmental Contamination and Toxicology

Effects of Zinc upon Tolerance and Heavy Metal Uptake in Alfalfa Plants (*Medicago sativa*)

J. R. Peralta-Videa,[1,2] J. L. Gardea-Torresdey,[1,3] J. Walton,[1,4] W. P. Mackay,[1,5] M. Duarte-Gardea[6]

[1] Environmental Science and Engineering, Ph.D. Program, University of Texas at El Paso, El Paso, TX 79968, USA
[2] ITESM Campus Ciudad Juárez, PMB 10482, El Paso, TX 79995, USA
[3] Chemistry Department, University of Texas at El Paso, El Paso, TX 79968, USA
[4] Civil Engineering Department, University of Texas at El Paso, El Paso, TX 79968, USA
[5] Biology Department, University of Texas at El Paso, El Paso, TX 79968, USA
[6] School of Allied Health, University of Texas at El Paso, El Paso, TX 79968, USA

Received: 28 September 2002/Accepted: 29 January 2003

Although the volume of information about single element or multi-element heavy metal hyperaccumulator plant species is abundant, the data related to the interactive effect of elements known as essentials at low doses, but toxic at higher concentrations, appear to change with the plant species. Overall, the interaction at root level among some transition elements could be antagonistic, synergistic or multiplicative. Beckett and Davis (1978) found that in barley, the toxic effect of Cu and Zn were antagonistic when the tissue concentration of these heavy metals surpassed a critical concentration. On the other hand, in vegetables grown in pots, high concentration of Zn in the soil solution was synergistic for Cd uptake (Gerritse et al. 1983). Furthermore, Ni combined with Cd, Mn, and Zn, presented a multiplicative effect in the reduction of wheat root growth (Taylor and Stadt 1990). Also, a five-year study showed that in lettuce, corn, and other plants the presence of Zn in the growing medium was antagonistic to Cd uptake (Smilde et al. 1992). Studies showed that in *Linum usitatisimum* the presence of Zn in the growing medium reduced the Cd concentration in the tissues by about 50% (Moraghan 1993). However, Luo and Rimmer (1995), found that the growth of barley was determined by the amount of bioavailable zinc in the soil; but the addition of copper increased the zinc uptake to a toxic level, which in turn, reduced the dry mass production. Previous research demonstrated that alfalfa was able to germinate and grow in solid medium individually contaminated with 0-40 mg/kg of Cd(II), Cr(VI), Cu(II), Ni(II), and Zn(II) (Peralta et al. 2001). Also, greenhouse experiments showed that alfalfa was able to germinate and grow in clay-soil contaminated with 80 mg/kg of Cd(II), Cu(II), Ni(II), or 160 mg/kg of Zn(II) (Peralta-Videa et al. 2002). Based on previous results, the present research was designed to evaluate the interactive effect of Zn (II) with Cd(II), Cu(II), and Ni(II) on alfalfa plant growth and uptake of heavy metals.

MATERIALS AND METHODS

The soil used in this investigation had a pH of 7.4 ± 0.2 and the textural analysis showed the following composition: fine sand (2.0 mm), 1.4%; very fine sand (850 μm), 1.6%; silt (425-45 μm), 93%; clay (< 45 μm), 4%. This indicated that this soil could be classified as silt soil. In order to have enough biomass for metal determination, approximately 75 seeds of alfalfa (Mesa variety) were placed in

Correspondence to: J. L. Gardea-Torresdey

each one of general-purpose plastic pots filled with 250 g of soil free of heavy metals. The seeds were previously soaked for 15 min in an antimycotic-antibiotic solution (Sigma A9909) at a concentration of 10%. At the time of planting, 25 mL of a nutrient solution previously described in the literature (Peralta et al. 2001), were added to each pot. The following salts, reagent grade, $Cd(NO_3)_2 \cdot 4H_2O$, $Cu(NO_3)_2 \cdot 2.5H_2O$, $Ni(NO_3)_2 \cdot 6H_2O$, and $Zn(NO_3)_2 \cdot 6H_2O$, were dissolved separately in deionized water and adjusted with HCl to pH 5.8. The stock solutions of Cd(II), Cu(II), and Ni(II) had a concentration of 5,000 $mg \cdot L^{-1}$. The stock solution of Zn(II) had a concentration of 1,000 $mg \cdot L^{-1}$.

In this study, at the growth stage of 15 days, a group of 12 pots (three by treatment) received 25 mL of the zinc solution described above. Previous investigation demonstrated that such amount of zinc in the soil favored the growth of alfalfa plants (Peralta-Videa et al. 2002). Five days later, a batch of three pots received 25 mL of the Cd(II), Cu(II), or Ni(II) solution and another three were kept as control treatment (no heavy metal added). The same concentration of each of the three heavy metals indicated above were also added individually to sets of three pots that did not receive the Zn(II) solution. The control treatment plants received 25 mL of deionized water at the time of the heavy metals application. All of the pots were randomly placed under a 12 h photoperiod at a photon irradiance of 19.5 $\mu mol \cdot m^{-2} s^{-1}$ and 25°C.

After two weeks, the plants were harvested and the size and mass of 10 plants per pots, selected at random, were measured (except for the Zn-Ni treatment that allowed only an average of 8 plants per pot). Subsequently, all the plants of each replicate were washed with deionized water, oven dried at 70°C for 72 h, weighed and digested. The digestion was accomplished using a microwave oven (CEM MarsX, Mathews, NC 28105-5044) at 110°C for 15 minutes with 10 mL of concentrated HNO_3, trace pure. Then, the samples were diluted with D.I. water in a 1:4 ratio in order to be analyzed with a Perkin-Elmer Optima 4300 Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES). The data were analyzed with analysis of variance (ANOVA) and the statistical significance of the differences between treatment means was determined through the least significant difference test (LSD). A sample of approximately one gram of dry soil was taken from each treatment-pot in order to determine the amount of elements present in the water-soluble fraction, as well as the final pH.

RESULTS AND DISCUSSION

Table 1 shows the growth characteristics of alfalfa plants exposed individually, at the age of 20 days, to 500 $mg \cdot kg^{-1}$ of Cd(II), Cu(II), or Ni(II). Table 1 also shows the effects of these heavy metals upon alfalfa plants that received 25 mL of the solution of 1000 $mg \cdot L^{-1}$ of Zn(II) five days before the application of the indicated heavy metals. As one can see in Table 1, the previous addition to the soil of the Zn(II) solution five days before the Ni(II) application, had a beneficial effect on the alfalfa plants. Ten percent of the plants treated by the Zn(II)-Ni(II)-treatment survived. There were no statistical differences among the size of the Zn(II)-Ni(II) treated plants and the size of the control treatment plants. The surviving plants developed significantly more biomass ($P < 0.01$) than the control or the Cd(II)-treated plants. It is clear that the presence of Zn(II) in the growing environment reduced the Ni(II) toxicity to the alfalfa plants. Yuji and Kazuo (1979) reported that in rice plants, the presence of zinc reduced the toxicity of nickel. Table 1 also shows that when Cd(II) was applied five days after the application of Zn(II), only 30% of the alfalfa plants survived to the Zn(II)-Cd(II) treatment. Comparing this result with the effect of Cd(II) applied alone (90% survival rate), and the results observed in the plant exposed to 100 mg/kg of Zn(II) (that showed a 100 percent survival rate), there was evidence of an additive or synergistic effect among these metals upon the alfalfa plants. However, the plants exposed to single Cd(II), or to the combination of 100 mg/kg Zn(II) followed by 500 mg/kg of Cd(II), grew significantly more as compared to the other treatments ($P < 0.05$). Furthermore, the surviving plants had the same size and developed similar biomass as those plants stressed by the single Cd(II)-treatment. These results were different from those reported by Sharma et al. (1999) who found that Zn/Cd exerted an antagonistic effect with respect to root growth in *Silene vulgaris*. Also, cadmium and zinc showed a synergistic interaction in the reduction of growth on *Phaseolus vulgaris* cultivated in hydroponics (Chaoui et al. 1997).

In the present investigation the single Cu(II)-treatment reduced the original population of alfalfa plants by 24%. However, when the treatment of 500 mg/kg of Cu(II) was preceded by the supplement of 100 mg/kg of Zn(II) to the soil, the alfalfa plant population was reduced by 80%, even though the surviving plants grew the same and the accumulation of biomass was similar as compared to the control treatment plants (Table 1). In an experiment conducted with spring barley (Luo and Rimmer 1995) found that a treatment of 100 mg/kg of zinc and 50 mg/kg of copper did not affect the dry biomass production of this plant. Nevertheless, in this investigation the biomass development of the plants exposed to the metal mixture could camouflage an effect of plant competition. Because of that, the original data were transformed to weighed averages using the surviving population/treatment/area and the population of the control treatment as converting parameter (Table 2). In this Table, it can be observed that the mass produced by the Cd(II) or Cu(II) treatments was similar to the biomass produced by the control plants, and statistically higher than that produced by the combined metals. However, the amount of biomass produced by these treatments was statistically significant ($P < 0.05$) as compared to the biomass produced by the binary mixture of metals.

As it can be seen in Table 2, alfalfa plants could be able to remove up to 2.2 kg/ha from a soil individually contaminated with 500 mg/kg of cadmium or copper. However, under the experimental conditions of this investigation, the presence of 100 mg/kg of zinc in the soil significantly reduces the capability of alfalfa plants to remove cadmium or copper from the soil (0.51 and 0.27 kg/ha, respectively). On the other hand, the presence of 100 mg/kg of zinc may allow the alfalfa plants to remove 0.23 kg /ha of Ni from the soil.

Figure 1a shows the accumulation of Cd, Cu, and Ni, in the root tissues of alfalfa plants exposed to Cd(II) and Cu(II) solely, or after the previous application of 100 mg/kg of Zn(II) in all the three elements. In this Figure one can observe that the presence of zinc increased cadmium uptake by alfalfa roots. The root of plants subjected to the Zn(II)-Cd(II) treatment, accumulated 7,409 mg/kg of Cd, a concentration that is significantly higher ($P < 0.01$) than the amount of cadmium accumulated by the root of the plants exposed to Cd(II) alone (4,150 mg/kg). Also, the concentration of cadmium accumulated by the root of the plants exposed to the Zn(II)-Cd(II) combination was statistically significant ($P < 0.01$) compared to the quantity of nickel or copper accumulated in the roots of the plants exposed to any treatment of Cu(II) or Ni(II). However, the presence of Zn(II) increased the toxicity of Cd(II) to alfalfa plants, since only 30% of the plants survived to the combined effects Zn(II)-Cd(II) treatment. According to Nan et al. (2002), in corn and wheat, the Cd-Zn interaction was synergistic, favoring the accumulation of Zn or Cd in the two crops. In the present investigation, the plants exposed to the single Cu(II)-treatment accumulated significantly ($P < 0.01$) more Cu (2,800 mg/kg) in the roots than the plant treated with the Zn(II)-Cu(II)-treatment (578 mg/kg). This could be an expression of the competition between the two metals for binding sites within the plant. It was impossible to compare the absorption of nickel from the single nickel treatment and the combination of zinc-nickel treatment, because in the former case, all the plants died.

Figure 1b shows the concentration of heavy metals found in the shoot tissues of the alfalfa plants exposed to the treatments described above. The translocation of cadmium from the Cd(II)-single treatment was higher (1,000 mg/kg) than the translocation from the Zn(II)-Cd(II) treatment (649 mg/kg), which was opposite of what was found in the roots. Chaoui et al (1997) found that the combination of 5 μM of Cd plus 25 μM of Zn significantly increased the Cd uptake in roots and shoots in *Phaseolus vulgaris,* as compared with the cadmium uptake by the same plant from single Cd or Zn-treatment. The result found with copper presented the same spectrum as that of cadmium and was similar to the effect seen in the roots. The amount of Cu found in the shoot tissues of the plants grown with the single Cu(II)-treatment was numerically higher (810 mg/kg) than the amount of the same element found in the shoot of the plants grown with the Zn(II)-Cu(II) treatment (351 mg/kg). This result could indicate that the high level of zinc induced the production of metabolites and free radicals (Alia et al. 1995) that might sequester Cd and Cu inside the alfalfa plant tissues, reducing the translocation of both metals. Moraghan (1993) reported that in *Linum usitatissimum,* the addition of extra zinc to the soil lowered by 50% the concentration of Cd in the seeds of this plant. Furthermore, McKenna and Chaney (1997) found that the lettuce leaves with high Zn concentrations, contained a high proportion of Zn bound to high molecular weight components, as well as higher proportion of amino acids cysteine, glutamine, glycine, and asparagine, which could lower the concentration of Cd in the leave tissues.

Table 1. Root and shoot length and mass of alfalfa plants exposed individually to 500 mg/kg of Cd(II), Cu(II), and Ni(II), and to the binary mixture of 100 mg/kg of Zn(II) and 500 mg/kg of the same heavy metals*.

Metal	Root length (mm)	Shoot length (mm)	Root mass (mg)	Shoot mass (mg)	Percent survival
Ni(II)	--	--	--	--	0.0
Control (ZnII)	$38.5 \pm 8_b$	$40.3 \pm 8_a$**	$2.2 \pm 1_{bc}$	$11.0 \pm 3_{bc}$	100
Cd(II)	$45.2 \pm 7_a$	$34 \pm 6_{bc}$	$2.8 \pm 1_b$	$13.3 \pm 6_b$	90
Cu(II)	$38.2 \pm 10_b$	$37.6 \pm 7_{ab}$	$4.6 \pm 1_{ab}$	$19.7 \pm 1.5_{ab}$	76
Zn(II)-Cd(II)	$46.4 \pm 8_a$**	$35.1 \pm 7_{bc}$	$2.9 \pm 1_b$	$14.3 \pm 7_b$	30
Zn(II)-Cu(II)	$39.2 \pm 10_b$	$38.6 \pm 7_{ab}$	$4.7 \pm 0.4_{ab}$	$20.7 \pm 1.7_{ab}$	20
Zn(II)-Ni(II)	$43.6 \pm 9_{ab}$	$38.9 \pm 6_{ab}$	$7.3 \pm 3_a^{**}$	$22.8 \pm 2_a$**	10

*Results are means ± SE. (n = 10, r = 3). Means with different letters are significantly different from one another according to LSD test. Comparisons are within columns. **Significant at 0.01. The zero values were not included in the ANOVA.

Table 2. Content of Cd, Cu, or Ni in alfalfa shoot biomass (kg/ha) as affected by the plant population and the biomass accumulation.

Treatment	Shoot biomass[1] (mg/plant)	Average plants No.	Percent survival	Shoot biomass[2] (g/m^2)	Cd, Cu, or Ni in shoots (mg/kg)	Cd, Cu, or Ni in shoots (kg/ha)
Control (ZnII)	11_{bc}	70	100	202.6_a	0.0	0.0
Cd(II)	13.3_b	63	90	220.5_a	$1,000_a$	2.21_a
Cu(II)	19.7_{ab}	53	76	275.8_a	810_a	2.23_a
Ni(II)	--	0	0.0	0.0	--	0.0
Zn(II)-Cd(II)	14.3_b	21	30	79.0_b	649_a	0.51_b
Zn(II)-Cu(II)	20.7_{ab}	14	20	76.2_b	351_b	0.27_c
Zn(II)-Ni(II)	22.8_a^{**}	8	10	42.0_b	546_{ab}	0.23_c

[1]Results are average of 30 plants per treatment
[2]Shoot mass = (shoot biomass)(percent)(70)/(area)(1000). Data with different letters are significantly different from one another (P< 0.05). Plot area for all the samples were 0.0038 m^2.

Figure 1a

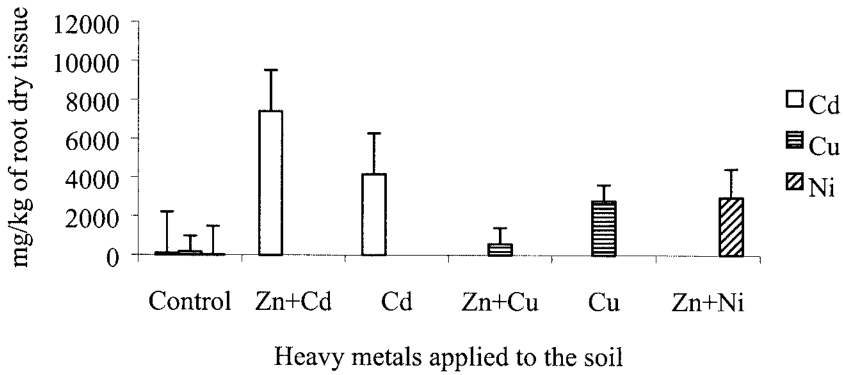

Figure 1b

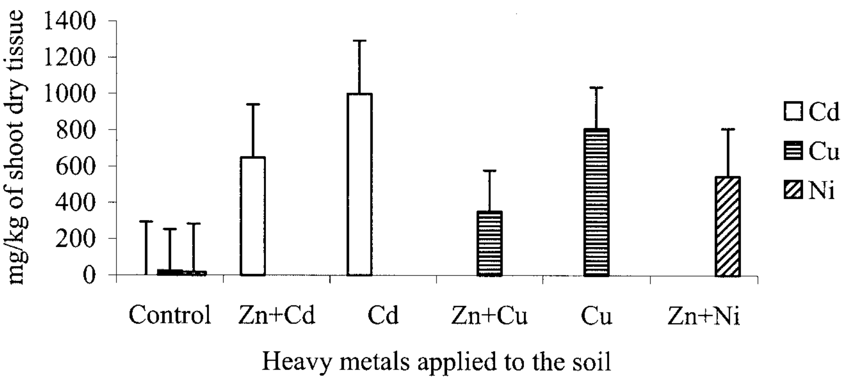

Figure 1. **(a)** Concentration of Cd, Cu, and Ni in alfalfa root tissues exposed individually to 500 mg/kg of Cd(II), Cu(II), or Ni(II), and to the combination of 100 mg/kg of Zn(II) plus 500 mg/kg of the same heavy metals. **(b)** Concentration of Cd, Cu, and Ni in alfalfa shoot tissues. Nickel alone is not shown, as the mortality was 100%. Error bars represent SE.

Figure 2a shows the concentration of Cd, Cu and Ni in the shoot tissues of alfalfa plants related to the concentration of the macroelements in the water soil-extract of Zn(II)-Cd(II), Zn(II)-Cu(II) or Zn(II)-Ni(II) combination-treatments. As one can see in the figure, the absorption of the three heavy metals mentioned above was significantly correlated to the concentration of Mg in the water extract ($P < 0.05$).

Figure 2a

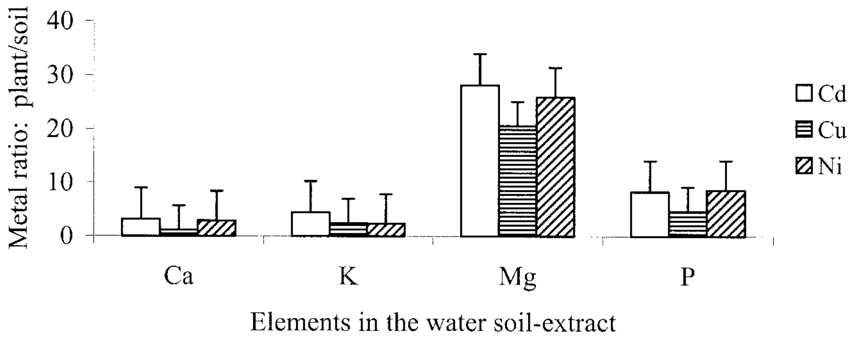

Figure 2b

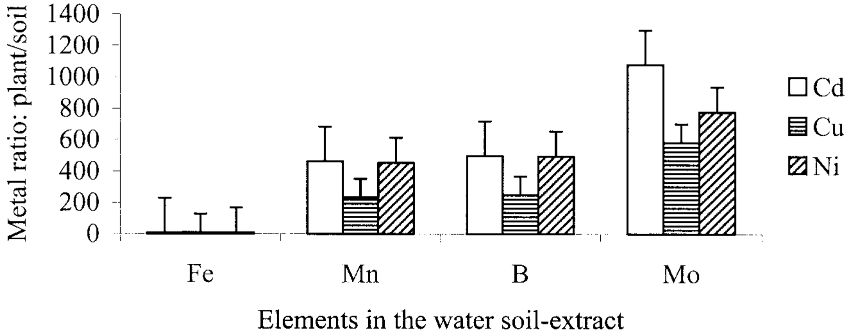

Figure 2. Concentration of Cd, Cu, and Ni in alfalfa shoot tissues related to the elemental concentration in the water-soil extract. (**a**) Ratio plant: soil for the macroelements in the respective heavy metal treatments, (**b**) ratio plant: soil for the microelements in the respective heavy metal treatment. Error bars represent SE.

Also, the concentration of Cd in the shoot tissues, related to the concentration of Ca, Mg and P in the water extract, was consistently higher as compared with the ratio found for Cu and Ni. Lin and Wu (1994) demonstrated that in *Lotus purshianus*, excessive copper concentrations cause a reduction in Ca uptake. These results meant a negative correlation among the concentration of Mg in the water extract and the translocation of the heavy metals to the alfalfa shoot tissues.

In relation to the microelement concentrations, Figure 2b shows that the ratio of Cd, Cu and Ni in the alfalfa shoot tissues/microelements in the water extract of the respective heavy metals combination was significantly correlated to Mo ($P< 0.05$), as compared to B, Fe or Mn. Also, in all the cases the ratio was higher for Cd than for Cu or Ni. In Cu-nontolerant plants of *Lotus purshianus*, an excess of Cu in soil reduced the Fe, Mn, and Zn uptake (Lin and Wu 1994). Various levels of interactions among micronutrients and heavy metals have been described in other plants such as *Triticum spp.* (Taylor and Stadt 1990) and *Phaseolus vulgaris* (Chaoui et al. 1997). Also, studies conducted with corn and wheat demonstrated that root exudates could affect significantly the absorption of cadmium and other trace elements (McLaughlin et al. 1998). The results of this greenhouse investigation showed that the uptake and translocation of Cd, Cu, and Ni was affected by the addition of 25 mL of a Zn(II) solution (1,000 mg/L) five days before the heavy metal contamination. The presence of Zn(II)-Cd(II), Zn(II)-Cu(II), and Zn(II)-Ni(II) reduced the alfalfa population by 70, 80, and 90 percent, respectively. However, the concentration of Cd in the roots of alfalfa plants stressed by the Zn(II)-Cd(II) treatment was significantly higher than the concentration of the same element in plants treated with Cd(II) solely. On the other hand, the absorption of copper by the roots of alfalfa plants exposed to the single copper treatment was significantly higher as compared to the absorption of copper from the Zn(II)-Cu(II) treatment. The treatment of 500 mg/kg of Ni(II) alone had a lethal effect to alfalfa plants. However when the same dose of Ni(II) was preceded by the addition of zinc solution, 10 percent of the plants survived. Furthermore, the concentration of Ni in the root tissues of alfalfa was comparable to the concentration of Cu and Cd found in the plants grown under the effects of 500 mg/kg of single concentrations of Cd(II) or Cu(II). It is important to point out that the results could be different if insoluble forms of these heavy metals are present in the soil. However, the outcome of these preliminary results demonstrated that Zn(II) reduced the toxic effects of Ni(II) to alfalfa plants. This information could be important for the use of alfalfa plants in the phytoremediation of nickel-contaminated soils.

Acknowledgments. We thank the National Institutes of Health (grant S06 GM8012-30), and the University of Texas at El Paso's Center for Environmental Resource Management (CERM) through funding from the Office of Exploratory Research of the U.S. Environmental Protection Agency (cooperative agreement CR-819849-01). We also acknowledge the support from the HBCU/MI Environmental Technology Consortium, which is funded by the Department of Energy.

REFERENCES

Alia KV, Prasad SK, Pardha Saradhi P (1995) Effect of zinc on free radicals and proline in *Brassica* and *Cajanus*. Phytochemistry 39: 45-47

Beckett PHT, Davis RD (1978) The additivity of toxic effects of copper, nickel, and zinc in young barley. New Phytol 8: 155-173

Chaoui A, Ghorbal MH, Ferjani EE (1997) Effects of cadmium-zinc interactions on hydroponically grown bean (*Phaseolus vulgaris* L.). Plant Sci 126: 21-28
Gerritse RG, Van Driel W, Smilde KW, Van Luit B (1983) Uptake of heavy metals by crops in relation to their concentration in the soil solution. 4th International Conference on Heavy Metals in the Environment, CEP Consult. Edinburgh, UK, pp 1057-1062
Lin S, Wu L (1994) Effects of copper concentration on mineral nutrient uptake and copper accumulation in protein of copper-tolerant and nontolerant *Lotus purshianus*. Ecotoxicol Environ Safety 29: 214-228
Luo Y, Rimmer DL (1995) Zinc-copper interaction affecting plant growth on metal-contaminated soil. Environ Pollut 88: 79-83
McKenna IM, Chaney RL (1995) Characterization of cadmium-zinc complex in lettuce leaves. Biol Trace Elem Res 48: 13-29
McLaughlin MJ, Smolders E, Merckx R (1998) Soil-root interface: physiological processes. In: Huang PM, Adriano DC, Logan TJ, Checkai RT (Eds) Soil Chemistry and Ecosystem Health. SSA Special Publication 52. Soil Sci Soc Am, Inc., Madison, WI, pp 233- 277
Moraghan JT (1993) Accumulation of cadmium and selected elements in flax seed grown on a calcareous soil. Plant Soil 150: 61-68
Nan Z, Li J, Zhang J, Cheng G (2002) Cadmium and zinc interactions and their transfer in soil-crop system under actual field conditions. Sci Total Environ 284: 187-195
Peralta JR, Gardea-Torresdey JL, Gomez E, Tiemann KJ, Arteaga S, Rascon A, Parsosn JG (2001) Uptake and effect of five heavy metals on germination and growth of live alfalfa plant (*Medicago sativa*) grown in solid media. Bull Environ Contam Toxicol 66: 727-734
Peralta-Videa JR, Gardea-Torresdey JL, Gomez E, Tiemann KJ, Parsons JG, de la Rosa G, Carrillo G (2002) Feasibility of alfalfa plant to phytoremediate individually contaminated montmorillonite soils with Cd(II), Cr(VI), Cu(II), Ni(II), and Zn(II). Bull Environ Contam Toxicol 69: 79-81
Sharma SS, Schat H, Vooijs R, Van Heerwaarden LM (1999) Combination toxicology of copper, zinc, and cadmium in binary mixture: concentration-dependent antagonistic, nonadditive, and synergistic effects on root growth in Silene vulgaris. Environ Toxicol Chem 18: 348-355
Smilde KW, Van Luit B, Van Driel W (1992) The extraction by soil and absorption by plants of applied zinc and cadmium. Plant Soil 143: 233-238
Taylor GJ, Stadt KJ (1990) Interactive effect of cadmium, copper, manganese, nickel, and zinc on root growth of wheat (*Triticum aestivum*) in solution culture. Develop Plant Soil Sc 41: 317-322
Yuji S, Kazuo T (1979) Studies on the heavy metal pollution in agricultural land. Part 5. Interaction between heavy metals in zinc, nickel, cobalt and copper absorption and translocation in rice plants. Shikoku Nogyo Shikenjo Hokoku 33: 23-31