Bull. Environ. Contam. Toxicol. (2002) 69:74–81

DOI: 10.1007/s00128-002-0012-y

Bulletin of Environmental Contamination and Toxicology

Potential of Alfalfa Plant to Phytoremediate Individually Contaminated Montmorillonite-Soils with Cadmium(II), Chromium(VI), Copper (II), Nickel(II), and Zinc(II)

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Received: 29 August 2001/Accepted: 6 April 2002

A few years ago, plant-based technology was considered to hold promise for cleaning up contaminated soils and waters (Cunningham et al., 1995). Gradually, this promise has become a reality for some types of contaminants such as radionuclides and heavy metals, and for several plant species. The main sources of phytoremediation-useful plants are metalliferous soils, metal smelters and metal refineries. Taxa such as: *Thlaspi, Viola* and *Silne* which are Zn accumulators (Reeves and Baker, 2000), *Armeria, Cardaminopsis* and *Agrostis*, which are effective cleaners of soils contaminated with Zn, Cd, and Pb, respectively (Dahmani-Muller et al., 2000), and *Larrea tridentata,* an inhabitant of the Chihuahuan Desert which is a Cu hyperaccumulator (Gardea-Torresdey et al., 1996) have been found near those areas.

Through out the world, researchers have found many other wild plant-species considered to be hyperaccumulators and have successfully used them in phytoremediation processes (Baker et al., 2000). Because phytoremediation of metal contaminated soils is a cost-effective methodology (Chaney et al., 1997), considerable efforts have been undertaken in order to search for new plant species able to be useful in the cleaning process. Ebbs et al., (1997) screened 300 accessions pertaining to 30 plant species with the specific aim of identifying those capable of removing Cd, Cu and Zn from contaminated soils. Some plants of agronomical value such as *Lycopersicon esculentum* (Brown et al., 1994), *Zea mays* (Ouzounidou et al., 1995), and alfalfa (Peralta et al., 2001), among others, have been studied in order to determine their phytoremediation potential. Previous studies conducted in agar with the alfalfa plant, describe the capabilities of this plant to germinate and to grow under the stress of Cd(II), Cr(VI), Cu(II), Ni(II), and Zn(II) when these heavy metal ions were completely bioavailable in the growing media (Peralta et al., 2001). This manuscript reports data concerning the potential of the alfalfa plant to clean up montmorillonite-clay contaminated with Cd(II), Cr(VI), Cu(II), Ni(II), and Zn(II).

MATERIALS AND METHODS

Gravel [0.81 mm of diameter (ϕ)] and sand (0.25 mm ϕ) were submerged in 0.01M HCl for two days, and then rinsed three times with deionized water and

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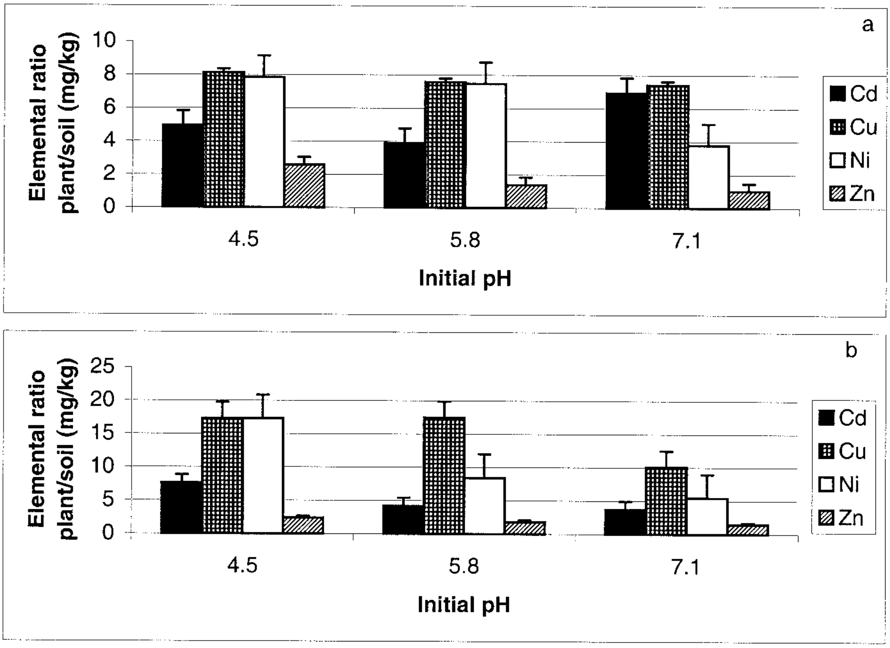

Figure 1. Magnitude of the ratio metal content in shoots: metal content in soil-water solution, (a) at 80 ppm, (b) at 160 ppm.

oven dried at 100°C. The gravel plus sand were then mixed with montmorillonite [(Super Gel-X, Colloid Environmental Technologies Company) (bentonite)] at 25%, 70% and 5%, respectively. Three hundred g of this mix were placed in plastic pots of 500 g capacity. The following compounds $Cd(NO_3)_2{\bullet}4H_2O$, $K_2Cr_2O_7$, $Cu(NO_3)_2{\bullet}2.5\ H_2O$, $Ni(NO_3)_2{\bullet}6H_2O$, and $Zn(NO_3)_2{\bullet}6H_2O$ were diluted separately in deionized water at the concentrations of 5, 10, 20, 40, 80, 160, and 320 ppm of Cd(II), Cr(VI), Cu(II), Ni(II), and Zn(II) ions. Four hundred ml of each concentration were prepared in triplicate and adjusted to pH 4.5, 5.8, and 7.1, respectively. The pots were arranged in a 3 x 5 x 8 factorial design with 120 treatments. Each treatment was replicated three times for statistical validity. The volume of 100ml of the corresponding solutions was slowly mixed with the soil-clay mix of each pot. After two days, each pot received 50 ml of a nutrient solution adjusted appropriately to pH 4.5, 5.8 and 7.1 (Peralta et al., 2001). Control treatments consisted of the nutrient solution adjusted to the same pHs. The deionized water used for watering was also adjusted at pH 4.5, 5.8, and 7.1.

Approximately 100 seeds of alfalfa Mesa variety were immersed in formaldehyde 3% v/v for 10 minutes in order to avoid fungal contamination, then washed three times with deionized water and planted in each pot. All of the pots were set under a 12 h photoperiod at 1500 luxes and 25°C. A sample of 5 plants per treatment was collected after 10 days in order to evaluate the effects of the heavy metals tested upon shoot growth. After two weeks the shoots were harvested and washed with deionized water, oven dried at 70°C for 72 h, weighed and digested. The digestion was accomplished using a microwave oven at 100°C for 15 minutes with 10 ml of concentrated HNO_3, trace pure. Subsequently, the volume was adjusted to 10 ml and the samples were analyzed using a Perkin-Elmer Optima 4300 Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES). The data were analyzed through analysis of variance (ANOVA) and the statistical significance of the differences between treatments means were determined through least significant difference (LSD) proofs. 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

The Mesa variety alfalfa plant was pretty sensitive to the effects of Cr(VI). Seedlings germinated at 5 and 10 ppm of Cr(VI) grew significantly less ($P < 0.01$) as compared with the seedlings of the other treatments (data not shown). We considered this a direct effect of Cr(VI) on the alfalfa plant, because montmorillonite does not readily adsorb Cr(VI) from solution due to the fact that Cr(VI) exists as an oxo-anion and will not interact well with the surface negative charges of the micelle (Brady and Weil, 1999). Furthermore, it is known that Cr(VI) manifests phytotoxicity at low levels, reducing chlorophyll biosynthesis and activity of several enzymes (Vajpayee et al., 2000).
The interaction upon alfalfa shoot growth of the doses of Cd(II), Cu(II), Ni(II), and Zn(II) and the original pH of their solutions are shown in Table 1. The plants in the control treatment showed significantly higher shoot development at pH 7.1 than at pH 4.5 ($P < 0.05$). In normal conditions, the alfalfa plant grows better at pHs higher than 6.5 (Brady and Weil, 1999). At the dose of 320 ppm and initial pH of 7.1, only Zn(II) allowed seed germination, whereas at pHs 4.5 and 5.8, both Zn(II) and Ni(II) allowed seed germination. There were no differences among the shoot length of the plants grown at pH 5.8 and 40 ppm dose, but at the same dose and pHs 4.5 and 7.1, plants stressed by Zn(II) showed shoots length significantly higher ($P < 0.05$) than the shoot size of the plants grown with Cd(II), Cu(II), and Ni(II). At 80 and 160 ppm, plants cultivated with Zn(II) grew significantly more ($P < 0.05$) than plants developed under the effects of Cd(II), Cu(II), and Ni(II). These results agree with previous results found on alfalfa cultivated in agar with these heavy metals (Peralta et al., 2001). The analysis of variance (ANOVA) for shoot weight (Table 2) showed statistical differences on the metals effect of metals in shoot weight ($P = 0.023$), for the dose ($P = 0.00$), and for the metal*dose interaction ($P = 0.00$). At pH 4.5, Zn(II) produced lighter shoots than the other metals, whereas at pH 7.1 and 5, 10 and 20 ppm, Ni(II) produced lighter shoots. In general, as the heavy metal dose increased, we found an opposite correlation among the plant size and the shoot weight.

When comparing the root growth of the control treatments plants, no pH effects were found, even though the roots size of those plants grown with initial pH of

Table 1. Shoot length (mm) of alfalfa plants Mesa variety after two weeks of exposure to heavy metals at three pHs. Data are average of five plants.

pH	Dose (ppm)	Metal			
		Cd	Cu	Ni	Zn
4.5	0	33 ± 3.9	33 ± 3.9	33 ± 3.9	33 ± 3.9
	40	32 ± 0.8 $_b$	31 ± 0.6 $_b$	35 ± 0.6 $_b$	55 ± 0.5$_a$
	80	30 ± 0.8 $_b$	31 ± 1.4 $_b$	26 ± 0.9 $_b$	52 ± 1.0$_a$
	160	20 ± 1.7	21 ± 1.2	21 ± 1.2	29 ± 0.3
	320	0	0	21 ± 1.0	26± 1.0
5.8	0	41 ± 1.9	41 ± 1.9	41 ± 1.9	41 ± 1.9
	40	35 ± 0.3	36 ± 0.5	32 ± 0.3	40 ± 0.8
	80	30 ± 0.2 $_b$	27 ± 0.7 $_b$	27 ± 0.5 $_b$	55 ± 1.2$_a$
	160	19 ± 0.8 $_b$	22 ± 0.7 $_b$	23 ± 0.6 $_b$	41± 2.0$_a$
	320	0	0	0	28 ± 1.3$_a$
7.1	0	52 ± 0.5	52 ± 0.5	52 ± 0.5	52 ± 0.5
	40	30 ± 1.2 $_b$	31 ± 0.8 $_b$	38 ± 1.0$_b$	53 ± 0.3$_a$
	80	30 ± 0.6 $_b$	32 ± 2.5 $_b$	28 ± 0.6 $_b$	53 ± 1.9$_a$
	160	22 ± 1.2 $_b$	29 ± 1.5 $_{ab}$	21 ± 0.6 $_b$	40 ± 0.5$_a$
	320	0	0	0	27 ± 0.6$_a$

Results are means ± SD. Means with different letters are significantly different from each other ($P < 0.05$) according to LSD-test (LSD = 13.4). Comparisons are among dose within pHs.

Table 2. Analysis of variance for shoot weight of alfalfa plants Mesa variety cultivated in montmorillonite-based soil contaminated with 0-320 ppm of Cd(II), Cu(II), Ni(II), and Zn(II).

Source	DF	SS	MS	F	P
pH	2	3.7694	1.8847	2.71	0.078
metal	3	7.3671	2.4557	3.53	0.023
dose	7	99.1813	14.1688	20.39	0.000
pH*metal	6	7.6373	1.2729	1.83	0.116
pH*dose	14	12.4656	0.8904	1.28	0.259
metal*dose	21	97.6479	4.6499	6.69	0.000
Error	42	29.1877	0.6949		
Total	95	257.2563			

DF = degree of freedom, SS = Square sum, MS = Mean square, F = Variance ratio, P = probability.

Table 3. Root length (mm) of alfalfa plants Mesa variety after two weeks of exposure to heavy metals at three pHs. Data are average of five plants.

pH	Dose (ppm)	Metal			
		Cd	Cu	Ni	Zn
4.5	0	49 ± 0.5	49 ± 0.5	49 ± 0.5	49 ± 0.5
	40	20 ± 0.3 $_b$	21 ± 0.6 $_b$	25 ± 0.6 $_b$	46 ± 0.5 $_a$
	80	8 ± 0.3 $_b$	13 ± 0.6 $_b$	17 ± 0.7 $_b$	43 ± 0.9 $_a$
	160	10 ± 0.3	10 ± 0.7 $_a$	11 ± 0.3 $_a$	23 ± 0.1 $_a$
	320	0	0	9 ± 0.8	11± 0.2
5.8	0	48 ± 0.4	48 ± 0.4	48 ± 0.4	48 ± 0.4
	40	20 ± 0.3 $_b$	21 ± 0.5 $_b$	25 ± 0.3 $_b$	46 ± 0.8 $_a$
	80	8 ± 0.2 $_b$	13 ± 1.6 $_b$	17 ± 0.5 $_b$	43 ± 2.2 $_a$
	160	10 ± 1.0	10 ± 0.7	11 ± 0.2	23 ± 0.7
	320	0	0	0	9 ± 0.5
7.1	0	57 ± 1.2	57 ± 1.2	57 ± 1.2	57 ± 1.2
	40	20 ± 1.2 $_b$	25 ± 0.8 $_b$	24 ± 1.0 $_b$	57 ± 0.3 $_a$
	80	15 ± 1.1 $_b$	15 ± 0.4 $_b$	21 ± 2.3 $_b$	51 ± 0.4 $_a$
	160	11 ± 1.2 $_b$	11 ± 1.4 $_b$	12 ± .1 $_b$	37 ± 0.2 $_a$
	320	0	0	0	10 ± 0.6

Results are means ± SD. Means with different letters are significantly different from each other (P< 0.05) according to LSD-test (LSD = 13.4). Comparisons are among dose within pHs.

7.1 were numerically higher (Table 3). The root size of plants exposed to 40 ppm and above for Cd(II), Cu(II), and Ni(II) were significantly lower (P< 0.01) as compared with the root length of the control treatment plants. Similar results were found with Ni(II) in *Brassica Lactuca, Raphanus* and *Triticum* by Claire et al. (1991).There were no differences among the root size of control treatment plants and the root length of plants cultivated with 40 and 80 ppm of Zn(II). One hundred sixty ppm of Zn(II) produced roots statistically shorter than the root size of the control treatment plants at any pH used in this experiment (P< 0.01), but still with agronomical possibilities. The maximum root size (57 mm) was reached by those plants exposed to 40 ppm of Zn(II) and the control treatment plants at pH 7.1 (Table 3). At the dose of 80 ppm of Zn(II) and pH 4.5 roots were produced at 438%, 230% and 153% larger than the roots obtained with CdII), Cu(II), and Ni(II), respectively; whereas at the same dose at pH 7.1, Zn(II) treatment formed roots 240%, 240%, and 143% bigger than the roots obtained with Cd(II), Cu(II),

Table 4. Heavy metal concentration in soil-water solution and plant shoot tissue as affected by the dose and the original pH.

pH	Dose applied (ppm)	Concentration in soil-water solution (ppm)			
		Cd	Cu	Ni	Zn
	0	0	0.2	0.1	0
4.5	80	$15 \pm 4_{b}$	$20 \pm 3_{b}$	$17 \pm 3_{b}$	$96 \pm 11_{a}$
	160	$41 \pm 4_{c}$	$29 \pm 12_{b}$	$26 \pm 4_{b}$	$167 \pm 4_{a}$
	0	0	0.2	0.2	0
5.8	80	$11 \pm 5_{c}$	$24 \pm 5_{b}$	$23 \pm 6_{b}$	$117 \pm 10_{a}$
	160	54 ± 14	30 ± 12	34 ± 13	207 ± 15
	0	0	0.2	0.1	1
7.1	80	$12 \pm 2_{c}$	$25 \pm 3_{b}$	$28 \pm 2_{b}$	$117 \pm 13_{a}$
	160	$55 \pm 1_{b}$	$32 \pm 4_{c}$	$50 \pm 1_{b}$	$201 \pm 18_{a}$
		Concentration in shoot plant tissue (ppm)			
4.5	0	0_{d}	$18 \pm 5_{bc}$	$31 \pm 9_{a}$	$25 \pm 6_{ab}$
	80	$76 \pm 9_{c}$	$164 \pm 11_{b}$	$30 \pm 24_{d}$	$249 \pm 21_{a}$
	160	$310 \pm 57_{d}$	$505 \pm 57_{a}$	$449 \pm 14_{b}$	$398 \pm 21_{c}$
5.8	0	$1 \pm 0.3_{b}$	$10 \pm 3_{b}$	$5 \pm 1_{b}$	$22 \pm 6_{a}$
	80	$42 \pm 7_{b}$	$178 \pm 14_{a}$	$174 \pm 51_{a}$	$162 \pm 42_{a}$
	160	$226 \pm 12_{d}$	$522 \pm 61_{a}$	$282 \pm 5_{c}$	$375 \pm 11_{b}$
7.1	0	$1 \pm 0.3_{bc}$	$17 \pm 3_{b}$	$8 \pm 2_{b}$	$70 \pm 10_{a}$
	80	$86 \pm 25_{d}$	$185 \pm 34_{a}$	$105 \pm 15_{c}$	$122 \pm 20_{b}$
	160	$205 \pm 30_{d}$	$322 \pm 33_{a}$	$270 \pm 15_{c}$	$293 \pm 41_{b}$

Results are means ± SD. Means with different letters are significantly different from each other ($P < 0.05$) according to LSD-test (LSD = 9.4). Comparisons are among dose within pHs.

and Ni(II), respectively. Based on the results of this experiment, we consider that a montmorillonite-clay-soil individually contaminated with 80 ppm of Cd(II), Cu(II), and Ni(II), and 160 ppm of Zn(II) allowed agronomicaly acceptable germination rate and growing of alfalfa plants (Mesa variety).

The concentration of Cd(II), Cu(II), Ni(II), and Zn(II) in the bentonite-water solution after the application of 0, 80 and 160 ppm, as well as the concentration of these heavy metals in alfalfa shoots Mesa variety are shown in Table 4. At pH 4.5, the amount of Zn(II) in shoots was statistically higher ($P < 0.05$) than those of Cu(II), Cd(II) and Ni(II), while at pH 5.8, the concentration of Cd(II) in shoots was statistically lower ($P < 0.01$) than the amounts of Cu(II), Ni(II), and Zn(II). Brown et al. (1994) also found some inconsistency with Cd(II) and Zn(II) in pennycress, bladder campion and tomato. At 160 ppm and pH 4.5, metal content in shoots was higher than the metal concentration found in shoots at pH 7.1 for the four heavy metals tested. At this dose and pH 4.5 the heavy metal concentrations in the shoots were 500 ppm of Cu(II), 450 of Ni(II), 400 of Zn(II), and 300 of Cd(II). At pH 5.8, we found 520 ppm of Cu(II), 380 ppm of Zn(II), 250 ppm of Ni(II), and 210 ppm of Cd(II), and at pH 7.1 there were 325 ppm of Cu(II), 300 ppm of Zn(II), 230 ppm of Ni(II), and 200 ppm of Cd(II). The results obtained at pH 4.5 agree with previous results reported in alfalfa cultivated in agar at pH 5.3 by Peralta et al. (2001). There was a strong and statistically significant interaction ($P < 0.01$) between the metal content in the soil-water solution, the concentration in the shoot tissue, and the original pH (Figure 1). At 80 ppm, the amount of Cu(II) in plant shoot tissue was 8 times higher than the amount in soil-water solution at pHs of 4.5 and 5.8, and 7 times at pH of 7.1, while at 160 ppm and pHs of 4.5 and 5.8, they were 17 times, and 10 times at pH of 7.1. Ni(II) presented the second highest ratios, being 8, 7, and 4 times at 80 ppm for the pHs of 4.5, 5.8 and 7.1, respectively. At 160 ppm, the ratios were 17, 8 and 5 times at the pHs of 4.5, 5.8 and 7.1, respectively. The ratios for metal in shoot tissue: metal in soils with Cd(II) at 80 ppm were 5, 4, and 7 times at the pHs of 4.5, 5.8 and 7.1, respectively, whereas with 160 ppm the relations were 8, 4, and 4 for the pHs of 4.5, 5.8 and 7.1, respectively. These ratios are considerable higher than those found in *Triticum* and *Lactaria* by Ptichel et al. (2000). Zn(II) presented the lowest ratio as compared to the other metals, its highest ratio being 3 times at pH 4.5. However, the contents of Zn(II) were significantly higher ($P < 0.01$) in the soil water-solution than the amount of Cd(II), Cu(II), and Ni(II).

Based on these results, we concluded that alfalfa plant has the capability to compete with the montmorillonite-clay for the individually sorbed cations Cd(II), Cu(II), Ni(II), and Zn(II) by virtue of the amount of heavy metals in the plant shoot tissue as compared with the concentration of these metal detected in the soil-water solutions. Live alfalfa plants have the potential to be sown and phytoremediate montmorillonite-soils, contaminated individually with 80 ppm as total concentration of Cd(II), Cu(II), and Ni(II), and 160 ppm of Zn(II). Using our current laboratory procedures, this plant did not show capabilities to tolerate Cr(VI) in the cultivation matrix. Alfalfa live plant is able to remove up to 86 ppm of Cd(II) and 185 ppm of Cu(II) at pH 7.1, as well as 174 ppm of Ni(II) at pH 5.8 and 398 ppm of Zn(II) at pH 4.5.

Acknowledgments. We thank the National Institute 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

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