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The influence of humic acids on the phytoextraction of cadmium from soil

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Abstract

Cadmium poses a major environmental and human health threat because of its constant release through anthropogenic activities. A need, therefore, exists for cost-effective remediation procedures. Phytoremediation, the use of plants to extract contaminants from soils and groundwater, has revealed great potential. However, it is limited by the fact that plants need time, nutrient supply and, moreover, have a limited metal uptake capacity. Synthetic chelators have shown positive effects in enhancing heavy metal extraction through phytoremediation, but they have also revealed a vast number of negative side-effects. The objective of this research was to investigate the use of humic acids as an alternative to synthetic chelators. Humic acids were applied to a cadmium-contaminated soil at various dosages, and the uptake of cadmium into *Nicotiana tabacum* SR-1 was determined in relation to the amounts of total and bioavailable cadmium in the soil. It was found that the theoretical bioavailability of cadmium, as determined by diethylenetriaminepentaacetic acid (DTPA) extraction, did not change, but its plant uptake was enhanced significantly, in some cases up to 65%. Humic acids added at a rate of 2 $g\,kg^{-1}$ soil increased the cadmium concentration in the shoots from 30.9 to 39.9 $mg\,kg^{-1}$. A possible reason for this enhancement is the decrease in pH, resulting in higher cadmium availability. Another possibility taken into account is that plants may take up cadmium complexes with humic acid fragments, which result from microbiological degradation or, self-dissociation.

Keywords: Phytoremediation; Cadmium; Humic acids; Chelate assisted; Tobacco

1. Introduction

Phytoremediation is defined as the use of green plants in removing pollutants from the environment, or in rendering them harmless (Raskin et al., 1997). In contrast to other remediation technologies, such as land filling, fixation and leaching, it is relatively cost-effective, aesthetically pleasing and requires smaller disposal facilities (Glass, 1999). Moreover, phytoremediation offers the great advantage of causing only minimal environmental disturbance, since it does not adversely alter the soil matrix. Thus after successful phytoremediation, the soil can directly be used for agricultural purposes.

Toxic heavy metals and organic pollutants are both targets for phytoremediation. Salt et al. (1998) summarised the following phytoremediation sub-groups:

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phytoextraction—the use of pollutant-accumulating plants to remove metals or organic pollutants from soil by concentrating them in harvestable parts; *phytodegradation*—the process whereby plants and associated microorganisms are used to degrade organic pollutants; *rhizofiltration*—whereby plant roots are used to absorb pollutants, mainly metals, from water and aqueous waste streams; *phytostabilisation*—whereby plants reduce the bioavailability of pollutants in the environment; and *phytovolatilisation*—the use of plants to volatilise certain pollutants and remove them from air.

All plants have the potential to extract metals from soil, but some plants have shown the ability to extract, accumulate and tolerate high levels of heavy metals, which would be toxic to other organisms. Such plants are termed hyperaccumulators. Hyperaccumulating plants are taxonomically widespread throughout the plant kingdom. Metal hyperaccumulation is an ecophysiological adaptation to metalliferous soils (Maywald and Weigel, 1997). Its function is not yet known, but experiments have been performed, which support the hypothesis that metal-hyperaccumulation works as a defence mechanism against plant pathogens (Boyd et al., 1994), and also prevents predation (Sagner et al., 1998). However, the potential for application of hyperaccumulators in bioremediation is limited by several factors. Such plants often accumulate only one specific element and are thus not applicable to multiple elements. For example the population of the hyperaccumulating plant *Thlaspi caerulescens* is divided in Zn and Cd hyperaccumulators. The Cd hyperaccumulating populations have the ability to accumulate concentrations of Cd in their aerial parts reaching 3000 $mg\,kg^{-1}$ (Schwartz et al., 2003). According to Brown et al. (1995), hyperaccumulator species are those plants whose leaves may contain >100 $mg\,kg^{-1}$ Cd. Additionally, most hyperaccumulators grow slowly and have a small biomass. Robinson et al. (2000) suggested that a plant used for phytoremediation should be fast growing, deep-rooted, easily propagated and accumulating the target metal. According to Römkens et al. (2002) it should also have a high biomass production. All the factors mentioned above are found in the tobacco plant, *N. tabacum*.

During the last decade, there has been much success in making phytoremediation a promising environmental technology. Nevertheless, there is still a great lack of knowledge concerning the plant mechanisms which are responsible for metal extraction, and the factors which influence the bioavailability of pollutants in soil. The bioavailability of metals in soil is affected by numerous factors, such as cation exchange capacity, pH values of the soil, excess amounts of fertilizers, and chelators. These may all be manipulated to improve cadmium phytoextraction. Chelators, such as EDTA increase the solubility of metal cations, and thus their bioavailability to plants. The positive effects of EDTA on the phytoextraction of metals are, however, accompanied by negative effects on the soil. Its non-selective nature in extracting metals is a disadvantage, since this agent extracts a wide variety of metals, including alkaline earth cations, such as Ca and Mg, which are necessary for plant growth (Barona et al., 2001). Moreover, EDTA is not easily biodegradable, and may remain adsorbed to soil particles even after soil cleaning (Wasay et al., 1998). EDTA has also the effect of decreasing severely the plant growth (Chen and Cutright, 2001).

As an alternative to these synthetic chelators widespread natural sources, such as humic substances, could be used. The term humic substances refers to a category of naturally occurring organic materials found in soils, sediments, and natural waters. They result from the decomposition of plant and animal residues (MacCarthy, 2001). Humic acids are those parts of humic substances which are not soluble in water under acidic conditions, but become soluble and extractable at higher pH values. Humic acids contain acidic groups such as carboxyl and phenolic OH functional groups, (Hofrichter and Steinbüchel, 2001) and, therefore, provide organic macromolecules with an important role in the transport, bioavailability, and solubility of heavy metals (Lagier et al., 2000).

The objective of this research was to investigate the ability of humic acids in enhancing the phytoextraction of cadmium from soil by the use of tobacco plants under laboratory conditions. A soil, already containing a natural cadmium content of approximately 2 $mg\,kg^{-1}$, was spiked with 0 (no added cadmium), 5, 10 and 15 $mg\,kg^{-1}$ cadmium. Thus, the cadmium concentrations of the experiments amounted to 2, 7, 12 and 17 $mg\,kg^{-1}$ soil. The humic acids concentrations amounted to 0 (no added humic acids), 10, and 20 $g\,kg^{-1}$.

2. Materials and methods

2.1. Soil characterisation

A loamy agricultural soil, defined with the guide of the Soil Texture Classes—The United States Department of Agriculture was collected from Melaten field in Aachen, Germany. The soil was air-dried at room temperature, sieved through a 2-mm sieve and characterized as follows. The sand, clay and silt fractions of the samples were determined by the hydrometer method (Bouyoucous, 1952). Sand particles amounted to 49.41%, silt to 42.10% and clay to 8.49%. Organic matter content, as determined by the Walkley–Black method (Nelson and Sommers, 1996), amounted to 4.3%. The soil pH of 7.2 was measured by the $CaCl_2$-method (Lewandowski et al., 1997). The initial total cadmium content of the soil, as determined by the aqua regia-method, was 2.33 $mg\,kg^{-1}$ and the initial bioavail-

able cadmium content, determined by diethylenetriaminepentaacetic acid (DTPA) extraction method amounted to 0.62 mg kg^{-1}.

2.2. Analysis of total and bioavailable cadmium in soil

The bioavailable cadmium was determined by the DTPA method (Risser and Baker, 1990). One litre of the DTPA extracting solution contains 14.9 g tetraethanolamine (TEA), 1.97 g of DTPA, and 1.47 g of $CaCl_2$2-H_2O and the pH was adjusted to 7.3 with 1 M HCl. The resulting solution contains 0.005 M DTPA, 0.01 M $CaCl_2$, and 0.1 M TEA. In a 125 ml flask, 20 ml of the DTPA solution were added to 10 g of air-dried soil. The flask was covered with a plastic stopper and shaken at 2 cycles s^{-1} for 2 h. Afterwards the suspension was gravity filtered through blue ribbon analytical filter paper.

Cadmium analysis in the filtrate was performed by flame AAS (Perkin-Elmer 1100B). Standards for the AAS calibration were prepared in the extraction solution by the addition of appropriate quantities of cadmium.

The total cadmium concentration was determined by the aqua regia-method (DIN 38414 Teil 7:1983-01). Three grams of air-dried soil were boiled with 28 ml of aqua regia for 120 min under reflux. Then 72 ml of deionised water were added to the suspension to make up for a total volume of 100 ml. The suspension was centrifuged at 3000*g* for 20 min and then decanted into an Erlenmeyer-flask. Cadmium was analysed by AAS.

2.3. Extraction of humic substances

Humic acids were extracted according to the method described by Swift (1996). Since there was no need to use highly purified humic acids in the experiments, some steps were changed in order to achieve the highest possible quantities of crude humic acids. An amount of about 70 g of peat was treated with 280 ml of an aqueous solution of 0.2 M NaOH (soil to solution ratio of 1–4). The resulting suspension was stirred for approximately 12 h at room temperature under nitrogen atmosphere, and then centrifuged at 2000*g* for 15 min. The purpose of the nitrogen atmosphere was to suppress oxidation of the humic acids in the alkaline milieu. The supernatant was filtered through qualitative filter paper (No. 595, Schleicher & Schuell Filters), and acidified to pH 1.0 with 10 mol l^{-1} HCl to precipitate the humic acids. The solution was stored at 4 °C for approximately 24 h to allow complete precipitation of the humic acids. The precipitate was separated from the soluble fraction (fulvic acids) by centrifugation at 2000*g* for 20 min, and washed 2–3 times with deionised water at a ratio of 1:3. The washed precipitate was transferred into a round bottom flask, freezed and lyophilised.

2.4. Soil preparation for the pot experiment

The pot experiments were conducted in a green house from May to September. One kilogram of air-dried and sieved soil was filled into 1.5 l plastic pots with six small holes at the bottom. A pot-plate was placed under each pot. To each pot the following amounts of fertilizer were applied: 1.686 g $Ca(NO_3)_2 4H_2O$, 439 mg KH_2PO_4 and 19.2 mg Fe(III)EDTA. The experiments included the control treatments (no addition of cadmium), and treatments with 5, 10 and 15 mg kg^{-1} cadmium applied as $3CdSO_4 8H_2O$. Each treatment was performed in triplicates. One day after the application of cadmium and the fertilizers, humic acids at 0, 10 and 20 g kg^{-1} were applied in a dry form to allow exact quantification. A uniform application was obtained by homogenization of the soil. The soil was subsequently incubated in the green house for 4 weeks. During these 4 weeks the soil was watered 1–2 times a week with 100 ml deionised water. In order to avoid leaching of cadmium, the water was not applied directly to the soil surface, but into the pot-plate.

2.5. Cultivar source, seedling preparation and plant growth

Tobacco plants (*N. tabacum* SR-1) were used for the pot experiments. This selection was based on the plant's ability to produce a great biomass in a very short time. Seeds of tobacco plants were germinated in a peat/sand mixture. After the 4-week incubation, seedlings with similar biomass were transferred into the pots with the metal and humic acids spiked soil. Four seedlings were planted into each pot and were thinned to one plant after 1 week. Thereafter, the experiment was initiated.

The work of Walch-Liu et al. (2000) was used as a reference for the growth of the tobacco plants with conditions adapted to the technical capabilities provided in our laboratory. All tobacco plants were grown under controlled environmental conditions with a 16 h light period (light intensity of 320 μmol $m^{-2} s^{-1}$), a 25/20 °C light/dark temperature regime, and 60% relative humidity. Plants were harvested after 4 weeks of growth.

2.6. Plant harvest and analysis

During harvest, plants were cut short above ground, and separated into stem, leaves and bloom. The subsequent steps were performed according to Jones and Case (1990). Plant samples (stem, leaves and bloom) were rinsed briefly in deionised water, dried between Kleenex tissues to remove surface contamination, and oven dried at 70 °C for 48 h to a constant weight. The dry weight

was determined and the samples were homogenised in particle size by grounding in a ball mill.

After milling, 200 ± 5 mg of dried plant tissue were weighed into a 15 ml high form porcelain crucible. The plant tissue was ashed at 500 °C for 5 h in a muffle furnace and cooled down. At 60 °C, 2 ml of 15% HCl were added and evaporated. Two ml of 15% HCl were again added, at room temperature. The ash was suspended with the assistance of a plastic stick, and the suspension subsequently filtered through a quantitative filter paper (blue ribbon filter 589/3, Schleicher & Schuell Filters). The filtrate was adjusted to 20 ml with deionised water and analysed for cadmium by AAS.

2.7. Statistical analysis

Each Cd concentration and each humic acid concentration was performed in triplicates ($n = 3$). The difference between specific pairs of means was identified by Student-Newman-Keuls test ($P < 0.05$). Statistical analysis of the data was performed by using SigmaStat 2.03 (SPSS Science, Chicago, IL).

3. Results

3.1. Plant growth

Dry matter yields of the shoots (sum of leaves and stem) are shown in Fig. 1. Compared to the dry weight of the leaves and stem, that of the blooms (data not shown) varied considerably among the various cadmium treatments, and even among the parallel treatments. The blooms' dry weight was, therefore, not taken into consideration. The application of cadmium or humic acids to the soil did not adversely affect dry matter production of the plants. Thus no difference in shoot biomass was visible, but toxicity symptoms, such as chlorosis and necrosis, were visible towards the end of the experiment when cadmium concentrations were above 10 mg kg^{-1}. The symptoms were first observed in older leaves of the cadmium treated plants, and later spread to the younger leaves. The humic acid-treated plants showed toxicity symptoms of an earlier stage than the control plants. Thus, indicating that the humic acid-treated plants reached their maximum tolerable cadmium concentration in the shoots more quickly than the plants grown without added humic acids.

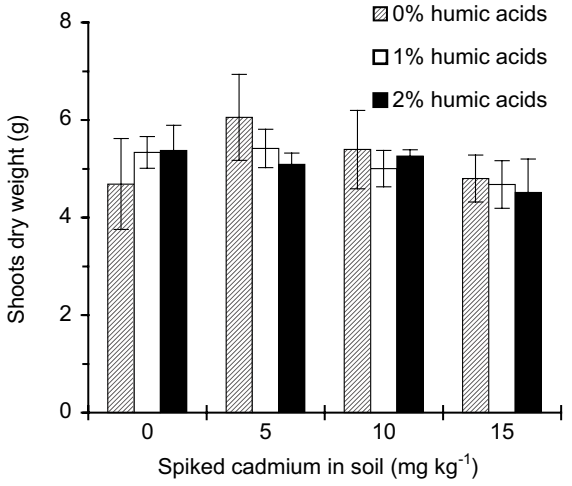

Fig. 1. Effect of the application of cadmium and humic acids to soil on shoot dry weight of tobacco. Error bars represent ±SE of triplicates ($n = 3$).

3.2. Soil analysis

The results from DTPA and aqua-regia extraction showed almost no variation in cadmium concentration between the replicates, as confirmed by low standard deviations. There was also very low variation in the cadmium soil concentrations amongst the three different humic acid treatments.

The total cadmium content of the soil, as determined by aqua regia extraction, showed no significant change between before planting and after harvesting, indicating that the amount of cadmium extracted by the plants was too small within the time frame of the experiment to be measurable (Table 1).

The potentially bioavailable cadmium concentrations, as determined by DTPA extraction, are shown in Table 1. Irrespective of the humic acid concentrations added to the soil before planting and after harvesting the potentially bioavailable cadmium concentration in soil was not affected significantly.

Soils containing spiked and natural aged cadmium behaved differently. The potentially bioavailable cadmium in the cadmium treated soil amounted to approximately 70% of the total concentration, whereas in the control treatment (neither cadmium nor humic acids added), it amounted to only 30%.

3.3. Cadmium concentrations in the plants

As presented in Fig. 2, cadmium concentrations in the shoots markedly increased with rising cadmium concentrations in the soil. In addition rising humic acids levels increased the cadmium uptake in the shoots at all cadmium concentrations except for the variant with 15 mg kg^{-1} cadmium and 1% humic acids. The treatment with 2% humic acids caused a significantly higher concentration of cadmium in the shoots at 0, 5, 10 and 15 mg kg^{-1} cadmium compared to the controls without the addition of humic acids.

Table 1
Concentrations of total and bioavailable cadmium (*) in soil before planting and after harvesting

Cadmium treatment dosage ($mg\,kg^{-1}$)	Cadmium concentration in soil ($mg\,kg^{-1}$)					
	Humic acids 0%		Humic acids 1%		Humic acids 2%	
	Before harvesting	After harvesting	Before harvesting	After harvesting	Before harvesting	After harvesting
0	2.4 ± 0.2	2.2 ± 0.2	2.7 ± 0.00	2.2 ± 0.2	2.4 ± 0.2	2.1 ± 0.4
*	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0
5	7.3 ± 0.3	7.3 ± 0.0	7.2 ± 0.0	6.8 ± 0.2	7.2 ± 0.0	7.3 ± 0.3
*	4.6 ± 0.2	4.1 ± 0.1	4.6 ± 0.2	4.3 ± 0.3	4.6 ± 0.0	4.1 ± 0.1
10	12.5 ± 0.2	12.1 ± 0.2	12.5 ± 0.5	12.2 ± 0.7	12.2 ± 0.2	12.7 ± 0.3
*	8.5 ± 0.2	7.0 ± 0.0	8.4 ± 0.2	7.8 ± 0.2	8.5 ± 0.1	8.3 ± 0.2
15	17.5 ± 0.5	17.0 ± 0.6	17.5 ± 0.2	18.0 ± 0.3	17.7 ± 0.7	17.6 ± 0.2
*	12.4 ± 0.0	10.0 ± 0.0	12.4 ± 0.7	11.7 ± 0.4	12.1 ± 1.1	12.1 ± 0.2

Values are given as means ± standard deviation.

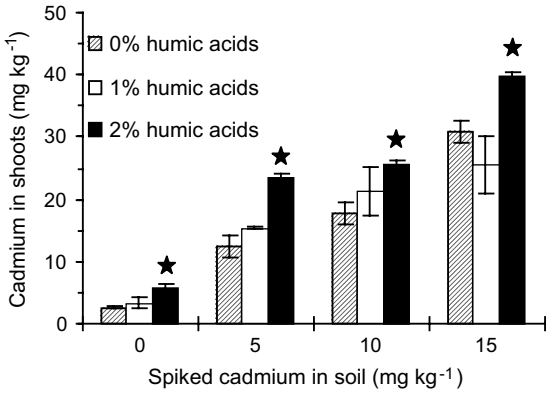

Fig. 2. Effect of cadmium and humic acids application to soil on cadmium concentration in shoots of tobacco. Bars marked with (★) are statistically different with the control ($P < 0.05$). Error bars represent ±SE of triplicates ($n = 3$).

4. Discussion

The addition of humic acids to soil increased the cadmium uptake of tobacco plants from an artificially contaminated soil, thus confirming the statement of Li and Shuman (1996). Also Evangelou and Marsi (2001) and Halim et al. (2003) came to similar results on the enhancement of the bioavailability and mobility of heavy metals in soil by humic acids. However, although humic acids revealed a positive effect on phytoextraction, the bioavailability of cadmium in the soil based on DTPA extraction was independent of the soil's humic acid content.

The concentrations in the shoots ranged from approximately 3 $mg\,kg^{-1}$ to approximately 39 $mg\,kg^{-1}$ (Fig. 2), whilst that of the soil contained between 2 and 17 $mg\,kg^{-1}$. The absolute uptake of Cd by the plants, which ranged from approximately 9–210 μg (data not shown), was too little within the time frame of the enhancement to produce a significant change in the cadmium content of the soil. The plants in the humic acid-treated soil reached their maximum cadmium concentrations more quickly than the non-humic acids treated soil as seen by their toxicity symptoms, above 10 mg $Cd\,kg^{-1}$. The effect of Fe-EDTA on the mobility of Cd can be excluded according to Chang et al. (2003), due to the fact that Cd does not alter its chemical form to Cd-EDTA in a significant amount. In addition a pH decrease was observed, from 7.2 to 6.6, after the amendment of the humic acids, which could have an affect on the mobility of Cd. The binding of Cd on the plastic pots was negligible.

The bioavailability of heavy metals in soil is influenced by many factors, such as the organic matter content (Li and Shuman, 1996), the cation exchange capacity (Alloway and Ayres, 1997), and, especially, the pH which is partially influenced by organic acids exudated by plants. Cieslinski et al. (1998) and Nigam et al. (2001) showed that organic acids had a positive effect on the metal extraction by plants. As in the case of EDTA (Grcman et al., 2001), it was shown that the corresponding metal complexes are translocated via xylem from the roots to the shoots. Humic acids are too large to permeate the root. Humic acid fragments, produced by microbial activity or by self-dissociation of the polymeric humic matter which is associated in part by hydrogen-bonding, hydrophobic interaction, ionic association and van-der-Waals forces (Piccolo, 2001), form metal chelates, which are resorbed by plants. Alternatively, humic acids could also form an enhancer through their functional groups, which is not resorbed by plants and delivers the heavy metals in a more available form to the exudates of the plants.

Current theories for the translocation of metals from plant roots to shoots propose that the responsible chelators are phytochelatins and organic acids, such as malic acid and citric acid, the latter translocating apparently via the xylem (Senden et al., 1990; Guo, 1995). The

reports concerning the phytochelatins mechanism in metal transport are controversial. According to Salt et al. (1995) translocation of cadmium is independent of the production of phytochelatin in roots. On the other hand, Guo (1995) does not exclude phytochelatins as translocation agents of cadmium from roots to shoots.

All carrier molecules, including chelators, regardless of their task and origin, have a limited binding capacity. Thus, they can carry only a restricted number of molecules or ions, depending on the number of binding sites. Assuming that the humic acids or humic acid fragments have a positive effect on the mobility of cadmium in soil, a larger amount of cadmium is extracted by the roots and translocated to the shoots by the carriers. The plants growing in the non-humic acid-treated soil received a much smaller cadmium amount due to its lower bioavailability in the soil and revealed much later toxicity symptoms, suggesting that there is a slower rise in cadmium concentration in the leaves. The lower bioavailability of cadmium, evident by the lack of toxicity symptoms, enabled the control plants to catch up with the humic acids treated plants in accumulating metals in a given period of time. The advantage of the humic acid-treated plants is that a larger amount of cadmium can be extracted in a shorter period of time. To use this advantage plants have to be selected which can tolerate high toxic metal concentrations i.e. revealing hyperaccumulator properties. Thus, a combination of using natural chelators and a plant with a high biomass and sufficient metal tolerance would enlarge the efficiency of phytoextraction significantly.

5. Conclusion

Humic acids do have a positive effect on metal bioavailability in soil and accelerate the phytoextraction efficiency. Moreover, they do not have the negative effects of synthetic chelators such as EDTA which severely decrease the plant growth. However, due to the high effort in obtaining humic acids in sufficient amounts for phytoextraction, humic acids will not serve as an economic alternative to synthetic chelators. We will continue research in investigating other natural chelators to replace synthetic chemicals for this purpose.

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