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Ability of *Agrogyron elongatum* to accumulate the single metal of cadmium, copper, nickel and lead and root exudation of organic acids

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Abstract: *Agrogyron elongatum* were grown in nutrient solution containing moderate to high amounts of separate heavy metal of Cd, Cu, Ni and Pb in a greenhouse for a 9 - day. Cd, Cu, Ni and Pb generally led to decrease in the elongation of roots although the length of seedlings exposed to Cd and Pb at 0.05 and 0.5 mg/L showed to be slightly greater than that of controls. Of the four metals in the experiment, Pb was absorbed and accumulated to the highest level, with the concentrations of 92754 mg/kg dry weight (DW) in roots and 11683 mg/kg DW in shoots. Cd was moderately accumulated in *Agrogyron elongatum*, but the maximum bioaccumulation coefficients (BCs) for roots and shoots were observed. The patterns for Cu and Ni uptake and distribution in plants differed from those of Pb and Cd, as it was showed that the shoot accumulation of Cu and Ni was significantly higher than in roots. *A. elongatum* had the highest Ni concentration in shoots (30261 mg/kg DW) at the external concentration of 250 mg/L. Cu ranked second, with a shoot concentration of 12230 mg/kg DW when 50 mg/L Cu in solution was applied. For the four trace elements tested, the highest concentrations in shoots decreased by the order of Ni > Cu > Pb > Cd (mg/kg DW), and those in roots were Pb > Cd > Ni > Cu (mg/kg DW). Malic, oxalic and citric acids exuded by roots exposed to 1 and 50 mg/L of the metals were detected. Release of organic acids from plants significantly differed among the metal treatments. Cu was most effectively in inducing root exudation of the three types of organic acids. Cd, and Ni were also the inducers of secretion of malic and oxalic acids. With reference of Pb, a small amounts of malic and oxalic acids were detected in the root exudates, but few quantities of citric acid were found. However, no correlation between alternations in root exudation of organic acids and metal accumulation could be established.

Key words: heavy metal; organic acid; *Agrogyron elongatum*; hyperaccumulation

Introduction

Contamination of soils with heavy metals becomes an increasing problem in many countries all over the world. This is much concerned because of their easy transferring from soils to plants, even to animals and human beings. In most cases, plants grown in heavy metal-polluted soils show metal accumulation. A number of plant species have been identified. They are endemic to metalliferous to soils, tolerant and can accumulate high levels of heavy metals, such as Zn, Cd, Pb, Cu, and Ni (Brooks, 1977; Baker, 1994; Miguel, 1998). As the plants have developed tolerance mechanism to heavy metals, they either involve the capacity to limit metal uptake and translocation or to accumulate these metals within the cells at high level in non-toxic forms (Baker, 1981; Wang, 1991). The later category of species, which takes up large amounts of metals and transport them to the above-ground parts, usually designed as a hyperaccumulator (Brooks, 1977). Unlike many other metal-tolerant plants, hyperaccumulators have an exceptionally high concentrations of metals, usually more than 100 times larger than those of many other metal-tolerant plants (Brooks, 1977). Baker *et al*. (Baker, 1994) reported that the natural population of *T. caerulescens* in the UK accumulated 21000 mg Zn kg^{-1} dry matter in the shoots. The ability to hyperaccumulate heavy metals from soil is one of the most important criteria in the selection of plant species for phytoremediation purposes (McGrath, 1997).

Agropyron elongatum, a perennial grass, is a metal hyperaccumulator and endemic to saline alkali soils of the USA. However compared to other metal hyperaccumulators, the nature of the species is less investigated.

Understanding the biology and chemistry of the rhizosphere is essential for determination of the mobility and availability of metals at the soil-root interface (Kafkorfietall, 1992; Uren, 1988). Exudation of organic compounds by roots may influence ion solubility and uptake through their indirect effects on microbial activity, rhizosphere physical properties and root growth dynamics and directly through acidification, chelation, precipitation and oxidation-reduction reactions in the rhizosphere (Marschher, 1995; Uren, 1988). Of these compounds, low-molecular-weight organic acids (LMWOAs) are of particular importance due to their metal chelation properties for mobilization of heavy metals (Mench, 1991; 1988; Cieslinski, 1998). Krishuamurti *et al*. (Krishuamurti, 1997) showed that various LMWOAs could influence the rate of Cd release from soils and therefore increase the solubility of Cd in bulk soils through the formation of soluble Cd-LMWOA complexes. Citrate has been proposal to be a major chelator for nickel in hyperaccumulators (Baker, 1990) and malate was the most abundant organic acid in Zn-hyperaccumulator of *Thlispi* caerulescens (Roser, 1996). However, little is known about the kinds and concentrations

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of LMWOAs excreted by roots from *Agrogyron elongatum*.

In the present study, we investigated the effects of Cd, Cu, Ni and Pb on the growth and metal concentration in *A. elongatum* to assess its tolerance towards these elements. We also determined the malic, oxalic and citric acids in root exudates.

1 Material and method

1.1 Material and culture

Seeds of *Agrogyron elongatum* were germinated on a mixture of vermiculite and sand moistened with deionized water. Two days later, the seedlings emerged and were grown in a greenhouse at ambient day /night temperature 28/20℃, with natural illumination supplemented daily with 10h of artificial light. After having grown for eight days in greenhouse seedlings were transferred to a variety of containers for hydroponics with a quarter strength modified Hoagland's nutrient solution consisting (μmol/L): KNO_3 5000; $Ca(NO_3)_2$ 2500; $MgSO_4$ 1000; $NH_4H_2PO_4$ 500; and micro-nutrients (μmol/L): NaCl 10; $ZnSO_4$ 2.5; $CuSO_4$ 0.5; $MnSO_4$ 1.0; Fe-EDTA 30. The solution was buffered to pH 5.8 with 1 mol/L HCl or NaOH and aerated continuously.

1.2 Treatment

After a two-day preculture, metal treatments began. On day 1, seedlings were transferred to each of a series of 500 ml containers filled with nutrient-free metal treatment solution (NFMTS). Four kinds of metals Cd, Cu, Ni and Pb were separately applied at different levels of concentrations as either $CdCl_2$ (0, 0.05, 1, 5, 10, 20, 50, 100 mg/L), $CuCl_2$ (0, 0.1, 0.5, 1, 10, 25, 50 mg/L), $NiCl_2$ (0, 0.5, 1, 10, 50, 100, 250 mg/L) or $PbCl_2$ (0, 0.5, 1, 5, 10, 50, 150, 300, 400, 500 mg/L). Each treatment was randomized with three replications.

The metal treatment began in the morning and lasted for 6h. Upon the end of treatment, the plants were removed to the containers with combined metal nutrient solutions (MNS) including half concentrations of above Hoagland medium and separate metal of different levels. The left metal treatment solutions (NFMTS) were collected for analysis of organic acids.

Once plants were introduced to the metal nutrient solution treatment, the pH of solution with different metal was maintained differently. For Cu, the pH was adjusted to 4.5 – 4.6, Cd was 6.5 – 6.6, and Ni and Pb was 5.8 – 5.9. Plants were growing in the metal nutrient solution. On day 5, the plants were again subjected to the nutrient-free metal treatment solution (NFMTS) for 6h. After that, they were moved back to containers with metal nutrient solutions (MNS). The left metal treatment solution (NFMTS) collected again for organic acid determination. This was done once again three days later (on day 9).

Plants were harvested on day 9. At harvest, two groups of plants of each treatment were divided. One group of plants was measured for the length of root, stem and leaf. All the roots and leaves in a single plant were measured and the mean values of root and leaf were expressed. For the second group, roots of intact plants were thoroughly rinsed with deionized water and blotted dry. Roots and shoots were separated. Samples were dried at 70℃ in a forced-air oven, weighted and digested with 1∶1 nitric/perchloric acid. Cd, Cu, Ni and Pb concentrations were determination by atomic absorption spectrometry.

1.3 Analysis of organic acids

For the analysis of root organic acid exudates, three collections on day 1, 5, and 9 were obtained and a portion of each collection was freeze dried. The dried exudates were resolved in 3 × 5 ml double-distilled water. The root exudates were run through a cation exchange resin column (16 mm × 25 cm) containing 5g Dowex 5W × 8 (100 – 200 mesh, H^+ form) in a cold room. The mixture of organic acids were eluted by 35 ml double-distilled water running at 2 ml/min. The eluent was collected and freeze-dried again. After the residue was re-dissolved

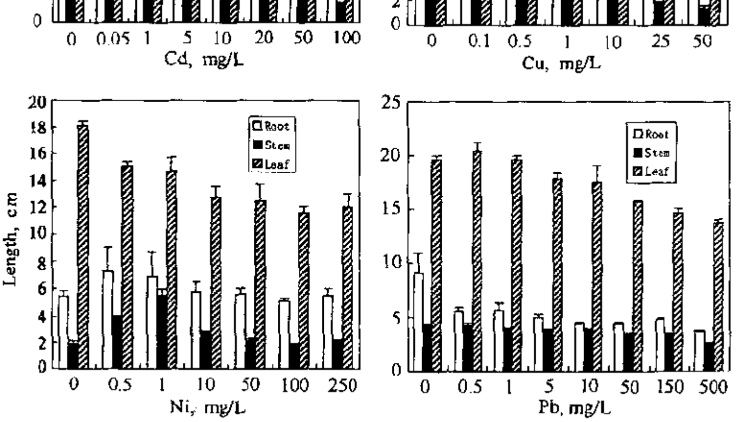

Fig.1 Root, stem and leaf length of *Agrogyron elongatum* seedling grown in the presence of Cu, Cd, Ni and Pb

in 3 ml Milli-Q water, the concentration of organic acids was analyzed by IC.

A Dionex DX 500 ion chromatography system, equipped with CD 20 electrochemical detestor in conductivity mode, GP 40 gradient pump, ASRA-ULTRA anion micromenbrane suppressor and computer interface with Dionex software (Peaknet), was used to quantify LMWOAs. Column used for determination of LMWOAs included an anion trap column (IonPac ATC-1), a guard column (IonPac AG-11 50 × 4 mm I.D) and an analytical column (IonPac As-11 250 × 4 mm I.D). The gradient pump mixed three different solutions: 5 mmol/L NaOH, 100 mmol/L NaOH and Milli-Q water, using an eluent flow rate of 2 ml/min.

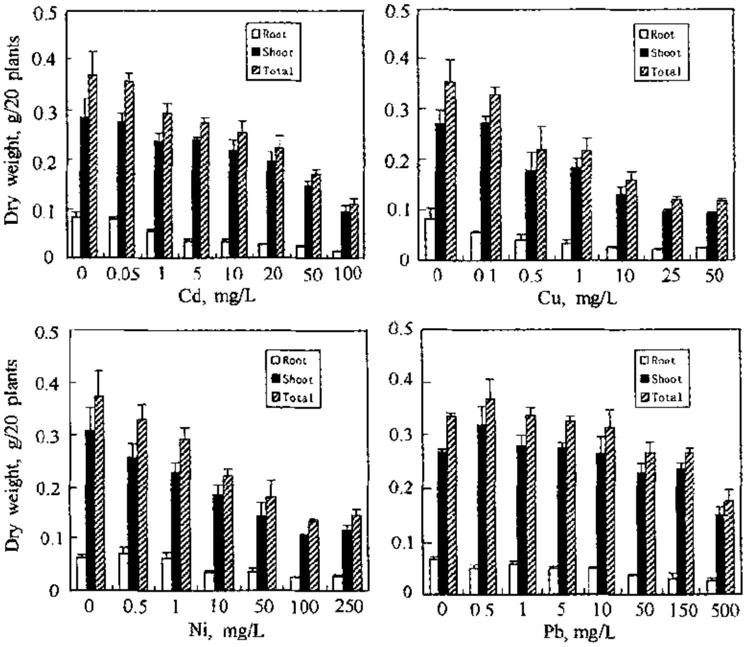

Fig.2 Root and shoot dry weight on a 20 plant basis for *Agrogyron elongatum* grown in the presence of Cd, Cu, Ni and Pb

2 Results

2.1 Effect of metals on the growth of *A. elongatum*

The results from the growth experiment showed that application of heavy metals of Cd, Cu, and Pb generally led to decrease in the elongation of roots, stems and leaves of *A. elongatum*, but the length of roots and shoots of seedlings exposed to Cd at 0.05 mg/L was showed to be slightly greater than those of control (Fig.1). The root and stem elongation of Ni-treated plants was hardly affected and plants incubated in 0.5, 1, 10, 50 μg/ml Ni-containing medium had greater development in their stems and leaves than control.

Over the whole range of Cd, Cu and Ni concentrations, both root and shoot biomass generally decreased (Fig.2). However, growth of plants was not affected at low and moderate Pb concentrations. Only at very high external concentration did the biomass production decrease.

2.2 Metal concentration and accumulation in *A. elongatum*

Concentrations of Cd, Cu, Ni and Pb within roots and shoots progressively increased with the external element levels (Fig.3). Of the four trace elements, Pb was uptaken and accumulated to a greatest degree, with the concentrations of 92754 mg/kg dry DW in roots and 11683 mg/kg DW in shoots. At all concentrations, Pb concentrations in roots were about 8 to 51 times higher than those in

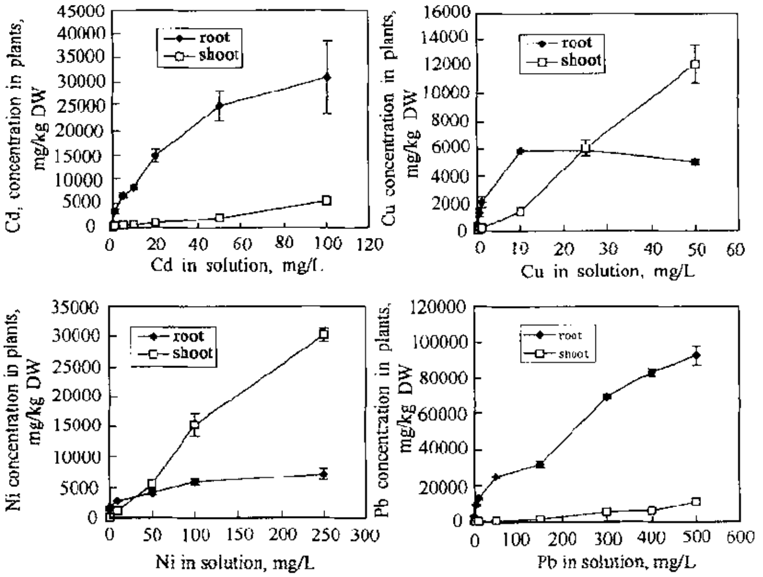

Fig.3 Cu, Cd, Ni and Pb concentration in *Agrogyron elongatum* grown in the presence of the metal

shoots. The bioaccumulation coefficients (BC), which measures the ratio of metal concentration in plant tissues to the concentration in the nutrient solution was highest at low Pb concentration (Fig.4). The maximum BCs for roots and shoots were 4856 and 175 respectively when 0.5 mg/L Pb was applied in the solution, while the minimum BCs for roots were observed when 500 mg/L Pb was supplied.

The pattern of Cu and Ni uptake and distribution in plants differed from that of Pb, Cu and Ni concentrations in roots and shoots increased with the external concentrations (Fig. 3). However, Cu concentration in roots did not reach maximum but slightly declined at the highest level of Cu in the solution. This was because the roots began to die of severe metal toxicity before the end of the treatment. Both Cu and Ni concentrations in roots were higher than in shoots at low external concentrations (0 - 25 mg/L), but at high concentrations, the two metal contents in shoots exceeded those in roots (Fig. 3) and most of elements absorbed by plants were accumulated in shorts (Table 1). The plants had the highest Ni concentrations in shoots, with the content of 30261 mg/kg DW. The plants of *A. elongatum* shoots showed no sharp decrease in BCs for Ni and Cu with the increasing external concentrations (Fig. 4). They showed U-shaped BC curve. That is, the BCs for Ni and Cu gradually decreased at low external concentration, then declined to the lowest point at medial concentration, and after that, increased at high concentration.

Concentrations and BCs curves for Cd were similar to those for Pb (Fig.3, 4). Cd contents in root and above-ground were much lower than those of Pb. The highest concentrations of Cd were 31004 mg/kg DW in roots and 5575 mg/kg DW in shoots. But the BCs for Cd at all concentrations were higher than in Pb (Fig.4).

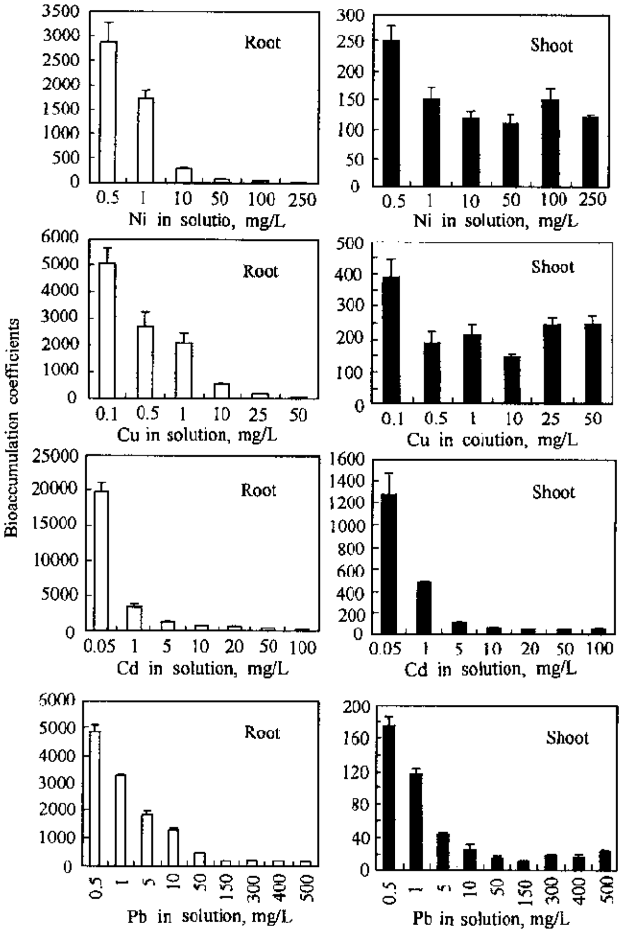

Fig.4 The ratio of Cu, Cd, Ni and Pb concentrations in plants tissues (DW) and those in nutrient solution

Table 1 Percentage of metal amounts in shoots to those in whole plants

Metal	Concentration in solution, mg/L	Root,	Shoots, μg/20 plants	Whole plants,	Shoot/whole plant, %
Cd	0.05	80.0 ± 9.0	17.7 ± 2.3	97.7 ± 6.5	18.1 ± 1.2
Cu	0.1	28.3 ± 1.3	10.9 ± 0.5	39.2 ± 2.1	27.8 ± 3.2
Ni	0.5	101.1 ± 9.8	32.8 ± 2.1	133.9 ± 11.2	24.5 ± 1.3
Pb	0.5	120.4 ± 1.3	28.0 ± 1.3	148.4 ± 10.8	18.9 ± 2.0
Cd	50	625.6 ± 59.9	304.9 ± 20.2	930.5 ± 83.0	32.8 ± 1.9
Cu	25	138.8 ± 8.6	593.6 ± 30.2	732.4 ± 36.2	81.0 ± 6.3
Ni	100	158.5 ± 16.3	1649.9 ± 111.2	1808.4 ± 200.5	91.2 ± 5.9
Pb	400	2185.6 ± 196.3	980.9 ± 46.3	3166.5 ± 333.2	30.9 ± 2.8

The net accumulation was used to illustrate the metal amount per unit plant or pot (one pot contained 20 plants). Generally, metal accumulation increased with external concentrations for the four elements, with an exception of Cd content decreasing at 100 mg/L (Fig.5). Ni was the metal which was mostly accumulated in shoots of *A. elongatum*, with the highest accumulation of 3.5 mg/pot. The rest of metals were accumulated in shoots in a decreasing order of Pb (1.7), Cu (1.1) and Cd (0.5) (mg/pot).

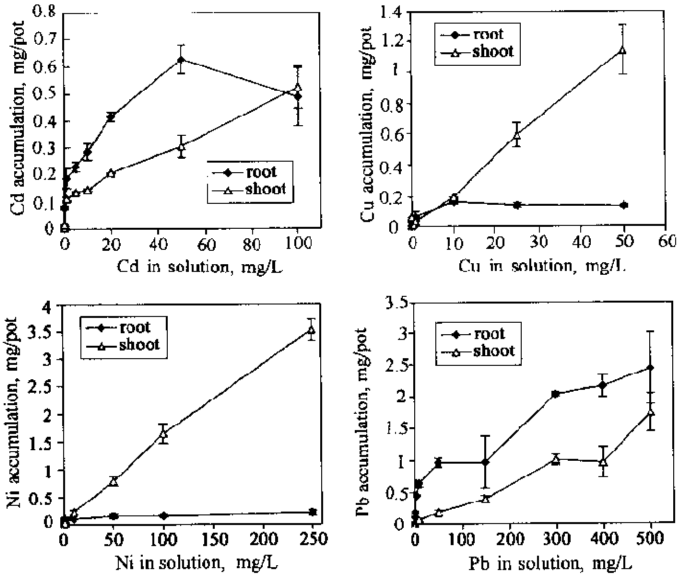

Fig.5 Cu, Cd, Pb and Ni accumulation in *Agrogyron elongatum* grown in the presence of the metal

2.3 Exudation of organic acids

Three kinds of organic acids malic, oxalic and citric acids exuded by roots of *A. elongatum* exposed to 1 and 50 μg/ml Cd, Cu, Ni and Pd were detected (Fig.6, 7). Release of organic acids from plants significantly differed among the metal treatments. Cu was most effectively in inducing root exudation of the three types of organic acids. Cd and Ni were also the strong inducers of excretion of malic and oxalic acids although their abilities of root exudation were weaker than that of Cu. For Pb, a small amounts of malic and oxalic acids were detected in the root exudates, but few quantities of citric acid were found. Exudation of citric acid by *A. elongatum* roots was metal-dependent as there was no citric acid detected in the control medium.

Although *A. elongatum* exuded different kinds of organic acids when exposed to Cd, Cu, Ni or Pb, the root exudation varied depending on the concentration applied and time of metal stress. For example, an exposure of plants to a relative low Cu concentration (1 μg/ml) induced the roots gradually to exude malic acid through the time course of day 1, 4, and 8, while a relative high (50 μg/ml) concentration of the metal initially lead to a dramatically high exudation of malic acid at initial treatment and it declined afterwards (Fig. 6).

For the total amount of organic acid secretion by root, there were no significant differences among the treatments of 1 and 50 mg/L Cu, Ni and Pb except for Cd (Fig. 7). The ability to exude organic acids by roots decreased by the order of Cu > Cd > Ni > Pb.

3 Discussion

Although the bioaccumulation of Cd, Cu, Ni and Pb by *A. elongatum* increased with the external metal concentrations, plants generally did not showed increased dry matter production except that maximum yields were achieved at 0.5 – 10 mg/L Pb concentrations (Fig.2). Pb was the most substantial element accumulated in *A. elongatum* tissue in this experiment. At designed concentrations, this species was tolerant to a Pb concentration up to 500 mg/L, where the maximum concentration in shoots was up to 11683 mg/kg DW. In previous limited experiments, the species *Thlaspi notundifolium* Gaud-Beaup was reported for its accumulation of Pb at 8500 mg/kg dry weight (Reeves, 1983). Cultivars of *Brassica juncea* Czern have also demonstrated the ability to accumulate as high as 1.5% Pb in shoot tissues when grown in nutrient solution with high concentrations of soluble Pb (Kuma, 1995; Blaylock, 1997). But it should be noted the high Pb concentration in these plants was achieved in a hydrophobic system. Under actual field conditions, however, Pb ion can not absorbed by 100 percent. Several other factors such as soil pH, precipitation of Pb with anion and content of organic matter may limit plant's ability to uptake Pb from soil solution (Ebbs, 1997).

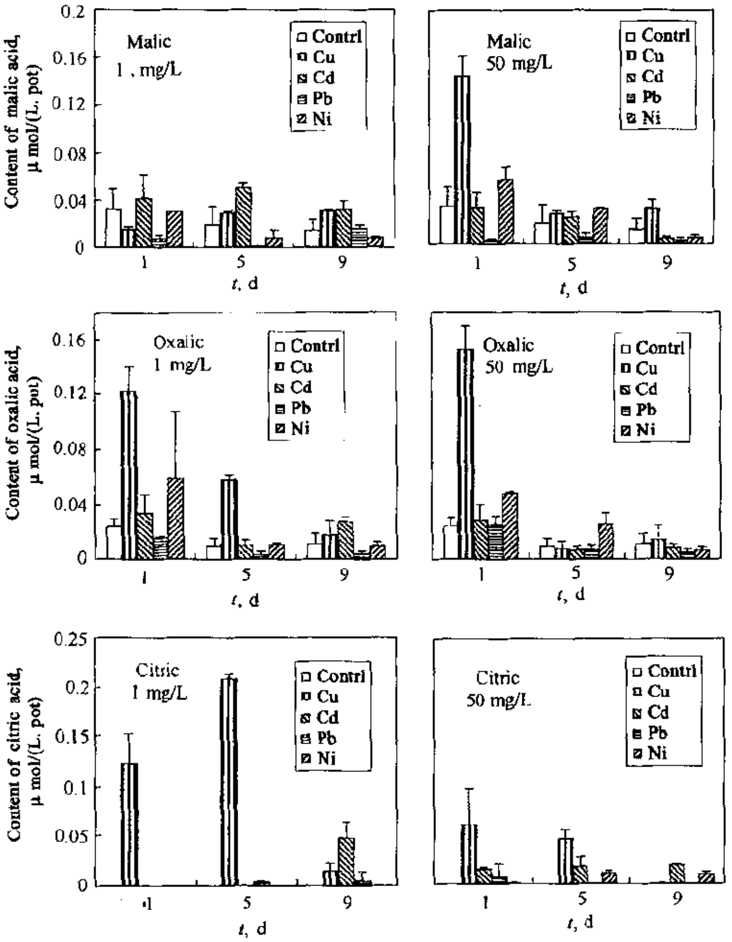

Fig.6 Exudation of malic, oxalic and citric acids by roots of *A. elongatum* exposed to 1 and 50 mg/L Cd, Cu, Ni and Pb in the medium

Tissue concentration has been used as a criteria for identification of hyperaccumalators (Reeves, 1996), but bioaccumulation coefficients (BC) or bioconcentration factors (BCF) may sometimes be better indicators because the use of element concentration does not take into account the trace element concentration in the substrate (Zhu, 1999). The results showed BCs were very high for Pb at low external concentration. Zayed *et al*. (Zayed, 1998) noted a good accumulator should have: (1) the ability to take up more than 0.5 % dry weight of a given element; and (2) the ability to bioconcentrate the elements in its tissue, with a bioconcentration factors of more than 1000. The results obtained from the Pb experiment met the criteria (Pb concentration = 9.3%, BC = 20000 in roots; Pb concentration = 1.2% in shoots). Compared to root concentration, foliar Pb concentration was much lower (Fig.3, Table 1). This pattern of Pb distribution in *A. elongatum* was attributed to lead retention in roots, as Pb is considered to be bond to ion exchangeable sites on the cell wall and extracellular precipitation in the form of lead carbonates deposited in the cell wall (Dushenkov, 1995). However, even from phytoremediation point of view, *A. elongatum* is a good Pb accumulator as the amounts of Pb in shoots based on unit plant was still significant (Fig 5), reaching the maximum bioaccumulation of 1298 mg/kg DW at 500 mg/L external concentration, and the greatest removal of Pb to plants

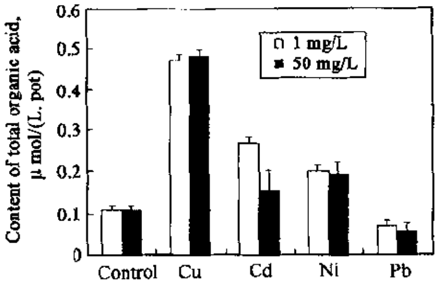

Fig.7 Total amount of exudation of malic, oxalic and citric acids (day 1, 5 and 9) by roots of *A. elongatum* exposed to 1 and 50 mg/L Cu, Cd, Ni and Pb in the solution

from solution per unit plant was observed.

A. *elongatum* had the similar pattern for Cd to Pb. Cd concentrations in plant roots were 5 – 15 times higher than in shoots (Fig.3). High Cd accumulation in plant roots is partially due to the binding of the cationic Cd to the anionic sites in the cell wall (Zhu, 1999). This fact suggests that translocation of the trace element from root to shoot could be a limiting factor for the bioconcentration of Cd in shoots. With regard to BC, Cd is a effective element most efficiently accumulated by *A*. *elongatum* as both root and shoot BCs for Cd were much higher than those for Pb (Fig. 4). However, the quantitative removal of Cd from solution per unit plant was much less effective compared to Pb (Fig.5).

The pattern of Ni and Cu accumulation and distribution differed from that of Pb or Cd. The most pronounced characteristics were their higher accumulation in shoots (Table 1, Fig. 3). The higher Ni concentrations in shoots than in roots in *A*. *elongatum* are typical pattern of a hyperaccumulator plant, which is more effective at translocating the metal from roots to shoots. A previous study conducted by Baker (Baker, 1981) showed that the ratio Ni concentration in shoot/Ni concentration in root in *A*. *murale* was between 2.0 – 2.4. In the present experiment, the ratio were 1.3 – 4.2 at the levels of 50 – 250 mg/L Ni in the solution. Of the two metals, Ni was more efficiently accumulated in the shoots of *A*. *elongatum* (Fig.3, 5). The maximum Ni concentration in shoots was 30260 mg/kg DW, while that of Cu was 12230 mg/kg DW. According to Baker and Brooks (Baker, 1989), Ni hyperaccumulator plants are defined as plants containing over 1000 mg/kg of Ni in the leaf dry matter, and they reported that in the family *Brassicaceae*, there were fourth-eight *Alyssum* species which were able to accumulate 1280 to 29400 mg/kg Ni in their dry leaves. The results agree with the previous reports and suggest that *A*. *elongatum* has a much higher potential for removal of Ni from contaminated soil or waters.

The least concentration of Cu was observed in *A*. *elongatum* (Fig.3), but the BCs for Cu in roots and shoots were comparable with those Pb and Ni. This suggests *A*. *elongatum* had the high efficiency in bioaccumulation of Cu without a great risk of being subjected to very high external concentration.

Plants tolerant to high metal concentrations and with high accumulation of metals must have efficient detoxification mechanisms. Various mechanisms have been proposed to account for the accumulation of these potentially toxic heavy metal ions, such as Cd^{2+}, Zn^{2+}, Pb^{2+}, Ni^{2+} and Cu^{2+} (Rauser, 1990; Wang, 1991). One of them proposed by Ernst *et al*. (Ernst, 1992) and Verkleij *et al*. (Verkleij, 1990) is that binding of the potentially poisonous metals by organic acids excreted by plant roots and thus reduce the activity of metal ions in the metabolically actively cell compartments. Mench and Martin (Mench, 1991) found that organic acids released by *Nicotiana tabacam*, *Nicotiana rustica* and *Zea mays* were able to increase Cd accumulation in plants, and also root exudates increased the solubility of Mn and Cu, whereas those of Ni and Zn were not affected. However, information on organic acid exudation of hyperaccumulator plants and how this is related to the uptake and accumulation of metals is rare.

In the present study, three kinds of organic acid were detected. Release of organic acid from plants was significantly different among the metal treatments. Cu was most effective in inducing malic, oxalic and citric acids (Fig 6, 7). The quantitative predominant exudation of organic acids by *A*. *elongatum* roots in the present of Cu might be responsible for the chelation of Cu in the solution, making it easier for Cu translocation to shoots. This mechanism may take advantage because soluble metal-organic acid complexes are considered to be a form of metal detoxification, and it is easy within the plants to translocate them into intracellular fractions for compartmentation. However, no correlation between alternations in root excretion of organic acids and Cu accumulation could be established in the present study.

Cadmium, nickel, and lead were less effective inducers of organic acids tested. There were similar patterns for Cd, Ni and Pb to exude malic and oxalic acids, and that was a relative high amount of exudates at the initial metal treatment, followed by decreasing exudation (Fig.6). It is noted that the blank concentration of metals showed the same pattern for exudation. This suggests that non-specific exudation of malic and oxalic acids by roots may exist in *A*. *elongatum*. For the quantitative exudation of the two organic acids, there was no significant difference between 1 and 50 mg/L treatments of Cd, Pb and Ni. Also, no definite correlation between the root exudes and metal accumulation was observed although Cd, Pb and Ni were accumulated more in plants. Compared to malic and oxalic acids, exudation of citric acid by roots of *A*. *elongatum* in the presence of Cd, Pb and Ni appeared much less. Very few amount of citric acid was detected from the Pb-treated plants either at 1 or 50 mg/L Pb rate. There was also very small amount of citric acid exuded by plant root in presence of Cd and Ni.

Results of the tested organic acids released from roots of *A*. *elongatum* in this experiment are preliminary. More further investigations will be required. The work includes at least: (1) characterization of specific exudation of organic acids by the plant roots exposed to these heavy metals; (2) the ability of organic acid exudation and its complexation with metals in soil-plant interface; (3) other chelators (amino acids, non-protein phytochlelatins etc.) in rhizosphere and plants to be identified and tested; (4) soil experiment because such a study are more close to field and natural conditions, where exudation of organic compounds by roots are influenced by physical properties, chemical and biological reactions in rhizosphere and varying plant growth state.

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