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The Low Molecular Weight Organic Acids in Root Exudates of *Leersia hexandra* Swartz and Its Role in Mobilization of Insoluble Chromium

Chancui Wu[1, 2], Jie Liu[3], Ying Liang[2], Yongrong Jiang[2] and Xuehong Zhang[2, *]

[1]Light industry and Food Engineering College, Guangxi University, Nanning, 530004, China;
[2]School of Life and Environmental Sciences, Guilin University of Electronic Technology, Guilin 541004, China;
[3]Guangxi Key Laboratory of Environmental Pollution Control Theory and Technology, Guilin University of Technology, Guilin, 541004, China
Email: wuchancui@163.com

Abstract. The root exudates of *Leersia hexandra* Swartz were collected to investigate their mobilization of chromic oxide (Cr_2O_3), lead chromate ($PbCrO_4$) and Cr-contaminated soil. It was observed that the root exudates can mobilize Cr_2O_3, $PbCrO_4$ as well as Cr-contaminated soil. Low molecular weight organic acids in the root exudates *L. hexandra* were analyzed by high performance liquid chromatography (HPLC). Six organic acids, including oxalic acid, tartaric acid, malic acid, lactic acid, maleic acid and citric acid, were detected in the root exudates, and their concentrations were 18.91±0.23, 130.90±1.44, 1031.34±4.38, 65.54±1.01, 0.96±3.67×10^{-3} and 201.50±1.13 μg (g root DW)$^{-1}$, respectively. Chemical reagents of these 6 acids were used to mobilize Cr_2O_3, $PbCrO_4$ and Cr-contaminated soil. The mobilization of different organic acids to Cr-contamination soil declined in the order of oxalic acid > malic acid > lactic acid > tartaric acid > maleic acid > citric acid. These results suggested that organic acids in the root exudates play an important role in uptaking a large amount of Cr from soil by *L. hexandra*. Information obtained from this study should develop the understanding of the accumulation mechanism of *L. hexandra* to Cr in the soil, and provide insights for improving the efficiency of phytoremediation.

1. Introduction

Root exudates are plant metabolites that are released to the root surface or into the rhizosphere to enhance plant nutrient uptake [1], [2]. They are generally classified into two types, high molecular weight (polysaccharides and proteins) and low molecular weight (i.e. amino acids, organic acids, sugars, phenolics) compounds [3]. Both of them play an important role in these rhizospheric processes [4], while low molecular weight organic acids (LMWOAs) in root exudates would affect metal availability in the soil. Moreover, heavy metal bioavailability is the most important factor to be monitored in the restoration process of a contaminated soil [3]. However, the composition and quality of root exudates vary from plant to plant. Therefore, study on LMWOAs in root exudates and its role in effect on heavy metal bioavailability could provide the basis to the phytoremediation of contaminated soil.

Hyperaccumulator is an efficient technology based on the use of plants to remove metals from contaminated sites, which are potential tools for phytoremediation [5]. There are specific physiological characteristics of metal uptake in the roots of hyperaccumulators. Most plants are capable of exuding some LMWOAs, such as citric, malic and oxalic acids [6]. These organic acids can react with metal ions in both the soil solution and solid phases, and increase metal mobility in the rhizospheric environment, thereby improving the phytoavailability of metals to plants [7]. Research on *Thlaspi caerulescens*, a Zn/Cd hyperaccumulator, indicates that the plant mobilizes Zn and Cd efficiently from soils [8]. Similar results were obtained for a Ni hyperaccumulator, *Thlaspi goesingense* [9]. However, there is still a vast gap in our understanding of the involvement of the LMWOAs in the root exudates of Cr hyperaccumulation.

Leersia hexandra Swartz has been found to be a Cr hyperaccumulator with an extraordinary accumulation capacity for both trivalent Cr and hexavalent Cr [10], [11]. A great bioaccumulation capacity for Cr was observed in the leaves, stems and roots of *L. hexandra*. The maximum Cr concentrations in the leaves, stems and roots were 5430 mg kg^{-1}, 1956 mg kg^{-1} and 40599 mg kg^{-1}, respectively [12]. *L. hexandra* is suitable for phytoremediation to Cr-contaminated soil [13]. It is hypothesized that a considerable amount of Cr uptaked by *L. hexandra* from the contamination soil owe to the LMWOAs in the root exudates. In order to investigate the role of root exudates in mobilizing Cr, hydroponics experiments have been conduct to study the root exudates of *L. hexandra*. The objectives of this research were: 1) to investigate the mobilization of insoluble chromium by the root exudates of *L. hexandra*; 2) to analyze the composition and quantity of the root exudates of *L. hexandra* by HPLC, and 3) to test the mobilization of insoluble chromium by organic acids. This study will provide scientific basis for improving phytoremediation efficiency to Cr-contaminated soil by *L. hexandra*.

2. Materials and Methods

2.1. Plant Culture and Root Exudates Collection

Seedlings of *L. hexandra* were collected from the riverside of Hua River in Guilin. The seedlings were washed thoroughly (at least three times) with redistilled water and placed in the round plastic pots filled with 1.5 L 25% Hoagland's nutrient solution which renewed every 3 days. Plants were grown in the pots for 20 days in a controlled environment growth cabinet (14 h photoperiod, 25 °C day/18 °C night, relative humidity 70-75%, and a light intensity of 300 μmol $(m^2\ s)^{-1}$).

In this work, the periods of 4 hours under sterile conditions was chosen to collect root exudates. The root exudates collection media used in this work was $CaCl_2$ (0.5 mmol L^{-1}). After being filtered to 0.45 μm with PTFE membrane filters, the collection solution of root exudates were concentrated on the Rotary Evaporator (45 °C, 80-90 r min^{-1}) to 25 ml, and concentrated solution of root exudates were reserved in the refrigerator (-20 °C) for mobilization experiments. In the meanwhile, 2-3 drops of 0.05% thymol were used in the bath, collecting and reserving to control the activities of microorganisms. Additionally, the concentrated solution of root exudates was vacuum-freeze dried then dissolved with 3 ml ultrapure water and filtered (0.45 μm) into the automatic sample bottles for HPLC analysis.

2.2. HPLC Analysis of Organic Acids In the Root Exudates

Common LMWOAs secreted by plant roots [14], [15] such as oxalic, tartaric, formic, malic, lactic, acetic, maleic and citric acids were prepared individually. Single stock solutions of 8 organic acids were mixed according to an optimum proportion and diluted by mobile phase to obtain the mixture standard solution.

The HPLC system, assembled from Waters' modules, was equipped with a 717 plus Autosampler; a 515 HPLC Pump operated under isocratic conditions; a 2996 Photodiode Array (PDA) Detector. The determination condition: XSelect HSS HPLC Columns (5 μm, 250 mm×4.6 mm, Waters, USA); mobile phase was: 40 mmol/L KH_2PO_4 (pH=2.40); wavelength was 205 nm; flow velocity was 1.0 ml/min; column temperature was 25 °C and the sample size was 10 μl. Qualitative and quantitative analysis of organic acids in the root exudates were performed by external standard method and measurement of peak area respectively.

2.3. Mobilization Experiments

The soils used in mobilization experiments were collected from the constructed wetland for treating chromium containing waste water. After drying (80 °C), grinding and sieving (100 mesh), the chromium content of the tested soil was determined, which has a value of 1.80 mg g^{-1}.

Concentrated root exudates of 25 ml were mixed with 0.200 g synthetic Cr_2O_3, $PbCrO_4$ and 2.000g Cr-contaminated soil respectively in a plastic centrifuge tube. After shaking samples for 1, 2, 3, 4 and 5 h (25 ºC, 150 r min^{-1}), and being filtered through the Syringe Filters (0.45 μm), the amount of mobilized Cr in root exudates were determined using atomic absorption spectrophotometer (AA-6300, Shimadzu, Japan). Working standard solutions for analysis of the elements were prepared with serial dilutions of stock standard solutions Cr (GBW (E) 080257). Ultrapure water was used to mobilize Cr_2O_3, $PbCrO_4$ and soil sample instead of the root exudates as the control. Chemical reagents of every organic acid are used to simulate root exudates and repeat the experimental steps described above. The mobilization of Cr was calculated as follows:

$$\text{mobilization}\left(\text{mg (kgCr)}^{-1}\right) = \frac{C_{\text{chromium}} \times V}{m_{\text{chromium}}} \quad (1)$$

Where C_{chromium} is the concentration of the Cr in the supernatant, V is the volume of the activator (root exudates, ultrapure water or organic acid solution) and m_{chromium} is the mass of chromium in the Cr_2O_3, $PbCrO_4$ and soil Cr added.

2.4. Statistical Analysis

The data presented in this paper are the average of three independent replicates ± standard error of means (SD). Each treatment was replicated at least three times. Analysis of variance (ANOVA) was performed on all data sets, and least significant difference (LSD) was used to compare treatments. Graphical work was performed using Origin Pro 8 and Excel 2003.

3. Results

3.1. Mobilization of Root Exudates on Cr-contaminated Soil And Insoluble Chromium

The effects of shaking time on Cr mobilization were investigated in Cr_2O_3, $PbCrO_4$ and soil Cr by the root exudates of *L. hexandra*. Results (Figure 1) showed that the mobilization of Cr_2O_3 and $PbCrO_4$ increased with the shaking time from 1 to 3 h and reached the greatest amount after 3 hours shaking. Moreover, the mobilization of soil Cr increased non-linearly with the shaking time and tended to steady. Shaking time ranges from 1 to 3 h enhanced the mobilization of soil Cr significantly. When the shaking time ranges from 3 to 5 h, there was no significant difference in the mobilization of soil Cr. Therefore, a shaking time of 3 h was selected for all other experiments.

The root exudates of *L. hexandra* were used to mobilize Cr_2O_3, $PbCrO_4$ and soil Cr with a shaking time of 3 h. Meanwhile, ultrapure water was used to replace the root exudates as the control. The result (Table 1) showed that the root exudates can mobilize Cr_2O_3 and $PbCrO_4$ as well as soil Cr. These tests revealed that the mobilization of Cr_2O_3, $PbCrO_4$ and soil Cr by the root exudates were all remarkably higher than that by water. The mobilization of $PbCrO_4$ by water was no detected. While the mobilization of Cr_2O_3 and soil Cr by the root exudates were 2.2 and 3.4 times higher than that by water respectively. The soil Cr is easier to be mobilized than Cr_2O_3 and $PbCrO_4$ by the root exudates.

Table 1. The mobilization capacity of chromium by root exudates of *L. hexandra* (*n*=3)

Insoluble Chromium	Mobilization (mg $kgCr^{-1}$)	
	ultrapure water	The root exudates of *L. hexandra*
Cr_2O_3	193.45±19.51	425.19±13.10
$PbCrO_4$	*n.d.*	292.12±16.05
soil Cr	201.39±78.26	694.44±133.94

3.2. Organic Acids In Root Exudates

Six organic acids in the root exudates of *L. hexandra* were measured by HPLC. The results showed (Table 2) that the contents of these 6 organic acids in root exudates were very different. The contents of malic acid was the greatest among the six organic acids assayed, which reached 1031.34 ± 4.38 μg (g root DW)$^{-1}$. The contents of the organic acids declined in the order of malic > citric > tartaric > lactic > oxalic > maleic. The contents of malic, citric, tartaric, lactic and oxalic acid are 1074.31, 209.89, 136.35, 68.27 and 19.69 times higher than that of maleic, respectively.

Table 2. The contents of organic acids in *L. hexandra* root exudates as determined by HPLC analyses (*n* = 5)

Organic Acids	Contents of organic acids ± SD (μg (g root DW)$^{-1}$)	Relative proportion
Oxalic	18.91 ± 0.23	19.69
Tartaric	130.90 ± 1.44	136.35
Malic	1031.34 ± 4.38	1074.31
Lactic	65.54 ± 1.01	68.27
Maleic	0.96 ± 3.67×10^{-3}	1.00
Citric	201.50 ± 1.13	209.89

3.3. Mobilization of Organic Acids on Cr-contaminated Soil And Insoluble Chromium

Results above indicated that there are organic acids in the root exudates of *L. hexandra* which can mobilize Cr_2O_3, $PbCrO_4$ and soil Cr. In order to verify the role of the organic acids in mobilizing insoluble chromium, chemical reagents of malic, citric, tartaric, lactic, oxalic and maleic acid were selected for simulation analysis of Cr mobilization of Cr_2O_3, $PbCrO_4$ and soil Cr. The results (Figure 2 to 4) showed that the Cr mobilization of three kinds of insoluble Cr are different, and vary from organic acid to organic acid. The mobilizations of soil Cr by all the 6 acids were higher than that of Cr_2O_3 and $PbCrO_4$. Considering the mobilization capacity of every organic acid, oxalic acid obtained the greatest, while that of citric acid was the lowest. The mobilization capacity of different organic acids to Cr-contamination soil declined in the order of oxalic acid > malic acid > lactic acid > tartaric acid > maleic acid > citric acid. On the other hand, the organic acids with concentration of 5mmol/L displayed the higher mobilization capacity compared to that of 1 mmol L^{-1}. The mobilization of Cr_2O_3, $PbCrO_4$ and soil Cr by 5mmol L^{-1} oxalic acid were 397.48±15.22 mg (kgCr)$^{-1}$, 431.18±18.53 mg (kgCr)$^{-1}$ and 1067.59±24.70 mg (kgCr)$^{-1}$, respectively.

According to the proportion of six organic acids in the root exudates of *L. hexandra*, a mixed solution of 6 organic acids are prepared for simulation analysis of Cr mobilization of Cr_2O_3, $PbCrO_4$ and soil Cr. The results of mobilization of insoluble chromium were compared with those of root exudates. The results (Figure 5) showed that the mobilization of Cr_2O_3 and soil Cr by the root exudates were 1.2, 2.0 and 1.4 times higher than that by the mixed organic acids respectively.

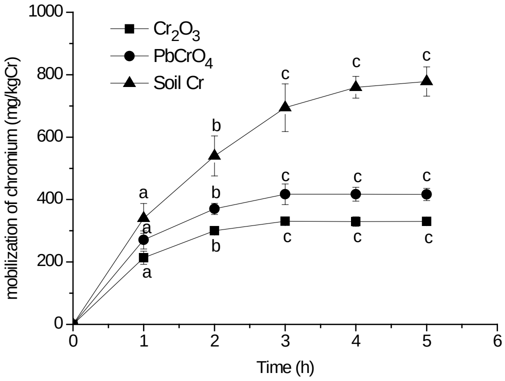

Figure 1. The effect of different shaking time on the mobilization of chromium by the root exudates of *L. hexandra* (n = 3)

Different letters above or below standard error bars indicated significant difference at P<0.05 (the same below)

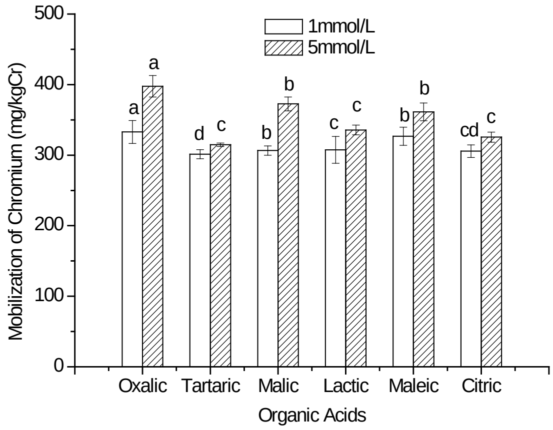

Figure 2. Mobilization of Cr_2O_3 by organic acids (n=3)

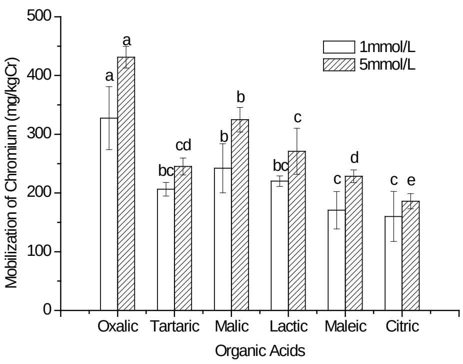

Figure 3. Mobilization of $PbCrO_4$ by organic acids (n=3)

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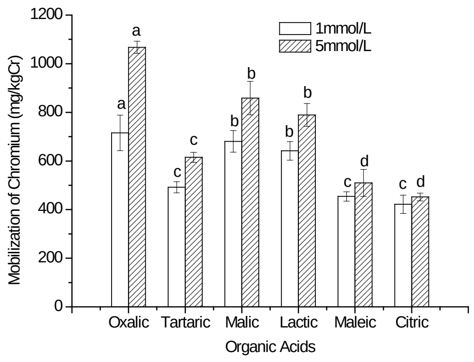

Figure 4. Mobilization of Cr-contamination soil Cr by organic acids (*n*=3)

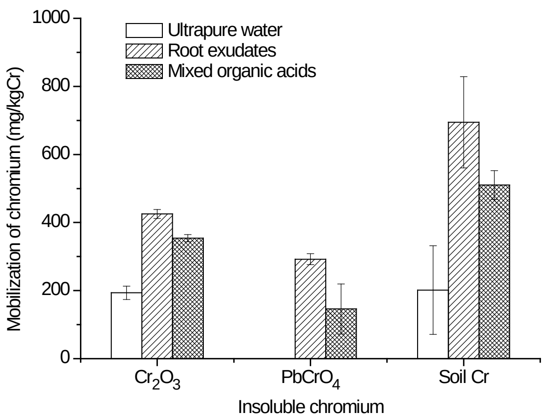

Figure 5. Comparison of mobilization of chromium by root exudates of *L. hexandra* and mixed organic acid solutions (*n*=3)

4. Discussion

In Cr-contaminated soil, most of the chromium exists in the forms of sparingly soluble mineral such as Cr_2O_3 and $PbCrO_4$. The available Cr concentration in soil is too low to be absorbed by plants. As a hyperaccumulator, *L.hexandra* shows a higher accumulation capacity for Cr than the non-hyperaccumulator. How is chromium in soil converted into the available form for use by plants? Our preliminary results indicated that the root exudates of *L. hexandra* can mobilize Cr_2O_3, $PbCrO_4$ and soil Cr effectively. The root exudates of *L. hexandra* solubilizing the insoluble chromium efficiently was probably attributed to the organic acids in the root exudates.

HPLC analysis identified that the dominating LMWOAs in the root exudates of *L. hexandra* were oxalic, tartaric, malic, lactic, maleic and citric acid (Table 2). Compared with findings in other plants, the amount of malic is the most in *L. hexandra*, which is agreed with that in rice [16] and *Zea mays L* [17]. but a little different from that in mangrove [15], which may be caused by the difference between grass and woody plants. Wang et al. [16] reported that the concentration of malic acid (3.9 μmol (g DW) $^{-1}$) was the greatest among the five organic acids assayed in rice, however, which was much lower than the amount of malic in *L. hexandra* (1031.34 μg (g DW)$^{-1}$). Aulakh et al. [18] also found that malic acid secreted by ten rice cultivars grown under soil conditions had the highest concentration followed by tartaric, succinic, citric and lactic acids. These findings suggest that malic acid secreted by roots may play an important role in adapting to the local environment. Oxalic acid is a typical LMW organic acid in plant root exudates. Since it possesses the capability of both proton donation and ion complexation, oxalate/oxalic acid has been widely used as an extractant for plant available nutrients, including phosphate, from soil [19]. Some grasses, such as rice [16], elephantgrass [20] and *Phragmites australis* [21], are known to contains oxalic acid. However, the contents of oxalic acid in these plants are lower than which in *L.hexandra*. It was interesting to note that the greatest Cr mobilization capacity of oxalic acid showed in simulation experiments. This may be due to both the greater acidity and stronger complexation capability of oxalic acid than other acids. In addition, there were significant differences in the amount of Cr-mobilization of different acids, which suggests that each organic acid plays different roles in rhizosphere chemistry and nutrient acquisition procedure. The process that was responsible for changes in rhizospheric pH involved the evolution of CO_2, root exudates, and the excretion or re-absorption of H^+ or HCO_3^-, as well as microbial production of organic acids [22]. According to the above view, in the case of mobilizing Cr from soil by *L.hexandra*, decrease pH in the rhizosphere is due to the organic acids secreted by the root. The results of mobilization experiments by the mixed acids indicated the mobilization of Cr_2O_3, $PbCrO_4$ and soil Cr by mixed organic acids were lower than that of the root exudates. Because root exudates of plants are varied in kinds and contents, In addition to the six organic acids detected in this study, the root exudates of *L. hexandra* also contain other components which may also play a role in mobilization of insoluble chromium. Thus, the components of the root exudates of *L. hexandra* should be studied further.

Although organic the role of acids in mobilization of insoluble chromium has been verified in this paper, study on the root exudates of *L.hexandra* under different culture condition has not been conducted. Therefore, other composition of the root exudates under different culture condition and their role in mobilization of Cr in soil require further study.

5. Conclusion

In summary, the root exudates of *L.hexandra* exhibited a considerable capacity in mobilizing Cr from Cr_2O_3 and $PbCrO_4$ as well as from Cr-contaminated soil. It's probably attributed to the organic acids secreted by the root of *L.hexandra*. The results of the simulation experiment which used the 6 organic acids detected in *L. hexandra* root exudates to mobilize the insoluble chromium and soil Cr provided further evidence for this hypothesis. This study demonstrated that the organic acids in the root exudates of *L.hexandra* play an important role in enhancing the bioavailability of Cr and are beneficial to improve phytoremediation efficiency.

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7. Reference

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