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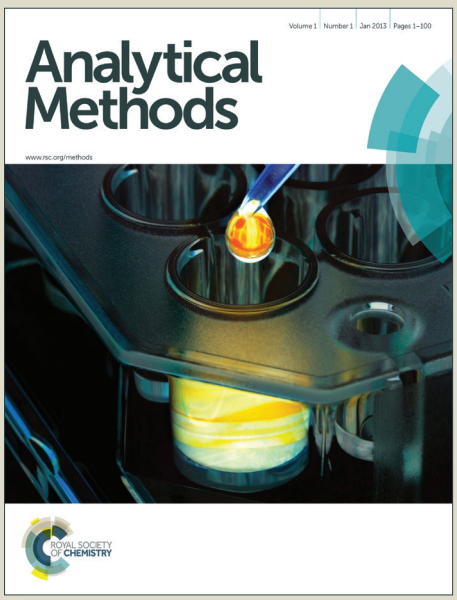

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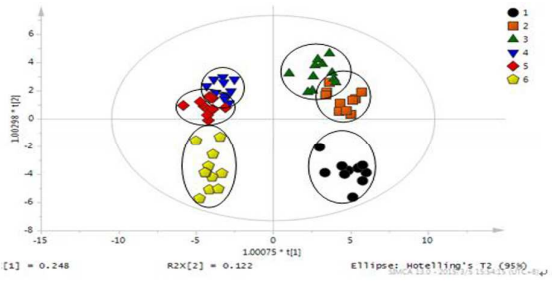

The variation of root exudates from the Cd hyperaccumulator *Sedum alfredii* under different Cd exposed concentration and time was researched.

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Metabolic profiling analysis of root exudates from the Cd hyperaccumulator *Sedum alfredii* under different Cd exposed concentration and time

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Abstract

Components of the Cd hyperaccumulator *Sedum alfredii* root exudates were surveyed by gas chromatography-mass spectrometry (GC-MS), the variation of root exudates from *S. alfredii* under different Cd exposed concentration and time was explored by metabonomics analysis, and the probable effect mechanism of *S. alfredii* tolerates or accumulates the heavy metal Cd was discussed. The root exudates were collected after 0, 5and 10 μmol/L Cd treated for 4 and 8 days. The collected solution was lyophilized and eluted with methanol, after derivatization with methoxyamine hydrochloride and N-methyl-N-trifluoroacetamide, the samples were analyzed by GC-MS. Principal component analysis and (PCA) and orthogonal partial least-squares discrimination analysis (OPLS-DA) were carried out for pattern recognition and a clear separation among the different treatments was achieved. 15 compounds resulting in the separation among the different treatments were found and identified. And the changing tendencies in the relative content of these 15 compounds under the different treatments were explored. These results indicated that the Cd hyperaccumulator *S.*

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alfredii could be able to adjust the secretion of root exudates to tolerate or accumulate the heavy metal Cd.

Key words: hyperaccumulator; *Sedum alfredii*; root exudates; GC-MS; metabonomics

Introduction

Plant root exudates are plant metabolites that are released to the root surface or into the rhizosphere to enhance plant nutrient uptake or copy with environment stresses, such as low-molecular-weight organic acids, amino acids, fatty acids and sugars.[1-4] They can be modifying the pH and Eh of the rhizosphere, chelating, complexing and depositing with heavy metals, altering the numbers and the activity of rhizoshperic microbes. Through these ways, root exudates can change the chemical existence of heavy metals and increase their bio-availability. [5]

Root exudates play an important role in the process of phytoremediation as an emerging green and in-situ remediation technology using plants to absorb, accumulate, stabilize or volatilize contaminant from soil.[6-8] However, previous studies were mostly focused upon the roles of root exudates, the changes in the total amount of the dissolved organic matter (DOM) or the dissolved organic carbon (DOC), or some specific organic acids and amino acids,[9-12] the components and variation of root exudates of hyperaccumulators under different stresses were rarely revealed.

In this study, the hyperaccumulator *Sedum alfredii*, a Zn/Cd hyperaccumulator native to China, with large biomass, rapid growth and asexual propagation,[13-14] was

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cultured in Cd stressed nutrition solutions. The analytical technique of gas chromatography- mass spectrometry (GC-MS), a mature method and has long been used for metabolite profiling of plant extracts,[15-17] was used to analyze the root exudates released from *S. alfredii*. And the metabonomics analysis method, a high throughput and unbiased comprehensive analysis method to study the dynamic change of endogenous metabolism of a biological system that is stimulated or disturbed,[18] was used to analyze the variation of root exudates from *S. alfredii* under the different Cd exposed concentration and time. Finally, we found out the compounds resulting in the separation among the different treatments. Then through the change trends of these compounds, we explored the possible mechanism of *S. alfredii* tolerates or accumulates the heavy metal Cd.

Material and methods

Chemicals and instruments

Methanol (HPLC grade, Fisher), pyridine (HPLC grade, Sinopharm), methoxamine hydrochloride (Sigma) and N-methyl-N-trimethylsilyl trifluoracetamide (MSTFA, Sigma) were employed in the study. Compositions of the nutrient solution and $CdCl_2$ were purchased from Sinopharm.

GC-MS (TRACE GC Ultra-PolarisQ, ThermoFisher, an ion trap mass spectrometry), nitrogen purging instrument (MG-2200, TOKYO RIKAKIKAI CO.LTD), and vacuum freeze drying system (FDU-1100, TOKYO RIKAKIKAI CO.LTD) were used in this study.

Plant material and growth conditions

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The plant materials of *S. alfredii* were collected from an old Pb/Zn mining area in Quzhou City, Zhejiang Province, China. The sampling site is located at 118°, 56' east longitude and 29°, 17' north latitude. It refers to the previous study for more information about this plant.[13] After having collected the plants, the rest of the experiments were undertaken in our laboratory. The shoot tops of *S. alfredii* were cut and cultured in a greenhouse in Shenyang University for 2 months. Healthy and uniform *S. alfredii* seedlings were selected and planted in the basal nutrient solution. The nutrition solution used was the half-strength Hoagland-Arnon solution,[19] which comprised of 3 mM KNO_3, 0.5 mM $NH_4H_2PO_4$, 2.0 mM $Ca(NO_3)_2$,1.0 mM $MgSO_4 \cdot 7H_2O$, 4.5 µM $MnCl_2 \cdot 4H_2O$, 23 µM H_3BO_3, 0.4 µM $ZnSO_4 \cdot 7H_2O$, 0.15 µM $CuSO_4 \cdot 5H_2O$, 0.05 µM $H_2MoO_4 \cdot H_2O$, and 22 µM EDTA-Fe. The nutrient solution was aerated continuously and renewed every 4 days, with its pH was adjusted to 6.0 using 0.1 M NaOH or HCl every day. The plants were grown under greenhouse conditions with natural light. The temperature varied from 10 to 20 °C. Until the relatively flourishing roots grow out, also were two weeks of pre-culture, *S. alfredii* were selected for 3 Cd treatments: 0 (control), 5, 10 µM Cd, and Cd was supplied as $CdCl_2$. There were 33 pots (1 piece per pot) in total, with 11 replicates for each Cd treatment.

Collection of root exudates

After having grown for 4 and 8 days in the nutrient solution spiked with Cd salts without renewal, the plants were transplanted to sterilized pots with 50 mL deionized water per pot to collect root exudates for 6 h. Sample preparation, derivatization and

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the GC-MS analysis were based on Suzuki et al.(2009)[16] and Lisec et al.(2006).[20] The root exudates from each pot were frozen in liquid nitrogen and freeze-dried for 2 days. The dried residue was resuspended in 100 mL of deionized water and freeze-dried again. The dried residue was redissolved in 10 mL of cold MeOH, then blown to dryness under a gentle nitrogen flow, and reconstituted in 1 mL of n-hexane used for the GC-MS analysis.

GC-MS analysis of root exudates

Samples were derivatized by 40 µL of methoxyamine hydrochloride (20 mg mL^{-1} in pyridine, 2 h, 37 °C) and 70 µL N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) (30 min, 37 °C). 1 µL of the sample was injected into the GC in the splitless mode. The GC analysis was carried out on a TR-5MS capillary column (30 m, 0.25µm, 0.25 mm, Thermo Fisher, USA). The injection, interface and ion-source temperatures were adjusted to 230, 250 and 210 °C, respectively. The gas flow rate was 1 mL min^{-1}, the column temperature was held for 1 min at 70 °C, 6 min ramp to 76 °C, 50 min ramp to 330 °C, 10 min at 330 °C. The column end was introduced into an ion trap mass spectrometer. Mass spectra were recorded at 2 scans s^{-1} with a m/z 50-600 scanning range.

Validation of the assay method

Because of the inhomogeneity of the plant growth, a large number of the same sample of root exudates is difficult to acquire, and this assay method is not aimed at the specific target compounds, the validation of this assay method is only involved the instrument performance. A post-preparative sample of root exudates was selected as

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the quality control (QC) sample to monitor the reproducibility and stability of the GC-MS analysis method. The intra-day precision was determined by detecting the QC sample seven times on one day. The inter-day precision was determined on six continuous days. During the analysis, QC sample is inserted at intervals and analyzed once every 10 samples during the analytical sequence to test the system stability.

Data analysis

The raw GC-MS chromatogram was automatically analyzed using the automatic mass spectral deconvolution and identification system (AMDIS, version 2.71) which obtained from NIST, and compared with the database of metabolites in plants (Fiehn and Golm Metabolome Database (GMD)). If the similarity was greater than 70%, the compounds were being identified. Then use the metabolomics ion-based data extraction algorithm (MET-IDEA, version 2.08) to extract and process the AMDIS output, 68 compounds were detected in one GC-MS scan. The parameters for MET-IDEA were: (i) chromatography: GC; average peak width, 0.1; minimum peak width, 0.3; maximum peak width, 6; peak start/stop slope, 1.5; adjusted retention time accuracy, 0.95; peak overload factor, 0.3; (ii) mass spec: trap; mass accuracy, 0.1; mass range, 0.5; (iii) AMDIS: exclude ion list, 73, 147, 281, 341, 415; lower mass limit, 50; ions per component, 1. The peaks of 11.09 min which detected in all samples were used for retention time calibration.

Normalize the peak area of the identified root exudates, the normalization was according to each compound based on the first appeared by the following and the normalization method is that the peak area values were divided by the average of the

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compound which first appeared, then import to the statistics software SIMCA-P 13.0. The principal component analysis (PCA) and orthogonal partial least-squares discrimination analysis (OPLS-DA) were used to analyze and explain the variation of root exudates from *S. alfredii* under the Cd stress.

Results

The results of GC-MS analysis

The GC-MS total ion chromatogram of root exudates from the hyperaccumulator *S. alfredii* at 0、5 and 10 μmol/L Cd treatment for 4 and 8 days, as show in Fig.1. Through analyzed the GC-MS data, 68 compounds were detected and identified. Including lactic acid, oxalic acid, succinic acid and other low-molecular-weight organic acids, l-valine, l-alanine, l-serine, l-glycine and other amino acids, xylose, fructose, glucose and other sugars, dodecanol, ribitol, d-pinitol, cholesterol and other alcohols, and other small-molecule metabolites, such as phosphoric acid, lauric acid and oleic acid.

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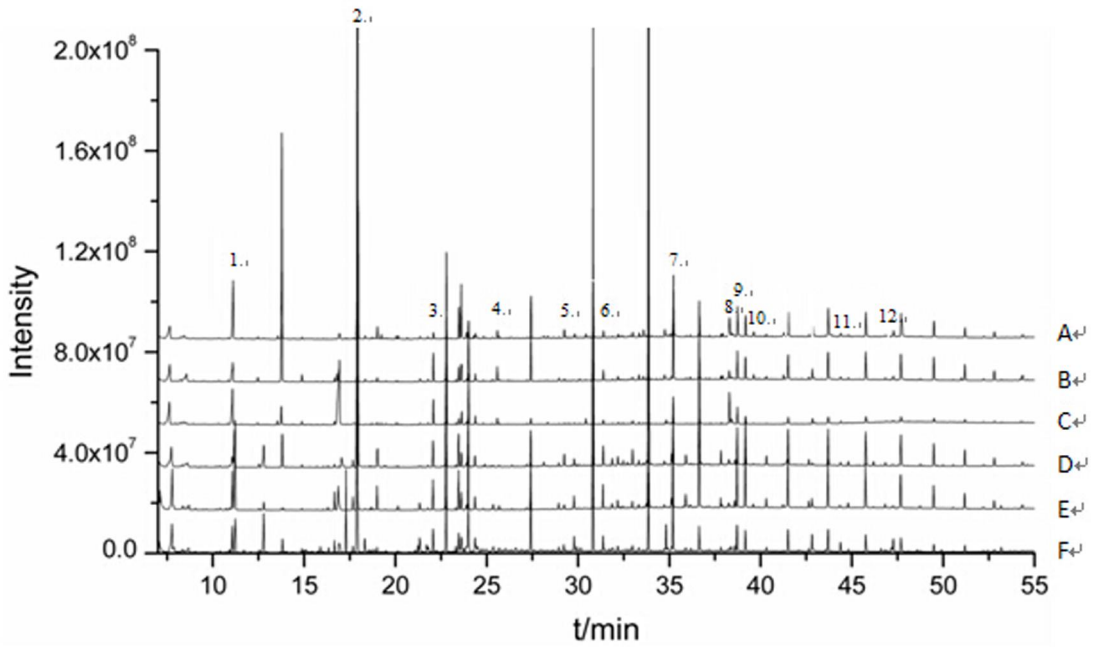

Fig.1 GC-MS TIC chromatogram of root exudates of the hyperaccumulator *S. alfredii* A. 0 μmol/L Cd treatment for 4 days; B.5 μmol/L Cd treatment for 4 days; C.10 μmol/L Cd treatment for 4 days; D. 0 μmol/L Cd treatment for 8 days; E. 5 μmol/L Cd treatment for 8 days; F. 10 μmol/L Cd treatment for 8 days

Some identified compounds：1. Lactic acid -2TMS; 2. Glycerol-3TMS; 3. Putrescine-4TMS; 4. Threonic acid-4TMS; 5. Ribitol-5TMS; 6. Tetradecanoic acid-1TMS; 7. Hexadecanoic acid-1TMS; 8. Oleic acid-1TMS; 9. Octadecanoic acid-1TMS; 10. n-Docosane; 11. 1-Monohexadecanoylglycerol-2TMS; 12. Trehalose-8TMS.

Assay validation

The intra-day precision, the absolute deviation of retention time and the relative standard deviation (RSD) of peak area, for the identified peaks was 0.01-0.15 min and 1.8-6.7 %, respectively. The inter-day precision, the absolute deviation of retention time and the RSD of peak area, for the identified peaks was 0.01-0.25 min and

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3.2-8.6 %. The system stability, the absolute deviation of retention time and the RSD of peak area, for the identified peaks was 0.01-0.3 min and 4.3-8.9 %, respectively. These indicators were all within acceptable levels and tolerances. All these results obtained indicated that the method was robust with good repeatability and stability.

Pattern recognition analysis of identified root exudates

The identified root exudates were analyzed using the unsupervised principal component analysis (PCA) method and supervised orthogonal partial least-squares discrimination analysis (OPLS-DA) method.

The results of the PCA on the identified root exudates were shown in Fig.2A. The representative points of the samples were mapped in the space spanned by the first two principal components PC1 versus PC2. This scores plot is illustrated a reasonable clustering appearing according to Cd exposed concentration and time. PCA unravelled the existence of differences in the composition of root exudates (24.8% of the variance was captured by the first PC) from *S. alfredii* under different Cd exposed concentration and time.

In order to enhance the separation effect obtained from the PCA model, the supervised clustering method OPLS-DA was used. The results of the OPLS-DA on the identified root exudates were shown in Fig.2B. As you can be seen in the scores plot, a better separation was attained after OPLS-DA for the identified root exudates under different Cd exposed concentration and time.

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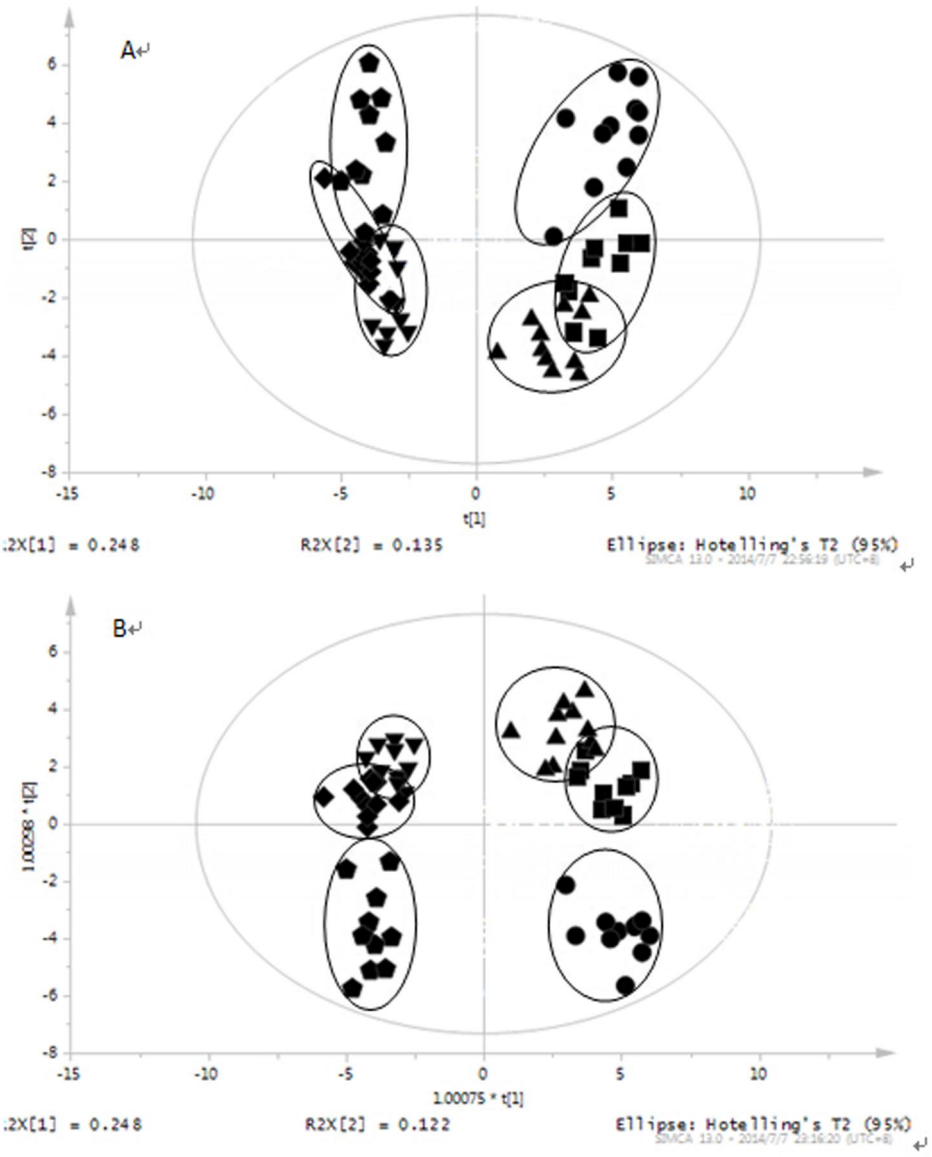

Fig.2 Principal component analysis (PCA) and orthogonal partial least-squares discrimination analysis (OPLS-DA) scores plots of root exudates of the hyperaccumulator *S. alfredii*

A. PCA scores plots, (●) 0 μmol/L Cd treatment for 4 days, (■) 5 μmol/L Cd treatment for 4 days, (▲) 10 μmol/L Cd treatment for 4 days, (▼) 0 μmol/L Cd treatment for 8 days, (◆) 5 μmol/L Cd treatment for 8 days，(⬟) 10 μmol/L Cd treatment for 8 days,; B. OPLS-DA scores plots

To find out the compounds of the identified root exudates resulting in the

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separation among the different Cd exposed concentration and time, the loadings plot of the related OPLS-DA model was conducted (Fig.3). Combined with the loadings plot, the variable importance factor (VIP, larger than 1, are the most relevant for explaining the group) values of the OPLS-DA and analysis of variance (ANOVA, $p<0.05$), 15 compounds caused the separation among the different Cd exposed concentration and time were found (Table 1).

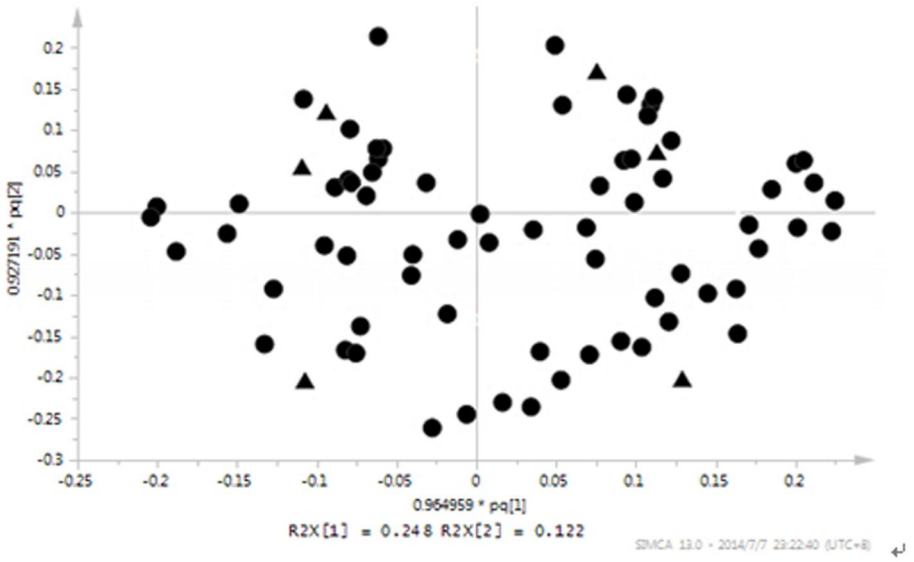

Fig.3 Orthogonal partial least-squares discrimination analysis (OPLS-DA) loading plots of root exudates of the hyperaccumulator *S. alfredii*

(●)X, the root exudate variable; (▲)Y, the group variable

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Table1 Potential biomarkers in root exudates of the hyperaccumulator *S.alfredii*

Compounds	0 μmol/L Cd treatment for 4 days (n=11)	5μmol/L Cd treatment for 4 days (n=11)	10 μmol/L Cd treatment for 4 days (n=11)	0 μmol/L Cd treatment for 8 days (n=11)	5 μmol/L Cd treatment for 8 days (n=11)	10 μmol/L Cd treatment for 8 days (n=11)
Oxalic acid	1.00±0.16	1.09±0.11	0.00±0.00	0.00±0.00	0.48±0.11	0.00±0.00
2-Hydroxyacetic acid	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.00±0.15	0.00±0.00
Octanol	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.00±0.41
Benzoic acid	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.00±0.08	0.00±0.00
2-Hydroxypentanoic acid	0.00±0.00	0.00±0.00	0.00±0.00	1.00±0.33	0.00±0.00	0.00±0.00
Succinic acid	1.00±0.31	0.83±0.09	0.77±0.11	0.83±0.11	0.83±0.11	0.00±0.00
Fumaric acid	0.00±0.00	1.00±0.12	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
L-Serine	0.00±0.00	1.00±0.23	0.43±0.12	0.00±0.00	0.00±0.00	0.00±0.00
Decanoic acid	0.00±0.00	0.00±0.00	0.00±0.00	1.00±0.12	0.00±0.00	0.00±0.00
Putrescine	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.00±0.95
Fructose	1.00±0.21	0.00±0.00	0.00±0.00	1.60±0.54	0.00±0.00	0.00±0.00
Mannitol	1.00±0.18	0.30±0.04	0.10±0.03	0.08±0.02	0.04±0.02	0.00±0.00
1-Monooctadecanoylglycerol	1.00±0.25	0.73±0.46	0.00±0.00	0.00±0.00	0.43±0.09	1.87±0.85
Octacosanol	1.00±0.25	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
beta-Sitosterol	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.00±0.37	0.00±0.00

The value is the ratio of the peak area of the compound which have the same retention time among the different treatments, mean ± standard deviation。

Change trends of the relative content of the potential biomarkers

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Under the Cd treated for 4 and 8 days, low concentration Cd can promote the secretion of oxalic acid, high concentration Cd can inhibit its secretion. But compared with 4 days, the secretion of oxalic acid reduced significantly at 8 days. This change trend indicated that oxalic acid might play an important role in mobilizing Cd. At the low Cd exposed concentration, the secretion of oxalic acid could increase the available Cd contents in the matrix, and then increased the adsorption and accumulation of Cd. At the high Cd exposed concentration, through reducing the secretion of oxalic acid to avoid Cd poisoning. Meanwhile, the secretion of oxalic acid is greatly influenced by the plant growth cycle.

When Cd treated for 4 days, the secretion of 1-monooctadecanoylglycerol reduced with an increase in the Cd exposed level, but increased when Cd treated for 8 days. This indicated that 1-monooctadecanoylglycerol may be attributed to mobilization of Cd. At the beginning of the Cd treatment, through reducing the secretion of 1-monooctadecanoylglycerol to decrease the available Cd contents in the matrix, and then reduced the toxic effect. With the increase of treatment duration, *S. alfredii* adapted the Cd stresses, and then through increasing the secretion of 1-monooctadecanoylglycerol to mobilize and accumulate Cd.

At the beginning of the Cd treatment, 2-hydroxyacetic acid, benzoic acid and beta-sitosterol are not secreted. But with the increase of treatment duration, namely Cd treated for 8 days, these three compounds are secreted in the low exposed concentration (5 μmol/L Cd). But these three compounds are not secreted in the high exposed concentration (10 μmol/L Cd). This indicated that these three compounds

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maybe play an important role in mobilizing Cd. At 5 μmol/L Cd treatment, through secreting these three compounds to mobilize Cd, and then adsorb and accumulate Cd. But at 10 μmol/L Cd treatment, though not secreting these three compounds to decrease the available Cd contents in the matrix, and then reduce the toxic effect.

Octanol and putrescine are secreted only at 10 μmol/L Cd treatment for 8 days. This might be because octanol and putrescine can stabilize Cd. Through the secretion of octanol and putrescine to reduce the toxic effect that *S. alfredii* growth under the high concentration for a long time.

At the beginning of the Cd treatment, Cd can promote the secretion of fumaric acid and l-serine. But at the high Cd exposed concentration (10 μmol/L Cd), the secretion of these two compounds are reduced. And when Cd treated for 8 days, *S. alfredii* not secreted fumaric acid and l-serine. This implied that fumaric acid and l-serine might play an important role in mobilizing Cd. The low concentration Cd and short time treatment can stimulate the secretion of fumaric acid and l-serine to mobilize Cd and increase the available Cd contents, and then promote the accumulation of Cd. The high concentration Cd or long time treatment can inhibit the secretion of fumaric acid and l-serine to decrease the available Cd contents and reduce the toxic effect.

Decanoic acid and 2-hydroxypentanoic acid are only secreted without Cd treatment for 8 days. This showed that the secretion of decanoic acid and 2-hydroxypentanoic acid is related to the plant growth cycle. And due to the secretion of these two compounds reduced to zero when Cd treated for 8 days, this implied that

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decanoic acid and 2-hydroxypentanoic acid may be can mobilize Cd. When Cd treated, *S. alfredii* decreased the secretion of these two compounds to reduce the available Cd contents and the toxic effect.

Fructose and octacosanol are only secreted without Cd treatment. When Cd treated, the secretion of these two compounds decreased to zero. This implied that fructose and octacosanol may be able to mobilize Cd. When Cd treated, *S. alfredii* decreased the secretion of these two compounds to reduce the available Cd contents and the toxic effect.

When Cd treated for 4 days, the secretion of mannitol and succinic acid were reduced with an increase in the concentration of Cd treatments. Also, the secretion of mannitol and succinic acid were reduced with an increase in the concentration of Cd treatments when Cd treated for 8 days. However, compared with 4 days, the secretion of these two compounds were lower when Cd treated for 8 days. This indicated that the secretion of mannitol and succinic acid is related to the plant growth cycle and the Cd stresses.

Discussions

In this study, through the PCA and OPLS-DA of the identified root exudates, we detected that the quantity or composition of root exudates released from *S. alfredii* under different Cd exposed concentration and time are obviously different. These results were accord with previous studies that the quantity and composition of root exudates could be influenced by many factors including the soil structure, the presence of microorganisms, the plant species as well as their developmental stage,

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nutritional status and environment stresses.[21, 22]

Through the analysis of the loadings plot, the VIP values of OPLS-DA and ANOVA, 15 compounds resulting in the separation among different Cd exposed concentration and time were found out. This indicated that these 15 compounds might play a main role in tolerating or accumulating heavy metal Cd. In these potential biomarkers, most are the organic acids and amino acids. Organic acids and amino acids play an extremely important role in the process of hyperaccumulator tolerates and accumulates the heavy metal.[23-25] Other potential biomarkers were rarely involved in the previous studies.

The change trends of the relative content of these potential biomarkers revealing their probable roles in the area of *S. alfredii* tolerates or accumulates the heavy metal Cd. Oxalic acid, 2-hydroxyacetic acid, benzoic acid, fumaric acid, decanoic acid, z-hydroxypentanoic acid, l-serine, fructose, octacosanol, beta-sitosterol and 1-monooctadecanoylglycerol might be mobilizing Cd. Octanol and putrescine may be able to stabilize Cd.

Oxalic acid can enhanced the adsorption and desorption of Cd on goethite, and can via an oxalate-bridge between the surface and the metallic cation to form a Cd-oxalate complex.[26] Organic acid, especially oxalic acid and citric acid, has strong metal-chelating properties and the ligand-promoted mechanism was the main mechanism of mineral dissolution, and indicated that oxalic acid was the main mineral-transforming agent.[27] The addition of exogenous oxalic acid increased the Cd accumulation in *Boehmeria nivea (L.) Gaud.* and improved the Cd translocation

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efficiency from root to shoot.[28] So oxalic acid is a very important root secreted substances, it can mobilize Cd around of rhizosphere, play an important role in the phytoremediation of Cd polluted soil.

Fumaric acid was found in the root exudates from the arsenic hperacuumulator *Pteris vittata* [29], 2-hydroxyacetic acid was detected in the tobacco and sunflower rhizosphere soils when they treated with Cd [30], benzoic acid was found in the root exudates of maize crop under 500 ppm Cr treatment [31] and l-serine was detected in the leaf of *Thlaspi* hyperaccumulators under Ni and Zn stress condition [32], but because of their concentration are little, did not arouse enough attention and in-depth analysis from past researchers. In this study, we do not pay attention to their absolute concentrations, through metabonomics analysis we found that these compounds are the potential biomarkers which resulted in the separation among different Cd treatments, so the role of these compounds in the phytoremediation of heavy metal polluted soil should arouse our attention.

Under heavy metal stress, the secretion of citric acid, malic acid and tartaric acid was increased [33,34]. Citric acid and malic acid can form complexes with Ni [35,36], and the addition of exogenous citric acid can promote uptake of Cd by indian mustard [37]. But in this study, these compounds were not selected as the potential biomarkers. Perhaps they were not detected or identified in this method, or the changes of their concentrations were not the main factors which resulting in the separation among different Cd treatments.

Previous researches of root exudates of hyperaccumulator did not pay attention

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to these compounds, such as decanoic acid, 2-hydroxypentanoic acid, fructose, octanol, and so on. But in this study, we thought that they may play a role in the process of hyperaccumulator tolerates and accumulates the heavy metal by metabonomics analysis, and we presumed their role, mobilize or stabilize heavy metal.

To our knowledge, the components of plant root exudates are multitudinous and complex.[38-40] But in this study, only 68 compounds were identified. Mainly it is because the limitation of the experiment method, the sensitivity of GC-MS and the library of MS. In the future, we will use the more excellent methods such as UPLC-Q-TOF or NMR to analyze the components of root exudates and verify the role of these potential biomarkers by leaching experiments and pot soil culture experiments. Then thoroughly research the role and mechanisms of root exudates from hyperaccumulators that can tolerate and accumulate heavy metals.

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