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RESEARCH PAPER

Metabonomics Study on Root Exudates of Cadmium Hyperaccumulator *Sedum Alfredii*

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Abstract: A metabonomics method based on gas chromatography-mass spectrometry (GC-MS) was developed to detect the significant differences root exudates of the Cd hyperaccumulator *Sedum alfredii* under different treatments conditions and study the effect mechanism of toleration or accumulation of *S. alfredii* to heavy metal Cd. The root exudates were collected after treatment with 0 and 40 μM Cd for 4 and 8 days. The collected solution was lyophilized and dissolved with methanol. After derivatization with methoxyamine hydrochloride and *N*-methyl-*N*-trifluoroacetamide, the samples were analyzed by GC-MS. Principal component analysis (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. Twelve compounds which caused the separation among the different treatments were found and identified. With the change of treatments, the relative amount of these 12 compounds revealed different trends, which indicated that the Cd hyperaccumulator *S. alfredii* could adjust the secretion of root exudates to tolerate or accumulate the heavy metal Cd.

Key Words: Hyperaccumulator; *Sedum alfredii*; Root exudates; Cadmium; Metabonomics; Gas chromatography-mass spectrometry

1 Introduction

Metabonomics is a scientific discipline on the dynamic change of endogenous metabolism of biological system caused by stimulation or disturbance[1]. It is utilized more and more widely in many important application fields such as disease diagnosis, drug discovery, toxicology and the mechanism of drug action[2–8]. At present, the main analytical techniques employed for metabonomic studies are based on nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS) and gas chromatography-mass spectrometry (GC-MS). GC-MS was a mature method for metabolite profiling of plant extracts with high sensitivity. Moreover, numerous readily available databases of GC-MS were established for the metabolite identification.

Root exudates from a plant are plant metabolites which 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. The root exudates can modify the pH and Eh of the rhizosphere, chelating, complexting 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[9].

Root exudates play an important role in the process of phytoremediation. However, previous studies mostly focused on the roles of root exudates, and the changes in the total amount of dissolved organic matter (DOM) or dissolved organic carbon (DOC), or some specific organic acids and amino acids[10–13], the detailed components and variation of root exudates from a hyperaccumulator under different stresses were rarely revealed.

In this study, the hyperaccumulator *Sedum alfredii* was

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cultured in Cd stressed nutrition solutions. The root exudates from *S. alfredii* were collected and analyzed by metabonomics based on GC-MS. Then, the variation of root exudates from *S. alfredii* was analyzed under the different stress of Cd, and the compounds resulting in the separation among the different treatments were found out. By investigating the changing tendencies of these compounds, the possible mechanisms of tolerates or accumulates of *S. alfredii* to the heavy metal Cd were explored.

2 Experimental

2.1 Instruments and chemicals

TRACE GC Ultra-PolarisQ GC-MS (ThermoFisher), MG-2200 nitrogen purging instrument and FDU-1100vacuum freeze drying system (Tokyo Rikakikai CO.LTD) were used in this experiment.

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

2.2 Plant material and growth conditions

The plant materials of *S. alfredii* were collected from an old Pb/Zn mining area at 118°56' east longitude and 29°17' north latitude in Quzhou City, Zhejiang Province, China. After the plants were collected, 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 basal nutrient solution. The nutrition solution used was the half-strength Hoagland-Arnon solution[14], 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, and its pH was adjusted to 6.0 with 0.1 M NaOH or HCl every day. The plants grew under greenhouse conditions with natural light. The temperature varied from 10 to 20 °C. The plants were pre-cultured for16 days until the relatively flourishing roots grew out. Then the *S. alfredii* plants were treated by 2 Cd concentration levels: 0 (control) and 40 μM Cd which was supplied as $CdCl_2$. There were 33 pots (1 piece per pot) in total, with 11 replicates for each Cd treatment.

2.3 Collection of root exudates

After growth for 4 and 8 days in the nutrient solution spiked with Cd salts without renewal, the plant roots were washed with deionized water for 3 to 5 times and then transplanted to sterilized pots with 50 mL deionized water per pot to collect root exudates for 6 h. Sample preparation, derivatization and the GC-MS analysis were carried out based on the study of Suzuki *et al*[15] and Lisec *et al*[16]. 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 with 10 mL of cold MeOH, then blown to dryness under a gentle nitrogen flow, and reconstituted in 1 mL of *n*-hexane for the GC-MS analysis.

2.4 Analysis of root exudates

The samples were derivatized by 40 μL of methoxyamine hydrochloride (20 mg mL^{-1} in pyridine at 37 °C for 2 h) and 70 μL *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) (at 37 °C for 30 min). 1 μL of the sample was injected into the GC in splitless mode. The GC analysis was carried out on a TR-5MS with integrated guard column (30 m × 0.25 μm, 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 controlled by a temperature programming as follows: held for 1 min at 70 °C, ramped to 76 °C in 6 min, then ramped to 330 °C in 50 min, held for 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 *m*/*z* 50–600 scanning range.

2.5 Data analysis

The raw GC-MS chromatogram was automatically analyzed using the Automatic mass spectral deconvolution and identification system (AMDIS), and compared with the database of metabolites in plants (Fiehn and GMD). If the similarity was greater than 70%, the compounds were identified. After the AMDIS output was extracted and processed using the MET-IDEA, 58 compounds were detected in one GC-MS scan. After normalized the peak area of the identified root exudates, the data were imported to a computer using 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. The loading plots, variable importance factor (VIP) and analysis of variance (ANOVA) were used to find the compounds which illustrated significant difference.

3 Results and discussion

3.1 GC-MS analysis

The GC-MS total ion chromatograms of root exudates from