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Response of edible amaranth cultivar to salt stress led to Cd mobilization in rhizosphere soil: A metabolomic analysis☆

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ABSTRACT

The present study aimed to investigate the metabolic response of edible amaranth cultivars to salt stress and the induced rhizosphere effects on Cd mobilization in soil. Two edible amaranth cultivars (*Amaranthus mangostanus* L.), Quanhong (low-Cd accumulator; LC) and Liuye (high-Cd accumulator; HC), were subject to salinity treatment in both soil and hydroponic cultures. The total amount of mobilized Cd in rhizosphere soil under salinity treatment increased by 2.78-fold in LC cultivar and 4.36-fold in HC cultivar compared with controls, with 51.2% in LC cultivar and 80.5% in HC cultivar being attributed to biological mobilization of salinity. Multivariate statistical analysis generated from metabolite profiles in both rhizosphere soil and root revealed clear discrimination between control and salt treated samples. Tricarboxylic acid cycle in root was up-regulated to cope with salinity treatment, which promoted release of organic acids from root. The increased accumulation of organic acids in rhizosphere under salt stress obviously promoted soil Cd mobility. These results suggested that salinity promoted release of organic acids from root and enhanced soil Cd mobilization and accumulation in edible amaranth cultivar in soil culture.

1. Introduction

Soil salinization can reduce crop growth and grain yield and is a long-standing environmental problem in the world (Wang and Li, 2013). Worse yet, a large proportion of salt-affected soil is simultaneously contaminated with heavy metals (Hu et al., 2013). Salinity could enhance the accumulation of heavy metals in crops (Acosta et al., 2011; Li et al., 2012; Weggler et al., 2004). Thus, co-occurrence of heavy metals and salinity in farmlands greatly threatens food safety. The impact of salinity on Cd mobilization can be reflected in changes of heavy metal species and distribution between the solid and solution phases of soil (Smolders et al., 1998), as well as changes in soluble heavy metal species in rhizosphere soil due to increased metal-Cl complexes. Salinity can increase soluble Cd concentration in soil by forming stable and soluble Cd-chloride complexes and/or desorbing Cd from solid soil and mineral surface via ion exchange (Acosta et al., 2011). Salt stress also induces osmotic stress and ion injury by disrupting ion homeostasis in plant cells (Shulaev et al., 2008). Stress resistance is primarily accomplished via metabolic adjustment through synthesis of enzymatic antioxidants and organic osmotic substances, such as proline, glycine betaine, soluble sugars, and free amino acids (Garg and Manchanda, 2009; Sleimi et al., 2015). Such synthesis would inevitably alter the release of exudates by metabolic adjustment in response to salt stress.

Exudates released from plants and microbes can alter the mobility of soil heavy metals by acidification (Li et al., 2013), changing soil redox potential (Wu et al., 2010), and complexation (Tao et al., 2016). A previous study indicated that cadmium (Cd)-dissolved organic matter complexes were the dominant Cd species in soil solutions after *Sedum alfredii* grew (Li et al., 2013). Differential pulse anodic stripping voltammetry measurement also indicated that Cd lability in the rhizosphere solution of durum wheat was mainly attributed to lability of organically-bound heavy metals (Bravin et al., 2012). However, the impact of salinity-induced exudates and the key metabolic pathway change on rhizospheric soil Cd mobilization has remained poorly understood. Metabolites

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in the rhizosphere can provide realistic insights into what happens under field conditions. Thus, acquiring a wide range of metabolites from roots and the rhizosphere is necessary for demonstrating plant metabolic responses to salt stress and its impact on Cd mobilization in the rhizosphere (van Dam and Bouwmeester, 2016). In recent years, metabolomics analysis has been developed as a powerful tool to investigate metabolites diversity (Obata and Fernie, 2012) and has been widely applied for metabolite profiles of plants (Gavaghan et al., 2011; Shulaev et al., 2008) and root exudates (van Dam and Bouwmeester, 2016).

As a toxic heavy metal, Cd may pose health risks to humans via consumption of vegetables. Edible amaranth (*Amaranthus mangostanus* L.), a favorite vegetable among consumers, is widely grown in Southeast Asia. Edible amaranth exhibits good adaptability in saline soil compared to other common vegetables (Li et al., 2009). Increased Cd accumulation in edible amaranth under salt stress treatment was also observed in a pot soil culture experiment (He et al., 2017). On the other hand, salinity (sodium chloride) in hydroponic cultures could reduce Cd accumulation in edible amaranth (Mei et al., 2014). Thus, increased Cd accumulation in edible amaranth grown in saline soil may be a result of increased Cd mobility in soil. The present study aimed to (1) determinate Cd mobilization directly or indirectly induced by salinity in rhizosphere soil of cultivars; (2) analyze the metabolic profiles of edible amaranth in response to salt stress; and (3) evaluate the impact of altered metabolites triggered by salinity on Cd mobilization in rhizosphere soil.

2. Materials and methods

2.1. Soil pot trial

Soil pot experiments were conducted in a greenhouse on the campus of Jinan University, Guangzhou (Guangdong Province, China). Soil used in pot culture was collected from a suburb farmland in Guangzhou that has long been contaminated with Cd. Fresh soil samples were air dried and passed through a sieve of 2.0 mm after removing coarse objects. The physicochemical properties of the soil have been described by previous study (He et al., 2015). In brief, soil pH, salinity, organic matter content, and cation exchange capacity were 6.38, 0.47 g kg^{-1}, 35.4 g kg^{-1}, and 20.86 cmol kg^{-1}, respectively. The total concentration of Cd was 1.81 mg kg^{-1}, which exceeded the Farmland Environmental Quality Evaluation Standard for Edible Agricultural Products (HJ332-2006, China).

Two contrasting Cd-accumulator edible amaranth cultivars, namely, *Quanhong* (low-Cd accumulator) and *Liuye* (high-Cd accumulator), were screened for pot experiments (He et al., 2017). The same two cultivars of edible amaranth were chosen for the present study. Seeds were sowed directly into rhizosphere bag containing 500 g sieved soil. Each pot had a diameter of 22 cm; and a depth of 15 cm, and contained three rhizosphere bags with 2.5 kg of sieved soil. After a 10-d seed germination period, the seedlings were thinned to one plant per rhizosphere bag. Soil was watered daily to maintain 75% maximum water holding field capacity. The plants were grown at temperature ranging from 25 to 35 °C and relative humidity ranging from 60% to 85%. Plants were treated with two different NaCl-level: control (no NaCl added) and salt stress (NaCl level: 3.0 g kg^{-1} dry soil). A soil treated with salt but without plant was used as bulk soil. Soils were thoroughly mixed with salt solution and then incubated for 4 weeks before used. Each treatment condition had six replicates per cultivar.

Plants were harvested after a 45-d soil culture, and rhizosphere soil and plant were carefully separated. After washing thrice with deionized water, fresh roots, shoot and whole plants in each pot were weighted and recorded. The roots were then immersed in 0.5 mmol L^{-1} $CaCl_2$ and sonicated for 10 min to remove sorbed metals. Take some part of fresh root and shoot were frozen with liquid nitrogen and stored at −80 °C in an ultra-low temperature freezer (Haier DW-86L626, China) for metabolomic analysis. The other part of roots and shoot were oven dried at 105 °C for 0.5 h, and then dried to a constant weight at 80 °C in preparation for Cd determination.

After plants were harvested, bulk soils and rhizosphere soils were collected separately. Soil adhering to the roots after shaking was defined as rhizosphere soil. About 100 g rhizosphere soil was collected per pot. Every two replicates were grouped into one to collect enough soil solution by centrifugation. Soil was directly saturated with deionized water and then maintained at equilibrium for extraction. The centrifuged rhizosphere soil was air dried and then their dry weight per pot was recorded. Extracted solutions were then centrifuged twice at 6000 rpm for 20 min and filtered through a 0.45 μm membrane filter. The centrifuged soil solution was divided into two parts, one for immediate Cd determination, and the other part was stored at −80 °C in an ultra-low temperature freezer (Haier DW-86L626, China) for metabolomic analysis.

2.2. Hydroponic culture trial

Seeds of two contrasting Cd-accumulator edible amaranth cultivars were sown in well-washed sand for germination. Three weeks after sowing, uniform seedlings were transplanted into 1.5 L Hoagland nutrient solution in a plastic vessel in a greenhouse. The growth condition was the same with as that for the pot experiment. The full Hoagland nutrient solution used in present study was agreement with Mei et al. (2014). The nutrient solution was continuously aerated and replaced every 3 days. After 6 days of full-nutrient culture, plant were treated with two different NaCl-level: control (0.25 μmol L^{-1} $Cd(NO_3)_2{\cdot}4H_2O$ + 0 NaCl) and salt stress (0.25 μmol L^{-1} $Cd(NO_3)_2{\cdot}4H_2O$ + 51.3 mmol L^{-1} NaCl). Six replicates per cultivar were grown in each treatment group. Plants were harvested after 10-d salt stress treatment. Part of the plants was used for root exudates collection (Lu et al., 2007). The roots rinsed in distilled water containing an antimicrobial agent to stop exudates degradation by microbes during collection. The rest part of plants was weighted for fresh roots and shoots. Cd adsorbed on the roots was washed with 0.5 mmol L^{-1} $CaCl_2$ before they were oven dried.

2.3. Determination of Cd

Before Cd analysis, 0.3 g of dry plant sample (root or shoot) was digested using HNO_3, and stored in bottles. The plant standard reference material [GBW07602 (GSV-1)] and blank were digested and analyzed as part of a quality control protocol. Cd concentrations were quantified by graphite furnace atomic absorption spectrometry (Shimadzu AA-7000, Japan).

2.4. Metabolites determination by GC-MS (gas chromatography-mass spectrometry)

The protocols of root and leaf sample collection, derivatization and GC-MS analysis were based on a method described by Lisec et al. (2006). A scheme about sample preparation was presented in Fig. S1 of Supplementary Material. In brief, 100 mg fresh root or leaf was rapidly frozen with liquid nitrogen and homogenized in ball mill. Then, 1.4 mL of 100% methanol was added as extractant. The mixture was shaken and the supernatant was transferred to an Eppendorf tube. Another 0.75 mL of chloroform and 1.5 mL of deionized water were added to the mixture, which was then vortexed and centrifuged. A total of 0.3 mL of supernatant was

transferred in a 2 mL tube and then freeze dried in a freeze-dryer (UATIL Coolsafe 55–4, China). The dried sample was further derivatized with 40 μL of methoxyamine hydrochloride (20 mg mL^{-1} in pyridine) and shaken for 2 h at 37 °C. Seventy microliters of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) were added, shaken for 30 min at 37 °C, and diluted to 1 mL with hexane. One microliter of each sample was injected into GC-MS set in splitless mode. Chromatographic separation was carried out on a DB-5 with integrated guard column (30 m × 0.25 mm ID, 0.25 mm film thickness; Shimadzu QP2010, Japan). The carrier gas flow rate was 1 mL min^{-1}. The column temperature was initially held for 1 min at 70 °C, followed by an 1 °C min^{-1} ramp to 76 °C, and then increased at 5 °C min^{-1} to 330 °C with a final hold of 6 min. Mass spectra were recorded at 20 scans per second with a 50–1000 m/*z* scanning range.

For analysis of rhizospheric soil solution samples and root exudates collected from hydroponic system, 1 mL of solution was freeze dried, the procedures for derivatization and GC-MS analysis are described as in root.

For the pooled biological quality control (PBQC) sample preparation, 15 μL of all prepared samples extracts was mixed in an Eppendorf tube under cold conditions. The pooled biological quality controls were used at the beginning, at the end, and randomly through the whole analysis batch in GC-MS systems. The retention time shift of the PBQC sample was less than 0.06 min, and the relative standard deviations (RSDs) of peak areas were less than 15% (Fig. S2). Thus, the GC–MS determination performed a good stability.

Spectral deconvolution and calibration were automatically analyzed by the automatic mass spectral deconvolution and identification system (AMDIS) and a set of alkane standards were used to obtain retention index values along with the corresponding mass spectra (Booth et al., 2011). The output from AMDIS was used as input to MET-IDEA to identify the components that are conserved in all the spectra. A list of ion/retention time pairs were built using MET-IDEA with a high quality representative spectrum (Broeckling et al., 2006). Appropriate model ions for each retention time were selected and a file containing abundance data for each metabolite in all samples was assembled before conducting statistical analysis.

2.5. Data analysis

Mobilization of soil Cd by salinity consists of two parts: one part involves chemical mobilization, such as complexing and/or desorption of salinity; while the other part involves biological mobilization, such as the mobilization resulting from change in exudates and microbes caused by salinity. Soil Cd chemical mobilization of salinity was calculated by

$$\mathrm{Cd_{chem-saline}} = (\mathrm{Cd_{salt}} - \mathrm{Cd_{control}}) \times \text{rhizospheric soil weight} \quad (1)$$

where $Cd_{chem\text{-}saline}$ indicates Cd amount chemically mobilized by salinity in rhizospheric soil per pot (μg pot^{-1}), Cd_{salt} indicates Cd concentration in bulk soil solution of salt stress treated soil (dry weight, μg kg^{-1}), and $Cd_{control}$ indicates Cd concentration in bulk soil solution of control soil (dry weight, μg kg^{-1}). Rhizospheric soil weight means dry weight of rhizospheric soil per pot (about 0.1 kg soil) (kg pot^{-1}).

The Cd amount accumulated in edible amaranth was calculated by

$$\mathrm{Cd_{accu}} = \mathrm{Cd_{root}} \times \mathrm{Weight_{root}} + \mathrm{Cd_{shoot}} \times \mathrm{Weight_{shoot}} \quad (2)$$

where Cd_{accu} indicates total Cd amount accumulated in edible amaranth per pot (dry weight, μg pot^{-1}), Cd_{root} (dry weight, μg g^{-1}) indicates Cd concentration in root, Cd_{shoot} (dry weight, μg g^{-1}) indicates Cd concentration in shoot, and $Weight_{root}$ (g pot^{-1}) and $Weight_{shoot}$ (g pot^{-1}) indicates the dry weight of root and shoot, respectively.

Total mobilized Cd amount in rhizosphere soil per pot was calculated by

$$\mathrm{Cd_{total}} = (\mathrm{Cd_{accu}} + \mathrm{Cd_{rhizo}} \times \text{rhizospheric soil weight}) \quad (3)$$

where Cd_{total} indicates total mobilized Cd amount in rhizosphere soil per pot (dry weight, μg pot^{-1}), Cd_{rhizo} indicates Cd concentration in rhizosphere soil solution (dry weight, μg kg^{-1}). Rhizospheric soil weight means dry weight of rhizospheric soil per pot (about 0.1 kg soil) (kg pot^{-1}).

Total Cd amount mobilized by salinity in rhizosphere soil per pot was calculated by

$$\mathrm{Cd_{saline}} = (\mathrm{Cd_{total}} \text{ in salt} - \mathrm{Cd_{total}} \text{ in control}) \quad (4)$$

where Cd_{saline} indicates total Cd amount mobilized by salinity in rhizosphere soil per pot (dry weight, μg pot^{-1}). Cd_{total} in salt (dry weight, μg pot^{-1}) indicates Cd_{total} in salt stress treated rhizosphere soil, and $\mathrm{Cd_{total}}$ in control (dry weight, μg pot^{-1}) indicates for $\mathrm{Cd_{total}}$ in control rhizosphere soil.

The amount of Cd biologically mobilized by salinity in rhizosphere soil per pot was calculated by

$$Cd_{\mathrm{bio-saline}} = Cd_{\mathrm{saline}} - Cd_{\mathrm{chem-saline}} \quad (5)$$

where $Cd_{bio\text{-}saline}$ indicates the Cd amount biologically mobilized by salinity in rhizosphere soil per pot (dry weight, μg pot^{-1}). Cd_{saline} indicates total Cd amount mobilized by salinity in rhizosphere soil per pot (dry weight, μg pot^{-1}). $Cd_{chem\text{-}saline}$ indicates Cd amount chemically mobilized by salinity in rhizosphere soil per pot (μg pot^{-1}).

Cd net uptake by root was calculated by

$$Cd_{\mathrm{net}} = \frac{Cd_{\mathrm{accu}}}{\mathrm{Weight_{root}}} \quad (6)$$

where Cd_{net} indicates Cd net uptake by root (dry weight, μg g^{-1}). Cd_{accu} indicates total Cd amount accumulated in edible amaranth per pot (dry weight, μg pot^{-1}). $Weight_{root}$ (dry weight, g pot^{-1}) indicates the dry weight of root.

Unsupervised principal component analysis (PCA) and supervised partial least-squares discrimination analysis (PLS-DA) were performed using Simca-P 12.0. The variable importance on project (VIP) was calculated by equation (7) to score the importance of *ak*th variables in explaining Y-variance in PLS regression model (Galindo-Prieto et al., 2015).

$$\mathrm{VIPAK} = \sqrt{\left(\sum_{a-1}^{A}\left(W_{ak}^{2} * (SSY_{a-1} - SSY_K)\right) * \left(\frac{K}{SSY_{a-1} - SSY_A}\right)\right)} \quad (7)$$

where A is the total number of components, K is the total number of X variables, W_{ak} is the PLS weight of the *ak*th term, SSY_a is the sum of squares explained by the ath component, $a = 1$ to A, and $k = 1$ to K.

The sum of squares of all VIP's is equal to the number of terms in the model. Hence, the average VIP is equal to 1. All VIPs can be compared with each other. Therefore, terms with VIP larger than 1 are the most relevant for explaining Y-variable. Hierarchical cluster analysis (HCA) was performed by R for Windows. Moreover,

Student's *t*-test was carried out to calculate significant change in variable at $p < 0.05$ using SPSS 19.0.

3. Results and discussion

3.1. Cd mobilization induced by salinity in rhizosphere soil of cultivars

Cd concentrations in soil and edible amaranth from soil trial cultures are listed in Tables 1 and 2, respectively. The Cd concentration in soil solution increased significantly ($p < 0.05$) in both the rhizosphere and bulk soils under salt stress treatment (Table 1). Rhizospheric acidization was observed in both LC and HC cultivars under salt stress treatment, with significantly decreased soil pH ($p < 0.05$). Cd concentration in the shoots of edible amaranth also increased significantly ($p < 0.05$) under salt stress treatment (Table 2). Although the biomass of two cultivars of edible amaranth was reduced by salinity, the total Cd amount accumulated in edible amaranth per pot increased under salt stress treatment ($p < 0.05$). The value of Cd net uptake by root was significantly higher in HC cultivar than in LC cultivar in both control and salt stress treatment. The total Cd amount mobilized by salinity per pot and the Cd amount biologically mobilized by salinity per pot were significantly higher in HC cultivar than those in the LC cultivar.

Cd concentrations in edible amaranth from hydroponic culture are listed in Table S1. Cd concentration in the root of the control was not obviously different compared to that under salt stress treatment. However, Cd concentration in the shoot under salt stress treatment was significant lower than that of the control. The net uptake of Cd by root and the total Cd amount accumulated in edible amaranth per pot in salt stress treatment were significantly lower than control sample in HC cultivar and were not significantly different in LC cultivar. Cd concentration in the shoot and the value of Cd net uptake by root was significantly ($p < 0.05$) higher in the LC cultivar than in the HC cultivar.

Previous studies demonstrated that salinity inhibited Cd uptake ability by reducing Cd root adsorption and Cd transportation across the root membrane in hydroponics culture (Lefèvre et al., 2009; Mei et al., 2014). Therefore, the increase in Cd accumulation resulting from salinity in edible amaranth in soil pot culture was attributed to increase in Cd mobilization in rhizosphere soil. Ondrasek et al. (2009) also reported that increased Cd accumulation in leaves after salinity exposure via increase in Cd mobility. The present study showed that the total mobilized Cd amount in rhizosphere soil under salinity treatment increased by 2.78-fold in LC cultivar and 4.36-fold in HC cultivar by biological mobilization of salinity compared with controls (Table 2). The increased soil Cd mobility under salt stress treatment included chemical mobilization ($Cd_{chem\text{-}saline}$) and biological mobilization ($Cd_{bio\text{-}saline}$) induced by salinity (Sharma and Dietz, 2006; Zhao et al., 2013). The chemical mobilization is resulted from chlorine complexing or sodium competition to the adsorption sites in solid soil and was directly induced by salinity. The biological mobilization is indirectly resulted from the changes of root and microorganism processes induced by salinity. The Cd amount chemically mobilized by salinity ($Cd_{chem\text{-}saline}$) in rhizosphere soil in both cultivars was similar and only about 2 μg per pot. Biological mobilization of Cd ($Cd_{bio\text{-}saline}$) by salinity was 2.14 and 7.75 μg per pot, accounting for 51.2% and 80.5% of the total amount of Cd mobilized by salinity (Cd_{saline}) in LC cultivar and in HC cultivar, respectively. The results implied that biological mobilization induced by salinity played a key role in Cd mobilization in rhizosphere soil of both cultivars, and further resulted in significant increase in Cd net uptake by root and Cd accumulation in shoot of both cultivars under salinity treatment.

3.2. Metabolic profiles of the root, leaf and root exudates of edible amaranth in response to salt stress

A total of 30 metabolites, including twelve carboxylic acids, eight amino acids, two alcohols, two amines, three sugars, two fatty acids, and urea, were identified in the roots of edible amaranth (Table S2). Results from multivariate analysis based on PCA are presented in Fig. 1a. Metabolite profiles of the four group samples (LC1, LC2, HC1, and HC2) were clearly separated, with the first two principal components explaining more than 94% of variance in the data set. HCA analyses performance on the same data also revealed that samples were divided into four groups (Fig. 2a). Our previous study (He et al., 2017; Xu et al., 2017; Mei et al., 2014) has confirmed that the Cd concentration used in soil pot trial and hydroponic trial of the present study was low enough and would not stress edible amaranth cultivar. Therefore, the responses of each metabolite detected were independent of the concentration of Cd level. These results indicated a clear difference in metabolic profiles between the control and salt stress treatment groups in both cultivars, as well as a large difference in metabolic profiles between LC and HC cultivars.

Four pairwise PLS-DA models were generated to examine the specific differences between the individual sampling groups, and the mean levels of 30 metabolites were also respectively calculated and compared (Table S3). A metabolite with VIP value exceeding 1 and Student's *t*-test $p < 0.05$ can be considered to have statistically significant contribution to the model and regarded as a significantly different metabolite. In Table S3, a total of 22 and 25 significantly different metabolites in HC and LC cultivars were identified resulting from such response to salt stress treatment. In the roots of HC cultivars, the level of 17 metabolites, including carboxylic acids (lactic acid, oxalic acid, succinic acid, malic acid, glyceric acid, threonic acid, hexadecanoic acid and citric acid), alcohols (inositol and glycerol), amino acids (serine, threonine, glutamic acid and 4-Aminobutyric acid) and sugars (ribose, fructose, and glucose), increased significantly as a coping mechanism against salt stress treatment. In the roots of LC cultivar, five metabolites including 3-hydroxybutyric acid, succinic acid, malic acid, lactic acid and fructose significantly increased under salt stress.

Table 1
Concentration of soil solution-Cd (dry weight, μg kg^{-1}) and rhizosphere soil pH.

Cultivars	NaCl-level	Soil solution Cd		$Cd_{chem\text{-}saline}$	Rhizosphere soil pH
		Rhizosphere	Bulk soil		
LC	Control	5.56 ± 0.09aB	2.20 ± 0.15aA		6.87 ± 0.02aA
	Salt stress	13.8 ± 1.7bA	22.6 ± 1.1bA	2.04 ± 0.11a	6.74 ± 0.03bB
HC	Control	3.25 ± 0.40aA	2.27 ± 0.10aA		6.85 ± 0.02aA
	Salt stress	19.1 ± 0.4bB	21.1 ± 1.63bA	1.88 ± 0.18a	6.55 ± 0.04bA

Different lowercase letters within the control and salt stress treatment of the same cultivar indicate significant differences at $p < 0.05$ level according to Student's *t*-test; Different uppercase letters within the HC and LC cultivar of the same treatment indicate significant differences at $p < 0.05$ level according to Student's *t*-test. $Cd_{chem\text{-}saline}$ indicates Cd amount chemically mobilized by salinity in rhizosphere soil per pot (μg pot^{-1}).

Table 2
Biomass (fresh weight, g pot^{-1}), Cd concentration (dry weight, μg g^{-1}) in edible amaranth, Cd net uptake by root (in μg per g of dry weight root), total Cd amount accumulated in edible amaranth per pot (dry weight, μg pot^{-1}), total mobilized Cd amount in rhizosphere soil per pot (dry weight, μg pot^{-1}), total Cd amount mobilized by salinity in rhizosphere soil per pot (dry weight, μg pot^{-1}), and Cd amount biologically mobilized by salinity in rhizosphere soil per pot (dry weight, μg pot^{-1}).

Cultivars	NaCl-level	Biomass	Cd concentration		Cd_{net}	Cd_{accu}	Cd_{total}	Cd_{saline}	$Cd_{bio\text{-}saline}$
			root	shoot					
LC	Control	18.2 ± 2.2aA	0.60 ± 0.12aA	0.49 ± 0.12aA	5.4 ± 1.3aA	0.94 ± 0.23aA	1.50 ± 0.23aA		
	Salt stress	15.5 ± 1.2aA	0.74 ± 0.02aA	2.89 ± 0.49bA	26.4 ± 4.4bA	4.32 ± 0.71bA	5.68 ± 0.69bA	4.18 + 0.79A	2.14 + 0.75A
HC	Control	23.2 ± 3.2aA	0.93 ± 0.07aB	0.78 ± 0.08aB	10.3 ± 0.9aB	1.89 ± 0.16aB	2.21 ± 0.15aB		
	Salt stress	20.7 ± 1.2aB	1.13 ± 0.02bB	4.98 ± 0.22bB	55.9 ± 2.4bB	9.93 ± 0.42bB	11.85 ± 0.42bB	9.63 + 0.57B	7.75 + 0.49B

Different lowercase letters within the control and salt stress treatment of the same cultivar indicate significant differences at $p < 0.05$ level according to Student's *t*-test; Different uppercase letters within the HC and LC cultivar of the same treatment indicate significant differences at $p < 0.05$ level according to Student's *t*-test. Cd_{net} indicates Cd net uptake by root. Cd_{accu} indicates total Cd amount accumulated in edible amaranth per pot. Cd_{total} indicates total mobilized Cd amount in rhizosphere soil per pot. Cd_{saline} indicates total Cd amount mobilized by salinity in rhizosphere soil per pot. $Cd_{bio\text{-}saline}$ indicates Cd amount biologically mobilized by salinity in rhizosphere soil per pot.

To gain insights into the key pathways activated in response to salt stress, all metabolites identified in the root of edible amaranth were imported into the online analysis platform Metaboanalyst (http://www.metaboanalyst.ca/). The metabolome map of the metabolites identified in the root of edible amaranth grown in soil pot is presented in Fig. S3. A pathway with impact values higher than 0.1 and enrichment pathways *p* value less than 0.05 can be considered as significantly relevant pathway. A total of five pathways were characterized, including alanine, aspartate and glutamate (AAG) metabolism, glycine, serine and threonine (GSTH) metabolism, glyoxylate and dicarboxylate (GD) metabolism, tricarboxylic acid (TCA) cycle metabolism, and arginine and proline (AP) metabolism. The integrated key metabolic pathways were further manually linked together based on our search results of key significantly relevant pathways and the general biochemical pathways in Plant Metabolic Network and KEGG (Kyoto Encyclopedia of Genes and Genomes), respectively (Fig. 3). The result showed that TCA cycles were up-regulated by salt stress treatment both in the HC and LC cultivars.

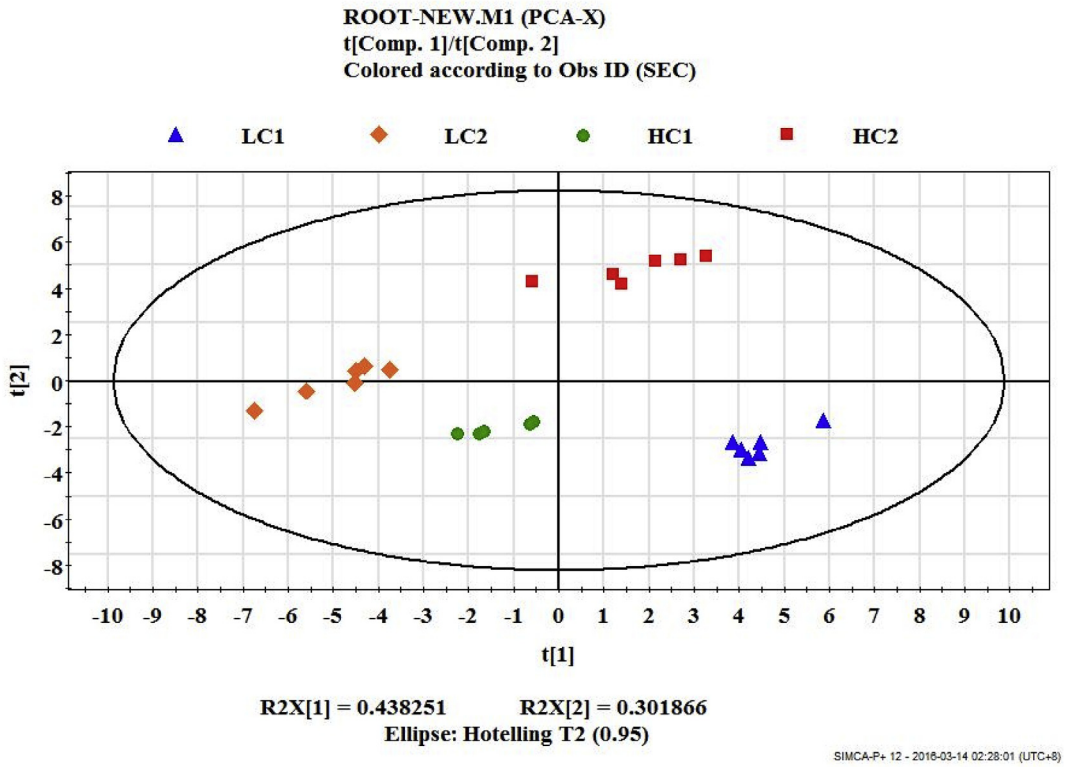
Our previous study demonstrated that the edible amaranth cultivars were not obviously stressed by Cd under the Cd concentration used in the experimental soil and hydroponic trial (Mei et al., 2014). Therefore, salinity was the major stress on crops in this study. Adjusting their metabolic pathways is a major strategy in plants to deal with salt stress (Kosová et al., 2011). Up-regulated TCA cycle could increase carboxylic acid accumulation in root, which could accommodate osmotic balance between cytoplasm and the ambient environment under salt stress treatment, and alleviate salt stress through neutralization of excess cations, such as Na^+ and Cd^{2+}(Patterson et al., 2009; Yang et al., 2007). Up-regulated TCA cycle has also been observed in the root of barley when suffering from salt stress (Shen et al., 2016; Wu et al., 2013). Taken together, our results suggest that up-regulated TCA cycle may be an important mechanism for edible amaranth to adapt to salt stress.

Comparisons of altered metabolite profiles under salt stress in root, leaf of soil pot culture and root exudates collected from hydroponic culture are presented in Table S3. A total of 23 metabolites were detected in leaf of soil pot culture (Tables S2 and S3). Except for alanine, tyrosine, ribonic acid, and galactose, all other metabolites were also detected in root. However, many metabolites directly or indirectly related to TCA cycle metabolism in leaf were down-regulated by salt stress. The result was not in agreement with that of Zhang et al. (2016), who reported that the ability of wild soybean to salt tolerance was mainly based on the synthesis of organic and amino acids, and the more active tricarboxylic acid cycle under salt stress in leaf. This was probably due to the inhibited photosynthesis in leaf of edible amaranth cultivars and the enhanced transport of glucose from leaf to root along phloem under salt stress. A total of 17 metabolites were detected in root exudates collected from hydroponic culture (Tables S2 and S3), which were also detected in root except for alanine and tyrosine. Levels of alanine and tyrosine were significantly up-regulated in root exudates, i.e., their release from root was significantly promoted, resulting in lower concentrations in root than the detected limits. The integrated key metabolic pathways of root exudates were presented in Fig. S4. It showed that most of the metabolisms related to organic acids were up-regulated by salt stress and significantly increased root release of organic acids.

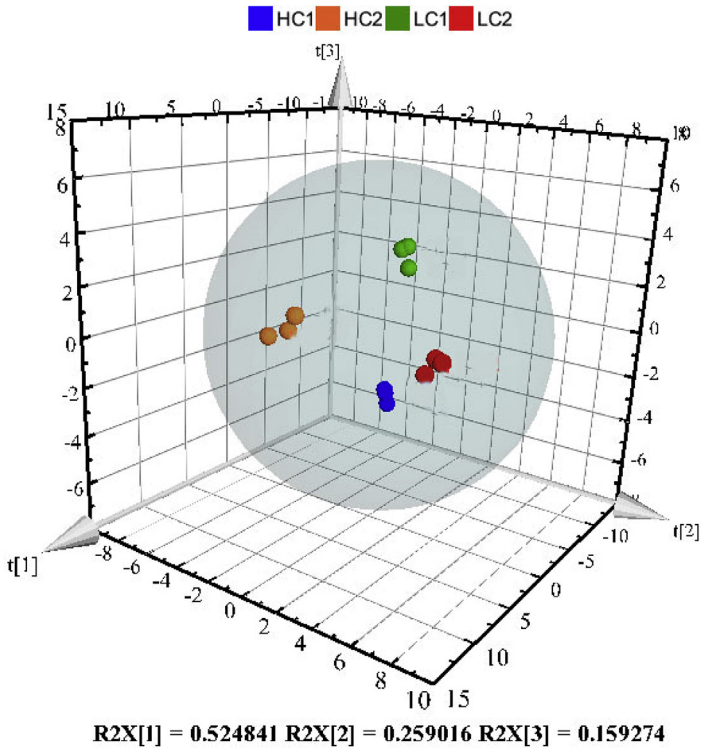
3.3. Metabolic profiles in rhizospheric soil in response to salt stress and its relationship with soil Cd mobilization

A total of 31 metabolites in rhizospheric soil solution were identified by GC-MS, including 10 carboxylic acids, 4 amino acids, 8 alcohols, 1 amines, 3 sugars, 4 fatty acids, and urea (Table S2). The results of PCA 3D-score plot (Fig. 1b) and HCA (Fig. 2b) revealed that the metabolic profiles of rhizosphere soil were different between the control and salt stress treated samples, and also varied across different cultivars. Metabolite changes in rhizosphere soil between individual sampling groups were listed in Table S3. For HC and LC cultivars, a total of 28 and 29 metabolites, respectively, were regarded as significantly different metabolites resulting from response to salt stress treatment. Among them, the level of 14 metabolites, including carboxylic acids (malic acid, fumaric acid, 3-hydroxybutyric acid, oxalic acid, glyceric acid, pipecolinic acid, and threonic acid), alcohols (inositol and glycerol), amino acids (aspartic acid, glutamic acid, and valine), ethanolamine and ribose, increased significantly in both cultivars, and they were also detected in root. In control samples, only the level of 7 metabolites such as valine, aspartic acid, glutamic acid, glyceric acid, malic acid, fumaric acid, and pipecolinic acid was significantly higher in the rhizosphere soils of HC cultivar than in LC cultivar (Table S3). However, as many as 15 metabolites had significantly higher level in HC cultivar than in LC cultivar under salt stress treatment (Table S3).

In general, metabolites in rhizosphere soil are mainly released from root, because up to 50% of the total plant photosynthetic production is exuded into the rhizosphere (van Dam and Bouwmeester, 2016). Among all 31 identified metabolites in rhizosphere soil solution, 23 metabolites were found both in the rhizosphere and the root, and 12 metabolites were found both in the rhizosphere of pot soil and the root exudates of hydroponic culture (Table S3). In HC cultivar, 10 metabolites including malic acid, 3-hydroxybutyric acid, oxalic acid, succinic acid, and threonic acid, inositol and glycerol, aspartic acid, 4-Aminobutyric acid and valine, significantly increased both in rhizosphere soil and root exudates under salt stress (Table S3). In LC cultivar, 5 metabolites

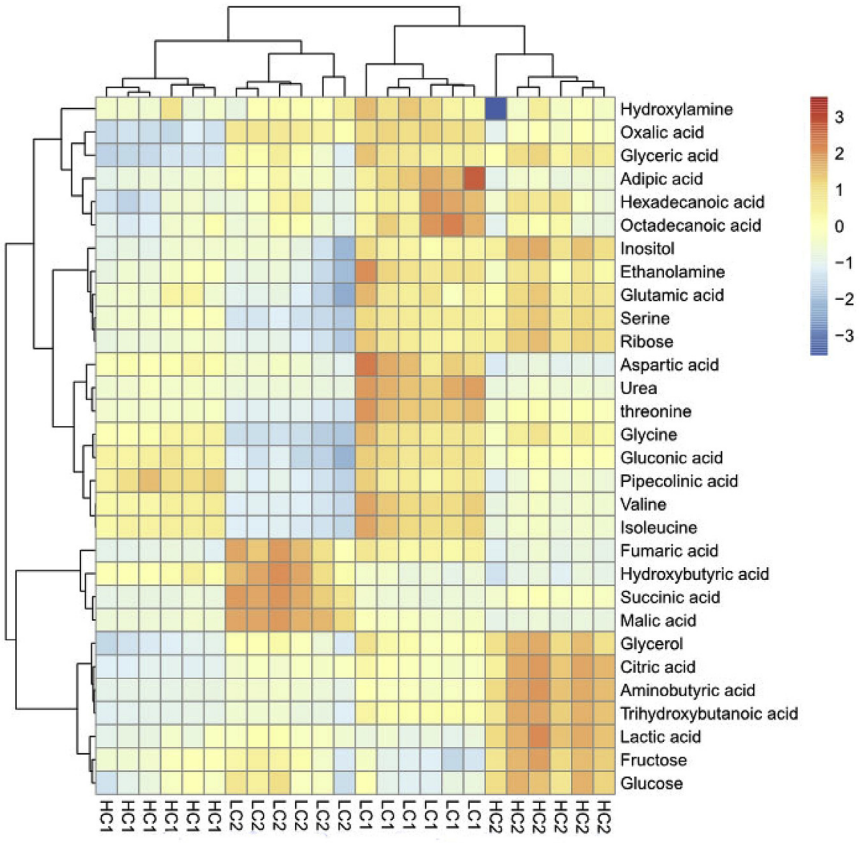


a



b

Fig. 1. Principal component analysis score plot based on metabolite variations in root of soil pot culture (a) and rhizospheric soil (b). HC1 and HC2 mean high-Cd-accumulator cultivar under control and salt stress treatment; LC1 and LC2 mean low-Cd-accumulator cultivar under control and salt stress treatment. Metabolite profiles of the four group samples (HC1, HC2, LC1, and LC2) were clearly separated.



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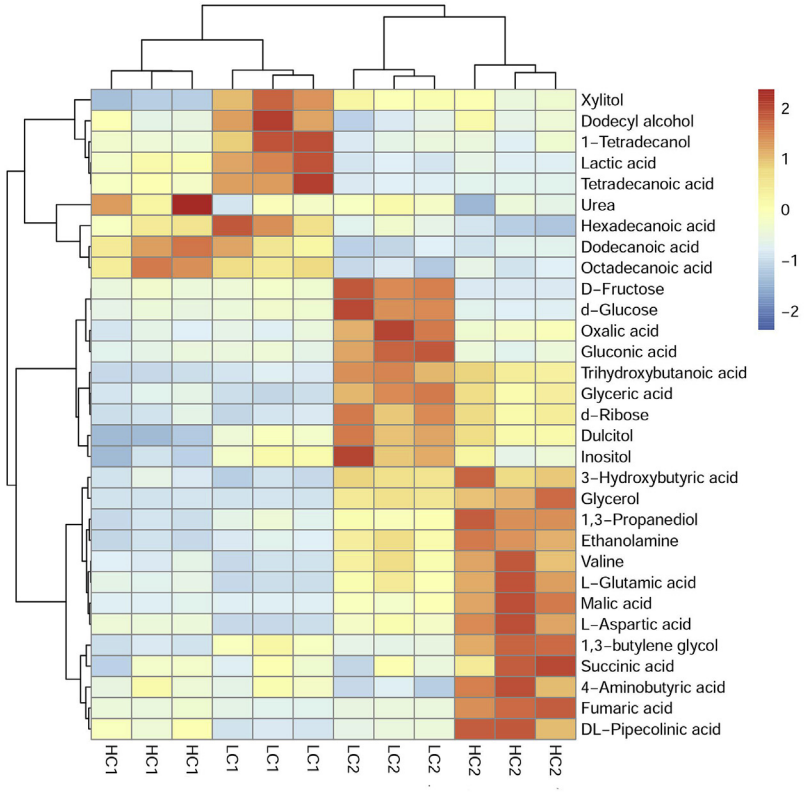

b

Fig. 2. Hierarchical clustering analysis based on the Spearman correlation coefficients of metabolites in the root of soil pot culture (a) and rhizospheric soil (b). HC1 and HC2 mean high-Cd-accumulator cultivar under control and salt stress treatment; LC1 and LC2 mean low-Cd-accumulator cultivar under control and salt stress treatment. A clear difference in metabolic profiles was presented between the control and salt stress treatment groups, as well as between LC and HC cultivars.

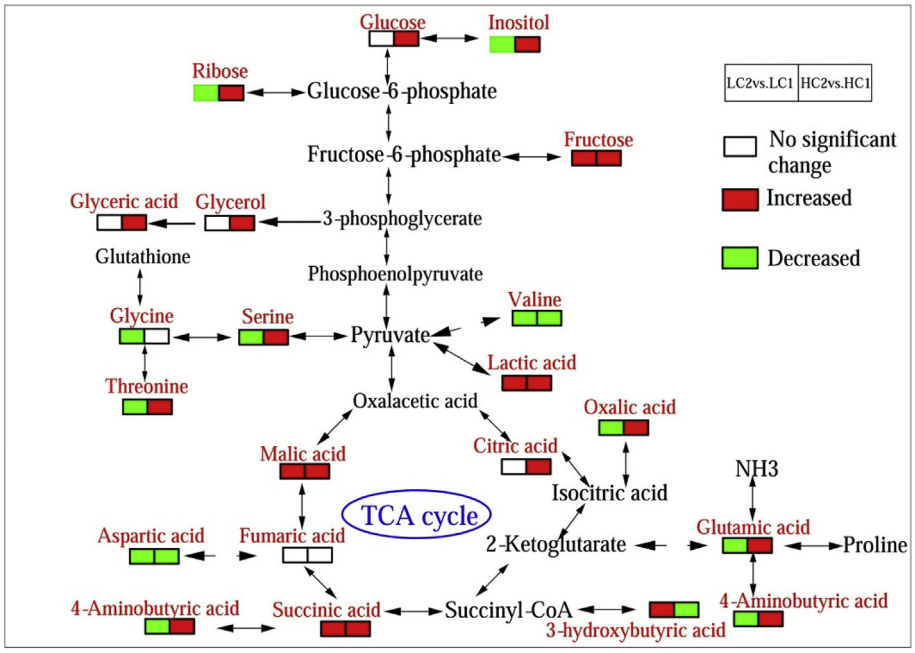

Fig. 3. Comparative visualization of identified metabolite levels in the pathways of root in soil pot culture. HC1 and HC2 indicate high-Cd-accumulator cultivar under control and salt stress treatment; LC1 and LC2 indicate low-Cd-accumulator cultivar under control and salt stress treatment.

including malic acid, threonic acid, inositol, glycerol, and aspartic acid, significantly increased both in rhizosphere soil and root exudates. However, among the total 17 detected metabolites in root exudates, 5 metabolites such as isoleucine, glycine, threonine, alanine, and tyrosine were not detected in rhizosphere soil, probably due to the different growth condition between soil pot culture and hydroponic culture.

Metabolic profiles of the root, the rhizosphere and root exudates were all significantly altered in response to salt stress and varied among different cultivars. This indicated that root's metabolic response to salt stress altered the level of metabolites in the rhizosphere soil. Among the major metabolites increased significantly in rhizosphere soil of both cultivars, the up-regulated TCA cycle directly increased the release of malic acid and fumaric acid from root (Fig. 3 and Table S3). The up-regulated TCA cycle might increase the precursors of oxalic acid, 3-hydroxybutyric acid, aspartic acid and glutamic acid, and indirectly resulted in their increased release from root (Fig. 3). The glycolysis metabolism in crops would be enhanced by stress such as drought and salinity (Fisher et al., 2016; Zhang et al., 2016). This would contribute to increase the root release of glycerol, glyceric acid, threonic acid, valine, inositol and ribose.

Eight out of 31 metabolites in rhizosphere, namely butylene glycol, dodecyl alcohol, dulcitol, 1,3-Propanediol, tetradecanol, xylitol, dodecanoic acid, and tetradecanoic acid, were only found in the rhizosphere, which suggests that metabolites in rhizosphere were affected by other factors, such as microbes and residues of anthropogenic input. More work should be done to further reveal changes in soil–microorganism–plant system triggered by salinity in future research.

Among the 14 metabolites increased significantly in rhizosphere of both cultivars (VIP > 1 and $p < 0.05$) and detected in root, a total of 10 organic acids possibly involved in soil Cd mobilization was listed in Table 3. All these organic acids except for oxalic acid showed significant ($p < 0.05$) negative correlation with pH values in rhizosphere soil solution and significant ($p < 0.05$) positive correlation with the values of biologically mobilized Cd amount induced by salinity ($Cd_{bio\text{-}saline}$) in rhizosphere soil per pot (Fig. 4, Fig. 5). The organic acids, such as carboxylic acid and amino acid, can form soluble Cd-organic complex via their carboxyl groups and/or acidize minerals by liberating H^+ (Chen et al., 2003). Thus, we deduced that an increase in accumulation of organic acid induced by salinity resulted in rhizosphere acidification and promoted soil Cd mobility. Our previous study reported the cadmium mobilization abilities by 44 organic acids commonly occurred in rhizospheric exudates of plants and found that malic acid, oxalic acid, and fumaric acid had the highest ability, aspartic acid, glutamic acid, 3-hydroxybutyric acid, glyceric acid and threonic acid had the middle ability, and pipecolinic acid and valine had the lowest ability (Wei et al., 2017). In this study, malic acid and fumaric acid increased the highest folds (29.5-folds and 6.36-folds) in rhizosphere organic acids of HC cultivar under salt stress (Table 3). Malic acid, aspartic acid and glutamic acid increased the highest folds (15.8-folds, 15.72-folds and 12.73-folds) in rhizosphere organic acids of LC cultivar (Table 3). Therefore, these four organic acids

Table 3
Fold changes of organic acid in rhizosphere soil between the individual sampling groups.

Category	Component	LC2/LC1 (folds)	HC2/HC1(folds)
carboxylic acid	Oxalic acid	3.70*	1.86*
	Malic acid	15.8*	29.5*
	Fumaric acid	2.01*	6.36*
	Pipecolinic acid	1.89*	2.60*
	Glyceric acid	7.99*	2.86*
	Threonic Acid	4.08*	4.07*
	3-Hydroxybutyric acid	3.60*	3.14*
Acidic amino acids	Aspartic acid	15.7*	4.12*
	Glutamic acid	12.73*	5.56*
	Valine	4.94*	4.93*

"*" indicates significant change at $p < 0.05$ level according to Student's *t*-test. HC1 and HC2 indicate high-Cd-accumulator cultivar under control and salt stress treatment; LC1 and LC2 indicate low-Cd-accumulator cultivar under control and salt stress treatment.

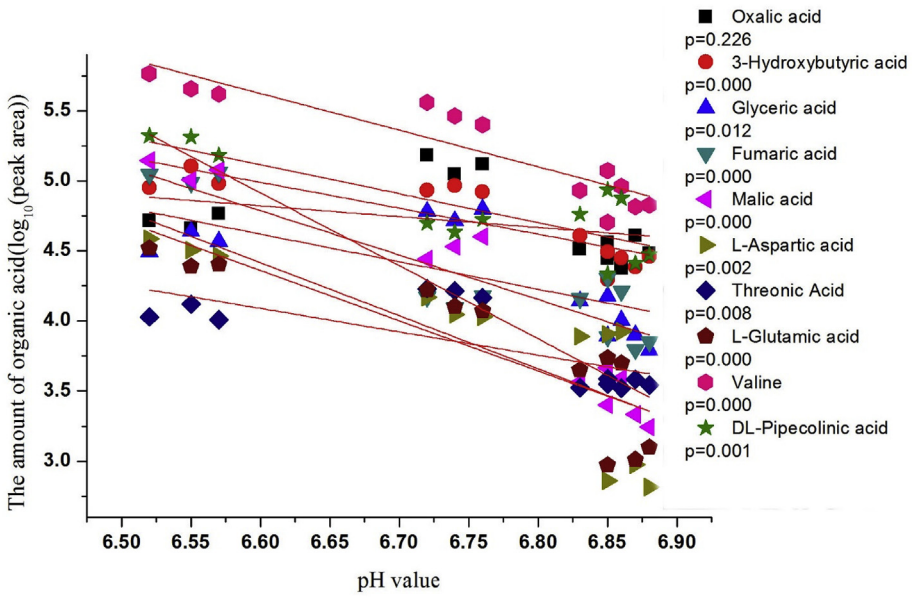

Fig. 4. The relationship between the amount of organic acid and pH values in rhizosphere soil solution.

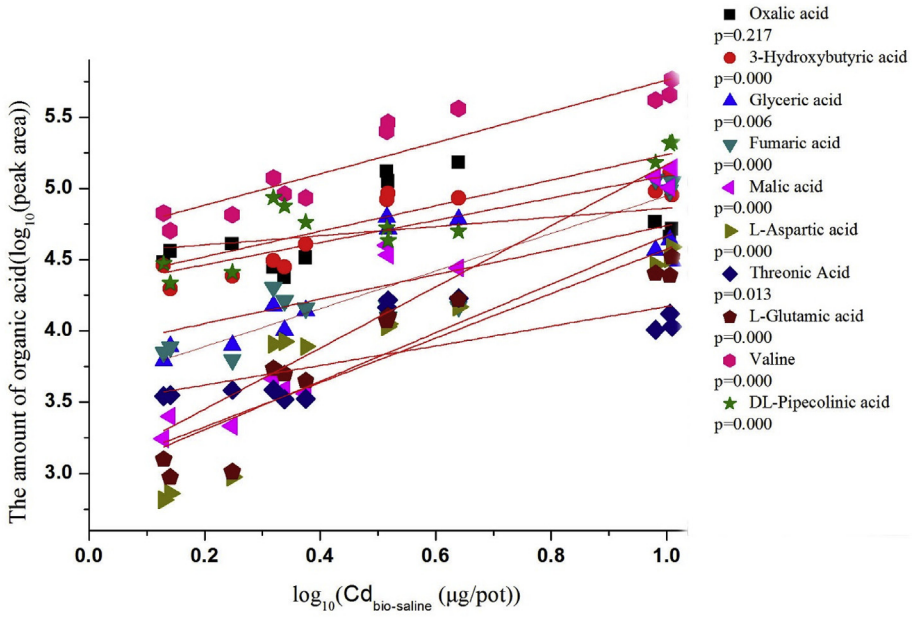

Fig. 5. The relationship between the amount of organic acid and total biologically mobilized Cd by salinity in rhizosphere per pot.

related to TCA cycle up-regulation, such as malic acid, fumaric acid, aspartic acid and glutamic acid, played a major role in soil Cd mobilization.

4. Conclusions

The increase of Cd accumulation in edible amaranth cultivars induced by salinity in soil culture was mainly attributed to the promotion of rhizosphere soil Cd mobility. Biological mobilization of soil Cd induced by salinity, but not its chemical mobilization, played a key role in Cd mobilization in rhizosphere soil. Salinity significantly changed the metabolic profiles in root, root exudates, and the rhizosphere soil of the two cultivars. The up-regulated TCA cycle in root directly increased the release of malic acid and fumaric acid from root, and indirectly increased release of aspartic acid and glutamic acid possibly through their precursor, resulting in rhizosphere acidification and soil Cd mobilization. Metabolomic analysis allowed us to acquire a comprehensive view of changes in metabolites *in vivo* and exudate composition in the rhizosphere soil, thus providing new insight into the response of plant to environmental stress and the underlying mechanisms in the rhizosphere at the metabolite level.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.05.018.

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