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Toxicological effects of environmentally relevant lead and zinc in halophyte *Suaeda salsa* by NMR-based metabolomics

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Abstract Lead (Pb) and zinc (Zn) are two typical metal contaminants with high levels in both seawater and sediment in the intertidal zones of the Bohai Sea. *Suaeda salsa* is the pioneer halophyte plant in the intertidal zones of the Bohai Sea. In the present work, the short (1 week) and long term (1 month) toxicological effects of environmentally relevant concentrations of Pb and Zn were characterized in *S. salsa* using NMR-based metabolomics combined with antioxidant enzyme activities. After metal exposure for 1 week, no significant metabolic responses were detected in root tissues of *S. salsa*. The significant metabolic responses included the increase of isocaproate, glucose and fructose, and decrease of malate, citrate and sucrose in root tissues of *S. salsa* exposed to Pb for 1 month. The increased phosphocholine and betaine, and decreased choline were uniquely found in Zn-exposed samples. The metabolic changes including decreased malate, citrate and sucrose were detected in both Pb and Zn-exposed groups. These metabolic biomarkers revealed that both Pb and Zn exposures could induce osmotic stress and disturbances in energy metabolism in *S. salsa* after exposures for 1 month. Overall, this work demonstrates that metabolomics can be used to elucidate toxicological effects of environmentally relevant metal contaminants using halophyte *S. salsa* as the bioindicator.

Keywords Metal · Toxicology · Halophyte · NMR · Metabolite

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Introduction

Marine metal pollution has been of great concern in ecotoxicology due to its high risk to the ecosystem and human health (Kennedy 2011; Rainbow 1995). Either essential or non-essential metal can cause toxicities, such as genotoxicity, neurotoxicity and immunotoxicity, to organisms when the dose is over the range of availability (Rainbow 1995). There are several typical metal contaminants including mercury (Hg), cadmium (Cd), arsenic (As), lead (Pb) and zinc (Zn) in both seawater and sediment along the Bohai coast (Mao et al. 2009; Zhang 2001). Since there are many smelting and electroplate factories of lead and zinc

located along the Bohai coast, the pollution of lead and zinc has been especially severe in both seawater and sediment. The lead concentrations have been up to 70 μg L^{-1} in seawater and 30 mg Kg^{-1} in sediment in some extremely polluted sites along the Bohai coast as reported by Mao et al. (2009). The concentrations of zinc have been higher than 120 μg L^{-1} in seawater and 150 mg Kg^{-1} in sediment (Mao et al. 2009; Zhang 2001). Lead is a non-essential element for organisms and is highly phytotoxic to plants because of the production of reactive oxygen species (ROS) damaging lipids, nucleic acids and proteins (Rucinska-Sobkowiak and Pukacki 2006). Zinc is essential and involved in the carbohydrate and phosphate metabolisms, proteins synthesis and ribosome's structural integrity in organisms (Cherif et al. 2011). However, excessive Zn can induce negative effects such as leaf chlorosis and oxidative stress in plant cells (Cherif et al. 2011). Therefore, it is necessary to assess the toxicological effects of metal contaminants in the intertidal organisms to elucidate the responsive mechanisms.

The Chenopodiaceae C3 halophyte, *Suaeda salsa*, is the pioneer plant species in saline soil and intertidal zones of the Yellow River Delta, where soil salt content is often higher than 3 % (Wang et al. 2007). As a halophyte, *S. salsa* has exhibited the potential as a bioindicator for environmental monitoring of contaminants in the intertidal zones and saline soil compared with nonhalophytes and animals (Zhu et al. 2005). Therefore it has been widely used in environmental sciences and applied for the phytoremediation of metal pollution due to its efficient accumulation of metals including Pb and Zn (Zhu et al. 2005). As a pioneer halophyte in the intertidal zones along the Bohai coast, the physiological and molecular responses of *S. salsa* to salinity have been extensively studied and hence been suggested as a bioindicator of saline soils (Han et al. 2005; Li et al. 2004; Zhang et al. 2001).

Metabolomics is a well-established system biology approach and has demonstrated its applicability on studying the interactions between organisms and environment at the molecular level (Bundy et al. 2009; Li et al. 2006). Therefore, metabolomics has been widely applied in ecotoxicology on the basis of metabolic responses to environmental contaminants (Liu et al. 2011; Santos et al. 2010; Sun et al. 2010). Previously, we reported the toxicological effects of environmentally relevant Cd in the above-ground part of seedling of *S. salsa* using NMR-based metabolomics (Liu et al. 2011). In the present study, ^{1}H NMR-based metabolomics was applied to the halophyte *S. salsa* exposed to the environmentally relevant lead (20 μg L^{-1}) and zinc (100 μg L^{-1}) to detect the metabolic changes (biomarkers) in root, and then to characterize toxicological effects induced by lead, zinc and their mixture in *S. salsa*. In addition, the antioxidant status was evaluated on the basis of enzyme activities in the above-ground part of *S. salsa.* Based on the combined biochemical parameters including the metabolic biomarkers and antioxidant enzyme activities, it was expected to elucidate the toxicological effects of two typical metal contaminants, lead and zinc, in the halophyte *S. salsa* in the Yellow River Delta.

Materials and methods

Cultivation of *S. salsa* under metal exposures

The seeds of *S. salsa* were collected from the Yellow River Delta in November, 2009 and stored in a refrigerator at 4 °C for 7 months. The seeds were surface sterilized using 0.5 % $HgCl_2$ for 10 min, and then washed in sterilized double distilled water for three times. Eighty seeds with similar size were sown in yellow sands (composition: silicon dioxide, size: 180 μm) in four plastic jugs (one control and three metal-exposed groups) with each containing 20 seeds. The yellow sands were collected from the intertidal zones of the Yellow River Delta and were rinsed in diluted nitric acid (1 %) to eliminate the impurities such as organic matters and metal ions before used for plant cultivation. The sown *S. salsa* seeds were irrigated with Hoagland's nutrient solution. The stock solution for Hoagland's nutrient was prepared in 0.5 L sterilized double distilled water containing following chemicals, $Ca(NO_3)_2 \cdot 4H_2O$ (47.25 g), KNO_3 (25.3 g), NH_4NO_3 (4 g), KH_2PO_4 (6.8 g), $MgSO_4 \cdot 7H_2O$ (24.65 g), KI (0.0415 g), H_3BO_3 (0.31 g), $MnSO_4$ (1.115 g), $ZnSO_4$ (0.43 g), Na_2MoO_4 (0.0125 g), $CuSO_4$ (0.00125 g), $CoCl_2$ (0.00125 g), $FeSO_4 \cdot 7H_2O$ (2.78 g) and Na_2EDTA (3.73 g). A volume of 500 μL of stock solution for Hoagland's nutrient was diluted to 25 L for plant cultivation. The concentrations of stock solutions of $Pb(NO_3)_2$ and $ZnCl_2$ were 200 mg L^{-1}. After sown in the jugs for 4 weeks without exposure, all the seedlings of exposed groups were irrigated with the Hoagland's nutrient solution containing 20 μg L^{-1} lead (0.1 ml of lead stock solution), 100 μg L^{-1} zinc (0.5 mL of zinc stock solution) and the mixed 20 μg L^{-1} lead and 100 μg L^{-1} zinc (0.1 mL of lead stock solution and 0.5 mL of zinc stock solution), respectively. The concentrations of metals were environmentally relevant to the real situation of pollution in the Yellow River Delta (Zhang 2001; Zhou and Yan 1997). In this work, the Hoagland's nutrient solution contained 20 mmol L^{-1} sodium chloride to maintain the growth of halophyte *S. salsa* (Zhao 1991). The culture condition was 28 ± 4 °C, photoperiod 12 h light/12 h darkness, relative humidity 70 % and photosynthetically active radiation 600 μmol m^{-2} s^{-1}. After exposure for 1 week and 1 month, the seedlings (n = 10)

of *S. salsa* from both control and exposed groups were randomly harvested. Before the determination of fresh weight and length, the seedlings were washed three times with distilled water, kept on filter paper for a few minutes to remove of excess liquid. After quick measure of the total length and weight of seedlings, all the roots and above-ground part of seedlings were flash-frozen in liquid nitrogen and stored at −80 °C. Among the 10 seedlings sampled at each time point, six of them were selected for metabolite extraction and enzyme assay, while the remaining four seedlings were used for metal quantification.

Measurement of antioxidant enzyme activities

In this work, the commercial enzyme kits (Jiancheng, Nanjing, China) were applied for the measurements of activities of antioxidant enzymes including superoxide dismutase (SOD, kit No. A001-1), peroxidase (POD, kit No. A084-3), glutathione *S*-transferases (GST, kit No. A004) catalase (CAT, kit No. A007) and glutathione peroxidase (GPx, kit No. A005). The antioxidant enzyme activities were assayed by a multiskan spectrum microplate spectrophotometer (Infinite M200, TECAN). Briefly, the above-ground part of seedling from *S. salsa* ($n = 6$, *approx.*. 100 mg) was ground in liquid nitrogen and transferred to homogenization medium (pH = 7.4, 0.1 mol L^{-1} Na_2HPO_4/NaH_2PO_4) with a proportion of 10 % (weight/volume). The homogenate was votexed for 30 s and centrifuged at 2,500 rpm for 10 min. The supernatant was transferred to an Eppendorf tube. Two hundred microliters of the supernatant was then transferred to a plate for the measurement of each antioxidant enzyme activity according to the manufacturer's protocols using enzyme kits. The absorbance wavelengths of measurement for SOD, POD, GST, CAT and GPx were 550, 420, 412, 405 and 412 nm, respectively. Protein concentration was determined by the Coomassie brilliant blue G-250 dye-binding method, using bovine serum albumin as standard (Bradford 1976). All the enzyme activities were expressed as U mg^{-1} protein.

Metal concentrations in plant tissues

The samples ($n = 4$) of root and above-ground part of *S. salsa* from 1 week and 1 month of exposure were rinsed in pure water and dried at 80 °C to the constant weights (weighing from 10 to 20 mg per sample). The dried tissue was accurately weighed and digested in 1 mL of concentrated nitric acid (70 %, Fisher Scientific) using a microwave digestion system (CEM, MAR5). The samples were heated in the microwave oven (program: heating at 15 min to 200 °C and holding at 200 °C for 15 min). Each completely digested sample was diluted with ultra pure water to 5 mL for the quantification of Pb and Zn using ICP-MS (Agilent 7500i, Agilent Technologies Co. Ltd, USA). GBW07605 tea leaves (State Bureau of Technical Supervision, People's Republic of China) were employed as certified reference materials for metal analysis to ensure internal quality assurance/quality control (QA/QC) practices (Li et al. 2012). The spike recovery of target elements, as tested by three individual spiking experiments, was restricted within 93.5–104.7 % for Pb and 95.4–106.5 % for Zn at the level of 5 ng g^{-1}. The concentrations of Pb and Zn in the certified standards were determined to be 4.2 ± 0.4 μg g^{-1} for Pb (certified value = 4.4 ± 0.3 μg g^{-1}) and 25.6 ± 2.8 μg g^{-1} for Zn (certified value = 26.3 ± 2.0 μg g^{-1}).

Metabolite extraction

Polar metabolites were extracted from the root tissues using the solvent system of methanol/water (1/1) as described previously (Kim and Verpoorte 2010; Liu et al. 2011). Briefly, the root tissue sample was ground in a liquid N_2-cooled mortar and pestle. The tissue powder (weighing from 50 to 100 mg per sample) was transferred to a tube containing ~50 ceramic beads with 1 mm diameter, and then thoroughly homogenized in 3.33 mL g^{-1} methanol/water (1/1) using a high throughput homogenizer, Precellys 24 (Bertin, France). After homogenization, the sample was transferred to an Eppendorf tube and vortexed for 15 s three times. Following centrifugation (3,000×*g*, 10 min, 4 °C), the supernatant was transferred to an Eppendorf tube and then lyophilized. It was subsequently resuspended in 600 μL of 100 mmol L^{-1} of phosphate buffer (Na_2HPO_4 and NaH_2PO_4, including 0.5 mmol L^{-1} TSP, pH 7.0) in deuterium oxide (D_2O). The mixture was vortexed and then centrifuged at 3,000×*g* for 5 min at 4 °C. The supernatant substance (550 μL) was pipetted into a 5 mm NMR tube prior to NMR analysis.

NMR analysis, spectral pre-processing and multivariate data analysis

NMR analysis of *S. salsa* root tissue samples and subsequent spectral pre-processing were performed based on the published methods (Liu et al. 2011).

Principal component analysis (PCA) was used in this work for the statistical multivariate analysis of control and various metal-exposed groups. PCA is a well-established unsupervised pattern recognition (PR) method calculating inherent variation within the data sets without use of the class membership. The algorithm of PCA calculates the highest amount of correlated variation along PC1, with subsequent PCs containing correspondingly smaller amounts of

variance. For each model built, the loading vectors for the PCs can be used for the identification of the contributive metabolites (metabolic biomarkers) for the clusters (Xu 2004).

Statistical analysis

Data of growth parameters, enzyme activities and metal concentrations were expressed as the mean ± standard deviation. The data were statistically analyzed using statistical software, Statistics toolbox in Matlab (version 7.0; The MathsWorks, Natick, MA). One way ANOVA (analysis of variance) with Tukey's test was conducted on the data, and significant difference was defined at $p < 0.05$.

To test the significance of separations between the control and metal-exposed groups, one way ANOVA with Tukey's test was performed on the PC scores of various groups For each model built, the loading vector for the PC could be examined to identify the metabolites which contributed to the clusters. SAM software (Katsiadaki et al. 2009; Santos et al. 2010) was then used to find significant metabolic differences among control and metal-exposed groups with appropriate false discovery rate (FDR) cutoffs. Bins that changed significantly (at FDR < 0.01) were subsequently identified using Chenomx software and isolated. These significant metabolites were contributive for the separation between control and metal-treated samples and hence were considered metabolic biomarkers induced by metals (Pb, Zn, or mixed Pb and Zn).

Results

Metal concentrations in root and above-ground part of *S. salsa*

Table 1 illustrates the accumulated metal concentrations in both root and above-ground part tissues of *S. salsa* following 1 week and 1 month exposures. The Pb concentrations in both root and above-ground part tissues of Pb-treated and mixed Pb and Zn-treated groups were approx. 1.5 times higher than that of both control and Zn-treated groups after 1 week exposure. The Zn concentrations in both tissues of Zn-treated and mixed Pb and Zn-treated groups were approx. 2.0 times higher than those of control and Pb-treated groups after 1 week exposure. After exposure for 1 month, the accumulations of both Pb and Zn in root tissue were not significantly different ($p > 0.05$) with those of corresponding metal-exposed groups for 1 week. However, the concentrations of Pb in above-ground part of Pb-treated and mixed Pb and Zn-treated samples were significantly higher than those in corresponding metal-exposed groups for 1 week exposure.

Table 1 The concentrations of Pb and Zn in *S. salsa* samples after exposure for 1 week and 1 month

Treatment	1 week				1 month			
	Pb (root)	Zn (root)	Pb (above-ground part)	Zn (above-ground part)	Pb (root)	Zn (root)	Pb (above-ground part)	Zn (above-ground part)
Control	0.95 ± 0.22	77.35 ± 20.26	1.08 ± 0.22	37.53 ± 15.34	0.88 ± 0.23	99.87 ± 22.21	0.68 ± 0.15	45.07 ± 8.03
Pb	1.50 ± 0.27*	82.46 ± 23.30	1.58 ± 0.27*	43.82 ± 17.45	1.51 ± 0.30*	107.57 ± 21.43	2.72 ± 0.41*,#	43.57 ± 8.21
Zn	0.82 ± 0.20	145.39 ± 27.46*	1.04 ± 0.16	96.46 ± 20.71*	0.77 ± 0.21	139.06 ± 20.75*	0.44 ± 0.13	63.73 ± 10.13*,#
Pb + Zn	1.46 ± 0.31*	140.76 ± 28.86*	1.49 ± 0.23*	79.27 ± 16.38*	2.03 ± 0.41*	135.92 ± 17.93*	2.21 ± 0.40*,#	57.56 ± 8.87*,#

Values are presented as the mean ± standard deviation (μg g^{-1} dry weight)

Significant difference (* $p < 0.05$) between control and metal-treated groups at each exposure time was tested by one-way analysis of variance

Significant difference (# $p < 0.05$) between two exposure time points from each metal-treated group was tested by one-way analysis of variance

The concentrations of Pb and Zn in mixed Pb and Zn-treated groups were not significantly different to those in single metal-treated groups (Table 1).

Antioxidant enzyme activities

After exposure for 1 week, the average antioxidant enzyme activities (except CAT) were slightly ($p > 0.05$) elevated in the above-ground part of seedlings from both Pb-treated and mixed Pb and Zn-exposed *S. salsa* samples (Table 2). However, the activities of SOD, POD, CAT and GPx were significantly ($p < 0.05$) increased in Zn-exposed samples. Interestingly, the activities of these SOD, POD, CAT and GPx were significantly ($p < 0.01$) decreased in Zn-exposed group compared with those of control group after 1 month exposure. Both Pb and metal mixture exposures did not induce significant alterations in the antioxidant enzyme activities after exposure for 1 month. However, the activities of SOD, POD, CAT and GPx in mixed Pb and Zn-treated group were significantly different with those in Zn-treated group after exposure for 1 month. The average values of total protein in chemical-exposed samples were lower than that of control samples after exposure for 1 week. However, the total protein content was significantly ($p < 0.01$) increased in Zn-exposed *S. salsa* after exposure for 1 month.

Plant growth

The weights and lengths of seedlings from *S. salsa* were measured (data not shown). The plant growth was significantly (ANOVA, $p < 0.05$) promoted by Pb and mixed Pb and Zn exposures in terms of the increased weight and length after exposure for 1 week. Zn exhibited no obvious effect on the plant growth of *S. salsa* with 1 week exposure. However, the growth of *S. salsa* was significantly ($p < 0.05$) inhibited by mixed Pb and Zn exposure after 1 month. The single metal exposure did not induce significant effects on the growth of *S. salsa* after exposure for 1 month.

Metabolic responses

Figure 1 presents one representative ^{1}H NMR spectrum of tissue extracts from root of *S. salsa* in control group. Various metabolite classes were identified in root tissue of *S. salsa*, including amino acids (branched chain amino acids: valine, leucine and isoleucine, alanine, glutamate, glutamine, etc.), carbohydrates (sucrose, fructose and glucose), organic osmolyte (betaine), and intermediates in the tricarboxylic acid (TCA) cycle (succinate, citrate, malate and fumarate). From the NMR spectrum, betaine (3.25 and 3.91 ppm) is visibly the most abundant

Table 2 The activities (U mg^{-1} protein) of SOD, GST, POD, CAT and GPx and total protein (mg g^{-1} fresh weight) in the above-ground part of seedlings from control, Pb, Zn and Pb + Zn-exposed *S. salsa* samples

Treatment	1 week				1 month			
	Control	Pb	Zn	Pb + Zn	Control	Pb	Zn	Pb + Zn
SOD	73.91 ± 37.22	103.40 ± 28.69	312.19 ± 184.05*	135.29 ± 52.41*	90.63 ± 21.45	77.72 ± 20.95	29.59 ± 3.82**	86.34 ± 28.45##
GST	171.75 ± 148.21	201.55 ± 57.81	781.51 ± 630.67	215.09 ± 169.52	179.77 ± 177.38	223.19 ± 166.99	78.03 ± 63.77	227.37 ± 193.21
POD	9.70 ± 4.80	12.03 ± 5.92	45.84 ± 30.09*	15.69 ± 7.80	20.90 ± 6.44	21.41 ± 11.20	6.24 ± 2.61**	28.77 ± 8.88##
CAT	491.36 ± 245.93	390.48 ± 130.34	1468.84 ± 836.69*	701.59 ± 376.16	1457.64 ± 303.78	1292.59 ± 349.28	471.90 ± 90.38**	1683.76 ± 554.66##
GPx	465.07 ± 164.49	527.20 ± 340.83	1101.01 ± 227.55**	696.86 ± 281.97	1329.91 ± 282.55	1113.57 ± 308.25	328.05 ± 120.17**	1328.46 ± 635.04##
Total protein	20.27 ± 10.04	12.34 ± 3.08*	4.63 ± 2.12**	11.08 ± 7.91	9.70 ± 2.66	11.14 ± 2.78	28.34 ± 4.14**	9.92 ± 2.95##

Values are presented as the mean ± standard deviation

Significant differences (* $p < 0.05$ and ** $p < 0.01$) between control and metal-treated groups at each exposure time were tested by one-way analysis of variance

Significant difference (## $p < 0.01$) between Zn treated and mixed Pb and Zn-treated groups after exposure for 1 month was tested by one-way analysis of variance

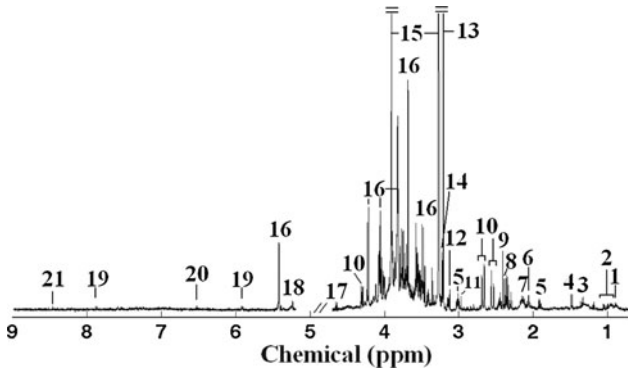

Fig. 1 A representative one-dimensional (1D) 500 MHz ^{1}H NMR spectrum of root tissue extracts from *S. salsa* using extraction solvent system of methanol/water (1/1). Metabolite assignments: (1) Isocaproate, (2) branched chain amino acids: leucine, isoleucine and valine, (3) lactate, (4) alanine, (5) γ-aminobutyrate, (6) glutamate, (7) glutamine, (8) malate, (9) succinate, (10) citrate, (11) unknown (2.97 ppm), (12) malonate, (13) choline, (14) phosphocholine, (15) betaine, (16) sucrose, (17) β-glucose, (18) α-glucose, (19) uridine, (20) fumarate and (21) formate

metabolite in root tissues from *S. salsa*. As a key secondary metabolite, betaine plays important role in osmotic balance in *S. salsa* and was approx. 10–100 times higher than other metabolites in the NMR spectral intensities.

Principal component analysis (PCA) was conducted on the ^{1}H NMR spectral data sets generated from the control and metal-exposed groups of *S. salsa* after exposures for 1 week and 1 month, respectively. After exposure for 1 week, the control and metal-treated groups were not significantly ($p > 0.05$) separated along either PC1 or PC2 axis from the PC scores plots (data not shown). In order to test the significance of NMR spectral bins, SAM software was then used to find significant metabolic differences between control and metal-exposed groups with appropriate false discovery rate (FDR < 0.01) cutoffs. However, no significant metabolic difference was found in the metal-exposed samples with 1 week exposure of Pb, Zn or mixed Pb and Zn. After exposure for 1 month, the separation between control and Pb or Zn-exposed groups was significantly found along PC1 axis (Fig. 2a, c). However, PCA did not resulted in significant separation between control and mixed Pb and Zn-treated *S. salsa* samples along any PC axis (Fig. 3). The significant metabolic differences including increased isocaproate, glucose and fructose, and decreased malate, citrate and sucrose were detected in

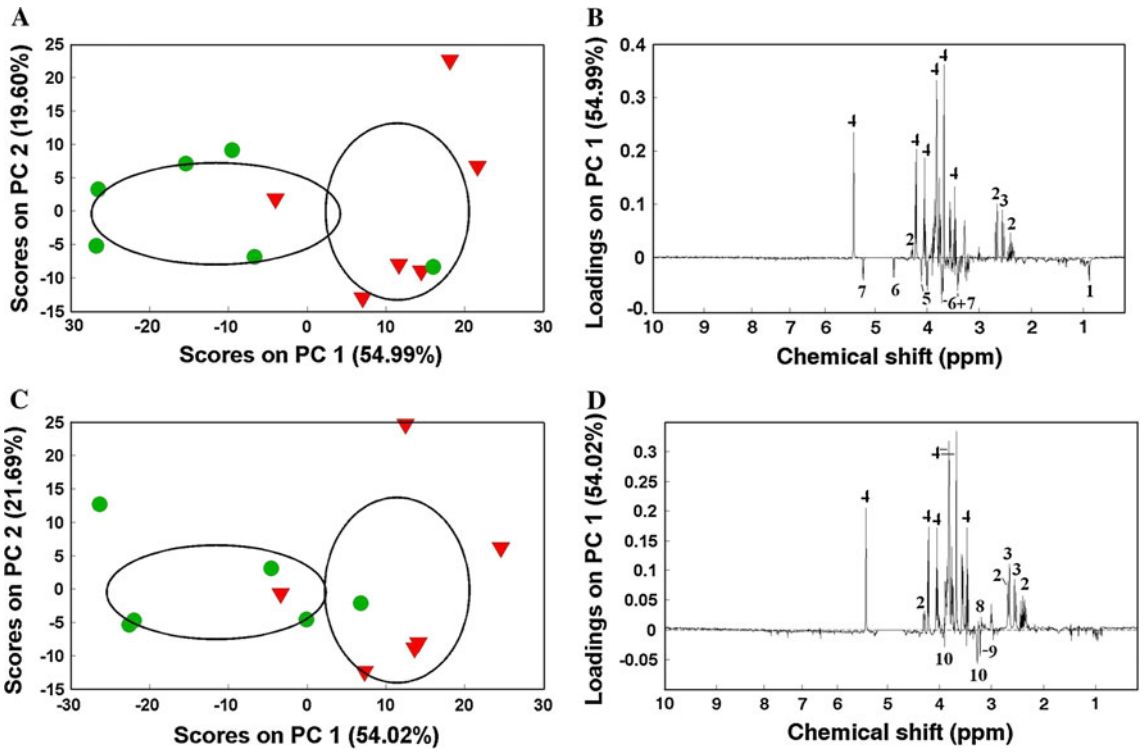

Fig. 2 PCA scores plots and corresponding PC1 loadings plots from the analysis of the 1D ^{1}H NMR spectra of root tissue extracts of *S. salsa* from control (*black down-pointing triangle*) and metal-exposed (*black circle*) groups including 20 μg L^{-1} Pb (**a**, **b**) and 100 μg L^{-1} Zn (**c**, **d**) after exposure for 1 month. *Ellipses* represented mean ± standard deviation of PC scores along both PC1 and PC2 axes for each group. Metabolite assignments: (1) isocaproate, (2) malate, (3) citrate, (4) sucrose, (5) fructose, (6) β-glucose, (7) α-glucose (8) choline, (9) phosphocholine and (10) betaine

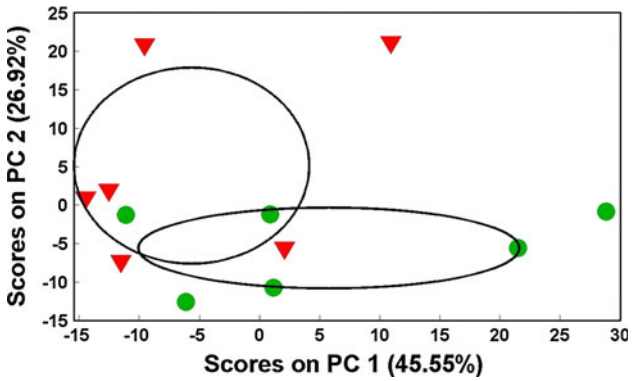

Fig. 3 PCA scores plot from the analysis of the 1D ^{1}H NMR spectra of the *S. salsa* from control (*black down-pointing triangle*) and combined 20 μg L^{-1} Pb and 100 μg L^{-1} Zn-exposed samples (*black circle*) after exposure for 1 month. *Ellipses* represented mean ± standard deviation of PC scores along both PC1 and PC2 axes for each group

Pb-treated *S. salsa* samples (Fig. 2b). The metabolic profile of Zn-exposed group was similar to that of Pb-exposed group (Fig. 2d). However, several metabolic changes, increased phosphocholine and betaine, and decreased choline were uniquely found in Zn-exposed samples. In addition, the level of glucose was not altered. For the group with combined exposure of Pb and Zn, there was no significant metabolic change induced in the *S. salsa* root samples.

Discussion

In this work, the toxicological effects of lead and zinc and the potential antagonistic and synergistic effects between lead and zinc were characterized on the basis of metabolic profiling, antioxidant enzyme activities and plant growth. As a known toxicant to plants, lead can induce phototoxic effects and inhibit the growth of aquatic plant, Lemnaceae *Wolffia arrhiza* (Piotrowska et al. 2009). However, there could be hermetic effects in plants induced by low concentrations of lead, which have been confirmed by the stimulation of plant growth of *Vicia faba* (Wang et al. 2010). In our case, it was found that environmentally relevant Pb stimulated the growth of *S. salsa* after exposure for 1 week. This ought to be associated with the hormesis induced by Pb in *S. salsa*. After exposure for 1 month, the growth of *S. salsa* exposed to Pb was similar to that of control, which suggested that the hormesis induced by Pb in *S. salsa* was eliminated due to the increasing accumulation of Pb (Table 1). Especially, the significant ($p < 0.05$) inhibition of plant growth caused by the combined exposure of Pb and Zn indicated the synergistic effects between Pb and Zn, since the single heavy metal exposures did not alter the growth of *S. salsa*.

After exposure for 1 week, the average antioxidant enzyme activities (except CAT) were slightly ($p > 0.05$) elevated in the above-ground part of seedlings from both Pb-treated and mixed Pb and Zn-exposed *S. salsa* samples (Table 2). However, the activities of antioxidant enzymes (SOD, POD, CAT and GPx) in Zn-exposed samples were significantly ($p < 0.05$) increased to approx. fourfold of control levels. It suggested that Zn could induce potential oxidative stress in *S. salsa* after exposure for 1 week. Interestingly, the activities of these antioxidant enzymes (SOD, POD, CAT and GPx) were significantly ($p < 0.01$) decreased in Zn-exposed group after 1 month exposure. In addition, these activities of these antioxidant enzymes were lower than those in the corresponding Zn-exposed samples after 1 week exposure. It could be accounted for the decrease of Zn accumulation in *S. salsa* samples, which was confirmed by the concentrations of Zn in the above ground part of *S. salsa* (Table 1).

From Table 1, both root and above-ground part of *S. salsa* accumulated significant levels of metals after exposure for either 1 week or 1 month. It seemed that root could accumulate more Zn than the above-ground part of *S. salsa*. However, it was interestingly found that the Pb concentrations in root from Pb-exposed *S. salsa* with 1 week and 1 month exposures did not vary significantly ($p > 0.05$), which indicated that there were no time-dependent effects of Pb accumulation in root within 1 month of exposure time. In the above-ground part tissue, the time-dependent accumulation of Pb was clearly found in both Pb-treated and mixed Pb and Zn-treated samples. It seemed there was a constant accumulation of Pb in root of *S. salsa*. The similar phenomenon was observed in cadmium-exposed *S. salsa* in our previous work (Liu et al. 2011).The detailed mechanism was unknown and further study is necessary to elucidate the mechanism of Pb accumulation in *S. salsa*. For the mixed Pb and Zn-exposed samples, the average concentrations of Zn in either root tissues or above-ground part tissues were lower than those in Zn-exposed samples, however, there were no statistical significances.

From the visual inspection, betaine was the dominant metabolite with a 10–100 time higher level than other metabolites in the ^{1}H NMR spectral intensities (Fig. 1). Betaine is the key metabolite that can be biosynthesized by cells for the protection against osmotic stresses, such as drought, high salinity and high temperature in organisms including both plants and marine animals (especially marine invertebrates) (Moghaieb et al. 2004). As a key secondary metabolite, the pathway of betaine biosynthesis in high plant is straightforward: choline monooxygenase (CMO) converts choline (a detectable metabolite in *S. salsa*, Fig. 1) to betaine aldehyde, and betaine aldehyde dehydrogenase (BADH) converts this product to betaine (Greenway and Osmond 1972; Lee et al. 2004; Peel et al. 2010). Since *S. salsa* is native to the saline soil containing a high salinity up to 3 %, therefore the organic osmolyte, betaine plays

important physiological role in osmotic regulation and hence was detected at high levels in *S. salsa* root tissues.

From the PCA scores plot, no significant separation along either PC1 or PC2 axis was found between control and metal-treated groups after exposure with metals for 1 week (data not shown). Moreover, SAM software (Katsiadaki et al. 2009) was used to find significant metabolic differences between control and metal-exposed groups with appropriate false discovery rate (FDR < 0.01) cutoffs. However, no significant metabolic changes were found in the metal-exposed samples with 1 week exposure of Pb, Zn or mixed Pb and Zn. It implied that the metal exposures (Pb, Zn and their mixture) did not severely induce toxicological responses at metabolic level within 1 week exposure time.

After exposure for 1 month, the separation between control and Pb or Zn-exposed groups was significantly ($p < 0.05$) found along PC1 axis (Fig. 2a, c). The significant metabolic differences including increased isocaproate, glucose and fructose, and decreased malate, citrate and sucrose were detected in Pb-treated *S. salsa* samples (Fig. 2b). In plant, sucrose can be commonly converted to glucose and fructose. The increased glucose and fructose and decreased sucrose in Pb-treated *S. salsa* indicated the elevated conversion of sucrose to glucose and fructose induced by Pb exposure. In *Silene cucubalus* exposed to Cd, however, only increased glucose was observed, while other carbohydrates (sucrose, fructose, etc.) were not altered (Bailey et al. 2003). Isocaproate is the main product from leucine under anaerobic conditions (Hamid et al. 1997). Therefore the increased isocaproate might imply the enhanced anaerobic metabolism in root tissue of *S. salsa* exposed to Pb for 1 month. Malate and citrate are two key intermediates in the TCA cycle. Therefore the decrease in malate and citrate indicated the disturbances in the TCA cycle that was related to the energy metabolisms induced by Pb exposures in *S. salsa*. The metabolic profile of Zn-exposed group was similar to that of Pb-exposed group (Fig. 2d). However, several metabolic changes, increased phosphocholine and betaine, and decreased choline were unique to Zn-exposed samples. Betaine is an osmolyte biosynthesized from choline as mentioned above. Sun et al. (2010) reported, in *Arabidopsis thaliana* exposed to Cd, some compatible solutes (alanine, proline, etc.) were increased to adapt osmotic stress induced by Cd. Since *S. salsa* is a halophyte that uses high level of betaine to regulate osmolarity. Hereby, the elevation of betaine clearly indicated the osmotic stress by Zn in root of *S. salsa*, which was different to osmoregulation of *A. thaliana* to Cd exposure. For the group with combined exposure of Pb and Zn, there were no significant metabolic responses induced in the *S. salsa* root samples (Fig. 3). It might mean that there existed antagonistic effects induced by the combined exposure of Pb and Zn at metabolic level. Contrarily, it seemed that Pb and Zn could induce synergistic effects based on the plant growth parameters, as mentioned above. The interactive mechanisms between Pb and Zn are unknown. In our previous work (Liu et al. 2011), Cd induced high levels of amino acids in above-ground part of *S. salsa*, which was related to the promotion of protein bio-degradation. In this study, however, neither Pb nor Zn induced significant changes in amino acids, which implied both Pb and Zn did not affect protein bio-degradation in root of *S. salsa*. The unique metabolic metabolites in *S. salsa*, such as amino acids for Pb and phosphocholine and choline for Zn, could be used as biomarkers for corresponding metal biomonitoring in the intertidal zones of the Bohai Sea.

Conclusions

The toxicological effects were characterized on the basis of metabolic profiles of root tissue extracts and antioxidant enzyme activities in above-ground part of seedlings of *S. salsa* exposed to environmentally relevant lead, zinc and combined lead and zinc exposures for 1 week and 1 month. The significant metabolic responses included the increased isocaproate, glucose and fructose, and decreased malate, citrate and sucrose in root tissues of *S. salsa* exposed to Pb. The increased phosphocholine and betaine, and decreased choline were uniquely found in Zn-exposed samples besides the similar metabolic changes such as decreased malate, citrate and sucrose. Our results revealed that Pb and Zn could induce osmotic and oxidative stresses, and disturbances in energy metabolism in *S. salsa* after exposure for 1 month.

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