



## Improved cadmium uptake and accumulation in the hyperaccumulator *Sedum alfredii*: the impact of citric acid and tartaric acid<sup>\*#</sup>

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**Abstract:** The elucidation of a natural strategy for metal hyperaccumulation enables the rational design of technologies for the clean-up of metal-contaminated soils. Organic acid has been suggested to be involved in toxic metallic element tolerance, translocation, and accumulation in plants. The impact of exogenous organic acids on cadmium (Cd) uptake and translocation in the zinc (Zn)/Cd co-hyperaccumulator *Sedum alfredii* was investigated in the present study. By the addition of organic acids, short-term (2 h) root uptake of <sup>109</sup>Cd increased significantly, and higher <sup>109</sup>Cd contents in roots and shoots were noted 24 h after uptake, when compared to controls. About 85% of the <sup>109</sup>Cd taken up was distributed to the shoots in plants with citric acid (CA) treatments, as compared with 75% within controls. No such effect was observed for tartaric acid (TA). Reduced growth under Cd stress was significantly alleviated by low CA. Long-term application of the two organic acids both resulted in elevated Cd in plants, but the effects varied with exposure time and levels. The results imply that CA may be involved in the processes of Cd uptake, translocation and tolerance in *S. alfredii*, whereas the impact of TA is mainly on the root uptake of Cd.

**Key words:** Hyperaccumulation, Cadmium, Organic acid, *Sedum alfredii*, Translocation, Uptake

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### 1 Introduction

The super ability of hyperaccumulators to extract excess metals from soil is being widely studied (Pence *et al.*, 2000; Zhao *et al.*, 2006; Hanikenne *et al.*, 2008), and transformed into a promising and fairly new field of phytoremediation which deals with sites contaminated with toxic

metallic elements (Chaney *et al.*, 2005). The efficiency of phytoremediation is largely dependent on the total amount of pollutants removed by the plants (Ebbs *et al.*, 1997). More information on the processes involved in metal uptake and translocation in plants will shed light on phytoremediation. Metal bioavailability in the soil-plant system is considered to be a key factor controlling plant uptake. Most plants are capable of exuding some low molecular weight organic acids (LMWOAs), such as citric, malic, oxalic, and succinic acids (Dakora and Phillips, 2002). These organic acids can react with metal ions in both the soil solution and solid phases (Jones *et al.*, 1994; Nigam *et al.*, 2000), and increase metal mobility in the rhizospheric environment, thereby improving the phytoavailability of metals to plants

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(Lopez-Bucio *et al.*, 2000; Ma *et al.*, 2001). Enhancement of cadmium (Cd) solubility by various organic acids in soils and subsequent metal uptake by plants has been widely reported (Nigam *et al.*, 2000; Han *et al.*, 2006). Due to its degradable nature, the use of LMWOA can be an efficient alternative to synthetic chelants used in phytoextraction technology.

In hyperaccumulator species, organic acid has also been suggested to be involved in tolerance and accumulation of toxic metallic elements (Boominathan and Doran, 2003; Kupper *et al.*, 2004; Sun *et al.*, 2006; Yang *et al.*, 2006). In mature and senescent leaves of *Thlaspi caerulescens*, most of the accumulated Cd was bound by some weak oxygen ligands such as organic acid (Kupper *et al.*, 2004). Nearly 13% of the total Cd accumulated in the hairy roots of *T. caerulescens* was associated with organic acids, and the rest was localized in the cell walls (Boominathan and Doran, 2003). Zn was predominantly coordinated and complexed with malate in the aerial parts of *Arabidopsis halleri* (Sarret *et al.*, 2004). Acetic and citric acids (CAs) might be related to Cd hyperaccumulation in leaves of the new Cd hyperaccumulator *Solanum nigrum* L. (Sun *et al.*, 2006). In case of nickel (Ni) hyperaccumulator *Thlaspi goesingense*, Ni was predominantly located in the vacuole as an organic complex, possibly CA (Kramer *et al.*, 2000).

*Sedum alfredii* Hance is one of the recently identified Cd hyperaccumulators native to China (Yang *et al.*, 2004; Sun *et al.*, 2008), and is of increasing interest for its potential use in phytoextraction (Li *et al.*, 2007; Huang *et al.*, 2008; Tian *et al.*, 2009). Our previous studies suggested an enhanced uptake and translocation of Cd, mostly through symplastic pathways (Lu *et al.*, 2008; 2009), in this particular plant species. The physiological mechanisms underlying uptake and accumulation of the metal in *S. alfredii*, however, are still unknown. Exudation of organic acids such as tartaric acid (TA) and oxalic acid has been observed from the roots of the hyperaccumulator *S. alfredii* under Cd exposure (Li *et al.*, 2007). Moreover, Cd in the shoots of hyperaccumulating ecotype (HE) *S. alfredii* was mainly coordinated with organic acids, mainly malic acid, and also CA (Tian *et al.*, 2011). Preliminary experiments showed that exogenous TA and CA

application increased Cd uptake and accumulation in plants of *S. alfredii*, whereas positive effects were not observed for malic acid or oxalic acid. The present study thereby aims to investigate the uptake and translocation of Cd in the hyperaccumulator *S. alfredii* when supplemented with a range of exogenous CA or TA. This may help to better understand and clarify the role of these two organic acids in the metal accumulation in this plant species, as well as to promote the use of organic acids in phytoremediation of contaminated soils by *S. alfredii*.

## 2 Materials and methods

### 2.1 Plant materials and growth conditions

Seedlings of the hyperaccumulator *Sedum alfredii* were cultivated hydroponically. Plants were originally obtained from an old plumbum (Pb)/Zn mine area in Zhejiang Province, China, and chosen to grow in non-contaminated soil for several generations to minimize the internal metal contents. Uniform and healthy shoots were selected and cultivated in the basal nutrient solution containing 2.0 mmol/L Ca(NO<sub>3</sub>)<sub>2</sub>, 0.7 mmol/L K<sub>2</sub>SO<sub>4</sub>, 0.1 mmol/L KH<sub>2</sub>PO<sub>4</sub>, 0.5 mmol/L MgSO<sub>4</sub>, 0.1 mmol/L KCl, 10 μmol/L H<sub>3</sub>BO<sub>3</sub>, 0.5 μmol/L MnSO<sub>4</sub>, 1.0 μmol/L ZnSO<sub>4</sub>, 0.2 μmol/L CuSO<sub>4</sub>, 0.01 μmol/L (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>, 100 μmol/L iron (Fe)-ethylenediaminetetraacetic acid (EDTA). All the chemicals (analytically pure) used in this study were provided by Sinopharm Chemical Reagent Co., Ltd., China. Nutrient solution pH was adjusted daily to 5.5–5.8 with 0.1 mol/L NaOH or HCl. Plants were grown under glasshouse conditions maintaining natural light, day/night temperature 26/20 °C and day/night humidity 70%/85%. The nutrient solution was continuously aerated and renewed every three days.

### 2.2 Radiotracer <sup>109</sup>Cd experiments

Intact roots of four-week old seedlings of *S. alfredii* were rinsed in deionized water, and then treated with a pretreatment solution containing 2 mmol/L MES-Tris (pH=5.8) and 0.5 mmol/L CaCl<sub>2</sub> (Lu *et al.*, 2008). After 12 h of pretreatment, the seedlings were used for two experiments as described subsequently.

### 2.2.1 Effects of organic acids on short-term $^{109}\text{Cd}$ uptake

The plants were transferred to custom-built hydroponic vessels (three seedlings in each 400 ml vessel) containing  $^{109}\text{Cd}$  labeled uptake solution. The uptake solution contained 0.5 mmol/L  $\text{CaCl}_2$ , 2 mmol/L MES-Tris (pH=5.8) and 10 or 100  $\mu\text{mol/L}$   $\text{CdCl}_2$  labeled with  $^{109}\text{Cd}$  (2.0  $\mu\text{Ci/L}$ ). Ten treatments were carried out as summarized in Table 1.

**Table 1 Concentrations of Cd, TA, and CA in the uptake solution for the treatments**

Treatment	Concentration ( $\mu\text{mol/L}$ )		
	Cd	TA	CA
T1	10	0	0
T2	10	10	0
T3	10	100	0
T4	10	0	10
T5	10	0	100
T6	100	0	0
T7	100	10	0
T8	100	100	0
T9	100	0	10
T10	100	0	100

Each treatment was replicated four times. Cd speciation in the nutrient solution was calculated by Visual-Minteq 3.0 (Table S1). After 2 h of uptake, the seedlings were quickly rinsed with the unlabeled pretreatment solution, and then transferred to identical vessels containing ice-cold desorption solutions (2 mmol/L MES-Tris, pH=5.8, 5 mmol/L  $\text{CaCl}_2$ , and 100  $\mu\text{mol/L}$   $\text{CdCl}_2$ ). After 15 min, the seedlings were separated into roots and shoots, blotted-dried and weighed. Plants were transferred into radioactivity counting vials, and  $^{109}\text{Cd}$  was assayed by gamma spectroscopy (Canberra Packard Auto Gamma 5780).

### 2.2.2 Effects of organic acids on $^{109}\text{Cd}$ translocation

The experimental procedure was similar as described above (Section 2.2.1). The uptake solution contained 0.5 mmol/L  $\text{CaCl}_2$ , 2 mmol/L MES-Tris (pH=5.8) and 100  $\mu\text{mol/L}$   $\text{CdCl}_2$  labeled with  $^{109}\text{Cd}$  (2.0  $\mu\text{Ci/L}$ ). Treatments include CA (0, 10, 50, 100, and 500  $\mu\text{mol/L}$ ) or TA (0, 50, 100, 250, and 500  $\mu\text{mol/L}$ ), respectively. Fresh  $^{109}\text{Cd}$ -labeled solution was added periodically in the uptake solution to maintain constant Cd concentrations. Each treatment

was replicated four times. After 24 h of uptake, the seedlings were desorbed by ice-cold solutions (the same as Section 2.2.1) and harvest. The radioactivity of  $^{109}\text{Cd}$  was quantified in both roots and shoots.

### 2.3 Long-term effects of organic acids on Cd accumulation

Four-week-old intact seedlings of *S. alfredii* were treated with 100  $\mu\text{mol/L}$   $\text{CdCl}_2$ , in addition with a range of CA (0, 10, 50, 100, or 500  $\mu\text{mol/L}$ ) or TA (0, 50, 100, 250, or 500  $\mu\text{mol/L}$ ) in basal nutrient solution (see Section 2.1). Each treatment was replicated three times. Cd speciation in the nutrient solution was calculated by Visual-Minteq 3.0 (Table S2). Nutrient solution pH was adjusted daily to 5.4 with 0.1 mol/L NaOH. The nutrient solutions with different treatments were continuously aerated and changed every three days. Plants were harvested after 7 or 14 d exposure, and rinsed, separated into roots and shoots, oven-dried and weighed. Dry plant samples (0.1 g) of each treatment were digested with 5.0 ml  $\text{HNO}_3\text{-HClO}_4$  (v/v, 4:1), and the digest was transferred to a 50-ml volumetric flask, made up to volume and filtered. Cd concentrations were determined by ICP-MS (Agilent 7500a, USA).

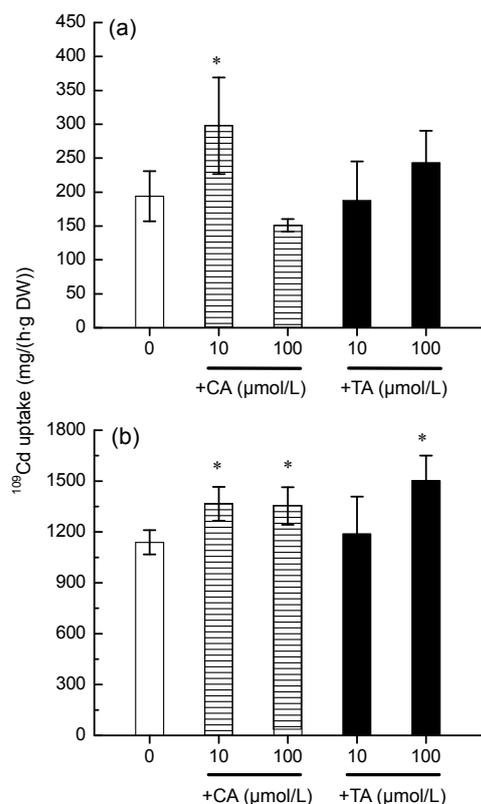
### 2.4 Statistical methods

All data were statistically analyzed using the SPSS package (Version# 11.0). Differences between treatments were determined by the least significant difference ( $P<0.05$ ) from the analysis of variance (ANOVA).

## 3 Results

### 3.1 Effects of CA or TA on $^{109}\text{Cd}$ uptake and translocation

The effects of CA or TA on  $^{109}\text{Cd}$  influx into the roots of *S. alfredii* were investigated by adding these two organic acids in the  $^{109}\text{Cd}$  uptake solution. Our previous studies suggested that the 2 h uptake period mostly represented the real Cd influx into the roots (Lu et al., 2008). As shown in Fig. 1, at 10  $\mu\text{mol/L}$  Cd, addition of equal molar CA significantly ( $P<0.05$ ) increased  $^{109}\text{Cd}$  influx into the roots of *S. alfredii* (Fig. 1a). When plants were exposed to 100  $\mu\text{mol/L}$  Cd, higher  $^{109}\text{Cd}$  influx into the roots was marked

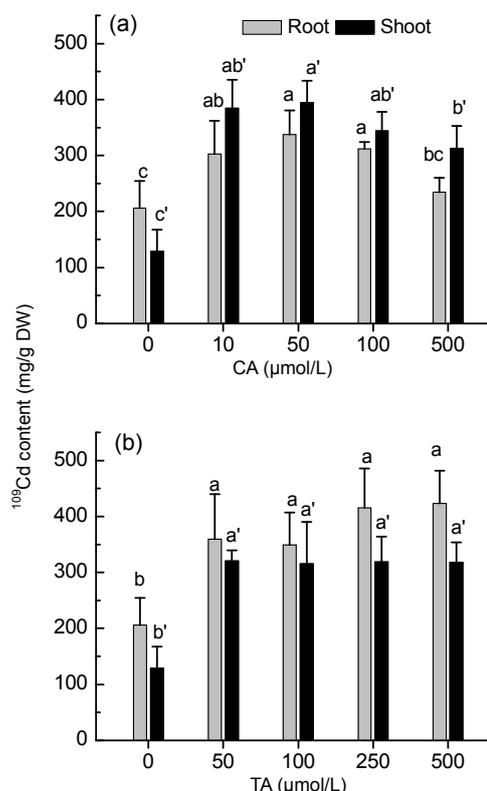


**Fig. 1** Effects of CA or TA on <sup>109</sup>Cd uptake by *Sedum alfredii*

Plants were exposed to 10 μmol/L (a) or 100 μmol/L (b) <sup>109</sup>Cd for 2 h, with addition of different levels (0, 10, and 100 μmol/L) of CA or TA. Data points and error bars represent means and standard errors (SEs) of four replicates. Means marked with one asterisks indicate significant difference between treatments and control at  $P < 0.05$ . DW: dry weight

( $P < 0.05$ ) with the addition of either low (10 μmol/L) or high (100 μmol/L) CA treatment (Fig. 1b). As compared with CA, the promoted Cd uptake of roots was only significant by the addition of TA at 100 μmol/L level (Fig. 1b).

Elevated <sup>109</sup>Cd contents in both roots and shoots of *S. alfredii* were noted by the application of exogenous CA or TA (Fig. 2). After 24 h of uptake, <sup>109</sup>Cd in the roots and shoots of *S. alfredii* increased by 1.5- and 3.0-fold ( $P < 0.05$ ), respectively, in addition with 50 μmol/L CA. However, the effect was less pronounced with elevated CA treatment (500 μmol/L) (Fig. 2a). Exposure of TA resulted in <sup>109</sup>Cd content elevations as high as 2.0- and 2.5-fold in the roots and shoots, respectively, but no dose-dependent increases were observed (Fig. 2b). A significant ( $P < 0.05$ ) increase in <sup>109</sup>Cd root-to-shoot translocation rate was observed for the addition of CA. For example, up to



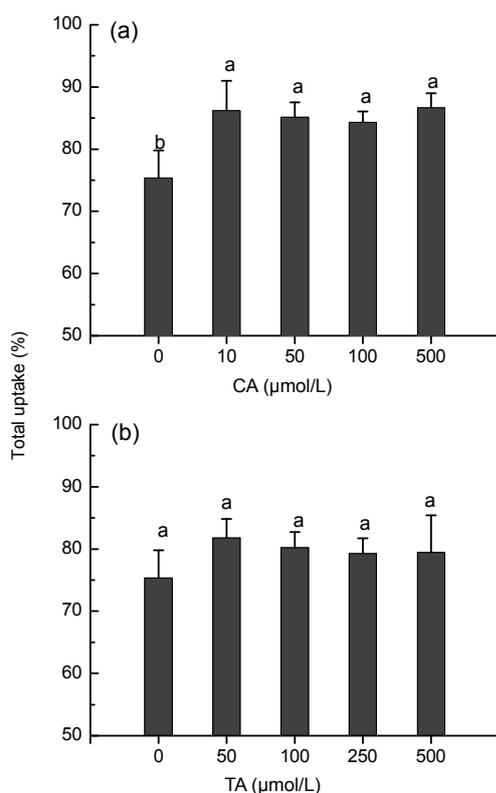
**Fig. 2** Effects of CA (a) or TA (b) on <sup>109</sup>Cd contents in root and shoot of *Sedum alfredii*

Plants were exposed to 100 μmol/L <sup>109</sup>Cd in nutrition solution for 24 h, with addition of different levels of CA (0, 10, 50, 100, and 500 μmol/L) or TA (0, 50, 100, 250, and 500 μmol/L), respectively. Data points and error bars represent means and SEs of four replicates. Different letters indicate significant difference between different treatments at  $P < 0.05$ . DW: dry weight

85% of <sup>109</sup>Cd taken up was distributed to the shoots in the plants with CA treatments, compared with 75% in those not treated with CA (Fig. 3a). In response to TA treatments, the <sup>109</sup>Cd translocation rate in *S. alfredii* increased to some extent, but not reached a significant level (Fig. 3b).

### 3.2 Effects of long-term CA/TA treatments on plant growth

Hydroponic experiments were employed to study the long-term growth response of the *S. alfredii* plants by application of CA and TA under Cd stress (Table 2). The Cd concentration in the nutrient solution was 100 μmol/L, as the report by Ye *et al.* (2003) suggested that HE *S. alfredii* grew healthy at this Cd level in solution. The results showed that no significant decreases in root biomass were observed in the



**Fig. 3** Effects of CA (a) or TA (b) on the percentage distribution of <sup>109</sup>Cd to shoots of *Sedum alfredii*. Plants were exposed to 100 μmol/L <sup>109</sup>Cd in nutrition solution for 24 h, with addition of different levels of CA or TA. Data points and error bars represent means and SEs of three replicates. Different letters indicate significant difference between different treatments at  $P < 0.05$

plants after 14 d of Cd exposure, and CA did not significantly affect root growth (Table 2). However, stem and leaf biomass decreased markedly ( $P < 0.05$ ) in the presence of high TA levels (100–500, 250–500 μmol/L, respectively, for stem and leaf) (Table 2). When plants were treated with 100 μmol/L Cd for 28 d, root biomass decreased significantly ( $P < 0.05$ ) as compared to the control (unstressed cultures). No pronounced effects were observed by the application of either CA or TA. Marked decreases of leaf and stem growth were also noted after 28 d of Cd exposure ( $P < 0.05$ ). However, application of low CA level significantly ( $P < 0.05$ ) alleviated Cd toxicity. After 28 d of exposure, the stem and leaf biomass of the plants under Cd stress increased by 22.5% and 24.9% ( $P < 0.05$ ), respectively, in the presence of 10 μmol/L CA, whereas such effect was not pronounced for TA (Table 2).

### 3.3 Effects of long-term CA/TA treatments on Cd accumulation

After 14 d of exposure to 100 μmol/L Cd, a majority of Cd taken up by *S. alfredii* was distributed to the shoots (Table 3). With low addition of CA (10–100 μmol/L), Cd increased significantly ( $P < 0.05$ ) in shoots, especially leaves, of *S. alfredii*, whereas the same effect was not found in roots. Exposure of TA resulted in root Cd content increases of up to 2.8-fold ( $P < 0.05$ ) and a 1.6-fold increase in Cd in the leaves ( $P < 0.05$ ).

**Table 2** Biomass of *Sedum alfredii* after 14 or 28 d treatments with or without 100 μmol/L Cd and different levels of CA or TA

Cd (μmol/L)	Treatment (μmol/L)	Biomass (g DW/plant)					
		14 d			28 d		
		Root	Stem	Leaf	Root	Stem	Leaf
0		0.86±0.08 a	2.45±0.23 a	5.23±0.62 a	1.56±0.15 a	4.91±0.51 a	8.03±0.64 a
100	CA						
	0	0.84±0.03 a	2.36±0.19 a	5.18±0.46 a	1.34±0.09 b	3.51±0.46 b	6.07±0.59 c
	10	0.84±0.02 a	2.12±0.16 a	4.81±0.70 a	1.42±0.08 b	4.30±0.59 ab	7.58±0.21 ab
	50	0.86±0.04 a	2.32±0.27 a	5.49±0.78 a	1.31±0.10 b	3.83±0.70 b	6.64±0.71bc
	100	0.87±0.04 a	2.14±0.22 a	4.66±0.48 a	1.31±0.17 b	3.92±0.46 b	6.06±0.40 c
	500	0.84±0.03 a	2.11±0.14 a	4.84±0.54 a	1.33±0.09 b	3.88±0.51 b	5.65±1.18 c
	TA						
	0	0.84±0.03 a	2.36±0.19 a	5.18±0.46 ab	1.34±0.09 b	3.51±0.46 b	6.07±0.59 b
	50	0.84±0.02 a	2.27±0.08 a	4.97±0.49 ab	1.23±0.12 b	3.78±0.61 b	6.35±0.58 b
	100	0.84±0.04 a	1.88±0.13 b	5.44±0.50 a	1.23±0.09 b	3.39±0.33 b	6.19±0.55 b
250	0.82±0.04 a	1.63±0.30 b	4.49±0.06 bc	1.34±0.04 b	3.50±0.56 b	6.33±0.37 b	
500	0.82±0.03 a	1.60±0.05 b	3.89±0.27 c	1.35±0.10 b	3.52±0.53 b	6.08±0.29 b	

Different letters among treatments indicate significant differences at  $P < 0.05$ . DW: dry weight

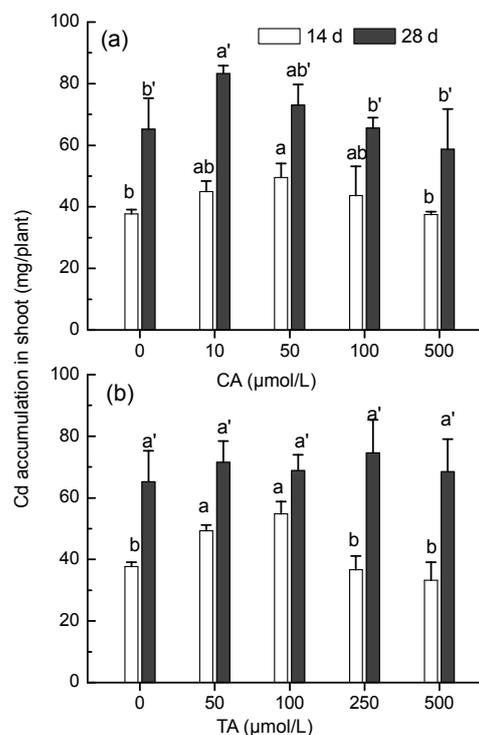
**Table 3** Cd concentrations in root, stem, and leaf of *Sedum alfredii* after 14 or 28 d treatments with 100  $\mu\text{mol/L}$  Cd and different levels of CA or TA

Treatment ( $\mu\text{mol/L}$ )	Biomass (g DW/plant)					
	14 d			28 d		
	Root	Stem	Leaf	Root	Stem	Leaf
CA						
0	464 $\pm$ 26 a	4740 $\pm$ 221 c	5152 $\pm$ 259 b	1096 $\pm$ 124 b	4882 $\pm$ 154 b	6958 $\pm$ 690 b
10	500 $\pm$ 20 a	5297 $\pm$ 186 ab	7054 $\pm$ 335 a	1516 $\pm$ 103 b	5288 $\pm$ 144 a	8008 $\pm$ 473 a
50	448 $\pm$ 108 a	5312 $\pm$ 135 ab	6596 $\pm$ 438 a	3328 $\pm$ 344 a	4906 $\pm$ 104 b	8410 $\pm$ 422 a
100	503 $\pm$ 80 a	5439 $\pm$ 268 a	6550 $\pm$ 764 a	3450 $\pm$ 305 a	4998 $\pm$ 338 ab	7915 $\pm$ 477 a
500	523 $\pm$ 52 a	5033 $\pm$ 311 bc	5387 $\pm$ 736 b	3297 $\pm$ 592 a	4862 $\pm$ 157 b	6883 $\pm$ 431 b
TA						
0	464 $\pm$ 26 b	4740 $\pm$ 221 c	5152 $\pm$ 259 b	1096 $\pm$ 124 b	4882 $\pm$ 154 b	6958 $\pm$ 690 b
50	1285 $\pm$ 65 a	5437 $\pm$ 567 a	7439 $\pm$ 110 ab	1640 $\pm$ 229 b	5084 $\pm$ 264 bc	7769 $\pm$ 392 bc
100	1193 $\pm$ 173 a	5895 $\pm$ 135 a	8048 $\pm$ 654 a	2268 $\pm$ 242 a	5352 $\pm$ 83 ab	8202 $\pm$ 352 ab
250	404 $\pm$ 133 b	5390 $\pm$ 199 a	6208 $\pm$ 1234 bc	2349 $\pm$ 238 a	5624 $\pm$ 326 a	8652 $\pm$ 332 a
500	567 $\pm$ 175 b	4711 $\pm$ 222 b	6559 $\pm$ 1031 bc	1453 $\pm$ 164 bc	5680 $\pm$ 106 a	7938 $\pm$ 462 ab

Different letters among treatments indicate significant differences at  $P < 0.05$ . DW: dry weight

As Cd exposure time prolonged to 28 d, the addition of CA increased Cd contents in the roots and leaves 3- and 1.5-fold, respectively, but the effect was less pronounced in the stems, as marked increase of Cd in the stems was only noted when 10  $\mu\text{mol/L}$  CA was added (Table 3). With addition of TA, Cd in the roots, stems, and leaves increased 2.2-, 1.2-, and 1.2-fold, respectively (Table 3). Regardless of the exposure time (14 or 28 d), no dose-dependent increase of Cd concentration in the plants was noted by the application of either CA or TA, and elevated Cd content was not observed with the treatment of high CA or TA level (500  $\mu\text{mol/L}$ ) (Table 3).

The total amount of Cd in the aerial parts of the plants is the key factor for the phytoextraction efficiency. Our results showed that low concentration application of CA or TA was able to increase the total amount of Cd in the shoots of *S. alfredii* (Fig. 4). When Cd-treated plants were exposed to 50  $\mu\text{mol/L}$  CA for 14 d, a statistically significant increase ( $P < 0.05$ ) of 28% in the Cd accumulation was observed in the shoots of *S. alfredii* (Fig. 4a). As treatment time was prolonged to 28 d, the addition of 10  $\mu\text{mol/L}$  CA resulted in enhanced Cd accumulation in the shoots ( $P < 0.05$ ), whereas no pronounced effect was noted in plants treated with higher CA levels (Fig. 4a). Cd accumulation in the shoots of *S. alfredii* ( $P < 0.05$ ) increased by 30% and 50%, respectively, with treatments of 50 or 100  $\mu\text{mol/L}$  TA for 14 d (Fig. 4b). No marked increase of shoot Cd was observed in plants treated with TA for 28 d (Fig. 4b).

**Fig. 4** Cd accumulation in shoots of *Sedum alfredii* after 14 or 28 d treatments with 100  $\mu\text{mol/L}$  Cd and different levels of CA (a) or TA (b)

Data points and error bars represent means and SEs of three replicates. Different letters among treatments indicate significant differences at  $P < 0.05$

#### 4 Discussion

LMWOAs are attractive alternatives to synthetic chelants for application in phytoextraction of contaminated environments due to their natural and

biodegradable properties (Romkens *et al.*, 2002). CA-enhanced phytoextraction of toxic metallic elements from contaminated soil has been previously reported (Turgut *et al.*, 2004; Quartacci *et al.*, 2005; Jean *et al.*, 2008). In this study, elevated Cd accumulation via the application of exogenous CA was observed in the hyperaccumulator *S. alfredii*, in response to either short-term or long-term application of exogenous CA (Table 3). The similar impacts of CA on Cd uptake and accumulation in non-accumulator plants have been reported in many papers (Senden *et al.*, 1995; Chen *et al.*, 2003; do Nascimento *et al.*, 2006; Duarte *et al.*, 2007). The positive effects of CA on the growth of *S. alfredii* and its heavy metal uptake and accumulation have been reported by using the pot-culture experiments (Sun *et al.*, 2009). Enhanced Cd uptake by CA, however, has been attributed to the increase of the metal mobility due to the decrease of the solution pH, as suggested by Chen *et al.* (2003).

In this study, the application of CA is enhanced the Cd accumulation in the shoots of *S. alfredii* (Table 3), despite the same adjusted solution pH level. This suggests that some other mechanisms, rather than decreased pH in the medium, may also be involved in CA-enhanced Cd uptake in plants. CA-induced elevation of Cd accumulation in the shoots of *S. alfredii* could be attributed to three processes. Firstly, CA might be involved directly in Cd uptake by the plants, possibly by complexation of the metal, rather than changes in rhizosphere environment. The significant increase of  $^{109}\text{Cd}$  influx into the roots of *S. alfredii* after 2 h application of exogenous CA (Fig. 1), supports this point of view. It is also supported by our previous analysis on Cd speciation in the hyperaccumulator *S. alfredii* (Tian *et al.*, 2011), which indicated that 24.9% of Cd in roots was associated with CAs. Moreover, with the addition of CA, a small amount of Cd was presented as complexation of Cd-citrate such as Cd-Citrate- in the uptake solution (Table S2). Secondly, citrate has been reported to play an important role in the xylem loading of metals such as aluminium (Al) and Fe (Ma and Hiradate, 2000; Durrett *et al.*, 2007). Elevated root-to-shoot translocation rate of  $^{109}\text{Cd}$  stimulated by CA (Fig. 3) suggests that this organic acid may also play an important role in xylem transport of Cd in the hyperaccumulator *S. alfredii*. Last but not the least,

application of CA may also contribute to enhanced Cd accumulation by increasing the storage capacity of Cd in the shoots of *S. alfredii*. Organic acids have been suggested to play an important role in the metal detoxification processes in the hyperaccumulators (Boominathan and Doran, 2003; Kupper *et al.*, 2004; Sun *et al.*, 2006; Yang *et al.*, 2006). In a newly discovered Cd hyperaccumulator *Solanum nigrum* L., a positive correlation was found between CA and water-soluble Cd in the leaves, suggesting that CA was responsible for the Cd accumulation in the leaves of the plants (Sun *et al.*, 2006). Yang *et al.* (2006) pointed out that CA might be involved in Zn tolerance and accumulation in the shoots of the Zn/Cd hyperaccumulator *S. alfredii*. Although it has been suggested that Cd was mainly associated with malic acid in shoots of *S. alfredii*, still a small proportion (about 9.2%–15.4% in leaves, and 21.7% in stems) of Cd was bound with CA (Tian *et al.*, 2011). In contrast with CA, only a few studies have reported on the role of TA in enhanced metal accumulation of plants. For example, Pb accumulation in the seedlings of *Zinnia elegans* Jacq. was markedly ( $P < 0.05$ ) increased by TA (Cui *et al.*, 2007). TA excretion by roots, however, has been reported to be positively correlated with the metal stress. Cd accumulation in the Fe-sufficient plants of *Solanum nigrum* L. had a significant ( $P < 0.01$ ) positive correlation with the exudation of TA (Bao *et al.*, 2011). TA was one of the two main constituents detected in the root exudates of *S. alfredii* over the 6 h period, and Cd addition had a significant effect ( $P < 0.05$ ) on the concentration of TA secreted by roots (Li *et al.*, 2007). However, Li *et al.* (2007) also suggested that the secretion of organic acids appeared to be a functional metal resistance mechanism that chelates the metal ions extracellularly, and reduces metal uptake and subsequent stresses on roots. This contrasts with the results observed in this study, in which a pronounced increase of  $^{109}\text{Cd}$  accumulation in plants of *S. alfredii* was observed in roots of *S. alfredii* by the addition of exogenous TA (Figs. 1 and 2). Nevertheless, the absence of enhanced root-to-shoot translocation rate by addition of TA suggests that the possible role of TA is mainly on the root uptake of Cd by the plants of *S. alfredii*.

Successful removal of metals from contaminated soils via phytoremediation largely depends on sufficient metal concentrations in the shoots of target

species. As highlighted by Ebbs *et al.* (1997), the emphasis for phytoremediation should be placed on the total amount of contaminant removed from soil. However, biomass losses caused by the negative effects of chelants on plants may decrease their total metal accumulation capacity (Jean *et al.*, 2008), despite the increasing removable metal concentrations by the application of chelants. This study suggested that CA and TA could be potentially be applied to contaminated soils where the hyperaccumulator *S. alfredii* is planted, since they both have positive effects on total metal accumulation in shoots when properly applied (Fig. 4).

## 5 Conclusions

This study suggests that CA may be involved in the processes of Cd uptake, translocation and tolerance in *S. alfredii*, whereas the impact of TA is mainly on the root uptake. Additionally, kinetics and organic acid application timing are important factors affecting Cd uptake success. The results of this study provide some theoretical reference for chelant-assisted phytoextraction by the hyperaccumulators *S. alfredii*. However, further studies, in particular under pot-culture and field condition, should be employed to optimize our understanding of their effects in field conditions.

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### List of electronic supplementary materials

Table S1 Cd speciation in the uptake solution with addition of different Cd and organic acid levels as calculated by Visual-Minteq 3.0

Table S2 Cd speciation in the nutrient solution with addition of 100 µmol/L Cd and different citric acid or tartaric acid levels as calculated by Visual-Minteq 3.0