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Identification of a new potential Cd-hyperaccumulator *Solanum photeinocarpum* by soil seed bank-metal concentration gradient method

Xingfeng Zhang[a,b,d], Hanping Xia[a,b,*], Zhi'an Li[a,b], Ping Zhuang[a], Bo Gao[c,a]

[a] South China Botanical Garden, The Chinese Academy of Sciences, Guangzhou 510650, China
[b] Key Laboratory of Vegetation Restoration and Management of Degraded Ecosystems, Chinese Academy of Sciences, Guangzhou 510650, China
[c] College of Horticulture and Gardening, Yangtze University, Jingzhou 434025, China
[d] Graduate University of Chinese Academy of Sciences, Beijing 100049, China

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ABSTRACT

A new method, soil seed bank-metal concentration gradient method was used to screen for heavy metal hyperaccumulators, and *Solanum photeinocarpum* was found to be a potential Cd-hyperaccumulator. The chlorophyll content and photosynthetic rate of *S. photeinocarpum* were not affected by Cd pollution, while leaf stomas and transpiration rate were significantly decreased by more than 60 mg kg^{-1} Cd, and leaf water use efficiency and shoot water content were significantly increased by more than 60 or 100 mg kg^{-1} Cd, respectively. In the seed bank-Cd concentration gradient experiment, the shoot biomass of *S. photeinocarpum* showed no significant reduction with soil Cd treatment as high as 100 mg kg^{-1}, but the root biomass was significantly reduced by more than 60 mg kg^{-1} Cd contamination. Plant tissues accumulated 544, 132 and 158 mg kg^{-1} Cd in roots, stems and leaves, respectively, and extracted 157 and 195 μg Cd plant^{-1} in roots and shoots at 100 mg kg^{-1} Cd in soil, respectively. In the transplanting-Cd concentration gradient experiment, plant shoot biomass and root biomass were unaffected by soil Cd as high as 60 mg kg^{-1}. Plant tissues accumulated 473, 215 and 251 mg kg^{-1} Cd in roots, stems and leaves, respectively, and extracted 176 and 787 μg Cd plant^{-1} in roots and shoots at 60 mg kg^{-1} soil Cd, respectively. Soil seed bank-metal concentration gradient method could be an effective method for the screening of hyperaccumulators.

1. Introduction

Phytoremediation is a promising clean-up technology, using plants to remedy soil heavy metal pollution [1,2]. The key to success of this technique is to find hyperaccumulators which can accumulate uniquely high quantities of heavy metals, for example, more than 1000 mg kg^{-1} for As, Pb, Cu, Ni, and Co, 10,000 mg kg^{-1} for Zn and Mn, and 100 mg kg^{-1} for Cd in plant shoots [3]. Though over 400 hyperaccumulators have been found so far worldwide [4], the majority of hyperaccumulating species discovered so far are restricted to tropical and subtropical areas [2,3,5–7]. The distribution of hyperaccumulators would severely restrict phytoremediation technology application worldwide. Meanwhile, the species that can be utilized for the purpose have limitations in practice, such as small biomass, slow growth, poor resistance and lack of seeds and competitiveness with local plants [8]. Therefore, finding efficient methods to identify new hyperaccumulators is necessary for phytoremediation to progress.

Nowadays, most researchers search for accumulators or hyperaccumulators by two methods. One is making a field survey in heavy metal contaminated places, like mining lands, refuse sites and industrial metallurgy areas [9]. The other is choosing some special species, for instance, flowers [10], crops [11], weeds [12], pastures [13], to identify their metal accumulation characteristics. Undoubtedly, these two methods have found many hyperaccumulators for phytoremediation. However, both of them miss or neglect a large percentage of species that have remediation potential for soil heavy metals. In this study, we postulate a new method of using the natural soil seed bank to screen for hyperaccumulators. The soil seed bank refers to all viable seeds present on or in the soil and associated litter/humus which act as a reservoir of plant propagules [14]. The soil seed bank is derived from a combination of stored seeds that have been incorporated over many years plus seeds input recently from the current species pool [15]. Thereby, the plant species in the soil seed bank are abundant and natural. Making use of the soil seed bank for the identification of hyperaccumulators can fully utilize the natural plant species and overcome the disadvantages of the existing methods.

* Corresponding author at: Key Laboratory of Vegetation Restoration and Management of Degraded Ecosystems, Chinese Academy of Sciences, Guangzhou 510650, China. Tel.: +86 20 3725 2737; fax: +86 20 3725 2711.
E-mail addresses: xiahanp@scib.ac.cn, xiahanping2010@126.com (H. Xia).

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The objectives of this study were to (i) use a soil seed bank experiment to find species that have basic properties of hyperaccumulators; (ii) use two metal concentration gradient experiments, seed bank and transplanting seedlings, to identify the hyperaccumulators; and (iii) determine physiology of hyperaccumulators response to metal pollution.

2. Materials and methods

2.1. Soil seed bank-metal concentration gradient method

The soil seed bank-metal concentration gradient method is described as follows: clean soils were collected from the field, and dried on the open concrete floor in fine weather. After sieving through 1 cm mesh and weighed, these soils were put into plastic pots. After the heavy metal solutions were added to the soils, the pots were placed in the greenhouse until the seeds germinated. Germination was a preliminary indication of species tolerance to the corresponding heavy metal. When the plants matured, those species with high biomass, high metal tolerance and their metal accumulation characteristics had not been previously documented were chosen to measure their heavy metal content. Those species with high accumulative abilities were selected for metal concentration gradient experiments (several metal concentrations were set up) to study their metal tolerance and accumulative characteristics, and verify their remediation capacity for heavy metals.

2.2. Soil seed bank-metal accumulator screening experiment

The pot culture experiment was conducted in the greenhouse at South China Botanical Garden from January to March of 2008. Top soil (0–20 cm) was collected from a local abandoned kale-yard with many plant species growing there, and soil properties as follows: soil texture of 70.5% sand, 12.2% silt, and 17.3% clay; soil pH of 6.09; organic matter of 3.9%; cation exchange capacity (CEC) of 79.7 mmol kg^{-1}; total Cd and Zn concentrations of 1.35 and 131 mg kg^{-1}, respectively; available Cd and Zn concentrations of 0.17 and 18.50 mg kg^{-1}, respectively [13].

Seven treatments were set up, which included a control (CK) (without heavy metal addition), two Cd spiking treatments (4 and 8 mg kg^{-1}) designated as Cd4 and Cd8, one Zn spiking treatment (100 mg kg^{-1}) designated as Zn100, two Pb spiking treatments (300 and 600 mg kg^{-1}) designated as Pb300 and Pb600, and two Cu spiking treatments (100 and 300 mg kg^{-1}) designated as Cu100 and Cu300. Dried soil (12.5 kg) sieved through 1 cm mesh was put into each plastic pot (30 cm in diameter and 25 cm in height), then saturated with heavy metal solution with the required amount of $CdCl_2 \cdot 2.5H_2O$, $ZnSO_4 \cdot 7H_2O$, $Pb(NO_3)_2$ or $CuSO_4 \cdot 5H_2O$. All pots were watered each day in order to keep soil moisture at 70–85%. After 65 days of cultivation, the plant species in each pot were identified and recorded (Table 1). Among all these species, *Solanum photeinocarpum* was found to have the highest biomass, best performance, and no toxic symptoms in almost all treatments (Fig. 1); furthermore, there is little documentation on its heavy metal tolerance and uptake characteristics. Therefore, *S. photeinocarpum* was chosen for further experiments. The plants of *S. photeinocarpum* were dug up, washed thoroughly firstly with tap water, followed by deionized water, and then dried at 75 °C until completely dry, weighed, ground to <0.5 mm, and sealed into plastic bags for analysis.

Fig. 1. Plants for different treatments in the soil seed bank-metal accumulators screening experiment (65 days after the heavy metal solution added to the soil; $n = 1$). The numbers marked on each pot represent different treatments (1, CK; 2, Cd4; 3, Cd8; 4, Zn100; 5, Pb300; 6, Pb600; 7, Cu100; 8, Cu300). The highest plant in each pot is *S. photeinocarpum*.

2.3. Seed bank-Cd concentration gradient experiment

In the above experiment, *S. photeinocarpum* grew normally without showing any toxic symptoms under various heavy metal treatments, and the shoot biomasses were near to that of the control, except Cu300 which had no seed germination (Figs. 1 and 2). *S. photeinocarpum* had a higher accumulation of Cd compared to Zn, Pb and Cu in roots and shoots. The BCFs for Cd and Zn were >1 while those of Pb and Cu were <1 (Table 2). The results indicated that *S. photeinocarpum* may have basic characteristics of a Cd-hyperaccumulator. The concentrations of Cd accumulated in stems and leaves were lower than 100 mg kg^{-1} may probably because the Cd contamination level in the soil (only spiked to 8 mg kg^{-1}) was not excessively high. In view of this fact, a further validation experiment based on the concentration gradient approach was conducted as described below.

The pot culture experiment was conducted in the same greenhouse from April to June 2008. Soil used for this experiment was the

Table 1
Plant species grown up from different treatments of pots in the soil seed bank-metal accumulators screening experiment (65 days after the heavy metal solution added to the soil; $n = 1$).

Treatment	Species (Family)
CK	*Solanum photeinocarpum* (Solanaceae); *Gnaphalium pensylvanicum* (Compositae); *Crassocephalum crepidioides* (Compositae); *Leonurus Artemisia* (Labiatae); *Polygonum chinense* (Polygonaceae); *Murdannia triquetra* (Commelinaceae); *Malachium aquaticum* (Caryophyllaceae); *Amaranthus viridis* (Amaranthaceae); *Oxalis corymbosa* (Oxalidaceae)
Cd4	*S. photeinocarpum*; *G. pensylvanicum*; *C. crepidioides*; *M. triquetra*; *M. aquaticum*; *A. viridis*; *O. corymbosa*
Cd8	*S. photeinocarpum*; *G. pensylvanicum*; *C. crepidioides*; *M. triquetra*; *M. aquaticum*; *A. viridis*; *O. corymbosa*
Zn100	*S. photeinocarpum*; *P. chinense*; *M. aquaticum*; *O. corymbosa*
Pb300	*S. photeinocarpum*; *L. artemisia*; *P. chinense*; *O. corymbosa*
Pb600	*S. photeinocarpum*; *L. artemisia*; *O. corymbosa*
Cu100	*S. photeinocarpum*; *P. chinense*; *M. triquetra*; *M. aquaticum*; *O. corymbosa*
Cu300	*O. corymbosa*

Table 2
Heavy metal concentration of *S. photeinocarpum* for different treatments in the soil seed bank-metal accumulators screening experiment (65 days after the heavy metal solution added to the soil; $n = 1$).

Treatment	Metal category	Heavy metal concentration of plants (mg kg^{-1})				BCF	TF
		Root	Stem	Leaf	Shoot		
CK	Cd	1.6	1.9	2.9	2.5		1.51
Cd4	Cd	84	15	22	19	4.8	0.23
Cd8	Cd	114	13	21	18	2.3	0.16
CK	Zn	32	39	40	40		1.23
Zn100	Zn	519	176	139	153	1.5	0.30
CK	Pb	31	21	25	24		0.77
Pb300	Pb	302	70	35	45	0.15	0.15
Pb600	Pb	734	87	43	57	0.10	0.08
CK	Cu	13	18	11	14		1.11
Cu100	Cu	LB	23	24	24	0.24	
Cu300	Cu	NSG	NSG	NSG	NSG	NSG	

LB means little biomass; NSG means no seed germination.

same as the above used for seed bank-metal accumulators screening experiment. Six Cd treatments (0, 8, 15, 30, 60 and 100 mg kg^{-1}) with 4 replicates were set up and designated as CK, Cd8, Cd15, Cd30, Cd60 and Cd100, respectively. Dried soil (2 kg) sieved through 1 cm mesh was put into each plastic pot (16 cm in diameter and 14 cm in height), and then saturated with heavy metal solution containing the required amount of $CdCl_2{\cdot}2.5H_2O$. All pots were watered each day in order to keep soil moisture at 70–85%. Three uniform-sized seedlings (about 5 cm in height) of *S. photeinocarpum* were left in every pot after seeds in the Cd contaminated soil had germinated for 10 days. After the plants were cultivated for 60 days and became mature, they were dug up, and treated according to the method described in Section 2.2.

2.4. Transplanting-Cd concentration gradient experiment

The pot culture experiment was conducted in the same greenhouse in April to June of 2009, and the same soils used for the soil seed bank-metal accumulators screening experiment described above were used here. Six treatments (0, 8, 15, 30, 60 and 100 mg kg^{-1}) with 4 replicates were set up and designated as CK, Cd8, Cd15, Cd30, Cd60 and Cd100, respectively. Dried soil (12.5 kg) sieved through 1 cm mesh was put into each plastic pot (30 cm in diameter and 25 cm in height), and then saturated with heavy metal solution containing the required amount of $CdCl_2{\cdot}2.5H_2O$. One month later, 3 uniform-sized seedlings (Seeds were collected from the mature *S. photeinocarpum* growing in the place where the soils were collected for the experiment. Then they were sown in the soil. After the seeds germinated, the seedlings were cultured in the greenhouse for 10 days waiting for experimental utilization) of *S. photeinocarpum* were transplanted into each pot. All pots were watered each day in order to keep soil moisture at 70–85%. After the plants became mature (60 days of cultivation), photosynthetic rate, stomatal conductance and transpiration rate were measured in one mature leaf of each pot with a portable photosynthesis system (LI6400P, South San Francisco, CA, American), and the SPAD values of chlorophyll were determined in 2 locations of each mature leaf and three leaves in each pot by the portable chlorophyll meter (chlorophyll meter SPAD-502, Osaka 590-8551, Japan). Then the plants were dug up and processed according to the method described in Section 2.2.

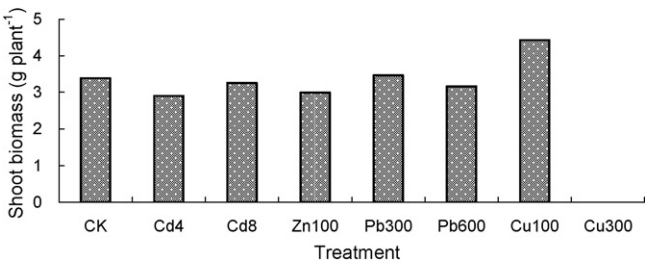

Fig. 2. Biomasses of *S. photeinocarpum* for different treatments in the soil seed bank-metal accumulators screening experiment (65 days after the heavy metal solution added to the soil; $n = 1$).

2.5. Sample analysis

Plant metal concentration (digested by concentrated HNO_3 and $HClO_4$ with 5:1 (v/v)) was determined by atomic absorption spectrophotometry (AAS, GBC932AA, Sydney, Australia). The measured values of heavy metals were checked by using certified standard reference material (GBW-07602, bush branches and leaves) obtained from the China National Center for Standard Reference Materials. The soil sample properties were measured according to Liu [16]. Soil total metal concentration (digested by concentrated HF, HNO_3 and $HClO_4$ with 24:3:4 (v/v)) and available metal concentration (extracted with 0.1 mol L^{-1} HCl) were determined by AAS.

2.6. Statistical analysis

Bioaccumulation factor (BCF), translocation factor (TF) and metal extraction amount (MEA) were used to evaluate plant phytoextraction efficiency. BCF is defined as the ratio of metal concentration in plant shoots to that in soil. TF is determined by the ratio of metal concentration in plant shoots to metal concentration in roots. MEA is referred to as the total amount of heavy metal in a plant [13].

Statistical analysis was performed using SPSS 13.0 statistical software. Data were analyzed by one-way ANOVAs with least significant difference (LSD) to determine any significant differences between treatments ($P < 0.05$), and one-tailed t-test was used to assess the significance of Cd contaminated treatment effects compared to the control.

3. Results and discussion

3.1. Effects of Cd stress on plant growth and physiology

In the seed bank-Cd concentration gradient experiment, during the period of observation, no toxic symptoms were manifest in any Cd treatments (8–100 mg kg^{-1}) and the shoot biomass showed no significant reduction relative to the control. However, the root biomass was significantly reduced in soils with more than 60 mg kg^{-1} Cd contamination, which indicated that

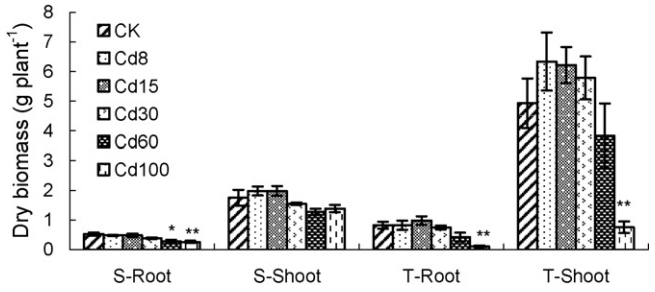

Fig. 3. Dry biomass of *S. photeinocarpum* for different treatments in the seed bank-Cd gradient experiment (S-Root and S-Shoot) and transplanting-Cd gradient experiment (T-Root and T-Shoot). Values represent mean ± S.E. ($n = 4$). One-tailed t-test was used to compare the significance between Cd contaminated treatments to control (Cd concentration was 0 mg kg^{-1}). Treatments significantly different from the control are marked with asterisks ($^{*}P < 0.05$, and $^{**}P < 0.01$).

roots were more affected by Cd pollution than shoot (Fig. 3). In the transplanting-Cd concentration gradient experiment, *S. photeinocarpum* exhibited signs of severe toxicity, such as chlorosis of mature leaves and reduction in plant height (data not shown), and its shoot biomass and root biomass were significantly decreased at 100 mg kg^{-1} Cd contamination (Fig. 3). Obviously, plants showed Cd toxicity symptoms including chlorosis, reddish veins and petioles, curled leaves, and severe reduction in biomass and height as described in previous documents [17–19].Chlorophyll SPAD value, photosynthetic rate, shoot water content, stomatal conductance, transpiration rate and water use efficiency of *S. photeinocarpum* for different treatments in the transplanting-Cd gradient experiment are shown in Fig. 4. Though plant biomass was significantly reduced by Cd pollution (Fig. 3), chlorophyll SPAD value (Fig. 4A) and photosynthetic rate (Fig. 4B) were not affected, which was reversed to some previous studies [18,19]. The decrease of plant biomass may result from other reasons, such as Cd-induced micronutrient deficiencies [20]. It has been recognized that the reduction of shoot water content is considered a toxicity symptom of heavy metals [21]. This is because when plants are contaminated by heavy metals, roots are severely damaged, and the water absorption capability is decreased, which leads to the reduction of shoot water content [22,23]. However, in the present study, although root biomass of *S. photeinocarpum* was significantly reduced when soils were contaminated by 100 mg kg^{-1} Cd (Fig. 3), shoot water content under such severe Cd pollution was significantly higher than that under the uncontaminated condition (Fig. 4C). This phenomenon may be caused by the closing up of leaf stomas when plants are intoxicated by heavy metals (Fig. 4D), leading to a decline in measured transpiration rate (Fig. 4E), and increase in apparent leaf water use efficiency (Fig. 4F). These events result in a rise of the shoot water content.

3.2. Cadmium uptake and phytoextraction potential of *S. photeinocarpum*

As shown in Fig. 3 and Table 3, *S. photeinocarpum* accumulated 132 and 158 mg kg^{-1} Cd in stems and leaves at a soil contamination of 100 mg kg^{-1} Cd in the seed bank-Cd concentration experiment and accumulated 215 and 251 mg kg^{-1} Cd in stems and leaves at the 60 mg kg^{-1} Cd soil contamination level in the transplanting-Cd concentration gradient experiment. The plants still grew well and shoot biomass was not decreased by such severe Cd contamination. Generally speaking, for most plant species, normal Cd concentration in leaf tissue ranges from 0.05 to 0.2 mg kg^{-1}, with 5–30 mg kg^{-1} considered excessive or toxic [24]. However, Cd-hyperaccumulating plants can accumulate above 100 mg kg^{-1} in plant shoots and show no toxic symptoms. Consequently,

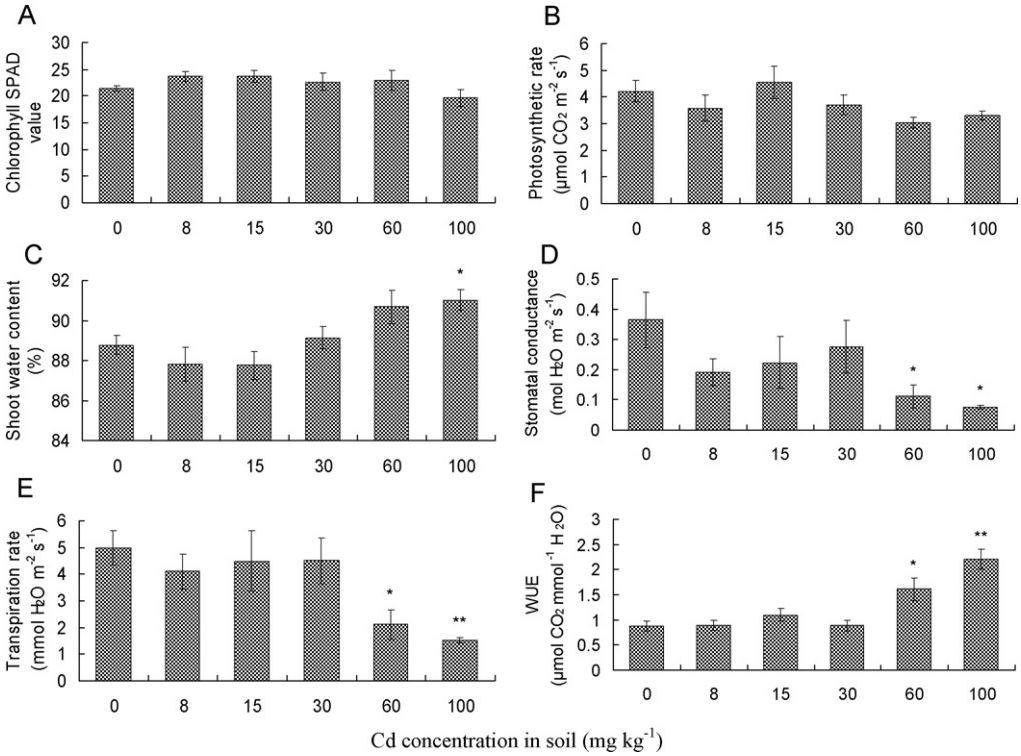

Fig. 4. Chlorophyll SPAD value (A), photosynthetic rate (B), shoot water content (C), stomatal conductance (D), transpiration rate (E) and water use efficiency (WUE) is defined as the ratio of photosynthetic rate to transpiration rate (F) of *S. photeinocarpum* for different treatments in the transplanting-Cd gradient experiment. Values represent mean ± S.E. ($n = 4$). One-tailed t-test was used to compare the significance between Cd contaminated treatments to control (Cd concentration was 0 mg kg^{-1}). Treatments significantly different from the control are marked with asterisks ($^{*}P < 0.05$, and $^{**}P < 0.01$).

Table 3
Cadmium accumulative characteristics in *S. photeinocarpum* in two gradient experiments.

Cd contraction in soil (mg kg^{-1})	Cd concentration in plant organs (mg kg^{-1})				BCF	TF	Cd extraction amount (μg plant^{-1})	
	Root	Stem	Leaf	Shoot			Root	Shoot
Seed bank-Cd concentration gradient experiment								
0	6.8 ± 2.0e*	3.8 ± 0.86e	4.8 ± 0.63e	4.2 ± 0.75e		0.78 ± 0.23a	3.5 ± 1.1d	6.8 ± 0.28e
8	129 ± 8.7c	26 ± 3.7d	51 ± 3.3d	36 ± 2.2d	4.5 ± 0.28a	0.29 ± 0.04b	61 ± 2.3c	70 ± 3.7d
15	174 ± 32c	30 ± 1.8d	48 ± 3.7d	38 ± 1.9d	2.4 ± 0.12b	0.23 ± 0.03b	85 ± 19bc	75 ± 8.9d
30	295 ± 39b	53 ± 7.0c	95 ± 5.6c	68 ± 6.9c	2.1 ± 0.22b	0.24 ± 0.01b	115 ± 21ab	107 ± 12c
60	561 ± 28a	75 ± 2.5b	146 ± 13b	99 ± 1.1b	1.8 ± 0.19bc	0.20 ± 0.01b	157 ± 25a	138 ± 7.3b
100	544 ± 16a	132 ± 7.9a	158 ± 21a	142 ± 7.5a	1.4 ± 0.08c	0.26 ± 0.02b	157 ± 22a	195 ± 13a
Transplanting-Cd concentration gradient experiment								
0	1.1 ± 0.54e	1.7 ± 0.21f	2.4 ± 0.67d	2.0 ± 0.16f		1.14 ± 0.06a	0.84 ± 0.42d	9.7 ± 1.7c
8	78 ± 11d	47 ± 1.6e	65 ± 3.7c	53 ± 1.7e	6.6 ± 0.22a	0.72 ± 0.12b	64 ± 16c	332 ± 43b
15	144 ± 12c	80 ± 2.3d	114 ± 5.2b	91 ± 2.7d	6.1 ± 0.18a	0.65 ± 0.07bc	137 ± 12ab	566 ± 48a
30	213 ± 19c	114 ± 13c	149 ± 19b	126 ± 12c	4.2 ± 0.14b	0.59 ± 0.01bcd	158 ± 18a	716 ± 85a
60	473 ± 59b	215 ± 24b	251 ± 36a	230 ± 29b	3.8 ± 0.49b	0.49 ± 0.02 cd	176 ± 36a	787 ± 114a
100	845 ± 92a	372 ± 56a	292 ± 36a	342 ± 45a	3.4 ± 0.45b	0.43 ± 0.09d	91 ± 26bc	231 ± 50b

* Mean ± S.E.; data followed by the same letters in the same column for the same experiment indicate no significant difference at $P = 0.05$ level by LSD test.

based on the results of tolerance and accumulation properties, *S. photeinocarpum* expressed strong tolerance to Cd pollution and may be a potential Cd hyperaccumulator.

To evaluate the efficiency of Cd phytoextraction in plants, the bioaccumulation factor (BCF), translocation factor (TF) and metal extraction amount (MEA) were calculated (Table 3). As depicted in Table 3, the BCF values of *S. photeinocarpum* in the seed bank-Cd concentration experiment were 1.4–4.5, but in the transplanting-Cd concentration gradient experiment, there were higher BCF values (3.4–6.6) for the plant under stress of Cd at the same concentration. Though the BCF values in both Cd concentration experiments decreased with increasing soil Cd concentrations, all of them were higher than 1.0 under different Cd treatments, suggesting that *S. photeinocarpum* has a stable feature of Cd accumulation. *S. photeinocarpum* accumulated substantial Cd in shoots, but its roots immobilized even more Cd. The TF values in the seed bank-Cd concentration experiment and transplanting-Cd concentration gradient experiment were 0.26–0.78 and 0.43–1.14, respectively. As the TF values were mostly lower than 1.0, they indicate the limited ability of Cd to translocate from roots to shoots. However, this can be improved by chemical treatment methods which increase its accumulation and translocation ability [2,25]. In terms of Cd removal, *S. photeinocarpum* performed surprisingly well. Its Cd extraction amounts in roots and shoots reached 157 and 195 μg plant^{-1} at 100 mg kg^{-1} Cd contamination in the seed bank-Cd concentration experiment and 176 and 787 μg plant^{-1} at 60 mg kg^{-1} Cd contamination in the transplanting-Cd concentration gradient experiment, indicative of strong potential to remedy Cd contaminated soil.

In addition to their metal accumulation ability, plants used for phytoextraction should be fast growing, deep rooted and easily propagated [26]. *S. photeinocarpum* is an annual weed and its height is about 1 m. It is widely distributed and easily found in China and Malaysia. It flowers all year round and is reproduced easily by its seeds. Due to its strong ecological adaptability, many habitats such as riversides, roadsides and forest land are good sites for its growth [27]. Hence, *S. photeinocarpum* has great potential remediation for high Cd pollution and the use of this species will undoubtedly broaden the applicability of Cd phytoremediation.

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