Journal of Hazardous Materials 197 (2011) 264–271

Contents lists available at SciVerse ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Lead tolerance and cellular distribution in *Elsholtzia splendens* using synchrotron radiation micro-X-ray fluorescence

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ARTICLE INFO

Article history:
Received 26 May 2011
Received in revised form
19 September 2011
Accepted 22 September 2011
Available online 29 September 2011

Key words:
Lead
Elsholtzia splendens
Chlorophyll fluorescence
Micro-XRF
Cellular distribution

ABSTRACT

Hydroponic experiments were conducted to investigate the tolerance and spatial distribution of lead (Pb) in *Elsholtzia splendens*—a copper (Cu) accumulator plant using synchrotron-based micro-X-ray fluorescence. According to chlorophyll concentration and chlorophyll fluorescence parameters, *E. splendens* displayed certain tolerance at 100 μM Pb treatment. Lead concentration in roots, stems and leaves of *E. splendens* reached 45,183.6, 1657.6, and 380.9 mg kg^{-1}, respectively. Pb was mostly accumulated in the roots, and there were also high concentrations of Pb been transported into stems and leaves. Micro-XRF analysis of the stem and leaf cross section revealed that Pb was mostly restricted in the vascular bundles and epidermis tissues of both stem and leaf of *E. splendens*. The correlation between distribution of K, Ca, Zn and Pb were analyzed. There were significant positive correlations ($P < 0.01$) among Pb and Ca, K, Zn distribution both in stem and leaf of *E. splendens*. However, among the three elements, Ca shared the most similar distribution pattern and the highest correlation coefficients with Pb in both stem and leaf cross section of *E. splendens*. This suggests that Ca may play an important role in Pb accumulation in stem and leaf of *E. splendens*.

1. Introduction

In the past decades, industrial and urban activities, transport and agricultural practices did lead to widespread heavy metal pollution. Lead (Pb) is one of the most common heavy metal pollutants in the environment [1], and has gained much attention due to its long persistence in soils and highly toxic effects on both plant growth and human health. Phytoremediation, a green technology that uses plants to clean up the environmental pollutants, such as organic pollutants, heavy metals, has attracted attention in recent years due to its low costs and significant environmental benefits [2]. Plants known as (hyper)accumulator, which can tolerate and accumulate high concentrations of heavy metals in their shoots [3], are of potential use in phytoremediation. To clarify the mechanism of tolerance and accumulation in these accumulators or hyperaccumulators is indispensable for increasing the capacity and efficiency of metal extraction, which could contribute to the development of phytoremediation. Until now, most researches about hyperaccumulation mechanism are focused on Ni, Zn and Cd hyperaccumulator plant species [3]. Over the recent years, plant species such as *Sesbania drummondii*, *Fagopyrum esculentum* Moench, *Elsholtzia argyi*, *Sedum alfredii* and *Blechnum niponicum* have been reported to be tolerant to high levels of Pb and accumulate high concentrations of Pb in their shoots [4–8]. Some of them have even been reported as Pb hyperaccumulators [7]. However, mechanisms of Pb tolerance and accumulation have not been well understood.

Photosynthesis is considered as one of the most sensitive metabolic processes to Pb toxicity. The tolerance of plants to heavy metals has been attributed to several mechanisms including maintenance of photosynthesis [9]. Unaffected photosynthesis indicates that the plant is not experiencing metal toxicity and, then, that it produces metabolites for further absorption, protection and growth [10]. Leaf chlorophyll fluorescence, an important parameter of photosynthesis efficiency, could be used to indicate heavy metal tolerance of plants. The metal compartmentation in less bioactive tissues such as epidermis is another mechanism for tolerance of heavy metals in plants. It is essential to investigate the distribution of accumulated heavy metals at the organ and cellular level. Synchrotron radiation based X-ray fluorescence spectroscopy (SRXRF) has become a powerful tool to investigate metal homeostasis in plants in recent years [11]. This technique could provide elemental distributions in plants with μm-order spatial resolution, and has been successfully used to analyze heavy metal distributions in various accumulators and hyperaccumulators [12–15].

Elsholtzia splendens is an endemic Cu tolerant and accumulating plant species native to China [16], which has also been found to exhibit ability to tolerate and accumulate considerable amounts

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doi:10.1016/j.jhazmat.2011.09.085

of Pb, and has a potential use for phytoextraction of heavy metals from multi-metal polluted soils [17]. Elucidating the biological mechanism on Pb tolerance in this plant species could help to enlarge our knowledge of Pb tolerance in plants. This study aimed to understand the fundamental mechanisms about Pb tolerance in *E. splendens* by investigating leaf chlorophyll parameters and metal distribution in leaf and stem tissues using μ-XRF imaging.

2. Materials and methods

2.1. Plant material and hydroponics culture

Seeds of *E. splendens* were collected from mature plants grown in copper mine waste deposits (Zhuji County of Zhejiang Province, China). Healthy seeds were surface sterilized by 1% NaOCl for 20 min. After sterilization, seeds were washed thoroughly with distilled water and sown in a substrate containing perlite and vermiculite 3:1 (v/v) moistened with distilled water. Upon the emergence of seedlings, 1/2 strength basal nutrient solution was supplied until seedlings with two-leaf pairs were established. Then these young seedlings were transplanted in plastic trays containing 1/2 strength basal nutrient solution continuously aerated with a pump.

After 2 weeks, seedlings with uniform size were selected and transferred to plastic pots containing about 2.5 L of modified nutrient solution, in which KH_2PO_4 concentration was adjusted to 0.01 mM in order to prevent precipitation of Pb. Seven plants were planted in each pot. The composition of modified nutrient solution was as follows (in μM): 2000 KNO_3, 50 KCl, 500 $Ca(NO_3)_2$, 200 $MgSO_4$, 100 NH_4NO_3, 10 KH_2PO_4, 12 H_3BO_3, 2.0 $MnSO_4$, 0.5 $ZnSO_4$, 0.2 $CuSO_4$, 0.1 Na_2MoO_4, 0.1 $NiSO_4$, 20 Fe-EDTA (iron-ethylene diamine tetraacetic acid). After 4 days, different Pb treatments were applied: (1) 0 μM (CK), (2) 100 μM and (3) 200 μM Pb and Pb was given as $Pb(NO_3)_2$. The experiment was randomly arranged with each treatment in triplicate. Plants were grown under glasshouse conditions with natural light, day/night temperature of 30/25 °C and day/night humidity of 70%/90%. Nutrient solution pH was adjusted to 5.5 with 0.1 M NaOH or 0.1 M HCl and was continuously aerated and renewed at every 4th day during the experiment.

2.2. Chlorophyll fluorescence measurement

Fluorescence measurements were carried out with an IMAGING-PAM chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany). Fluorescence was measured with relatively weak measuring light pulses (<1 μmol m^{-2} s^{-1}) at a low frequency (2 Hz) for measurement of Fo (minimum fluorescence yield of dark-adapted leaf). Fm (maximal fluorescence yield of dark-adapted leaf) was measured by a 0.8 s pulse light at about 4000 μmol m^{-2} s^{-1}. The intensity of continuous actinic illumination was adjusted to 185 μmol m^{-2} s^{-1}. All fluorescence measurements were started after an additional 30 min dark adaptation. When performing a measurement, an area of interest (AOI, diameter: 1 cm) was selected in the middle of the whole leaf. Values of the chlorophyll fluorescence parameters Fo, Fm, Fv/Fm (maximal PS II quantum yield), ΦPSII (effective PS II quantum yield) and NPQ (nonphotochemical quenching) were the average of the AOI. In addition, their images were simultaneously derived from the IMAGING-PAM software. After chlorophyll fluorescence measurement, the leaves were immediately wrapped in aluminum foil for chlorophyll content measurement. Chlorophyll was extracted by 80% acetone for 24 h. Chlorophyll content was determined spectrophotometrically and calculated according to Lichtenthaler [18].

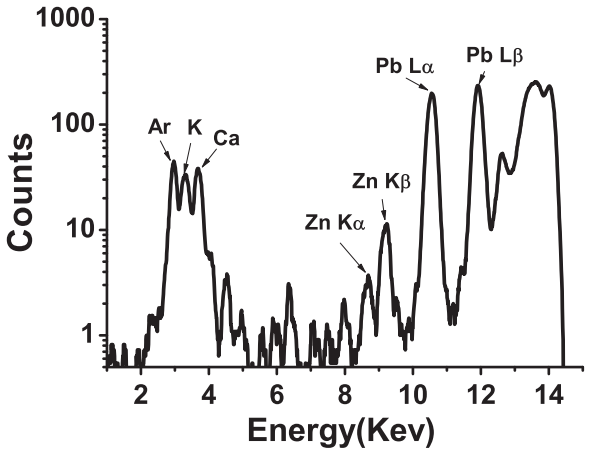

Fig. 1. Typical SRXRF microprobe spectra in cross-sections of the stems of *E. splendens*. The main Pb Lα peak located at 10.6 KeV, and the secondary Pb Lβ at 11.3 KeV.

2.3. SRXRF analysis

Micro-X-ray fluorescence imaging of Pb in the *E. splendens* leaf and stem cross-sections was carried out on beam line BL15U1 at the Shanghai Synchrotron Radiation Facility (SSRF), Shanghai, China. Stems and leaves were cut from plants after 30 days exposure to Pb and rinsed. The mid-transverse areas of stem and mature leaf samples were selected. Sections (50 μm thick) were cut with a freezing microtome (Shandon FE) at an ambient temperature of −20 °C, and subsequently freeze-dried at −20 °C for 1 day. The incident beam was monochromatized by a Si (1 1 1) double-crystal monchromator, and then focused by a K–B mirror system into a micron sized X-ray beam at the sample position, with energy ranges from 3.5 to 22.5 KeV. The electron energy in the storage ring was 3.5 GeV, with an average current of 300 mA, and the detector was Si (Li) SSD. The beam energy was set to 15 KeV during mapping. The focused X-ray beam was adjusted by horizontal slits, and a beam size of 3×3 μm^2 was obtained. The step size was set to 10 μm. The measured fluorescent X-rays were Pb Lα (10.6 KeV), K Kα (3.3 KeV), Ca Kα (3.6 KeV), Zn Kα (8.5 KeV). As K Kβ line overlaps the Ca Kα line, the contribution of K in the Ca ROI (Region of Interest) window was subtracted based on the ratio of the relative intensities of the Kα and Kβ emission lines from a pure K spectrum. Fig. 1 showed the typical SRXRF microprobe spectra in cross-sections of the stems of *E. splendens*. The maps were produced using the software Igor pro 5.

2.4. Measurement of Pb and other elements

The Pb-treated plants were harvested on 30th day after the treatment. At harvest, roots of intact plants were washed with distilled water, and then immersed in 20 mM Na_2EDTA (Ethylene Diamine Tetraacetic Acid Disodium Salt) for 15 min to remove Pb adhering to the root surface. After that, plants were washed three times with distilled water and finally with de-ionized water. Different plant parts were separated and their fresh weights were recorded.

The dried plant materials were ground using a stainless steel mill and passed through a 0.25 mm sieve for analysis of Pb and other elements. Dry plant samples (0.1 g) of each treatment were digested with HNO_3–$HClO_4$ (4:1, v/v), and the digest was transferred to a 50 ml volumetric flask, made up to volume with water and filtered. Concentrations of Pb and other elements (i.e. K, Ca and Zn) in the filtrates were analyzed using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, iCAP 6000, Thermo Scientific).

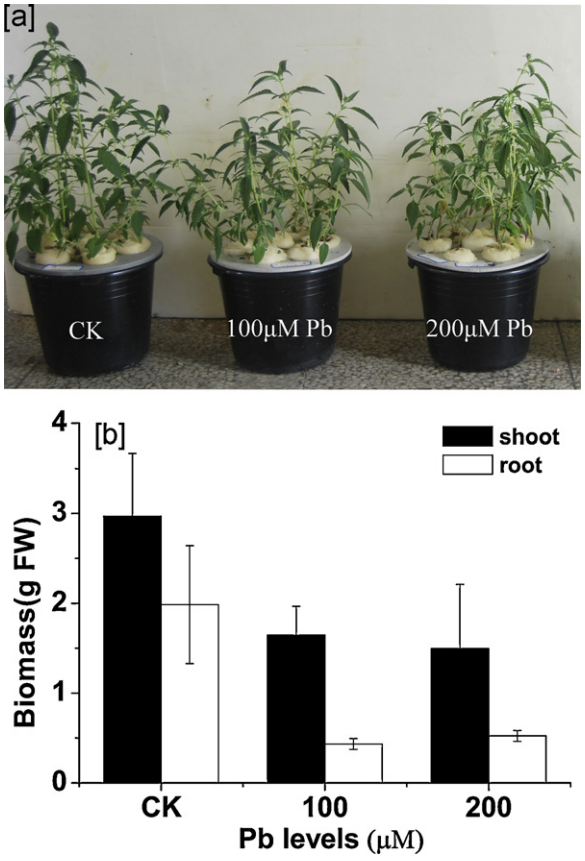

Fig. 2. Plant growth (A) and fresh weight (B) of *E. splendens* exposed to different levels of Pb for 30 days. Data represent means ± SD of three replicates ($n = 3$). Different letters indicate significant differences ($P < 0.05$) among the treatment and control (CK).

2.5. Statistical analysis of data

All data were statistically analyzed using the SPSS. Analysis of variance (ANOVA) was performed on the data sets and the mean and standard error (SE) of each treatment as well as least-significant difference (LSD) ($P < 0.05$ and $P < 0.01$) for each set of corresponding data were calculated.

3. Results

3.1. Effect of Pb on plant growth, leaf chlorophyll concentration and chlorophyll fluorescence parameters

In the hydroponics experiment, *E. splendens* grew at lead levels up to 200 μM Pb, although significant reduction of both shoot and root biomass were observed in response to Pb exposure as compared with the control ($P < 0.05$, Fig. 2a). No significant differences were observed within the different Pb levels (Fig. 2b).

(Table 1) showed the effects of Pb exposure to leaf chlorophyll concentration. It can be seen that there were no significant differences between the control and 100 μM Pb treatment, but at 200 μM Pb treatment, chlorophyll *a* and total chlorophyll showed slightly decrease compared with the control. There were no significant differences of chlorophyll *b* among all the treatments. The image of chlorophyll fluorescence parameters of *E. splendens* was shown in Fig. 3. Under 100 μM Pb treatment all these parameters showed no significant differences with the control. ΦPSII showed a slight decrease at 200 μM Pb level. NPQ appears to show an increase in the center of leaf for 200 μM Pb compared to 100 μM Pb as well as the control.

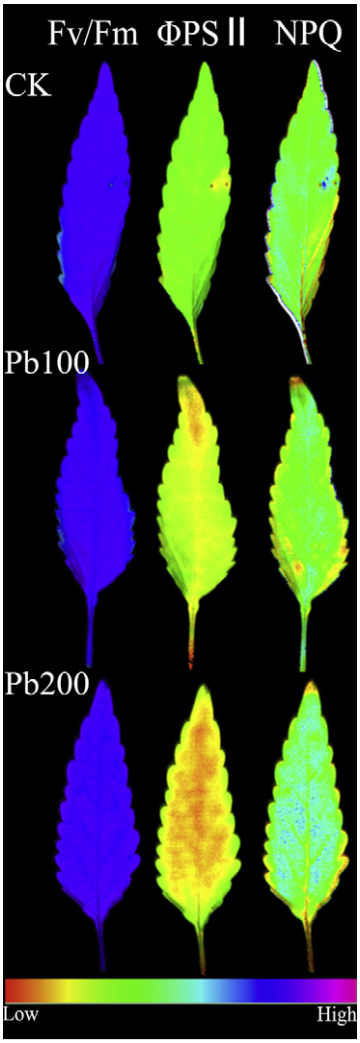

Fig. 3. Image of the maximum quantum efficiency of PSII photochemistry (Fv/Fm), maximum quantum yield of PSII (ΦPSII) and nonphotochemical quenching (NPQ) of *E. splendens* grown under different levels of Pb for 30 days.

3.2. Metal concentrations in plant tissues

The concentration of Pb in leaves, stems and roots of *E. splendens* increased significantly ($P < 0.05$) with increasing external Pb supply levels (Fig. 4). The maximum Pb concentration in roots and stems reached 45,183.6 and 1657.6 mg kg^{-1} (DW), respectively, when the plants were grown at 200 μM Pb for 30 days, while Pb concentration in leaves reached its maximum as 380.9 mg kg^{-1}, when the plants were grown under 100 μM Pb treatment for 30 days. The results showed that Pb was mainly restricted in roots, but the concentration of Pb in the shoots also reached a high level.

Table 1
Chlorophyll concentration of *E. splendens* grown under different levels of Pb for 30 days. Data and error bars represent means ± SD of three replicates ($n = 3$). Different letters in the same column indicate significant differences ($P < 0.05$) among the treatments and control.

Treatment	Chlorophyll concentration (mg g^{-1})		
	Chl *a*	Chl *b*	Total Chl
CK	3.22 ± 0.27a	1.10 ± 0.35a	4.32 ± 0.63a
Pb100	3.62 ± 0.06a	1.05 ± 0.09a	4.67 ± 0.09a
Pb200	2.42 ± 0.32b	0.73 ± 0.11a	3.16 ± 0.43b

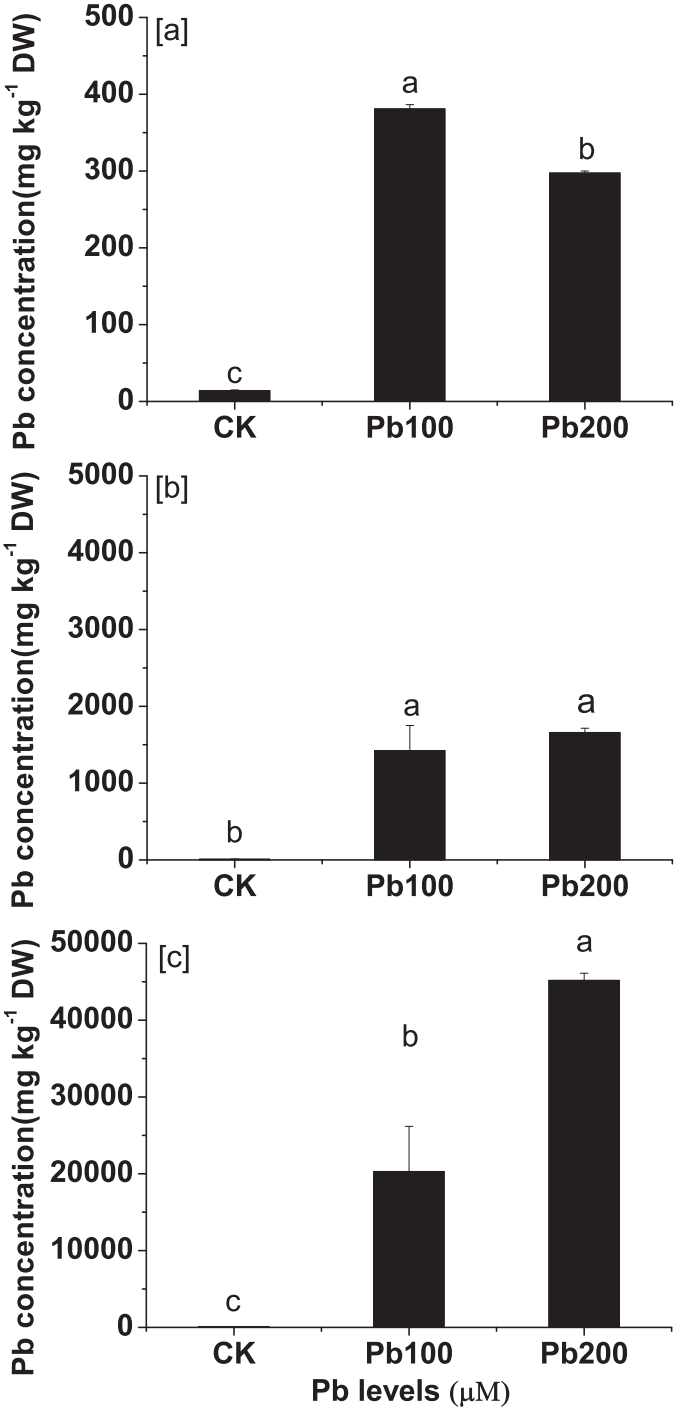

Fig. 4. Pb concentrations in the leaf (a), stem (b) and root (c) of *E. splendens* grown under different levels of Pb for 30 days. Data and error bars represent means ± SD of three replicates ($n = 3$). Different letters indicate significant differences ($P < 0.01$) among the treatment and control.

The concentrations of several other elements (Ca, K and Zn) showed significantly differences in response to the Pb treatment (Table 2). Pb exposure significantly decreased concentrations of Ca and K in root, stem and leaf tissues of plants ($P < 0.05$). Zn concentration in the leaves was also significantly decreased due to the Pb exposure ($P < 0.05$), but there were no significant differences between Pb exposure and Zn concentration in the roots and stems of the plants (Table 2).

3.3. Distribution of elements in stems and leaves

Synchrotron X-ray fluorescence was used to investigate the distribution of Pb in the stems and leaves of *E. splendens*. The elemental distribution maps of Pb, Ca, K and Zn in the scanned area of stem and leaf cross sections together with photographs taken using an optical microscope are presented in Fig. 5. The stem cross-section image of *E. splendens* (Fig. 5a) displayed that Pb was distributed mainly in the stem vascular bundles and epidermis. The distribution pattern of Ca was similar to that of Pb, while Zn and K had different distribution patterns and were mainly accumulated in the epidermis.

In preliminary experiments, results showed that Pb was mainly accumulated in the veins of the leaves, and so the primary vein of the leaf cross section was chosen to do the μ-XRF mapping. The μ-XRF maps for the *E. splendens* leaf cross section (Fig. 5b) showed that Pb was mainly distributed in the lower epidermis, little in the vascular tissues and upper epidermis, and there were almost no Pb signals observed in the cortex. Ca, K and Zn distribution patterns appeared to be similar to that of Pb in the leaf cross section, but the concentration of Zn in the leaf cross section was apparently low according to the counts of the signals. In order to demonstrate the accuracy and sensitivity of the SRXRF technique for trace elements analysis, the average counts of each element measured by SRXRF were chosen to compare the concentrations measured by ICP-OES. There was a positive correlation between the counts measured by SRXRF and the concentrations measured by ICP-OES (data not shown).

3.4. Correlations of Pb distribution with other elements

From the distribution maps of Pb, K, Ca and Zn in stem and leaf cross sections of *E. splendens,* it could be seen that the distribution patterns of these elements were very similar. Fig. 6 indicated that Ca, Zn and K distributions all showed significantly positive correlation relationship ($P < 0.01$) with Pb distributions in both stem and leaf cross sections. Among the three elements, Ca shared the most similar distribution pattern and the highest correlation coefficients with Pb in both stem and leaf cross sections of *E. splendens* (correlation coefficient: $r^2 = 0.7428$ and 0.7560, data number: $n = 4473$ and 2236 for stem and leaf, respectively) (Fig. 6a and d).

Table 2
Effects of Pb treatments on the concentrations of K, Ca and Zn in the root, stem and leaf tissues of *E. splendens*. Plants were exposed to different Pb concentrations for 30 days. Data and error bars represent means ± SD of three replicates ($n = 3$). Different letters in the same column indicate significant differences ($P < 0.05$) among the treatments and control.

Treatment level (Pb^{2+}, μM)	Metal concentrations								
	K (g kg^{-1} DW)			Ca (g kg^{-1} DW)			Zn (mg kg^{-1} DW)		
	Root	Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf
CK	20.8 ± 2.6a	25.0 ± 1.7a	28. 5 ± 0.7a	5.3 ± 0.6a	10.9 ± 0.7a	7.4 ± 0.4a	74.3 ± 19.1a	141.65 ± 14.42a	117.33 ± 18.67a
100	8.1 ± 0.0b	19. 7 ± 2.3b	26.0 ± 0.7b	3.3 ± 0.7b	7.8 ± 0.5b	6.9 ± 0.3a	54.46 ± 2.42a	106.51 ± 24.83a	63.86 ± 0.07b
200	6.1 ± 0.1b	13.4 ± 0.6c	18.2 ± 0.1c	2.2 ± 0.3b	4.4 ± 0.4c	4.6 ± 1.0b	57.30 ± 6.55a	101.20 ± 1.14a	51.63 ± 4.29b

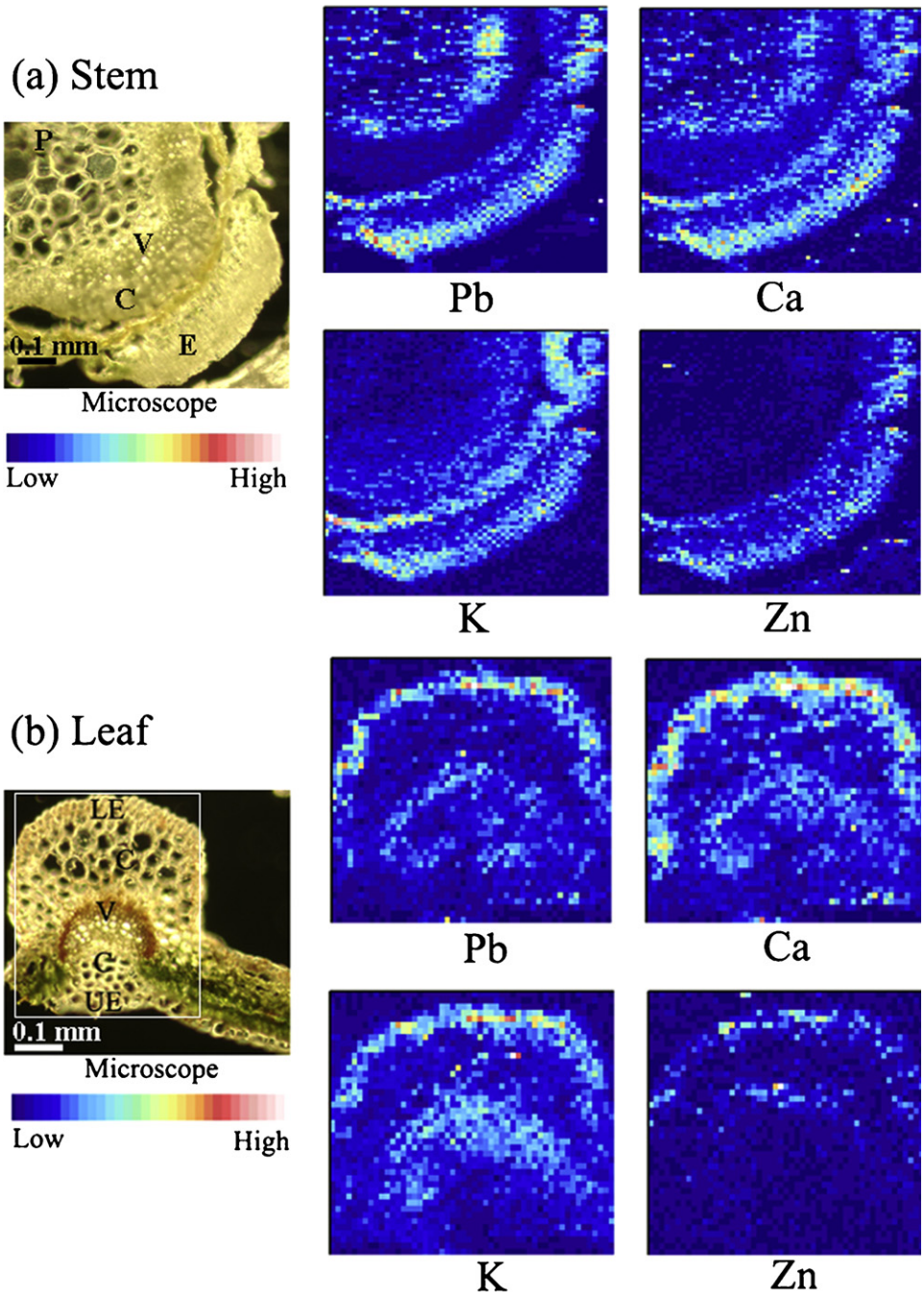

Fig. 5. μ-XRF elemental maps for Pb, K, Ca and Zn of stem (a) and leaf (b) cross sections from *E. splendens* treated with 100 μM Pb for 30 days. The number of fluorescence yield counts was normalized by I_0 and the dwell time. E, epidermis; UE, upper epidermis; LE, lower epidermis; C, cortex; V, vascular tissues; P, pith.

4. Discussion

Lead is one of the major heavy metals in the environment and is extremely toxic to plants. It inhibits photosynthesis, upsets mineral nutrition and water balance, changes hormonal status and affects membrane structure and permeability [19]. Chlorophyll content and chlorophyll fluorescence parameters are widely used indicators of heavy metal stress responses, such as Pb or Cd [20]. In the present study, these parameters were also analyzed as an indicator of the tolerance of *E. splendens* to Pb stress. The result showed that no significant effects was detected on both chlorophyll concentration and chlorophyll fluorescence parameters of *E. splendens* at 100 μM Pb treatment, which revealed that the photosynthesis system of *E. splendens* was not damaged under this level of Pb. This also indicated that *E. splendens* could tolerate at least 100 μM Pb under hydroponic condition. There were slightly decrease on chlorophyll *a* and ΦPSII at 200 μM Pb treatment, which might be due to higher sensitivity of Chl *a* to Pb [21] and some impaired energy trapping efficiency in PSII of *E. splendens* leaves, respectively. Increasing $Pb(NO_3)_2$ concentration in the culture solution also increased the concentration of NO_3^{-1}. This might be benefit to the plant growth as N is a macronutrient [22]. Nitrogen was also reported to be effective for increasing heavy metal accumulation in plants [23–25]. However, the increased NO_3^{-1} concentration in the

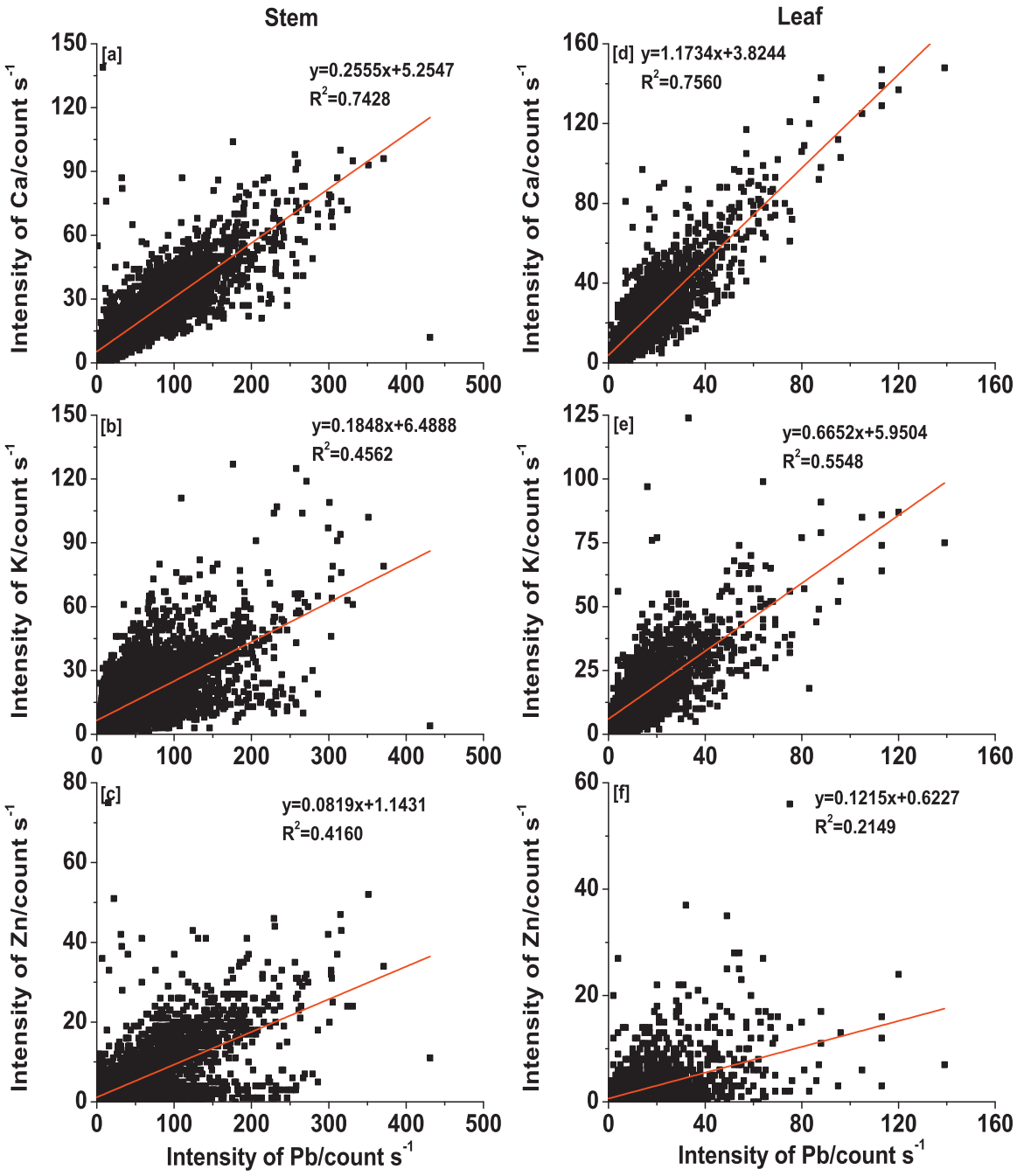

Fig. 6. Correlation between XRF intensities of Pb and K, Ca, and Zn distributed in stem (a–c) and leaf (d–f) cross section of *E. splendens*.

treatment solution was very low (100 or 200 μM) compare to these in the nutrient solution (2600 μM), and would have less effect on the growth of plants.

The mobility of Pb in plants is relatively low. As has been reported that Pb is unevenly distributed in roots, where different root tissues act as barriers to apoplastic and symplastic Pb transport and hence, Pb transport to shoot gets restricted [26]. In this study, Pb concentrations were as high as 45,183.6, 1657.6 and 380.9 mg kg^{-1} (DW) in the roots, stems and leaves of *E. splendens,* respectively (Fig. 4). It revealed that Pb was mostly restricted to root tissues and that the Pb accumulation ability of *E. splendens* was similar to that of *E. argyi* [6], another plant species belonging to genus *Elsholtzia* that is a Pb accumulator [27].

Metal hypertolerance and hyperaccumulation in plants require complex alterations in the plant metal homeostasis network and need a series of mechanisms to cope with them [3]. Over the last few years, a number of researches have been done on the distribution of elements in various plants and their correlations. However, most of them were focused on the elements such as Ni, As, Cd and Zn, but few on Pb. These studies revealed that these toxic metals were mostly localized at less bioactive tissues such as epidermis and vascular tissues [14,15,28–30]. Metal compartmentation in less bioactive tissues such as epidermis has been hypothesized as one of the possible mechanisms for tolerance and detoxification of heavy metals in plants [31]. In the present study, to investigate the cellular compartmentation of Pb in *E. splendens,* the distribution of Pb and

other elements were analyzed using micro-XRF mapping, which could not only provides the nondestructive spatial image of metal abundance but also can be used to simultaneously detect multiple elements [11,14,32,33]. The results indicated that Pb was mostly accumulated in the vascular tissues and epidermis both in stems and leaves of *E. splendens*. It was consistent with the hypothesis that sequestration of heavy metals in epidermis tissues is an important strategy of metal detoxification in accumulators and hyperaccumulators [34,35]. On the other hand, Pb deposited in the vascular tissues can be thought to be primarily transported from roots to shoots by xylem vessels. Recently, Tian et al. [33] have reported that Pb is primarily accumulated in the vascular tissues of *S. alfredii*, and Kodera et al. [5] have also revealed that Pb is restricted to vascular bundles, but in this study Pb was not only distributed in vascular tissues but also largely accumulated in the epidermis tissues. So maybe there is a different mechanism to transport Pb from vascular tissues to epidermis in *E. splendens*.

On the basis of micro-XRF results, the correlation of the distribution of Pb and other elements was analyzed. There was a significantly positive correlation between Pb and Ca distribution in both stems and leaves of *E. splendens* (Fig. 6a and d). However, there is no such correlation between Pb and Ca distribution in *S. alfredii*—a Pb accumulator [33]. Calcium is a very important nutrient element that is involved in nearly all aspects of plant development and participates in many regulatory processes [36]. For years, calcium has been reported to be involved in heavy metal tolerance, especially Cd and Pb [37–39]. Therefore these results of strongly positive correlation between Pb and Ca distribution suggested that Ca might play an important role in Pb tolerance and accumulation in *E. splendens* and this needs further studies.

5. Conclusion

In this research, we investigated the tolerance and distribution of Pb in *E. splendens*. Through the results of the biomass, chlorophyll concentration and chlorophyll fluorescence parameters, it can be seen that *E. splendens* did not show toxic symptom at 100 μM Pb level under hydroponic condition. Pb was mostly retained in the roots, but also up to more than 1000 mg kg^{-1} of Pb was transported to the shoots. Pb was mainly distributed in the vascular bundles and epidermis of both stems and leaves of *E. splendens*. The distribution pattern of Ca was most similar with that of Pb. These results provide important information for better understanding of the mechanisms of Pb tolerance and accumulation by plants.

Acknowledgements

This work was supported by Projects from the National Natural Science Foundation of China (30871589, 30630046), and Key Project from Ministry of Environmental Protection of China (2011467057). This research was carried out at the Shanghai Synchrotron Radiation Facility (SSRF), China.

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