



Effect of EDTA and NTA on cadmium distribution and translocation in *Pennisetum purpureum* Schum cv. Mott

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Abstract

The primary objective of this research was to investigate the cadmium (Cd) distribution in *Pennisetum purpureum* (Napier grass) in the presence of 30 mg/L of Cd and different types and concentrations of chelating agents (ethylenediaminetetraacetic acid disodium dihydrate (EDTA), nitrilotriacetic acid (NTA), and EDTA-NTA mixtures). Plant samples were collected every 15 d during a 105-d experimental period. Accumulation of Cd in each part of the plant was determined using atomic absorption spectrometer (AAS), and the distribution of Cd was determined by laser ablation inductively coupled plasma mass spectrometer (LA-ICP-MS) and synchrotron radiation micro X-ray fluorescence (SR-micro-XRF). The highest concentrations of Cd accumulation of 889 ± 53 mg kg⁻¹ in the underground part (roots) and 265 ± 26 mg kg⁻¹ in the aboveground part (stems and leaves) in the presence of 1:1 M ratio of Cd:EDTA after 30 d of exposure were observed. Plants grown in the presence of either NTA or EDTA-NTA mixtures showed significant lower Cd accumulation levels. The LA-ICP-MS analysis showed that Cd was primarily accumulated in the aboveground part (stems and leaves), especially in the xylem and intercalary meristem. In addition, translocation factor was very low. Thus, *P. purpureum* could be considered as a candidate plant for cadmium phytostabilization.

Keywords Napier grass · Mechanism · Transportation · Distribution · Laser ablation inductively coupled plasma mass spectrometry · Synchrotron X-ray fluorescence

Introduction

Heavy metal contamination in the environment is primarily caused by the expansion of heavy industries, such as mining, metal coating, and leather tanning, and has become one of the

major environmental concerns (Pendias and Pendias 2001). In Thailand, zinc (Zn) mining activities and open-land agriculture in the Mae Sot district, Tak province (Northwestern Thailand), have caused severe cadmium (Cd) contamination in both the soil and surface water for several decades (Alloway 2011). Concentrations of Cd in the surface water were approximately six to 16-fold higher than the standard level of Cd in the natural surface water (National Environment Board 1994). As such, alternative crops and other hyperaccumulation plants instead of rice have been introduced in the area to remediate the high Cd levels (Natural Resources Infrastructure and Disasters Management Research Center 2017). However, high Cd concentrations are still detected in this area.

Generally, heavy metal contamination can be remediated using ex situ and in situ techniques, including physical, chemical, and biological treatments (Krishna 2008; Akkajit 2015). Although the removal efficiencies of both physical and chemical treatments are relatively high, the high remediation cost of both technologies is a major barrier (Ensley 2000; Uera et al. 2007).

Phytoremediation is a more economically feasible and environmentally friendly remediation technology to correct

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contaminated surface water, sediment, and soil. It could, therefore, be an appropriate choice for Cd remediation in the area given budget constraints and lack of environmental remediation expertise (USEPA 2000; Peer et al. 2007; Tananonchai and Sampanpanish 2014; Sampanpanish 2015). However, a critical disadvantage of this technique is the long treatment time (Aisien et al. 2012). Therefore, several research studies have recently used Cd hyperaccumulating plants, such as the *Pennisetum purpureum* Schum cv. Mott (Napier grass), to remediate heavy metal contaminated sites (Ishii et al. 2000; Abdel Salam 2012; Sabeen et al. 2013). In addition, several chelating agents have been used to enhance the Cd uptake rates of these hyperaccumulating plants. Among the various chelating agents, ethylenediaminetetraacetic acid disodium dihydrate (EDTA) and nitrilotriacetic acid (NTA) were observed to be the most effective in enhancing Cd accumulation levels within plant tissues (Evangelou et al. 2007). According to Anjum et al. (2013), it was suggested that the LA-ICP-MS technique and the synchrotron radiation technique are new techniques to identify and describe the distribution of heavy metals in plant.

Therefore, this study was conducted to (i) determine the effects of different chelating agents (EDTA, NTA, and EDTA-NTA mixtures) on the Cd accumulation level in *P. purpureum* and (ii) investigate the Cd distribution and uptake mechanism in *P. purpureum*. This study used the laser ablation inductively coupled plasma mass spectrometer (LA-ICP-MS) technique and the synchrotron radiation micro X-ray fluorescence (SR-micro-XRF) to determine the distribution and localization of Cd in plants. The results obtained were expected to extend the scientific knowledge on Cd distribution and accumulation in plant concerning plant biology. This is the first quantitative and qualitative study on Cd accumulation in parts of *Pennisetum purpureum* Schum cv. Mott.

Material and methods

Experimental design

Three replications of each treatment, as shown in Table 1, were performed in this work. Before the start of this study, we studied the phytolerance and phytotoxicity of plants with Cd, EDTA, and NTA additions (Tananonchai and Sampanpanish 2018). The result shows that a Cd concentration of 30 mg Cd/L was the most suitable condition because *P. purpureum* had shown the highest relative growth rate (RGR), lowest phytotoxicity, and highest Cd accumulation. In the case of EDTA, there was no significant negative effect of EDTA on RGR and phytotoxicity. For the molar ratio, EDTA and NTA formed complexes with most cations in a 1:1 M ratio (Kolodynska 2011).

Chemical reagents

The Cd, as cadmium nitrate tetrahydrate ($\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$), EDTA and NTA solutions were prepared from analytical grade reagents. Before starting the experiment, sedimentation tests were carried out with the addition of nutrient solutions (calcium nitrate, ethylenediamine-N, N'-bis (2-hydroxyphenylacetic acid), mono potassium phosphate, magnesium sulphate, potassium nitrate, and manganese chelate) with cadmium nitrate, EDTA, and NTA. The result showed that no sedimentation was observed in the experiments.

Plants preparation and cultivation

One-month-old *P. purpureum* rootstock, which was previously grown in uncontaminated soil, was used in all the experiments. Prior to each experiment, the rootstock was washed in distilled water (DW) and placed into a pre-cleaned plastic bottle containing 1.2 L of the mixed solution of Cd, essential plant nutrients (to control the factors affecting the Cd accumulation of plants), and chelating agents (Table 1). All containers were cleaned with a 10% (v/v) aqueous nitric acid overnight and rinsed thoroughly with DW before use. Air was continuously supplied throughout the 105-d experimental period using an aquarium air pump in order to provide adequate oxygen to the plant roots. The nutrient solutions (2 mL) were added every 2 d until the end of the experiment.

Sample collection and analyses of Cd accumulation and distribution

A summary of the sample preparation and analyses of Cd accumulation is presented. Plant samples were collected every 15 d during the 105-d experimental period. After collection, whole plant samples were rinsed in tap water and then DW. Samples were subsequently divided into two subsamples. Each subsample was dried at room temperature for 2–3 h and weighed to obtain the wet weight. The plant was then dried in a hot-air oven at 105 °C for 24–48 h until reaching a constant weight and then were all measured in their dry weight. Next, the RGR was calculated following Eq. 1. Direct observation was used to evaluate the phytotoxicity in *P. purpureum* as shown in Eq. 2. The score of each symptom is exhibited in Table 2.

$$\text{RGR} = [\ln W_2 - \ln W_1] / (t_2 - t_1) \quad (1)$$

Where t_1 is the first date of the experiment, t_2 is the last date of the experiment, W_1 is the weight of *P. purpureum* in t_1 (g), and W_2 is the weight of *P. purpureum* in t_2 (g).

Table 1 Summary of the experimental conditions

Experimental set no.	Cd concentration (mg/L)	Mole ratio of chelating agent to 1 mol of Cd	
		EDTA	NTA
Blank	–	–	–
1 (control)	30	–	–
2	30	0.5	–
3	30	1	–
4	30	–	0.5
5	30	–	1
6	30	0.25	0.25
7	30	0.5	0.5

– denotes the absence of that particular chelating agent

$$\text{Phytotoxicity (\%)} = \frac{(A_0 \times B_0) + (A_1 \times B_1) + (A_2 \times B_2) + (A_3 \times B_3) + (A_4 \times B_4) + (A_5 \times B_5)}{(A_r \times B_r)} \times 100 \quad (2)$$

Where A_0 is the number of normal leaves with no change in color, A_1 is the number of pale yellow leaves (< 20% of leaf area), A_2 is the number of pale yellow leaves (< 40% of leaf area), A_3 is the number of burned dark yellow leaves (leaf blight; < 60% of leaf area), A_4 is the number of burned dark yellow leaves (leaf blight; < 80% of leaf area), A_5 is the number of burned dark yellow leaves (leaf blight; > 80% of leaf area), A_r is the total number of leaves, and $B_0, B_1, B_2, B_3, B_4, B_5,$ and B_r are the scores of toxicity which are equal to 0, 1, 2, 3, 4, 5, and 5, respectively (Brown et al. 1991; Pangta 2009).

Afterward, the subsample was oven-dried at 105 °C for 2–3 d to determine the dry matter yield. One subsample was treated following the USEPA method 3052 (USEPA 1996) and analyzed for the total Cd concentration using atomic absorption spectrometer (AAS). The percentage of remediation factor (RF) was calculated using Eq. 3. The RF values for each cut and then the values of the sum of the three cuts were used in preparing the data illustrated in the figures. The translocation factor (TF) was calculated using Eq. 4. The TF value explains the ability of *P. purpurem* to translocate the metal from roots through shoots which is primarily responsible for phytoextraction.

$$\text{Remediation factor (RF)} = (\text{Conc Cd}_{\text{shoot}} \times \text{DW} \times 100) / (\text{Conc Cd}_{\text{water}} \times \text{volume of water in the pot}) \quad (3)$$

Where $\text{Conc Cd}_{\text{shoot}}$ is the concentration of Cd in shoot as milligram per gram, DW is the dry weight of shoot as gram per pot, and $\text{Conc Cd}_{\text{water}}$ is the concentration of total Cd in water. (Eissa 2016; Neugschwandtner et al. 2008)

Where $\text{Conc Cd}_{\text{shoot}}$ is the mean Cd concentration in shoot of *P. purpurem* (mg kg^{-1}), $\text{Conc Cd}_{\text{root}}$ is the mean Cd concentration in root of *P. purpurem* (mg kg^{-1}) (Eissa 2016; Radulescu et al. 2013).

$$\text{Translocation factor TF} = \text{Conc Cd}_{\text{shoot}} / \text{Conc Cd}_{\text{root}} \quad (4)$$

For the evaluation of the Cd distribution in plant tissue, each plant sample was first cut into the five different parts of

Table 2 Guidelines for evaluating the phytotoxicity of *P. purpurem* based on direct observation

Score of toxicity	Appearance and symptoms
0	Normal, green leaf
1	Slight toxicity, small area of pale yellow on leaf
2	Slight moderate toxicity, large area of pale yellow on leaf
3	Moderate toxicity, small area of blight leaf was observed
4	High toxicity, large area of blight leaf was observed
5	Critical toxicity, whole area of blight leaf was observed

the (i) root tip, (ii) root trunk, (iii) trunk base, (iv) middle trunk, and (v) leaves, and then dried at the ambient pressure and temperature for 2–3 h. Next, samples were pressed and preserved for 4 weeks to obtain herbarium specimens. Finally, the Cd distribution in each part of the plant was analyzed by LA-ICP-MS and SR-XRF. *P. purpurem* uncontaminated with Cd was washed, dried at 105 °C, ground by a mortar and pestle, and sieved to obtain a particle size of < 250 µm. After that step, different concentrations of Cd solution (0, 0.022, 20, 100, and 200 mg kg⁻¹) were spiked into 3 g of sieved samples and left at room temperature for 2 weeks and dried and compressed into a pellet form under a pressure of 4 ton/m² to yield the Cd standard concentrations in *P. purpurem*. These standards were analyzed to obtain response signals from the LA-ICP-MS and the response signals were subsequently used to generate a standard curve of Cd concentrations and analytical signals.

To validate the response signals obtained from the spiked samples, the procedure described previously (Becker et al. 2010; Wang and Specht 2011) was conducted. In brief, a response signal from the spiked sample with a Cd concentration of 0.022 mg kg⁻¹ was compared to the response signal obtained from the standard reference material (SRM) of rice flour (NIST SRM 1568a) with a known Cd concentration of 0.022 mg kg⁻¹. As the response signals obtained from both the spiked sample and the SRM were consistent, the LA-ICP-MS was finally used to analyze the Cd level in the plant tissues in this study qualitatively and quantitatively.

Statistical analyses

Statistical package for the Social Sciences (SPSS) version 13 was used to analyze the significant differences in the effects of different types and concentrations of chelating agents in relation to the accumulation and distribution of Cd in plant. In addition, Duncan's new multiple range test (DMRT) was applied to compare the significant difference in the mean concentrations of Cd accumulated in plant tissues with different cultivation times. A *p* value of < 0.05 was considered to be significantly different.

Results and discussion

Relative growth rate and phytotoxicity of Cd on plants

The increase in *P. purpurem* biomass (wet weight) was used to indicate plant growth. The maximum growth rate of 0.0678 ± 0.0013 was observed at the beginning of the experiment, especially on the 1st and 15th day of exposure to 1:1 mol ratio of Cd:EDTA in the solution. After 15 d of exposure, the relative growth rate tended to decrease in most experimental sets. The

plant exposed to Cd only experienced a steady relative growth rate of 0.01 to 0.02 throughout the experimental period. The presence of EDTA and/or NTA in the Cd solution did not retard the plant growth (Fig. 1a). Rather, the presence of EDTA and/or NTA increased the biomass of *P. purpurem* throughout the 105-d experimental period. This finding corresponds to the effects of Cd on water hyacinth biomass and its growth rate reported by Lu et al. (2004) and Cheng (2003).

In the case of Cd-mediated phytotoxicity, *P. purpurem* cultivated in both Cd and EDTA solutions (1:0.5 and 1:1 mol ratio of Cd: EDTA) showed symptoms of Cd phytotoxicity in the range of 4.00 to 23.17% throughout the entire test period (Fig. 1b). This was lower than in the presence of Cd alone, where the phytotoxicity ranged from 6.67 to 42.67% but, in the presence of Cd alone, were 54.67%. The significantly lower phytotoxicity with EDTA in the Cd solution than with Cd alone corresponds to a study by Wu et al. (2004) which demonstrated the ability of EDTA to reduce the toxic effects of heavy metals on cabbage.

Effect of chelating agents on Cd accumulation in plants

As clearly shown in Fig. 2, Cd was primarily accumulated in the underground part (roots) of *P. purpurem*. These findings are similar to those reported by Abrantes et al. (2007), who found the direct effect of a chelating agent on Cd accumulation in *Halimione portulacoides* occurred primarily in the underground part (roots). In our study, the use of EDTA as a chelating agent resulted in a significantly higher Cd accumulation in the underground part (roots) and aboveground part (stems and leaves) (Fig. 2) than with Cd alone for the whole 105-d experimental period (*p* < 0.05). The maximum Cd concentration in the plant tissues when cultivated in a 1:1 mol ratio of Cd:EDTA was 889.31 ± 52.73 mg kg⁻¹ in the underground part (roots) after 30 d of treatment (Fig. 2a) and 264.50 ± 25.86 mg kg⁻¹ in the aboveground part (stems and leaves) after 45 d of treatment (Fig. 2b). Similar positive effect of EDTA on heavy metal (Pb, Zn, and Cd) accumulations were previously reported in *Rorippa globosa*, *Cynara cardunculus*, and *Bidens maximowicziana* (Hernandez et al. 2006; Wei and Zhou 2005), while EDTA showed a greater enhanced Cd accumulation in *Typha angustifolia* than citric acid (Muhammad et al. 2009). This result was also in accordance with the result reported by Eissa (2017) that EDTA application can enhance Cd solubility. Therefore, Zaier et al. (2014) suggested that EDTA was suitable for enhancing phytoremediation.

Additional positive effects of NTA on the Cd accumulation level were also found, where the presence of NTA with the Cd, especially at a 1:1 mol ratio of Cd:NTA, significantly enhanced the Cd accumulation level in underground part (roots) as well as aboveground part (stems and leaves) (*p* < 0.05) over that of Cd alone (Fig. 2c, d). Maximum Cd

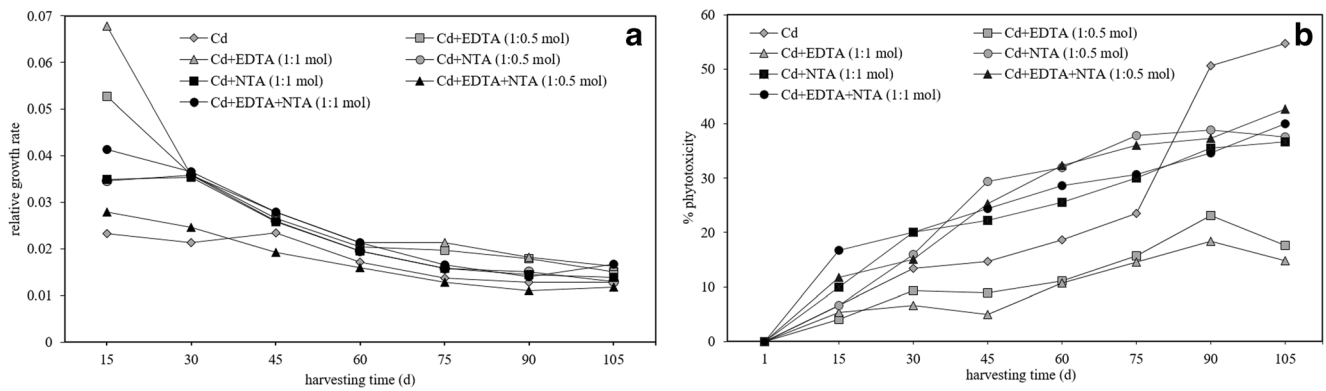


Fig. 1 Depiction of **a** relative growth rate (RGR) and **b** phytotoxicity of Cd with and without chelating agents on *P. purpurem*

concentrations of 638.98 ± 16.24 and $255.25 \pm 6.63 \text{ mg kg}^{-1}$ were found after 30 and 45 d of treatment, respectively. This finding is in good agreement with the increased Cd accumulation in corn after the addition of NTA to the contaminated soil, as previously reported by Xu et al. (2013).

When the EDTA-NTA mixture was added, highest Cd accumulation levels in the underground part (roots) ($606.25 \pm 25.77 \text{ mg kg}^{-1}$) were observed in the presence of a 1:0.5:0.5 mol ratio of Cd:EDTA:NTA after 30 d of treatment (Fig. 2e), while the highest Cd accumulation levels in the

aboveground part (stems and leaves) ($263.25 \pm 4.18 \text{ mg kg}^{-1}$) were observed after 45 d (Fig. 2f).

Based on the biomass growth rate and Cd accumulation level, EDTA appeared to be the most promising chelating agent to enhance the Cd accumulation and reduce phytotoxicity on the growth of *P. purpurem*. This finding corresponds to observations reported by Schor-Fumbarov et al. (2005), Muhammad et al. (2009), and Song et al. (2017). The optimal mole ratio of Cd to EDTA of 1:1 in our study resulted in a significantly higher Cd accumulation level in plants than all the other

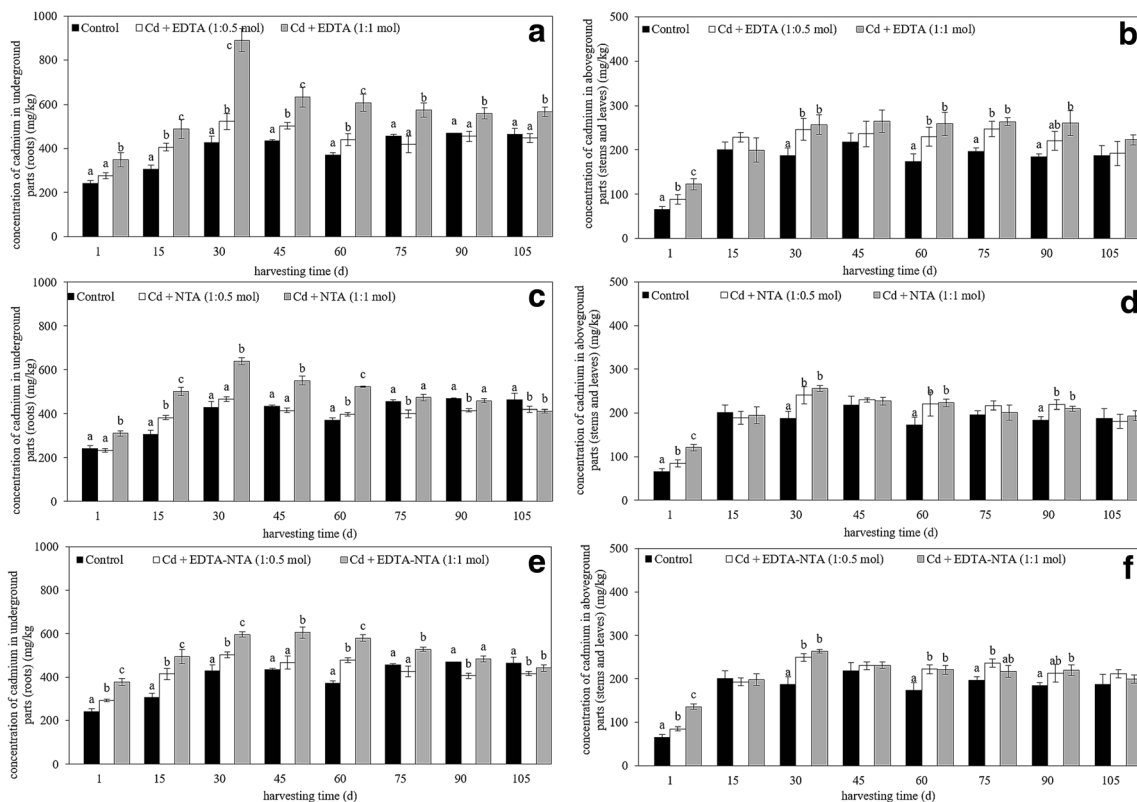


Fig. 2 Effects of **a, b** EDTA, **c, d** NTA, and **e, f** EDTA/NTA mixtures on the Cd accumulation in the underground part (roots) and aboveground part (stems and leaves) of *P. purpurem*. Data are shown as the mean \pm 1SD derived from independent trials. Means with a different letter are significantly different

treatments ($p < 0.05$), with the maximum total Cd level of 20.71 mg Cd/plant being observed at the 105th day, as shown in Fig. 3. Similar results have also been found in rice saltbush and barley (Assawadithalerd et al. 2014; Hirzel et al. 2017).

The assessment on the phytoremediation effectiveness in this study focused on the Cd concentration which was removed from the synthetic contaminated water. A level of remediation factor (RF) was used to indicate the ability of *P. purpurem* on Cd phytoremediation. Values of RF in this study are shown in Fig. 4a. These RF value which ranged from 1.51 to 19.20% indicates the efficiency of *P. purpurem* on Cd removal from water. In general, RF value tend to increase over the harvesting time. At the end of the experiment, the RF values ranged between 8.89 and 18.30%. The highest RF value was recorded in the case of *P. purpurem* in Cd and EDTA at molar ratio 1:1. This result was similar to Eissa (2016) who found the positive effect of EDTA addition on the RF value. In addition, translocation factor (TF) as shown in Fig. 4b was also used to determine the efficiency of EDTA on the enhancement of Cd uptake and translocation from the root to shoot of *P. purpurem*. All TF values were lower than 1. Thus, it is possible to imply that the higher amount of Cd was accumulated in root than that in aboveground part. According to Sun et al. (2009), plant with TF value lower than 1 is considered to be a phytostabilizing plant. These results were in line with the low TF on Cd removal by *P. purpurem* with the presence of Cd-resistant bacterial inoculation (Wiangkham and Prapagdee 2018). Therefore, *P. purpurem* could be considered as a candidate plant for Cd phytostabilization.

Accumulation and distribution of Cd in plants tissue

To investigate the accumulation and distribution of Cd in the *P. purpurem* tissue, only the samples from the experimental set with 30 mg kg⁻¹ of Cd and a 1:1 mol ratio of Cd:EDTA

were considered (experimental set no. 3). Plant samples were collected after 30 d, as these showed the highest Cd accumulation. The accumulation and distribution of Cd in five different parts of the plant (root tip, root trunk, trunk base, middle trunk, and leaves) was initially determined by SR-XRF and used to describe the effect of time on Cd accumulation in plant tissues. However, due to the low emission energy of 1.2 keV, only major elements, such as Ca and K, could be detected in the samples (Fig. 5). The limitation of low emission energy to determine the levels of trace metal accumulation in the tissue of *Solanum lycopersicum* has been reported previously (Trebolazabala et al. 2017). Therefore, the LA-ICP-MS technique was used for the analysis of Cd accumulation in the plant tissues. However, when the emission energy of the SR-XRF could reach and activate the energy level of the K shell of the Cd atom, the quantitative accumulation of Cd in plant tissue could be determined. This accumulation is based on the absence of any significant difference in the response signals of the Cd contained in the SRM Cd pellet and the spiked plant samples. For example, Fukuda et al. (2008) utilized this method and found that the high energy level (37 keV) of SR-XRF could determine the highest Cd accumulation in the epidermal tissue and leaf surface of *Arabidopsis halleri* ssp. *gemmaifera*.

The LA-ICP-MS technique was recently introduced for both quantitative and qualitative analyses of metal distribution (Pozebon et al. 2017). Compared to the results of AAS, which only provides the total concentration of each metal of interest in singular parts of the plant, LA-ICP-MS analysis can show the level and site where the Cd is accumulated (Hamilton et al. 2016). However, specific sample preparation must be conducted according to the methods delineated in Fernandez (2012). The LA-ICP-MS analysis in our study indicated the accumulation of Cd outside the root system with Cd concentrations ranging from 250 to 2000 mg kg⁻¹ for the root tip and 1000 to 8000 mg kg⁻¹

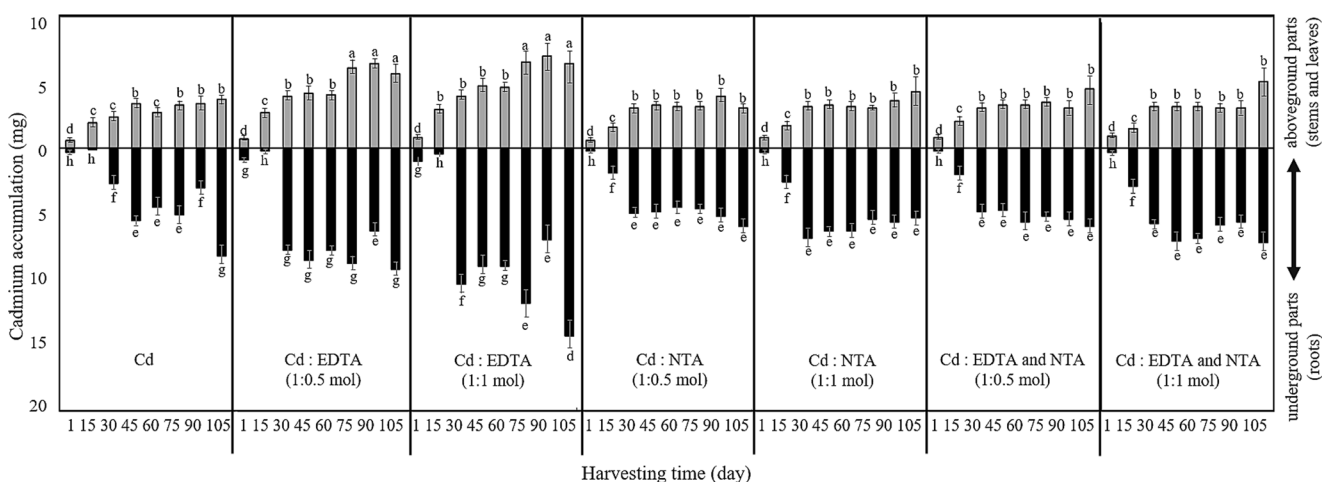


Fig. 3 Effects of EDTA, NTA, and EDTA-NTA mixtures on the Cd accumulation level in *P. purpurem*. Data are shown as the mean \pm 1SD, derived from independent trials. Means with a different letter are significantly different

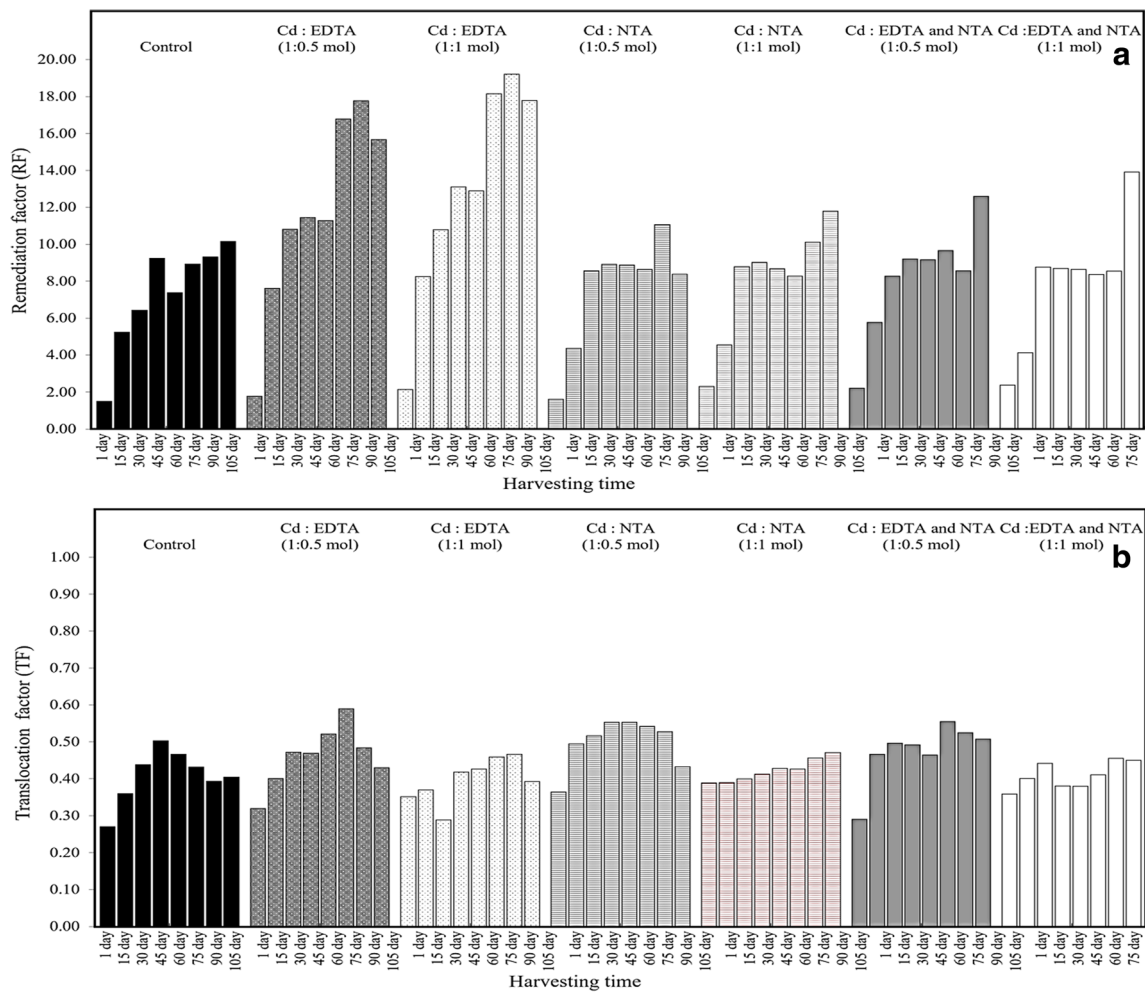


Fig. 4 **a** Remediation factor (RF) of *P. purpurem* with the presence of EDTA, NTA, and EDTA-NTA mixtures. **b** Translocation factor (TF) of Cd in *P. purpurem* with the presence of EDTA, NTA, and EDTA-NTA mixtures.

for the root trunk (Fig. 6 a and b). For the trunk base, approximately 5000 to 20,000 mg kg⁻¹ of Cd was found in the epidermal or meristematic tissue (Fig. 6c). A long sectional cut of the middle trunk showed a high Cd accumulation (4000–16,000 mg kg⁻¹) in the intercalary meristem (Fig. 5d). Examination of the leaves revealed that Cd was mainly accumulated in the leaf vein at a concentration of 600 to 1600 mg kg⁻¹ (Fig. 6e).

It can be concluded that Cd primarily accumulates in the stem and stolon of the *P. purpurem* where the meristematic tissues are located and plant transpiration occurs. This result is comparable to the findings of Jarvis et al. (1976), Jalil et al. (1994), and Yang et al. (1995), who found that the major site of Cd accumulation in tomato, barley, and corn was the intercalary meristem and xylem. Relatedly, other studies conducted by Tian et al. (2011)

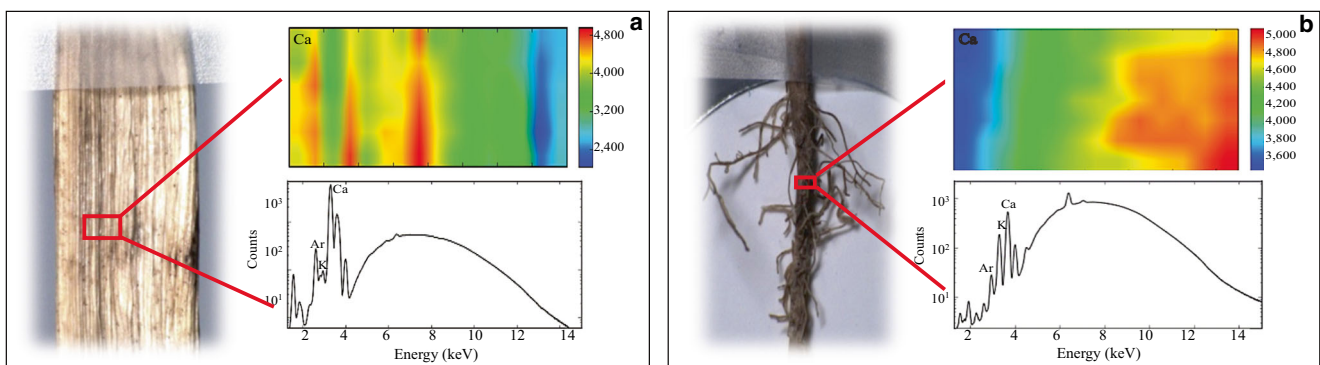


Fig. 5 Distribution of the major elements, such as Ca and K, in the **a** aboveground part (stems and leaves) and **b** underground part (roots) of *P. purpurem*, as determined by SR-XRF analysis

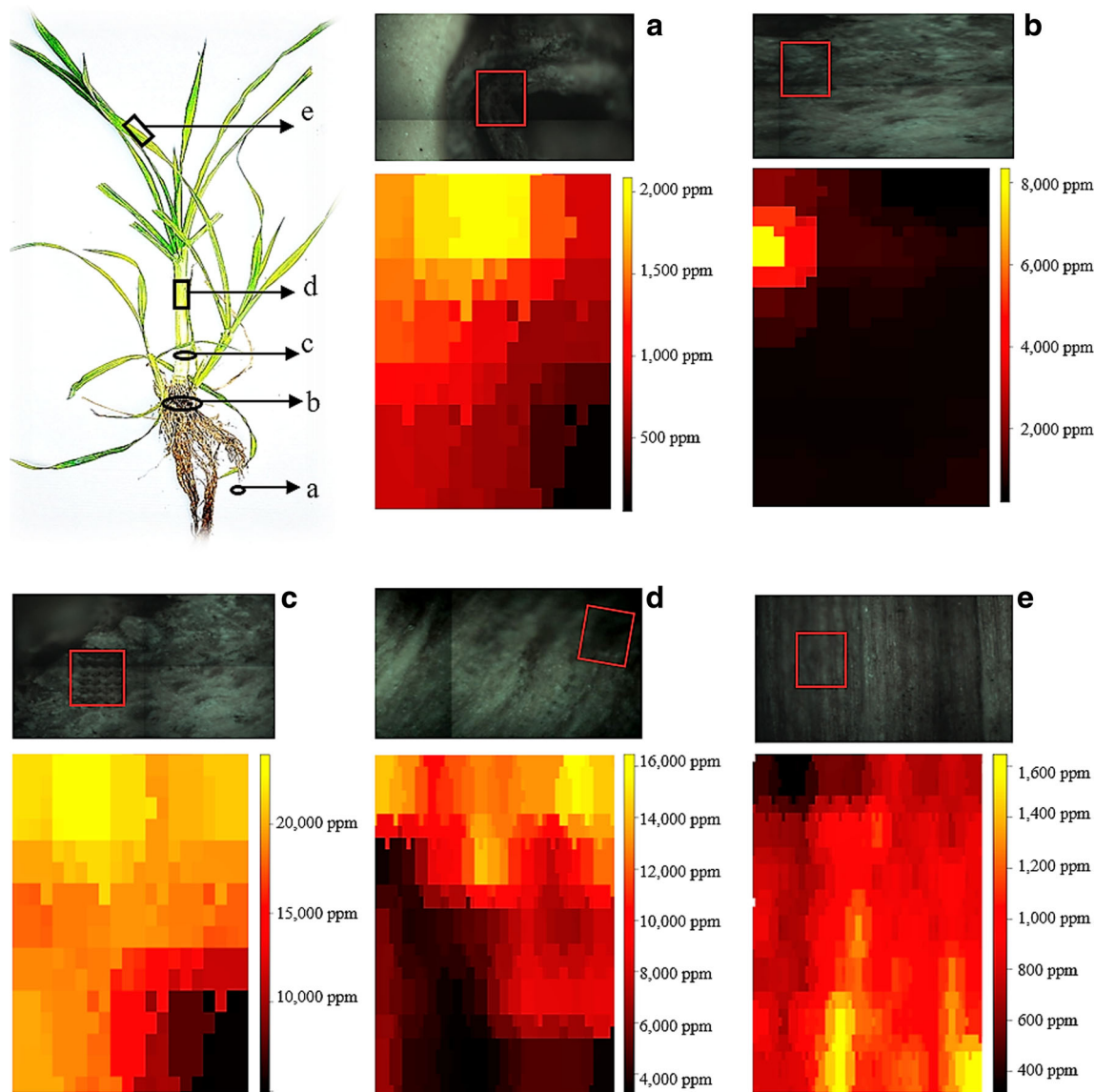


Fig. 6 Distribution of Cd in the **a** root tip, **b** root trunk, **c** trunk base, **d** middle trunk, and **e** leaves of *P. purpurem*, as determined by LA-ICP-MS analysis. Images shown are representative of those seen from independent samples

and Lefevre et al. (2014) which concluded that the mechanism of Cd hyperaccumulation in *Sedum alfredii* and *Zygophyllum fabago* is either dominated by (i) ligand formation with oxygen, which resulted in approximately 47% of the Cd accumulation in the aboveground part (stems and leaves) or (ii) bonding with organic malic acid within the plant's intercalary meristematic tissues. However, it is worth noting that Cd accumulation in the aboveground part (stems and leaves) might be reduced once the budding process commences (Salt et al. 1995).

Conclusions

As more than 1000 mg kg⁻¹ of Cd was detected in the intercalary meristematic tissues of plants, it is possible

to use *P. purpurem* as a hyperaccumulator plant to bioremediate Cd from contaminated areas. The AAS analysis indicated that the Cd primarily accumulated in the underground part (roots) followed by the aboveground part (stems and leaves). The translocation factor was lower than 1. *P. purpurem* could be considered as a candidate plant for cadmium phytostabilization. The LA-ICP-MS results confirmed that Cd was predominantly distributed in the aboveground part (stems and leaves), particularly within the meristematic tissues. These two analytical techniques suggest that differences in the Cd accumulation within *P. purpurem* might be caused by the deposition of Cd on the surface of plant tissues. However, further research will be needed to confirm this finding.

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