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**Published on:** 30 Sep 2017 - Plant and Soil (Springer International Publishing)

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# The accumulation and fractionation of Rare Earth Elements in hydroponically grown *Phytolacca americana* L.

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## Abstract

**Background and Aims** The widespread use of Rare Earth Elements (REEs) has resulted in localized soil pollution. *Phytolacca americana* L. has potential for REE phyto-extraction, but the related mechanism is not clear.

**Methods** In this study, the uptake and fractionation of REEs, and the influence of REEs on biomass production was investigated in hydroponically grown plants. Furthermore, the effects of  $\text{Ca}^{2+}$  and  $\text{Al}^{3+}$  on REE uptake, and the role of organic acids in REE translocation were also examined.

**Results** Results showed that biomass and accumulation of REEs in *P. americana* were enhanced at low REE concentrations, but inhibited at higher concentrations in solution. Significant heavy REE (HREE) enrichment was observed during the stem-to-leaf transport, with a quotient of  $\sum\text{LREE}/\sum\text{HREE}$  decreasing from 0.75 to 0.23.  $\text{Ca}^{2+}$  and  $\text{Al}^{3+}$  treatments diminished REE accumulation. The  $\sum\text{LREE}/\sum\text{HREE}$  ratio decreased from 0.84 to 0.62 with increasing input of  $\text{Ca}^{2+}$ , but increased from 0.83 to 0.92 with higher Al addition.

**Conclusions** LREEs appear to enter into the root of *P. americana* through  $\text{Ca}^{2+}$  ion channels, whereas HREEs may share pathways with  $\text{Al}^{3+}$ . Finally, citrate plays an important role in the translocation of REEs in *P. americana*.

## Introduction

Rare earth elements (REEs) are a homogenous group of chemical elements with similar physical and chemical properties, including 15 lanthanides together with scandium (Sc) and yttrium (Y) (IUPAC 2005). In fact, REEs are not rare in nature, but widely distributed in the environment (Chen 2011). For example, the average abundances of lanthanum (La, 30 mg kg<sup>-1</sup>) and cerium (Ce, 60 mg kg<sup>-1</sup>) in the crust are comparable to the common metals such as zinc (Zn, 70 mg kg<sup>-1</sup>) and copper (Cu, 55 mg kg<sup>-1</sup>) (Tyler 2004). Over the past decades, rapid urbanization and industrialization led to an increased demand for REEs because of their utilization in modern technology, especially 'green technologies', such as high strength magnets, electric vehicles and medical devices (Long et al. 2010). In addition, several different REE compounds are widely used as specialty fertilizers in agriculture to improve yield and quality of crops in China (Liu et al. 2012a; Zeng et al. 2006) and in other countries (Redling 2006). Although REEs are not generally considered toxic, compared to cadmium (Cd) or arsenic (As) for example, they can have long-term negative human health effects (Zhu et al. 1996, 1997a, b). The toxic effects of REEs have also been observed in bacteria (Ozaki et al. 2006), plants (Babula et al. 2008) and animals (Che et al. 2011). Due to worldwide use of REEs in technology and agriculture, and a lack of recycling, REEs are considered emerging pollutants (Yang et al. 2012; Kulaksiz and Bau 2011) and have been classified as a major contaminant in China since the 1990s (NNSFC 1996).

As an in-situ, low cost remediation means, phyto-extraction consists of the use of hyperaccumulator plants to remove contaminants from polluted soils by growing and repeatedly harvesting hyperaccumulator plant biomass, thereby exhausting soil concentrations of the target element (Brooks et al. 1977; Chaney et al. 1997). To date, 22 plant species have been reported to (hyper)accumulate REEs through field and/or laboratory investigations; these species belong to 11 families and 11 genera, with the greatest numbers being pteridophytes (ferns) (Liu et al. 2017), such as *Pronephrium simplex* (up to 1200 mg kg<sup>-1</sup>, Lai et al. 2005) and *Dicranopteris dichotoma* (up to 3358 mg kg<sup>-1</sup>, Wang et al. 1997). *Phytolacca americana* is a known manganese hyperaccumulator (Min et al. 2007; Xu et al. 2009; Pollard et al. 2009; Xue et al. 2010); but also has REE accumulation abilities (Ichihashi et al. 1992; Wu et al. 2013). Moreover, *P. americana* is a high-biomass plant that is naturalized worldwide, and is able to accumulate REEs up to 623 mg kg<sup>-1</sup> in dry leaves, although plant REE concentrations vary substantially among sampling sites (Ichihashi et al. 1992). Our recent field survey reported a high abundance of *P. americana* at an REE mine site in south Jiangxi Province (China) with foliar concentration of REEs as high as 1040 mg kg<sup>-1</sup> (Yuan et al. 2017). Most research effort to date has focused on the pteridophyte *D. dichotoma* elucidating REE accumulation, distribution and effects on photosynthesis (Wang et al. 1997, 2006; Wei et al. 2001), in contrast the mechanisms of REE uptake and translocation in the dicot *P. americana* are largely unknown. There are reports about the accumulation mechanisms of REEs in 'normal' plants (Sun et al. 1994). For example, it is known that La<sup>3+</sup> can be used as a Ca<sup>2+</sup>-channel blocker in plant root cell membranes (Lewis and Spalding 1998), on which La<sup>3+</sup> replaces Ca<sup>2+</sup> sites in enzymatic reactions (Squier et al. 1990). The antagonistic effects of La and Ca have been reported in rice (Liu et al. 2012b), and in *Hordeum vulgare* (Han et al. 2005). Also, the plant Ca<sup>2+</sup> content can be decreased by other REEs, such as Ce<sup>3+</sup> or Eu<sup>3+</sup> in rye (Shtangeeva 2014), but is increased by Gd<sup>3+</sup> in soybean (de Oliveira et al. 2015). Therefore, REEs are defined as super-calcium because of their analogous characteristics with Ca (Brown et al. 1990). Except Ca, a high correlation between foliar Al and La has been found in *Rhynchanthera grandiflora* (Olivares et al. 2014). La<sup>3+</sup> can compete for the Al<sup>3+</sup> binding sites on the plasma membrane of buckwheat (Ma and Hiradate 2000). Interestingly, some REEs, such as La<sup>3+</sup>, Gd<sup>3+</sup> and Sc<sup>3+</sup>, have been used as Al<sup>3+</sup> analogues to study Al<sup>3+</sup> toxicity in plants (Clarkson 1965; Kopittke et al. 2009; Reid et al. 1996), but different effects were found depending on the REE (Kopittke et al. 2009). These findings imply that Ca<sup>2+</sup> and Al<sup>3+</sup> can influence the REE accumulation process in plants which may partly share uptake pathways of these

cations. Organic acids play an ambiguous role in metal uptake and root-shoot translocation in plants (Han et al. 2006; Mnasri et al. 2015; Ghnaya et al. 2013). REEs have also been reported to bind with organic ligands which contribute to REE translocation in plants (Ding et al. 2006). It has been reported that acetic, malic and citric acids can enhance the  $\text{La}^{3+}$  uptake and transport in *Hordeum vulgare* (Han et al. 2005). Moreover, acetic or malic acids can also increase La accumulation in wheat roots (Wang et al. 2004). In addition, La can form complexes, such as  $\text{La}(\text{CH}_3\text{COO})_3$  in the roots of *H. vulgare* (Han et al. 2005) and those of wheat (Ding et al. 2006). Europium has also been found to combine with carboxylate groups in roots of *Eichhornia crassipes* (Kelley et al. 1999).

In the past decades, REEs have been widely used as tracers in geosciences because of their generally coherent behavior. Meanwhile, small but systematic chemical dissimilarities of these REEs can cause fractionation, i.e. departure from the expected pattern, which can be applied to trace sources or biogeochemical processes within a system. For example, based on the REE pattern, Wei et al. (2001) suggested that soluble REEs in the soil might be a significant source of REE uptake for the naturally grown REE hyperaccumulator *D. dichotoma*. In the solution cultured wheat, the roots were characterized by a middle REE (MREE, Gd-Sm) enrichment, which was ascribed to a combination effect of phosphate precipitation and absorption by root cell walls (Ding et al. 2005). However, root-to-shoot translocation favored a conspicuous fractionation toward heavy REE (HREE, Gd-Lu), indicating that organic complexation in the xylem vessels may play a role in REE translocation in wheat (Ding et al. 2005, 2006), but the specific organic ligands involved remain unknown. Recently, our field survey found that *P. americana* exhibited a preferential accumulation of LREEs (La-Eu) during soil-to-root assimilation, and a preferential accumulation of HREEs during stem-to-leaf translocation (Yuan et al. 2017). However, the accumulation, transport and fractionation mechanisms of REEs in this plant are still inadequately understood. Therefore, the main objective of this study was to elucidate key mechanisms involved in REE uptake and translocation in *P. americana*, as well as the link with REE fractionation. Specifically, we aimed to investigate: (i) REE accumulation and fractionation properties in *P. americana*; (ii) the effects of  $\text{Ca}^{2+}$  and  $\text{Al}^{3+}$  on REE accumulation in *P. americana*; and (iii) the possible role of organic acids in REE translocation.

## Materials and methods

### Seed collection of *Phytolacca americana* in China

Our previous work has located a natural population of *P. americana* in Dingnan County, a key REE mining area in the southern province of Jiangxi in China, with a relatively high REE background (Yuan et al. 2017). Seeds of *P. americana* were collected at site P-5 in Yuan et al. (2017).

### Plant growth and REE treatment

Seeds were treated with 98%  $\text{H}_2\text{SO}_4$  for 30 s to destroy the seed coat to break dormancy, subsequently they were immersed in 15%  $\text{H}_2\text{O}_2$  solution for 15 min to sterilize the seeds, then rinsed and soaked for 10 min in deionized water. The seeds were germinated on folded layers of filter paper moistened with deionized water for 10 days at 24 °C. Healthy and uniform seedlings were transferred to plastic pots containing 1 L of a modified Hoagland nutrient solution (1000  $\mu\text{M}$   $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , 1000  $\mu\text{M}$   $\text{KNO}_3$ , 500  $\mu\text{M}$   $\text{MgCl}_2 \cdot 7\text{H}_2\text{O}$ , 50  $\mu\text{M}$   $\text{KH}_2\text{PO}_4$ , 50  $\mu\text{M}$   $\text{KCl}$ , 10  $\mu\text{M}$   $\text{H}_3\text{BO}_3$ , 1  $\mu\text{M}$   $\text{MnSO}_4$ , 0.2  $\mu\text{M}$   $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.2  $\mu\text{M}$   $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 20  $\mu\text{M}$   $\text{Fe}(\text{III})\text{-EDDHA}$ ). The solution pH was adjusted to  $5.3 \pm 0.1$  with  $\text{HCl-KOH}$  throughout the experimental period. The nutrient solution was aerated continuously. Two weeks after transplantation, the seedlings were exposed to a nutrient solution containing REE added as chloride salts (La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb and Lu). Six concentrations of total REEs were tested: 0, 1, 10, 50, 100 and 250  $\mu\text{M}$  (each

REE with equal molar concentration). Each treatment was prepared in triplicate with one plant per pot. The REE solution was replaced every 3 d. The plants were placed in a growth chamber under controlled conditions (350  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR-light, 14/10 h day/night, 25 °C and 75% relative humidity). Plants were harvested individually after 28 d of culture. In order to remove any adsorbed REEs, roots were rinsed with deionized water, then transferred to 5 mM ice-cold  $\text{CaCl}_2$  solution and desorbed for 15 min. (Han et al. 2005). Roots and shoots were finally washed with running deionized water, then blotted dry with a soft paper towel, and dried in a forced-air drying oven at 65 °C until constant weight.

### **Sample preparation and element analyses**

After grinding to pass a 100-mesh sieve, each plant material sample was weighed to  $100 \pm 1$  mg and then placed in Teflon containers. The samples were wetted with 1–2 drops of high purity water, and then digested with a mixture of concentrated  $\text{HNO}_3$  (4 mL) and  $\text{HClO}_4$  (1 mL) at  $220 \pm 5$  °C for 8 h. The samples were then dried at 115 °C, and the residue was dissolved in 2 mL  $\text{HNO}_3$ . The crucibles were removed from the oven and dried on a hot plate at 115 °C. After evaporation to dryness, the residue was dissolved again in 2 mL  $\text{HNO}_3$ . In order to compensate for signal drifting and matrix suppression, 1 mL of 20  $\mu\text{g mL}^{-1}$  indium (In) was added to the solution as an internal standard, and a certified leaf reference material (GBW07603) was used as quality control according to the method described in Yuan et al. (2017). The concentrations of REEs in the solutions were determined by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS, Model NexION 300D, Perkin Elmer, Waltham, MA, USA) (Cao et al. 1998). The macro and micronutrients (Ca, Mg, Fe, Zn, P, Mn, Cu, K, S) were analyzed using atomic absorption spectrophotometer (PerkinElmer, San Jose, CA, USA) (de Oliveira et al. 2015).

### **Effect of $\text{Ca}^{2+}$ inhibitor on REE concentration and fractionation patterns in *Phytolacca americana***

Verapamil (a  $\text{Ca}^{2+}$ -uptake inhibitor) was purchased from Sigma Ltd. The *P. americana* seedlings at the five-leaf stage were exposed to the following treatments: control, 100  $\mu\text{M}$  REEs, 100  $\mu\text{M}$  REEs +100  $\mu\text{M}$  Verapamil. Each treatment had three replicates. The plants were harvested after 72 h and prepared for REE analysis as described above.

### **Effect of $\text{Ca}^{2+}$ and $\text{Al}^{3+}$ on REE concentrations and fractionation in *Phytolacca americana***

Seedlings at the five-leaf stage were exposed to a range of  $\text{CaCl}_2$  (0, 0.1, 1, 5, 10 mM) or  $\text{AlCl}_3$  (0, 0.05, 0.1, 0.5 mM) solutions in the presence of a mixture of 50  $\mu\text{M}$  REEs. The solutions were renewed every day. After 72 h of exposure, the root, stem and leaf were sampled and analyzed for REE concentrations.

### **Collection of xylem sap of *Phytolacca americana* and organic acid analysis**

The five-leaf stage seedlings of *P. americana* were cultured in 0.1 mM  $\text{CaCl}_2$  overnight and then exposed in low (1  $\mu\text{M}$ ), medium (50  $\mu\text{M}$ ) and high (250  $\mu\text{M}$ ) level of mixed REEs in 1 mM  $\text{CaCl}_2$  solution. After 24 h, stems were severed 4 cm above the root, and the xylem sap was collected for 8 h with a micro-pipette under low pressure created by a vacuum pump (Morita et al. 2004). Each sample was filtered through a 0.22  $\mu\text{m}$  membrane filter and divided into two parts. One part was used for the determination of REEs and nutrient elements in the xylem sap, and the other part was used for the determination of organic acids by ion chromatography (Dionex-600, USA) according to the methods from Han et al. (2005). In order to model the REE chemical species in the xylem sap of *P. americana*, Visual Minteq 3.0 was used to simulate the complexation of organic acids with REEs.

### **The calculation of REE fractionation**

The  $\Sigma\text{LREE}/\Sigma\text{HREE}$  value represents the ratio of the sum of concentrations of LREEs to the sum of HREEs ( $\Sigma\text{LREE}/\Sigma\text{HREE} = \text{sum concentrations [La to Eu]} / \text{sum concentrations [Gd to Lu]}$ ). The ratio of  $\Sigma\text{LREE}/\Sigma\text{HREE}$  was used to depict variations in REE abundance in different REE pools (e.g. in solution or plant). The higher value of  $\Sigma\text{LREE}/\Sigma\text{HREE}$  means a relative higher LREE-enrichment, and lower value shows a relative HREE-enrichment. To elucidate the REE accumulation and transportation into the plants grown hydroponically, the REE fractionation was investigated for three key steps: i) the REE uptake by roots from the solution; ii) the translocation from roots to stems, and; iii) the trans- location from stems to leaves. In order to decipher the REE fractionation for these steps, they were calculated and displayed as accumulation coefficients. Accumulation coefficients are considered to be normalized REE concentrations; for example, the fractionation of REEs from solution to root can be considered as the REE concentration in the root normalized to (divided by) that in the solution (Henderson 1984). For instance, La accumulation coefficients from solution to root is La concentration in root/La concentration in solution.

### **Statistical analyses**

SPSS 19.0 statistical analysis software was used. The significant difference among the treatments was determined by post hoc Fisher Least Significant Difference test ( $p < 0.05$ ). All results are presented as arithmetic means with standard deviations. Values with the same letter in the same organ of *P. americana* are not significantly different ( $p < 0.05$ ).

### **Results**

#### **Influence of REEs on the growth of *Phytolacca americana***

Biomass of *P. americana* under REE treatments (0– 250  $\mu\text{M}$ ) exhibited clear dose-response effects (Fig. 1). A significant increase of 161% in dry weight was found for roots (1–50  $\mu\text{M}$  REEs), and 43% in shoots (1– 10  $\mu\text{M}$ ). However, with higher REE concentrations; at 250  $\mu\text{M}$  REEs, growth was inhibited by 72% for roots and 70% for shoots, compared to the control (0  $\mu\text{M}$  REEs).

#### **REE and nutrient elements concentrations in *Phytolacca americana***

The concentration of total REEs (from La to Lu) in organs of *P. americana* increased significantly when exposed to increasing REE concentrations (1–100  $\mu\text{M}$  for root and stem, 1–50  $\mu\text{M}$  for leaves). It was decreased with the highest REE concentration in solution (250  $\mu\text{M}$  for stems and leaves and >100  $\mu\text{M}$  for roots, Fig. 2). The increasing concentration of REEs in plants resulted in a significant decrease in Ca and Mg, but an increase in P in the roots of *P. americana* (Table 1).

#### **REE patterns and fractionation in *Phytolacca americana***

Roots of *P. americana* were characterized by slightly higher LREE and lower HREE concentrations, but the stems and leaves characterized by opposite phenomenon (Fig. 3a–c). The ratio of  $\Sigma\text{LREE}/\Sigma\text{HREE}$  in the organs of *P. americana* decreased in the order root > stem > leaf, except for the 1 and 10  $\mu\text{M}$  treatments (Fig. 4d). The average ratio of  $\Sigma\text{LREE}/\Sigma\text{HREE}$  in whole plant of *P. americana* ranged from 0.70 to 0.80. The REE fractionation patterns for the three steps were shown in Fig. 4a, b and c. A slight LREE enrichment was found in the accumulation process from solution to root (Fig. 4a). There was no observable fractionation from the roots to the stems (Fig. 4b). However, significant HREE enrichment was found in the fractionation from the stems to leaves (except for 1  $\mu\text{M}$  REE treatment) (Fig. 4c) and the ratio of  $\Sigma\text{LREE}/\Sigma\text{HREE}$  ranged from 0.23 to 0.41 (Fig. 4d).

### **The effect of Verapamil to the REE accumulation and fractionation in *Phytolacca americana***

Total REE accumulation significantly decreased in organs of *P. americana* when treated with Verapamil (Fig. 5a). In addition, the REE concentrations in the roots of *P. americana* decreased by 23.9%, but no preferential accumulation of LREEs or HREEs in stems and leaves was observed after treatment with Verapamil (Fig. 5b, c and d).

### **The effect of $\text{Ca}^{2+}$ / $\text{Al}^{3+}$ to the REE accumulation in *Phytolacca americana***

The total REE concentrations in the organs of *P. americana* decreased when treated with  $\text{Ca}^{2+}$  ( $>0.1 \mu\text{M}$ ) (Fig. 6a). The ratio of  $\sum\text{LREE}/\sum\text{HREE}$  in roots of *P. americana* decreased from 0.84 to 0.62 with increasing concentrations of  $\text{Ca}^{2+}$  (0–10 mM). No particular REE distribution patterns were observed in the stems and leaves of *P. americana* when treated with increasing  $\text{Ca}^{2+}$  concentrations (Fig. 6b, c and d). Similarly, treatment with  $\text{Al}^{3+}$  significantly decreased the accumulation of REEs in *P. americana* (Fig. 7a). However, the ratio of  $\sum\text{LREE}/\sum\text{HREE}$  in roots of *P. americana* increased from 0.84 to 0.94 with increasing doses of  $\text{Al}^{3+}$  (Fig. 7b, c and d).

### **The role of organic acids in the REE transporting in *Phytolacca americana***

The concentration of HREEs was greater than concentration of LREEs in xylem sap of *P. americana* (Fig. 8). The  $\sum\text{LREE}/\sum\text{HREE}$  ratio decreased from 1.11 (1  $\mu\text{M}$  REE) to 0.15 (250  $\mu\text{M}$  REE). Concentrations of lactic, acetic, formic, oxalic, citric, malic and tartaric acids in plants treated with the high REE concentrations (250  $\mu\text{M}$ ) were higher (by 238%) than that in plants treated with the low REEs concentrations (1  $\mu\text{M}$ ) (Table 2). However, malic acid and tartaric acid were not detected in the xylem sap regardless of the REE concentrations in solution. The results show that most of the lactic acid, acetic acid and formic acid does not complex with REEs. Oxalic acid can complex a few elements (Tm) (Table 3), but citric acid can ligate with most of the REEs, and the percentage of combination with HREEs was higher than that with LREEs (by 483%). The concentrations of REEs in xylem sap had a significant positive correlation with the percentage of

## **Discussion**

### **REEs uptake mechanisms by *Phytolacca americana* and fractionation between plant and media**

Even though REEs have similar chemical and geochemical properties (Brookins 1989), their relative concentrations in plant tissues varied in *P. americana*. A slight LREE enrichment was observed in the fractionation of REEs from solution to roots (Fig. 4b). In addition, the  $\sum\text{LREE}/\sum\text{HREE}$  ratio in roots was higher than in stems and in the leaves (Fig. 4d), which also suggests a relative higher LREEs accumulation ability in roots of *P. americana*. In this study, the results show competitive interaction between REEs and  $\text{Ca}^{2+}$  (Table 1 and Fig. 6a), and the inhibition of REE uptake by Verapamil (a  $\text{Ca}^{2+}$  ion inhibitor), which confirmed a shared uptake and transport system in *P. americana*. It is likely that REEs and  $\text{Ca}^{2+}$  use the same channels in *P. americana*, because  $\text{Ca}^{2+}$  ions have similar ionic radius (9.9 nm) as REEs (9.6–11.5 nm) (Das et al. 1988).  $\text{Ca}^{2+}$  can be replaced by REE ions in many enzymatic reactions, and thus normal physiological functions of  $\text{Ca}^{2+}$  may not be disturbed (Squier et al. 1990). A previous study found that some REEs, such as  $\text{La}^{3+}$ , are  $\text{Ca}^{2+}$  – channel blockers of cell membranes in plants (Lewis and Spalding 1998). It has been reported that  $\text{La}^{3+}$  and  $\text{Eu}^{3+}$  can enter into the plant cell through  $\text{Ca}^{2+}$  channels by binding with membranes of the mitochondria, chloroplasts and cytoplasm (Gao et al. 2003). The application of increasing concentrations of  $\text{Ca}^{2+}$  not only decreased the REE concentrations in root, but also limited the REE fractionation patterns. The ratio of  $\sum\text{LREE}/\sum\text{HREE}$  in root significantly decrease with the increasing concentrations of  $\text{Ca}^{2+}$ , which demonstrates that the uptake of LREEs is preferentially inhibited by  $\text{Ca}^{2+}$  compared to uptake of HREEs. This

may be partly explained by the fact that the ionic radius of LREEs are closer to  $\text{Ca}^{2+}$  than those of the HREEs (Das et al. 1988).

The concentrations of REEs in *P. americana* can be diminished by treatment with  $\text{Al}^{3+}$ , especially in the case of the HREEs, and the ratio of  $\sum\text{LREE}/\sum\text{HREE}$  in root has a significant increase. This suggests that Al competes with REEs during the uptake process in *P. americana*. As Al and REEs are trivalent elements, it is reasonable that REEs have the same properties with  $\text{Al}^{3+}$ . In addition, REE and  $\text{Al}^{3+}$  have similar characteristics, such as their behavior at the cell surface (Hou et al. 2010), and the same inhibition effect to cell division in root tip cells (Clarkson and Sanderson 1969). The inhibitory effect of  $\text{Al}^{3+}$  on REEs accumulation, and the increased ratio both indicate that REEs, especially HREEs, compete with the same pathways of  $\text{Al}^{3+}$  in root of *P. americana*. Lanthanum has an inhibitory effect on the uptake of  $\text{Al}^{3+}$  (Ma and Hiradate 2000). It has been reported that  $\text{Al}^{3+}$  uptake was accompanied by substantial increases of low-molecular-acids (Martins et al. 2013). Organic acids can ligate with  $\text{Al}^{3+}$  and increase its uptake in the plant root (Xu et al. 2016). This is also consistent with the REE transport system in plants, since it was shown that the chelation with organic ligands plays an important role in REE transportation (Ding et al. 2006). Because of the increasing stability constants of ligand-REE complexes for REE with increasing atomic number (Byrne and Li 1995; Millero 1992), HREEs bind stronger than LREEs. Therefore, the uptake of HREEs is preferentially inhibited by  $\text{Al}^{3+}$ . LREE enrichment was found in the average patterns of the whole plant of *P. americana* (Fig. 4d), which suggests more LREEs enter into the root of *P. americana* during the uptake from solution to plant. In general, the REE fractionation during uptake is caused by at least two processes: REE chelation in media, and REE transport across root cell membrane. As the most of REEs were present as free ions in the solution (Table S1), which should have no significant impact on the REE patterns in the whole plant. Therefore, REE uptake by root is likely the key process controlling REE patterns in the whole plant. As both pathways of  $\text{Ca}^{2+}$  and  $\text{Al}^{3+}$  contribute to the average REE fractionation patterns in *P. americana*, the ion channel of  $\text{Ca}^{2+}$  plays a more important role than  $\text{Al}^{3+}$  in REE uptake. This is similar to the isotope theory, which shows low-affinity transport such as ion channels or electrogenic pumps would favor light isotopes, while high-affinity transport like ion carriers favor the heavier isotopes (Weiss et al. 2005). Consequently, LREEs likely enter into *P. americana* through  $\text{Ca}^{2+}$  ion channels, which behaves similar as the light isotope.

It is noteworthy that *P. americana* showed similar REE patterns in the leaves, but a different pattern in the root compared with hydroponically grown wheat (Ding et al. 2006). The significant MREEs (Sm–Gd) enrichment in the roots of wheat was partly the result from the REE phosphate precipitates (Ding et al. 2005). The same precipitation of Gd and phosphate has also been reported in the extracellular space of maize roots (Quiquampoix et al. 1990). In addition, it was found that 72% of the total REEs was in the cell walls of the wheat roots. Therefore, the effect of both phosphate precipitation and selective absorption to cell walls resulted in the enrichment of MREEs in the root of wheat (Ding et al. 2005). However, REEs accumulation plays a significant role in the plant of *P. americana*. Higher content of REEs enter into the root of *P. americana* through ion channels (such as  $\text{Ca}^{2+}$ ), which can cover the same processes in wheat and the MREE enrichment phenomenon, and therefore resulting LREE enrichment in the root.

Furthermore, after entering into the root of plant, REEs are transported to the shoot. Accordingly, the fractionation patterns of REEs in root of *P. americana* not only result from the pathways of  $\text{Ca}^{2+}$  and  $\text{Al}^{3+}$ , but also from the REE translocation process. HREEs in the root of *P. americana* have a higher translocation ability than LREEs in xylem sap (Fig. 8). Therefore, the relative LREE enrichment and HREE depletion in root of *P. americana* mainly results from the accumulation process by ion channels ( $\text{Ca}^{2+}$ ) and the efficient REEs translocation from root to shoot.



### REEs fractionation from root to shoot in *P. americana*

Compared to the roots, the REEs patterns in the shoots of *P. americana* differed, and HREE enrichment was found in the translocation process from stems to leaves. This implicates other processes contribute to REE transport from the roots to the shoots in *P. americana*. In order to learn more about the mechanism of REE transporting in *P. americana*, the xylem sap of *P. americana* under REEs was collected and analysed. The relative higher concentrations of HREEs than LREEs in xylem sap (Fig. 8) may help to understand the HREEs enrichment in shoot of *P. americana*. The ratio of  $\sum\text{LREE}/\sum\text{HREE}$  in xylem sap was lower than in stems and leaves, which shows that *P. americana* has a strong HREE transporting ability resulting in the enrichment of HREEs in leaves. Studies indicated that transition metals can ligate with low molecular weight organic acids (LMWOA), such as oxalate, malate, citrate, malonate (Haydon and Cobbett 2007). Among these, citrate plays an important role in the metal transporting in plant. It was reported that the citrate had a positive correlation with  $\text{Pb}^{2+}$  in xylem sap of *Sesuvium portulacastrum*, and had potential to accumulate and translocate  $\text{Pb}^{2+}$  in shoot of this plant (Ghnaya et al. 2013). Similarly, citric acid was the main complexing ligand of  $\text{Ni}^{2+}$  in the leaves of *Noccaea goesingense* (Krämer et al. 2000) and was responsible for the long-distance transporting of  $\text{Ni}^{2+}$  in *Alyssum murale* (Montarges-Pelletier et al. 2008).

Organic acids can also ligate with REEs (Johannesson et al. 2004), and increased the  $\text{La}^{3+}$  uptake in *Hordeum vulgare* (Han et al. 2005). In this study, citric acid preferred to bind to HREEs (Table 3), which may partly due to the increasing stability of REE-ligand complexes with increasing REE atomic number (Byrne and Li 1995). The other reason may be that citrate is a ligand which can effectively alleviate the excess transition element toxicity in plants (such as Ni, Cu, Zn and Al) (Ma and Hiradate 2000; Ma 2000). In addition, a field study also found a correlation (correlation coefficient 0.73–0.96) between the concentrations of REEs by citric acid extraction from soil and from plants (Zhang et al. 2000).

Considering both the accumulation and translocation processes together, HREEs have similar properties compared to Al in plants, which may also help to explain the ligation of citrate with HREEs in translocation process, because Al prefers citrate complexation in the xylem sap (Wang et al. 2015). In addition, the coprecipitation of  $\text{Al}^{3+}$  and P in root tissues relates to  $\text{Al}^{3+}$  resistance (Zheng et al. 2005), and behaved similarly as the relationship between REE and P for fractionation (Ding et al. 2006). Therefore, the REE fractionation in organs of *P. americana* can help to understand the uptake and translocation mechanisms in this plant. However, the mechanisms of organic ligands in REE transporting in plants needs further research.

### The effects of REEs on biomass and REE accumulation in *Phytolacca americana*

In general, most plants contain low concentrations of REEs (Horovitz et al. 1974), and REE concentrations of the grains and vegetables range from  $10^{-4}$  to  $10^{-2}$  mg  $\text{kg}^{-1}$  (Sun et al. 1994). However, the total REEs concentrations in *P. americana* were much higher than in normal plants (Fig. 2), which is why it has potential for REE phyto-extraction (Yuan et al. 2017). In addition, a significantly higher concentration and rate of accumulation of REEs in roots in comparison to the shoots suggests that REEs have limited translocation in *P. americana* (Fig. 2). This may be explained by the fact that the root was in direct contact with the REEs in the nutrient solution, and the binding of REEs to root surfaces (Quiquampoix et al. 1990), resulting in limited REE translocation to shoots (de Oliveira et al. 2015). REEs also have high affinities to carboxylic groups and can be bound in the apoplast (cell walls), which can serve as a storage pool for polyvalent cations (Marschner 1995).

The concentrations of total REEs in roots and shoots increased with increasing REE concentrations under  $<100$   $\mu\text{M}$  REEs, but decreased for higher concentrations of REEs in solution ( $>100$   $\mu\text{M}$  REEs) (Fig. 2). The

same trend has been observed in biomass of *P. americana* (Fig. 1), which implies that low concentrations of REEs can stimulate plant growth, but that high REEs treatment has an adverse effect on plant biomass production. This kind of typical biphasic concentration-response relationship has been reported in rice (Liu et al. 2012b), tobacco (Chen et al. 2001) and soybean (de Oliveira et al. 2015) under various treatments with La. The growth stimulation may be explained in part due to the inducement of cell proliferation (de Oliveira et al. 2015), as well as due to the increase in the efficiency of photosystem II photo chemistry and electron transport rate by REEs (Wang et al. 2006). However, the damage to plant growth in higher REEs treatments results in the inhibition of the mitotic index (Qin et al. 2015; d'Aquino et al. 2009) and the inducement of DNA breakage and DNA-protein crosslinks (DPCs) (Wang et al. 2012). The decrease in the uptake of major elements, such as Mg and P, under the high REE treatments (Table 1) may also be partly responsible for the reduction in biomass of *P. americana*. Magnesium is required for the synthesis of chlorophyll, whereas P is required for the formation of ATP. Therefore, the decrease of these two elements imply that the excess of REEs may disturb the photo-synthesis and metabolic processes in the plant.

### Conclusions

In this paper, the basic REE uptake and translocation properties and fractionation patterns have been studied. The biomass and REE concentrations in hydroponically grown plant were stimulated by low REE concentrations but were decreased by high REE concentrations, which is a typical biphasic concentration-response. Significant HREE enrichment in the REE fractionation was induced by the translocation from the stems to the leaves indicating a strong HREE translocation ability in shoots of *P. americana*. The ratio of LREE/HREE decreased by exogenous  $\text{Ca}^{2+}$  treatment but increased by Al treatment, implying that LREEs are more likely to enter into the plant through  $\text{Ca}^{2+}$  ion channel but HREEs are more likely to share the same pathways with  $\text{Al}^{3+}$ . In the xylem sap of *P. americana*, the combination of citrate with REEs shows a significant positive correlation with the REE fractionation patterns, which suggests citrate plays an important role in REE translocation process. Therefore, the insights in the REE accumulation and translocation in *P. americana* in this study contribute to better understanding plant REE uptake mechanisms, information that may ultimately assist in developing REE phyto-extraction.

### Acknowledgements

This work was supported by National Natural Science Foundation of China (NSFC) under Grant No. 41225004, No. 41771343 and No. 41371315; Science and Technology Planning Project of Guangdong Province under Grant No. 2016A020221013; Fundamental Research Funds for the Central Universities under Grant No. 15lgjc36 and Science and Technology Transfer Program of SYSU. A. van der Ent is the recipient of a Discovery Early Career Researcher Award (DE160100429) from the Australian Research Council. The International Joint Lab ECOLAND was acknowledged for support of this work.

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## FIGURES AND TABLES

**Fig. 1** Root and shoot biomass of *Phytolacca americana* with mixed REEs (La to Lu) (0, 1, 10, 50, 100, 250  $\mu\text{M}$  with total concentrations) (means  $\pm$  SD, n = 3). Values with the same letter in the same organ of *P. americana* are not significantly different within different REEs concentrations ( $p < 0.05$ ).

**Fig. 2** REE concentrations in 30000 roots, stems and leaves of *Phytolacca americana* with increasing concentrations of mixed REEs (La to Lu) (0, 1, 10, 50, 100, 250  $\mu\text{M}$  with total concentrations) (means  $\pm$  SD, n = 3). Values with the same letter in the same organ of *P. americana* are not significantly different within different REEs concentrations ( $p < 0.05$ ).

**Fig. 3** REE concentration distribution patterns in roots (a), stems (b) and leaves (c) of *Phytolacca americana* (means  $\pm$  SD, n = 3) (the linkage of data points does not indicate dependence).

**Fig. 4** REE fractionation patterns from solution to roots ( $[\text{REE}]_{\text{root}} / [\text{REE}]_{\text{solution}}$ ) (a), from roots to stems ( $[\text{REE}]_{\text{stem}} / [\text{REE}]_{\text{root}}$ ) (b) and from stems to leaves ( $[\text{REE}]_{\text{leaf}} / [\text{REE}]_{\text{stem}}$ ) (c) (means  $\pm$  SD, n = 3) and ratios of  $\Sigma\text{LREE} / \Sigma\text{HREE}$  in organs and fractionation processes in *Phytolacca americana* (d) (mean values). Black dash line is the standard value of LREE/HREE (mass ratio of LREEs to HREEs, each element under equal molar concentration), higher than standard value means LREE enrichment and vice versa (d).

**Fig. 5** The effects of Verapamil to the total REEs concentrations (a) and to the REE concentration distribution patterns in root (b), stem (c) and leaf (d) of *Phytolacca americana*. Values with star in the same organ of *Phytolacca americana* are significantly different within the corresponding controls (a) ( $p < 0.05$ ).

**Fig. 6** The effects of Ca to the total REEs concentrations (a) and to the REE concentration distribution patterns in root (b), stem (c) and leaf (d) of *Phytolacca americana*. Values with the same letter in the same organs of plant are not significantly different within different Ca concentrations (a) ( $p < 0.05$ ).

**Fig. 7** The effects of Al to the total REEs concentrations (a) and to the REE concentration distribution patterns in root (b), stem (c) and leaf (d) of *Phytolacca americana*. Values with the same letter in the same organs of plant are not significantly different within different Al concentrations (a) ( $p < 0.05$ ).

**Fig. 8** Concentrations of REEs in the xylem sap of *Phytolacca americana*.

**Table 1** Nutrient accumulation in root and shoots of *Phytolacca americana* exposed to increasing concentrations of REEs (Mean values  $\pm$  SD, n = 3).

**Table 2** Concentrations of organic acids in xylem sap of *Phytolacca americana* (Mean values  $\pm$  SD, n = 3).

**Table 3** Percentage of organic acids ligated with REEs in the xylem sap of *Phytolacca americana* (%).

FIGURE 1

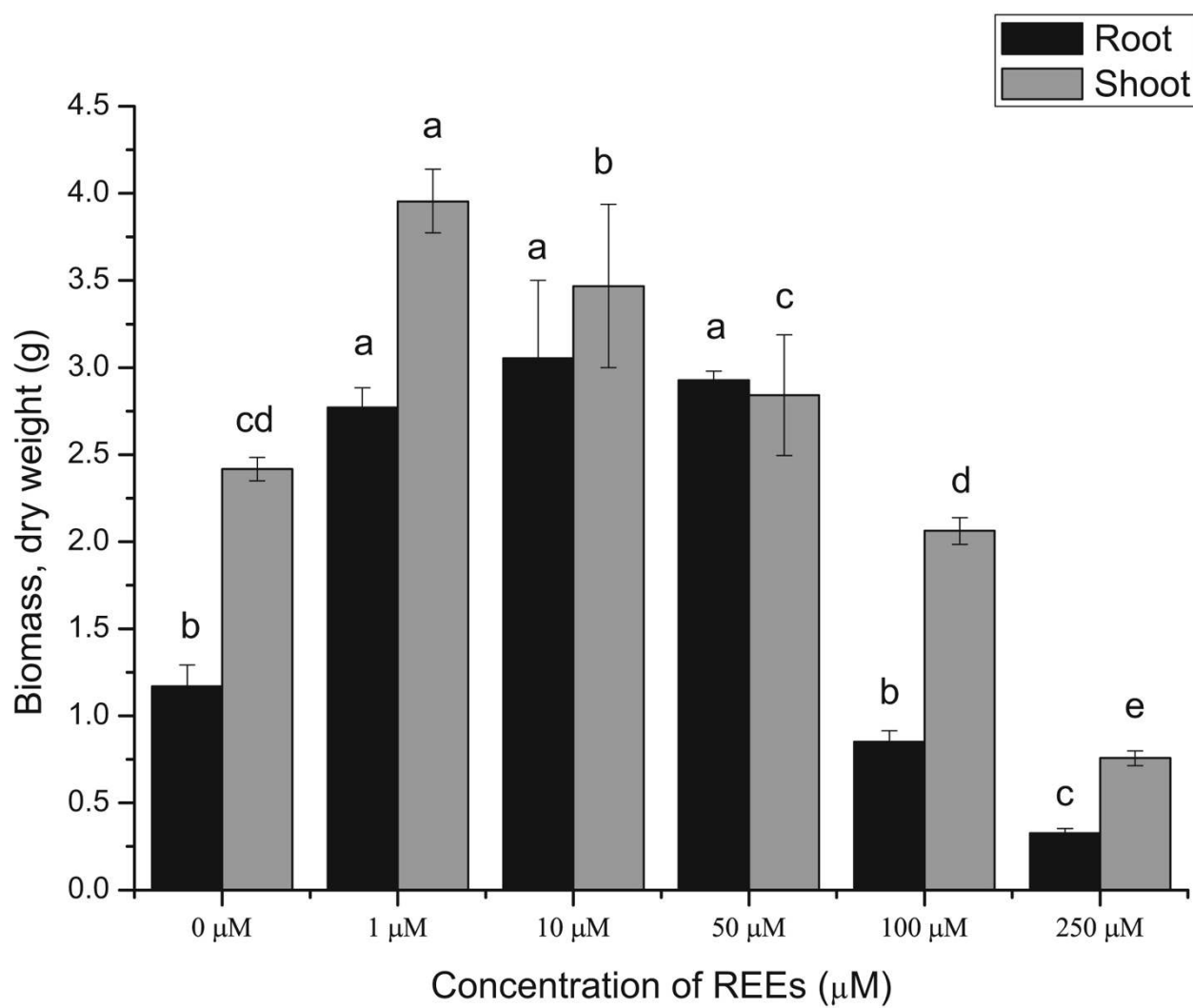


FIGURE 2

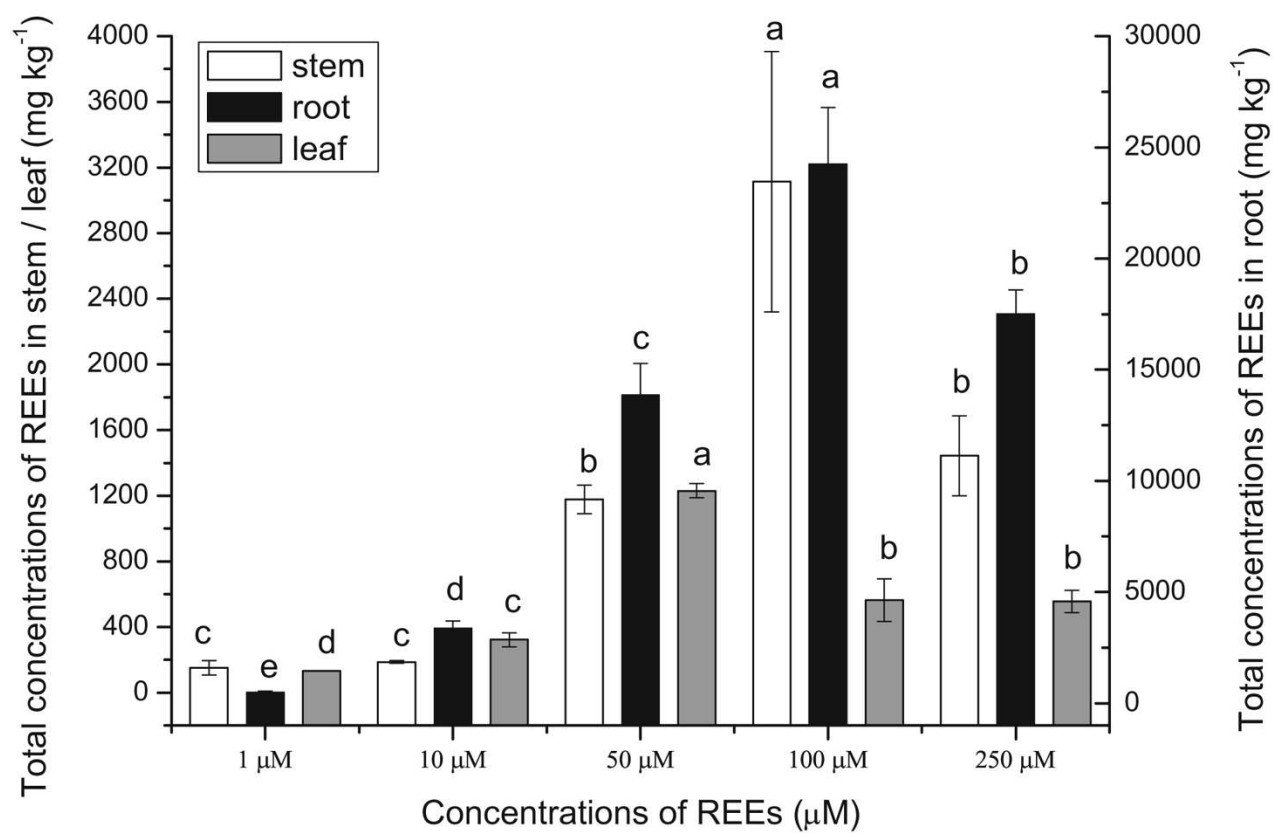


FIGURE 3

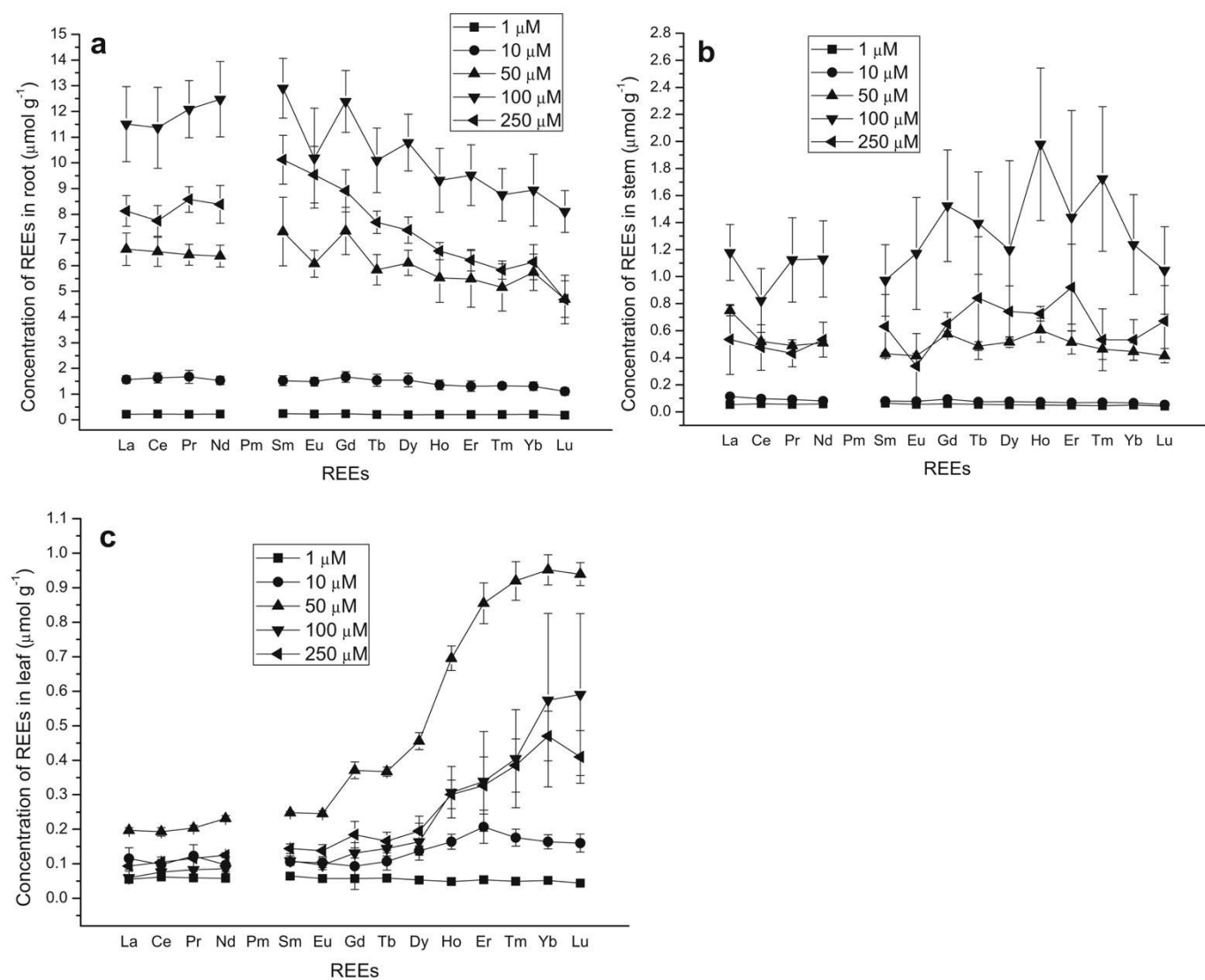
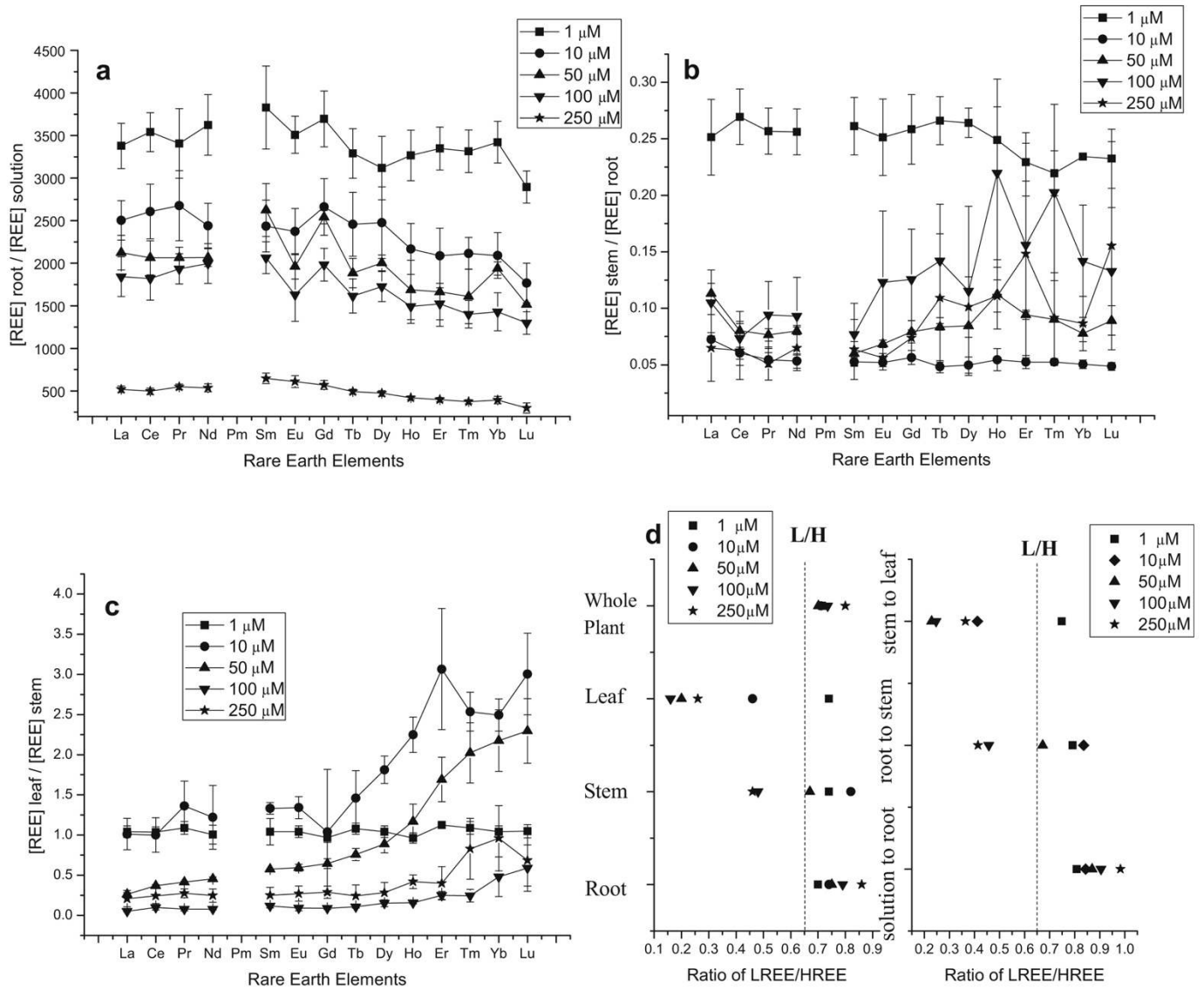
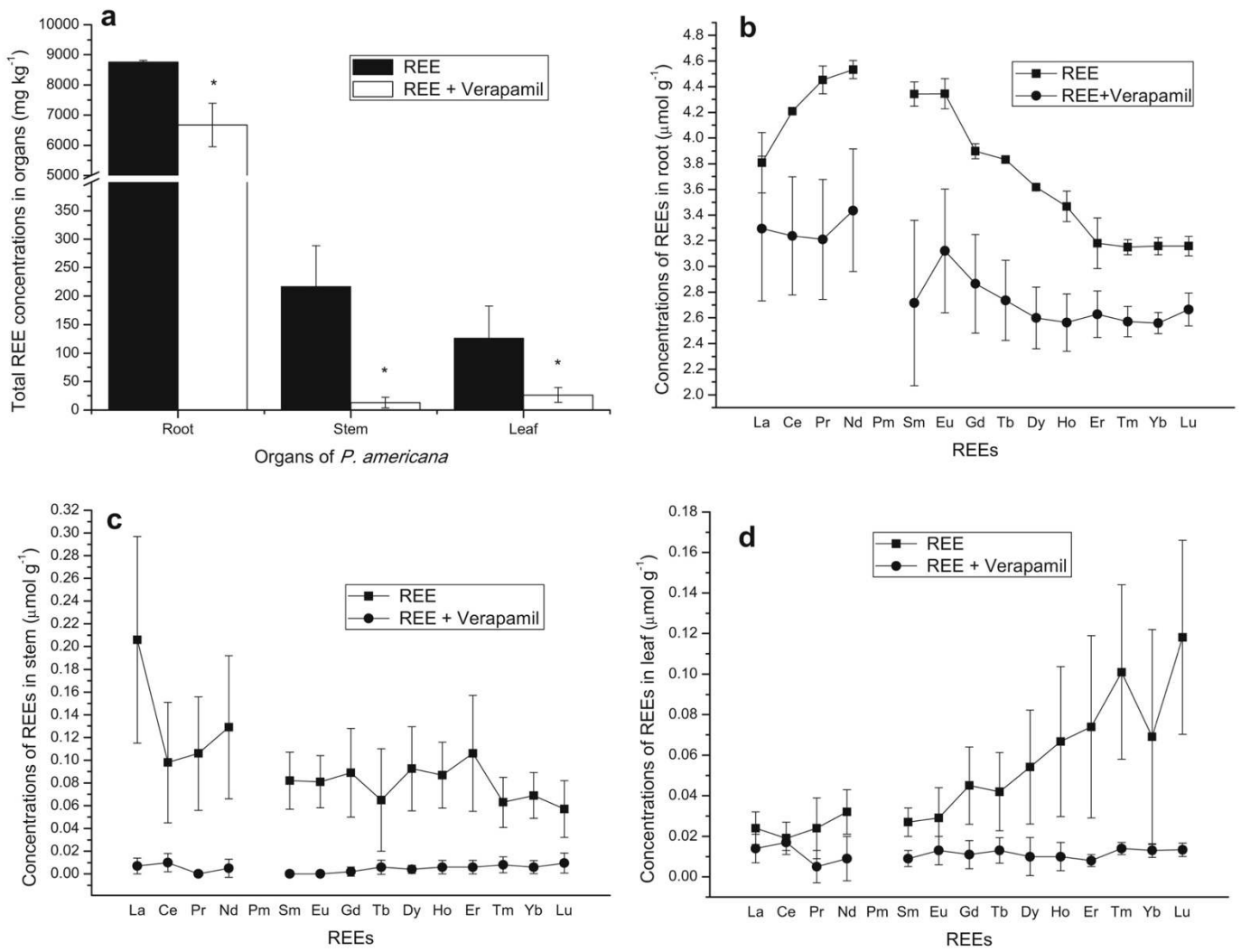


FIGURE 4



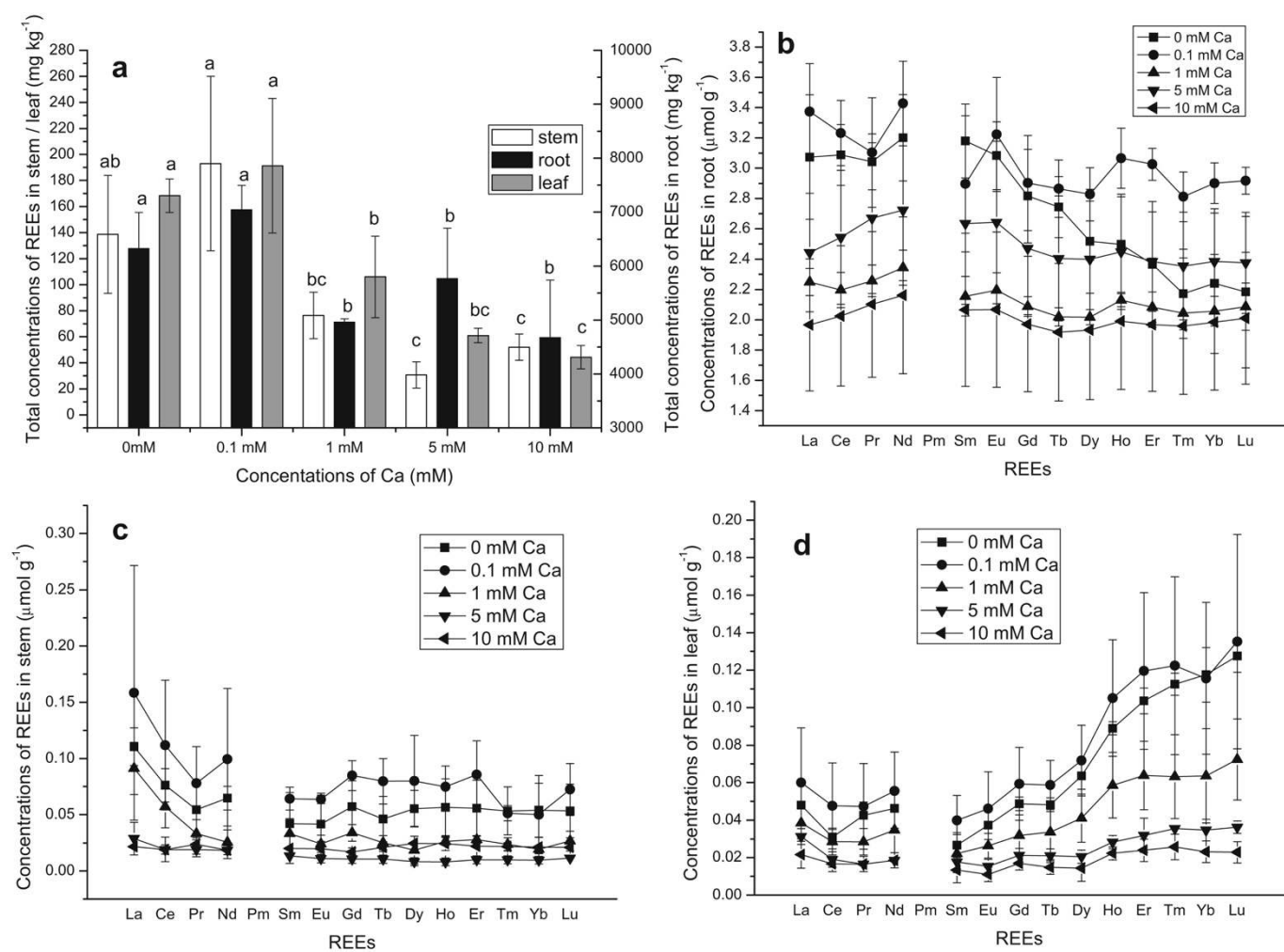
ACCEPTED

FIGURE 5



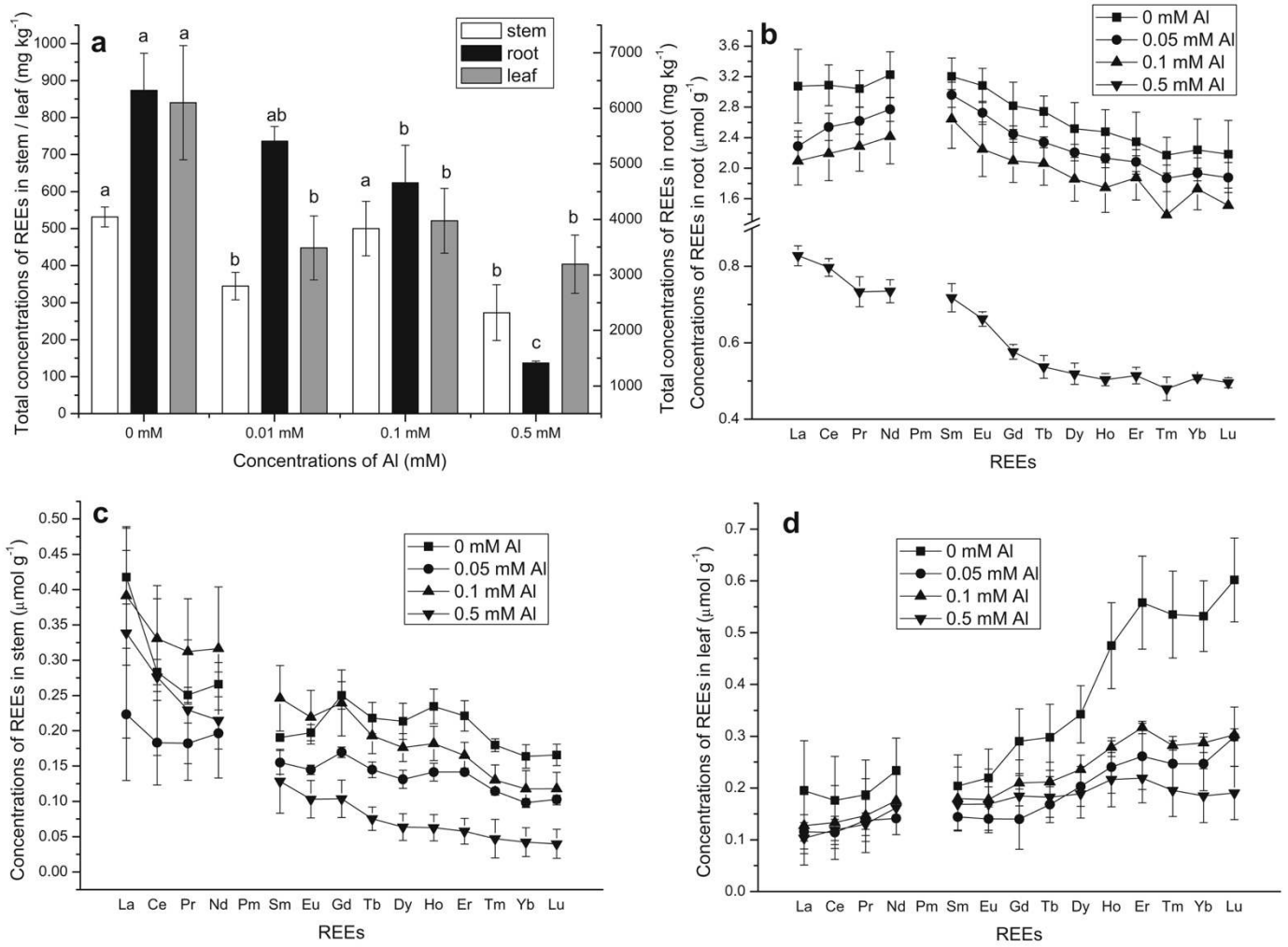
ACCEPTED

FIGURE 6



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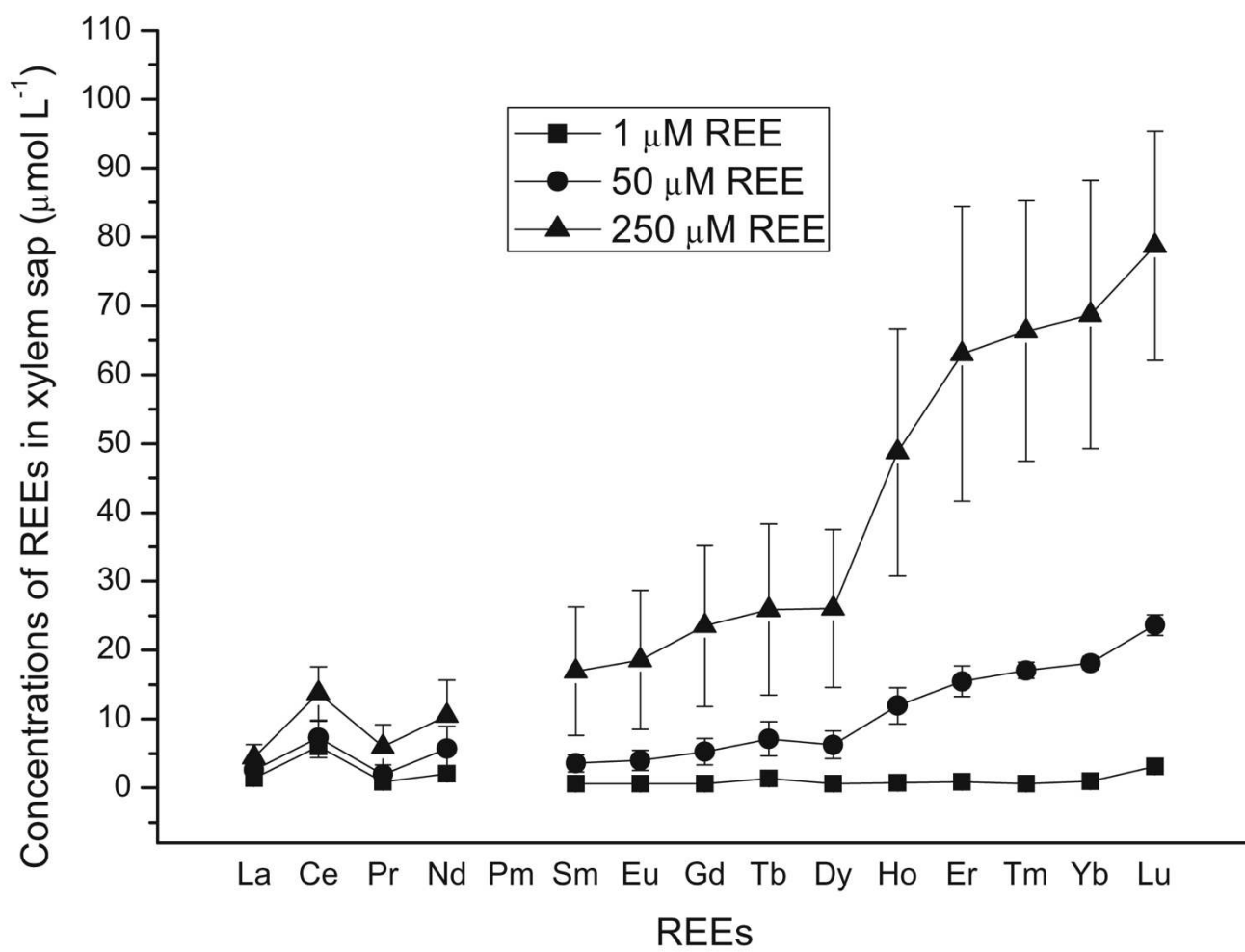
**FIGURE 7**



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FIGURE 8



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**TABLE 1**

Nutrients mg g <sup>-1</sup>	Nutrient accumulation in roots for each REE concentration ( $\mu M$ )					
	0	1	10	50	100	250
Ca	13.4 ± 2.3 <sup>a</sup>	11.6 ± 0.5 <sup>a</sup>	8.7 ± 0.9 <sup>b</sup>	6.5 ± 0.1 <sup>bc</sup>	6.3 ± 0.9 <sup>bc</sup>	4.5 ± 2.0 <sup>c</sup>
Mg	13.3 ± 1.8 <sup>a</sup>	9.4 ± 0.9 <sup>b</sup>	13.4 ± 3.4 <sup>a</sup>	9.9 ± 2.2 <sup>ab</sup>	5.0 ± 1.9 <sup>c</sup>	2.4 ± 1.1 <sup>c</sup>
Fe	0.41 ± 0.02 <sup>b</sup>	0.57 ± 0.06 <sup>b</sup>	0.39 ± 0.04 <sup>b</sup>	0.42 ± 0.02 <sup>b</sup>	1.01 ± 0.03 <sup>a</sup>	0.97 ± 0.33 <sup>a</sup>
Zn	0.49 ± 0.16 <sup>a</sup>	0.53 ± 0.18 <sup>a</sup>	0.40 ± 0.12 <sup>a</sup>	0.34 ± 0.21 <sup>a</sup>	0.39 ± 0.15 <sup>a</sup>	0.32 ± 0.08 <sup>a</sup>
P	3.2 ± 0.7 <sup>c</sup>	3.3 ± 0.5 <sup>c</sup>	3.4 ± 0.4 <sup>c</sup>	4.3 ± 1.1 <sup>bc</sup>	9.3 ± 4.2 <sup>a</sup>	8.3 ± 4.0 <sup>ab</sup>
Mn	0.055 ± 0.031 <sup>c</sup>	0.065 ± 0.029 <sup>bc</sup>	0.082 ± 0.028 <sup>ab</sup>	0.094 ± 0.011 <sup>a</sup>	0.069 ± 0.01 <sup>b</sup>	0.067 ± 0.007 <sup>b</sup>
Cu	0.017 ± 0.012 <sup>a</sup>	0.018 ± 0.009 <sup>a</sup>	0.019 ± 0.006 <sup>a</sup>	0.019 ± 0.008 <sup>a</sup>	0.021 ± 0.007 <sup>a</sup>	0.017 ± 0.008 <sup>a</sup>
K	52.6 ± 15.1 <sup>a</sup>	40.6 ± 15.0 <sup>a</sup>	51.1 ± 3.8 <sup>a</sup>	50.3 ± 11.9 <sup>a</sup>	30.8 ± 9.1 <sup>a</sup>	29.3 ± 6.6 <sup>a</sup>
S	2.2 ± 0.3 <sup>ab</sup>	2.1 ± 0.1 <sup>ab</sup>	1.6 ± 0.3 <sup>b</sup>	1.9 ± 0.1 <sup>ab</sup>	3.0 ± 1.1 <sup>a</sup>	2.4 ± 0.8 <sup>ab</sup>
Nutrients mg g <sup>-1</sup>	Nutrient accumulation in shoots for each REE concentration ( $\mu M$ )					
	0	1	10	50	100	250
Ca	24.4 ± 7.1 <sup>a</sup>	22.1 ± 1.8 <sup>a</sup>	23.8 ± 0.6 <sup>a</sup>	19.7 ± 3.2 <sup>ab</sup>	8.7 ± 1.2 <sup>c</sup>	13.4 ± 3.7 <sup>bc</sup>
Mg	14.0 ± 3.6 <sup>a</sup>	12.8 ± 1.9 <sup>ab</sup>	13.7 ± 2.8 <sup>a</sup>	11.2 ± 2.2 <sup>ab</sup>	6.4 ± 3.7 <sup>b</sup>	7.8 ± 5.4 <sup>ab</sup>
Fe	0.10 ± 0.03 <sup>a</sup>	0.11 ± 0.03 <sup>a</sup>	0.10 ± 0.04 <sup>a</sup>	0.10 ± 0.04 <sup>a</sup>	0.08 ± 0.01 <sup>a</sup>	0.10 ± 0.03 <sup>a</sup>
Zn	0.14 ± 0.02 <sup>a</sup>	0.12 ± 0.01 <sup>a</sup>	0.11 ± 0.01 <sup>a</sup>	0.13 ± 0.05 <sup>a</sup>	0.05 ± 0.05 <sup>a</sup>	0.12 ± 0.03 <sup>a</sup>
P	2.4 ± 0.7 <sup>a</sup>	1.7 ± 0.3 <sup>ab</sup>	1.7 ± 0.4 <sup>ab</sup>	0.9 ± 0.2 <sup>c</sup>	0.7 ± 0.1 <sup>c</sup>	1.4 ± 0.4 <sup>bc</sup>
Mn	0.134 ± 0.048 <sup>b</sup>	0.134 ± 0.041 <sup>b</sup>	0.165 ± 0.069 <sup>a</sup>	0.129 ± 0.010 <sup>bc</sup>	0.075 ± 0.069 <sup>cd</sup>	0.087 ± 0.004 <sup>d</sup>
Cu	0.005 ± 0.005 <sup>a</sup>	0.009 ± 0.004 <sup>a</sup>	0.010 ± 0.003 <sup>a</sup>	0.008 ± 0.001 <sup>a</sup>	0.007 ± 0.001 <sup>a</sup>	0.005 ± 0.002 <sup>a</sup>
K	68.2 ± 10.7 <sup>a</sup>	57.3 ± 8.5 <sup>a</sup>	62.8 ± 19.5 <sup>a</sup>	53.6 ± 20.5 <sup>a</sup>	45.4 ± 27.1 <sup>a</sup>	45.2 ± 19.6 <sup>a</sup>
S	1.9 ± 0.2 <sup>a</sup>	1.5 ± 0.2 <sup>ab</sup>	1.7 ± 0.3 <sup>ab</sup>	1.5 ± 0.3 <sup>ab</sup>	1.2 ± 0.4 <sup>b</sup>	1.2 ± 0.2 <sup>b</sup>

**TABLE 2**

REEs	Organic Acids ( $\mu M$ )						
	Lactate	Acetate	Formate	Oxalate	Citrate	Malate	Tartrate
1 $\mu M$	81.4 $\pm$ 0.4	26.8 $\pm$ 2.5	12.3 $\pm$ 1.5	36.8 $\pm$ 2.7	45.6 $\pm$ 0.8	n.d.	n.d.
50 $\mu M$	64.5 $\pm$ 9.3	15.0 $\pm$ 6.0	13.1 $\pm$ 1.7	19.0 $\pm$ 6.9	142 $\pm$ 35	n.d.	n.d.
250 $\mu M$	139 $\pm$ 56	30.1 $\pm$ 8.6	16.6 $\pm$ 0.6	252 $\pm$ 20	248 $\pm$ 30	n.d.	n.d.

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TABLE 3

Percentage of organic acids bind with REEs or in other species (%)

Free organic acids	La	Ce	Pr	Nd	Sm	Eu	Gd	Tb	Dy	Ho	Er	Tm	Yb	Lu	Other species
Oxalic acid	0.248	0	0	0	0	2.03	2.21	4.33	0	0	0	11.3	8.21	6.54	64.5
Formic acid	0	0	0	0.017	0.029	0.041	0.052	0.037	0.058	0.087	0.096	0.047	0.046	0.051	0.326
Acetic acid	0.038	0	0.054	0.095	0.206	0.162	0.178	0.167	0.155	0.243	0.272	0.254	0.191	0.355	0.435
Lactic acid	0.16	1.18	0.195	0.606	1.10	0.989	1.56	1.46	2.57	4.37	8.51	3.69	6.53	6.72	4.18
Citric acid	0	0.658	0	1.45	2.48	2.99	3.62	2.73	4.95	9.91	12.2	8.86	11.4	15.6	19.0