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An investigation of cellular distribution of manganese in hyperaccumlator plant *Phytolacca acinosa* Roxb. using SRXRF analysis

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Abstract: *Phytolacca acinosa* Roxb. (*P. acinosa*) is a recently discovered manganese hyperaccumulator plant from southern China. It is a good candidate for phytoremediation of manganese(Mn) polluted soil for its high biomass and fast growth. Knowledge of the tissue localization and identification of heavy metals can provide essential information on metal toxicity and bioaccumulation mechanisms. Synchrotron radiation X-ray fluorescence spectroscopy (SRXRF) microprobe was used in this study to investigate the cellular distributions of Mn and other elements in root, stem, leaf, petiole and midrib of *P. acinosa*. The highest Mn content was found in the vascular tissues of root, stem, petiole and midrib. Cortex in root played a key role in Mn absorption and Mn was limited in the vascular bundle during the process of transportation in stem. Moreover, Mn content in leaf epidermis was higher than that in mesophyll, which suggested that the sequestration of Mn in leaf epidermis might be one of the detoxification mechanisms of *P. acinosa*. The significance of other elemental (such as P, S, K, Ca, Fe, Zn and Cu) distribution patterns and the correlation with Mn were also discussed.

Keywords: hyperaccumulator; *Phytolacca acinosa* Roxb.; manganese; element distribution; SRXRF

Introduction

Manganese (Mn) is an essential nutrient to all plants, but it is a toxicant when in excess. Mn is a common metal in the earth crust and its presence in soils mainly results from the parent material. However, the intensive human activities at the present have led to the increase of Mn content availability in many soils. Mine tailings and metal smelters, chemical and industry, long-term heavy application of sewage-sludge (biosolids) or other organic amendments to soils all lead to an increase in the content or availability of Mn in soil, then the subsequent effects of Mn on plant will appear (Wong *et al.*, 1983; Zheljazkov and Nielsen, 1996; Ramachandran and D'Souza, 1997).

Metal-contaminated soils are notoriously hard to remediate. The possibility of using specific plants which hyperaccumulate metals to selectively remove and recycle excessive soil metals was introduced by Chaney (1983). Phytoremediation has been defined by Salt *et al.*(1995) as an emerging technology using specially selected and engineered metal-accumulating plants for environmental clean-up. However, the prerequisite for phytoremediation is developing studies on the hyperaccumulaor plant (Chen *et al.*, 2003).

Phytolacca acinosa Roxb(*P. acinosa*) is a recently discovered Mn hyperaccumulator plant from southern China, the Mn content in leaves of *P. acinosa* is very high, 19300 mg/kg dw was found during field investigation, and in greenhouse hydro-cultured with 12000 (mol/L of Mn, it can reach 36380 mg/kg (Xue *et al.*, 2004), while in normal plants only 20—500 mg/kg (Reeves and Baker, 2000). *P. acinosa* is a prospective plant in phytoremediation for its fast growth, substantial biomass, broad geographic distribution and ecological amplitude (Xue *et al.*, 2004; Xue *et al.*, 2005).

Studies on uptake and accumulation of Mn by hyperaccumulator plants and various aspects of the biology of *P. acinosa* have been reported (Memon, 1980; Memon and Yatazawa, 1982, 1984; Bidwell *et al.*, 2002; Xue *et al.*, 2004), but little is known about elemental distribution in *P. acinosa* tissues. Knowledge of the tissue localization and identification of heavy metals can provide essential information on metal toxicity and bioaccumulation mechanisms. Deleterious amounts of metals can also be translocated and stored in certain cell organelles, where metabolic activities do not take place (Verkleij and Schat, 1990). For example, Zn and As has been found to be accumulated in leaf epidermis of *Thlaspi caerulescens* and *Pteris vittata*, respectively (Küpper *et al.*, 1999; Lombi *et al.*, 2002a).

The element location and distribution in plants have been studied by using EDXS (Küpper *et al.*, 2000; Monni *et al.*, 2002; Wójcik *et al.*, 2005), PIXE (Krämer *et al.*, 1997; Ager *et al.*, 2002) or other microanalytical techniques (Liu and Kottke, 2004). Compared with these microanalytical techniques mentioned above, synchrotron radiation X-ray fluorescence spectroscope (SRXRF) has higher detective sensitivity and less destruction to sample, SRXRF can also measure multi-elements rapidly and simultaneously (Sánchez *et al.*, 2000; Chen *et al.*, 2003; Shi *et al.*, 2004), therefore SRXRF can be used to analyze the distribution of some elements, usually such elements have lower content in plants. The present

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study is a further example of the application of SRXFS microprobe and is the first detailed investigation of the distribution of major and trace elements within *P. acinosa* tissues. The specific objective of this study was to investigate elements localization in the tissues of *P. acinosa* by using SRXFS and obtain more information about possible absorption and tolerance mechanisms of *P. acinosa*.

1 Materials and methods

1.1 Sample preparation

Seeds of *P. acinosa* were collected from the tailings wasteland at the Xiangtan manganese mine areas at Xiangtan City, Hunan Province, China (Xue *et al.*, 2004). Seeds were soaked in distilled water overnight, and then germinated in a plastic basin filled with sand. After germination, the seedlings were grown in a controlled environment room with a 16-h, 25℃ light and an 8-h, 20℃ dark regime, and 60%—70% relative humidity. Mn treatments were performed by addition of $MnCl_2$ to Hoagland medium (Hoagland and Arnon, 1950) to 2000 μmol/L final concentration. This is due to that *P. acinosa* can grow normally without Mn toxicity symptom at 2000 μmol/L Mn, and at the same time plant tissue has a certain amount of Mn content which help to SRXRF detection. The solutions were aerated for 24 h a day, and adjusted daily to pH 4.5 with 0.1 mol/L NaOH or 0.1 mol/L HCl. The solution was renewed every 3 d during the culture.

After a 3-week cultivating, the plants were harvested and washed with tap-water, followed by 3 times rinses with deionized water, then root, stem, leaf tissues were sampled. Plant samples were quickly frozen at -20℃, and embedded in frozen deionized water. Slices (20 μm in thickness) of root, stem, leaf, petiole and midrib were cut with a cryotome (HM505E, MICROM Co.) at an ambient temperature of -20℃ and subsequently were attached to polyethylene film of sample holder, and air dried at -20℃ for SRXRF scanning.

1.2 SRXRF measurements

X-ray fluorescence (XRF) analysis of plant slices was performed at the XRF microprobe station of Beijing Synchrotron Radiation Facility (BSRF). The electron energy in the storage ring is 2.2 GeV, with a current range from 60 to 120 mA. The size of exciting X-ray beam was 10 × 10 μm^2. XRF spectra were collected by PGT Si (Li) solid detector, positioned at 90° to the beam line, 7 mm from the target. The apparatus of SRXRF in BSRF were reported in detail elsewhere by Huang *et al.* (2001). The scanning points of the samples were selected and observed by a microscope (Fig.1), 3 replicated slices of each tissue were performed. Spectra data were processed by AXIL program to integrate the area of elements

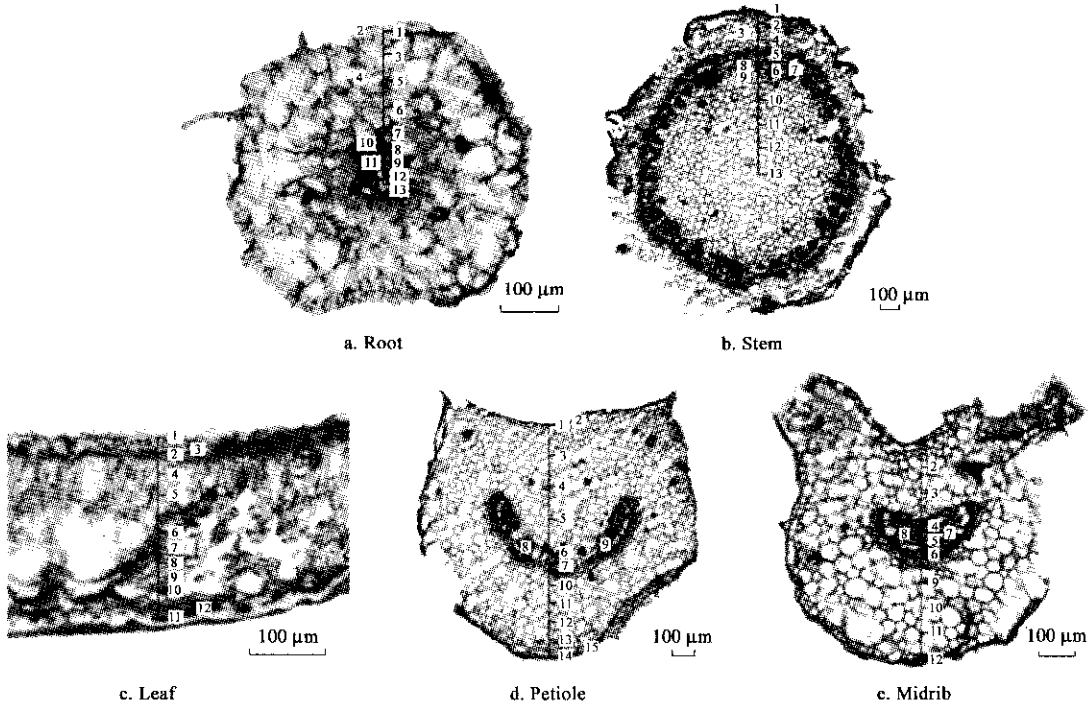

Fig.1 Anatomical structure of *P. acinosa* tissues and SRXRF scanning points

The SRXRF scanning points in root section include epidermis (1—2), cortex (3—6), endoderm (7, 13), and vascular bundle (8—12); The scanning points in stem section include epidermis (1), cortex (2—4), phloem (5—6), xylem (7—9), and pith (10—13); The scanning points in leaf section include upper (adaxial) epidermis (1—3), palisade tissue (4—5), sponge tissue (6—10), and lower (abaxial) epidermis (11—12); The scanning points in petiole section include adaxial epidermis (1—2), cortex near axis (3—5), vascular bundle (6—9), cortex away axis (10—13), and abaxial epidermis (14—15); The scanning points in midrib section include adaxial epidermis (1), cortex near axis (2—3), vascular bundle (4—8), cortex away axis (9—11), and abaxial epidermis (12)

excited peak. Relative contents of elements were calculated by calibrating the peak area with electron current, followed by normalization with Compton scattering intensity.

2 Results and discussion

2.1 Mn microdistribution in plant tissue

Fig.2 is a typical SRXRF spectrum of *P. acinosa* slice. The characteristic peaks of element such as P, S, Cl, K, Ca, Mn, Fe, Cu, Zn, and Br were checked by SRXRF. The "counts" in the ordinate (logarithmic coordinates) of Fig.2 means fluorescence counts of SRXRF and stands for the relative concentration of different elements at a single scan point. There has no comparability between different elements at the same scan point due to that different elements may have different signal sensitivitity. However the fluorescence counts of the same element obtained by SRXRF at different scan points (e.g. different locations in the root slice of *P. acinosa*) were comparable.

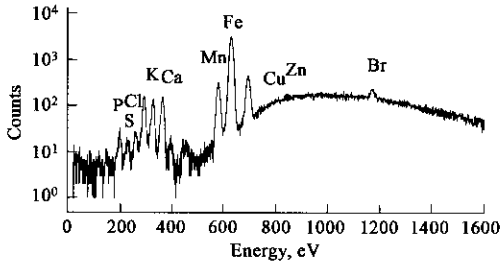

Fig.2 Typical SRXRF spectrum for a single scan point of *P. acinosa* slice

From Figs.3 and 4 it could be inferred that the element distribution in cross-section of the root, stem, leaf, petiole and midrib of *P. acinosa* varied with different kinds of elements or tissues. The SRXRF point measurements were carried out in epidermis, cortex, endoderm and vascular bundle of the root cross-section (Fig.1a and Fig.3a). The Mn content decreased from epidermis to cortex, and increased from cortex to vascular bundle via endoderm. The highest Mn content was found in vascular bundle. However, cortex in cross section of root has the least Mn, where the Mn content was about 1/4 of that in epidermis and 1/6 of that in vascular bundle. The Mn content increased from cortex to vascular bundle, indicating that the translocation of Mn from cortex to vascular bundle was a process of "from lower concentration to higher concentration", which showed some similarities with an active transportation.

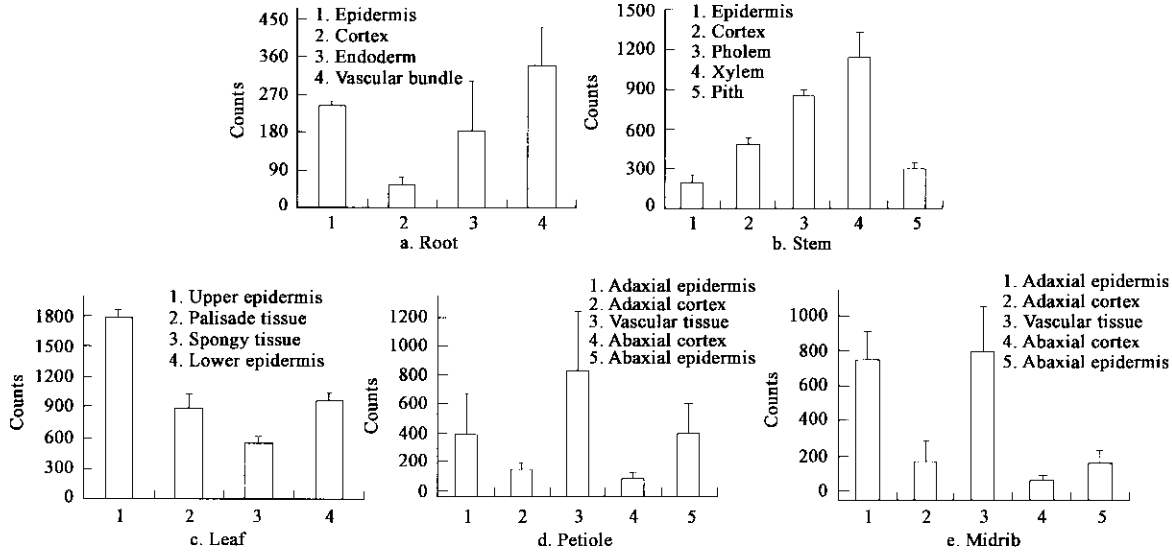

Fig.3 Relative content of Mn in *P. acinosa* tissues at cellular level
Values are means of three replicated slices, bar represents standard error of the mean

Considering the distribution of Mn in the whole cross section of root, two different Mn transportation processes can be found in different locations of the root, and meantime cortex acted as the division of these two processes, which suggested that cortex might act as a key role in Mn transportation in root. The epidermis also has a higher Mn content, which means that epidermis can intercept and capture some Mn, but once Mn get to cortex, it mainly transport by active process.

In the case of the stem cross-section (Fig.1b and Fig.3b), the Mn content in the vascular tissue was the highest, then the cortex and pith, and the lowest was in the epidermis, which demonstrated that Mn was mainly restricted in vascular tissues, especially in xylem tissue during the up-translocation process, only very limited Mn transported to pith, cortex and epidermis. In other study, it was found some Cu present in xylem tissue, through efflux or via the endodermal structural gap, allowing translocation to other plant organs (MacFarlane and Burchett, 2000).

The *P. acinosa* leaf consists mainly of mesophyll

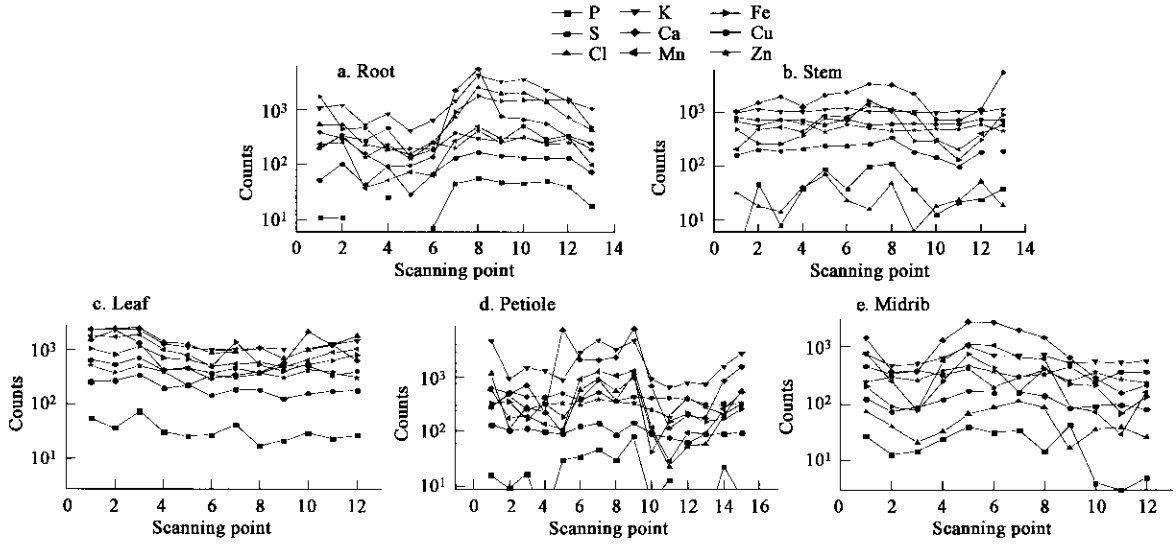

Fig.4 Relative element content in tissue cross-section of *P. acinosa* measured by SRXRF

and epidermis in its cross section (Fig.1c). The relative Mn content in epidermis was higher than that in mesophyll, and for the epidermis itself, the upper (adaxial) epidermis has higher Mn content than the lower (abaxial) epidermis (Fig.3c). The Mn content decreased from palisade tissue to spongy tissue in mesophyll. The cellular compartmentation of Zn and As in the leaf epidermis was found in *Thlaspi caerulescens* and *Pteris vittata*, respectively, and the sequestration of metals in epidermis of leaf was considered one of the detoxification and tolerance mechanisms (Küpper *et al.*, 1999; Lombi *et al.*, 2002a). However, more Cd and Zn were found in mesophyll than those in epidermis in hyperaccumulator *Arabidopsis halleri* (Küpper *et al.*, 2000). All these studies indicated that the cellular compartmentation of heavy metals in leaf epidermis was not a universal detoxification mechanism for all the plants. But the sequestration of Mn in leaf epidermis was one of the detoxification mechanisms in hyperaccumulator *P. acinosa.*

Vascular bundle, cortex and epidermis in the cross section of petiole ranked from stele to epidermis with a radial direction (Fig.1d). However, adaxial-abaxial differentiation existed in the petiole tissue, vascular bundle located in the central region of petiole and shaped as a capital "V", which was very different from the central symmetry in stem. The highest Mn content was found in vascular tissue, then in the epidermis, the lowest in the cortex. Mn amounts were twice higher in the adaxial cortex than in the abaxial cortex, whereas the Mn contents in adaxial epidermis is almost equal to that in abaxial epidermis (Fig.3d).

Adaxia-abaxial differentiation also exists in the midrib of the *P. acinosa* leaf (Fig.1e) and the changes of Mn contents in midrib cross section have the similar trend with cross section of the petiole. The highest Mn contents in midrib cross section were also found in the vascular bundle, but the Mn content in epidermis is higher than that in cortex, and the Mn contents in adaxial cortex and adaxial epidermis were more than twice and four times than those in the corresponding abaxial tissues (Fig.3e). When the anatomical differences between adaxial and abaxial cortex were considered, the adaxial cortex and epidermis have higher Mn content than those in abaxial cortex and epidermis, it might be due to that the adaxial tissue was close to the mesophyll tissue on both sides of midrib, so that the transportation of Mn to adaxial tissue in midrib led to the accumulation of Mn in leaf.

2.2 Element microdistribution and correlations between Mn and other elements

The Mn, Cu, Fe, Zn, K, Ca, P, S and Cl contents in the cross-section of the root, stem, leaf, petiole and midrib measured by SRXRF were presented in Fig.2 and Fig.4, which showed that SRXRF is an ideal technique for localizing the sites of metal accumulation in plants. There is significant difference among the microdistribution of different kinds of element in different tissues of *P. acinosa* (Fig.4). Cu, Fe, Zn, K, Ca, P, S and Cl in vascular bundle of the root all have the highest content levels, then in the epidermis, and the lowest was in the cortex. Such trends were very similar to the distribution of Mn in root tissue (Fig. 4a).

In the stem cross-section, the distribution of K, Cu and Zn were relatively homogeneously, whereas P, S, Ca and Fe have the similar distribution trend with Mn, namely the highest content levels were found in vascular tissue, then in cortex and pith, and the lowest were in the epidermis of stem (Fig.4b).

The content levels of Cu, Zn, K, Ca, P, S and Cl in epidermis of *P. acinosa* leaf were also the highest, then in the mesophyll. Moreover, the content levels of these elements in adaxial epidermis and palisade

tissue were higher than those in abaxial epidermis and spongy tissue, respectively (Fig.4c). The distribution of Fe has the similar distribution except at point 8. Distributions of these elements showed the similarities with Mn.

The Cu and Zn contents were relatively uniform, whereas the other elements, such as P, S, Cl, K and Fe, varied greatly in cross section of petiole. The highest levels were in vascular tissue, then in epidermis, the lowest in cortex (Fig.4d). In the cross section of midrib (Fig.4e), all the elements except Cu, have the similar distribution with Mn.

There was a significant correlation between Mn and P, S distributed in the cross-sections of root, stem, leaf, petiole and midrib (Table 1), which suggested P and S probably play an important role in Mn absorption and accumulation of *P. acinosa*. P is a constituent of nucleic acids and ATP, while S is an important component of amino acids, proteins (Marschner, 1995) and heavy metal binding phytochelatins (Marschner, 1995; Keltjens and van Beusichem, 1998; Clemens, 2001). The complexes of Cu, Cd and Ag with metallothioneins (MTs) and phytochela-tins (PCs) have already been identified in some plants (Cobbett, 2000), but the proteins of MTs and PCs combining Mn have not been reported. Mn is mainly in ionic form as it forms unstable complexes with organic ligands (Marschner, 1995). As a cofactor for different enzymes, Mn was abundant in the cytoplasm of plants (Burnell, 1988).

K is very mobile and mainly occurs in the symplasm, the cytosolic K content being usually relatively constant (Hsiao and Läuchli, 1986; Marschner, 1995). Most K present in the epidermis of leaf and vascular bundle of root, petiole and midrib, while the K distribution in the stem tissue of *P. acinosa* was homogeneous. Stress conditions to which material was submitted could be responsible for alterations in K^+ channels activity probably inducing the inward-rectifier channels present in plants (Zimmerman *et al.*, 1998; Grabov and Blatt, 1999). In *E. nigrum*, K occurred mainly in the cytoplasm, but the amounts varied due to the site or tissue (Monni *et al.*, 2002).

Ca is mainly bound to structural material, and should be most abundant in cell walls (Kirkby and Pilbeam, 1984), while the amount of Ca in the cytoplasm is usually very low (Marschner, 1995). Ca concentration must be a general physiological response of the plant against metal toxicity (Ouzounidou *et al.*, 1992), and Ca content in plant tissues of maize has a significant reduction (Ouzounidou *et al.*, 1995). The significant reduction of Ca content caused by Mn was also found in our other study (data not shown). However, in present study the significant correlation between Ca and Mn showed that they probably have very similar absorption and accumulation.

Several studies suggested that zinc transporter (ZIP1, ZIP2, ZIP3), iron transporter1 (ITR1) and related inducible transporters may transport potentially toxic metals, such as Zn (II), Fe (II), Mn (II), Co (II), Cd (II), and/or Cu (II), as well as nutrients (Meagher, 2000; Lombi *et al.*, 2002b). Significant correlation between Mn and Fe, Zn was also found in this study, but Mn and Cu were only correlated by each other in leaf (Table 1). All these findings suggested that heavy metals such as Mn, Fe and Zn probably be transported with a broad substrate range by the same transporters.

Table 1 Correlation coefficient between manganese and other elements distributed in the cross-section of root, stem, leaf, petiole and midrib of *P. acinosa*

	P	S	Cl	K	Ca	Fe	Cu	Zn
Root (n=13[a])	0.798**	0.842**	0.853**	0.855**	0.726**	0.849**	0.303	0.725**
Stem (n=13)	0.842**	0.809**	0.039	0.203	0.577*	0.789**	-0.501	-0.44
Leaf (n=12)	0.767**	0.885**	0.982**	0.987**	0.581*	0.496	0.729**	0.66*
Petiole (n=15)	0.805**	0.721**	0.84**	0.917**	0.456	0.84**	0.025	0.283
Midrib (n=12)	0.598*	0.905**	0.745**	0.887**	0.968**	0.783**	-0.163	0.627*

Notes: a. The number of scanning point; * means the correlation is significant when $p<0.05$; ** means the correlation is significant when $p<0.01$

Considering that Mn usually exists in a cation form in plant, but it appeared positive correlation with some cations, such as Fe, Zn, Ca and K, which showed that they have similar absorption and/or accumulation mechanism; Mn also positively correlate with anion, such as P, S and Cl, which indicates that some ionic balance function might operate in *P. acinosa*, however, further study is necessary for proving this conclusion.

3 Conclusions

The elements such as P, S, Cl, K, Ca, Mn, Fe, Cu, Zn in root, stem, leaf, petiole and midrib cross-section of *P. acinosa* can be checked by SRXRF microprobe. SRXRF is a valuable technique to study the element's distribution for its higher detective sensitivity, less destruction to samples, measure multi-elements rapidly and simultaneously.

There was a trend that Mn in *P. acinosa* be transported from cortex tissue to vascular tissue in root, and kept in vascular during transportation in stem, petiole and midrib. Cortex played a key role in