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BRIEF COMMUNICATION

# Spatial distribution and speciation of copper in root tips of cucumber revealed by μ-XRF and μ-XANES

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## Abstract

The localization, biotransformation, and chemical speciation of copper in root tips of cucumber (*Cucumis sativus*) were investigated using synchrotron-based micro X-ray fluorescence (μ-XRF) and micro X-ray absorption near edge structure (μ-XANES). The highest content of Cu was found in a root cap and meristematic zone whereas low Cu content in elongation and maturation zones. There was a dramatic increase of Cu content in a root cap and meristematic zone after treatment with 100 μM $CuSO_4$ for 72 h. The μ-XANES analysis revealed that most Cu in a root tip was bound with alginate, citrate, and cysteine-like ligands whereas rarely deposited in form of CuO. From a root cap to maturation zone, the proportion of Cu bound with alginate-like ligands increased whereas that bound with citrate-like ligands decreased. The proportion of Cu bound with cysteine-like ligands increased from a root cap to elongation zone but sharply declined in maturation zone. The results suggest that Cu was chelated by S ligands in the cell walls which protect protoplasm against possible damage caused by Cu excess.

*Additional key words*: *Cucumis sativus*, metallothioneins, phytochelatins, X-ray fluorescence.

Heavy metal pollution especially in the mine area is one of the major environmental problems. Pb, Cu, Hg, Cd, and Cr mainly account for the pollution (Bai *et al.* 2011). Although copper is an essential nutrient element for plants and plays an important role in photosynthesis and cell metabolism, excessive uptake of Cu may bring about great risks to plants and through the food chain to animal and human health (Lin *et al.* 1994). For example, the reduction in growth and fresh mass of roots was observed in plants exposed to Cu, *e.g.*, recently in *Aechmea blanchetiana* (Giampaoli *et al.* 2012). Thus, researchers begin to pay attention to the mechanisms of Cu accumulation in plants and Cu tolerance (Clemens 2001, 2006, Hall 2002). It has been previously reported that metals are more accumulated in roots than in shoots, and much Cu in root tissues is accumulated in cell walls (Nishizono *et al.* 1987). Excess Cu can be detoxified by chelating to organic acids or proteins (Lee *et al.* 1978, Cobbett 2000). Besides, Cu can be bound to amino acids, such as asparagine, histidine, and glutamine (Liao *et al.* 2000). In some plants, Cu tolerance is related to amount of metallothioneins (MTs) and phytochelatins (PCs) (Murphy *et al.* 1997, Cobbett 2000). However, most of these studies are focused on tolerant plants and hyperaccumulators (Verbruggen *et al.* 2009, Kramer 2010) and crops and vegetables have been referred less often.

Plant roots play essential roles in heavy metal uptake, accumulation and detoxification. The root tip, which is the most vigorous part of plant root, is also most sensitive to heavy metals (Pahlsson 1989, Kahle 1993). Structures and

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*Abbreviations*: EXAFS - extended X-ray absorption fine structure; MTs - metallothioneins; PCs - phytochelatins; SRXRF - synchrotron radiation X-ray fluorescence; SSRF - ShanghHai synchrotron radiation facility; μ-XRF - micro X-ray fluorescence; μ-XANES - micro X-ray absorption near edge structure; XAS - X-ray absorption spectroscopy.
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physiological functions of cells largely differ in different root tip growth zones (root cap, meristematic zone, elongation zone, and maturation zone). Therefore, accumulation of heavy metals and detoxification mechanisms of cells in different zones of the root tip may also be different. Clarifying the absorption and transformation mechanisms of heavy metal in different zones is critically important for modeling and understanding its dynamics (Tao *et al.* 2004). Thus, it is necessary to elucidate the typical spatial distribution and molecular speciation of Cu in different zones of the root tip.

In recent years, significant improvement have achieved in the utilities of synchrotron-based techniques such as synchrotron radiation X-ray fluorescence (SRXRF) and X-ray absorption spectroscopy (XAS) for biological samples, *e.g.*, for localization of elements including heavy metals or metalloids (Pickering *et al.* 2003, Fukuda *et al.* 2008, Tian *et al.* 2010). Synchrotron techniques are sensitive but injurious to cells and excellently characteristic in high brightness, collimation, polarization, and low bremsstrahlung. SRXRF has been extensively applied for quantitative elemental analysis with minimal sample preparation (Kang *et al.* 2002). The distributions of Cu in the leaf epidermis and cross-section of the stem of *Elsholtzia splendens* has been successfully analyzed by micro X-ray fluorescence (μ-XRF; Shi *et al.* 2004) whereas little information is available on the Cu localization in root tips. XAS consists of two complementary techniques: X-ray absorption near edge structure (XANES) and extended X-ray absorption fine structure (EXAFS) (Barrett *et al.* 2010). Both methods have been used to determine the chemical speciation of metals or metalloids in many fields (Kang *et al.* 2002). Moreover, XAS has been applied to investigate Pb, Zn, and Cu speciation in soil and plant samples (Sarret *et al.* 2001, Tao *et al.* 2004). Recently, the third-generation synchrotron radiation facility allows much smaller X-ray spot that could detect micro-regions and even nano-regions. For example, μ-XRF imaging and μ-XAFS is able to study the accumulation mechanism of cadmium in rice (Yamaoka *et al.* 2010). Therefore, the spatial distribution and speciation of Cu in different zones of the root tip are studied here using μ-XRF imaging and μ-XANES.

A cucumber (*Cucumis sativus* L.) cv. Jinlv No. 1 (*Tianjin Lvfeng Co.*, Tianjin, China) was used in this study. After soaked in distilled water overnight, the seeds were germinated in darkness at 25 °C for 3 d. The 1/4 Rorison solution, which was composed of 1.0 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 0.5 mM $K_2HPO_4$, 23 μM $H_3BO_3$, 4.5 μM $MnSO_4$, 0.5 μM $Na_2MoO_4$, 0.8 μM $CuSO_4$, 27 μM Fe(III)-EDTA, and 0.5 μM $ZnSO_4$ (pH 6.0), was used for cultivation. Nutrient solutions were renewed every 3 d. After 7 d, the uniform seedlings were transferred to 1/2 Rorison solution without (control) or with 100 μM $CuSO_4$ for 72 h. All plants were grown in a growth chamber with a 16-h photoperiod, irradiance of 400 μmol $m^{-2}$ $s^{-1}$, day/night temperatures of 25/20 °C, and relative humidity of 60 - 70 %.

Straight roots were cut after 72 h exposure to 100 μM Cu and were surface rinsed. Approximately 12 mm long root tips and 10 mm long root hairs were cut off and pasted on a *3M tape* in liquid nitrogen. The samples were freeze-dried under -40 °C for 3 d, and then stored at -75 °C. Relative concentrations of elements in root tips were determined directly by SRXRF microprobe.

Elemental distribution was determined at beamline *15 U1* of the *Shanghai Synchrotron Radiation Facility* (SSRF) with the energies ranging from 5 to 20 keV. The storage ring operated at 3.5 GeV with the currents ranging from 210 to 190 mA. 10-keV incident energy of X-rays was monochromatized with a *Si[III]* monochromator and focused on a measured spot size of 3 × 3 $μm^2$ using Kirkpatrick-Baez mirror system. The scanning zone of the cucumber root is shown in Fig. 1. The selected areas were scanned in 10 μm steps. The fluorescence spectra of Cu, Fe, Mn, K, and Ca were collected for 5 s of dwell times by a liquid nitrogen cooled energy dispersive one-element Si drift detector. The signals were normalized with ionization chamber signals which were collected simultaneously. The image-processing was performed with *Matlab v. 6.5*.

Cu XANES in different sections of a cucumber root tip were also analyzed at 15 U1 beamline of SSRF with the storage ring containing 170 to 190 mA at 3.5 GeV. XANES data of samples were recorded using a Si(III) detector. A Cu foil internal reference was used for energy calibration with the first inflection point set at 8.979 keV. The samples were scanned at the step width of 0.0010 keV ranging from 8.95 to 9.06 keV and the scan time of each step was 5 s. Spectra of standard copper species including Cu, CuCl, $CuCl_2$, CuO, $Cu(Ac)_2$, CuS, $Cu_2S$, $Cu(NO_3)_2$, $Cu(OH)_2$, $Cu_3(PO_4)_2$, $CuSO_4$, Cu-citrate, Cu-oxalate, Cu-cysteine, Cu-histidine, and Cu-alginate were collected. Spectra of root samples and standard solution samples were recorded in fluorescence mode whereas those of solid samples were recorded in transmission mode.

The fitting and plotting package *WINXAS v. 3.1* was used for analysis (Ressler 1997). All of the spectra were normalized by fitting first- and second-order polynomial functions to the pre- and post-edge regions, respectively. Quantitative edge fitting analysis was conducted with the program *LISFIT XAFS* using principal component analysis (Paktunc *et al.* 2004). The nor XANES spectrum of the sample was fitted utilizing a least squares from the library of Cu reference compounds to obtain a linear combination of edge spectra. The best fitted results were derived by incrementally increasing the number of fit components and minimizing the fit residual. Fits were optimized by minimizing the residual of the fit which were defined as the normalized root square difference between the data and the fit. Range for the fit was varied as a function of data quality to examine the contributions from minor components and the fractional contribution was directly proportional to the percentage of Cu that was chelated by the respective compound in the samples.

Elemental distribution maps of Cu, Fe, Mn, Ca, and K in the scanned area of different sections of a cucumber root tip are presented in Fig. 2. For the control roots, the highest content of Cu was found in the root cap and the front of meristematic zone whereas much lower content was found in the elongation zone and maturation zone (Fig. 2*A*). Cu was evenly distributed in the elongation zone whereas in the maturation zone, it was mainly concentrated in the middle of a root with the vascular cylinder. Distribution patterns of Fe resembled those of Cu in the entire root and XRF intensities of Cu and Fe were positively correlated (correlation coefficient: $R^2 = 0.957$, $n = 4257$, $P < 0.0001$). K and Ca were also positively correlated ($R^2 = 0.962$, $P < 0.0001$). However, correlations between Cu and other elements (Mn, K, and Ca) were much lower ($R^2 = 0.382$, $R^2 = 0.175$, and $R^2 = 0.174$, $P < 0.0001$, respectively).

For the roots exposed to Cu for 72 h, the distributions of elements, especially Cu and Fe in the cucumber root had changed (Fig. 2*B*). Content of Cu in a root cap and meristematic zone increased sharply whereas no obvious accumulation was observed in the maturation and elongation zones. Content of Fe which rapidly decreased in root cap and meristematic zone was maximal in the maturation zone and minimal in the elongation zone. Obviously, correlation between the distribution patterns of Cu and Fe in the whole root was lower ($R^2 = 0.404$, $n = 4158$, $P < 0.0001$) but between Cu and other elements (Mn, K, and Ca) significantly increased up to $R^2 = 0.914$, $R^2 = 0.899$, and $R^2 = 0.937$ ($P < 0.0001$), respectively. Notably, K and Ca were still highly positively correlated ($R^2 = 0.846$, $P < 0.0001$).

XANES is able to provide basal information about Cu complexes in the small zones of plant tissues, thus it was employed to investigate Cu speciation in different sections of a cucumber root tip. Linear combination arithmetic was employed to determine the possible Cu speciation in various sections of root tips utilizing the *LSFITXAFS* program (Table 1). XANES analysis revealed that after Cu treatment for 72 h, most Cu in a root tip was bound with alginate, citrate, and cysteine-like ligands, and little Cu deposited in the form of CuO. The proportion of cysteine-Cu-like species gradually increased from the root cap to the elongation zone (from 17.7 to 28.7 %) but it sharply declined in the maturation zone (5.1 %). The proportion of Cu bound with alginate-like ligands increased from the root cap to the maturation zone (from 25.7 to 71.2 %) whereas that bound with citrate-like ligands decreased along the same direction (from 53.1 to 17.7 %). CuO-like species only accounted for a small proportion which also tended to increase from the root cap to the maturation zone (from 3.4 to 6.0 %).

The root cap and meristematic zone are the most active regions for the absorption of mineral elements (Walker *et al.* 2003). The root cap consists of loosely arranged cells with thin walls and mucosal outer walls. The mucus comprise highly hydrated polysaccharides and some organic acids which can not only protect the root tip from wear and tear but also adsorb and chelate certain minerals to facilitate their absorption by root. Therefore, the highest concentrations of mineral elements are found in the root caps under normal conditions (Fig. 2*A*) (Newman 2001, Walker *et al.* 2003). These results for Cu are in line with the known affinity between the cell wall and Cu (Kopittke *et al.* 2011). Meristematic zones also absorbed mineral

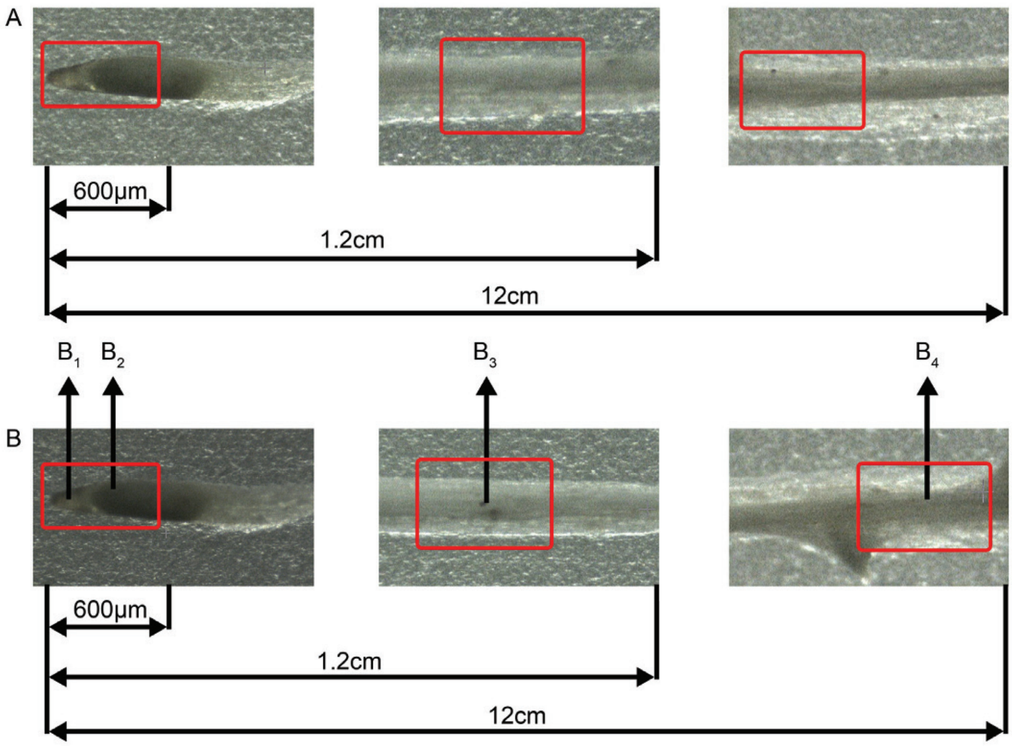


Fig. 1. The SRXRF scanning zones of the *C. sativus* root untreated (*A*) or treated (*B*) with 100 μM $CuSO_4$ for 72 h; the XANES test point in the root cap ($B_1$), meristematic zone ($B_2$), elongation zone ($B_3$), and maturation zone ($B_4$).

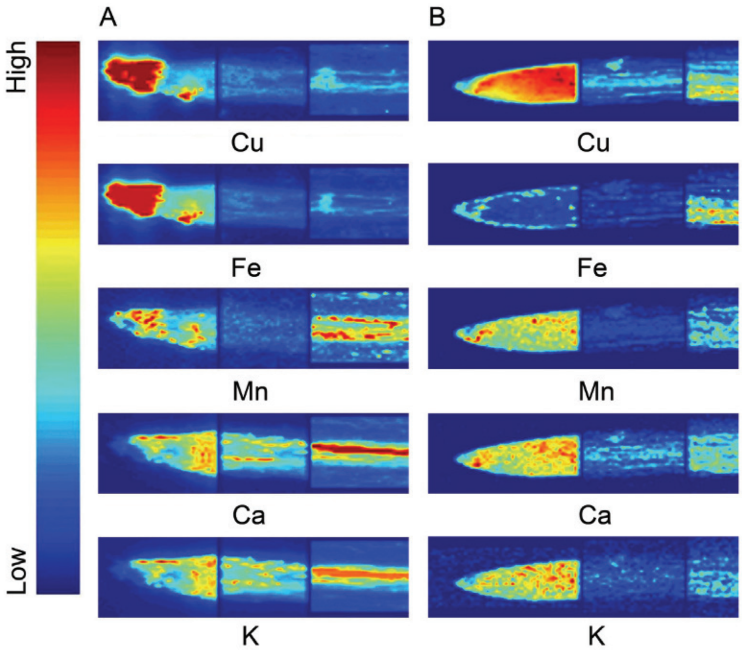


Fig. 2. The μ-XRF elemental maps for Cu, Fe, Mn, Ca, and K in different zones of the *C. sativus* root tip untreated (*A*) or treated (*B*) with 100 μM $CuSO_4$ for 72 h. The number of fluorescence yield counts was normalized by D0 (D0 represented the fluorescence counts of pre-ionization chamber) and the dwell time. The red colour depicting elemental content in each map was scaled to the maximum value for each map.

Table 1. Cu species [%] in different zones of the cucumber root tip after Cu treatment for 72 h identified by XANES.

| Zone | Cu-cysteine | Cu-alginate | Cu-citrate | CuO |
|---|---|---|---|---|
| Root cap | 17.7 | 25.7 | 53.1 | 3.4 |
| Meristematic | 19.8 | 44.8 | 31.6 | 3.9 |
| Elongation | 28.7 | 46.3 | 20.2 | 4.8 |
| Maturation | 5.1 | 71.2 | 17.7 | 6.0 |

elements sufficiently. These cells were small, tightly packed, and filled with high concentration of cytoplasm (Fig. 2). Cells elongated and cell volumes increased rapidly in the elongation zone, so this zone grew fastest in the whole root. Mineral elements accumulated at the speed corresponding to their absorption resulting in the lowest concentrations of Cu and other elements in this part (Fig. 2). Cells in the maturation zone ceased elongating. Some cells differentiated to form vessels that functioned in ion transport, thus mineral elements were likely to be accumu- lated in this zone. As a result, the concentrations of elements were relatively high (Fig. 2). Correlation between Cu and Fe in the whole root was lower after Cu treatment for 72 h. Cu and Fe competed during transport in the roots, so increased Cu absorption reduced the absorption of Fe similarly as was reported recently (Shi *et al.* 2011). In our study, correlation between Cu and Ca increased whereas the accumulation of Cu after Ca treatment declined in *Elodea canadensis* (Min *et al.* 2012). These suggest an antagonistic interaction between Cu and Ca due to their similar ionic form (Maksymiec and Baszyński 1998).

Furthermore, thiol (HS-) contained in cysteine could form complexes with Cu called phytochelatins (PCs). The procedure can significantly reduce the bioavailability of Cu thereby reducing the toxicity of Cu excess (Murphy *et al.* 1997). The region rich in cysteine occurred in the root tip and Cu-cysteine content increased from the root cap to the elongation zone whereas decreased in the mature region (Lee *et al.* 2003). After Cu exposure for 72 h, substantial quantity of Cu was bound with poly-galacturonic acid which is the skeleton component of pectin. Alginic acid is a kind of natural polysaccharide with multiple hydroxyl groups. These hydroxyl groups can easily combine with numerous divalent cations and reduce their mobility (Jeon *et al.* 2002). Because the structure of alginic acid is very similar to that of the cell wall, it could be used to model cell wall like ligands in this study. XANES analysis herein shows that high proportions of Cu-alginate appeared in all sections of the root tip. Cells in the root cap, meristematic zone, and elongation zone were mostly thin-walled. The thickness of cell walls gradually increased from the root cap and reached the maximum in the maturation zone. Therefore, the proportion of Cu-alginate like speciation increased from the root cap to the maturation zone (Table 1). These results are in accordance with the known affinity of Cu with a cell wall. In addition, citric acid is an important intermediate in Krebs cycle and the main component of root exudate. The reaction products of citric acid and Cu were highly mobile. Although CuO only accounted for a small proportion, its amount also tended to increase from the root cap to the maturation zone (Table 1). In summary, we speculated that

Cu was chelated by S ligands in cell walls of the growing zone. Cu was predominantly restricted to the cell wall in the mature region which primarily contributed to the accumulation and detoxification of Cu in the root tip.

In conclusion, this work demonstrates that in the cucumber, Cu mainly accumulated in the root cap and the meristematic zone. It largely retained in the cell walls of the mature region and SH-compounds may be involved in the Cu accumulation in the root tip.

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