Combining cryo-scanning electron microscopy, µX-ray fluorescence spectroscopy and X-ray absorption spectroscopy to probe copper speciation in a bamboo.

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Abstract
Copper uptake, localization and speciation were studied in hydroponically-grown *Phyllostachys fastuosa* plants at different increasing concentrations of Cu (0.2, 1.5 and 100 µM). The spatial distribution and speciation of Cu in tissues of the bamboos were investigated using cryo-scanning electron microscopy, coupled with an energy dispersive spectrometer (SEM-EDS), µX-ray fluorescence spectroscopy (µXRF) and X-ray absorption spectroscopy (XAS). Inhibitory effects of Cu on plant growth were observed only at higher Cu concentration. Copper concentration in roots and shoots increased with increasing Cu concentration in the growth solution. Copper accumulation followed the pattern roots>stems>leaves. Analysis by SEM and µXRF studies revealed the presence of Cu mainly in the epidermis of the root. A significant amount of Cu seems to be adsorbed and/or precipitated at the surface of the roots i.e. into apoplast material. Cu K Edge X-ray absorption near edge structure (XANES) showed that the Cu speciation depends on the part of the plant: in roots most Cu is divalent, whereas in stems and leaves Cu is monovalant.

Introduction
Water and organic wastes recycling is a critical environmental issue. In order to take up this scientific technologies and economic challenges, plantation for wastewater disposal are an interesting solution. Among various plants used by this technology (reed, willow...), bamboo is particularly relevant. Bamboo is a fast-growing plant whose rate of biomass generation is unsurpassed by any other plants (10-30 % annual increase in biomass versus 2-5 % for trees). It has a high absorption capacity of water and mineral elements, which allow the growth of bamboos plantations on very restricted areas compared to conventional agricultural wastes recycling. But some organic wastes contain high concentration of copper (Cu) and the information about Cu tolerance of bamboo is still very limited. Copper is an essential element for plant growth and development since it is a constituent of many enzymes and other proteins. However, high levels of Cu in soil can be phytotoxic, causing deleterious effects at the morphological and the physiological levels (Kabata-Pendias and Pendias, 2001).
To better understand the mechanisms employed by bamboo to uptake and protect against the presence of excess copper, an hydroponic study was carried out. The objectives of the present study were (1) to investigate the effect of Cu on the growth, (2) to determine the uptake of Cu, (3) to evaluate the localization of Cu using µX-ray fluorescence spectroscopy (µXRF) and scanning electron microscopy coupled to energy dispersive X-ray analysis (SEM-EDS), (4) to investigate the speciation of Cu in the tissues by using X-ray absorption spectroscopy.

Materials and Methods
Plant culture and treatments.
The bamboo *Phyllostachys fastuosa* was grown in a continuously aerated 1/4 Hoagland nutrient solution during 11 weeks. Plants were subjected to three free copper (i.e. Cu^{2+}) levels, 0.2 1.5 and 100 µM. Each treatment was run with 4 replicates. Nutrients solutions were renewed every 7 days. The number of leaves and stems was determined to evaluate the development of each bamboo. Samples were taken from several plant organs (roots, stems and leaves). For each sample, one fraction of the material was ground and pressed as 5 mm diameter pellet in liquid N_2 and the frozen pellet was stored until XAS measurements; a second fraction was freeze and kept intact for µXRF and SEM-EDS investigations, and the last fraction was dried at 60°C for chemical analysis.
XAS data collection
Copper K-edge X-ray absorption near edge structure (XANES) spectra were recorded at liquid helium temperature on FAME beamline (BM 30B, ESRF) in fluorescence mode using a 30 elements solid-state detector.
Micro X-ray fluorescence spectroscopy (µXRF)
The measurements were carried out on a HORIBA XGT-5000 microscope equipped with an Rh X-ray tube producing a finely focused and high-intensity beam with a 10-µm spot size. X-ray emission from the irradiated sample was detected with an energy dispersive X-ray spectrometer.
Cryo-SEM-EDS
Samples of roots were freeze-fractured in a cryo specimen chamber, sputter-coated with gold in argon athmosphere, and examined using a Philips XL30 SFEG STEM at nitrogen liquid temperature coupled with an EDS. Chemical micro-analysis results can be obtained with a spatial resolution of about 2 to 5 µm.

Results
Plant growth and accumulation of copper
The 1.5 µM Cu^{2+} treatment had no impact on the growth of bamboos whereas strong Cu phytotoxicity symptoms (chlorosis and 50% decrease of root elongation) were observed at lower concentration (0.6 µM) in wheat (Bravin et al., 2010). The 100 µM Cu^{2+} treatment led to a significant reduction in plant growth rate. In this treatment, copper accumulation follows the pattern root>stem>leaf with concentration of 3915 $mg.kg^{-1}$ dry weight (DW), 122 $mg.kg^{-1}$ DW and 35.2 $mg.kg^{-1}$ DW respectively. This

accumulation is important compared to the treatment 0.2 µM in which concentrations of root, stem and leaf are 14.7 mg.kg^{-1} DW, 3.7 mg.kg^{-1} DW and 2.4 mg.kg^{-1} DW respectively.

SEM-EDS and µXRF analysis

SEM is a powerful imaging technique because of its nanometer-scale spatial resolution and very large depth of field. Individual 'spot' analysis can be applied to compare concentrations in different cells. SEM imaging can be coupled with µXRF, which has a better detection limit for Cu, allowing 2D imaging of elemental distributions and semi-quantification along transect. The examination of root cross-sections showed that heavy deposition of Cu is mostly in the epidermis of the root (Fig.1). SEM-EDS analysis allows the Cu detection exclusively at the root surface (fig1.B). However Cu is also detected into the root, as we can see in the Cu profil intensity (Fig1.A), suggesting a symplastic pathway.

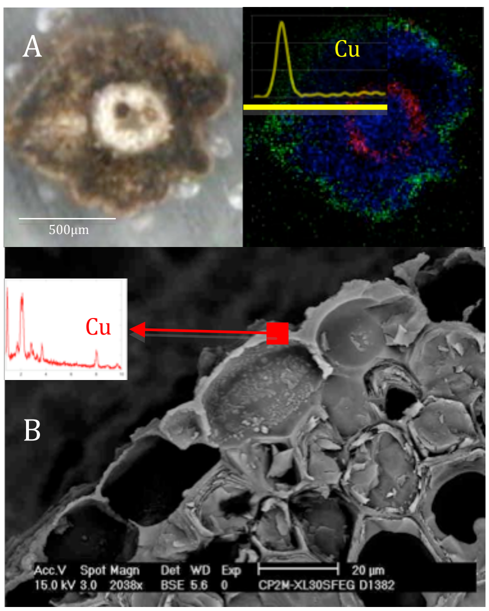

Fig.1. A. µXRF maps showing the distribution of Cu (green), K (blue), Si (red) in the root cross-section, with a 8x8 µm pixel size. The spectra in yellow represents the Cu intensity along the transect line indicated below. B. cryo-SEM image with an EDS spectra taken in the epidermis cells.

XANES spectra analysis

Figure 2 presents the normalized Cu K-edge XANES spectra of bamboo samples and two selected reference compounds: Cu(I)-acetate and Cu(II)-acetate. We can identify different inflections in the absorption edge. Feature A corresponds to the 1s➔4p transitions for Cu(I) compounds and feature B corresponds to 1s➔4p and 1s ➔continuum transitions for Cu(II) compounds. Therefore Cu in the leaves and in the stems was found as Cu(I) and Cu(II) oxidation states whereas in roots, Cu is mostly as Cu(II).

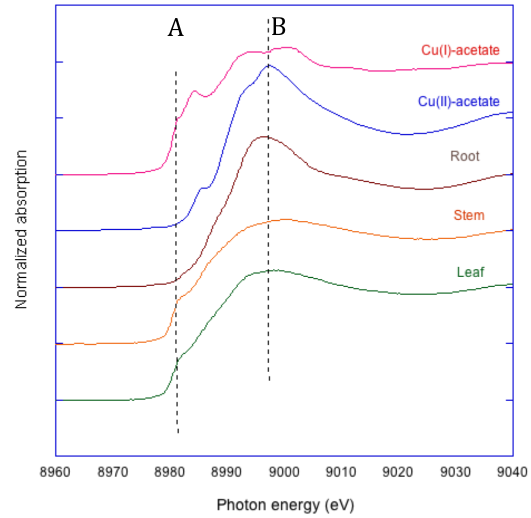

Fig. 2. Copper K-edge XANES spectra from Cu reference compounds (Cu(I)-Acetate and Cu(II)-Acetate) and *P. fastuosa* samples (root, stem, leaf) treated with 100 µM Cu for 11 weeks.

Discussion

A large amount of Cu is adsorbed and/or precipitated at the roots surface i.e in the root apoplast. The root apoplast is apparently a major barrier of Cu which limits the symplastic pathway of Cu and root-to-shoot translocation. In roots, Cu is only in oxidized form whereas in the aerial part it is in two oxidated states Cu(I) and Cu(II). The reduction of Cu(II) to Cu(I) was only described once for creosote bush. Polette et al, (2000) measured copper in leaves at both Cu(I) and Cu(II). However, in the Cu-tolerant plant E. Splendens, most copper in roots, stems and leaves exist as Cu(II) (Sahi et al., 2007; Shi et al., 2008). The oxidation state of Cu may reveal differences in mechanisms employed by the plant to tolerate high Cu concentration.

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