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Comparing analysis of elements sub-cellular distribution in *Kandelia obovata* between SEM-EDX and chemical extraction

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ABSTRACT

The mangrove plant *Kandelia obovata* Sheue, Liu & Yong sp. nov. can sequestrer and inherently tolerate high metal concentrations in polluted estuary wetlands. Sequential extraction and scanning electron microscopy coupled with energy-dispersive X-ray microanalysis (SEM-EDX) were used to determine the cellular distribution and chemical forms of Cu, Zn, Pb and Cd in this mangrove. Metals were largely confined to roots compared to the aerial parts. All plant tissues of *K. obovata* followed a gradient with the sequence: cell wall > intercellular space. The root epidermis provided a major barrier to the transport of metals. In roots of 50 mg L^{-1} Cd(II) treatment, SEM-EDX confirmed that the highest Cd concentration was found in epidermal cells. Cd was distributed mainly along the walls of epidermis, cortex, endodermis and some xylem parenchyma. Other metals showed a similar distribution trend. The sub-cellular distribution pattern of metals is probably involved in tolerance mechanisms of *K. obovata*.

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1. Introduction

Mangrove forests, a complex intertidal ecosystem distributed in the tropics and subtropics, appear to possess a remarkable capacity to retain heavy metals in their sediments (Tam and Wong, 1993; Zhou et al., 2010). The cycling of heavy metals, because of their toxicity, bio-accumulation capacity and persistence, is an important research subject in estuarine mangrove swamps (Agoramoorthy et al., 2008; Maanan, 2008). The processes of uptake, accumulation, distribution and detoxification have been studied in a wide range of mangrove species (Lacerda, 1998; Joshi et al., 2007; Cheng et al., 2010). Techniques like chemical extraction with analysis by inductively coupled plasma mass spectrometry (ICP-MS), scanning electron microscopy coupled with energy dispersive X-ray analysis (SEM-EDX) give the opportunity to study the internal distribution of the metals (MacFarlane and Burchett, 2000; Scott and Ritchie, 2009), and can help in our analysis of how cellular and sub-cellular distribution of metals is involved in the capacity of metal detoxification by mangrove plants.

The red mangrove plant *Kandelia obovata* Sheue, Liu & Yong sp. nov. is widely distributed in tropical and sub-tropical estuaries of Eastern Asia and commonly used for the revegetation of disturbed coastal wetlands (Sheue et al., 2003). *K. obovata* is considered a plant with high detoxification and phytoremediation potential and has been widely used to construct wetlands for the treatment of industrial wastewater containing heavy metals (MacFarlane et al., 2007; Rai, 2008). Therefore, much attention has recently been focused on the response of this plant to heavy metal stresses (Liu et al., 2009). Compared to the metals such as Cu, Zn and Pb accumulated in sediment Cd is well known to be toxic to plants (Shaw et al., 2004; Xu et al., 2011). As a non-essential element for living organisms, Cd has a high mobility in soil-plant systems, with propensity to adversely effect both human health and the functioning of mangrove ecosystems (Perronnet et al., 2000; Liu et al., 2007). We hypothesized from earlier work (MacFarlane and Burchett, 2000) that root cell walls would be major zone of accumulation and defuse line of the plant. In this work we studied how cellular distribution under polluted conditions deal with an excess of metal. The main objectives of this study were to assess metal distribution pattern in the *K. obovata* using chemical extraction methods and SEM-EDX.

2. Materials and methods

2.1. Sampling site description

The mangrove forest studied is located in the estuary of Jiulong River (24°24′ N, 117°55′ E), Fujian Province, Southeastern China.

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The area studied is about 233 ha, dominated by mature *K. obovata* community, and mixed with some other mangrove species (*Avicennia marina*, *Aepiceras corniculatum*). It is one of the best mangrove reserves in China. Metallic enrichment of this mangrove environments arises from urban and agricultural runoff, industrial effluents, boating and recreational use of water bodies, chemical spills, sewage treatment plants, leaching from domestic garbage dumps and mining operations (Zhang et al., 2007). The previous studies in this area have indicated that there is heavy metal pollution (Liu et al., 2007). The physical and chemical properties of the sediments among the roots of *K. obovata* were measured in the laboratory as follows: pH 6.63, moisture content 49.5%, total organic carbon (TOC) 2.1%, and total nitrogen amount 0.90 g kg^{-1} dry sediment, total phosphate amount 0.62 g kg^{-1} dry sediment and cation exchange capacity 15.8 cmol kg^{-1}, Cu 27.3 mg kg^{-1}, Cd 1.1 mg kg^{-1}, Pb 67.9 mg kg^{-1}, Zn 375.3 mg kg^{-1}, K 630 mg kg^{-1} and Fe 3.5 g kg^{-1}.

2.2. Sample collection and plant culture

Mangrove samples were collected in monotypic stands during a single low tide period. Leaves, stems and roots from *K. obovata* were collected within a 5 m × 5 m area. Collected plant samples were stored separately in polythene bags and transported to the laboratory for processing. Sediment in contact with the plant roots and rhizomes was collected simultaneously. Mature and healthy propagules of *K. obovata* collected from site were cultivated in sand irrigated by 0.5 strength Hoagland nutrient solution under greenhouse conditions. Nutrients were weekly added to the solution to replace those removed by the plants. The plants were grown under greenhouse conditions with natural illumination (from April 8, 2009 to October 8, 2009, and the average length of sunshine was 14.5 h) and a relative humidity of 85%, temperature ranged from 26 to 32 °C throughout the experiments.

After six months cultivation, Cd was added to the solution at a concentration of 50 mg kg^{-1} in the form of $CdCl_2$, in order to reach the detection limits for SEM-EDX microanalysis. This Cd concentration was chosen based upon preliminary experiments and earlier published literature (MacFarlane and Burchett, 2000) which had identified that this was the range in which plant root detected clear signals for SEM-EDX microanalysis. Cultivated plants without Cd supply were used for controls ($n = 3$). Greenhouse treatment groups including the control were arranged in a completely randomized design. Seven days after the start of metal treatment, the seedlings were harvested.

2.3. Procedure for chemical extraction

A sequential metal extraction scheme was conducted according to Sousa et al. (2008) (Table 1). Different plant organs (leaves, stems and roots; 1 g DW; $n = 3$) were homogenized and processed individually in a Soxhlet by successive extractions. Metals bound to pectic, polysaccharidic, ligninic and cellulosic fractions are those bound to the cell wall. The different types of proteins cannot be determined using this extraction method, which implies that an exact location in the cell cannot be defined. The metals bound to some amino acids, chlorophyll, low weight compounds (all extracted by ethanol) and those extracted in the aqueous fraction were designated as soluble metals (Farago and Pitt, 1977). Extract metal concentrations of mangrove samples were determined by ICP-MS (PerkinElmer Inc., Elan Drc-e, USA).

2.4. Procedure for SEM

After harvesting from the greenhouse, root samples were excised and immediately submerged in1% (w/v) Na_2S for 30 min to precipitate Cd. Samples were then fixed in 2.5% glutaraldehyde in 0.1 mmol L^{-1} phosphate buffer (pH 7.2) for 8 h. After dehydration in an ethanol series, tertiary butyl alcohol was used to exchange ethanol. Samples were then fractured transversely in liquid N_2 using a sterile knife in order to obtain new intact cryofractured surface. The cryofractured specimens were freeze-dried and coated with a thin layer of gold. The above samples were examined using a SEM (JSM-6390/LV, JEOL) combined with energy dispersive spectrometer (INCA, Oxford, Great Britain) with 10 or 20 kV accelerating voltage. A high accelerating voltage was chosen to minimize hydrated sample charging and to obtain a sufficient signal to noise ratio for X-ray line profiles (MacFarlane and Burchett, 2000).

2.5. Statistical analyses and calculations

Our experiments are performed in triplicate and the results presented were average values of the three replicates. Data were analyzed statistically using analysis of variance (ANOVA) and the Least Significant Difference (LSD) test was employed to determine the significance of the differences between the parameters. The Bonferroni correction was used to counteract the problem of multiple comparisons. The statistical package used was SPSS statistical software package (Version 11.0) and the confidence limit was 95%.

The translocation factor (TF) was calculated as the ratio of $metal_{leaves}/metal_{roots}$ and also by the ratio $metal_{stems}/metal_{roots}$, expressing the metal's translocation within the plant, from the roots to the leaves and the stems. The TF from sediment to roots was also calculated.

3. Results

3.1. Metals compartmentalization in tissues from sequential extraction

Total metal concentrations (sum of metals from all extracted fractions) from different tissues (roots, stems and leaves) of *K. obovata* show a common pattern: Zn > Pb > Cu > Cd, ranging between 210.21 μg g^{-1} DW of Zn in the roots to 0.84 μg g^{-1} DW of Cd in the leaves (Table 2). Roots present significantly higher metal concentrations than stems and leaves. Most metals were found to be located in the cell wall (sum of metals quantified in the pectic, polysaccharidic, ligninic and cellullosic fractions) of the leaves, stems and roots. Roots were found to accumulate 53% of Zn, 66% of Cu, 58% of Pb and 70% of Cd in the cell wall, 21% of Zn, 13% of Cu, 32% of Pb and 20% of Cd was found to be intracellularly (sum of the metal extracted with ethanol and demineralised water; average for all metals) and 26% of Zn, 21% of Cu, 10% of Pb and10% of Cd was retained in the proteic fraction. Hence, metals accumulated predominantly in cell walls of roots, stems and leaves of *K. obovata* (Fig. 1).

3.2. Metals accumulation and translocation

Concentrations of metals in roots, stems and leaves of *K. obovata* are shown in Fig. 2. The roots present significantly higher metal concentrations than the stems and the leaves, for all studied metals (Fig. 2). Zn, Cu, Pb and Cd were accumulated in root tissue in concentrations equal to or less than adjacent sediment concentrations (Table 3, TFs 0.34–0.97). Little translocation was observed, with metal concentrations up to an order of magnitude lower in leaf tissue. Translocation of Pb was minimal and the lowest of all metals, with concentrations of accumulated Pb in leaf tissue only being 26% that of roots (Table 3).

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