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Does ammonium nitrogen affect accumulation, subcellular distribution and chemical forms of cadmium in *Kandelia obovata*?

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ABSTRACT

Heavy metals and nutrients are commonly found in mangrove sediments, but the effect of nutrients on heavy metals in mangrove plants is not clear. A study quantifying the effects of ammonium nitrogen (NH_4^+-N) on the accumulation, subcellular distribution and chemical forms of cadmium (Cd) in *Kandelia obovata* seedlings were conducted. The experiment consisted of four levels of NH_4^+-N (0, 10, 50 and 100 mg L^{-1}) in each of which consisted of four Cd levels (0, 1, 5 and 10 mg L^{-1}). The results showed that NH_4^+-N magnified the Cd toxicity due to reduced plant biomass, especially with 10 mg L^{-1} Cd and 100 mg L^{-1} NH_4^+-N supply. NH_4^+-N, especially at 100 mg L^{-1}, enhanced the concentration and accumulation of Cd in root but its role on Cd translocation from root to stem and leaf was limited, probably due to low translocation factor. At subcellular level, Cd mainly accumulated in root cell wall but its fractionation depended on Cd levels. Under the stress of 1 and 5 mg L^{-1} Cd, 50 mg L^{-1} NH_4^+-N supply improved transfer of Cd from root cell wall into cell, and increased pectate and protein integrated forms of intracellular Cd to alleviate Cd toxicity. Under the stress of 10 mg L^{-1} Cd, NH_4^+-N supply promoted the deposition of Cd on root cell wall to restrain its transfer to root cell, which was verified by the reduced levels of pectate and protein integrated forms of Cd in root cell. Thus, NH_4^+-N supply improved immobilization of Cd in roots and alleviated Cd toxicity through integration with pectate and protein as well as cell wall combinations in root of *K. obovata*.

1. Introduction

Mangroves are diverse communities distributed along tropical and subtropical coastal lines, and they play vital roles in protecting embankments and maintaining coastal ecological balance (Himes-Cornell et al., 2018). In recent decades, mangroves have suffered from contamination due to effluent discharge, urban and agricultural runoff, and dumping of solid wastes (Rog et al., 2017). Among these anthropogenic contaminants, heavy metals have received increasing attention (Usman et al., 2013). Cadmium (Cd), a non-essential element, can easily be taken up by plants and resulted in chlorosis (Sterckeman et al., 2015), wilting (Hu et al., 2016) and cell death (Farooq et al., 2016). On the other hand, mangrove plants have developed various mechanisms to reduce Cd toxicity, including cell wall compartmentalization, and combination with protein and organic acids (Weng et al., 2012; Dai et al., 2018).

Not only heavy metal contamination, but large amounts of nutrients including nitrogen (mainly in the form of ammonium nitrogen, NH_4^+-N) from domestic sewage also accumulate in mangrove sediment, and change its oligotrophic state (Lovelock et al., 2012) and pH (Tam et al., 1995). For example, in Futian mangrove forest (the only mangrove swamp in the heart of Shenzhen, China), the levels of NH_4^+-N changed from 1.03 to 12.36 mg L^{-1}, and accounted for 60% of the total nitrogen (Niu et al., 2018). It has been reported that the uptake of NH_4^+-N by plants with the release of H^+ ions decreased the pH in rhizosphere and enhanced the solubility of Cd in the soil (Zaccheo et al., 2006). Although previous studies showed that NH_4^+-N improved the accumulation of Cd in *Solanum tuberosum* (Larsson Jönsson and Asp, 2011), *Rorippa globosa* (Wei et al., 2015) and *Carpobrotus rossii* (Cheng et al., 2017), opposite findings were found in rice (Alpha et al., 2009). It is obvious that heavy metal pollution and eutrophication are serious problems in mangrove forests, and excess amounts of Cd and NH_4^+-N are commonly found and interacted in mangrove sediments. Arias et al. (2010) showed that nitrogen fertilization helped the phytoextraction of heavy metals in contaminated soils. Nitrogen fertilization also enhanced plant growth and allowed the production of various kinds of

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proteins to detoxify heavy metals in plants (Sady and Kowalska, 2006; Zhang et al., 2008). However, the interaction between these two groups of elements on mangrove plants and the mechanisms related to the plant uptake and tolerance of heavy metals with the supply of NH_4^+-N have seldom been researched.

Once enter into a plant, Cd mobility and toxicity was associated with its subcellular distribution and chemical forms in cells (Lai, 2015). In root, cell wall deposition and vacuolar compartmentation were important for the detoxification and accumulation of Cd (Zhao et al., 2015). The immobilization of Cd at cell wall was the main mechanism to alleviate Cd toxicity in maize (Lozano-Rodriguez et al., 1997) and lettuce (Ramos et al., 2002), whereas vacuolar sequestration was important in tobacco (Vogeli-Lange and Wagner, 1990) and bean (Vazquez et al., 1992). *Kandelia obovata*, a dominant mangrove species along the coast of south China, was reported to have tolerance to Cd stress due to cell wall compartmentalization and conjugation with protein and organic acids (Sheue and Liu, 2003; Weng et al., 2012). However, the role of NH_4^+-N in cadmium (Cd) tolerance of mangrove plants and their involved mechanisms has not been reported.

Based on the above, it was hypothesized that NH_4^+-N could alter the subcellular distribution and chemical forms of Cd in root, and limited the root-to-shoot translocation of Cd. To this end, the aim of the present study was to (1) investigate the effect of NH_4^+-N on the growth of *K. obovata* and the translocation of Cd from root to aboveground parts under different levels of Cd stress, and (2) evaluate the influence of NH_4^+-N on the subcellular distribution and chemical forms of Cd in the root of *K. obovata*. Our results will provide new insights into the role and the potential mechanism of NH_4^+-N on the uptake, translocation and detoxification of Cd in *K. obovata*.

2. Materials and methods

2.1. Plant culture and experimental design

Mature propagules of *K. obovata* were collected from the Futian National Nature Reserve (22°32′N, 114°03′E), Shenzhen, China in July 2014. Uniform propagules (20 cm height) were transplanted into plastic pots, each of which with a diameter of 15 cm and 24 cm depth was filled with 3 kg of dry, clean sand. All pots were placed outdoors and protected from the rain (daily temperatures of 25–30 °C). Distilled water was added daily to ensure the water level was not lower than the sand surface for 30 days to let the propagules grow up. After 30 days, when the uniform seedlings grew into 22 cm tall with four leaves, they were ready to be used for the experiment. To simulate NH_4^+-N and Cd stresses, a total of 16 factorial combination treatments, including four NH_4^+-N treatments (0 mg L^{-1}(N0), 10 mg L^{-1}(N10), 50 mg L^{-1}(N50) and 100 mg L^{-1}(N100)) and four Cd treatments (0 mg L^{-1}(Cd0), 1 mg L^{-1}(Cd1), 5 mg L^{-1}(Cd5) and 10 mg L^{-1}(Cd10)), were prepared by dissolving different amounts of NH_4Cl and $CdCl_2$ into 50 ml distilled water, then added and mixed thorough with the sand. For each individual pot, three seedlings were transplanted and weekly irrigated with 50 ml½ Hoagland's nutrient solution (pH 6.5) to provide basic nutrients for plant growth and simulate the weak acid environment in the field conditions of *K. obovata*. The composition of Hoagland's nutrition solution were (mg L^{-1}): $Ca(NO_3)_2$, 945; KNO_3, 506; KH_2PO_4, 136; $MgSO_4$, 493; $FeSO_4{\cdot}7H_2O$, 13.9; Na-EDTA, 37.3; KI, 4.15×10^{-3}; H_3BO_3, 3.1×10^{-2}; $MnSO_4$, 0.11; $Na_2MoO_4{\cdot}2H_2O$, 1.25×10^{-3}; $CuSO_4$, 1.25×10^{-4}; and $CoCl_2$, 1.25×10^{-4}. Distilled water was added to each pot daily to ensure the water level to be even with the sand surface and the watering-amount depended on the weight difference (due to evapotranspiration loss) in the pot. The experiment lasted six months and seedlings were collected at the end of the experiment. The collected seedlings were then washed with distilled water and separated into leaves, stems and roots. Some samples were frozen by liquid N_2 and kept frozen until use, and some were dried at 75 °C.

2.2. Determination of total Cd in different plant tissues

The 75 °C dried samples of leaves, stems and roots were ground into fine power and digested with $HNO_3/HClO_4$ (10:1, v/v, USEPA, 1996). The Cd contents were determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES, Prodigy XP, Leeman, USA). The translocation factor (TF) was calculated according to Hart et al. (1998) as follows: TF(root to stem) = C_{stem}/C_{root}; TF(root to leaf) = C_{leaf}/C_{root}, where C_{leaf}, C_{stem} and C_{root} are the concentrations of Cd in the leaf, stem and root of *K. obovata*, respectively. The bioconcentration factor (BCF) was calculated as BCF = CP/CS, where CP is the metal content in the plant root, and CS is the metal content in the sand in this study.

2.3. Subcellular fractions of Cd in roots

Root cells are divided into three fractions, namely cell wall, organelle-containing and soluble fractions, according to Weigel and Jäger (1980). Frozen roots were weighted and homogenized in cooled extraction buffer which consisted of 50 mM Tris-HCl, 250 mM sucrose and 1.0 mM DTE ($C_4H_{10}O_2S_2$) at pH 7.5. The homogenate was centrifuged at 3000*g* for 30 s. The precipitate was designated as the cell wall fraction and consisted mainly cell walls and cell wall debris. The supernatant solution was further centrifuged at 20,000*g* for 45 min. The resultant pellet and supernatant were referred to the organelle-containing fraction and the soluble fraction, respectively. All steps were performed at 4 °C. Each fraction was wet-digested with HNO_3:$HClO_4$ and Cd contents were determined using ICP-AES (Prodigy XP, Leeman, USA).

2.4. Chemical forms of Cd in roots

Chemical forms of Cd were determined using the method described by Xin et al. (2017) with five extraction solutions: (1) 80% ethanol, extracting inorganic Cd giving priority to nitrate, chloride, and aminophenol cadmium, (FE); (2) distilled water, extracting water-soluble Cd with organic acids and $Cd(H_2PO_4)_2$, (FW); (3) 1 M NaCl, extracting pectate- and protein-integrated Cd, (FNaCl); (4) 2% HAC, extracting insoluble $CdHPO_4$, $Cd_3(PO_4)_2$, and other Cd-phosphate complexes, (FHAC); and (5) 0.6 M HCl, extracting cadmium oxalate (FHCl). In brief, frozen roots were weighted and homogenized in the extraction solution (1) with a mortar and a pestle at a ratio of 1:10 (w:v). The homogenate was shaken for 18 h at 30 °C, centrifuged at 5000*g* for 10 min, and the first supernatant solution was collected. The precipitate was re-suspended in the same amount of extraction solution and centrifuged, and this procedure was repeated three times. The supernatants of the four centrifugations were then pooled. The residues were subjected to the next extraction (2) with the same procedures of solution (1). This repeated until solution (5). Each fraction was evaporated on an electric plate at 70 °C until getting a constant weight and then digested with an 11 ml of $HNO_3/HClO_4$ mixture. Cd content in each extractant was determined with ICP-AES (Prodigy XP, Leeman, USA). All reagents were analytical grade or better. The reagents with no sample addition were regarded as blank. Internal standard method was used to test the recovery of heavy metals in samples. In this study, the recovery of Cd was 96.55%.

2.5. Statistical analysis

All data were expressed as the mean ± standard deviation of three replicates. One-way ANOVA was performed to determine significant differences among Cd levels at the same NH_4^+-N concentration, as well as among different NH_4^+-N levels at the same Cd concentration. The data fulfilled the assumptions of the ANOVA test and no data transformation was needed. All statistical analyses were run by using SPSS 16.0 software (SPSS, Chicago, USA).