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The influence of flavonoid amendment on the absorption of cadmium in *Avicennia marina* roots

Jian Li, Haoliang Lu, Jingchun Liu, Hualong Hong, Chongling Yan *

Key Laboratory of Ministry of Education for Coastal and Wetland Ecosystems, Xiamen University, Xiamen 361102, China

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ABSTRACT

Flavonoid is a key factor for the tolerance to cadmium in plants. Concentration-dependent kinetics experiment was conducted to investigate the influence of flavonoid amendment on the Cd^{2+} uptake in *Avicennia marina (Forsk) Vierh.* roots. We found that compared with the control, saturation concentration and maximal absorption rate of Cd was higher under flavonoid amendment ($p < 0.05$). When roots were exposed to ion transport inhibitor ($LaCl_3$), flavonoid amendment also facilitated Cd transport in roots. Flavonoids had no influence on Cd^{2+} uptake in root cell walls. In conclusion, flavonoids enhance the tolerance to Cd and have a significant stimulative effect on symplasm transport of Cd in *A. marina* roots. Ca^{2+}-channel was not the unique means of symplasm transport for Cd^{2+} absorption.

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1. Introduction

Mangrove forests located in the intertidal zone of tropical and subtropical regions, are known as specific ecological habitats, and have been regarded as playing a key role in maintaining the coastal ecological balance (Du et al., 2013; Liu et al., 2010). However, mangroves are subjected to environmental pollutants, especially heavy metal contaminants, such as Cd, Cr, Cu, Pb, Hg (Cuong et al., 2005; Wu et al., 2011). *A. marina*, being the pioneer mangrove species in the southeast of China, was found to accumulate Cd, Cu, Pb and Zn in root tissues under field conditions with levels equal to or greater than the surrounding sediment concentrations (MacFarlane and Burchett, 2002; MacFarlane et al., 2003; Nowrouzi et al., 2012). Cd has drawn more attention because of its high activity and bioavailability, which can interfere with plant metabolic processes, resulting in poor growth and lower biomass. The plants absorb Cd^{2+} through apoplastic and symplasm transport, nevertheless, there are specific or generic ionophore or channel proteins into root cells. Cd^{2+}can be taken in through Ca^{2+} and Mg^{2+}/Fe^{2+} channels, and compete with divalent cations (Welch et al., 1999).

Mangrove plants have a high content of phenolic compounds (Rahim et al., 2007; Rahim et al., 2008) mainly containing flavonoids and tannins. As one kind of secondary metabolites, flavonoids account for 37% of secondary metabolites (Narasimhan et al., 2003). Besides being free radical scavengers, flavonoid can chelate heavy metal depending on their different molecular structure (Korkina, 2007), thus inhibit lipid peroxidation and Fenton reaction (Chen et al., 1990), where heavy metals cause lipid peroxidation and free radical imbalance (Korkina, 2007). Stingu et al. (2012) indicated that phenolic compounds made a critical difference in tolerance and bioaccumulation of *Avena sativa L.* as chelator and solubilizer of heavy metals (Stingu et al., 2012); Keilig and Ludwig-Muller (2009) made a similar conclusion, pointed out that flavonoids were a key factor for the tolerance in *Arabidopsis thaliana*. Arora et al. (1998) tested the inhibition of flavonoids in the liposome membrane system by lipid peroxidation induced by Fe^{3+} or peroxide radical. They found that all the tested flavonoids had higher antioxidation efficiency to peroxidation induced by metal ion than those induced by peroxy radical (Arora et al., 1998). The substituent group mode of flavonoids B-ring influences its antioxidation ability significantly. Certain hydroxy substituents on the A-ring may compensate and become a larger determinant of the antioxidant efficacy when B-ring cannot contribute to the antioxidant activity of the flavonoids. These are determining factors for flavonoids' antioxidation ability (Arora et al., 1998). All these authors have suggested that the chelation of flavonoids makes much critical difference to their antioxidation. The anti-lipid peroxidation capability of flavonoids is the cause of the activity of scavenging free radical and metal chelation.

The absorption of Cd^{2+} is impacted by divalent cations (Zn^{2+}, Ca^{2+}, Fe^{2+} etc.) (Lombi et al., 2002; Zhao et al., 2002) and root exudates (flavonoids and low molecular weight organic acids etc.) (Roth et al., 2012). Cd^{2+} is absorbed through an iron transporter

* Corresponding author.
E-mail address: ycl@xmu.edu.cn (C. Yan).

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protein in the plasma membrane; where the absorption and transport of Cd^{2+} in roots is increasingly involved. Fe^{2+} affect the absorption of Cd^{2+} by competing binding site and transport agent with Cd^{2+} (Charlatchka and Cambier, 2000; Davranche and Bollinger, 2000; Pi et al., 2011). Flavonoids as chelators take on a transporter role to influence the absorption of Cd^{2+}; with the same effect on Zn^{2+}, Fe^{2+} etc. A large number of studies reported that flavonoids are able to function as chelators for metals (Aherne and O'Brien, 2000; Brown et al., 1998; Korkina, 2007; Michalak, 2006; Soczynska-Kordala et al., 2001). They are the main components of root exudates in response to Fe-deficiency (Cesco et al., 2010; Olsen et al., 1981), which act as a Fe^{2+}-carrier to transport Fe^{2+}.

The classic Michaelis–Menten enzyme kinetics model has been widely applied to absorption of metals in plant roots. The Michaelis constant *Km* (half-saturation constant) shows the affinity of roots system with metal, while *Vmax* (maximal rate of velocity) represents the capability of roots for absorbing metal (Zhao et al., 2002, 2006). The model is described as

$$V = (Vmax[S])/(Km + [S])$$

Zhao et al. (2002) utilized the classic Michaelis–Menten equation to study concentration-dependent kinetics of Cd^{2+} uptake under Fe-deficiency in *Thlaspi caerulescens*. They suggested that *Vmax* was usually more focused on than *Km*, for the reason of absorption velocity deciding the uptake and transport ability of Cd^{2+} in plants.

Cd can replace necessary elements to plants growth, e.g. Cu, in various cyto-plasmic and membrane proteins (Yan et al., 2015) and then cause plant poisoning. However, Cd accumulation in leaves of *A. marina* is much less than that in root, even undetectable (Wu et al., 2014). *A. marina* accumulate heavy metals Cd in root tissues with levels equal to or greater than the surrounding sediment concentrations, especially in fine nutritive roots (Wu et al., 2014). Thus the researches on the absorption of Cd in *A. marina* roots are especially crucial. To our knowledge, few researches have reported on the influence of flavonoids on concentration-dependent kinetics of Cd^{2+} absorption in *A. marina* roots.

In the present study, *A. marina*, one of the dominant mangrove species in the southeast of China was selected as the representative species because of its high content of phenolic compounds, abundance and well-developed root system. The aim being: (i) to investigate the concentration-dependent kinetics of Cd^{2+} absorption in *A. marina* roots; (ii) to evaluate the influence of flavonoids on concentration-dependent kinetics of Cd^{2+} absorption in *A. marina* roots, and (iii) to determine the physiological role of flavonoids in Cd^{2+} uptake and accumulation in mangrove seedlings.

2. Material and methods

2.1. Plant material

Mature *A. marina* propagules were collected from the Jiulong River mangrove natural reserve (24°24' N, 117°55' E), Fujian China, in September 2013. Complete undamaged propagules with testa intact and high vitality were chosen for pre-cultivation in sea sand. Sea sand used was prewashed with concentrated HCl and rinsed thoroughly with tap water (Liu et al., 2009). All propagules selected had comparable sizes (25 ± 2 g). The propagules were inserted into the 4 L polyethylene seedling pots. Polyethylene seedling pots were filled with nutrient solution and black polyethylene beads to prevent light exposure of the nutrient solution. Cultured the propagules for 2 months to be 3–4 euphyllas. In general, seedlings were grown in a modified Hogland nutrient solution containing the following macronutrients in mg/l: KNO_3, 707.70; $NH_4H_2PO_4$, 230.04; $MgSO_4$, 240.72; H_3BO_3, 2.868; $CuSO_4 \cdot 5H_2O$, 0.08; $ZnSO_4 \cdot 7H_2O$, 0.22; $MnSO_4 \cdot H_2O$, 1.55; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.61; $FeSO_4$, 5.57; EDTA.2Na, 7.45; $Ca(NO_3)_2$, 1180.76. The nutrient solution was renewed once every 3 days. The seedlings were placed in a greenhouse with daily temperature of 20–29.5 °C, relative humidity of 59–80% and light intensity of 800–1400 umol photons $m^{-2}s^{-1}$ (Yan and Tam, 2011).

2.2. Experimental design and sample culture

All seedlings were divided into two groups, group-A treated without flavonoids and group-B treated with flavonoids respectively, were used for the following 5 processes. The group just treated with different Cd concentration was stated as "control".

Step 1. Seedlings in group-A were inserted into 18 black polyethylene 1 L cups (four seedlings per cup) filled with isometric Hogland nutrient solution. This was adjusted to a concentration of 5 mg/l Cd^{2+}. The same operation was performed on group-B with added flavonoids (Catechin: gallic acid = 1:2) to 5 mg/l. Three duplicate cups for group-A and group-B were cultivated for 1, 2, 4, 8, 16, 32 h, respectively. Roots were rinsed thoroughly, then cut out and freeze dried in a vacuum for 48 h.

Step 2. Seedlings in group-A were inserted into 18 black polyethylene cups (four seedlings per cup) filled with isometric Hogland nutrient solution. This was adjusted to a concentration of Cd^{2+} to 0, 2.5, 5, 10, 20, and 40 mg/l. The same operation process was performed on group-B with adjusting flavonoids concentration (Catechin: gallic acid = 1:2) to 5 mg/l. Three duplicate cups for group-A and group-B were cultivated. The period was decided by step 1. Roots were rinsed thoroughly, then cut out and freeze dried in a vacuum for 48 h.

Step 3. Root cell wall preparations that maintained the morphologic and geometric characteristics of intact roots were obtained. Root systems were immersed in methanol: chloroform (2:1, v/v) solutions for 3 d and rinsed in several changes of distilled water for 1 d. This treatment has been shown to yield lipid-free cell wall preparations in maize while maintaining the structure and morphologic characteristics of an intact roots (Hart et al., 1992). Culture and harvest was consistent with step 2.

Step 4. Root systems were immersed in a solution containing 0.2 mmol/l $LaCl_3$ for 30 min (Cohen et al., 1998), then rinsed thoroughly. Culture and harvest was consistent with step 2.

Step 5. The root were immersed in a solution containing 20umol/l CCCP (Carbonylcyanidem-chlorophenyl-hydrazone) for 30 min (Cohen et al., 1998), then rinsed thoroughly. Culture and harvest was consistent with step 2.

Cd concentration was determined by atomic absorption spectrophotometry (AAS, Model AA-6800, Shimadzu, Kyoto) with 90–94% percentage recovery and 1 ug/kg detection limit after digestion operated by the description of Soto-Jimenez and Paez-Osuna (2001). The concentration factor was calculated as the ratio of the metal concentrations in the plants (in micrograms per gram dry weight (DW)) and the concentrations in the solution (in milligrams per liter).

Reagent blanks and standard references of plant material (GBW-07603) (from the National Research Center for Standards in China) were included to verify the accuracy and precision of the digestion procedure and subsequent analysis. All reagents were Merck analytical grade or Suprapur quality, and all materials (bottles, filters etc,) were acid-cleaned (14% (v/v) nitric acid) and rinsed with deionized water prior to use. Deionized water was

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