Archives of Environmental Protection
Vol. 47 no. 1 pp. 35–40

PL ISSN 2083-4772
DOI 10.24425/aep.2021.136446

Influence of Chloride Salinity on Cadmium uptake by Nicotiana tabacum in a Rhizofiltration System

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Keywords: cadmium, Chloride-complexes, root surface area, leaf surface area, hydroponics, Biotic Ligand Model

Abstract: A hydroponic trial was conducted to study the effect of chloride salinity in simulated effluent on Cd accumulation by tobacco. Leaf surface area (LSA) and root surface area (RSA) measurements were incorporated as possible determinants of Cd uptake rate by plants. Results showed that individual plant differences in Cd content were normalized when including RSA to express Cd uptake rates by plants but not including LSA. A biotic ligand model (BLM) fitted to predict Cd uptake, estimated active and almost linear uptake of the free Cd^{2+} ion by tobacco plants, while virtually no changes in the chloride complex ($CdCl^{+}$) uptake were predicted, presumably due to a rapid saturation of the hypothetical root sorption sites at the concentrations used in this trial. *Nicotiana tabacum* var. K326 is evidenced to be a species potentially suitable for biological wastewater treatment using rhizofiltration at concentrations commonly found in salt-affected wastewater, with high Cd accumulation (185 to 280 mg/$kg_{d.m.}$) regardless of water salinity and tolerance up to 80 mmol/L NaCl.

Introduction

Since metal pollution affects the quality of drinking water, great efforts have been made in the last two decades to reduce pollution of water resources by wastewater discharges in order to contribute to environmental sustainability (goal 6 of the Sustainable Development Goals) (UN 2021). A promising cost-effective alternative to conventional clean-up methods is rhizofiltration, a phytotechnology in which plants grown in water absorb, concentrate and precipitate potentially toxic metals (such as Cd) from polluted effluents (Yadav et al. 2015). On the other hand, salinity has been shown to affect numerous bioprocesses applied to wastewater treatments such as biodegradation (He et al. 2011a) and biosorption (Green-Ruiz et al. 2008) but the effects of salinity on rhizofiltration are still poorly understood.

In general, the free uncomplexed cadmium ion (Cd^{2+}) form has been widely reported to be the determinant for phytoavailability by plants (e.g. Elouear et al. 2014). In a previous hydroponic trial maintaining constant Cd^{2+} in solution, it has been demonstrated that chloride salinity greatly influenced both Cd speciation in solution and Cd accumulation in *Brassica juncea* (López-Chuken and Young 2005) due to the formation, increased diffusion and uptake of the positively charged ion $CdCl^{+}$ ion (Weggler-Beaton et al. 2000). In a rhizofiltration system where all Cd in solution is uncomplexed in the absence of Cl^{-}, it would be reasonable to assume that the individual uptake characteristics of the free Cd^{2+} ion by tobacco plants could be isolated to then extrapolate them to a system where equivalent Cd^{2+} ion activities are in the presence of $CdCl^{-}$ complexes, notwithstanding variation in ionic strength.

Plants used for rhizofiltration should be able to tolerate and accumulate the target metals in high amounts. Recent reports using *Populus* x *canescens* have suggested that Cd tolerance, uptake and detoxification are tissue-specific (He et al. 2011b, 2013, 2015). For the present rhizofiltration trial,

tobacco (*Nicotiana tabacum* var., K326) was chosen due to its easy adaptation to hydroponic conditions, in conjunction with high production of biomass and high transpiration rates from leaves. Tobacco plants have been shown to accumulate Cd from agricultural soil (Lin et al. 2016) and to develop large hairy roots when cultivated under hydroponic conditions (Candelario-Torres 2014) suggesting its potential use to treat metal-affected waste water. In contrast to some other plants, tobacco plants, having big leaves that can reach up to 60 cm length, offer the possibility of expressing Cd uptake as a function of the leaf surface area (LSA). For these reasons, the present study was aimed to (A) investigate the effect of $CdCl^+$ complexes activity on Cd uptake by tobacco under conditions of variable Cd^{2+} activity, incorporating measurements of LSA and root surface area (RSA) as possible determining factors of Cd uptake by tobacco, and (B) fitting a biotic ligand model (BLM) to predict Cd uptake using datasets with variation in Cd^{2+} activity in the presence or absence of $CdCl^-$-complexes.

Methodology

Experimental Design

Seeds of *N. tabacum* var. K326 were germinated in peat-moss and irrigated for 7 weeks with a complete nutrient solution (pH = 4.9) containing KNO_3 10 mM, KH_2PO_4 2 mM, $MgSO_4 \cdot 7H_2O$ 4 mM, $Ca(NO_3)_2 \cdot 4H_2O$ 13 mM, H_3BO_3 92 µM, $MnCl_2 \cdot 4H_2O$ 18.3 µM, $ZnSO_4 \cdot 7H_2O$ 1.53 µM, $CuSO_4 \cdot 5H_2O$ 640 nM, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 30 nM, $FeSO_4 \cdot 7H_2O/Na_2EDTA$ 40 µM. After this period, seedlings selected for homogeneity (average height 12.78cm ± 1.89, n=36) were thoroughly washed with deionized water and transplanted to a 27.5-L hydroponic tray filled with aerated 12.5 mM $Ca(NO_3)_2$ solution for another week. One plant was transplanted per experimental rhizofiltration pot (800 mL filled with aerated 12.5 mM $Ca(NO_3)_2$ solution), and treatments (Table 1) were applied at the same time. These treatments were chosen to be at concentrations commonly found in salt-affected wastewater (Li et al. 2002).

Treatment solutions were replaced after initial 12 hours to allow for rapid Cd depletion caused by an assumed initial equilibration between the root cation exchange sites and the treatment solutions. In order to maintain constant Cd concentrations in the Cd-treated solutions throughout the exposure time of the plants, 5 mL samples from the treatment cups were taken at the intervals of 4, 6, 11 and 12 days after treatment and, following analysis, Cd concentrations in the nutrient solutions were immediately replenished to return the solutions to the original concentrations shown in Table 1. Plants were harvested after 12 days' growth in treatment solutions. The trial was conducted under glasshouse controlled conditions (16 h, 26°C /8 h, 24°C during day/night, respectively).

Nutrient Solution and Plant Analysis

Immediately after solution sampling, pH was measured. After harvest, shoots and intact root systems were cut apart at the transition point between the hypocotyl and root and washed thoroughly with deionized water. Roots were additionally desorbed in a 5 mM $CaCl_2$ solution and excess water removed (blotted) before analyses. Plant roots were analyzed for RSA using the software WinRIZHO® (Regent Instruments, Inc.) which is an image analysis system specifically designed for root measurement in different forms (*e.g.*, morphology, topology, architecture, color). Additionally, the leaf surface area (LSA, cm^2) for each individual plant was measured assuming rhomboid--shaped leaves based in a non-destructive methodology (Pandey and Singh 2011). Measurements of the LSA were undertaken both before treatment application and after harvest, in order to determine changes during treatment exposure and express Cd uptake by tobacco plants including LSA as a possible determinant of uptake rate. Fresh and dry biomass was determined. Plant material was finely milled prior to digestion in hot concentrated HNO_3 and the analysis of Cd was conducted by Flame Atomic Absorption Spectrometry (F-AAS) (Varian® Australia Ltd.) or Graphite Furnace Atomic Absorption Spectrometry (GF-AAS) (Varian® Australia Ltd.) depending on concentrations.

Modeling Cd uptake by tobacco with a 'BLM'

Metal uptake by plants was modeled using a 'biotic ligand model' (BLM) based on the one widely described by López-

Table 1. Rhizofiltration experimental design: Cadmium concentration (added as $Cd(NO_3)_2 \cdot 4H_2O$) and predicted speciation as modeled by WHAM-VI in a 12.5 mM $Ca(NO_3)_2$ solution containing different $NaCl^-$ concentrations

Treatment			Activity (nM)		
NaCl (mM)	Cd (µM)	Ionic strength	(Cd^{2+})	($CdCl^+$)	($CdCl_2^{(0)}$)
0	0.36	0.03	193	0.15	0.00
	0.55	0.03	299	0.24	0.00
	0.72	0.03	390	0.31	0.00
	0.89	0.03	482	0.39	0.00
40	1.25	0.07	193	576	75
	1.93	0.07	298	889	116
	2.53	0.07	391	1166	152
	3.12	0.07	482	1438	187
80	2.24	0.11	193	1098	273
	3.47	0.11	299	1701	423
	4.54	0.11	391	2226	553
	5.59	0.11	481	2741	681

-Chuken et al. (2010). This approach has proven advantages in studies dealing with metal accumulation by plants since it assumes initial sorption of free metal ions (*e.g.* Cd^{2+}), or defined metal complex species (*e.g.* $CdCl^{+}$) from solution (or soil pore water) onto hypothetical plant root sorption sites considering competition between cations and protons for sorption sites.

This specific experiment was intended to implement the BLM to separately model Cd uptake by tobacco as a function of the free ion Cd^{2+} and the $CdCl^{+}$ complexes as modeled by WHAM (VI). In this way, the Cd uptake constants for the free ion Cd^{2+}, (using Cd uptake data from Cl^{-}-free treatments), and subsequently of the $CdCl^{+}$ complex (using data from the 40 and 80 mM Cl^{-} treatments) could be independently determined. This approach was intended to enable the independent fitting of ion reaction constants.

Data Quality Measurements

All treatments were replicated three-fold in a randomized block design. Plant and solution variables were analyzed using ANOVA/Tukey´s test. The Kruskal-Wallis/MSD (minimum significant difference) test was used. Data were not normally distributed according to the Anderson-Darling data normality test. A standard reference material (1573a tomato leaves by the National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA), containing a certified concentration of Cd (1.52 mg/kg ± 3%) was used to ensure the quality of the data. This quality standard was run with each plant digestion batch and averaged 1.48 ± 0.08 mg/kg over the whole trial. For all analyses, blanks and known standard samples were analyzed to ensure consistency. If these standards disagreed (≥5%), the analysis was repeated.

Results and Discussion

Cadmium speciation in nutrient solution

As suspected, a rapid initial decrease (9.97%) in the average Cd concentrations in solution was observed during the first 12 h caused by an immediate approach to a pseudo-equilibrium state between root surface sorption sites (and onto the experimental pots internal surface) and the treatment solutions. On the other hand, after the treated solutions were replaced and Cd concentrations were replenished 4, 6 and 11 days after treatment, only a low variation (±4.23%) for the rest of the 12 days rhizofiltration trial was detected. The simplification of the nutrient solution (containing only 12.5 mM $Ca(NO_3)_2$) used for this experiment ensured that virtually all (>99.9%) Cd present in solution was computed to be either as Cd^{2+}, $CdCl^{+}$ or the uncharged $CdCl_2^0$ complex, while minimal organic Cd-complexation in the nutrient solution was calculated (<0.01 of the total Cd) as modelled by WHAM-VI. The calculated post-trial activities of Cd^{2+} (Fig 1.) were kept convincingly constant between replicates, in spite of a noticeable general decrease (20.0%±0.84) compared to the original calculated pre-trial activities (Table 1). This could be reasonably attributable to insufficient precision caused by manual adjustments of Cd in treatment solutions in each sampling time and also to a rapid approach to a pseudo equilibrium state between root surface sorption sites (and onto the experimental pots internal surface) and the treatment solutions (López-Chuken et al. 2012). On the other hand, the activities of the NaCl-treated solutions $CdCl^{+}$ and $CdCl_2^0$ increased (P<0.01) with increasing Cl^{-} concentrations (Fig. 1).

Plant development and biomass

The 36 tobacco plants used for this trial weighed within the range of 13.1 g±0.38 (fresh biomass) when treatments were applied. The treatments had no statistical effect (P>0.05) on shoot and root biomass, indicating both good tolerance of tobacco plants to Cd and salinity. Similarly, the treatments had no statistical effect (P>0.05) on RSA (total average 111.0 cm^2±16.2, n=36). The LSA values had an initial average value of 207.1 cm^2 (n=36) and showed an average increment of 20.8 during the trial period. Erdem et al. (2012) reported antagonistic effects of Cd on 2 cultivars of tobacco yield (up to 39.2% reductions) when added to soil at concentrations up to 10 mg/kg, however, there is a lack of the available literature data on Cd-affected tobacco yield under hydroponic culture to compare our results.

Plant Cd concentrations

Tobacco is regarded as an efficient accumulator of Cd in its tissues (Lugon-Moulin et al. 2004) and may well be suitable for rhizofiltration because they produce longer, extensive, often fibrous root systems with large surface areas for metal sorption (López-Chuken & Young 2010) from wastewater effluents. In our study, the Cl^{-} treatments led to significant but irregular differences in Cd uptake among tobacco plants (ranging from 185 to 280 mg/kg) (P<0.05). In general, Cd uptake by tobacco plants was consistently better explained by the activity of the $CdCl^{-}$-complexes in solution compared to the activity of the free ion Cd^{2+}, and the best correlations were obtained when expressing Cd uptake by plants per unit of RSA (µmol/m^2 root) (correlation coefficient R = 0.39, P<0.05). Similar tendencies have been found in wheat (Berkelaar and Hale 2000) and maize (López-Chuken and Young 2010) as differences between Cd accumulations were reduced when expressing Cd uptake as µg/cm^2 RSA.

The disruption of plant water status after Cd exposure has been addressed in a number of studies (*e.g.* Perfus-Barbeoch et al. 2002), showing a decrease in transpiration, as well as reduction of the moisture content (Wani et al. 2005). Reduced transpiration may result from reduced LSA caused by decreased leaf growth (Durand et al. 2010, Garg and Chandel 2012). Nevertheless, it has been also shown that in the presence of toxic metals (*i.e.* Cd), some plants are capable of coping with reduced evapotranspiration by increasing the stomatal density on leaf surface (Hetherington and Woodward 2003), which is thought to be an evolutionary adaptation of plants to environmental stress (Xu and Zhou 2008). Conversely, for the current trial, including LSA to express Cd uptake by plants resulted in a very weak correlation with any form of Cd in solution (R= -0.06 to 0.4, P>0.05), thus suggesting that mechanisms controlling Cd uptake by tobacco plants under the current experimental conditions were independent of leaf morphology and/or evapotranspiration rate.

Figure 1 shows a consistent increase (P<0.05) in the Cd uptake rate (µmol/m^2 root) by tobacco plants calculated when the free Cd^{2+} ion was the only Cd ion species in solution (*i.e.* 0 mM Cl^{-} treatments). However, when Cd-Cl^{-}-complexes were present in the nutrient solution (40 and 80 mM Cl^{-} treatments),

an initial increase in Cd uptake over equivalent Cd^{2+} concentrations for both Cl^- treatments was modelled followed by no further significant variation (Fig. 1). While the increase in Cd uptake from both Cl^- treatments at low Cd levels gives indication of $CdCl^+$ uptake, the lack of further Cd uptake with increasing Cd concentration and the similar trend followed by both the 40 and 80 mM Cl^- concentration treatments strongly suggest that at both Cl^- levels, the root sorption sites for $CdCl^+$ complexes were saturated (see later in Fig. 3). Therefore, further increase in the activity of $CdCl^-$-complexes in solution was not reflected in greater Cd uptake by plants. This suggests that after this assumed saturation point, Cd-Cl^--complexes are not being taken up with the same efficiency as the free ion Cd^{2+} (Fig. 1).

Testing a biotic ligand model 'BLM'

The best-fit BLM predicting the uptake of Cd by tobacco plants was parameterized (equation 1) including the Cd^{2+} and $CdCl^+$ activity (as it was shown to be the principal determinant for Cl^--enhanced Cd uptake by tobacco plants (López-Chuken et al. 2012)) and expressed as µmol/m² of root (Fig. 2).

$$Cd_{Tobacco} = \frac{KtRt_{Cd^{2+}}(Cd^{2+})}{1+K_{Cd^{2+}}(Cd^{2+})} + \frac{KtRt_{CdCl^+}(CdCl^+)}{1+K_{CdCl^+}(CdCl^+)} \quad \text{(eq. 1)}$$

This BLM formulation assumed two root sorption sites (R_t), K_{Cd}^{2+} and K_{CdCl}^{+} are the absorption reactions for the (Cd^{2+}) and ($CdCl^+$) respectively, and 'K_tR_t' is a proportionality constant which expresses the assumption that metal concentrations in plant shoots reflect the concentration of metal ions adsorbed on root sites (*i.e.* Cd^{2+} and $CdCl^+$) integrated over the growing time of the plant, in this case meaning no competition between Cd^{2+} and $CdCl^+$ for the same sorption sites at root level (López--Chuken and Young 2010).

For the present dataset, the constants describe Cd^{2+} uptake that resulted from modeling Cd uptake by tobacco plants (µmol Cd/m² of root) at 0 m*M* NaCl were $KtRt_{Cd}^{2+} = 7.97\times10^8$ and $K_{Cd}^{2+} = 5.40\times10^6$. A strong regression coefficient for the Cd^{2+} uptake modeling was obtained (R^2= 0.92) (P<0.05) (Fig. 2; filled circles). Modeling $CdCl^+$ uptake from the 40 and 80 mM NaCl treatments resulted in the constants $KtRt_{CdCl}^{+} = 2.48\times10^{11}$ and $K_{CdCl}^{+} = 1.65\times10^{10}$. A correlation coefficient of R = 0.56 (P<0.05) between the measured and modeled Cd in shoots (µg/cm² of root) was observed for the best-fit BLM (Fig. 2).

Again, the greater scatter associated with data from the Cl^- treatments arises primarily because the uptake rate of the $CdCl^+$ complex is assumed to be close to maximal across the range of conditions. In the context of the BLM this would be interpreted as implying that the occupancy of the sites associated with $CdCl^+$ uptake are close to saturation.

Figure 3 illustrates the hypothetical Cd uptake by tobacco plants when using the Cd^{2+} and $CdCl^+$ uptake constants resulting from the best-fit BLM (equation 1 and Fig. 2). According to the parameterized BLM resolved from the data, the uptake of Cd^{2+} would continue to higher concentrations than the uptake of the $CdCl^+$ complex (*i.e.* uptake sites are saturated at 15.03 µmol m^{-2} root in the case of the latter). The lack of variation on Cd uptake rates with varying activity of $CdCl^+$ in solution may explain the considerable scatter shown in the best-fit BLM (Fig. 2) when including uptake of Cd complexes. While other studies have found exceptions to the BLM when including various $Metal^{2+}$-ligand combinations (Berkelaar and Hale 2003), we demonstrated, under this hypothetical scenario, that the levels of chloride (or $CdCl^+$) chosen appeared to saturate the uptake rate of this complex, which could be translated, for rhizofiltration purposes and under the present conditions, that after roots become saturated with metals, plants can be harvested for further treatment/disposal (Sas-Nowosielska et al. 2004).

Conclusions

- Root surface area (RSA) was found to be an important source of variation of Cd accumulation, while leaf surface area (LSA) had little influence on mechanisms controlling Cd uptake by tobacco plants.

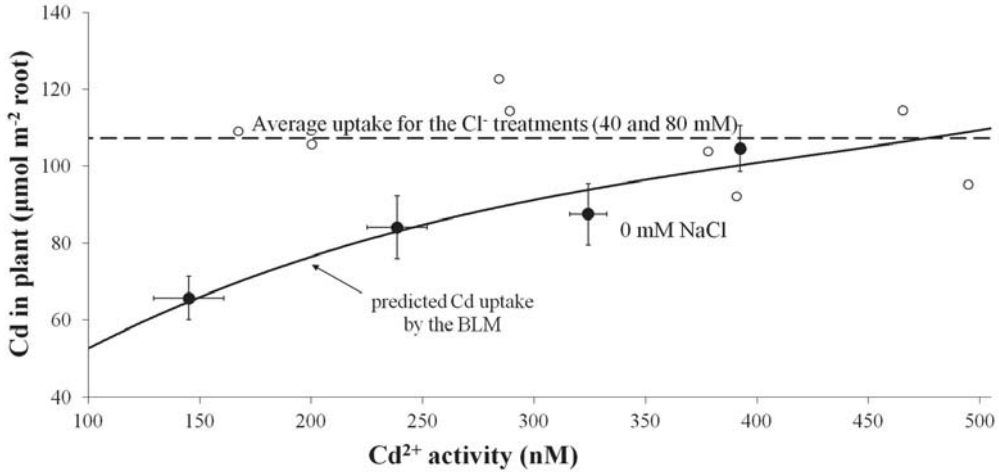

Fig. 1. Relationship between the Cd uptake rate (µmol/m² root) in *Nicotiana tabacum* var. K326 plants and the modeled activity of the free ion Cd^{2+} in nutrient solution with no added Cl^- (●). The Cd uptake rates within the Cl^--free treatments were statistically different (P<0.05). Values are means of replicates (n=3) with standard error bars. The solid line shows predicted uptake rates by plants at 0 mM Cl^- based on the BLM parameterisation shown in equation 1. Cadmium uptake rates from the Cl^- t reatments (○) were not affected (P<0.05) and the average Cd uptake rate from these treatments is illustrated with the broken line.

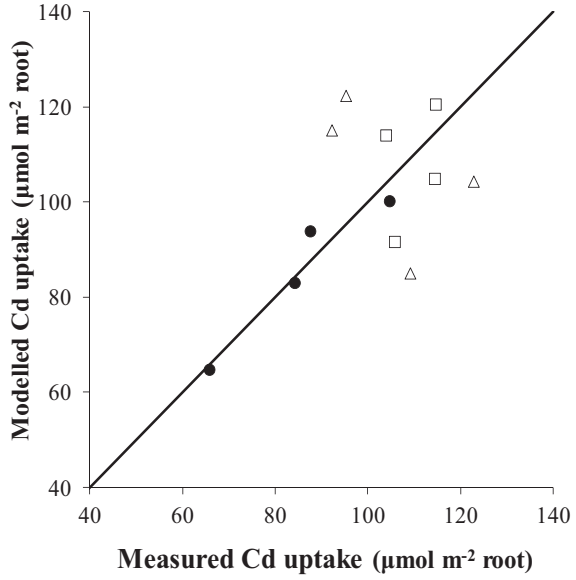

Fig. 2. Cadmium uptake (µmol m^{-2} of root) by tobacco (whole plants) modeled by the BLM assuming two absorption sites for Cd^{2+} and $CdCl^{+}$ ions respectively. Values are means of the replicates (n=3). The solid line represents a 1:1 relation.

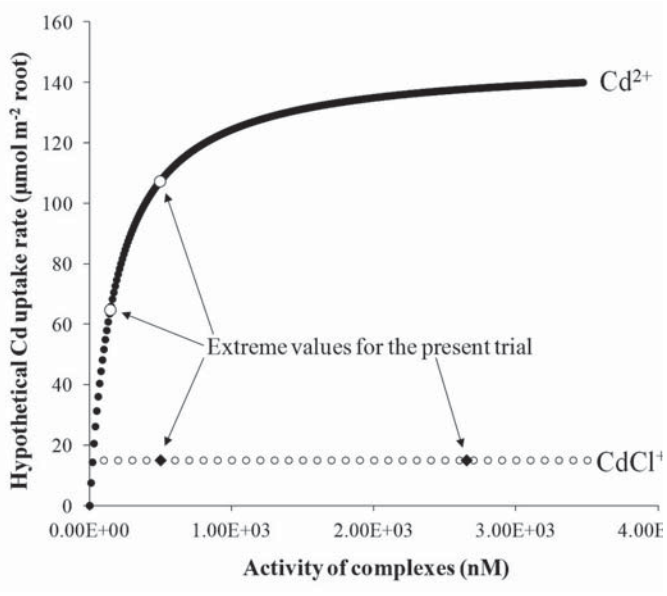

Fig. 3. Hypothetical uptake (µmol m^{-2} of root) of Cd^{2+} and $CdCl^{+}$ by *Nicotiana tabacum* var. K326 plants using the Cd^{2+} and $CdCl^{+}$ uptake constants resulting from the best-fit BLM (equation 1 and Fig. 2). The actual experimental activity values (nM) for the free ion Cd^{2+} and the predominant Cd-Cl^{-}-complex ($CdCl^{+}$) are shown between arrows.

- A systematic improvement of the predicted Cd uptake in tobacco plants (µmol/m^2 root) by the BLM was achieved when Cd^{2+} and $CdCl^{+}$ uptakes were separately modeled. An active and almost linear uptake of the free Cd^{2+} ion by tobacco plants was predicted while virtually there were no changes in $CdCl^{+}$ uptake within the range of activities present in solution, indicating that $CdCl^{+}$ saturated the hypothetical root sorption sites at the concentrations used in this rhizofiltration trial.
- *Nicotiana tabacum* var. K326 was evidenced to be a potential species suitable for biological wastewater treatment using rhizofiltration. In simultaneous Cd and salt-affected effluents, tobacco showed to have a high Cd accumulation rate (185 to 280 mg/kg) while showing non-statistically significant decrease on biomass productivity caused by Cd and/or water salinity (P>0.05).

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