Greenhouse Assessment of Citric Acid-Assisted Phytoremediation of Cadmium by Willows (*Salix* spp.) – Effect on Photosynthetic Performances and Metal Tolerance

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Abstract

The aim of this study was to define effects of cadmium (Cd) applied alone and in combination with citric acid, on the plant tolerance, accumulation, translocation and photosynthesis in willows (*Salix viminalis*, *S. matsudana* and *S. alba*). Low metal bioavailability in soil is often the limiting factor for phytoextraction, thus citric acid was used as a chelating agent aiming to improve metal solubility and therefore accelerates phytoextraction. Willows were grown by soil culture method in semi-controlled conditions (greenhouse) with two different Cd concentrations (3 and 6 ppm), applied separately and in combination with citric acid (20 mM/kg of dry soil), followed by control plants. The reduction of plant growth, biomass, photosynthesis parameters, chlorophyll and carotenoids were induced by Cd supplied. The addition of citric acid (CA) showed beneficial effects on different morpho-physiological levels through alleviate stress conditions as well as enhancing overall phytoextraction. Citric acid has increased transport of the accumulated Cd from the roots to aerial part in *S. viminalis* and *S. alba* in comparison with same treatments without CA, as well as tolerance of analyzed clones. Significant depressive effect on photosynthetic CO_2 assimilation was evident in plants grown in soil with Cd applied. A significant negative correlation between biomass production, leaf area, and photosynthesis due to the presence of Cd in plant tissue was observed in *S. viminalis*. Bioaccumulation factor among selected willow clones was higher than 1, reliably suggesting good potential of selected genotypes for phytoextraction.

Keywords: willows, cadmium, citric acid, soil phytoextraction, photosynthesis, accumulation, translocation, tolerance

Introduction

Being a worldwide problem, soil pollution by heavy metals has been rapidly increased and attracts more attention due its negative impact on the environment on a global scale. Among heavy metals, cadmium (Cd) is one of the most abundant toxic metal and major environmental pollutants. High level of Cd in plant tissue can cause several physiological and biochemical disorders: plant growth and yield reduction, disrupt nutrients and water uptake, changes in chloroplast ultrastructure, decreasing in chlorophyll content, and initiate oxidative stress (Seregin and Ivanov 2001, Mleczek et al. 2010, Pajević et al. 2016).

One of the most common technique for diminishing soil pollution is phytoremediation. In recent years, phytoremediation attracts wide attention because it is considered as a green, eco-friendly, noninvasive and relatively cheap technique (Zhang et al. 2018). The selection of plant species is crucial for effective cleaning of heavy metals polluted sites. According to Baker et al. (2000) Cd hyperaccumulator species are defined as plants which accumulated more than 0.01 % Cd (100 mg kg^{-1}) of dry mass. In regard to this definition, woody species are not included in this category, however the use of fast-growing trees in phytoremediation is favoured by the possibility of long-term growth, high biomass production, deep root system, high rates of evapotranspiration during the growth season, as well as easily vegetative propagation (Trakal et al. 2013, Borišev et al. 2018). A large number of studies highlighted that among the fast-growing trees, *Salix* species are leading candidates in removal of Cd from soil (Vysloužilová et al. 2003, Mleczek et al. 2010, Pietrini et al. 2010). At the same time, several studies revealed clonal variation regarding metal accumulation and tolerance emphasizing importance of plant selection for phytoremediation purpose (Wang et al. 2014, Yang et al. 2015). Plants from Salicaceae family, *Salix* and *Populus*, are widespread woody species which are well established in different climate regions and they are recognized as the most commercially exploited forest trees with great economic importance (Pajevic et al. 2016, Jasinskas et al. 2017). Willows are intensively used in reforestation due to their ability to survive in degraded soil, as well as on agricultural land which is being intensively managed. Further, fast-growing tree plantation based on willows are well established in northern Europe and can be used in short rotation copies providing a feedstock of wood biomass for different industries, energy from biomass and paper production (Mola-Yudego et al. 2016).

Although phytoextraction is the most commonly used technique of phytoremediation, the main drawback of this approach is the low metal bioavailability (Dickinson et al. 2009). Metal bioavailability mostly depends on physico-chemical properties of soil such as pH values, content of organic matter, and aeration (Singh et al. 2015). During the recent years, numerous studies showed that the addition of different chelating agents can enhance metal mobility and bioavailability, thereby accelerate phytoextraction potential of different plant species (Römkens et al. 2002, Chen et al. 2003, Johnson et al. 2009, Ehsan et al. 2014). However, synthetic chelating agents are non-degradable and can reach to unpolluted area due to the high solubility and mobility and can cause environmental risk (Meers et al. 2005, Sun et al. 2005). On contrary to that, natural low-molecular-weight organic acids (LMWOA) such as citric, malic, etc., are distinguished by rapid biodegradation and low phytotoxicity, thus potential risk of leaching of metals to groundwater is minimized (Kim and Lee 2010). Further, application of soil amendments enhances metal uptake and accumulation making stabilized metal-chelate complexes that are predominantly sequestrated in vacuole thereby reducing the heavy metals effect on plant metabolism. However, despite various investigations, the effect of citric acid, as chelating agent, on different physiological processes under the metal pollution is still unclear. Understanding the role of citric acid in plant tolerance and detoxification mechanisms is necessary for its implementation in assisted phytoremediation. Further, the plant response to heavy metal stress is complex and varies depending on plant species, even clone selection, metal concentration and soil properties (Montiel-Rozas et al. 2016).

Based on above discussion, the aim of this study was to compare the ability of three *Salix* species to uptake and accumulate Cd alone and in combination with CA. The parameters regarding plant biomass production, accumulation, translocation and tolerance index, gas exchange parameters, as well as photosynthetic pigment contents were analyzed in order to elucidate the role of citric acid as a chelating agent. The results of this pilot study carried out in the greenhouse can provide the information for development of good management in chelating assisted decontamination of moderately polluted soil. Data obtained in this work could be used to evaluate CA-assisted remediation by willows.

Materials and Methods

Plant growth conditions and treatments

In order to compare the ability of various *Salix* clones to absorb and accumulate Cd in plant tissue, as well as to test the effectiveness of citric acid on selected genotypes, we analyzed three willow clones. Plant material contained the 1-year-old woody cuttings of the willow species *Salix viminalis* L. (clone SV068), *Salix matsudana* Koidz. (clone SM4041) and *Salix alba* L. (clone V-158). The clones were selected according to our previous study (Arsenov et al. 2017) since these clones showed good characteristics in Cd removal of moderate polluted soil. Willows were grown by soil culture method in a semi controlled conditions (greenhouse) with around 12 h light: 12 h dark photoperiod, 20-30°C temperature and 55-60 % relative humidity. Unrooted woody cuttings were set up in the Mitscherlich pots and plants were grown for two months, from May to July (Spring-Summer vegetation season). Each pot contained six cuttings, approximately 20 cm long and 1 cm wide, with one shoot per cutting.

Every pot contained 5 kg of soil. Soil type was small sandy clay, with pH ranged from 7.6 to 8.4 depending on the pot (treatment). Content of total N, P, and K were 0.32 %, 2.45 % and 2.85 %, respectively, $CaCO_3$ was 18.45 %, while humus was 1.23 %. Soil was irrigated permanently to maintain optimal soil humidity. Cadmium and citric acid were dissolved in deionized water and sprayed onto the soil, which was homogenized by mixing. Cadmium was supplied as $CdNO_3 \cdot 4H_2O$ solution in 3 ppm Cd concentration (MPC, maximum permitted concentrations) and 6 ppm Cd (2MPC), according to Pravilnik o količinama (2010, 2011), following the same treatments with addition of citric acid in concentration of 20 mM per kilogram of soil. The plants were divided into 6 treatments: control (without Cd nor citric acid), Cd_3 (addition of 3 mg per kg dry soil), Cd_6 (addition of 6 mg per kg dry soil), CA (addition of citric acid 20 mM per kg dry soil), CA+Cd_3 (combined treatment of 3 mg per kg Cd + citric acid, 20 mM per kg dry soil) and CA+Cd_6 (combined treatment of 6 mg per kg Cd + citric acid, 20mM per kg dry soil). Plants were harvested after two months of applied treatments. Total fresh weight of leaves, stem and root were measured. Leaf area (cm^2) was measured with an "ADC Bioscientific Ltd. AM350 Portable Leaf Area Meter".

Plant Cd Accumulation

Prior to analyses, plants were washed with deionized water to ensure complete removal of Cd and soil from roots. Leaves were divided into two groups, young leaves consisted of the seven youngest leaves, and other group was mature leaves. The division has been carried out in order to analyze impact of cadmium with and without CA due to the leaves age. Dry weight of leaves, stem and roots (expressed in g per plant) were estimated.

All chemicals used for analysis were purchased at Sigma (St. Louis, USA). Plant material was dried at 80°C, for 24 hours, milled and weighed. Plant samples were dry-ashed at 450°C following the acid digestion with 25 % HCl and mineralization with 33 % H_2O_2. Total amounts of Cd in various plant parts (young leaves, mature leaves, shoots and roots), as well as in soil were determined using an employing flame atomic absorption spectrophotometry (Varian, AAS240FS). Triplicate extracts of each sample were analyzed. The concentrations of Cd in young leaves, mature leaves, shoots and roots were calculated by the following formula:

Cd concentration (μg/g) = reading × dilution factor/dry weigh of plant part.

Bioconcentration Factor (BCF), Translocation Factor (Tf) and Tolerance Index (Ti) Calculation

The cadmium bioconcentration factor (BCF) of aerial plant parts (young leaves + mature leaves + stem) and roots was calculated according to Zacchini et al. (2009). The bioconcentration factor (BCF) was calculated as follows:

BCF = Cd concentration in harvested plant tissue (μg/g)/Cd concentration in the soil, (μg/g).

The translocation factor (Tf) was calculated to evaluate the capacity of plant to translocate the metal, absorbed by roots, to the aerial plant part:

Tf = Cd concentration in the aerial parts (μg/g)/ Cd concentration in the roots (μg/g).

The tolerance index (TI) was calculated according to Wilkins (1978) using fresh weight of Cd treated and control plants as follows:

TI = fresh weight of Cd treated plants × 100/fresh weight of control plants.

Photosynthetic Parameters

Net photosynthetic rates (Pn), transpiration (E), water use efficiency (WUE), stomatal conductance (gs) and substomatal CO_2 concentration (ci) were measured using LC pro$^+$ Portable Photosynthesis System, (ADC BioScientific Ltd). Photosynthetic activity was measured in young leaves. Measurements were conducted for each treatment, on 3 different plants. Three replicates were recorded for each plant. Light conditions were set using the LCpro$^+$ light unit, which emitted photosynthetic active radiation (PAR) at 1000 μmol/(m^2 s). The air supply unit provided a flow of ambient air to the leaf chamber at a constant rate of 100 μmol/(m^2 s). Temperature, CO_2 concentration and humidity were at ambient levels. Parameter WUE (water use efficiency) was calculated as the ratio of photosynthetic and transpiration rate and expressed in μmolCO_2/(m^2 s)/mmolH_2O/(m^2 s). Stomatal conductivity of water vapour was expressed in unit molH_2O/(m^2 s).

Concentration of photosynthetic pigments

The content of photosynthetic pigments was measured in young and mature leaves. The concentration of chlorophyll a (Chl a), chlorophyll b (Chl b) and total carotenoids were extracted in with absolute acetone 100 percent (v/v) aqueous solution (Von Wettstein 1957). Light absorbance was determined at 662 nm, 644 nm and 440 nm by spectrophotometer (Beckman, DU® Series 65, Scotland). The concentration of photosynthetic pigments was expressed as mg per g of fresh weight.

Statistical Analysis

Analyses were performed in three replicates (*n* = 3). The obtained data were expressed as mean ± standard deviation (SD). Data were subjected to the analysis of variance (ANOVA) and followed by the Fisher multiple range test at a significance level of $p < 0.05$. The mean values showed in the tables and figures followed by the same character did not differ significantly. The least significant difference (LSD) is presented in the tables. Correlation coefficient was performed between accumulation, biomass and leaf area production, photosynthesis, transpiration and photosynthetic pigments. Correlation was performed for each willow species using Statistica 13.0 software package (Dell 2015).

Results

Willow plants revealed different response in growth parameters (leaves area and dry weight) when exposed to Cd alone and in combination with CA. Slightly reduction to no significant changes in biomass production was observed in plants treated with Cd in comparison to control plants (Figure 1). The addition of CA in the soil did not induce reduction in biomass production among the tested willows. On contrary, *S. alba,* showed increase in biomass production of roots in plants treated with citric acid, in comparison to control plants.

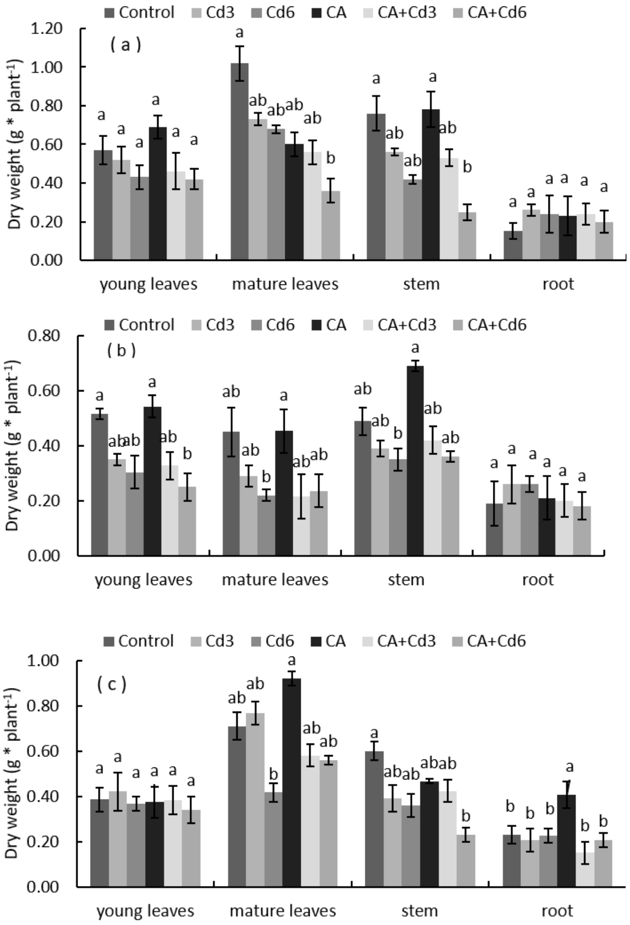

Figure 1. Plant dry weight yield (g DW plant^{-1}) of young and mature leaves, stem and roots among the *Salix* species exposed to Cd and CA. Values are mean ± SD (n=3). Different letters indicate that values are significantly different at p≤0.05 (a) *S. viminalis*; (b) *S. matsudana*; (c) *S. alba*

In general, willows were resistant to elevated Cd content in soil, therefore neither chlorosis nor necrosis were observed in *Salix* plants (Figure 2). The results showed variation in leaf area in plants exposed to Cd in comparison to plants exposed to combined treatment. *S. viminalis* and *S. alba* showed statistically significant decrease in leaf area in mature leaves in Cd supplied alone in comparison to combined treated plants. The same pattern was observed in young leaves of *S. matsudana* (Table 3).

The accumulation of Cd among different plant organs in tested *Salix* species is presented in Figure 3. Accumulation of Cd in plant tissue was proportional to the increase of Cd content in soil. Cadmium was mainly accumulated in roots, followed by leaves and stem, respectively (Figure 3). The highest Cd content was recorded in roots of *S. matsudana* plants under higher Cd dose applied regardless citric acid applied. The addition of CA showed species specific, as well as plant organ specific response. Significant elevation of Cd concentration was evident in *S. matsudana* plants grown under combined treatments with CA addition (Figure 3).

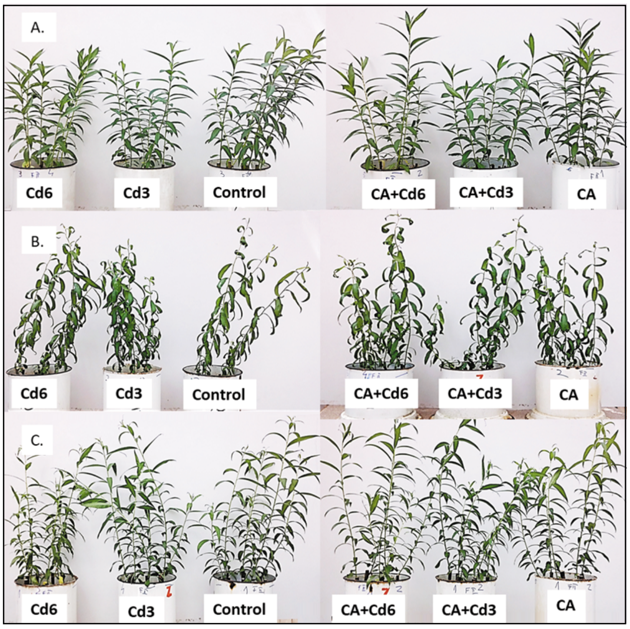

Figure 2. *Salix* plants: (a) *S.viminalis*; (b) *S. matsudana*; (c*) S. alba* after 60 days of exposure to different Cd concentration (Cd6 – 6 ppm, Cd3 – 3 ppm and Control – without Cd) and combined treatments of Cd with citric acid (CA+Cd6, CA+Cd3 and CA – citric acid, 20 mmol/kg soil). Plants were grown in soil culture in greenhouse

Bioconcentration factor in aerial plant parts revealed an increase accumulation of Cd in plants exposed to CA in all analyzed *Salix* species (Figure 4). Furthermore, the data present in this work showed that the addition of citric acid increases translocation of Cd absorbed by plants in *S. viminalis* and *S. alba* comparing with same treatments without CA (Figure 4). The highest translocation factor was observed in *S. viminalis*. The Cd treatments applied alone showed a tolerance index ranged from 60.57 (in *S. viminalis* plants exposed to higher Cd concentration) to 76.67 (in *S. matsudana* plants exposed to lower Cd concentration). Correlation coefficient revealed high negative correlation between biomass production and Cd accumulation in *S. viminalis* and *S. matsudana* plants (Table 1). In contrary to this, *S. alba* showed no significant correlation between biomass production of young leaves in response to the presence of Cd in plant tissue.

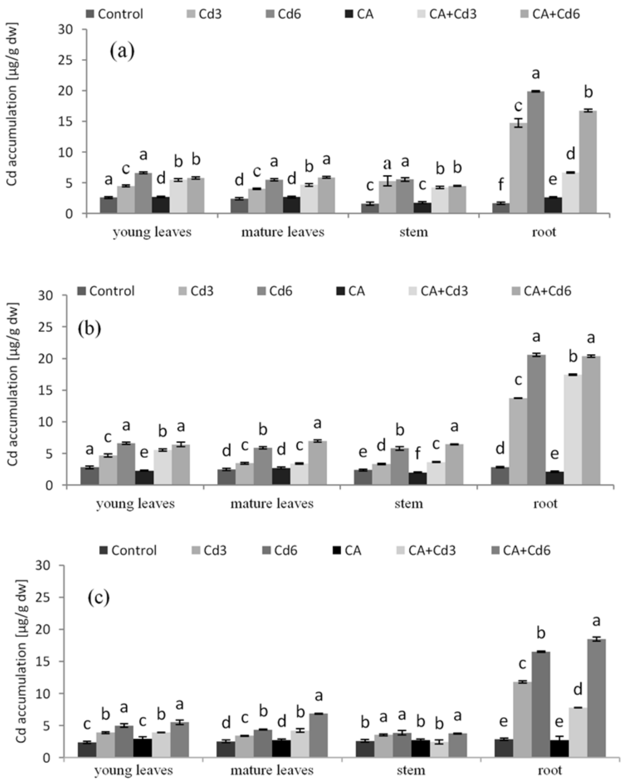

Figure 3. Accumulation of Cd in different plant organs among the *Salix* species exposed to Cd and CA. Values are mean ± *SD* (*n*=3). Different letters indicate that values are significantly different at *p*≤0.05 (a) *S. viminalis*; (b) *S. matsudana*; (c) *S. alba*

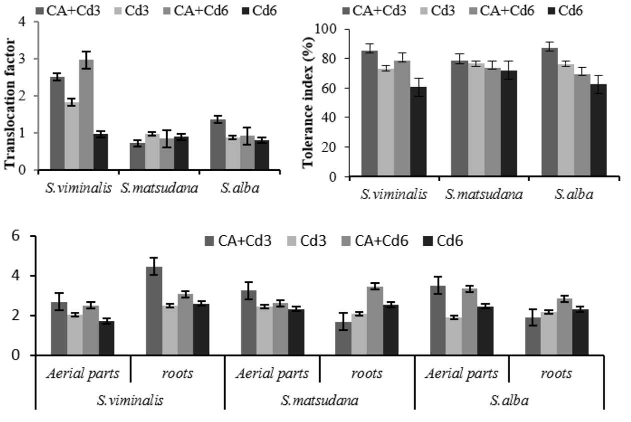

Figure 4. Translocation factor (a), Tolerance index (b) and Bioconcentration factor (c) among the *Salix* species exposed to Cd and CA. Values are mean ± *SD* (*n*=3)

Table 1. Correlation coefficient between Cd accumulation and morpho-physiological parameters

	Mean	stdev	Pn*	E*	accumulation	biomass	Chl a	Chl b	carotenoids	leaf area
					S.viminalis					
Pn*	10.76	3.15	1.00							
E*	2.20	0.46	0.77	1.00						
accumulation	4.61	1.66	-0.81*	-0.57	1.00					
biomass	0.61	0.10	0.59	0.33	-0.91*	1.00				
Chl a	1.70	0.15	0.72	0.47	-0.78	0.51	1.00			
Chl b	0.44	0.12	0.36	-0.17	-0.67	0.65	0.68	1.00		
carotenoids	0.53	0.05	0.70	0.89*	-0.39	0.06	0.59	-0.16	1.00	
leaf area	41.62	5.18	0.67	0.46	-0.95*	0.95*	0.70	0.64	0.29	1.00
					S. matsudana					
Pn*	11.62	1.60	1.00							
E*	2.57	0.18	0.33	1.00						
accumulation	4.73	1.82	-0.58	-0.20	1.00					
biomass	0.47	0.14	0.58	0.08	-0.98*	1.00				
Chl a	1.48	0.48	0.66	0.17	-0.57	0.49	1.00			
Chl b	0.50	0.25	-0.55	-0.07	0.42	-0.31	-0.90*	1.00		
carotenoids	0.55	0.09	-0.78	-0.37	0.51	-0.44	-0.95*	0.80	1.00	
leaf area	0.55	0.09	-0.78	-0.37	0.51	-0.44	-0.95*	0.80	-0.93*	1.00
					S. alba					
Pn*	13.92	2.11	1.00							
E*	3.08	0.30	0.78	1.00						
accumulation	3.94	1.19	-0.89*	-0.66	1.00					
biomass	0.45	0.04	0.51	0.63	-0.77	1.00				
Chl a	2.13	0.12	-0.22	-0.43	0.16	-0.46	1.00			
Chl b	1.11	0.04	0.91*	0.67	-0.99*	0.76	-0.24	1.00		
carotenoids	0.34	0.02	-0.23	0.29	0.17	0.08	0.20	-0.27	1.00	
leaf area	36.98	7.39	0.26	0.01	-0.58	0.68	-0.15	0.60	-0.49	1.00

*Net photosynthetic rates (Pn), transpiration (E), marked correlations (*) are significant at p≤0.05

The photosynthetic responses of the analyzed willow clones induced by Cd and CA applied alone and in combination are presented in Table 2. This work showed that net photosynthetic rate (Pn) was significantly decreased following increasing of Cd in plants. Net photosynthetic rate was decreased by 59 % in *S. viminalis*, 33 % in *S. matsudana*, and 35 % in *S. alba* respectively, as compared to control plants. At the same time, in plants treated with combination of Cd and CA, the reduction of Pn was 19 % in *S. viminalis*, 17 % in *S. matsudana*, and 20 % in *S. alba* in comparison with control plants. A significant negative correlation between biomass production, leaf area, and photosynthesis due to the presents of Cd in plant tissue was observed in *S. viminalis* (Table 1). The transpiration rate showed relatively high values in all analyzed genotypes, and was not affected in most treatments. The highest transpiration rate was recorded in *S. alba*, ranged from 2.57 to 3.35 mmol$H_2O/(m^2$ s). The

Table 2. Photosynthetic activity in young leaves of *S. viminalis*, *S. matsudana* and *S. alba* exposed to cadmium and citric acid. Values are mean ± *SD* (n=3). Different letters indicate significant differences at *p*≤0.05

Treatments	Pn*	E*	WUE*	gs*	ci*
			S. viminalis		
Control	14.83±24.90^{a}	2.93±0.12^{a}	5.3±1.86^{ab}	0.33±0.12^{a}	261.89±0.11^{a}
Cd3	13.46±2.61^{ab}	2.53±0.48^{ab}	5.2±0.99^{ab}	0.27±0.03^{ab}	211.78±0.48^{bc}
Cd6	6.05±3.34^{c}	2.06±0.68^{ab}	4.46±1.02^{ab}	0.17±0.11^{ab}	216.89±0.68^{bc}
CA	11.11±2.46^{abc}	1.98±0.94^{ab}	6.94±3.24^{a}	0.19±0.12^{ab}	201.89±0.93^{c}
CA+Cd3	10.09±3.28^{abc}	2.06±0.7^{ab}	5.02±0.88^{ab}	0.18±0.09^{ab}	211.56±0.69^{bc}
CA+Cd6	9.01±3.36^{bc}	1.64±0.67^{b}	3.83±1.38^{b}	0.13±0.12^{b}	245±0.66^{ab}
LSD$_{p < 0.05}$	5.35	1.06	2.9	0.09	21.08
			S. matsudana		
Control	13.89±2.81^{a}	2.65±0.31^{a}	5.37±1.50^{a}	0.27±0.09^{a}	205.11±19.61^{bc}
Cd3	10.76±0.80^{bc}	2.78±0.77^{a}	4.05±0.77^{a}	0.25±0.03^{a}	220.22±12.44^{ab}
Cd6	9.43±1.04^{ab}	2.50±0.39^{a}	3.85±0.72^{a}	0.2±0.02^{a}	193±16.03^{c}
CA	12.10±1.66^{c}	2.55±0.35^{a}	4.88±1.21^{a}	0.29±0.05^{a}	229.44±11.73^{a}
CA+Cd3	10.82±1.33^{bc}	2.26±0.32^{a}	4.93±1.13^{a}	0.21±0.03^{a}	206±9.5^{abc}
CA+Cd6	12.71±2.09^{ab}	2.67±0.55^{a}	4.96±1.19^{a}	0.2±0.11^{a}	197.78±19.61^{bc}
LSD$_{p < 0.05}$	2.89	0.79	1.84	0.11	24.31
			S. alba		
Control	16.3±0.99^{a}	3.13±0.66^{a}	4.38±0.40^{a}	0.29±0.05^{bc}	224.33±7.44^{ab}
Cd3	13.5±2.25^{abc}	3.35±0.48^{a}	4.79±0.97^{a}	0.35±0.04^{ab}	190.0±11.35^{c}
Cd6	10.66±3.31^{c}	2.57±0.29^{a}	4.19±0.50^{a}	0.18±0.04^{d}	214.78±11.2^{b}
CA	15.63±1.72^{ab}	3.34±0.63^{a}	5.08±1.26^{a}	0.39±0.05^{a}	232.78±5.38^{a}
CA+Cd3	14.88±1.17^{ab}	3.18±0.55^{a}	4.85±1.36^{a}	0.33±0.04^{abc}	221±13.85^{ab}
CA+Cd6	12.55±0.54^{bc}	2.91±0.89^{a}	4.39±0.65^{a}	0.25±0.08^{cd}	216.44±7.93^{ab}
LSD$_{p < 0.05}$	3.12	1.01	1.53	0.01	16.34

*Net photosynthetic rates (Pn), transpiration (E), water use efficiency (WUE), stomatal conductance (gs) and substomatal CO_2 concentration (ci) decrease in transpiration rate was observed in *S. viminalis* at a higher dose of Cd applied.

The concentration of photosynthetic pigments showed variation between treatments and leaves age in all analyzed *Salix* species (Table 3). The reduction of chlorophyll and carotenoids contents was observed in Cd treated plants mainly at the higher dose of Cd applied. The highest reduction was recorded in *S. matsudana* in both young and mature leaves. The addition of CA reduced the negative effect of Cd through maintaining the concentrations of photosynthetic pigments at similar levels compared to control plants in both young and mature leaves.

Table 3. Photosynthetic pigments content and leaf area in young and mature leaves of *S. viminalis*, *S. matsudana* and *S. alba* exposed to cadmium and citric acid. Values are mean ± *SD* (n=3). Different letters indicate significant differences at $p \leq 0.05$

		Chlorophyll a	Chlorophyll b	Carotenoids	Leaf area
			S.viminalis		
young leaves	control	1.67±0.21^{ab}	0.37±0.11^{bc}	0.56±0.12^{a}	46.37±14.69^{a}
	Cd3	1.60±0.02^{ab}	0.36±0.02^{bc}	0.54±0.08^{ab}	40.84±5.91^{a}
	Cd6	1.70±0.03^{ab}	0.52±0.04^{ab}	0.48±0.13^{c}	36.21±6.89^{a}
	CA	1.75±0.05^{a}	0.61±0.07^{a}	0.54±0.13^{a}	48.93±15.77^{a}
	CA+Cd3	1.71±0.02^{ab}	0.40±0.07^{bc}	0.47±0.04^{c}	41.04±8.49^{a}
	CA+Cd6	1.50±0.01^{b}	0.27±0.05^{c}	0.50±0.15^{bc}	36.32±6.35^{a}
	LSD$_{p<0.05}$	0.26	0.2	0.06	13.54
mature leaves	control	1.96±0.01^{a}	0.65±0.08^{ab}	0.54±0.04^{b}	77.50±24.03^{a}
	Cd3	1.94±0.01^{a}	0.49±0.16^{a}	0.61±0.17^{ab}	54.79±19.15^{b}
	Cd6	2.01±0.02^{a}	0.71±0.05^{ab}	0.54±0.04^{b}	43.02±13.95bc
	CA	2.04±0.06^{a}	0.46±0.14^{ab}	0.61±0.02^{ab}	51.40±15.61^{bc}
	CA+Cd3	1.93±0.02^{a}	0.54±0.06^{b}	0.57±0.06^{ab}	46.76±8.99^{bc}
	CA+Cd6	1.98±0.03^{a}	0.44±0.18^{ab}	0.62±0.14^{a}	32.74±9.82^{c}
	LSD$_{p<0.05}$	0.21	0.26	0.09	20.83
			S.matsudana		
young leaves	control	1.76±0.03^{a}	0.98±0.05^{a}	0.72±0.01^{a}	60.42±6.11^{a}
	Cd3	1.72±0.05^{a}	0.45±0.05^{bc}	0.50±0.01^{b}	40.09±8.73^{b}
	Cd6	0.52±0.01^{b}	0.54±0.02^{b}	0.47±0.01^{b}	27.28±4.61^{d}
	CA	1.66±0.16^{a}	0.91±0.01^{a}	0.54±0.02^{b}	38.69±3.75^{bc}
	CA+Cd3	1.70±0.13^{a}	0.39±0.15^{bc}	0.54±0.07^{b}	33.16±10.41^{bcd}
	CA+Cd6	1.55±0.01^{a}	0.35±0.01^{c}	0.51±0.08^{b}	31.84±5.92^{cd}
	LSD$_{p<0.05}$	0.21	0.17	0.18	7.15
mature leaves	Control	1.47±0.14^{c}	0.82±0.16^{a}	0.73±0.01^{a}	31.30±6.65^{a}
	Cd3	0.46±0.05^{d}	0.38±0.14^{bc}	0.54±0.01^{b}	24.87±12.33^{a}
	Cd6	0.39±0.01^{d}	0.81±0.01^{ab}	0.41±0.01^{c}	24.07±8.11^{a}
	CA	2.08±0.14^{a}	0.87±0.17^{a}	0.72±0.03^{a}	35.61±11.15^{a}
	CA+Cd3	1.98±0.13^{ab}	0.64±0.05^{abc}	0.61±0.01^{b}	29.17±9.08^{a}
	CA+Cd6	1.79±0.01^{b}	0.34±0.01^{c}	0.59±0.04^{c}	26.12±8.06^{a}
	LSD$_{p<0.05}$	0.23	0.43	0.01	12.18
			S.alba		
young leaves	control	2.09±0.03^{a}	1.11±0.11^{a}	0.55±0.08^{a}	49.36±4.47^{a}
	Cd3	1.97±0.48^{a}	1.07±0.1^{a}	0.35±0.03^{b}	39.63±4.48^{ab}
	Cd6	2.23±0.26^{a}	1.17±0.03^{a}	0.34±0.04^{b}	38.89±11.42^{b}
	CA	2.28±0.1^{a}	1.11±0.06^{a}	0.47±0.08^{a}	34.28±6.97^{b}
	CA+Cd3	2.02±0.23^{a}	1.06±0.1^{a}	0.33±0.03^{b}	29.81±8.31^{b}
	CA+Cd6	2.19±0.12^{a}	1.14±0.03^{a}	0.27±0.04^{b}	29.89±9.38^{b}
	LSD$_{p<0.05}$	0.51	0.16	0.09	10.29
mature leaves	control	2.31±0.07^{a}	1.13±0.02^{a}	0.36±0.02^{a}	95.55±31.14^{a}
	Cd3	2.18±0.2^{ab}	1.02±0.04^{c}	0.33±0.01^{a}	53.31±15.66^{b}
	Cd6	1.86±0.07^{c}	0.92±0.01^{d}	0.26±0.02^{bc}	60.20±18.06^{b}
	CA	2.18±0.05^{ab}	1.05±0.01^{bc}	0.30±0.02^{ab}	52.93±13.18^{bc}
	CA+Cd3	1.70±0.12^{c}	1.00±0.02^{c}	0.20±0.05^{c}	47.39±6.19^{bc}
	CA+Cd6	2.12±0.08^{b}	1.11±0.05^{ab}	0.31±0.02^{ab}	30.23±11.09^{c}
	LSD$_{p<0.05}$	0.17	0.09	0.06	22.84

Discussion and Conclusions

Results of this work are contribution to understanding the effects of exogenous supplied organic chelators in the soil contaminated with Cd. A slight decrease of plant biomass was observed in willows exposed to elevated Cd concentration. The toxic concentrations of Cd in plants can induce reduction of plant growth as a result of combined effect on photosynthesis and mineral nutrition (Trakal et al. 2013). Various literature data showed variation in biomass production as a response to different Cd concentration present in soil (Zacchini et al. 2009, Nikolic et al. 2015, Yang et al. 2015). The root length retardation and decrease in shoot biomass, as the primary toxic effect of heavy metals is confirmed in several *Salix* species and genotypes (Vysloužilová et al. 2003, Pajevic et al. 2009). Besides that, plant productivity is closely related to the photosynthetic potential, relatively high and stabile WUE, and balanced mineral nutrition. Positive effect of citric acid applied was observed in the production of root biomass in *S. alba* plants which showed slightly increase in comparison with no CA treated plants. Similar to this, Kim and Lee (2010) reported no reduction in biomass of *Echinochloa crus-galli* treated with CA in combination with Cd, compared with control plants.

The bioconcentration factor (BCF), as soil/plant ratio, was measured in order to evaluate the tested genotypes as proper candidates for the decontamination of soils polluted by heavy metals. In general, the plant is defined as an accumulator, indifferent or excluder when that ratio is >1, =1 or <1, respectively. BCF values were higher than 1 in all analyzed species, reliably suggests a good potential of selected willow clones for phytoextraction (Figure 4). Furthermore, the BCF was decreased by increasing the Cd concentrations in soil. In addition, citric acid applied promoted Cd accumulation and bioconcentration factor showed higher value in combined treatments (CA+Cd) in all analyzed genotypes. These results support the enrollment of CA in elevation of Cd bioaccumulation in *Salix* clones. Similar to this was observed by Gao et al. (2011) who revealed that CA applied under Cd and Pb combined pollution could significantly increase the phytoextraction potential of *Solanum nigrum*, compared to no CA treatment. The allocation of heavy metals in plant tissues is a valuable feature for determination of various remediation goals. Therefore, the translocation factor was estimated to analyze the effectiveness of plants to translocate Cd from roots to aerial plant parts. Cadmium was mostly retained in roots, but translocation to aboveground plant parts was sufficient to confirm the good phytoextraction potential of analyzed genotypes. This pattern of Cd distribution could be associated with activation of protective mechanisms of plants and avoidance of negative influence of heavy metals in photosynthetic tissue.

Heavy metal bioavailability has a great influence on the successful decontamination of soil. Different soil characteristics such as pH, soil structure, and mineral and organic matter content can effect on metal removal (Farrag et al. 2012). The influence of soil properties on metals uptake and biomass production is particularly evident in calcareous soil where strong ions bonding at alkaline pH limits the absorption of heavy metals (Lesage et al 2005, Meers et al. 2005). Furthermore, the uptake of heavy metals by roots and their translocation to the above plant part could varied between species, amount of heavy metal content in soil, duration of exposure, age and nutritional status of the plant, transpiration conditions and other morpho-physiological characteristics of plants and experiment design (Zupunski et al. 2016).

Many studies have shown that some organic acid could promote accumulation and translocation to aboveground plant parts in various species (Chen et al. 2003, Sun et al. 2005, Han et al. 2016). Exogenous supplied organic acids, such as malic and citric, could be involved in metal xylem loading and transport of heavy metals from root to shoot, and thus in elevation of phytoextraction. (Chen et al. 2003). This observation is in agreement with results of this work (Figure 4). The effect of citric acid relies on enhanced transport of Cd ions from the roots to aerial parts by diffusion. A similar effect of exogenous applied CA in reduction of Cd stress was confirmed in previous studies on different species *Helianthus annus* (Lesage et al. 2005), *Solanum nigrum* (Gao et al. 2011) and *Brassica juncea* (Kaur et al. 2017). Contrary to our findings, Qiu et al. (2009) showed a reduction of Ni uptake by *Brassica juncea* and *Alyssum corsicum* exposed to citric acid, suggesting that Ni is more easily taken up in the form of the free ion instead of as a chelate-Ni(II) complex. Possible explanation for that rely on nickel role an essential nutrient for the plant, thus it is normally transported to all the organs, while Cd is not an essential nutrient thus addition of different chelators can improve availability and mobility of the metal (Duarte et al. 2007). Furthermore, CA has shown positive role in phytoextraction causing enhanced mobility of heavy metals by lowering pH values of soil (Chen et al. 2003). In the contrary to this, Gao et al. (2011) showed that citric acid has no effect on soil pH and indicates the involvement of CA in metal uptake despite changes of pH in rhizosphere. In context to that, data in this work revealed that the addition of CA improved mobilization of Cd from roots to aerial parts in *S. viminalis* and *S. alba*, while in *S. matsudana* that was not the case. Furthermore, the addition of CA did not cause acidification of soil; only slightly decrease of pH in soil, which is in line with other studies (Quartacci et al. 2005, Gao et al. 2012). In addition to the aforementioned, it could be concluded that the possible reason for enhanced mobilization and solubility of Cd in plants relies on combined effect of CA through decreasing pH values of soil and forming mobile complex with ligands. These organometallic complexes are relatively stable in nutrient solution and root surface, therefore free Cd ions can be easily taken up from the soil by plant roots and accumulated in different plant organs (Wuana et al. 2010). The beneficial effects of citric acid on *Salix* plants exposed to Cd stress were estimated in roots as well as in young leaves in *S. alba* and *S. matsudana*, while in mature leaves only in lower applied Cd concentration (3 ppm Cd). Addition of chelating agents may cause activation of ATPases in the root plasma membrane, and therefore elevated ions transport, as well as Cd uptake through symplastic or apoplastic pathways. Furthermore, different studies (Meers et al. 2005, Ehsan et al. 2014, Han et al. 2016) recorded that chelating agents, including citric acid, improves absorption of heavy metals and the plant ability to detoxify Cd. These findings are related to increased activation of antioxidative defense system and enhanced compatible solute accumulation in presents of CA (Kaur et al. 2017). Besides the positive effect in improving Cd uptake, citric acid is attractive for phytoextraction studies, due to its natural and biodegradable properties and thus it does not provoke secondary pollution.

Metal tolerance as a combination of high metal accumulation with no reduction of plant biomass is a very valuable feature for phytoextraction process. A number of studies illustrate differences in tolerance within *Salix* genus, toward heavy metals, which is associated to high genetic variation among willows (Zacchini et al. 2009). In general, all analyzed genotypes showed Ti > 60, and therefore could be defined as tolerate species under moderate Cd stress (Zacchini et al. 2009). The addition of CA has been advantageous in increasing tolerance potential under both applied Cd concentrations (Figure 4). The effect of CA on tolerance varied among the tested willow clones. The highest increase of Ti was recorded in *S. viminalis*, 16.82 % at Cd_3+CA treatment and 30.21 % at Cd_6+CA in comparison to plants exposed to Cd applied alone. Besides biomass production, the removal of heavy metals from contaminated soil is closely relate to photosynthetic and transpiration rates. A number of studies have confirmed the variation in the photosynthetic response of different willows due to the applied Cd concentration, genotypic characteristics, and experiment duration (Vassilev et al. 2005, Pajević et al. 2009). The data from recent study showed that exogenously applied CA could minimize heavy metals stress, through maintaining photosynthetic properties on high level (Han et al. 2016). Results of this study revealed that the photosynthetic response of the tested genotypes was species specific under different treatments and was dose dependent. Correlation coefficient revealed significant negative correlation between photosynthetic rate and Cd content in the leaves of *S. viminalis* and *S. alba* (Table 1), which confirmed the strong impact of cadmium to photosynthesis. Similar to these findings, negative influence of Cd on photosynthetic parameters was recorded in different fast-growing tree species (Lunackova et al. 2003, Mleczek et al. 2010, Nikolic et al. 2015, Župunski et al. 2016). Moreover, Pietrini et al. (2010) observed that the willows had efficient strategy for protecting and maintaining high net photosynthetic rate, with respect to poplar clones. Different response among species was the result of special distribution of Cd in leaf, in willows Cd was mostly distributed in areas near the main vein, causing no damage of photosynthetic tissue. In this work, the addition of CA reduced the negative effect on CO_2 assimilation influenced by Cd contamination. This is in agreement with Gao et al. (2011) who reported a complete restoration of assimilation of CO_2 at Cd–Pb contamination with the addition of CA in soil, in comparison to no CA. These findings point out the involvement of CA in photosynthetic response under Cd pollution.

According to the literature data willows are well known as a 'water pump' with high evapotranspiration rate (Pajevic et al. 2009). The water regime is closely related to biomass production, and has an influence on photosynthetic activity. The presence of various heavy metals in plant tissue can cause disturbance in water and ion uptake which consequently has negative effects on the plant water balance in different species (Pietrini et al. 2010), thus high WUE (water use efficiency) is important feature for determining the plant ability to cope with heavy metals contamination in soil. In this study, WUE in most cases was not significantly influenced by Cd stress. Furthermore, plants grown in the presence of CA showed no statistically significant differences in WUE comparing with no CA added. This pattern occurs in all tested willows and could be explained by the fact that selected willow clones have good strategy to tolerate accumulated Cd. Furthermore, high photosynthesis is associated with stomatal opening as well as with high transpiration rate which enable sufficient CO_2 content (Pietrini et al. 2010). Substomatal CO_2 concentration showed reduction under the Cd contamination. The partial closure of stomata under the heavy metal presence in plants might be related with reduction of photosynthesis. The results obtained in this study are in correlation with Ehsan et al. (2014) who reported that Cd suppressed photosynthetic properties in hyperaccumulator species *Brassica napus*. The stomatal conductance (gs) was decreased in *Salix alba* and *S. viminalis* plants exposed to a higher Cd dose applied. In addition, CA showed a positive effect by alleviating the reduction of stomatal conductance of *S. alba* when compared to Cd applied alone (Table 2). The low gs value limits assimilation rate by restricting CO_2 diffusion into the leaf, and causing changes in carbohydrate status, consequently leading to reduced biomass production (Lawson and Blatt 2014).

Presents of Cd can alter the concentration of photosynthetic pigments in different willow species (Lunáčková et al. 2003, Nikolic et al. 2015). The decrease in chlorophyll content could be contributed by both, the inhibition of its biosynthesis and the induction of its degradation. The presence of Cd can cause distortion of chloroplast ultrastructure leading to changes in thylakoids structure, manifested by a notable decrease in chloroplast number and size, grana stacking, and starch grain content. Among the tested species *S. alba* showed good potential to maintains the concentration of photosynthetic pigments under the Cd stress. It is well known that Cd stress induced reduction in chloroplast content leading to decrease in plant growth (Jiao et al. 2015). Since, young leaves of *S. alba* plants did not shown reduction in fresh weight of young leaves, it could be concluded that this clone has a good adaptation strategy to tolerate moderate levels of Cd in plant tissue.

Results of this study pointed out genotypic specificity in analyzed morpho-physiological parameters in willows exposed to different concentrations of Cd in soil. The presence of Cd caused reduction of leaf area in mature leaves of *S. viminalis* and *S. alba* in comparison to combined treated plants. The same pattern was observed in young leaves of *S. matsudana*. The addition of citric acid showed positive effects in alleviating heavy metal stress. The beneficial effect of applied citric acid in elevation of Cd bioavailability, uptake and therefore in overall phytoextraction by willows is confirmed in this work. The highest influence of citric acid on Cd translocation from roots to aerial parts was observed in *S. viminalis*. Moreover, citric acid showed positive role through maintaining photosynthetic response in Cd contaminated soil revealing its beneficial potential as a chelating agent. However, these preliminary results should be implemented in field trials and the effectiveness of this chelating agent should be confirmed in future research.

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