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Exogenously Applied Citric Acid Enhances Antioxidant Defense and Phytoextraction of Cadmium by Willows (*Salix* Spp.)

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Abstract The effect of exogenously applied citric acid (CA) on phytoextraction and antioxidant defense was analyzed using willow species (*Salix viminalis*, *S. alba*, and *S. matsudana*) grown in soil contaminated with cadmium (Cd). Citric acid has been used as a chelating agent for the purpose of accelerating the solubility of Cd in soil and enhancing the phytoextraction of selected plants. Willows were exposed to 6 mg/kg of Cd, following the same with citric acid (20 mM/kg soil). Results revealed a positive effect of citric acid in mobilization of accumulated Cd from roots to shoots and leaves. The addition of citric acid alleviated Cd toxicity by helping plants to overcome oxidative stress, through CA's chelating properties and the increased activity of antioxidant enzymes. Different protection strategies were evident through modification of activities of antioxidant enzymes such as catalase (CAT), ascorbate-peroxidase (APx), and guaiacol peroxidase (GPx) in young versus mature leaves in plants exposed to Cd. Furthermore, results revealed that addition of citric acid may be beneficial in the reduction of the negative effect of Cd stress on photosynthesis. The efficiency of coupling phytoextraction with the chelating agents represents a good strategy for decreasing damages caused by cadmium and has good potential in decontamination of a polluted environment.

Keywords Willows · Antioxidant enzymes · Soil phytoremediation · Cadmium · Stress tolerance

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1 Introduction

The worldwide distribution of heavy metals is one of the most abundant global problems due to their high environmental and health risk. Among various heavy metals, cadmium (Cd) is one of the leading toxic elements with high mobility, solubility, and bioavailability in the environment (Pietrini et al. 2015). Cadmium occurs naturally in soil, but problems arise with different anthropogenic activities such as mining, waste water management, intensive use of phosphate fertilizers, and urban traffic which are main sources of increased Cd content in the environment (Gallego et al. 2012). Cadmium is a non-essential element, which is persistent in soil for very long period, and thus, it is very toxic for living organisms. The presence of Cd in plant tissue is evident on overall metabolism, leading to decrease in plant biomass production, imbalance in mineral nutrition, and inhibition of photosynthesis and respiratory processes, disturbing synthesis of protein and activation of enzymes (Hawrylak-Nowak et al. 2015; Yang et al. 2015; Nogueirol et al. 2016). Besides the fact that Cd is not a redox active element, it can be involved in the production of reactive oxygen species (ROS) and

formation of free radicals, causing imbalance of redox homeostasis (Gill and Tuteja 2010), cellular damage, and lipid peroxidation (Gao et al. 2012). In order to survive, plants have developed different strategies to cope with heavy metal stress through activation of defense mechanisms. Based on the chemical and physiological properties of metals and plant species, these mechanisms could involve biochemical changes to adjust plant metabolism to stress condition. These mechanisms involved activation of the ascorbate-glutathione cycle enzymes and antioxidant enzymes such as catalase, peroxidases, etc. Other non-enzymatic substances can also be involved in plant defense mechanisms, like ascorbate, glutathione, some amino acids, and carotenoids (Mourato et al. 2012). Therefore, the understanding of mechanisms involved in stress tolerance is extremely important for understanding plant adjustment to polluted environment.

Phytoextraction is known as one of the widespread techniques for decontamination of polluted soil due its low cost, eco-friendly properties, and good effectiveness (Pajević et al. 2016). In this context, recent studies showed that chelate-assisted phytoextraction is proposed as a new technology with a high rate of decontamination of heavy metal-polluted soil (Gao et al. 2012; Ehsan et al. 2014). Different chelating agents have been used to enhance metal solubility in the soil and thus enhanced plant uptake (Wuana et al. 2010). The ability of low molecular weight organic acids (LMWOAs) such as citric acid to accelerate removal of heavy metals has attracted increasing attention during recent years (Habiba et al. 2014; Agnello et al. 2016). The importance of chelating processes with organic acid is their high biodegradability (Quartacci et al. 2005), low phytotoxicity (do Nascimento et al. 2006), and bonding with heavy metals and thus the prevention of leaching of heavy metals to groundwater (Souza et al. 2013). Due to those mentioned above, citric acid has the appropriate characteristics for chelate-assisted phytoextraction. On the other hand, the physiology-chemical effectiveness of citric acids like chelating agents is still unclear. A few recent studies showed that citric acid can mitigate the toxic effects of heavy metals through activation of antioxidant systems in plants (Sun and Hong 2011; Ehsan et al. 2014). Furthermore, the selection of plant species and genotype is crucial for remediation process. Numerous studies highlighted the use of fast-growing woody plants for phytoremediation process (Pajević et al. 2016). The *Salix* species are the leading candidate for phytoremediation according to their properties such as high biomass production, rapid growth, deep root system, as well as the ability to accumulate large quantities of different heavy metals (Landberg and Greger 2002; Yang et al. 2015). The wide distribution of these species over the northern hemisphere and their adaptability to different ecological conditions are advantageous features for their use in the decontamination of polluted areas, notably along watercourses (Greger and Landberg 1999; Dickinson and Riddell-Black 2014). Furthermore, willow species have great potential for vegetative propagation; thus, they have the ability to form roots from stem cuttings. The rapid and efficient vegetative propagation from stem cuttings leads to the production of large number of new shoots and high biomass production in a short period of time (Pajević et al. 2016). Concerning all the above mentioned, the aim of this study was to determine the role of citric acid in activation of defense mechanisms as well as the possibility of citric acid to promote decontamination of Cd-polluted soil through elevation of accumulated Cd in aboveground parts among *Salix* species.

2 Materials and Methods

2.1 Experiment Design

Plant material consisted of three willow genotypes *S. viminalis* L. (clone SV068), *S. matsudana* Koidz. (clone SM4041), and *S. alba* L. (clone V-158) which was selected at the Institute of Lowland Forestry and Environment in Novi Sad. Plants were grown in a greenhouse in semi-controlled conditions as follows: 12 h light/12 h dark photoperiod, 20–25 °C temperature, and 55–60% relative humidity. Six woody cuttings, approximately 20 cm long and 1 cm wide, with one shoot per cutting, were grown by soil culture method. Plants were grown in the Mitscherlich pots containing 5 kg of soil. Soil was irrigated with water permanently to maintain optimal soil humidity. Water used for irrigation was tap and drinking water, free of cadmium. Cadmium was supplied as nitrate salt dissolved in deionized water ($CdNO_3 \times 4H_2O$) in concentration of 6 mg/kg applied alone (Cd_6), following the same with addition of citric acid (Cd_6 + CA) in concentration of 20 mmol per kilogram of dry soil. Concentration of cadmium was selected according to Official Gazette of Republic of Serbia, and present doubled level of permitted level of

Cd (2MPC) in soil (Official Gazette of the Republic of Serbia 2010 and 2011). Plants were harvested after 90 days and divided into roots, shoots, young leaves, and mature leaves. The four fully expanded leaves from the top of each plant in each treatment were selected as young leaves, and the other group is defined as the mature leaves.

2.2 Analysis of Metal Content

Plant material was dried at 80 °C, for approximately 48 h to constant mass (Zupunski et al. 2016), and then it was powdered in a mixer grinder. The dried samples were heated and mixed with 33% H_2O_2. The dry-aching process was performed at 450 °C for 4 h to complete mineralization, followed by addition of 25% HCl. The total amount of Cd was determined in roots, stem, and young and mature leaves using an employing flame atomic absorption spectrophotometry (Varian, AAS240FS). The analysis of each sample was performed in three independent replicates.

Enrichment factor (Ef) was calculated by dividing the ratio of metal content in the plant to the metal content in the soil. Translocation factor (Tf) was calculated by dividing cadmium concentration in the aerial parts (μg/g) by cadmium concentration in the roots (μg/g) according to Al-Qahtani (2012).

2.3 Soil Characterization and Analyses

Soil was obtained from a forest plantation nursery near Novi Sad, Serbia (45.3025°N 19.9385°E). The tested soil was small sandy fluvisol. The main soil parameters ($CaCO_3$, N, P, K, humus) were calculated by methods officially accepted by YSSS (1966). Total N, P, and K contents were 0.25%, 2.25, and 2.74%, respectively. The content of humus was 1.12%, while $CaCO_3$ content was 19.78%. Prior to analysis, the soil samples were air dried, crushed, and sieved through a 2-mm mesh. Soil pH was measured potentiometrically in suspensions. The pH of soil was measured using 10 g of soil and mixed with 25 ml of 1 M KCl, as well as in H_2O. Samples were agitated on a shaker for 30 min. The pH was 7.8 (in KCl) and 8.4 (in H_2O). The content of Cd was measured in soil in the beginning of the experiment after the addition of Cd, as well as in the moment of harvest of plants (Table 1). Bioavailability of Cd from soil was calculated according to Takáč et al. (2009) using 10 g of air-dried soil and mixed with 100 ml of 0.1 M $CaCl_2$ and shaken for 1 h. Cd content was determined in extract using AAS.

Free proline accumulation Proline was determined in both young and mature leaves by the standard method of Bates et al. (1973). The procedure was based on proline's reaction with ninhydrin reagent. The 0.5 g of plant tissue was extracted using 5 ml 3% sulfosalicylic acid. After centrifugation (10 min at 12,000 rpm), 2 ml of supernatant was mixed with ninhydrin acid and glacial acetic acid and was incubated at 100 °C for 15 min. The reaction was stopped on ice and the cromophore was extracted with 4 ml toluene. The absorbance was measured using a spectrophotometer at 520 nm and expressed on fresh weight of leaves as μg proline g^{-1}.

2.4 Lipid Peroxidation

Lipid peroxidation (LPx) measurement by thiobarbituric acid assay was analyzed in young and mature leaves by measuring the malondialdehyde content (MDA). MDA is one of the final products of lipid peroxidation, and it is quantified according to Devasagayam et al. (2003). Leaf extract (0.5 ml) was homogenized with 4.5 ml of 2-thiobarbituric acid (TBA). Samples were mixed with the TBA reagent and incubated in a boiling bath at 95 °C for 20 min. After cooling, the solution was centrifuged at 3000 rpm for 10 min. The absorbance of supernatant was measured at $\lambda = 532$ nm. The blank contained the reagent without biological samples. The MDA concentration was calculated using an extinction coefficient of 1.56×105 mol^{-1} cm^{-1}.

2.5 Reduced Glutathione

Content of reduced glutathione was evaluated using the method of Kapetanović and Mieyal (1979). The procedure was estimated using Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid) or DTNB) (0.1 mM) prepared with 0.1 M potassium phosphate buffer (pH 7.0). The plant extract was prepared using 0.5 g of plant material with addition of 5% sulfosalicylic acid, and the mixture was centrifuged for 10 min at 200 rpm. The supernatant (200 μl) was mixed with 2 ml of Ellman's reagent, and absorbance was measured using a spectrophotometer at 420 nm.

Table 1 Cd availability and total metal content in soil (μg/g) in *Salix* spp. in control plants (Control), plants treated with citric acid (CA), plants exposed to Cd applied alone (Cd_6), and in combination with citric acid (CA + Cd_6)

Treatment/species	Initial Cd content in soil in the beginning of experiment	Cd content in soil after plant harvest			Cd bioavailability in soil ($CaCl_2$ method)
		S. viminalis	*S. matsudana*	*S. alba*	
Control	0.39	0.36	0.35	0.35	nd
Cd6	6.25	5.36	5.42	5.35	0.26
CA	0.38	0.35	0.34	0.36	nd
CA + Cd_6	6.39	5.13	5.33	5.62	0.46

nd Not detected Cd in soil samples

2.6 Plant Preparation for Enzyme Assays

Approximately 10 g of plant material (young and mature leaves, separately) was ground with liquid nitrogen and stored in −80 °C. Crude leaf extracts were crushed with a mortar and pestle and extracted in 5 volumes of 50 mM phosphate buffer, pH 7.0, containing 1 mM EDTA, and 1% polyvinyl pyrrolidone (PVP). The extract was centrifuged at 4 °C at 15,000 rpm for 15 min, and the supernatants were used for biochemical analyses. All enzyme activities were measured in triplicate for each sample using a Beckman DU–65 spectrophotometer. The total protein concentration in each leaf extract was measured according to Bradford (1976).

Catalase (CAT, EC 1.11.1.16) activity was assayed by the method of Claiborne (1984), which is based on H_2O_2 degradation by following decrease in absorbance at 240 nm. The reaction mix contained 50 mM potassium phosphate buffer (pH 7.0), 12.5 mM H_2O_2, and 100 μl enzyme extract.

Ascorbate peroxidase (APx, EC 1.11.1.11) activity was determined by oxidized ascorbate observing with the decrease in absorbance 290 nm, as described by Nakano and Asada (1981). The reaction mix contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 10 mM ascorbate, 0.25 mM H_2O_2, and 25 μl enzyme extract for a total volume of 1 ml. Calculations of activity was made using an extinction coefficient of 2.8 $mmol^{-1}$ cm^{-1}.

Guaiacol peroxidase (GPx; 1.11.1.7) activity was evaluated according to Simon et al. (1974). The reaction was observed as increasing of absorbance at 436 nm as oxidation of guaiacol and transformation to tetraguaiacol. The reaction mix contained 0.1 M potassium phosphate buffer (pH 7.0), 0.1 mM guaiacol, 12.3 mM H_2O_2, and 100 μl enzyme extract. The activity of GPx was calculated using an extinction coefficient of 25.6 $mmol^{-1}$ cm^{-1}.

2.7 Photosynthetic Parameters

Photosynthetic activity of plants was measured on the fourth fully expanded leaf. Measurements were conducted on three different plants per treatments, and three repetitions were recorded for each. Net photosynthetic rates (A), 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). Light conditions were set using the $LCpro^+$ light unit, which emitted photosynthetic active radiation (PAR) at 1000 μmol m^{-2} s^{-1}. The air supply unit provided a flow of ambient air to the leaf chamber at a constant rate of 100 μmol m^{-2} s^{-1}. Temperature, CO_2 concentration, and humidity were at ambient levels. Parameter water use efficiency (WUE) was calculated as the ratio of photosynthetic and transpiration rate and expressed in μmol of CO_2 m^{-2} s^{-1}/mmol of H_2O m^{-2} s^{-1}.Stomatal conductivity of water vapor was expressed in unit mol H_2O m^{-2} s^{-1}. Measurements were performed in the middle of the daily photoperiod at temperature (23 ± 2 °C) conditions and atmospheric CO_2 concentration.

2.8 Statistical Analyses

The obtained data were expressed as mean of three replicates ± standard deviation (SD). Data were subjected to the analysis of variance (two-way ANOVA) and followed by post hoc Fisher multiple comparison test at a significance level of $p < 0.05$.

3 Results and Discussion

3.1 Effect of CA on Cd Uptake, Accumulation, and Translocation

In order to analyze whether CA has positive role in accelerating uptake of Cd, the content of Cd was measured in different plant organs and summarized in Fig. 1. Results revealed the highest cadmium content in young leaves, followed by those in roots, mature leaves, and stem, respectively. In general, young leaves showed higher ability to accumulate Cd in comparison to mature leaves in all tested species; therefore, it could be concluded that the growing phase is an important character in Cd accumulation (Fig. 1). The Cd distribution trend was in line with previous research; Yang et al. (2015) have compared 39 clones of willow species grown hydroponically and revealed the highest content in roots, followed by leaves and stem. In general, the authors recorded significantly higher concentrations of Cd accumulated in plants when compared to our results. The data from different studies are often difficult to compare because many factors are involved in heavy metal uptake, such as type and pH of grown media, duration of experiment, and selection of plant species (Pajević et al. 2016). The plant bioavailability of metals in soils is influenced by several factors such as pH, cation exchange capacity, organic matter content, and soil texture (Gallego et al. 2012). The major factor with limiting effect on absorption of Cd from soil to roots was the alkaline pH of the present soil. Besides that fact, slightly increased Cd content was evident in the root of *S. matsudana* and *S. alba* plants exposed to CA in comparison to plants treated only with Cd (Fig. 1). It is well known that lower pH of soil stimulates the desorption of heavy metals in soil (Ali et al. 2013), thus promoting that the role of citric acid in alkaline soil has great importance for increasing the bioavailability of Cd in soil for uptake by willows. The effectiveness of citric acid in enhancing the uptake of heavy metals from root to shoots is lower in comparison to synthetic chelating agents, such as EDTA (Souza et al. 2013; Chigbo and Batty 2015). This might be contributed to the biodegradability of citric acid in soil, indicating that the formation of complex between citric acid and metals is relatively stable. At the same time, this is one of the major advantageous features of citric acid, that it can be applied during vegetative growth stage without the addition of consequences or damage to the environment, in comparison to synthetic chelating agent (Souza et al. 2013). Further, the beneficial effect of exogenously applied CA in phytoextraction ability was recorded in young leaves of *Salix* spp., as well as in mature leaves of *S. alba*, while *S. viminalis* and *S. matsudana* did not show statistically significant elevation of Cd accumulation in plant tissue when CA is applied. In general, significant differences in Cd accumulation due to the CA applied were evident among willow species (Fig. 1). *S. viminalis* showed the highest capacity to accumulate Cd in young leaves under conditions in which citric acid is added, which was 38% higher when compared to Cd accumulation in *S. alba* plants exposed to the same treatment. This result indicates that the effectiveness of citric acid varied depending upon the concentration of the selected heavy metal, plant species, organ, and growth stage (Ehsan et al. 2014; Agnello et al. 2016).

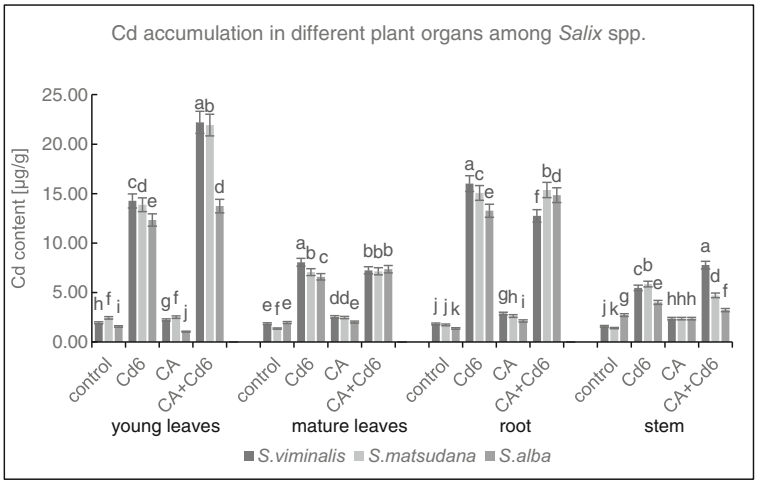

Fig. 1 Cadmium accumulation in different plant organs among *Salix* spp. exposed to 6 mg/kg Cd (Cd_6) and 20 mM/kg CA in dry soil (CA + Cd_6). Values are mean ± SD ($n = 3$). Different *letters* within the group (plant organ) indicate that values are significantly different at $p \leq 0.05$

Enhanced Cr uptake and accumulation was evident in *Brassica napus* plants treated with different concentrations of chromium in combination of citric acid (Afshan et al. 2015).

In addition to the total metal content, the distribution of Cd within the plant is an indirect feature which shows detoxification mechanisms in plants. Thus, enrichment factor and translocation factors were calculated in order to quantify the effectiveness of the selected willow genotypes for decontamination of polluted soils (Fig. 2). According to literature data, enrichment factor in moderate Cd-accumulated soil range from 1 to 10 (Irfan et al. 2012). Analyzed *Salix* plants showed high enrichment factor ranging from 6.31 to 8.17. Presence of citric acid caused increase of Ef values in comparison to no CA addition (Fig. 2). Furthermore, citric acid was associated with translocation of accumulated Cd in plant tissue, thus translocation factor (Tf) showed higher values in plants treated with combined treatments (Fig. 2). In comparison to non-CA-treated plants, translocation factor was enhanced in *S. matsudana* (37%), followed by *S. alba* (32%) and *S. viminalis* (30%), respectively. Plants with Tf values higher than 1 are classified as high-efficiency plants for decontamination of polluted soils (Al-Qahtani 2012). Findings of this study confirmed the positive effect of citric acid in mobilization of accumulated Cd from roots to shoots and leaves. This might be attributed to the bioavailability of Cd in soil treated with citric acid which was almost twofold higher than in soil with Cd applied alone (Table 1). The highest potential for Cd removal in moderately polluted soil in the presence of citric acid was recorded by *S. viminalis* in comparison with other tested species. However, application of citric acid did not induce elevation of Cd removal from soil using *S. alba* plant. Exogenously applied citric acid has a dominant role in heavy metal detoxification mechanisms causing desorption of heavy metals from the soil and promotes entering of Cd ions to roots and therefore improves metal transport into the xylem (Xie et al. 2014). According to the abovementioned, citric acid has a positive impact in chelating process and could promote the translocation of heavy metals to the aerial plant parts.

3.2 Effect of CA and Cd on Free Proline Content

In response to heavy metal stress, plants can accumulate different amino acids, such as proline, which plays an important role in osmoregulation and osmotolerance and has beneficial effect in heavy metal detoxification processes (Nikolić et al. 2008; Bauddh et al. 2016). Thus, the accumulation of proline is a very useful tool for assessing plant tolerance mechanisms in stress conditions. The results showed that proline accumulation depended on cadmium concentration, species, and leaf age (Table 2). Higher proline accumulation was estimated in young versus mature leaves which may be due to its protective role in plant metabolism. An important role of proline is its ability to reduce metal toxicity by metal bonding to the cell wall, by reducing transport across the cell membrane, and by active efflux (Bauddh et al. 2016). Proline content was markedly increased in young leaves of *S. viminalis*, while the exogenously applied CA showed beneficial effects through maintaining stress on lower level by reducing proline production. The high level of proline production in young leaves is correlated to its role in protection of thylakoid membranes by quenching ROS. Proline is a well-known indicator of stress conditions and has multiple functions, such as osmoprotectant, by helping to maintain sufficient cell turgor for growth (Sun and Hong 2011), as scavenger of ROS and stabilizer of membrane, and sink for energy to regulate redox potential (Semida Wael et al. 2015), as well as source of nitrogen and carbon for post-stress growth (Sharma and

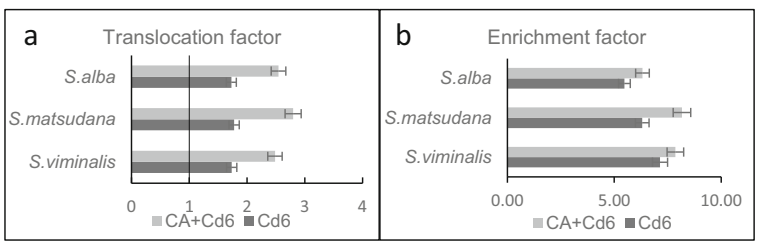

Fig. 2 **a** Translocation factor and **b** enrichment factor of *Salix* spp. exposed to Cd applied alone (Cd_6) and in combination with citric acid applied (CA + Cd_6). Values are mean of three independent samples ± SD

Table 2 Proline content, antioxidant enzymes, reduced glutathione, and MDA content in *Salix* spp. exposed to Cd applied alone (Cd_6) and in combination with citric acid (CA + Cd_6)

	CAT activity (Units/mg)					
	Young leaves			Mature leaves		
Treatments	*S. viminalis*	*S. matsudana*	*S. alba*	*S. viminalis*	*S. matsudana*	*S. alba*
Control	0.50 ± 0.05bcd	0.08 ± 0.11fg	0.02 ± 0.01g	0.15 ± 0.12efg	0.04 ± 0.01fg	0.01 ± 0.01g
Cd_6	0.87 ± 0.05ab	0.64 ± 0.25abcd	0.38 ± 0.05def	0.39 ± 0.13cd	1.19 ± 0.23a	0.76 ± 0.07b
CA	0.44 ± 0.24cdef	0.28 ± 0.09efg	0.17 ± 0.05efg	0.33 ± 0.14c	0.25 ± 0.03de	0.24 ± 0.05de
CA + Cd_6	0.97 ± 0.12a	0.83 ± 0.25abc	0.78 ± 0.17abc	0.69 ± 0.01b	0.86 ± 0.03b	0.41 ± 0.1cd
APx activity (Units/mg)						
Control	0.34 ± 0.29bcd	0.02 ± 0d	0.21 ± 0.12bcd	0.26 ± 0.06bc	0.27 ± 0.09bc	0.02 ± 0.01d
Cd6	1.11 ± 0.21a	0.12 ± 0cd	0.22 ± 0.06bcd	0.37 ± 0.06b	0.36 ± 0.09b	0.25 ± 0.09bc
CA	0.08 ± 0.01cd	0.10 ± 0.04cd	0.02 ± 0.01d	0.25 ± 0.06bc	0.76 ± 0.19a	0.06 ± 0.01cd
CA + Cd_6	0.36 ± 0.04bc	0.47 ± 0.12b	0.06 ± 0.01cd	0.24 ± 0.04bcd	0.32 ± 0.16b	0.06 ± 0.01cd
GPx activity (Units/mg)						
Control	0.09 ± 0.04c	0.14 ± 0.03bc	0.04 ± 0.02c	0.11 ± 0.04cd	0.12 ± 0.08cd	0.04 ± 0.04cd
Cd6	0.65 ± 0.5a	0.73 ± 0.03a	0.26 ± 0.2bc	0.10 ± 0.05cd	0.34 ± 0.12ab	0.1 ± 0.06cd
CA	0.05 ± 0.02c	0.06 ± 0.04c	0.04 ± 0.03c	0.01 ± 0.01d	0.10 ± 0.01cd	0.19 ± 0.09bc
CA + Cd_6	0.75 ± 0.01a	0.44 ± 0.02ab	0.03 ± 0.023c	0.16 ± 0.09 cd	0.44 ± 0.09a	0.25 ± 0.15bc
MDA content (nmol/mg)						
Control	49.83 ± 2.71cde	25.60 ± 3.25fgh	12.98 ± 0.34h	75.19 ± 0.02cd	58.70 ± 1.25de	22.27 ± 7.89fg
Cd_6	69.49 ± 0.26ab	58.82 ± 4.83bcd	43.41 ± 2.58def	126.62 ± 14.83a	92.76 ± 12.68bc	65.70 ± 12.9de
CA	34.62 ± 5.71ef	36.17 ± 3.17efg	22.54 ± 2.57gh	56.40 ± 5.72de	33.65 ± 7.98f	14.60 ± 1.94g
CA + Cd_6	66.70 ± 2.76abc	79.38 ± 9.89a	62.20 ± 2.98abc	96.97 ± 5.09b	60.78 ± 4.83de	55.30 ± 5.62e
GSH (μmol/mg)						
Control	30.07 ± 5.13e	26.54 ± 2.91e	31.43 ± 1.59e	81.37 ± 5.13d	24.87 ± 2.91e	35.20 ± 1.59e
Cd_6	60.53 ± 4.31d	153.03 ± 7.35b	171.63 ± 24.29b	187.67 ± 4.31b	132.20 ± 7.35c	176.47 ± 24.29b
CA	17.23 ± 1.42e	56.67 ± 8.45d	16.53 ± 3.57e	69.13 ± 1.42d	20.53 ± 8.45e	32.17 ± 3.57e
CA + Cd_6	221.27 ± 27.42a	208.47 ± 19.55a	121.9 ± 20.78c	115.27 ± 27.24c	124.07 ± 19.55c	236.54 ± 20.78a
Proline (μg proline g^{-1} FW)						
Control	35.99 ± 0.44bc	24.11 ± 2.61c	32.34 ± 5.30bc	19.27 ± 1.85bcd	7.5 ± 0.38e	15.73 ± 2.95cde
Cd6	101.66 ± 29.33a	31.82 ± 1.53bc	48.95 ± 5.98bc	27.5 ± 1.16ab	18.44 ± 3.86bcde	33.13 ± 6.69a
CA	24.38 ± 4.33c	26.82 ± 7.47c	23.12 ± 9.4b	11.56 ± 1.33de	10.36 ± 1.94de	9.01 ± 0.53de
CA + Cd_6	39.79 ± 6.05bc	22.70 ± 1.73c	64.38 ± 1.8b	24.84 ± 8.11abc	13.44 ± 0.67cde	20.10 ± 3.65bcd

Values are mean ± S.D. Statistical analyses was done with two-way ANOVA and followed with Fisher's LSD test. Different lowercase letter within the group (young or mature leaves) indicates statistically significant differences.

Dietz 2009). The accumulation of proline is related to disturbed water regime under heavy metal stress. Since water balance was pretty stable in almost all treatments, the content of free proline confirmed good adaptation and tolerance potential of the analyzed willows. Furthermore, literature date revealed correlation in proline synthesis with the reduction state of glutathione (GSH) (Sharma and Dietz 2009) which might be attributed to the strategies of plants to cope up with heavy metal toxicity. The chelates are reported to promote uptake and translocation of different heavy metals regardless of plant species, as well as protect plants from oxidative damage (Afshan et al. 2015; Agnello et al. 2016). The metabolic profiling of bermudagrass confirmed that the production

of proline and citric acid in Cd-exposed plants has a dominant role in defense pathways, and these metabolites are involved in the adaptation to Cd stress (Xie et al. 2014).

3.3 Effect of CA on Activities of Antioxidant Enzymes, Reduced Glutathione, and Malondialdehyde Contents Exposed to Cd

It is well known that heavy metals can alter plant metabolism through generation of free radicals and reactive oxygen species. However, there is lack of data on the effect of citric acid on plant cell oxidative properties. In order to analyze the impact of citric acid in Cd-contaminated soil on the production of antioxidant enzymes and non-enzymatic antioxidants, the activities of CAT, APx, GPx, MDA, and GSH contents were measured (Table 2). Overproduction of ROS can lead to oxidative injury such as membrane lipid peroxidation, protein oxidation, enzyme inhibition, and DNA and RNA damage (Mittler 2002). However, different antioxidant enzymes and non-enzymatic constituents such as glutathione are responsible for ROS scavenging under stress conditions. Furthermore, it is still unknown if citric acid has the same effect during plant growth; thus, it was separately analyzed in young versus mature leaves. ROS scavenging mechanisms include CAT and APx as leading detoxifying enzymes in plants which convert H_2O_2 to water and molecular oxygen (Smiri et al. 2013). Under presence of cadmium, catalase activity was significantly increased in all tested genotypes in both young and mature leaves. The addition of CA in Cd-treated plants caused increase of catalase activity in young leaves, while in mature leaves of *S. matsudana* and *S. alba*, there was a reduction in CAT activity when compared to the no CA treatment. The elevation of CAT activity in young leaves of CA-treated plants could be contributed to higher Cd content in CA-treated plants. Previously reported data showed that CA could be useful for plants to overcome oxidative stress by enhancing their antioxidant enzyme activities under metal stress (Najeeb et al. 2011). The overexpression of antioxidant enzymes is a powerful tool for plant survival in conditioning of elevated heavy metals (Ehsan et al. 2014; Nogueirol et al. 2016). The important role of APx is to mitigate the harmful effects of free radical in oxidative stress conditions caused by the presence of heavy metals, high salinity, high alkalinity, or drought (Sun and Hong 2011). In the present study, the highest increase of APx activity (threefold higher than control) was evident in young leaves of *S. viminalis* plants exposed to Cd_6 treatment (Table 2). The predominant role of APx in the neutralization of H_2O_2 was most evident in young leaves in comparison to mature leaves, which is contributed to the higher Cd content in young leaves. However, in mature leaves of *S. matsudana*, reduction in APx activity was recorded in the presence of CA in combination with Cd applied. Specific response in activity of APx is combined to the complexity of the interactions of APx with other antioxidants. Citric acid caused reduction of APx activity in young leaves of *S. viminalis* and *S. matsudana* in Cd-treated plants, while that was not the case in the mature leaves of the same plants. The beneficial effect of CA applied in significant reduction in the APx activity was recorded in spinach exposed to different Pb regimes (Khan et al. 2013). Guaiacol peroxidase is widely accepted as stress enzyme whose activation could be induced by Cd exposure (Bauddh et al. 2016). The presence of Cd initiates elevation of GPx activity mainly in young leaves versus mature leaves, with higher influence on *S. viminalis* and *S. matsudana* than in *S. alba* plants. However, exogenous application of citric acid did not cause changes in GPx activity neither in control nor in Cd-exposed plants. In agreement to our research, Afshan et al. (2015) recorded significant increase of catalase, guaiacol, and ascorbate peroxidase activity in *B. napus* plants exposed to 100 μM of Cr content in both leaves and roots. On the other hand, Smiri et al. (2013) observed that both, catalase and guaiacol peroxidase, showed lower activity during germination seeds of pea at 5 mM concentrations of Cd. The authors concluded that the activity of antioxidant enzymes are not correlated to Cd tolerance (Smiri et al. 2013). In addition to that, it could be concluded that different mechanisms are involved in tolerance to heavy metal stress, with regard to the applied metals and their concentrations and plant species.

Lipid peroxidation is an effective indicator of cellular oxidative damage causing the oxidative deterioration of lipids resulting in damage of biological membrane (Khan et al. 2013). This non-specific oxidation of lipids results in an increase of malondialdehyde (MDA) content. In the present study, the level of MDA production was significantly increased in both young and mature leaves among willow genotypes in the presence of Cd. Mature leaves showed higher production of MDA indicating higher damages of cell membrane. At the same

time, addition of CA caused statistically significant changes in MDA production depending on plant species, level of Cd accumulated, and maturity of leaves. Exogenous citric acid had potential to promote defensive mechanisms to avoid stress conditions.

The results of many studies have suggested that the tripeptide glutathione (GSH) is an important metabolite with a dominant role in the tolerance to heavy metals (Foyer et al. 2001; Gao et al. 2012). GSH is an important compound for the synthesis of phytochelatins, which are involved in the long-distance transport in the xylem and detoxification in the cytoplasm and in vacuole. The sequestration of heavy metals by phytochelatin complexes in the vacuole may cause transformation of accumulated metals into metabolically less harmful forms. Treatment with CA led to increased production of GSH mostly in young leaves (Table 2) while that pattern was observed only in mature leaves of *S. alba*. Under heavy metal stress, plants are able to produce high doses of acid-soluble thiols for binding Cd (Gao et al. 2012); thus, production of GSH in our study was correlated to the presence of Cd in plant tissue. Although considerable changes in biomass production and phytotoxic effect such as chlorosis and necrosis were not recorded, changes in production of antioxidant enzymes and non-enzymatic compounds were evident as a consequence of the presence of Cd and CA in plants.

3.4 Effect of CA and Cd on Photosynthetic Activity

Stable photosynthetic activity under heavy metal stress is a beneficial feature for plant survival and growth potential, resulting in successful and efficient phytoextraction. Gas exchange parameters in willows exposed to Cd applied alone and in combination with CA are presented in Table 3. Net photosynthetic rate (A) did not differ in control in comparison to CA-treated plants. At the same time, plants exposed to Cd showed significant decrease of net assimilation rate of CO_2 with the highest reduction (34%) recorded in *S. viminalis* plants. Disruption of photosynthetic apparatus under Cd stress is related to reduce chlorophyll content and reduced activity of enzymes involved in CO_2 fixation (Anjum et al. 2016). As observed for photosynthetic

Table 3 Photosynthetic activity in *Salix* spp. exposed to Cd applied (Cd_6) and in combination of citric acid (CA + Cd_6)

Net assimilation rate of CO_2 (A) [μmol of CO_2 m^{-2} s^{-1}]				
Species/treatments	control	Cd6	CA	Cd6 + CA
S. viminalis	21.65 ± 0.88b	19.75 ± 0.74c	25.39 ± 0.16a	19.82 ± 0.06cd
S. matsudana	21.82 ± 0.32b	19.3 ± 1.06cd	21.19 ± 0.51b	19.1 ± 0.37cd
S. alba	18.65 ± 0.55d	17 ± 0.49e	18.78 ± 0.21d	16.42 ± 0.44e
Transpiration rate (T) [mmol of H2O m^{-2} s^{-1}]				
S. viminalis	3.14 ± 0.01c	2.89 ± 0.02d	3.36 ± 0.2b	2.96 ± 0.02d
S. matsudana	3.55 ± 0.01a	3.09 ± 0.03c	3.15 ± 0.04c	3.14 ± 0.04c
S. alba	2.62 ± 0.03e	2.56 ± 0.03ef	2.48 ± 0.05f	2.49 ± 0.01f
Water use efficiency (WUE) [μmol of CO2 m−2 s−1/mmol of H2O m−2 s−1]				
S. viminalis	7.56 ± 0.24c	7.72 ± 0.2c	10.26 ± 0.82a	8.68 ± 0.13b
S. matsudana	6.25 ± 0.1de	6.15 ± 0.13e	6.73 ± 0.34d	6.08 ± 0.34ef
S. alba	5.93 ± 0.22g	5.88 ± 0.06efg	5.59 ± 0.21fg	5.55 ± 0.13g
Stomatal conductance of CO2 (gs) [mol of CO2 m^{-2} s^{-1}]				
S. viminalis	0.49 ± 0.1d	0.28 ± 0.02g	1.16 ± 0.04a	0.57 ± 0.04b
S. matsudana	0.35 ± 0.04f	0.48 ± 0.1d	0.52 ± 0.1c	0.43 ± 0.13e
S. alba	0.35 ± 0.01f	0.29 ± 0.01g	0.35 ± 0.02f	0.29 ± 0.015g
Intercellular CO2 concentration (ci) [μmol/mol]				
S. viminalis	221 ± 9.64a	176 ± 3g	205 ± 2b	218 ± 4.16a
S. matsudana	185 ± 8.08efg	182 ± 6.02fg	197 ± 2.08bcd	202 ± 3.51bc
S. alba	191 ± 3.58def	200 ± 4.5bcd	182 ± 3.6fg	194 ± 9.53cde

Values are mean ± S.D. Statistical analyses was done with two-way ANOVA and followed with Fisher's LSD test. Different lowercase letter within the group (A, E, gs, or ci) indicates statistically significant differences

activity, similar pattern was evident for transpiration rate. However, in *S. matsudana* and *S. alba*, addition of CA reduced the toxic effects of Cd causing no reduction of transpiration rate in plants exposed to Cd along with CA applied. (Table 3). Excessive heavy metal presence in plant tissue leads to inhibition of photosynthetic activity and suppressed gas exchange in different woody plants (Pietrini et al. 2010; Pietrini et al. 2015). This may be attributed with high Cd ion mobility and potential to be transported in large quantities into leaves. Furthermore, *S. viminalis* and *S. matsudana* plants exposed to combine treatment (CA + Cd_6) showed significant increase of WUE, gs, and ci in comparison to Cd applied alone. Considering these results, it could be concluded that exogenously applied citric acid may have beneficial effect on overall photosynthesis. However, different genotypes showed species-specific response in presence of Cd along with CA addition in plant tissue. Results of this work are in line with Gao et al. (2012), who revealed enhanced CO_2 fixation in plants exposed to combined treatment of CA with cadmium and lead (Cd + Pb)-treated plants in respect to non-CA-treated plants of *Solanum tuberosum*. Application of CA can promote activation of antioxidant defense mechanisms and thus can reduce negative effect of Cd stress on photosynthesis. Observation from this work suggests the need for further analyses with the aim to explain strategies how citric acid affects photosynthetic properties.

4 Conclusion

Results of this study imply that improvements of phytoextraction could be obtained by the addition of chelating agents such as citric acid. The promoting role of citric acid was observed in increasing the bioavailability of Cd in soil which was almost twofold higher with addition of citric acid. Further, application of citric acid showed promoting effect on Cd accumulation and translocation to above plant parts and thus to overall phytoextraction potential. On the other hand, effectiveness of citric acid varied depending upon the plant species, organ, and leaf age. The highest potential for Cd removal from soil in the presence of citric acid was recorded by *S. viminalis*, while the highest translocation was enhanced in *S. matsudana*. Citric acid showed beneficial effect through maintaining stress on lower level by reducing proline production. Besides that, favorable effect of citric acid was evident in activation of antioxidant defense mechanisms. Elevation of activity of antioxidant enzymes was observed in young leaves, which was correlated with higher Cd accumulation. Further, the tolerance potential of analyzed *Salix* spp. was confirmed through stable photosynthetic activity. Our results highlighted the positive role of citric acid in phytoextraction, and at the same time, they point out genotypic specificity in mechanisms of antioxidant defense. The data from this work indicate that exogenous application of citric acid could be recommended as chelate-assisted approach for decontamination of polluted environment using willow species.

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