HortScience 54(7):1249–1257. 2019. https://doi.org/10.21273/HORTSCI14018-19

Phytoremediation of Cadmium-, Copper-, and Lead-contaminated Soil by *Salix mucronata* (Synonym *Salix safsaf*)

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Additional index words. accumulation, antioxidant, contaminating ions, reclamation, Safsaf willow

Abstract. **Phytoremediation is an environmentally friendly and effective method of reducing contaminating ions to very low levels. In this study, the effects of different concentrations of cadmium (Cd), copper (Cu), and lead (Pb) on vegetative growth and the chemical and biochemical compositions of *Salix mucronata* as well as the potential for phytoextraction of these metals by plant organs were investigated. *S. mucronata* had the highest survival percentage (100%) in the presence of $CdCl_2$, $CuCl_2$, and Pb acetate up to 80, 200, and 850 mg·kg^{-1} in soil, respectively. A negative influence of these metals on vegetative and chemical parameters was observed relative to the control plants. The potential role of antioxidant enzymes in protecting plants from oxidative injury was examined by analyzing the antioxidant enzyme activities of plants grown in contaminated and control soils. Enzymatic activities and electrolyte leakage were higher in the plants grown in soil with increasing heavy metals than in the control plants. The bioconcentrating efficiency of Cd, Cu, and Pb in plant organs was estimated to be medium [bioconcentration factor (BCF) of 1–0.1]; an exception was the BCF of Cu in the roots, which was estimated to be intensive (BCF < 1). Concentrations of 60 mg·kg^{-1} $CdCl_2$, 50 mg·kg^{-1} $CuCl_2$, and 650 mg·kg^{-1} Pb acetate caused significantly higher translocation compared with other levels of each pollutant. The biomass tolerance index was less than 1. Additionally, *S. mucronata* accumulated Cd, Cu, and Pb in the following order: roots > stems > leaves. Therefore, the risk of contamination through leaf fall can be minimized. Therefore, *S. mucronata* could be a good candidate for phytoremediation of Cd-, Cu-, and Pb-contaminated soil.**

Heavy metal–contaminated agricultural soil is a complex and serious phenomenon that has hazardous effects on the environment and, consequently, on humans, animals, plants, and beneficial microorganisms by influencing and tainting food chains, soil, irrigation or potable water, aquifers, and the surrounding atmosphere (Wuana and Okieimen, 2011). Cadmium (Cd) is an important poisonous element that is not known for any essential biological function. Furthermore, Cd may cause malfunctioning of metabolic processes (Campbell, 2007). In plants, Cd causes various types of damage, such as the disturbance of metal homeostasis resulting in iron deficiency in the shoot (Fodor et al., 2005). As a result, the biosynthesis of chlorophylls, the formation of Chl–protein complexes, and the development of thylakoid membranes are highly disturbed (Basa et al., 2014). Copper (Cu) is an important essential micronutrient that participates in many vital physiological functions of plants, including acting as a catalyzer of redox reactions in mitochondria, chloroplasts, and the cytoplasm of cells (Fargasova, 2004) or as an electron carrier during plant respiration (Yruela, 2009). Uptake of Cu by plants and its toxicity are contingent on the nutritional condition of the plant, Cu^{2+} concentration in the soil, exposure time, and plant species (Nicholls and Mal, 2003). Increased Cu can damage membranes and produce free radicals in different plant parts (Chen et al., 2000). Lead (Pb) accumulation in plant tissue impairs different morphological, physiological, and biochemical functions in plants, either directly or indirectly, and induces a range of deleterious effects. Pb causes phytotoxicity by changing cell membrane permeability and reacting with active groups of various enzymes involved in plant metabolism (Pourrut et al., 2011).

Phytoremediation has become an effective and affordable technological solution used to extract or remove metal pollutants from contaminated soil using plants. Plants possess a sophisticated and interrelated network of defense strategies to avoid or tolerate heavy metals or facilitate their de-toxification (Harada et al., 2010). To reduce the harmful impact of free radicals resulting from heavy metal stress, plant cells have developed an antioxidant defense mechanism (Sharma et al., 2012). Tree species have been suggested as appropriate plants for phytoremediation of heavy metal–contaminated soil because they provide several beneficial attributes such as large biomass, genetic variability, established management practices, economic value, public acceptability, and site stability (Pulford and Waston, 2003). To achieve good phytoremediation efficiency, plants should accumulate a significant amount of heavy metals, tolerate soil pollution, and produce a great quantity of biomass under contaminating conditions (McGrath et al., 2002). The advantages of phytoremediation are mainly due to its effectiveness in reducing contaminant ions to very low levels. Moreover, it involves the use of an inexpensive bio-sorbent material (Rakhshaee et al., 2009) applicable to a wide range of toxic metals and radionuclides (Liu et al., 2000) and is a low-cost and environmentally friendly method.

Salix spp. trees are important as a source of biomass for energy purposes (Zalesny

et al., 2016). Additionally, there has been a growing interest in their use for different environmental purposes and land reclamation, including phytoremediation and phytoextraction of contaminated soils (Dimitriou et al., 2012). Willows are recognized for their phytoremediation potential and phytoextraction of soils contaminated by heavy metals (Vervaeke et al., 2003). Safsaf willow (*Salix mucronata*), a member of the Salicaceae family, is a popular commercial tree in Egypt that is used for many industrial purposes (Fahmy, 1948). It is indigenous to northern and tropical Africa and widely distributed throughout Egypt and Sudan. It is used for wind screening, planting in damp woodlands, waterside planting, coppicing, and remediation of contaminated sites. *S. mucronata* can absorb some heavy metals; therefore, it has been planted in reclaimed industrial sites and used for small-scale water treatment systems in which wastewater is passed through willow beds, which cleans the water for re-use (Schmidt, 2003). However, there are 552 accepted species names under the *Salix* genus, with 963 intraspecific ranks for the genus worldwide, including 3 species in Egypt (Fahmy, 1948). A previous study indicated that 34 species of *Salix* were used for phytoremediation (Kersten, 2015). Of these, phytoremediation of heavy metal–contaminated soil involves *S. eriocephala* and *S. discolor* for manganese (Mn^{++}), iron (Fe^{++}), aluminum (Al^{+++}), sulfur (S^{++}), and magnesium (Mg^{++}) (Mosseler and Major, 2017), Salix viminalis for sodium (Na^{+}), Mg^{++} (Hegedus et al., 2009), and Cu^{++} (Çsecka et al., 2012), *Salix polaris* for Cd^{++}, chromium (Cr^{+++}), Fe++, zinc (Zn^{++}), and molybdenum (Mo^{++}) (Krajcarova et al., 2016), *Salix purpura* ×*viminalis* for Cu^{++} and nickel (Ni^{++}) (Drzewiecka et al., 2017), and *S. matsudana* for Pb (Tang et al., 2017). In general, these studies concluded that although growth and chemical parameters were negatively affected in the presence of heavy metals, these species could be used as phytoremediators to clean contaminated soil. However, there is no report of using *S. mucronata* as a phytoremediator of heavy metal–contaminated soil. Additionally, most previous studies involved acidic soil. Therefore, this study used alkaline soil to study the effects of Cd, Cu, and Pb on the growth and chemical and biochemical compositions of *S. mucronata*. The relationships between the concentrations of these metals in plant organs and their concentrations in the soil were determined to facilitate the use of *S. mucronata* to remove these metals from soil.

Received for publication 6 Mar. 2019. Accepted for publication 23 Apr. 2019.
We thank the International Scientific Partnership Program (ISPP) of King Saud University for funding this research (ISPP# 0064) and the Research Support & Services Unit (RSSU) for their technical support.
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Materials and Methods

Plant material. Mature shoot cuttings (1-year-old wood) 15 cm in length and 0.5 cm in diameter were procured from a 10-year-old mother tree of *S. mucronata* that was grown in the nursery of the Faculty of Agriculture at Kafrelshikh University. The cuttings were cultured in plastic bags 10 cm in diameter (one cutting per bag) filled with clay soil (Table 1). The cultured bags with cuttings were kept in an air-conditioned plastic greenhouse adjusted to 25 ± 2 °C, with 40% to 50% relative humidity, a photoperiod of 16 h of light and 8 h of dark, and light intensity of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The cuttings were watered manually every 10 d using 10-L watering cans; the same water volume was applied to each bag. After 3 months, homogeneous transplants with an average height of 35 cm and stem diameter of 0.9 cm (at the soil surface) were used in this study.

Pollutant treatments and media preparation. Different concentrations of cadmium chloride [$CdCl_2*H_2O$] (20, 40, 60, and 80 $mg \cdot kg^{-1}$ soil), copper chloride [$CuCl_2 2H_2O$] (50, 100, 150, and 200 $mg \cdot kg^{-1}$ soil), and lead acetate trihydrate [$(CH_3COO)_2Pb3H_2O$] (250, 450, 650, and 850 $mg \cdot kg^{-1}$ soil) were used in separate treatments. The clay soil used in this study was placed in plastic pots 40 cm in diameter with 9 kg of air-dried soil per pot, sprinkled with solutions of the aforementioned concentrations of metals and incubated for 60 d before being planted outdoors under a waterproof tarpaulin. Soil without heavy metal contamination served as a negative control.

Pot experiments. Homogeneous 3-month-old plants were transplanted to previously prepared plastic pots (one transplant per pot) on 1 May 2015. The experiment consisted of 13 treatments (three heavy metals × four concentrations and the negative control) with three replicates and three plants per replicate; therefore, nine plants were used for each treatment. The plants were placed in an open field after planting and were irrigated with tap water using 10-L watering cans to reach field capacity when required. The experiment continued for 27 months.

Soil analysis. Soil analysis was performed before and after completion of the experiment (Table 1). Both physical and chemical analyses were performed; the particle size distribution was analyzed using a hydrometer method (Gee and Balder, 1986) before planting only. The soil had a clay-like texture consisting of 24.03% sand, 22.92% silt, and 50.05% clay. Soil pH was measured in a 1:1 ratio (soil: deionized water suspension) using a calibrated pH meter 3510 (Jenway, Staffordshire, UK). Soil salinity [electrical conductivity (EC)] was measured in a 1:5 ratio (soil: deionized water) using an EC Meter (MI 170; Italy). Soluble ions in saturated extracts were measured according to the methods of Jackson (1973). Total carbonate was determined using a volumetric calcimeter (Nelson and Sommers, 1996). Organic matter content was determined using the dichromate oxidation method (Nelson and Sommers, 1996). Available nitrogen (NH_4^+) was determined using the micro Kjeldahl method (Bremner and Mulvaney, 1982), and available phosphorus ($P_2O_5^-$) was determined (Olsen and Sommers 1982). Calcium (Ca^{++}) and magnesium (Mg^{++}) were also measured (Jackson, 1973). The concentrations of cadmium (Cd^{++}), copper (Cu^{++}), and lead (Pb^{++}) were quantified using an atomic absorption spectrophotometer (Page et al., 1982). Sodium (Na^+) and potassium (K^+) were extracted according to the methods described by Black (1965), and concentrations were determined using a Flame photometer PFP7 (Jenway, Staffordshire, UK). Chloride (Cl^-) was determined by titration with a standard solution of silver nitrate (Jackson, 1973).

Variables measurements. At the end of the experiment on 1 Aug. 2017, six samples (plants) from each treatment (for three replicates) were chosen randomly to determine the following growth parameters: plant height (measured from the medium surface to the shoot apex); number of branches per plant; stem diameter (measured 5 cm from the soil surface); leaf area using a C1–202 laser area meter (CID Bio-Science, Camas, WA); fresh and dry weights of vegetative growth and roots (recorded after drying in oven at 60 °C for 48 h); and root lengths (the longest root). The degree of greenness was measured on the fifth leaf from the apical meristem using a portable leaf chlorophyll meter (SPAD-501; Minolta Corp., Osaka, Japan) according to the methodology described by Markwell et al. (1995).

Biochemical assays of antioxidant enzyme activities. To determine antioxidant enzyme activities, 0.5 g of fully expanded young leaves were homogenized in liquid nitrogen with 3 mL of extraction buffer [50 mM TRIS buffer (pH 7.8) containing 1 mM EDTA-Na2 and 7.5% polyvinylpyrrolidone)] using a prechilled mortar and pestle. The homogenate was filtered through four layers of cheesecloth and centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant, which was re-centrifuged at 12,000 rpm for 20 min at 4 °C, was used for the total soluble enzyme activity assay using an ultraviolet-160A spectrophotometer (Shimadzu, Japan).

Catalase assay. Catalase (CAT; EC 1.11.1.6) activity was measured by following the consumption of H_2O_2 at 240 nm (Aebi, 1984). A total of 1 mL of the reaction mixture contained 20 µg total protein, 50 mM sodium phosphate buffer (pH 7.0), and 10 mM H_2O_2. The reaction was initiated by adding the protein extract. For each measurement, the blank corresponded to the absorbance of the mixture at time zero, and the actual reading corresponded to the absorbance after 1 min. One unit of CAT activity was defined as a 0.01 decrease in absorbance at 240 nm/mg of protein/min.

Polyphenol oxidase assay. Polyphenol oxidase (PPO; EC 1.10.3.1) activity was determined according to the method described by Malik and Singh (1980). The reaction mixture contained 3.0 mL of

Table 1. Chemical analysis of the soil used for growth of *Salix mucronata* before plantation and 27 months after plantation.

Parameter	Soil before plantation	Soil after plantation (Treatments mg·kg^{-1} soil)												
			Cadmium				Copper					Lead		
		Control	20	40	60	80	50	100	150	200	250	450	650	850
pH	7.84	7.84	7.82	7.82	7.81	7.79	7.84	7.83	7.81	7.81	7.80	7.82	7.77	7.78
EC (dS·m^{-1})	3.30	2.36	4.38	4.56	4.75	5.00	3.38	4.69	4.79	4.87	3.44	3.63	4.06	4.69
$CaCo_3$ (%)	3.26	3.15	3.12	3.19	3.19	3.19	3.16	3.19	3.18	25.17	2.89	3.12	3.17	3.17
Organic matter (%)	1.31	1.24	1.25	1.27	1.28	1.28	1.27	1.27	1.28	1.28	1.26	1.27	1.27	1.27
Soluble cations (meq·L^{-1})														
Ca^{++}	7.71	5.56	11.35	12.21	13.46	15.53	7.92	11.80	13.89	13.85	7.98	9.46	11.33	12.71
Mg^{++}	4.82	3.97	8.41	8.52	8.69	9.43	4.90	9.80	8.80	9.52	4.99	5.20	7.09	9.55
Na^{+}	20.12	13.92	22.54	23.17	23.92	23.38	20.14	23.77	23.33	23.42	20.92	20.67	20.87	22.96
K^{+}	0.35	0.17	1.42	1.70	1.63	1.66	0.82	1.53	1.88	1.91	0.59	0.97	1.31	1.68
Soluble anions (meq·L^{-1})														
Cl^{-}	19.73	13.16	23.44	24.20	24.44	25.92	20.20	23.20	23.44	24.47	21.68	21.44	22.44	22.68
CO_3^{-}														
HCO_3^{-}	2.50	2.35	4.38	4.96	5.15	5.30	3.31	4.69	4.79	4.87	3.44	3.13	4.06	4.69
SO_4^{-}	10.77	8.09	15.90	16.44	17.84	18.78	10.27	19.01	19.70	19.36	9.27	11.73	14.10	19.53
Available minerals (mg·kg^{-1})														
N	2.75	1.89	2.36	2.39	2.60	2.60	2.35	2.38	2.42	2.56	2.41	2.43	2.44	2.46
P	3.51	2.45	2.43	2.48	2.98	3.11	2.62	2.64	3.23	3.33	2.73	2.83	2.85	3.06
K	216	144	166	172	183	189	169	191	198	201	126	179	198	201
Cd	00		2.74	5.47	7.84	10.00								
Cu	3.6	1.87					5.08	9.59	16.48	28.21				
Pb	00										10.85	13.2	19.28	25.61

Ca = calcium; Mg = magnesium; Na = sodium; K = potassium; N = nickel; P = phosphorus; Cd = cadmium; Cu = copper; Pb = lead.

buffered catechol solution (0.01 M) freshly prepared in 0.1 M phosphate buffer (pH 6.0). The reaction was initiated by adding 100 µL of the crude enzyme extract. Changes in the absorbance at 495 nm were recorded at 30 s for 3 min. Enzyme activity was expressed as an increase in the absorbance min^{-1}·g^{-1} fresh weight.

Peroxidase assay. Peroxidase (POD; EC 1.11.1.7) activity was determined according to the procedure proposed by Hammerschmidt et al. (1982). The reaction mixture consisted of 2.9 mL of a 100-mM sodium phosphate buffer [pH 6.0 containing 0.25% (v/v) guaiacol (2-methoxy phenol) and 100 mM H_2O_2]. The reaction was started by adding 100 µL of crude enzyme extract. Changes in absorbance at 470 nm were recorded at 30-s intervals for 3 min. Enzyme activity was expressed as an increase in the absorbance min^{-1}·g^{-1} fresh weight.

Electrolyte leakage. Measurements were performed as described by Szalai et al. (1996), with some modifications. Twenty leaf discs (1 cm^2) were placed individually into flasks containing 25 mL of deionized water (Milli-Q 50, Millipore, Bedford, MA). Flasks were shaken for 20 h at an ambient temperature to facilitate electrolyte leakage from injured tissues. Initial EC measurements were recorded for each vial using an Acromet AR20 EC meter (Fisher Scientific, Chicago, IL). Flasks were then immersed in a hot water bath (Fisher Isotemp, Indiana, PA) at 80 °C (176 °F) for 1 h to induce cell rupture. The vials were again placed on the Innova 2100 platform shaker for 20 h at 21 °C (70 °F). Final conductivity was measured for each flask. The percentage of electrolyte leakage for each bud was calculated as the initial conductivity/final conductivity × 100.

Chemical composition. Plant samples (leaves, stems, and roots) were oven-dried at 80 °C for 24 h. Dry samples were ground to obtain a homogenous powder in a metal-free mill (IKa-Werke, M 20 Germany). Concentrated sulfuric acid (95%, 5 mL) was added to the sample (0.2 g), and the mixture was heated for 10 min on a sand hotplate. Then, 0.5 mL of perchloric acid was added, and heating was continued until a clear solution was obtained. The solution was left to cool before it was filtered and diluted to 50 mL with distilled water (Evenhuis and de Waard, 1980). The digested samples were prepared for nitrogen measurements (N% using a modified micro-Kjeldahl method as described by Chemists and Horwitz, 1990). Phosphorus (P%) was extracted according to the methods described by Murphy and Riley (1962) and detected by colorimetrically in a spectrophotometer (GT 80^{+}, UK). Potassium (K%) was extracted according to the methods described by Cottenie et al. (1982) and detected using an atomic absorption spectrophotometer (Avanta E; GBC, Victoria, Australia). The total carbohydrate percentage in leaves was determined according to the methods described by Herbert et al. (1971). In addition, N, P, and K leaf uptake (mg) were calculated as described for the percentage of N, P, and K in dry leaves. The Cd, Cu, and Pb concentrations (mg·kg^{-1} dry weight) were determined using the methods of Page et al. (1982) in different plant parts (leaves, stems, and roots) using atomic absorption spectrophotometry (Avanta E; GBC).

Determination of the phytoextraction potential of S. mucronata for removing Cd, Cu, and Pb from contaminated soil. The bioconcentration factor was determined using the following formula:

$$\textit{Bioconcentration factor}\ (\mathrm{BCF}) = \frac{\text{Metal concentration in the plant organ}\ (\mathrm{mg\cdot kg^{-1}D.W.})}{\text{Metal concentration in the soil}\ (\mathrm{mg\cdot kg^{-1}soil\ D.W.})}$$

The Cd, Pb, and Cu concentrations per kg of soil were calculated (regardless of the calculated BCF) as follows: added Cu concentration/kg soil in treatment + Cu concentration/kg in soil before contamination. Depending on the BCF values, the accumulation efficiency was estimated as one of four groups: intensive, BCF >1; medium, BCF = 1–0.1; weak, BCF = 0.1–0.01; and no accumulation, BCF = 0.01–0.001 (Kabata-Pendias and Pendias, 1999)

$$\textit{Translocation factor}\% = \frac{\text{Metal content in the shoots}\left(\mathrm{mg\cdot kg^{-1}D.W.}\right)\times 100}{\text{Metal content in the roots}\left(\mathrm{mg\cdot kg^{-1}D.W.}\right)}$$

The TF% was calculated to estimate the metal ion transport efficiency from the roots to aerial plant organs (Maiti and Jaiswal, 2008), whereas shoots were considered equivalent to leaves and stems.

The biomass tolerance index (TI_b) was calculated to estimate the resistance of *S. mucronata* to Cd, Cu, and Pb phytoextraction. According to Wilkins (1978), there are three values: TI_b <1, indicating a net decrease in biomass and stressed conditions of plants; TI_b = 1, indicating no difference relative to control treatment; and TI_b >1, indicating a net increase in biomass and correct plant development.

Statistical analysis. The experiment used a completely randomized design. Data were subjected to an analysis of variance using the SAS program (version 6.12; SAS Institute Inc., Cary, NC). The mean separations were performed using Duncan's multiple range testing method, and significance was determined at $P \leq 0.05$.

Results and Discussion

Soil analysis and contamination. A physiochemical analysis of the soil used for the growth of *S. mucronata* demonstrated that the texture was clay-like and had an organic matter level of 1.31 and a pH level of 7.84 (Table 1). In addition, changes in organic matter and $CaCO_3$ content before and after

Table 2. Effects of different levels of cadmium (Cd), copper (Cu), and lead (Pb) pollutants on vegetative growth traits of *Salix mucronata* 27 months after plantation.

Treatments (mg·kg^{-1} soil)		Plant ht (cm)	Stem diam (cm)	Branches (no./plant	Leaf area (cm^2)	Vegetative fresh wt (g/plant)	Vegetative dry wt (g/plant)	Root length (cm)	Root fresh wt (g/plant)	Root dry wt (g/plant)	Degree of greenness (SPAD)
Control		234.83 ± 1.15 a	2.65 ± 0.170 a	14.33 ± 0.67 a	9.94 ± 0.09 a	200.25 ± 0.69 a	71.59 ± 0.24 a	80.19 ± 0.13 a	116.14 ± 1.46 a	34.84 ± 0.44 a	43.12 ± 0.06 a
$CdCl_2$	20	198.00 ± 0.50 d	2.43 ± 0.010 b	12.67 ± 0.33 b	9.40 ± 0.09 b	192.01 ± 0.4 b	70.27 ± 0.23 a	75.68 ± 0.26 b	113.21 ± 0.46 b	33.96 ± 0.14 b	41.48 ± 0.81 b
	40	176.07 ± 0.55 f	2.08 ± 0.003 cd	8.33 ± 0.33 c	7.89 ± 0.04 d	155.69 ± 0.66 d	58.15 ± 0.55 b	68.36 ± 0.09 c	95.27 ± 0.62 c	28.58 ± 0.19 d	36.90 ± 0.70 c
	60	156.47 ± 1.70 h	1.95 ± 0.010 f	6.00 ± 0.58 d	7.12 ± 0.00 e	116.18 ± 0.39 g	42.63 ± 0.12 e	62.90 ± 0.31 e	82.95 ± 0.45 d	24.89 ± 0.14 f	30.07 ± 0.16 d
	80	135.93 ± 1.50 i	1.76 ± 0.012 g	4.00 ± 0.00 e	4.76 ± 0.13 g	85.23 ± 0.18 i	31.66 ± 0.15 g	54.07 ± 1.04 g	69.61 ± 0.26 g	20.89 ± 0.08 g	26.17 ± 0.03 e
$CuCl_2$	50	231.72 ± 0.70 b	2.46 ± 0.003 b	13.00 ± 0.00 b	9.90 ± 0.05 a	200.45 ± 0.69 a	71.90 ± 0.22 a	76.90 ± 0.38 b	114.91 ± 0.11 ab	34.47 ± 0.03 a	42.85 ± 0.29 a
	100	196.07 ± 0.38 d	2.12 ± 0.003 c	9.33 ± 0.33 c	8.26 ± 0.01 c	160.92 ± 0.77 c	58.48 ± 0.22 b	66.20 ± 0.42 d	97.15 ± 0.48 c	29.15 ± 0.14 d	37.40 ± 0.53 c
	150	174.60 ± 1.65 f	2.02 ± 0.020 de	6.67 ± 0.33 d	7.42 ± 0.01 e	136.53 ± 0.74 e	51.66 ± 0.23 c	58.03 ± 0.29 f	83.76 ± 0.02 d	25.13 ± 0.01 f	29.19 ± 0.54 d
	200	151.30 ± 1.32 i	1.80 ± 0.010 g	4.33 ± 0.33 e	5.44 ± 0.30 f	117.93 ± 0.29 g	47.24 ± 0.22 d	48.78 ± 1.15 h	68.26 ± 0.68 e	20.48 ± 0.20 g	24.90 ± 1.15 e
$(CH_3COO)_2Pb$	250	226.05 ± 0.29 c	2.46 ± 0.003 b	12.67 ± 0.00 b	9.73 ± 012 a	197.31 ± 0.67 a	71.31 ± 0.58 a	75.55 ± 0.20 b	114.21 ± 0.21 ab	33.88 ± 0.40 b	40.51 ± 0.05 b
	450	189.73 ± 0.56 e	2.10 ± 0.010 cd	9.00 ± 0.01 c	8.22 ± 0.03 c	157.28 ± 0.44 cd	59.10 ± 0.14 b	65.20 ± 0.61 d	96.07 ± 0.22 c	29.95 ± 0.14 c	37.07 ± 0.57 c
	650	165.27 ± 0.47 g	2.00 ± 0.020 ef	6.00 ± 0.02 d	7.28 ± 0.12 e	126.71 ± 0.61 f	48.25 ± 0.24 d	56.70 ± 0.76 f	83.58 ± 0.33 d	26.60 ± 0.03 e	29.79 ± 0.52 d
	850	140.97 ± 1.09 j	1.77 ± 0.030 g	4.33 ± 0.03 e	5.68 ± 0.17 f	97.83 ± 0.67 h	38.01 ± 0.35 f	48.12 ± 0.92 h	62.91 ± 1.12 f	20.50 ± 0.39 g	26.73 ± 0.20 e
Significance		**	**	**	**	**	**	**	**	**	**

Means followed by a similar letter within each column are not significantly different at $P \leq 0.05$ according to Duncan's Multiple Rang Test.

planting were limited. Conversely, cation and anion concentrations increased after planting using the Cd, Cu, and Pb treatments, except for control treatments, which were decreased. Regardless of the treatments, the changes in pH values were slight, but we noted a slight decrease in EC values after control planting. However, EC values increased in soil contaminated with heavy metals to 5 dS·m^{-1} at 80 mg·kg^{-1} $CdCl_2$. The available concentrations of N, P, and K decreased in the control soil after planting compared with the soil before planting.

In contrast, the available concentrations of N, P, and K were only slightly decreased in soil contaminated with high heavy metal concentrations. Furthermore, Cd, Cu, and Pb concentrations were deceased after planting relative to their concentrations before planting. There were negligible changes in pH values with the treatments used in this study. Accordingly, Vysloužilová et al. (2006) found that soil pH did not differ among rhizobox compartments of Cd-, Pb-, and Zn-polluted soil under *Salix* ×*robens* clones. Soil characteristics such as pH, organic matter (O.M.), and cation exchange capacity were positively correlated with Cu retention (King, 1988) and Cd and Pb retention (Jopony and Young, 1994). The increased EC values may be due to the addition of metals, resulting in increased cations and anions. However, larger quantities of ions and soluble salts have resulted in increased EC values in industrial effluent-treated soil samples (Sharma and Raju, 2013). Additionally, the reduction in available N, P, K, soluble cations, and Cd, Cu, and Pb concentrations after planting may refer to the uptake by plant roots or the loss to irrigation water. It is known that plants were growing in soil with a pH of 7.84; at this pH, the uptake of essential nutrients by roots, especially N, P, K, Mg, and Zn, among others, is poor. These nutrients are extremely important for many components such as carbohydrates, proteins, amino acids, phospholipids, and energy components. In addition, Cd and Pb negatively impact the permeability of the plasma membrane (Pourrut et al., 2011; Sharma et al., 2010).

Effect of heavy metal–contaminated soil on vegetative growth. Using different concentrations of Cd, Cu, and Pb significantly reduced most vegetative traits compared with control plants (Tables 2). The highest values for plant height, stem diameter, and the number of branches were reported for the negative control treatment group as 234.83 cm, 2.65 cm, and 14.33, respectively. Leaf area and vegetative fresh and dry weight exhibited the same trend for all treatments, showing a decrease with increasing heavy metal concentrations. Additionally, the values of root lengths, root fresh and dry weights, and greenness degrees were significantly reduced for all Cd, Cu, and Pb levels compared with controls, except 50 mg·kg^{-1} $CuCl_2$ and 250 mg·kg^{-1} Pb acetate. Therefore, the reduction in root parameters was parallel to increasing heavy metal levels in the soil. The results showed that low heavy metal concentrations had the same significant effect as the controls on most traits. In addition, 50 mg·kg^{-1} $CuCl_2$ and 250 mg·kg^{-1} Pb acetate had the same effect on all traits except plant height and degree of greenness.

Conversely, high concentrations of heavy metals had negative effects on all studied traits. Our findings indicated that vegetative growth and root traits were drastically inhibited, especially with medium and high heavy metal concentrations. Tauqeer et al. (2016) revealed that root fresh and dry weights of *Alternanthera bettzickiana* significantly decreased at 0.225 mg·L^{-1} Cd and 0.414 mg·L^{-1} Pb. Additionally, Cd stress deleteriously affects the photosynthetic rate and intracellular CO_2 concentration and can interfere with photosynthetic pigments by substituting Mg^{2+} ions with Cd^{2+} ions in chlorophyll molecules, producing much lower fluorescence quantum yields compared with magnesium chlorophylls (Jing et al., 2005). These two toxic effects reduce the production of chlorophyll and, consequently, photosynthesis, which can lead to senescence and cell death (Santos et al., 2010). Vegetative trait values gradually decreased with the increasing concentrations of each element in the soil. However, there was no plant lethality at any of the tested concentrations of the elements. Some toxic effects appeared on the adult leaves, such as yellow coloration and drying of the leaf edges after treatments with high heavy metal concentrations. The reduction in growth and abscission of leaves were observed in Willow Tangio (*S. mastudana* × *S. alba*) when grown in soil containing 0.6–60.6 µg·g^{-1} Cd (Robinson et al., (2000). Additionally, the total leaf area of Willow clones was affected by 38.5 mg·L^{-1} Cd sulfate (Zacchini et al., 2009) and 0.19 mg·L^{-1} Cu in *S. viminalis* (Gasecka et al., 2012). In the present study, high concentrations of heavy metals suppressed root development of *S. mucronata.* Moreover, Yuan et al. (2013) indicated that excess Cu in the root zone and more uptake by plant roots have hazardous effects on elongation and meristem zones because they alter auxin distribution, which is responsible for Cu-mediated inhibition of primary root elongation. In addition, Pb toxicity leads to the inhibition of photosynthesis, oxidative stress, DNA damage, and defects in mitosis (Kupper, 2017). All these effects lead to reduced growth rates of the aerial or root parameters and leaf chemical composition. These toxicity symptoms were increased in the youngest plant at the beginning of the experiment, but they were decreased in older plants at the end of the experiment. Therefore, we inferred that older *S. mucronata* plants had greater tolerance for heavy metals compared with younger plants. Tolerance increased with increasing plant age, and some toxic symptoms decreased in older plants (Tu et al., 2004).

Effects of heavy metal–contaminated soil on enzyme activities and electrolyte leakage. Significantly higher activities of CAT, PPO,

and POD were observed in plant leaves grown on soil contaminated with different Cd, Cu, and Pb levels compared with control plants (Fig. 1a-c). The results indicated that 40 mg·kg^{-1} $CdCl_2$, 100 mg·kg^{-1} $CuCl_2$, and 450 mg·kg^{-1} Pb acetate induced a significant increase in antioxidant enzyme activities compared with other treatments and the negative control treatment. The maximum activity of PPO and POD was observed in 100 mg·kg^{-1} $CuCl_2$ and 450 mg·kg^{-1} Pb acetate treatments, respectively, whereas the highest value of CAT activity was recorded for 100 mg·kg^{-1} $CuCl_2$ and 450 mg·kg^{-1} Pb acetate. Previous reports confirmed the relationship between heavy metal–contaminated soil and oxidative stress in *Euplotes crassus* for Cu, Pb, and Zn (Kim et al., 2011) and in *Nasturtium officinale* for Cr, Cu, and Cd (Ercan et al., 2018). According to these studies, the maximum antioxidant enzyme-coding genes (Ec-GR, Ec-GPx, and Ec-GST theta) were observed in *E. crassus*, and catalase, superoxide dismutase, and increased levels of malondialdehyde in *N. officinale* were observed with increasing heavy metal concentrations. Biosynthesis of several cellular biomolecules is the primary mechanism of tolerating or neutralizing metal toxicity. This includes the induction of many components such as amino acids, organic acids, hormones, and phenolic compounds (Viehweger, 2014). Previous studies have mentioned that increased antioxidant enzyme activity in *N. officinale* has an important role in alleviating the toxicity of Cr, Cu, and Cd (Ercan et al., 2018).

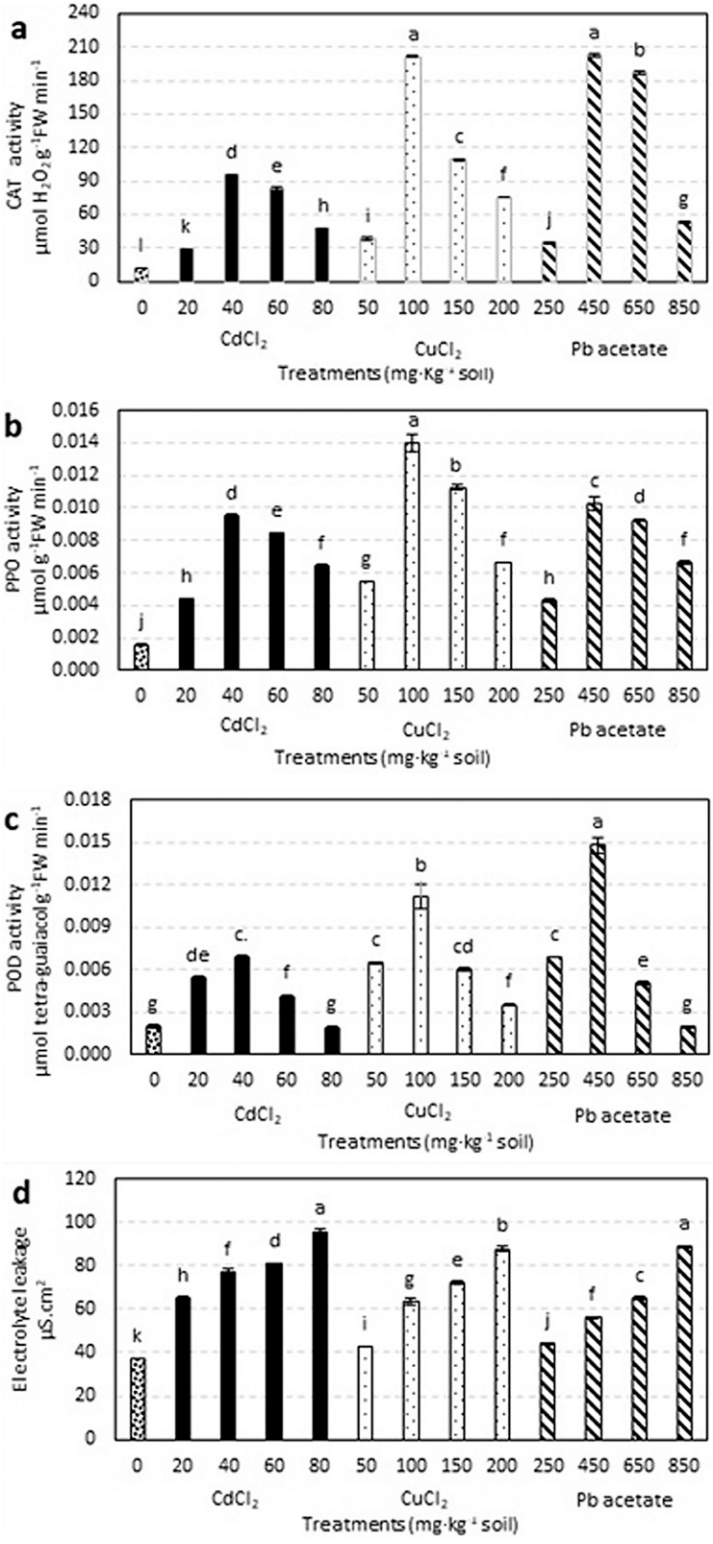

Fig. 1. Effects of different levels of cadmium (Cd), copper (Cu), and lead (Pb) on antioxidant enzyme activity and electrolyte leakage of *S. mucronata*. (**A**) Catalase, (**B**) polyphenol oxidase, (**C**) peroxidase, and (**D**) electrolyte leakage (similar letters within a figure are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test).

In contrast, the aforementioned strategies are not sufficient to restrain metal poisoning, and the equilibrium of the cellular redox system in plants is negatively affected, leading to increased induction of reactive oxygen species (Mourato et al., 2012). To mitigate the harmful effects of free radicals, plant cells have developed an antioxidant defense mechanism composed of enzymatic antioxidants such as CAT (Sharma et al., 2012). Increased CAT, PPO, and POD activities occur in the presence of low heavy metals concentrations; these decrease when high levels are encountered (but still higher than the control). This phenomenon was observed by Zou et al. (2017) in *S. matsudana* treated with Cd at 1.124 mg·L^{-1} as a low concentration and 11.24 mg·L^{-1} as a high concentration. Emamverdian et al. (2018) observed a similar effect in *Indocalamas latifolius* treated with Cu, Pb, and Zn at four different concentrations (0, 500, 1000, and 2000 mg·kg^{-1})

Concerning the results of EL, it should be noted that with increasing concentrations of each heavy metal in the soil, the value of EL is significantly increased (Fig. 1D). Overall, the maximum significant values of EL resulted from treatment with 80 mg $CdCl_2$ and 850 mg Pb acetate compared with other heavy metal concentrations. The lowest EL value was found in the control plants. This finding suggests that levels of heavy metals have negative impacts on cell membranes. Ion leakage is a well-known parameter for the evaluation of oxidative damage to cell membranes (Liu et al., 2008) that expresses membrane dysfunction as the increase in permeability and electrolyte leakage from the cell. Membrane damage can be inferred from an increase in EL because of Cu (Liu et al., 2004); additionally, increased Cd levels markedly increase EL, along with enhanced activities of antioxidant enzymes (Ahmad et al., 2016). Tauqeer et al. (2016) revealed that with lower Cd and Pb levels, POD and CAT activities increased, whereas

Table 3. Effects of different levels of cadmium (Cd), copper (Cu), and lead (Pb) in soil on leaf N, P, and K, and total carbohydrates percentage and BCF, TF%, and TI_b of *Salix mucronata* 27 months after plantation.

Treatments (mg·kg^{-1} soil)		N (%)	P (%)	K (%)	Total carbohydrate (%)	Bioconcentration factor (BCF)			Translocation factor TF (%)	Tolerance index biomass (TI_b)
						Leaves	Stems	Roots		
Control		2.53 ± 0.01 a	0.20 ± 0.012 a	2.88 ± 0.01 a	12.90 ± 0.57 a	0.12 ± 0.017 e	0.09 ± 0.010 c	0.27 ± 0.012 d	76.91 ± 6.35 d	
$CdCl_2$	20	2.13 ± 0.03 ab	0.15 ± 0.006 c	2.34 ± 0.02 c	11.91 ± 0.01 c	0.10 ± 0.011 d	0.04 ± 0.011 c	0.49 ± 0.010b c	28.08 ± 3.55 c	0.98 ± 0.010 a
	40	1.71 ± 0.02 bc	0.09 ± 0.006 f	2.02 ± 0.01 e	10.32 ± 0.01 e	0.18 ± 0.002 c	0.12 ± 0.004 b	0.46 ± 0.010 c	65.48 ± 1.68 b	0.81 ± 0.004 b
	60	1.12 ± 0.01 cd	0.05 ± 0.002 i	1.77 ± 0.02 g	9.46 ± 0.01 fg	0.22 ± 0.004 b	0.17 ± 0.004 a	0.52 ± 0.002 b	76.68 ± 0.64 a	0.63 ± 0.004 c
	80	0.83 ± 0.01 de	0.03 ± 0.001 k	1.37 ± 0.02 i	8.86 ± 0.01 i	0.26 ± 0.003 a	0.17 ± 0.002 a	0.61 ± 0.020 a	69.12 ± 2.09 b	0.49 ± 0.010 d
$CuCl_2$	50	2.22 ± 0.01 ab	0.17 ± 0.006 b	2.45 ± 0.01 b	12.25 ± 0.02 b	0.73 ± 0.015 a	0.61 ± 0.012 a	1.14 ± 0.001 b	117.68 ± 2.17 a	1.00 ± 0.005 a
	100	1.74 ± 0.01 bc	0.12 ± 0.006 d	2.27 ± 0.02 d	10.65 ± 0.02 d	0.47 ± 0.012 d	0.32 ± 0.010 b	1.22 ± 0.030 a	65.34 ± 0.68 c	0.82 ± 0.004 b
	150	1.15 ± 0.01 cd	0.08 ± 0.001 g	1.86 ± 0.01 f	9.55 ± 0.02 f	0.65 ± 0.010 b	0.34 ± 0.012 b	1.09 ± 0.012 c	91.12 ± 2.35 b	0.72 ± 0.010 c
	200	0.89 ± 0.02 de	0.05 ± 0.001 ij	1.50 ± 0.02 h	8.99 ± 0.03 hi	0.59 ± 0.010 c	0.34 ± 0.010 b	1.05 ± 0.011 c	89.09 ± 2.71 b	0.64 ± 0.010 d
$(CH_3COO)_2Pb$	250	2.16 ± 0.04 ab	0.17 ± 0.006 b	2.40 ± 0.01 bc	12.21 ± 0.02 b	0.31 ± 0.012 ab	0.19 ± 0.010 a	0.60 ± 0.011 a	84.11 ± 3.42 b	0.99 ± 0.013 a
	450	1.65 ± 0.02 bc	0.11 ± 0.009 e	2.23 ± 0.01 d	10.48 ± 0.03 de	0.33 ± 0.010 a	0.21 ± 0.010 a	0.51 ± 0.014 b	104.86 ± 3.06 a	0.84 ± 0.004 b
	650	0.93 ± 0.01 de	0.07 ± 0.002 h	1.89 ± 0.12 f	9.24 ± 0.03 gh	0.32 ± 0.020 a	0.23 ± 0.012 a	0.49 ± 0.020 b	112.53 ± 1.98 a	0.70 ± 0.002 c
	850	0.72 ± 0.01 e	0.04 ± 0.002 k	1.46 ± 0.01 h	8.89 ± 0.02 i	0.28 ± 0.010 b	0.22 ± 0.005 a	0.48 ± 0.020 b	103.78 ± 5.35 a	0.55 ± 0.011 d
Significance		**	**	**	**	**	**	**	**	**

Means followed by a similar letter within each column are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test.

with higher levels the opposite occurred, and EL increased with increasing Cd and Pb levels.

Effects of heavy metal–contaminated soil on the chemical analysis of leaves. N, P, K, and total carbohydrate percentages of plants grown on the heavy metal–contaminated soil were significantly lower than those of controls (Table 3). The impact of applied Cd, Cu, and Pb concentrations on these parameters showed a similar decreasing trend in the presence of increasing heavy metal concentrations. In contrast, the N% of plants grown in soil with a low heavy metal content was not significantly different from that of control plants. In addition, our results showed that the reduction in values of N% depended on the level of each metal in the soil. Gasecka et al. (2012) reported that the inhibition of carbohydrate transport to different organs resulted from the high Cu accumulation in the roots of *S. viminalis* and was similarly lower in leaves and shoots. Furthermore, Cd has a negative impact on the permeability of the plasma membrane, thus interfering with nutrient uptake (Sarwar et al., 2010). Additionally, lead accumulation in plant tissues reacts with the phosphate groups of ADP or ATP and replaces essential ions, thus impairing the uptake of essential elements such as Mg and Fe, and induces CO_2 deficiency resulting from stomatal closure (Pourrut et al., 2011). Growth inhibition or stimulation of willow species or clones is dependent on metal concentrations in the nutrient solution (Krajcarova et al., 2016). Drzewiecka et al. (2017) revealed that significantly decreased growth rate values of *Salix purpurea* × *S. viminalis* in relation to control plants were noted for all Cu^{2+}-treated plants, as was a strong affirmation of negative effects of Cu^{2+} in the root system. Overall, foliage element concentrations differ according to their concentration in soil or soil chemistry and according the plant species or clones (Mosseler and Major, 2017).

Effects of heavy metal–contaminated soil on Cd, Cu, and Pb concentrations in plant parts. Cd, Cu, and Pb levels in plant tissues grown on heavy metal–contaminated soil were significantly higher than those in control plant tissues (Fig. 2A–C). The metal content in the different plant parts gradually increased with increased levels of the corresponding metal in the soil. The results indicated that the highest significant metal concentrations in the plant parts were recorded for the highest concentrations of each heavy metal used. The content of Cd, Cu, and Pb in the plant parts was in the order of roots > leaves > stems. Our previous results indicated that the content of Cd, Cu, and Pb increased with increasing levels in the root zone. In this regard, Mleczek et al. (2013) found a general increase in Cu accumulation in *S. viminalis* organs with increased Cu concentrations in the medium. Additionally, *S. polaris* organs treated with Cd have shown different concentrations between plant parts (Krajcarova et al., 2016). Our results confirmed that the concentrations of Cd, Cu, and Pb were higher in roots than in aerial parts. Tang et al. (2017) reported Pb levels in all *S. mastudana* organs with the following order: roots > cutting > twigs > leaves. Therefore, *S. mucronata* roots accumulated much more Cd, Cu, and Pb than the leaves; in this case, the risk of contamination of the wider environment through leaf fall can be considered minimal. Therefore, these data suggest that *S. mucronata* is a suitable alternative to deciduous hyperaccumulators.

Relationship between metal concentrations in soil and concentrations in plant organs. The phytoextraction efficiency of plant organs for Cd, Cu, and Pb is dependent on soil concentrations (Table 3). The estimated BCF indicated that the majority of Cd, Cu, and Pb accumulations in plant organs were considered medium (BCF = 1–0.1). Conversely, the Cd content in leaves and stems on treatment with 20 mg·kg^{-1} $CdCl_2$ was estimated as weak (BCF = 0.1–0.01), and the Cu content in roots was estimated as intensive (BCF >1). In general, BCF values were higher in roots, followed by leaves and stems, in Cd-contaminated, Cu-contaminated, and Pb-contaminated soil. The BCF values for plant organs are dependent on the type of metal ion and its concentration in the soil, as well as the plant organ. Kabata-Pendias and Pendias (1999) reported that in the majority of cases, BCF values for leaves, stems, and roots are medium (1–0.01) for Cd-contaminated, Cu-contaminated, and Pb-contaminated soil, with some exceptions, especially BCF values of roots in Cu-contaminated soil, which is estimated as intensive (BCF >1). Heavy metal mobility decreases with increasing soil pH (pH ≥8) due to the precipitation of hydroxides and carbonates or the formation of the insoluble organic complex (Smith and Giller, 1992). Therefore, BCF <1 for Cd and Pb indicated that *S. mucronata* selected these metals, whereas BCF <1 for Cu indicated that *S. mucronata* is nonselective for Cu in contaminated soil. Accumulations of Cd, Cu, and Pb are dependent on their concentrations in the root zone, soil pH, and plant organs. Moreover, Dos Santos Utmazian et al. (2007) revealed that willow growth and phytoextraction efficiency were significantly dependent on the planted species, and that there were differences in plant biomass, metal tolerance, and metal phytoextraction of willow clones. In addition, BCF values of different organs (leaf, bark, shoots, and roots) of *S. viminalis* in Cu concentrations of different Ca/Mg ratios were estimated as weak in most cases and as moderate in some cases, particularly in roots (Mleczek et al., 2013).

The TF% from the roots to the aerial parts was significantly increased with increasing Cd and Pb concentrations in the soil until the (Table 3). In addition, the TF% for Cu did not exhibit a clear trend with various concentrations of Cu. However, the highest values of TF% were 76.68%, 117.68%, and 112.53% observed with 60 mg·kg^{-1} Cd Cl_2, 50 mg·kg^{-1} $CuCl_2$, and 650 mg·kg^{-1} Pb

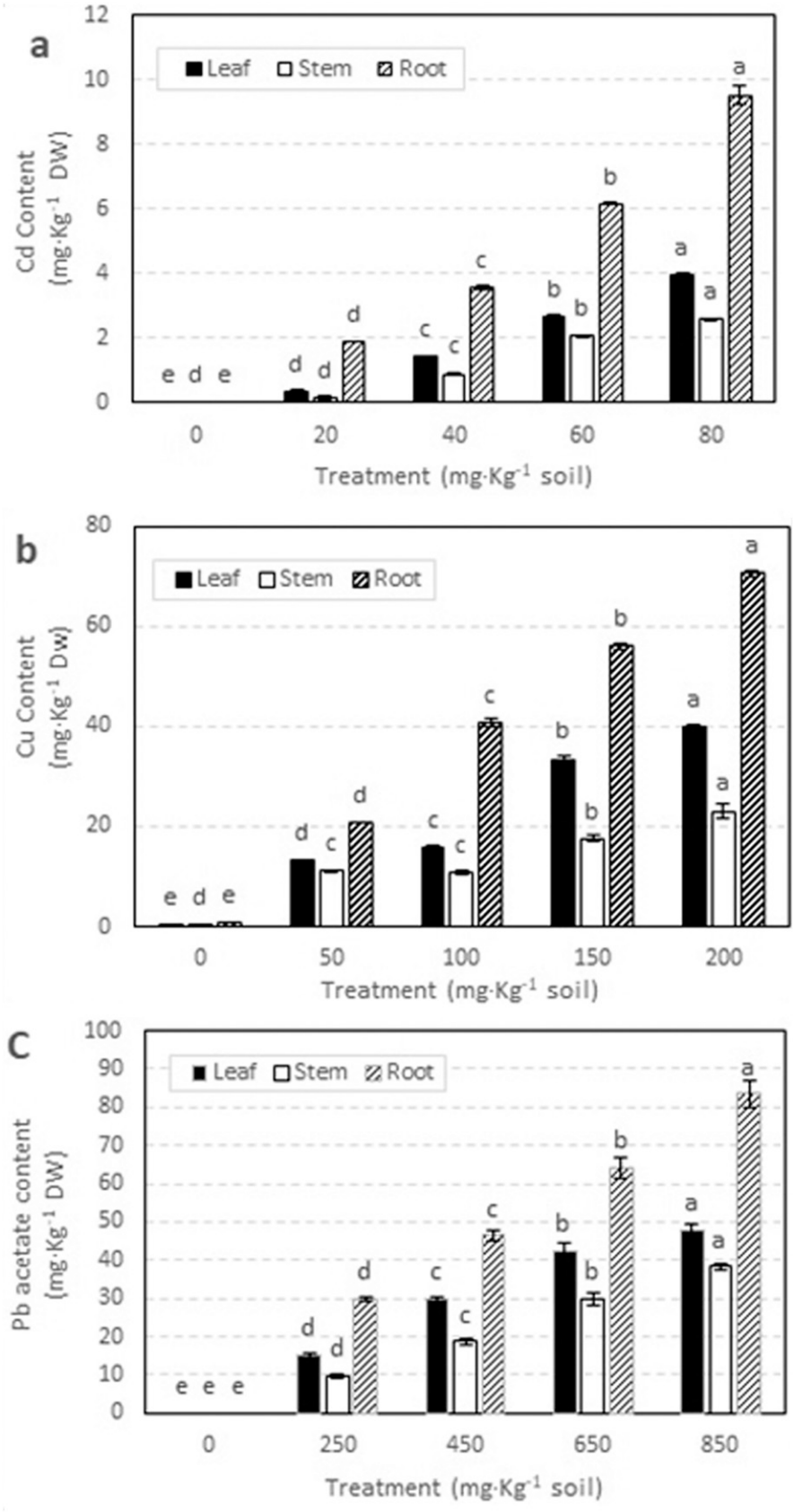

Fig. 2. Heavy metal contents in the leaf, stem, and roots of *Salix mucronata.* (**A**) Cadmium (Cd) content, (**B**) copper (Cu) content, and (**C**) lead (Pb) content (similar letters within a figure are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test).

acetate, respectively. Based on these observations, TF% values are dependent on the type of metal ion and its concentration in the root zone. The natural levels of free Cd and Pb can be highly influenced by cellular sequestration of these metals, which affects their movement throughout the plant (Niu et al., 2007). Our results indicate that TF% values of Cd at different levels were not higher. Supporting this result, Lux et al. (2011) showed that the TF% of Cd is often restricted due to the ability to create a Cd–phytoextraction complex by sequestration in the vacuole. Despite the excessive Cd concentrations in soil and even in the *S. polaris* leaves and stems that were identified (Krajcarova et al., 2016), the plant can activate some protection mechanisms against the excessive intake of Cd, resulting in decreased TF%.

TI_b values were significantly reduced with increasing Cd, Cu, and Pb concentrations in the soil (Table 3). Therefore, the highest values of TI_b (0.98, 1.00, and 0.99) were observed with 20 mg·kg^{-1} $CdCl_2$, 50 mg·kg^{-1} $CuCl_2$, and 250 mg·kg^{-1} Pb acetate, respectively. Conversely, the lowest TI_b values were 0.49, 0.64, and 0.55 for 80 mg·kg^{-1} $CdCl_2$, 200 mg·kg^{-1} $CuCl_2$, and 850 mg·kg^{-1} Pb acetate, respectively. According to these data, TI_b values were less than 1 for Cd, Cu, and Pb levels (a net decrease in biomass and stressed conditions of plants); however, the TI_b value was 1 for the treatment of 50 mg·kg^{-1} $CuCl_2$ (no difference relative to control treatment). According to TI_b values, *S. mucronata* has suitable tolerance against $CdCl_2$, $CuCl_2$, and Pb acetate up to 40 mg, 150 mg, and 650 mg·kg^{-1}, respectively, whereas the TI_b values for the aforementioned concentrations of these metals are more than 0.70 (or 70%). Metal tolerance and uptake were found to be species-dependent and willow clone–dependent (Dickinson et al., 1994). Additionally, plant biomass, metal tolerance, and metal accumulation patterns in roots and leaves varied greatly between 20 different clones of willow and poplar species (Dos Santos Utmazian et al., 2007).

In conclusion, *S. mucronata* tolerated $CdCl_2$, $CuCl_2$, and Pb acetate up to 80, 200, and 850 mg·kg^{-1}, respectively, with 100% survival. Accumulations of Cd, Cu, and Pb in the roots were greater than those in the aerial parts; therefore, the risk of contamination of the wider environment from falling leaves can be considered minimal. Based on BCF, TF%, and TI_b data, *S. mucronata* is suitable for use as a phytostabilizer for Cd and Cu and as a phytoextractor for Pb-contaminated soil.

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