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Assessment of differences in anatomical and hydraulic properties of the root and xylem of three willow (*Salix* L.) clones during phytostabilization after exposure to elevated cadmium

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Abstract: An anatomical study of adventitious roots of three *Salix* clones, 'B-44', 'SV068' and 'SM4041', treated with 3 and 6 mg Cd kg^{-1} dry weight in soil was performed in a greenhouse experiment. The aim was to analyze the anatomical characteristics of roots in response to pollution by cadmium and to assess the potential application of anatomical and hydraulic characteristics in the selection of the most suitable *Salix* clones for phytostabilization of pollutants in soils. Anatomical parameters measured in this study included root cross-sectional area, root diameter, the proportion of periderm, secondary phloem (cortex) and wood (secondary and primary xylem), and parameters of the vessels (lumen area, diameter and frequency). Based on the measurements of individual vessel lumens and the number of vessels, the theoretical hydraulic conductivity (k_h) of roots was calculated. The effects of applied Cd concentrations on root traits were studied in clones and control plants. Following treatments with both Cd concentrations, plants of clone 'B-44' had the highest values of most parameters and significantly higher k_h in comparison with control samples due to the significantly larger root cross-sectional area and lumen area of vessels. It was concluded that these characteristics can serve for effective evaluation and selection of studied clones for remediation of sites contaminated with cadmium.

Keywords: *Salix*; cadmium; root xylem; hydraulic conductivity; phytostabilization

INTRODUCTION

Continual anthropogenic activities lead to the accumulation of potentially toxic elements in soil, water and air [1,2]. Among potentially toxic elements, cadmium (Cd) is one of the most hazardous metals whose increased concentrations in the environment pose a serious threat to soil quality, food security and health of all living organisms [3,4]. Therefore, in order to improve the overall condition of soil and plant health, it is necessary to find the most suitable approach for the rehabilitation of soil contaminated with Cd. Such soils require special refining techniques, convenient for long-term management and based on environmentally friendly and low-cost processes. Phytoremediation is an emerging green technology that uses living plant organisms to remove potentially toxic elements from soils or to stabilize them into a harmless form, which is achieved by two techniques, phytoextraction and phytostabilization. Phytoextraction is the most commonly used technique in phytoremediation that involves the absorption of potentially toxic elements from the soil, their transport and accumulation in the biomass of harvestable organs, shoots and leaves. With phytostabilization, potentially toxic elements are immobilized in the soil by plant roots whose excluded metabolites reduce the solubility, mobility and bioavailability of contaminants in the rhizosphere, thereby neutralizing their harmful effect [2,5,6]. Using plants for phytoremediation/phytostabilization of polluted soils has proven to be a widely accepted alternative to traditional soil remediation techniques such

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as soil flushing, solidification and vitrification since it is feasible, cost-effective, ecologically beneficial, aesthetically preferable and generally improves the quality of contaminated soils [5,6]. To achieve successful reclamation of soils with high metal content, plants must possess efficient mechanisms of exclusion, detoxification, or compartmentation of potentially toxic elements [6,7]. Over decades, many studies have found that woody species from the genus *Salix* L. (willows) have important traits that make them suitable for phytoremediation. These traits include the ability to grow on nutrient-poor and metal-polluted soils, deep and extensive root systems, high biomass production, easy vegetative propagation by cuttings and a high evapotranspiration rate [8-12]. The high genetic variability of *Salix* species is suitable for different plant responses such as accumulation and tolerance to metal exposure and consequently for phytoremediation [13-14]. Given that phytostabilization is the phytoremediation technique used to reduce the mobility and bioaccumulation of heavy metals in soils through processes of metal precipitation, adsorption, complexation and vacuole sequestration of metal ions within plant roots [4-5], *Salix* clones suitable for phytostabilization should combine high metal resistance and tolerance with increased metal accumulation in the roots [13-15].

Since the root is the first plant organ in contact with soil pollutants [11,16], its resistance to pollution by non-essential metals such as Cd is reflected in the changes of overall root growth and production of biomass, uptake and translocation of potentially toxic elements and mechanisms of tolerance and detoxification [17]. When plants are grown on contaminated substrates, greater concentrations of Cd most often accumulate in willow roots than in aboveground organs [9,10,18,19]. Root systems of willow species and clones can accumulate up to 10-fold more Cd than aboveground organs [9-11,20]. This is because of the ability of roots to modify the area of the rhizosphere, which in the case of toxic bioavailable metals leads to their absorption by the roots and restricted translocation to the aboveground plant parts [21]. This process involves plant defense mechanisms, such as the activation of enzymes involved in chelation, vacuolar sequestration and compartmentalization, synthesis of phytochelatins and osmolytes, leading to increased accumulation of the majority of metals, including Cd, in the root and limited root-to-shoot translocation [16]. The rate of Cd accumulation in root tissues significantly depends on both exogenous and endogenous factors that include differences in metal speciation and solubility in soils, the interactions between nutrients and Cd, soil pH, temperature, aeration and ion competition in the rhizosphere, duration of exposure to the metal, age and nutritional status of the plant, transpiration conditions and architecture of entire root system [17-19, 21]. In this regard, it is necessary to better understand the role of the root tissues of *Salix* species in the uptake, accumulation and translocation of Cd. However, only a few studies have observed impaired morphology and anatomy of the root system in several *Salix* species as the consequence of retention and accumulation of excess Cd. According to published research, the greatest changes in root structure were observed in the cortex and vascular tissue parameters [11,20,22,23]. For example, a considerable decrease in the cross-sectional area of the xylem elements in the roots of *Salix caprea* L. became evident after exposure to 0.5 mg Cd/L [11]. In addition, a significant reduction was noted for a number of layers of root cortical parenchyma and cross-sectional area of exodermal cells in *Salix alba* L. clone ‘68/53/1’ after treatment with 10^{-5} M Cd and 10^{-4} M Cd [23]. Exposure of *Salix* roots to toxic concentrations of Cd indicated the development of the exodermis and endodermis, root apoplastic barriers that represent important anatomical adaptations to the presence of this metal [11,16].

Toxic metals can also negatively affect the root absorbing area for water and its movement into the plant vascular system [18]. There are several reasons to pay attention to the soil-root water regime. Water transport and the hydraulic conductivity of the root xylem are a factor that has a great impact on plant drought tolerance, productivity and survival [24,25]. In many tree species, the number and diameter of xylem vessels are the main traits affecting root hydraulic conductivity [24,26]. Variations in xylem anatomy and hydraulic traits occur at both inter- and intraspecific levels [24,25]. Root vessels are considered the best determinant of hydraulic differences among and within species [27]. Variations in vessel diameter can thoroughly affect the plant conducting system according to the fourth power ratio between the radius and flow through a capillary tube, as described by the

Hagen-Poiseuille law [24]. Even a small increase in the mean diameter of vessels has an exponential effect on hydraulic conductivity [28]. Potentially toxic elements can affect root hydraulic conductivity [29]. Nevertheless, only a few studies have paid attention to the anatomical changes that underlie potentially toxic element stress, such as Cd stress, on the hydraulic properties of roots [18,30,31]. They concluded that water absorption by the plant is indirectly regulated by changes in root morphology and anatomy that are affected by polluting metals. Decreased elongation of the primary root, impaired secondary growth, increased root dieback, reduction of root hairs, thickened cell layers of the root's endodermis and exodermis, the reduced amount of xylem and decreased hydraulic conductivity in the root, are considered to be the deleterious effect of Cd on the root-absorbing area [18,30]. Investigations on plant-water relations under metal toxicity revealed drought as a frequent stress factor in metal-enriched soils [30,32]. Accumulation of Cd ions in root tissues and related structural variations result in insufficient root-soil interaction and complex and deleterious changes of water transport across the entire plant, consequently disturbing all physiological processes [18]. Moreover, several studies have suggested that a plant's response to toxic metals is essentially a response to drought due to a reduced water-uptake capacity of roots exposed to metal. The authors state that the effects of metal and drought stresses on xylem characteristics in roots resembled each other, with a reduced proportion of xylem tissue and conduit size [18,30]. However, there are little data on the hydraulic conductivity of willow roots and their connection with potentially toxic elements [33]. To the best of our knowledge, more extensive anatomical analyses of willowroot hydraulic traits have not been conducted. Therefore, in the present study we performed a detailed anatomical analysis of roots and calculated the theoretical hydraulic conductivity in three *Salix* clones grown in a soil culture with different concentrations of Cd. The main objectives of this study were to analyze the differences in root anatomical properties (xylem characteristics in particular) among different *Salix* clones and under treatment with two Cd concentrations, to calculate the theoretical hydraulic conductivity of the roots of control and Cd-treated plants, and to evaluate the possible application of xylem anatomical characteristics and values of theoretical hydraulic conductivity in the selection of clones that could have a potential role in the phytostabilization of Cd pollution.

MATERIALS AND METHODS

Plant material and experimental design

The anatomical study was performed on 1-year-old stem cuttings of clones of three willow species: *Salix alba* L. clone 'B-44', *Salix viminalis* L. clone 'SV068' and *Salix matsudana* Koidz. clone 'SM4041', obtained from the Institute of Lowland Forestry and Environment (ILFE), Novi Sad, Republic of Serbia. The experiment was conducted in a greenhouse of the Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad, Republic of Serbia (45.245411 E, 19.852786 N). Unrooted willow cuttings (20 cm long, with one shoot per cutting) were grown under semi-controlled conditions by the soil culture method. The temperature in the greenhouse was maintained between 20 and 30°C, with a 12 h light/dark photoperiod and 55-60% relative humidity.

Plants were planted in Mitscherlich pots containing 5.0 kg of sandy fluvisol obtained from a forest nursery (19.9385 E, 45.3025 N) with pH 7.6-8.4. Cd was applied to the soil as a nitrate salt dissolved in deionized water ($CdNO_3 \cdot 4H_2O$ solution) in two different concentrations to a final concentration of 3 and 6 mg Cd kg^{-1} dry soil each. The plants were then divided into three groups as follows: control (0 mg Cd kg^{-1}), Cd 6 (treatment containing 6 mg Cd kg^{-1}) and Cd 3 (treatment containing 3 mg Cd kg^{-1}). Plants were harvested after two months of treatment. Prior to further analysis, the plants were washed with deionized water to ensure the complete removal of soil from the root surface.

Microscopic assessment of the root anatomical traits

Adventitious roots were separated from five replicate plants per clone and treatment. Plant material was fixed and preserved in 50% ethanol. Cross sections of the roots were made 4 cm from the root neck using a Leica CM 1850 (Leica Microsystems, Nussloch, Germany) cryostat at temperatures ranging from -18 to -20°C, in 60-μm-thick sections. Cross sections were examined, and measurements were made using a light microscope and the Image Analyzing System Motic Image Plus 3.0 (Motic Microscopes, Germany). Root cross sections of three willow clones were measured and relative proportions calculated for each tissue of interest.

Detailed anatomical analyses included measurements of the root cross-sectional area, root diameter, percentages of periderm, secondary phloem (cortex) and wood (secondary and primary xylem). Vessel lumen area (VL) and vessel diameter (VD) of each vessel, as well as vessel frequency (VF; number of vessels/mm^2 xylem cross section) were measured and calculated. All vessels in every root cross section were measured, yielding 497-1112 measured vessels per clone. The total area of vessels was given as the sum of areas of all vessels in each observed root cross section. The relative vessel lumen area was obtained as the percentage of total vessel lumen area in the corresponding wood area. The hydraulic diameter (or hydraulically weighted vessel diameter), D_H (μm), in which every individual vessel is weighted according to its contribution to the total hydraulic conductivity, was calculated from single vessel diameters [34] as $D_H=\Sigma D^5/\Sigma D^4$, where D is an individual vessel diameter at a given cross section. Based on their diameter, root vessels were divided into three frequency classes of 20 μm, as described [35,36]: vessels with a diameter smaller than 20 μm (I group); vessels with a diameter in the range of 20 to 40 μm (II group); vessels with a diameter greater than 40 μm (III group). The percentage of vessels in each class, expressed as the percentage of the total number of vessels (100%), was calculated.

Based on anatomical measurements, the theoretical root hydraulic conductivity (k_h) was calculated per visual field of cross sections, according to the modified equation [24] based on the Hagen-Poiseuille law:

$$k_h = \frac{\pi \cdot \rho}{128 \cdot \eta} \sum_{i=1}^{n} d_i^4$$

where d is the diameter of the vessels in meters, ρ is the density of water (1,000 kg m^{-3}) and η is the viscosity of water (1.002 10^{-9} MPa s), both at 20°C [37].

Statistical analyses

Data were statistically processed using STATISTICA for Windows ver. 12.0 (StatSoft, Tulsa, OK, USA). To determine whether differences in quantified root anatomical and hydraulic traits were significant among control plants as well as between Cd-treated and control plants, one-way ANOVA was performed, followed by Duncan's multiple range test ($P<0.05$). The general structure of sample variability was established by principal component analysis (PCA) based on a correlation matrix.

RESULTS

Anatomical traits of willow adventitious roots

The roots of three *Salix* clones (both control and Cd treatments) underwent secondary thickening, and the rhizodermis and primary cortex completely collapsed and shed (Figs. 1-3). Three zones of root tissues could be distinguished: the periderm, the secondary cortex (phloem) and the wood (primary and secondary xylem). The tangential groups of fiber cells were observed in the secondary cortex.

Anatomical analyses showed significant differences in root anatomical parameters among the three analyzed clones (Table 1): clone 'B-44' differed significantly from clones 'SV068' and 'SM4041' in most measured parameters. The clone 'B-44' was characterized by the significantly highest values of root cross-sectional area, root diameter, VF and relative VL, as well as by the significantly highest values of individual vessel parameters (VL, VD and D_H) and the percentage of vessels in the second diameter class. On the other hand, clone 'SV068' exhibited the significantly smallest values of many anatomical parameters when all three clones were compared. This clone was characterized by the significantly smallest root cross-sectional area, root diameter and individual vessel parameters. Clone 'SM4041' was to some extent positioned between the other two clones; it only had a significantly highest total VL area. Considering the proportion of vessels in each class of vessel diameters, clones 'B-44' and 'SM4041' had the highest diameters in the range of 20 to 40 μm (group II). The roots of clone 'SV068', with the smallest values of most parameters,

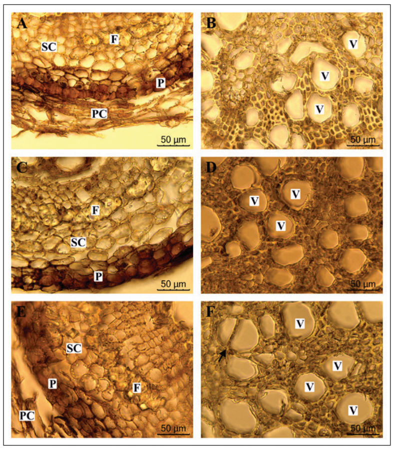

Fig. 1. Anatomical traits of willow adventitious roots. Light micrographs of root cross sections of *Salix alba* L. clone 'B-44': **A**, **B** – control; **C**, **D** – treatment with 3 mg kg^{-1} Cd; **E**, **F**–treatment with 6 mg kg^{-1} Cd. P – periderm. PC – shed primary cortex; SC – secondary cortex; F – fibers; V – vessels. Arrows point to grouping vessels.

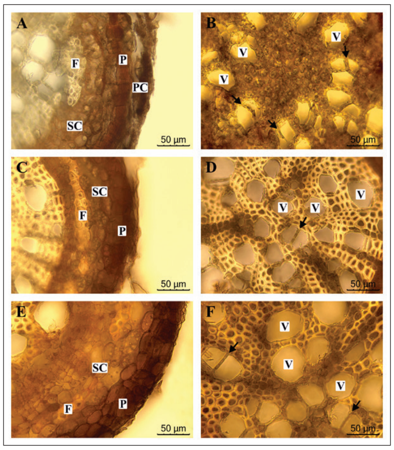

Fig. 2. Anatomical traits of willow adventitious roots. Light micrographs of root cross sections of *Salix viminalis* L. clone 'SV068': **A**, **B** – control; **C**, **D** – treatment with 3 mg kg^{-1} Cd; **E**, **F** – treatment with 6 mg kg^{-1} Cd. P – periderm. PC – shed primary cortex; SC – secondary cortex; F – fibers; V– vessels. Arrows point to grouping vessels.

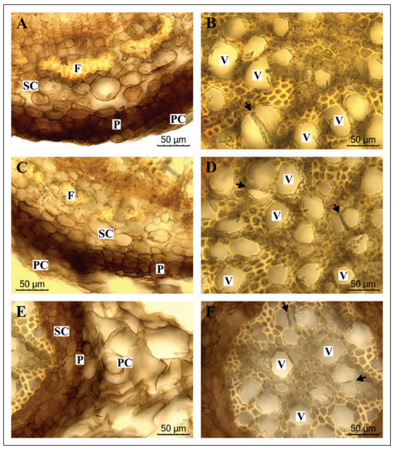

Fig. 3. Anatomical traits of willow adventitious roots. Light micrographs of root cross sections of *Salix matsudana* Koidz. clone 'SM4041': **A**, **B** – control; **C**, **D** – treatment with 3 mg kg^{-1} Cd; **E**, **F** – treatment with 6 mg kg^{-1} Cd. P – periderm; PC – shed primary cortex; SC – secondary cortex; F – fibers; V– vessels. Arrows point to grouping vessels.

Table 1. Root anatomical traits of three *Salix* clones (control plants) grown in soil culture, with mean values±standard error (n=5).

Root traits	**'B-44'**	**'SV068'**	**'SM4041'**
Root cross–sectional area (µm^2 10^5)	6.06±1.83a*	1.82±0.09c	3.91±0.75b
Root diameter (µm)	849.71±134.15a	478.19±10.42c	683.92±65.38b
% periderm thickness	3.51±0.53a	4.64±0.32a	5.42±0.44a
% secondary cortex	19.19±3.01b	20.94±2.26b	33.24±5.18a
% wood (primary and secondary xylem)	32.03±3.20a	34.05 ±2.40a	21.69±1.69b
Wood/cortex ratio	1.74±0.12a	1.70±0.19a	0.71±0.11a
VF (N/mm^2)	81.00±19.76a	42.80±5.21b	41.60±7.68b
Total VL (µm^2 10^4)	6.31±2.55c	1.32±0.17b	2.38±0.69a
Relative VL (%)	29.14±2.06a	20.88±1.66b	27.20±1.75a
Individual VL (µm^2)	780.29±25.66a	307.70±12.20c	572.60±31.37b
Individual VD (µm)	29.87±0.50a	18.94±0.40c	24.93±0.72b
D_H (µm)	34.07±3.77a	23.98±1.11c	31.55±4.08b
% vessels I group	21.98±7.05c	53.96±9.33a	29.37±16.51b
% vessels II group	66.85±3.63a	46.04±9.34c	62.06±14.92b
% vessels III group	11.17±6.60a	0.0±0.0c	8.57±6.76b

* Differences among the means marked with the same letter were not significant according to Duncan's test ($P < 0.05$)
% periderm; % secondary cortex; % wood – proportion of tissues in the root cross-section; VF – vessel frequency; VL – vessel lumen area; VD – vessel diameter; D_H – hydraulic mean vessel diameter; % vessels in I group – % vessels with a diameter <20 µm; % vessels II group – % vessels with a diameter 20-40 µm; % vessels III group – % vessels with a diameter >40 µm

had a diameter peak in group I with the smallest diameter values (in the range of 0-20 µm).

Principal component analysis (PCA) of root traits of willow clones

The results of PCA analysis showed that the first principal component explained 51.39% of the total variation. The root variables loaded on the first principal component axis were root cross-sectional area and root diameter, as well as most of the vessel parameters, including VF, total VL, individual VL and VD, D_H, and percentages of vessels belonging to groups I and III (Table 2). The second principal component accounted for a further 19.18% of the total variance and was defined by the percentages of peridermal thickness and secondary cortex along the wood/cortex ratio. The scatterplot showed a clear separation and high homogeneity of clone 'SM4041' specimens along the first axis. Clones 'B-44' and 'SV068' were more heterogenous, without specific and distinct clusters (Fig. 4).

Table 2. Principal component analysis of root anatomical parameters of three *Salix* clones.

Anatomical traits	Factor 1	Factor 2	Factor 3	Factor 4
Root cross–sectional area (μm^2 10^5)	-0.9394*	0.1175	-0.1747	0.1564
Root diameter (μm)	-0.9220*	0.1723	-0.1021	0.2527
% periderm thickness	0.1864	-0.8246*	0.2616	-0.3335
% secondary cortex	-0.1352	-0.9203*	-0.1102	-0.2059
% wood (primary and secondary xylem)	-0.3387	0.2847	-0.0186	-0.8893*
Wood/cortex ratio	-0.0384	0.8996*	-0.0256	-0.3413
VF (N/mm^2)	-0.7009*	0.1284	-0.6515	-0.0642
Total VL (μm^2 10^4)	-0.9195*	0.1212	-0.3412	-0.0691
Relative VL (%)	-0.6091	-0.3853	-0.3345	0.0997
Individual VL (μm^2)	-0.9594*	-0.1158	0.2085	0.0549
Individual VD (μm)	-0.7713*	-0.3084	0.2833	-0.1874
D_H (μm)	-0.9628*	-0.0581	0.1956	0.0461
% vessels I group	0.7971*	-0.1085	-0.5822	-0.0312
% vessels II group	-0.4894	0.2493	0.7903*	0.0778
% vessels III group	-0.8841*	-0.2260	-0.1746	-0.0765
Eigenvalue	7.7084	2.8774	1.9313	1.2213
% Total variance	51.39	19.18	12.88	8.14

Values marked by an asterisk (*) are >0.7000 and significant for the axis

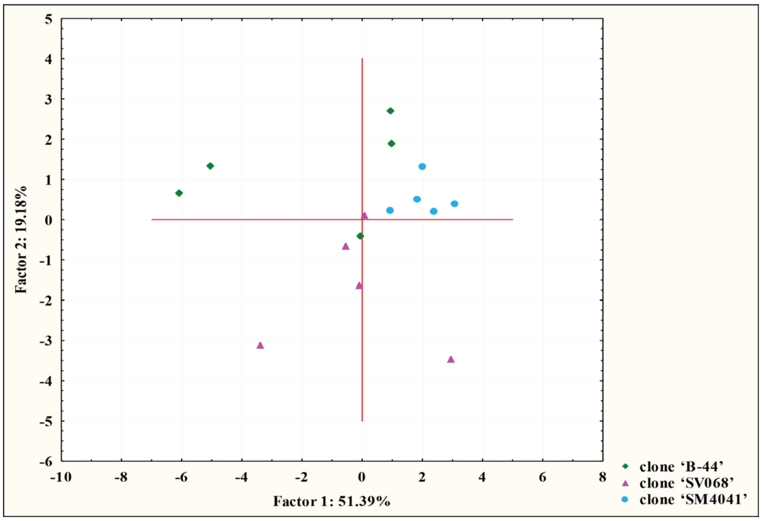

Fig. 4. Principal component analysis (PCA) of anatomical traits of willow roots. PCA scatterplot based on root anatomical parameters of three different *Salix* clones.

Variations in root anatomical traits of Cd-treated willow clones

When compared with the control plants, the applied Cd elicited different anatomical responses in the roots of the three *Salix* clones (Fig. 5A-C). In the roots of clone ‘B-44’, many anatomical traits presented significantly increased values under Cd treatments. The most prominent were the root cross-sectional area, root diameter, percentages of periderm and secondary cortex, and total VL. Parameters of individual vessels (VL, VD and D_H) significantly increased in response to both Cd treatments (Fig. 5A). Percentages of vessels in the I and II groups were significantly lower, but in group III significantly higher when compared to control plants. Both Cd concentrations produced opposite effects on the percentage of xylem and relative VL in root tissue of clone ‘B-44’.

The two treatments with Cd produced the reverse effects on root traits in clone ‘SV068’ when compared to control samples (Fig. 5B). The concentration of 6 mg Cd kg^{-1} led to significantly increased values of the root cross-sectional area, percentage of secondary cortex and total VL, while the concentration of 3 mg Cd kg^{-1} produced a decreasing effect on the same root anatomic traits. Both concentrations of Cd caused a significant decrease in the percentage of secondary xylem and VF. Similar to clone ‘B-44’, the values of individual vessel lumens and diameters in the roots of clone ‘SV068’ increased after both Cd treatments. In addition, the percentage of vessels belonging to group I significantly decreased, but in groups II and III, significantly increased in clone ‘SV068’ when compared with the control. In the roots of clone ‘SM4041’, both Cd concentrations produced the most significant increase in the root cross-section area. The Cd treatments caused significant but opposite changes in most root parameters of this clone. Percentages of secondary phloem and wood (primary and secondary xylem), total VL, and individual VL and VD significantly decreased after treatment with 6 mg Cd kg^{-1}, but significantly increased after treatment with 3 mg Cd kg^{-1} with respect to the control (Fig. 5C). A significant increase in the percentage of vessels belonging to group II was observed in the root of clone ‘SM4041’,

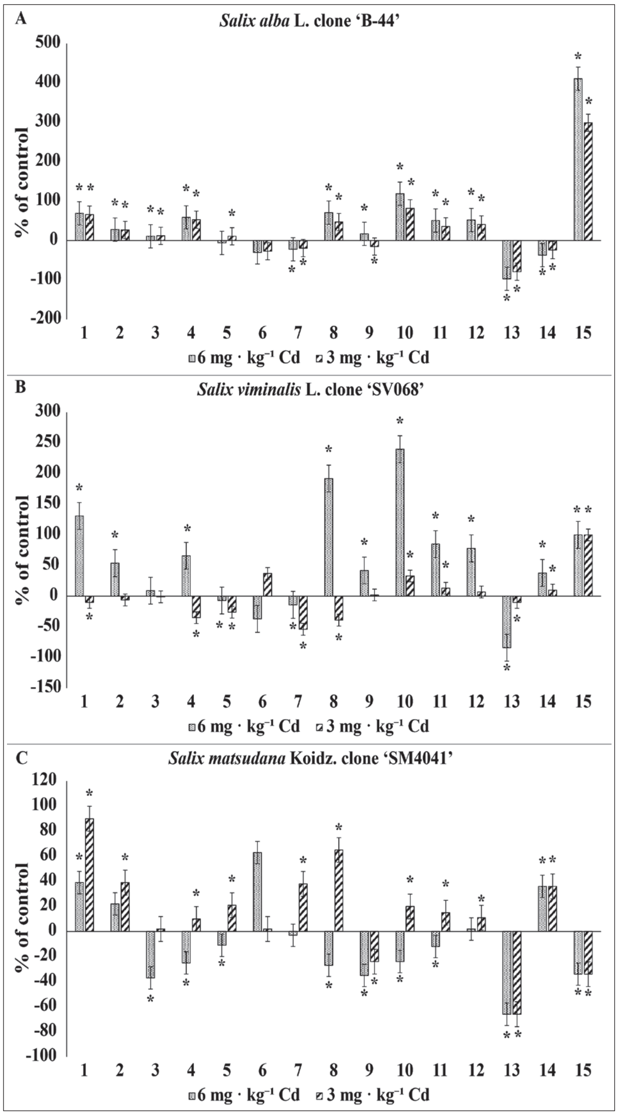

Fig. 5. Variations of roots anatomical traits. Values were compared to control plants for *Salix* clones 'B-44' (**A**), 'SV068' (**B**), 'SM4041" (**C**). 1 – root cross-sectional area; 2 – root diameter; 3 – percentage of periderm thickness; 4 – percentage of secondary cortex (phloem); 5 – percentage of wood (primary and secondary xylem); 6 – wood/cortex ratio; 7 – VF; 8 – total VL; 9 – relative VL; 10 – individual VL; 11 – individual VD; 12 – hydraulically weighted vessel diameter (D_H); 13% of vessels, I group; 14% of vessels, II group; 15% of vessels, III group. Duncan's test indicates significant differences between Cd-treated and control plants. *P<0.05.

which had the highest diameter in the same vessel group. In contrast, Cd treatments caused a significant decrease in vessels belonging to the I and III diameter classes in comparison with the control.

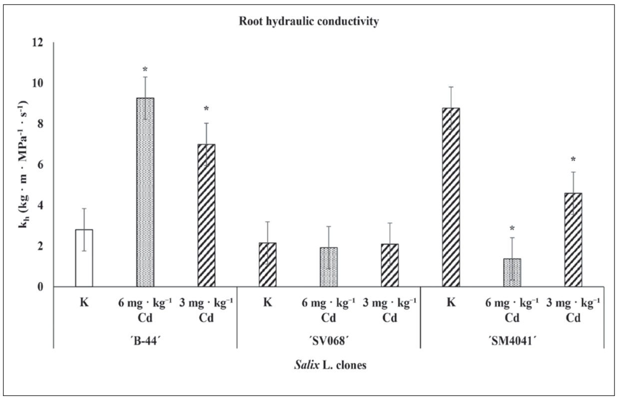

Fig. 6. Root hydraulic conductivity (k_h) of three willow clones. The values represent the means of three visual fields of five sections per root±SE. Differences between the means with an asterisk are significant in comparison with control plants according to Duncan's test at P<0.05.

Overall, the root cross-sectional area of clone 'B-44' plants treated with both doses of Cd significantly positively correlated with the root diameter (r=0.98 and 0.99, respectively), VF (r=0.96 and 0.98, respectively), total VL (r=0.98 and 0.98, respectively), and the percentage of vessels belonging to group III (r=0.93 and 0.84, respectively). The cross-sectional area negatively correlated with the percentage of vessels in group II (r=-0.93 and -0.84, respectively). In clone 'SV068' plants treated with 6 mg Cd kg^{-1}, the root cross section displayed positive significant correlations with the root diameter (r=0.94) and total VL (r=0.89), while the root cross section of plants treated with 3 mg Cd kg^{-1} exhibited a significant positive correlation with the root diameter (r=0.98), but a significantly negative correlation with relative VL (r=-0.89). In clone 'SM4041', root cross sections of plants treated with 6 mg Cd kg^{-1} displayed a significantly positive correlation with the root diameter (r= 0.89), VF (r= 0.96) and total VL (r= 0.89). In addition, in the roots of this clone treated with 3 mg Cd kg^{-1}, the root cross-sectional area had a significantly positive correlation with the root diameter (r= 0.94), percentage of secondary cortex (r=0.92) and VF (r=0.93), but a significantly negative correlation with the wood/cortex ratio (r=-0.92).

Root hydraulic conductivity

Theoretical hydraulic conductivity (k_h) was significantly higher in the roots of clone 'B-44' treated with both Cd concentrations but was significantly lower in clone 'SM4041' after both Cd treatments in comparison with the control (Fig. 6). In clone 'SV068', significant differences in hydraulic conductivity between treated and control plants were not observed.

DISCUSSION

The willow clones assessed in this experiment were selected according to high biomass production, the property of good Cd removal from moderately polluted soils, a high Cd content in roots in comparison with aboveground organs, and a clone-specific response to high Cd concentrations [3,12]. Anatomical examination of root tissues is significant in the assessment of root development and distinguishing different types of roots [38], and their functioning in stress-effect studies [39]. The examined *Salix* roots underwent secondary growth. Unlike primary roots with the main absorption function, roots characterized by the production of secondary tissues are known as transport roots [38,40,41]. Senescence of root primary tissues, particularly the primary cortex, and the development of secondary tissues, notably increases the abundance and size of metaxylem vessels, resulting in increased root transport capacity [41,42]. Functionally, such roots are referred to as structural roots that serve as conduits for long-distance transport of water, nutrients and metabolites [43]. Considering the measured diameters (Table 1), the roots in the three *Salix* clones can be classified as fine and small roots, as described previously [44]. The anatomical and hydraulic traits of secondary roots and wood, i.e. primary and secondary xylem as its central part, have been observed in several different tree species [44-46]. In our experiment, the results of anatomical analyses indicated that the *Salix* clones had different measured values of most traits of secondary roots. The most significant differences were observed in the percentages of individual root tissues and in the parameters of vessels. Clone 'B-44' had the greatest values of most measured root parameters, primarily the root cross-sectional area and individual vessel traits, VL and VD. On the other hand, 'SV068' had the smallest values of these root parameters among the clones. Vessels in *Salix* clone roots were usually solitary, only rarely in radial multiples of two. A similar arrangement of vessels is observed in the roots of tree species such as *Populus nigra* L. [40] and *Betula platyphylla* Roth. [47].

The root is the primary plant organ for metal uptake, detoxification and tolerance, and it plays an important role in limiting metal translocation to aboveground organs by metal stabilization and metal removal [6,11]. The ability of willows to grow on soils with high Cd concentrations is generally related to the ability of their roots to accumulate high concentrations of Cd [10,48]. Despite its importance to plant functioning, the anatomical structure of the root is rarely described in stress-effect studies [6,30,38]. The structural differences in willow root anatomy and the related changes under elevated Cd stress [23] are important for sustaining plants exposed to the effects of metal toxicity [49]. Data on the significant negative effects of elevated Cd concentrations on the size and shape of root cells, and the relative proportions of tissues contributing to the primary root structure of different *Salix* species, varieties and clones have been presented [6,20,23]; many of these changes are specific to *Salix* clones that display different characteristics of Cd accumulation and Cd tolerance [20]. However, data on the impact of Cd concentrations on secondary root anatomy in willow are scarce. Our results showed that the roots of willow clones, although grown under conditions of increased concentrations of Cd, did not express necrotic changes. The obtained data indicated that the applied Cd concentrations caused different anatomical responses in willow roots that were both clone- and Cd concentration-dependent. For *Salix alba* clone 'B-44' and *Salix matsudana* clone 'SM4041' plants that grew under both Cd concentrations, the root cross-sectional area was significantly increased when compared to control samples. In the first willow clone, the increase in this value can be related to elevated total VL and individual VD and VD when compared to the control. In the second willow clone, a similar increase in the root cross section was caused by the elevated percentage of vessels from group II. For comparison, a related study described a significant increase in the root cross-sectional area of *Salix nigra* clone '0408' exposed to 10^{-4} M Cd [23]. The significant positive correlation of the root cross-sectional area with root diameter, VF and total VL recorded in clones 'B-44' and 'SM4041' indicated that the wider roots of these clones possessed more vessels in comparison with the control. A small but significant decrease in the root cross-sectional area in response to 3 mg kg^{-1} Cd was observed in *Salix viminalis* clone 'SV068' and was caused by the reduction in the percentages of the secondary cortex and wood, VF and total VL. Similarly, a significant reduction in the cross-sectional area after Cd treatment was recorded in roots of *Salix alba* clone '68/53/1' in the presence of 10^{-4} M and 10^{-5} M Cd [23], and in poplar clone 'B-81' in the presence of 10^{-7} M Cd [50].

Given this data, it can be concluded that relatively scarce information is available about the development and anatomy of roots of tree species from the family Salicaceae exposed to elevated metal concentrations in the environment and in the laboratory [11,16,23]. This topic requires more attention, given the importance of root-to-shoot translocation of potentially toxic elements, notably Cd [51], the impact of Cd on root xylogenesis and the distribution of Cd in secondary root tissues [11]. The anatomical structure of root wood in the three *Salix* clones pointed to interspecific differences in response to Cd toxicity. The results of the present study showed that concentrations of 6 and 3 mg Cd kg^{-1} produced opposite effects on the parameters of wood and vessels in the roots of all tested clones when compared to control plants (0 mg Cd kg^{-1}). In the roots of clones 'B-44' and 'SV068', both Cd treatments led to increased values of the lumen and diameter of individual vessels. Poorer results were obtained for the individual parameters of vessels in clone 'SM4041', where the presence of 6 mg Cd kg^{-1} led to their significant decrease, and 3 mg Cd kg^{-1} caused a significant increase in VL and VD. Previous studies confirmed that the traits of root xylem that are affected by an excess of Cd include earlier differentiation of xylem [52], smaller xylem vessel size and lower vessel density [30]. In *Salix* roots, VD appeared to be a more sensitive root parameter to Cd stress than VF, unlike earlier findings [39,53].

The hydraulic conductivity of the root xylem of *Salix* clones is a parameter of special interest. Most research has focused on the anatomical and hydraulic properties of stems and branches of tree species, while these properties in roots have been examined in fewer studies [27,45,46,54]. Short- and long-distance transport of water and other molecules are significantly affected by the anatomical traits of the root xylem [44,45]. It was reported [38] that the anatomical changes in root structure, especially changes in the size and diameter of vessels in the xylem, are important for the survival of trees under stress. Environmental stresses acting on root anatomy include potentially toxic elements, but their impact on the hydraulic properties of roots in tree species was observed in only a few studies [18,30,50]. The negative effects of metals on the xylem characteristics of roots included a reduced proportion of xylem tissue, reduced conduit size and consequently restricted water uptake in metal-stressed plants, which can produce and aggravate the drought-stress effect [30]. Very little data about this topic refer to the genus *Salix* [33]. The roots of *Salix* clones grew under the same conditions, and the root values of k_h represent a response to the elevated Cd concentrations and correspond to the variations in secondary xylem traits. The higher hydraulic conductivity of roots of 'B-44' plants treated with Cd relative to the control was due to the increase in VL and VD values when compared to the matching control. In the roots of Cd-treated 'SV068' plants, despite the higher values of VL and VD, the k_h did not differ significantly from the control plants. In the roots of 'SM4041', lower values of k_h were measured under both conditions of Cd exposure. This is in accordance with data for several cherry rootstocks and variations in vessel characteristics [46], which were probably driven by genetic factors [55]. The results provided by Loval et al. [56] show that six *Salix* clones derived from different crossings showed differences at the level of the xylem in the structural, functional and growth response to different abiotic stresses and that these responses depend on the clone, regardless of the crossing. In general, plant roots have a wider conduit diameter than the stem [45]. Additionally, the hydraulic conductivity depends on the number and diameter of vessels [30,57]. In the three *Salix* clones examined herein, the highest percentage of vessels was observed in the group with a diameter ranging from 20 to 40 μm, which shows the strong impact of metal stress on the largest diameter category. In contrast, xylem and vessel traits and the related k_h in roots of *Acer rubrum* L. decreased in the presence of metal stress, and the roots had fewer small conduits [30]. The negative impact of Cd stress on root conductivity expressed through the reduction in the size of vessels had a pronounced negative effect on the efficiency of water transport through the xylem and on the hydraulic properties of plant roots [1,58]. Even a small increase in vessel diameter significantly increases water conductivity but can increase the risk of embolism due to drought [57]. A larger number of small vessels seems to be an adaptive response since smaller vessels are less susceptible to embolism [59]. Therefore, the effects of Cd on root anatomy in different willow genotypes is of considerable interest, and the results from our study could be used to optimize phytoremediation efficiency.

CONCLUSIONS

The results obtained in the present study revealed that elevated Cd concentrations in the soil affected the anatomical structure and hydraulic traits of roots in *Salix* clones 'B-44', 'SV068' and 'SM4041', especially the lumen and diameter of the individual vessels. This indicates that the anatomical structure of adventitious roots of different tree genotypes can serve as a useful indicator of soil pollution. Our results also revealed the genotype-specific response of three *Salix* clones to elevated concentrations of soil Cd, indicating the need for further research associated with the possible use of clone 'B-44' in strategies for remediation of Cd-contaminated soils by phytostabilization. Based on the results obtained in this study, it can be concluded that basic anatomical and hydraulic characteristics of roots of the examined *Salix* clones can be used in the future selection of genotypes with desirable features required for efficient phytoremediation of soils polluted with Cd. Furthermore, knowledge of the hydraulic conductivity of tree roots, along with anatomical, morphological and physiological traits can be used to predict plant vulnerability to abiotic stress such as water deficiency, since excess metal ions in soil can lead to a decrease in the water content in roots.

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Data availability: All data underlying the reported findings have been provided as part of the submitted article and are available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Hrkic-Ilic%20et%20al_7554_Data%20Report.pdf

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