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Biochar improves the morphological, physiological and biochemical properties of white willow seedlings in heavy metal-contaminated soil

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Abstract: Biochar is an efficient soil amendment used for promoting plant resistance to heavy metal (HM)-contaminated soils. There is a need for further investigation of its impacts on plants and soil. This study was undertaken as a pot experiment to assess the effect of biochar (0, 2.5, and 5% mass fractions) on the morphological, physiological and biochemical responses of white willow seedlings (*Salix alba* L.) cultured in uncontaminated soil and mixed soil contaminated with HM (Cu, Pb, and Cd). Additionally, some chemical properties and HM bioavailability were evaluated. Biochar increased height and diameter, root elongation, leaf area and dry biomass of the seedlings in both soils. Its addition to the contaminated soil reduced electrolyte leakage, the malondialdehyde and proline contents but increased the chlorophyll content, net photosynthesis rate, intercellular CO_2 concentration and transpiration rate in the leaf. Use of biochar (especially at 5% rate) in both soils, increased soil pH, total nitrogen, soil organic carbon and available P and K, while in the contaminated soil the availability of Cu, Pb, and Cd decreased. The results showed that biochar is a suitable amendment to contaminated soils that improves plant properties by improving soil chemical features and immobilizing HMs.

Keywords: heavy metal bioavailability; phytoremediation; soil amendment; soil properties; biochar

Abbreviations: Brunauer-Emmett-Teller (BET); Barrett-Joyner-Halenda (BJH); intercellular CO_2 concentration (Ci); transpiration rate (E); electrolyte leakage (El); stomatal conductance (Gs); leaf area (LA); heavy metal (HM); malondialdehyde (MDA); total nitrogen (TN); scanning electron microscopy (SEM); specific leaf area (SLA); soil organic carbon (SOC); water use efficiency (WUE)

INTRODUCTION

Human population growth and anthropogenic activities, which have led to a decrease in soil quality and yield production, have become an important issue throughout the world, especially in developing countries [1]. Soil pollution by HM is the result of human activities such as mining, the smelting industry, burning fossil fuels, industrial and municipal waste disposal, industrial and municipal wastewater discharge, irrigation with polluted water and use of pesticides and agricultural fertilizers [2]. High concentrations of HM such as cadmium (Cd), lead (Pb), chromium (Cr), copper (Cu), and mercury (Hg) produce toxic effects on soil processes [3]. To eliminate HM from contaminated soils, metal-accumulator plants have been used as a novel eco-friendly and cost-effective approach, referred to as phytoremediation [4].

Willows are multipurpose trees [5] that have high biomass production, rapid regeneration, an extensive root system and tolerance to changed environmental conditions [6]. Recent studies have confirmed the potential of willows to survive in metal-enriched soils and their use in phytoremediation [7-10]. Although willows are not hyperaccumulator plants, their tolerance to HM toxicity, rapid growth and high biomass production makes them suitable for phytoremediation [8-12]. Willows are utilized in short rotation coppicing systems for both biofuel production and soil remediation purposes [12]. In order to ensure the optimal performance of willows, amendments such as biochar could be considered a good option [6].

Biochar can be produced from carbon-rich feedstock through the process of pyrolysis at high tem-

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peratures in the absence of oxygen. During pyrolysis, volatile matter production creates a porous structure in biochar and holes on its surface [14]. In some cases, the biochar pore size is lower than 100 nm [15], which makes biochar a nanostructure according to the International Union of Pure and Applied Chemistry (IUPAC) standard. Recent studies indicate that biochar can increase soil essential nutrients and provide a habitat for soil microorganisms [6,13]. Biochar is widely used in biomass waste management [16] by improving the physical and biological properties of soil [17], by reducing the adverse effects of pollutant soils [18], and particularly for increasing crop yield [19].

The biochar porous nanostructure acts as adsorption sites for soil HM and enables water retention [14]. According to earlier findings, biochar could improve the HM uptake by plants through growth increment [3,20-21] or absorption of HM and reducing their bioavailability [22]. Regarding biochar-assisted phytoremediation with willow, Lebrun et al. [23] showed that adding pinewood biochar to Pb- and As-contaminated soil increased the growth and biomass production of willow and the metal content in willow trees. In addition, a positive effect of biochar was noticed on soil quality [23]. In contrast, Lebrun et al. [6] found no positive effect after the addition of pinewood biochar on willow growth parameters and on the physicochemical properties in a multi-contaminated soil. In these studies, willow growth parameters and their ability to accumulate HM were investigated and different results were obtained for different conditions.

Since there are inconsistent results from previous investigations, further studies need to explore useful information regarding the impact of biochar on white willow with different feedstock (broadleaf wood biochar) in HM-contaminated soils. Furthermore, the analysis of physiological and biochemical properties of the plants as stress indicators is considered to be useful in describing plant tolerance to HM stress as it provides information on how plants are affected by contaminants [24]. Along with these properties, growth analysis could also provide new insights into the plants' response to HM-contaminated soil amended by biochar.

The objective of the present study was to analyze the effects of biochar amendment and HM contamination (Cu, Pb, and Cd) on selected morphological (dry biomass, height and diameter growth and leaf area), physiological (photosynthetic rate, transpiration rate, stomatal conductance, internal CO_2 concentration and water use efficiency), and biochemical (electrolyte leakage, malondialdehyde and proline content) properties of white willow seedling growth. Furthermore, the effect of soil amendment on certain soil chemical properties (pH, total nitrogen, soil organic carbon and available K and P) and availability of Cu, Pb, and Cd were investigated.

MATERIALS AND METHODS

Preparation and planting of cuttings

Cuttings of white willow were collected from the Ardabil Agriculture and Natural Resources Research and Education Center, AREEO (northwest Iran). Uniform cuttings with a 20-25 cm height and 1.5-2 cm diameter were transplanted at the end of February 2016 into 1-kg cylindrical plastic pots (10 cm diameter, 15 cm height) that were filled with sand. The pots were kept at the Research Greenhouse of the Faculty of Natural Resource, Tarbiat Modares University (northern Iran). The greenhouse temperature was adjusted to 18-25°C with 12 h of light. Pots were watered with an equal amount of water twice a week for two months before transplantation of the seedlings into pots with clean and mixed HM-contaminated soils.

Preparation and analysis of soil samples

A bulk soil sample was collected (depth of 0-20 cm) from an uncontaminated area located in the Mazandaran province, northern Iran. The collected soil was air-dried and sieved through a 2-mm mesh and mixed thoroughly with sand at a 2:1 ratio (sand/soil). The pots (10 cm diameter and 20 cm height) were filled with 3 kg of prepared soil. Half of these pots (9 pots) were spiked with 500, 200, and 20 mg of Pb, Cu, and Cd kg^{-1} soil as Pb $(NO_3)_2$, Cu $(NO_3)_2$, and Cd $(NO_3)_2$ [25], and the other half were not spiked with HM (uncontaminated soil). The pots were kept in the greenhouse (18-25°C, 12 h light) for at least 8 weeks before transplanting the seedlings.

The chemical properties of the contaminated and uncontaminated soils were analyzed before transplanting the seedlings. The soil pH was determined in a 1:2.5

soil/distilled water suspension after 1.5 h shaking and 1 h equilibration using an Orion Ionalyzer Model 901 pH meter. Organic carbon (SOC) and total nitrogen (TN) of the soils were determined according to the Walkley-Black [26] and Kjeldahl [27] methods. Nitric-perchloric acid digestion method in a metal digestion apparatus (HotBlock, VELP Scientifica, DK8S, Italy) was used to determine the macro- and microelements (Pb, Cu, Cd, Ca, P, K, Mg, Mn, Fe, Al, Zn, and Ni) of the soil [28]. The concentrations were determined using inductively coupled plasma optical emission spectrometry (ICP-OES, Optima 2100, Perkin Elmer, USA) with a five-point calibration standard curve. The accuracy of the metal recovery reached 97-117% for CRM 277. The available phosphate-phosphorus (PO_4^{3-}-P) content was measured by the Olsen method [29]. Available K was determined by the ammonium acetate extraction method (pH=9) and atomic absorption spectroscopy (AAS, Shimadzu aa-6300, Japan) [30]. The main characteristics and HM concentrations of the soils are presented in Supplementary Table S1.

At harvesting time, soil samples were taken from the pots, air-dried and sieved through a 2-mm mesh. The soil pH, TN, SOC, and available K and P were determined as described above. The acid-extractable concentrations of Cu, Pb, and Cd, indicating the available forms of the HM, were determined according to the BCR 1 method in the contaminated soil. According to Liu et al. [31], BCR 1 is a suitable method for identifying the bioavailable form of HM for plant uptake. Based on this method, a 20-mL acetic acid solution (0.11 M) was added to 1 g of soil and shaken for 16 h at 250 rpm at room temperature. The samples were centrifuged for 20 min at 3000 x *g* and the supernatants were filtered using Whatman no.43 ashless filter paper and adjusted to 25 mL with deionized water [31]. The accuracy of the metal recovery of Cu, Pb, and Cd reached 96.3, 112, and 97% for CRM 277, respectively. The Cu, Pb, and Cd availability in the soil are shown in Supplementary Table S2.

Preparation and analysis of biochar samples

In order to produce biochar, the waste biomass of hornbeam (*Carpinus betulus* L.) was used as feedstock. The feedstock was cut into fine particles (<4 mm) to minimize heat transfer differences and heated in a reactor from room temperature to 400°C at a rate of 10°C min^{-1} under a nitrogen flow of 200 mL min^{-1}; then the temperature was maintained at 400°C for 2 h. Prior to carbonization, the feedstock was dried at 105°C overnight in an oven. The biochar was then cooled down to room temperature in the absence of air and the obtained particles were sieved to obtain <0.4 mm particles [32].

The surface area and structure of the produced biochar were determined using the BET method by N_2 adsorption analysis (at 77 k with Specific Surface Area and Porosity Analyzer (PHS-1020, PHSCHINA). The pore size distributions of the biochar were measured by the BJH method (Fig. 1). The surface morphology of the biochar was determined by SEM. The elemental composition (C, H, N, and O%) of the biochar was determined with the CHNOS element analyzer (Elementar, Germany). Biochar pH was determined in a 1:5 biochar/distilled water suspension after 1.5 h of shaking and 1 h equilibration using an Orion Ionalyzer Model 901 pH meter. Biochar macro- and microelements were determined and are described in the above section. Table S1 shows the main characteristics and HM concentrations in the prepared biochar.

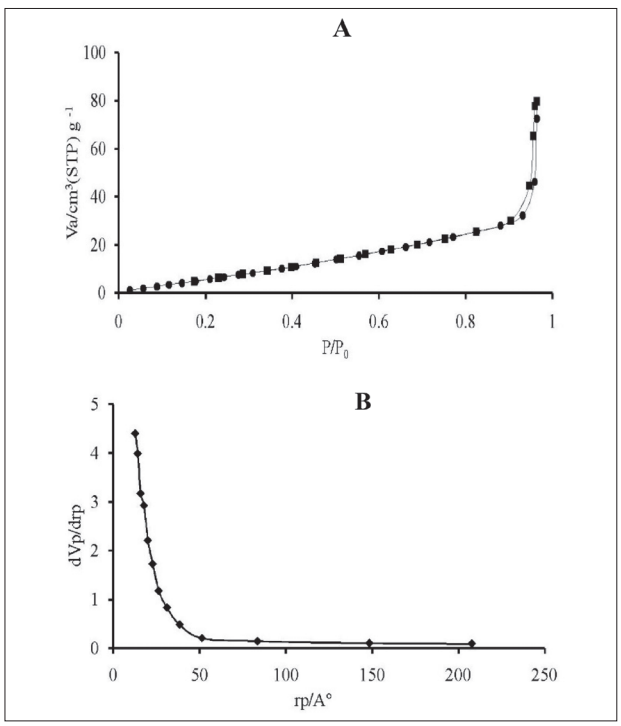

Fig. 1. N_2 adsorption (-•-) and desorption (-▪-) isotherm with a corresponding pore size distribution of the produced biochar according to the BET method (**A**); pore distribution of the produced biochar according to the BJH method (**B**).

Experimental setup

After three months, uniform seedlings (15 to 20 cm in height) grown in sandy beds were selected and transplanted into pots with uncontaminated and mixed HM (Cu, Pb, and Cd)-contaminated soils. Simultaneously with seedling transplantation, the produced biochar was added to each pot at 0, 2.5 and 5% of the mass. Six treatments were defined as: uncontaminated soil+0% biochar (uncontaminated control), uncontaminated soil+2.5%, uncontaminated soil+5% biochar, mixed HM-contaminated soil+biochar 0% (contaminated control), mixed HM-contaminated soil+2.5% biochar, and mixed HM-contaminated soil+5% biochar. The experimental design was a completely randomized design with three replicates. The pots were maintained in the greenhouse at 20-25±2°C and 12 h light for 160 days.

Cu, Pb and Cd accumulation in seedlings

In order to determine the Cu, Pb, and Cd concentrations in the seedling biomass, 1 g of oven-dried plant samples (70°C for 72 h) were digested in a mixture of HNO_3 and $HClO_4$ (4:1) using the metal digestion apparatus [33]. Next, the digested samples were passed through Whatman no. 43 filter paper and adjusted to 25 mL with deionized water. The concentrations of HM in the plant and soil samples were determined in extracts using atomic absorption spectrophotometry (SavantAA, AAS, GBC, Australia) with five-point calibration standard solutions of Cu, Pb, and Cd. The recovery of Cu, Pb, and Cd was 98, 105, and 98% for CRM 100, respectively. The HM concentrations in the seedling biomass are shown in Supplementary Table S2.

Willow seedling morphological, physiological and biochemical properties

Morphological properties

After measuring the gas exchange parameters, pots were harvested and the stem diameter, plant height and root length were measured for individual seedlings using a_Vernier caliper and steel ruler, respectively. Plant samples were transferred to the laboratory and separated into root, stem, and leaf tissues, washed with running tap water and then with deionized water. The leaf area (LA, cm^2) was measured using a leaf area meter (C1-202 Area Meter, CID Inc., USA). To measure the dry biomass, different parts of the plant, including the leaves, stems and roots, were oven-dried at 70°C for 72 h and weighed. The specific leaf area (SLA) was obtained by dividing the LA by the leaf dry weight.

Physiological properties

At harvest time, non-destructive analysis was conducted to measure gas exchange on randomly selected, fully expanded leaves (n=5; one measurement per plant) using a portable gas exchange device (LI-6400, LI-COR Inc., Neb., USA). Gas exchange parameters, including photosynthetic rate (Pn; μmol m^{-2} s^{-1}), transpiration rate (E; mmol H_2O m^{-2} s^{-1}), stomatal conductance (Gs; mmol H_2O m^{-2} s^{-1}) and internal CO_2 concentration (Ci; μmol m^{-2} s^{-1}) were measured. Water use efficiency (WUE) was calculated by dividing the photosynthetic rates by the transpiration rate.

Biochemical properties

In order to determine leaf electrolyte leakage (EL), four intact and fully expanded leaves were selected and rinsed with deionized water. Foliar circular plates (0.5 cm diameter) were cut and placed in a tube containing 10 mL of deionized water [34]. After 24 h, the electrical conductivity (EC1) of samples was measured using an Orion Ionalyzer Model 901 EC meter. The samples were autoclaved at 121.5°C and 15 Pa for 15 min and after cooling the samples, electrical conductivity (EC2) was measured. EL (%) was calculated using the following formula:

$$EL\ (\%) = \frac{EC1}{EC2} \times 100 \quad (1)$$

To evaluate the MDA, proline and Chl contents, parts of the fresh leaves were homogenized in liquid N_2 and stored at -80°C. The MDA content was determined as described by Rao et al. [35]. Thereafter, 0.3 g of frozen leaves was mixed with 10 mL of 0.25% 2-thiobarbituric acid. The mixtures were placed in a water bath at 95°C for 30 min and cooled on ice. The cooled samples were centrifuged at 3000 × *g* for 10 min. The absorbance of the supernatant extracted samples was measured at 532 and 600 nm. The concentration of MDA was estimated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

The proline content was estimated based on the method of Bates et al. [36]; 0.3 g of frozen leaf samples were extracted in 5 mL of 3% sulfosalicylic acid and centrifuged at 3000 × *g* for 20 min. Two mL of each centrifuged sample was mixed with 2 mL of ninhydrin acid and 2 mL of glacial acetic acid and incubated at 100°C for 1 h. After cooling the samples in an ice bath, 4 mL of toluene was added to the samples and shaken for 1 min. A standard curve prepared using L-proline (0-100 mg mL^{-1}; absorbance at 520 nm) was used to determine the proline content [36].

Chl '*a*' and '*b*' pigments were estimated according to the Arnon method [37]. To this end, 0.2 g of N_2-frozen leaves were homogenized in 80% chilled acetone in the dark and centrifuged (10000 × *g* for 10 min at 4°C). The absorbance of the prepared extracts was measured at 645 and 663 nm. Finally, the Chl '*a*' and '*b*' levels were calculated using the following formulas:

$$\text{Chl } a = \frac{(19.3A_{663} - 0.86A_{645})V}{100W} \quad (2)$$

$$\text{Chl } b = \frac{(19.3A_{643} - 0.86A_{665})V}{100W} \quad (3)$$

Statistical analyses

All data are expressed as the mean±SD of three independent experiments with three replications. The Shapiro-Wilk's and Levene's tests were used to test the normality and homogeneity of variances of the data, respectively. Significant differences in data were analyzed using two-way and one-way ANOVA at 95% confidence level. Tukey's test was used as *post-hoc* for mean comparisons of the data. The IBM SPSS 19 software package was used for analyses.

RESULTS

Biochar properties

According to BET analysis (Fig. 1A), the biochar absorption/desorption isotherm is fitted to a type III isotherm based on the IUPAC standard. Type III isotherm points to an almost multilayer formation. The isotherm hysteresis indicates bottleneck or slit-shaped pores. The BET of the prepared biochar was 34.87 $m^2 g^{-1}$.

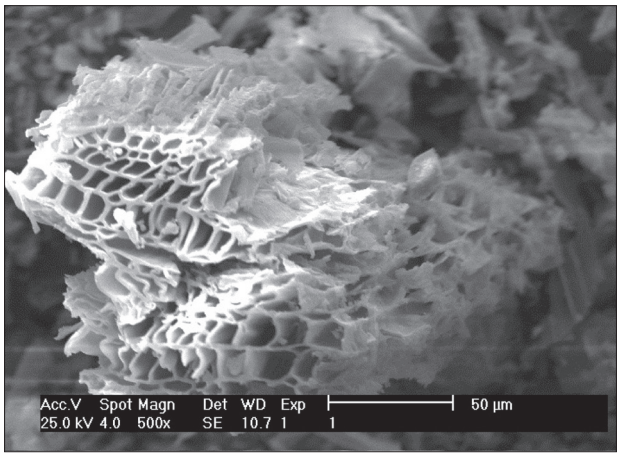

Fig. 2. SEM image of the porous structure of biochar.

The BJH isotherm (Fig. 1B) indicated that the pore size was less than 50 nm and the pores were almost micro- and mesoporous. The SEM image illustrates the porous structure of the biochar (Fig. 2). The produced biochar has a higher content of nutrients such as phosphorus and potassium than contained in the soil (Supplementary Table S1). The concentrations of Cu (11.61 mg kg^{-1}), Pb (7.42 mg kg^{-1}) and Cd (0.19 mg kg^{-1}) in the produced biochar (Table S1) were lower than the minimum threshold values (Cu=63, Pb=70, and Cd=1.4 mg kg^{-1}; Supplementary Table S3), according to the IBI biochar guidelines [38].

Soil properties and HM accumulation in seedling tissues

Applications of the biochar led to a significant ($p<0.001$) alkalization in the contaminated and uncontaminated soils in comparison to matching controls, resulting in an increase of 0.08-0.12 unit of pH (Table 1). However, soil alkalinization was not significantly different between the contaminated and uncontaminated soils. Biochar application caused a considerable increase in the soil TN, SOC, and available K, as follows: 2.5% biochar 63, 64, and 88%, respectively, and 5% biochar 116, 140, and 174%, respectively. However, these increments were not different between the contaminated and uncontaminated soils. The soil C/N ratio increased significantly by biochar 5% in both soils in comparison to the controls. The available P of the soil decreased in the contaminated control soil by 25% as compared to the uncontaminated control soil. However, the available

Table 1. Biochar effects on the chemical properties of uncontaminated and mixed HM-contaminated soils.

Soil type	Biochar (%)	pH	TN (%)	SOC (%)	C/N	Available P (mg kg^{-1})	Available K (mg kg^{-1})
Uncontaminated	0	8.07±0.03c	0.057±0.002c	0.52±0.01c	9.06±0.37c	24.03±1.22d	103.19±6.04c
	2.5	8.13±0.01a	0.084±0.003b	0.84±0.04b	9.96±0.28bc	42.59±1.51b	184.67±16.25b
	5	8.15±0.01a	0.120±0.01a	1.26±0.05a	10.75±0.63ab	48.82±1.62a	228.47±15.73a
Mixed HM Contaminated	0	8.02±0.04c	0.052±0.001c	0.52±0.01c	10.01±0.13bc	14.10±1.03e	92.33±5.13c
	2.5	8.12±0.01ab	0.086±0.002b	0.87±0.02b	10.19±0.55abc	33.06±1.98c	164.33±10.69b
	5	8.14±0.01ab	0.111±0.008a	1.23±0.01a	11.61±0.95a	48.01±1.52a	232.2±5.29a
	B	**	***	ns	**	***	ns
	S	***	***	***	***	***	***
	B×S	ns	ns	ns	ns	***	ns

S – soil type; B – biochar; S×B – interaction of soil type and biochar; TN – total nitrogen; SOC – soil organic carbon.

Different letters within columns indicate significant differences according to Tukey's multiple range tests. The significant F-test value was obtained by two-way ANOVA and indicated at the end of each column at p< 0.01 (**), p< 0.001 (***), and ns= not significant.

Table 2. Biochar effects on selected morphological properties of willow seedlings grown in clean and mixed HM-contaminated soils.

Soil type	Biochar (%)	Leaf biomass (g)	Shoot biomass (g)	Root biomass (g)	Total dry biomass (g)	Height increase (%)	Diameter increase (%)	Root elongation (%)	LA (cm^2)	SLA (cm^2 g^{-1})
Uncontaminated	0	1.03±0.03bc	1.08±0.07bc	1.24±0.13cd	3.35±0.12c	56.98±4.98b	39.74±3.66bc	61.54±11.37bc	5.85±0.05c	278.29±1.71ab
	2.5	1.47±0.06b	1.31±0.06ab	1.73±0.04bc	4.51±0.05b	86.39±3.63a	45.94±0.29ab	94.08±35.48ab	6.09±0.04b	285.21±3.16a
	5	2.16±0.05a	1.53±0.06a	2.47±0.12a	6.15±0.14a	110.10±14.33a	51.56±4.38a	139.28±10.85a	6.32±0.05a	291.88±2.41a
Mixed HM Contaminated	0	0.76±0.22c	0.85±0.12c	1.08±0.13d	2.69±0.31d	29.58±4.31c	22.05±4.39d	24.34±10.54e	4.53±0.09e	261.17±13.09b
	2.5	1.23±0.16bc	0.93±0.23c	1.26±0.23cd	3.42±0.16c	56.03±4.91b	14.90±0.95d	107.32±38.04ab	5.58±0.03d	257.55±8.48b
	5	1.49±0.43b	1.16±0.09bc	1.90±0.33b	4.56±0.30b	88.87±13.85a	33.69±5.61c	85.84±23.83abc	5.71±0.3d	259.98±9.39b
	B	***	***	***	***	***	***	**	***	***
	S	***	***	***	***	***	***	***	***	ns
	B×S	ns	ns	ns	***	ns	***	**	***	ns

S: soil type, B: biochar. S×B: interaction of soil type and biochar, SLA: leaf area, SLA: specific leaf area.

Different letters in the columns indicate significant differences according to Tukey's multiple range tests. The significant F-test value was obtained by two-way ANOVA and is indicated at the end of each column at p<0.01 (**), p<0.001 (***) and ns=not significant.

P of the soil increased with the application of biochar (2.5 and 5%) in the contaminated soil (by about 73 and 151%, respectively) and in the uncontaminated soil (by about 77 and 103%, respectively). In the mixed HM-contaminated soil, biochar addition significantly (p<0.01) decreased the availability of Cu, Pb, and Cd by about 13-23%, 38-57%, and 28-30%, respectively, in comparison to the contaminated control treatment (Supplementary Table S2). Furthermore, biochar application significantly decreased the concentration of Cu, Pb, and Cd in white willow dry biomass (p<0.01, Supplementary Table S2).

Plant morphological, physiological and biochemical responses

Except for the root, stem and root biomass and SLA, other morphological properties of the seedlings exhibited a significant (p<0.01) response to soil types. In the mixed HM-contaminated soil, the total dry biomass, root elongation, diameter and height, and LA significantly decreased (p<0.001) by 19, 60, 44, 48, and 22%, respectively, when compared to the uncontaminated soil (without biochar addition) (Table 2).

Apart from the SLA, other growth and morphological properties were significantly (p<0.01) improved by biochar addition to both uncontaminated and mixed HM-contaminated soils. In the uncontaminated soil, the addition of 2.5% biochar significantly increased the total dry biomass, height and LA (about 34, 51, and 4% respectively); however, the 5% biochar amendment increased the leaf (110%), stem (41%), root (98%) and total (83%) biomass, root elongation (126%), diameter (29%) and height (93%) and LA (8%) in comparison to the uncontaminated soil (p<0.001).

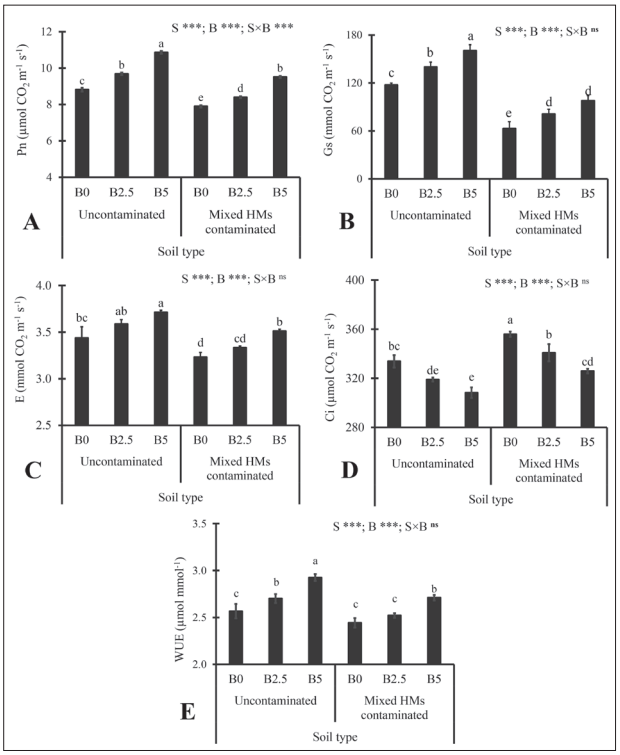

Fig. 3. Mean and standard deviation of Pn (**A**), Gs (**B**), E (**C**), Ci (**D**) and WUE (**E**) of white willow seedlings treated with biochar in uncontaminated and mixed HM-contaminated soils from three replicates. Different letters indicate significant differences according to Tukey's multiple range tests after performing two-way ANOVA multifactor analysis of biochar 0, 2.5, and 5% mass fractions (B0, B2.5, and B5, respectively) and uncontaminated and mixed HM-contaminated soils. The significant F-test value was indicated at the top of each figure at p<0.01 (**) and p<0.001 (***), ns – not significant, S – soil type factor, B – biochar factor, S×B – interaction of soil type and biochar.

Gas exchange (Pn, E, Gs, Ci, and WUE), analyzed in fresh leaves of the seedlings, is presented in Fig. 3. The Pn, Gs, and E rates in leaves declined in seedlings grown in the contaminated soil as compared to the uncontaminated soil, while the biochar amendment significantly enhanced the rates of Pn, Gs, and E in the leaves of seedlings grown in both soils (Fig. 3A to E). The highest values of Pn, Gs, and E were noticed for the highest added level of biochar (5%) in both soils. The interaction effect of the biochar treatment and soil type for the Pn content was found to be significant (p<0.05); however, this interaction was not significant for the Gs, E, Ci and WUE. The WUE increased by increasing the biochar level in the uncontaminated soil; however, in the contaminated soil WUE was high only after 5% biochar addition.

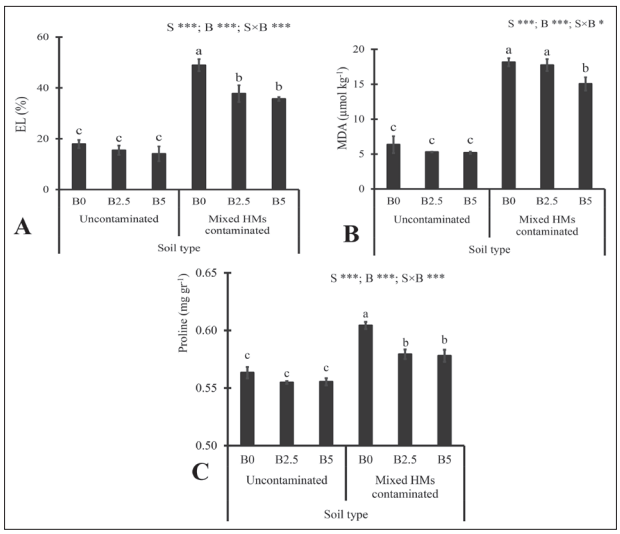

Fig. 4. Mean and standard deviation of EL (**A**), MDA (**B**), and proline content (**C**) of white willow seedlings induced by biochar treatment in clean and mixed HM-contaminated soils from three replicates. Different letters indicate significant differences according to Tukey's multiple range tests after performing two-way ANOVA multifactor analysis of biochar 0, 2.5, and 5% mass fractions (B0, B2.5, and B5, respectively) and uncontaminated and mixed HM-contaminated soils. The significant F-test value is indicated at the top of each figure at p<0.05 (*), p<0.01 (**) and p<0.001 (***), S – soil type factor, B – biochar factor, S×B – interaction of soil type and biochar.

In the mixed HM-contaminated soil, the EL increased by 173% (Fig. 4A). However, by adding biochar into the mixed HM-contaminated soil, a significant decrease was observed in the EL value, about 22 to 27% in the 2.5% and 5% biochar applications, respectively (Fig. 4A). The EL value of seedling leaves grown in the uncontaminated soil was 60% less than that in the mixed HM-contaminated soil. On the other hand, a significant variation in the EL value was not observed at different biochar doses in seedlings grown in the uncontaminated soil.

The leaves of seedlings grown in the mixed HM-contaminated soil had a significantly high MDA content (about 186%) in comparison to the uncontaminated control (Fig. 4B). On the other hand, only the high dose of biochar significantly decreased the MDA content in the leaves of willow seedlings grown in the contaminated soil. No significant variation was observed in the uncontaminated soil treatments after biochar addition (Fig. 4A to C), indicating that the biochar did not have a phytotoxic effect on the

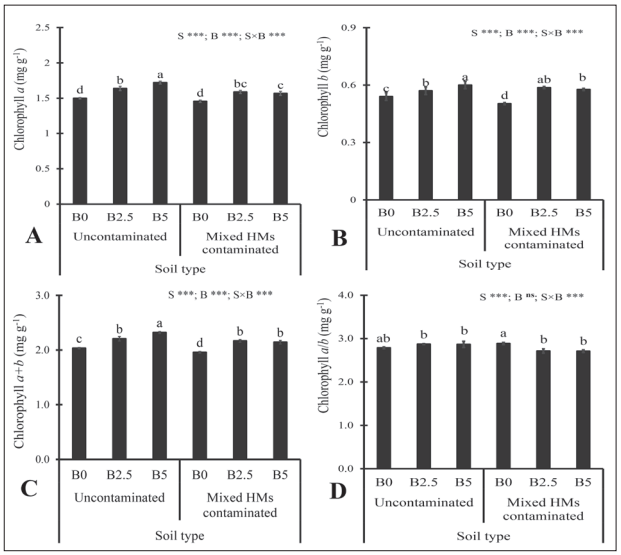

Fig. 5. Mean and standard deviation of Chl *a*, *b*, *a*+*b*, and *a*/*b* (**A**, **B**, **C**, and **D**, respectively) of white willow seedlings induced by biochar treatment, grown in clean and mixed HM-contaminated soils from three replicates. Different letters indicate significant differences according to Tukey's multiple range tests after performing two-way ANOVA multifactor analysis of biochar 0, 2.5, and 5% mass fractions (B0, B2.5, and B5, respectively) and uncontaminated and mixed HM-contaminated soils. The significant F-test value is indicated at the top of each figure at $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***), S – soil type factor, B – biochar factor, S×B – interaction of soil type and biochar

seedlings. According to Fig. 4C, in the contaminated soil the proline content of the leaf increased by about 65% as compared to the uncontaminated treatment. The proline content decreased by 6% after 5% biochar addition to the mixed HM-contaminated soil. There was no significant difference in the proline content in the uncontaminated soil treated with biochar.

The concentration of Chl *b* and *a*+*b* significantly decreased in the mixed HM-contaminated soil in comparison to the uncontaminated control soil by 6.8 and 3.8%, respectively (Figs. 5B and C). The addition of biochar to the mixed HM-contaminated soil increased the Chl *a* (5%), *b* (8%), *a*+*b* (6%), and decreased the Chl *a*/*b* (2%) ratio (Figs. 5A to D) in comparison to the contaminated control. There were no significant differences in these parameters between the effects of high and low levels of biochar. The results also indicated that 5% biochar increased the Chl *a*, *b,* and *a*+*b* content of white willow leaves by 15, 11, and 14%, respectively, in the uncontaminated soil. Also, 2.5% biochar significantly increased the Chl *a* (9%), *b* (5%), and *a*+*b* (8%) contents; however, these increments were lower than observed in the 5% biochar uncontaminated control soil (Figs. 5A to C).

DISCUSSION

The present study evaluated the effect of biochar on the morphological, physiological and biochemical properties of white willow in mixed HM-contaminated and uncontaminated soils. The biochar application improved almost all soil properties (increased soil pH, TN, SOC, available P, and K) both in mixed HM-contaminated and uncontaminated soils, which is in line with the findings of Adekiya et al. [39], who reported a similar effect of biochar in terms of improving soil chemical properties. The TN, SOC, available K, and P were higher in the 5% biochar treatments than the 2.5% treatments. In addition, the prepared biochar had a substantial content of nutrients like C, N, P, and K and its amendment to soil increased these nutrient contents. Soil pH increment might be explained by a higher pH of the prepared biochar than those in the soil. Jin et al. [40] reported that a higher content of alkaline materials (e.g. wood ash) and negatively charged carboxyl groups and phenolics in biochar could make the soil more alkaline.

Additionally, the biochar application decreased Cu, Pb, and Cd availability in the mixed HM-contaminated soil, corroborated by previous investigations [6]. The reduction in Cu, Pb, and Cd availability in soil could be attributed to pH increment due to the biochar addition to soil. This is in line with the recent finding of Yang et al. [41] who reported that the addition of biochar to soil resulted in a decreased availability of Cd and Zn. Also, the prepared biochar has a porous structure that is favorable for HM immobilization by trapping in the porous structure, as stated by Lahori et al. [42]. The Cu and Pb immobilization was more in the 5% biochar rather than the 2.5% biochar. This could be due to the higher pore amount that entered the soil by the 5% biochar addition. The morphological properties of white willow, such as the dry biomass of leaf, stem, root, diameter growth and root elongation had a negative response to the mixed HM contamination. Similar to the morphological properties, the gas exchange of white willow leaves was adversely affected by HM toxicity (Fig. 3). Low Pn, Gs, and E and high

accumulation of CO_2 in white willow leaves indicates stomatal closure due to HM stress that was also proved by dos Reis et al. [43].

Similar to the findings of Chen et al. [44] and Yang et al. [45], in our study biochar addition significantly improved the morphological and gas exchange properties of white willow seedlings both in the uncontaminated and contaminated soils. The 5% biochar improved the white willow morphological and gas exchange properties in comparison to the 2.5% biochar, indicating that soil quality improvement by biochar addition could facilitate plant growth. In addition to the nutrients' enhancement of soil, the HM immobilization that occurred by the biochar application could improve white willow growth and its physiological properties. The 5% biochar was more effective in soil nutrient increment and HM immobilization as well as in the white willow morphological and gas exchange parameters than the 2.5% biochar. Lebrun et al. [6] observed a similar beneficial biochar effect on biomass production and reducing Pb availability in soil pore water when growing *Salicaceae* species on Pb- and As-contaminated soils amended by biochar obtained from lightwood-pinewood and harboring. Furthermore, Rodríguez-Vila et al. [46] also reported soil chemical improvement and Cu, Ni, Pb, Zn, and Co immobilization by the addition of biochar to *Brassica juncea* L. seedling.

Significantly, higher values of EL concentration in leaves could be attributed to HM stress [47]. Similar to the EL, the concentrations of MDA and proline were high in white willow seedlings. Under such conditions, increasing the MDA and proline contents could protect plants against oxidative damage and act as a defense mechanism in stressful conditions [48-49]. The insignificant EL, MDA and proline contents in the leaves of seedlings grown in uncontaminated soil and amended with biochar (both 2.5 and 5%) indicated no phytotoxicity of the prepared biochar for white willow. Parallel to the increases in MDA and proline, the Chl *a* and *b* content decreased in white willow leaves, confirming that chlorophyll content can be a useful biomarker of HM abiotic stress [50]. The reduction in the leaf Chl *a*, *b,* and *a*+*b* could negatively affect the photosynthesis and gas exchange in white willow seedlings. It can be stated that damage to the respiration pathway and then disorder in photosynthesis finally led to decreases in the concentration of Chl *a* and *b* of the white willow seedlings. Biochar addition increased the Chl *a* and *b* concentrations in the white willow seedlings grown in both soils. Furthermore, in the contaminated soil, the biochar application decreased the EL, proline and MDA contents in the seedlings' leaves. These results are in agreement with the findings of Mehmood et al. [51] and Zhang et al. [52]. We also found that, similar to the morphological and physiological properties, the application of 5% biochar had a more important role in improving the chemical properties of white willow leaves than the 2.5% biochar, since it can provide better growth conditions for the seedlings.

The biochar addition to the mixed HM-contaminated soil significantly decreased the uptake of Cu, Pb, and Cd in white willow seedlings that could be attributed to the immobilization of HM in the contaminated soil. This finding is in line with Sarwar et al. [53]. Globally, this study indicated that in both uncontaminated and mixed HM-contaminated soils, biochar could supply the essential nutrients in soil and enhance plant growth. These conditions finally led to an enhancement in plant resistance to HM stress in contaminated soil beyond the immobilization of HM. The high dose of biochar (5%) had more positive effects on improving the investigated properties of white willow than the low dose (2.5%).

CONCLUSION

The results obtained in this study revealed that Cu, Pb, and Cd soil contamination had an adverse effect on the growth, gas exchange and biochemical properties of white willow. However, biochar application increased its growth parameters as well as physiological and biochemical properties. In addition, biochar application improved the chemical characteristics in mixed HM-contaminated and uncontaminated soils and decreased Cu, Pb, and Cd availability in the mixed HM-contaminated soil. HM impose great stress to plants. Biochar could be used as an effective and eco-friendly amendment to ameliorate HM stress and to improve soil quality. Further comprehensive research with different plants, feedstock and doses is needed to achieve optimal results.

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Supplementary Data

Available at: http://serbiosoc.org.rs/NewUploads/Uploads/Mokarram-Kashtiban%20et%20al_3422_Supplementary%20Data.pdf