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Interaction of lead and cadmium on growth and leaf morphophysiological characteristics of European hackberry (*Celtis australis*) seedlings

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Abstract

Background: In the present study, different concentrations of lead (factor A; 0, 15, and 30 mg L⁻¹) and cadmium (factor B; 0 and 5 mg L⁻¹) were applied via irrigation water during 6 months to evaluate their effects on growth of European hackberry (*Celtis australis* L.) plants. The experiment was arranged in factorial with completely randomized design and four replications under greenhouse conditions.

Results: Application of 5 mg L⁻¹ Cd or the lead levels (15 and 30 mg L⁻¹) significantly reduced new shoot growth, plant leaf area, SPAD value, leaf water conductance and leaf photosynthesis, whereas significant increase in number of chlorotic and necrotic leaves, leaf transpiration rate, leaf proline and soluble sugars was observed. Higher reduction in new shoot growth and leaf water conductance and higher increase in leaf soluble sugars and proline was observed over the interaction of 5 mg L⁻¹ Cd and 30 mg L⁻¹ Pb. The highest soluble sugars and proline were in Pb30Cd5 (a3b2) treatment. Leaf Pb or Cd concentration was increased following their treatment. Application of cadmium significantly reduced leaf Pb, and similarly leaf Cd was significantly reduced by application of lead at both levels compared to untreated plants.

Conclusion: The results showed that the hackberry growth was influenced by positive and negative interactions of Pb and Cd applied in irrigation water. The extent of growth reduction indicates that hackberry represents a relatively tolerant ornamental tree to high Pb and Cd levels.

Keywords: Environment, Heavy metal, Proline, Soluble sugars, Stress, Urban landscape, Wastewater

Background

Heavy metal pollution of environment has been a matter of significant concern in recent decades. Cadmium (Cd) and lead (Pb) are two major heavy metals with strong toxicity effects on all types of life including vegetations [7, 18]. From environmental and biological point of view, Pb and Cd are more important than many other

heavy metals [6, 16]. Cadmium and lead as nonessential elements, can readily be taken up by plant roots and induce adverse effects on plant metabolism, growth, and development [3, 8]. These two metals are potential contaminants of agricultural foods resulting in many human health problems [4, 11, 14].

Heavy metals can significantly reduce the plant growth and its quality [2, 3, 7, 16]. Plant species may differently respond to heavy metals; however, reduction in plant growth is frequently observed under lead and cadmium treatment [1, 2, 20]. So, there is economic and quality loss as well as various health risks when edible crops are

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cultivated in heavy metal-polluted soils [17]. Nevertheless, ornamentals and flowers are non-edible crops and more suitable for cultivation in polluted area.

European hackberry (*Celtis australis*) is a medium-to-large size, long-living deciduous perennial tree from *Ulmaceae* family that is native to a vast area, from India and Pakistan to France, with the Mediterranean as its main origin. It is an ornamental and forest tree species that grows in sub-tropical to temperate climates. Hackberry represents a good candidate for landscape planning and cultivation purposes due to its tolerant to air pollution and drought stresses; however, there is not enough information regarding the heavy metals effects on its growth. In the present study, effects of different levels of lead and cadmium in irrigation water were evaluated on growth of seedlings of *Celtis australis* under greenhouse conditions.

Materials and methods

Experimental setup

This study was performed using 1-year-old seedlings of European hackberry (*Celtis australis*) during 2016 and under greenhouse conditions. The seedlings were prepared from a local nursery supplier, and uniform seedlings were transplanted on 15 April 2016 into pots containing a mix soil composed of two-third field soil and one-third fine sands. The soil was mixed thoroughly and passed across a 2-mm sieve and then its physicochemical characteristics were analyzed. The soil texture was silty-loam with electrical conductivity (EC) of 0.41 dS m⁻¹ and pH value of 7.17. It contained 1.2% organic matter with an average level of mineral nutrients of nitrogen (78 mg kg⁻¹), phosphorus (15.2 mg kg⁻¹) and potassium (256 mg kg⁻¹). In addition, an amount of 150 mg kg⁻¹ soil of a NPK chemical fertilizer (N:P:K 20:8:10) was mixed uniformly into the soil before seedling transplantation. Black plastic pots of 30 cm height and 24 cm diameter (9–10 L volume) were filled with the soil. After transplantation, plants were irrigated for 2 weeks with tap water (without Pb or Cd treatment), thereafter different lead and cadmium combinations were applied to the pots.

Treatment application

In this study, hackberry seedlings were irrigated with water containing different concentrations of lead and cadmium, and for 6 months during growing season from May to November. The experimental layout was factorial based on completely randomized design with four replications. Each replication was a pot (about 10 L volume) containing one seedling. Treatments were the factorial combinations of lead levels (factor A: 0, 15 and 30 mg L⁻¹) and cadmium levels (factor B: 0 and

5 mg L⁻¹). Lead was used from lead chloride (PbCl₂) and cadmium was used from cadmium chloride (CdCl₂) source. The treatments were prepared using stock solution of lead (3000 mg L⁻¹) and cadmium (1000 mg L⁻¹). Plants were generally irrigated (with water containing heavy metals levels) 2 times per week during May and October; while during June, July, August and September plants received 3 irrigations per week. In each irrigation, the pots were irrigated to a water content of 80% of soil field capacity (FC).

Measurements

During plant growth period, the seedlings were irrigated with water containing different levels of Pb and Cd. Various morphophysiological traits were recorded during growth season. The leaf SPAD value (the soil and plant analysis development) of plants was determined using a portable SPAD meter (Model SPAD-502 Plus, Illinois, USA). The average of 30 reading of leaves, generally in middle part of plant shoots, was recorded. The cumulative new shoot growth of plant branches was determined using a ruler and expressed in the results as cm per plant. The average plant leaf area was determined using leaf area meter (Model CI 202, Germany), by recording the average area of 4–5 randomly detached leaves from the middle part of plant's branches. The number of leaves with chlorotic and necrotic symptoms were counted and recorded per plant before autumn senescence. The leaf photosynthesis rates were determined using a portable photosynthesis meter of Li Core (Li 6100, Li Core CO. USA), at full light condition and on 11 o'clock in the morning from a well-developed leaf in the middle part of the new shoot growth. Leaf soluble carbohydrates were determined using anthrone reagent, in which 0.5 g of leaf fresh tissues was extracted twice in 2.5 mL ethanol 80% at 95 °C for 60 min. The two extracts were then combined, filtered and the alcohol was removed by evaporation. The anthrone reagent was used for preparation of samples, and their absorption was measured at 625 nm. A standard glucose curve was also used for calculation of carbohydrate content of leaves. The leaf proline concentration was also determined using ninhydrin method, in which 0.5 g fresh leaves were homogenized in 10 mL of 30% ninhydrin solution, and the absorption of leaf extract samples was measured against different standard proline concentrations of 0, 5, 10, 20 and 40 mg L⁻¹ and by spectrophotometer determination at 520 nm. Soil available lead and cadmium was determined using atomic absorption spectrometer.

Statistical analysis

Data were analyzed using SPSS Software. For each measured parameter, the effect of the treatments was analyzed

by analysis of variance (ANOVA1) and comparison of means was performed with LSD test at 5% level.

Results

The results of ANOVA (Table 1) showed that the interaction effect of lead and cadmium (AB) for new shoot growth, leaf soluble sugars, leaf water conductance and soil available Pb was significant at $P=0.05$, and their interaction effect for other traits was not significant. The effect of lead (factor A) on new shoot growth, leaf SPAD value, leaf area, number of chlorotic and necrotic leaves, leaf soluble sugars concentration, leaf transpiration rate, leaf water conductance, leaf photosynthesis rate and soil available Pb concentration was significant at $P=0.01$, and on leaf proline concentration was significant at $P=0.05$, and on soil available Cd concentration was not significant (Table 1). The effect of cadmium (factor B) on leaf area, leaf soluble sugars concentration, leaf transpiration rate, leaf water conductance and soil available Cd concentration was significant at $P=0.01$, and on new shoot growth, number of chlorotic and necrotic leaves, and leaf proline concentration was significant at $P=0.05$, and on leaf SPAD, leaf photosynthesis rate and soil available Pb concentration was not significant (Table 1).

Comparison of means showed that the growth of new shoots (Fig. 1) in control (a1b1) and in those plants treated with 5 mg L⁻¹ cadmium (a1b2) or 15 mg L⁻¹ (a2b1) lead was significantly higher than plants that treated with 30 mg L⁻¹ lead, with (a3b2) or without 5 mg L⁻¹ cadmium (a3b1), as well as than those plants treated with 15 mg L⁻¹ lead with 5 mg L⁻¹ cadmium (a2b2).

The interaction of lead and cadmium was not significant on leaf morphological characteristics including leaf area, leaf SPAD value, and number of chlorotic and necrotic leaves in plant (Table 2); however, these traits were influenced by simple effects of lead and cadmium. Plant leaf area (Table 2) was significantly reduced by application of cadmium or lead, particularly by higher lead level of irrigation water. Application of 5 mg L⁻¹ cadmium, as well as increasing lead levels of irrigation water from zero to 30 mg L⁻¹ significantly reduced leaf SPAD value, whereas the number of chlorotic and necrotic leaves in plant was increased. The highest leaf SPAD value or the lowest number of chlorotic or necrotic leaves were in plants received no cadmium or lead in irrigation water (Table 2).

Application of lead and cadmium significantly reduced the leaf stomatal conductance (Fig. 2). There was a reducing trend in leaf stomatal conductance with increasing levels of heavy metals and over their interactions

compared to untreated control (a1b1) plants. Similarly, leaf transpiration rate was significantly reduced by lead application compared to untreated plants, and there was no significant difference among lead levels of 15 and 30 mg L⁻¹ in this regard. Similarly application of 5 mg L⁻¹ cadmium significantly reduced leaf transpiration rate compared to untreated plants (Table 3). Leaf photosynthesis rate was not influenced by application of 5 mg L⁻¹ cadmium; however, there was a reducing trend of this trait with increasing the lead levels in irrigation water (Table 3).

Application of all heavy metals treatments significantly increased leaf proline concentration compared to control plants (Table 3). The highest leaf proline was in plants treated with 5 mg L⁻¹ cadmium or 30 mg L⁻¹ lead. Similarly, application of lead and cadmium alone or in combination significantly increased leaf soluble sugars concentration compared to untreated control plants (Fig. 3). There was an increasing trend in leaf soluble sugars with increasing the concentration of heavy metals in irrigation water. The highest concentration of leaf soluble sugars was in those plants treated with Pb30Cd5 (a3b2) that showed no difference with Pb30Cd0 (a3b1) or Pb15Cd5 (a2b2) treatments, and the lowest leaf soluble sugars was in control (a1b1) plants.

Determination of leaf heavy metals (Pb and Cd) showed that leaf lead (Fig. 4) and cadmium (Fig. 5) concentrations were significantly increased following 15 and 30 mg L⁻¹ lead, or 5 mg L⁻¹ cadmium applications, respectively. Application of lead or cadmium negatively influences the leaf concentration of other heavy metal (Figs. 4, 5). A sharp increase in leaf Pb was observed following Pb application regardless of the applied lead levels (Fig. 4); however, under 30 mg L⁻¹ treatment despite leaf Pb was significantly higher than those of 15 mg L⁻¹ treatment, but it was not a sharp increase. Application of 5 mg L⁻¹ cadmium also significantly reduced leaf Pb concentration compared to untreated plants (Fig. 4). Similarly, leaf cadmium concentration was significantly reduced by lead treatments at both levels (Fig. 5). There was no significant difference between 15 and 30 mg L⁻¹ lead levels in this regard.

Determination of soil available concentrations of lead and cadmium (Fig. 6) showed that there was an increasing trend in soil available Pb following application of 15 and 30 mg L⁻¹ lead in irrigation water. Similarly, application of 5 mg L⁻¹ cadmium in irrigation water significantly increased soil available Cd concentration. While nearly 1000–2000 mg lead and 350 mg cadmium was added to the pot soil during experimental period.

Table 1 Analysis of variance of plant growth traits

Source of variation	df	New shoot growth	Leaf SPAD value	Leaf area	No. of chlorotic leaves	No. of necrotic leaves	Leaf soluble sugars	Leaf proline	Leaf transpir. rate	Leaf water conduct.	Leaf photosyn. rate	Soil available Pb	Soil available Cd
Factor A (Pb)	2	767.5**	818.56**	132.64**	71.37**	30.04**	230032.4**	7.08*	0.33**	0.00034**	1.28**	38171**	0.72 ns
Factor B (Cd)	1	140.16*	73.85 ns	93.61**	22.04*	7.04*	183680.0**	11.9*	0.48**	0.00047**	0.26 ns	3.47 ns	4566.8**
A*B	2	88.29*	2.62 ns	8.56 ns	5.54 ns	1.79 ns	63489.4*	2.26 ns	0.13 ns	0.00011*	0.055 ns	131.6*	0.61 ns
Error	15	19.41	54.58	5.14	1.73	1.45	10157.8	1.43	0.051	0.000026	0.068	39.44	2.68
CV		17.22	11.63	14.56	33.9	49.02	14.68	23.3	22.7	31.5	19.1	15.9	20.0

***, * and ns mean significant effect at 1%, 5% and not significant effect, respectively

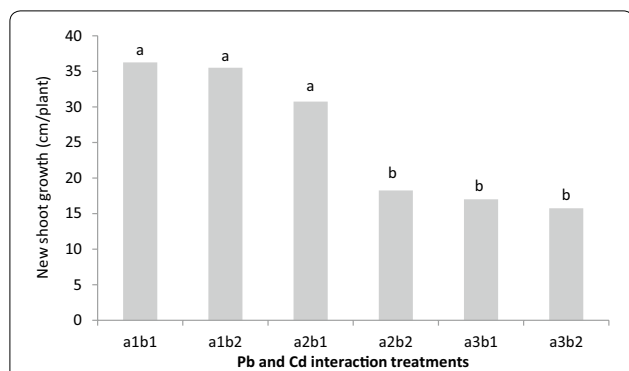


Fig. 1 New shoot growth of European hackberry plants under the interaction of different lead and cadmium levels in irrigation water. Data are average of four replicates ± SD. Means with the same letter are not significantly different at 5% level of LSD test

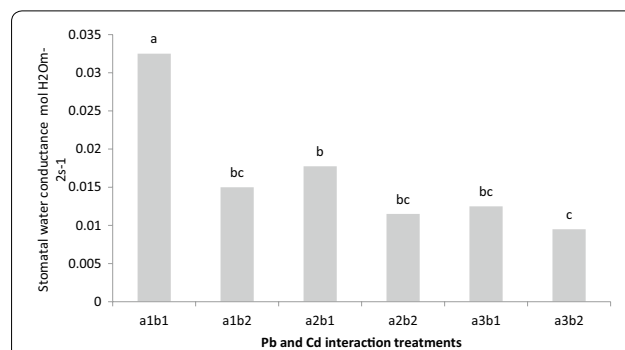


Fig. 2 The stomatal water conductance of hackberry leaves under the interaction of different lead and cadmium levels in irrigation water. Data are average of four replications ± SD. Means with the same letter are not significantly different at 5% level of LSD test

Discussion

The results showed that plant growth characteristics were adversely influenced by 6 months application of lead and cadmium in irrigation water. The reduction in plant growth is a common response of many plant species exposed to toxic levels of heavy metals [3, 16]. Various mechanisms may be involved in growth reduction of plants under heavy metal toxicity. There might be a general reduction in leaf morphophysiological characteristics including leaf area expansion. Cell division and cell enlargement are the major processes in leaf area expansion and plant growth, and probably both of these physiological processes are adversely affected by lead and cadmium in the present study, and resulted in reduced shoot growth under high levels of lead and/or cadmium [9, 10]. Adverse effects of high lead and cadmium on hydraulic conductance and water potential of plant tissues may also contribute to reduce the shoot growth under heavy metal treatment [1, 10].

Leaf morphophysiological characteristics are among those traits that are very sensitive to stress conditions particularly to heavy metals. Our data show that despite

reduction of leaf greenness by application of heavy metals, hackberry leaf greenness was relatively tolerant to high lead and cadmium concentrations. Heavy metal toxicity is known to reduce leaf chlorophyll index, leaf area expansion, greenness and many other leaf metabolic processes [3, 16]. Higher levels of lead and cadmium, alone or in combination, reduced plant leaf area, probably due to restriction of cell division and cell expansion [5, 8]. The reduction in major nutrient elements such as potassium and nitrate could also result in reduced leaf area due to heavy metal application [5, 9, 16]. Sensitivity of various enzymes in chlorophyll biosynthesis processes is probably involved in reduction of reduced leaf greenness under heavy metal toxicity [16]. On the other hand, increasing the necrotic points of leaves with increasing the Cd and Pb levels could be a mechanism of heavy metal tolerance in this plant species.

Application of Pb and Cd significantly increased leaf proline and soluble sugars concentrations compared to control plants. The increase in leaf soluble sugars and proline amino acid is a common physiological response of many plant species under heavy metal toxicity [16,

Table 2 Leaf area, leaf SPAD value, and number of leaves with chlorotic and necrotic symptoms of plants under lead and cadmium treatments

Treatments	mg L ⁻¹	Leaf area (cm ² /leaf)	Leaf SPAD value	No. of chlorotic leaves/plant	No. of necrotic leaves/plant
Lead levels	0	19.3a	73.1a	1.2c	0.5c
	15	16.2b	64.4b	3.2b	2.5b
	30	11.2c	52.9c	7.1a	4.3a
Cadmium levels	0	17.5a	65.2a	2.9a	1.9b
	5	13.6b	55.7b	4.8b	3a

The lead and cadmium levels were applied to pots via irrigation water, 2–3 times per week for 6 months

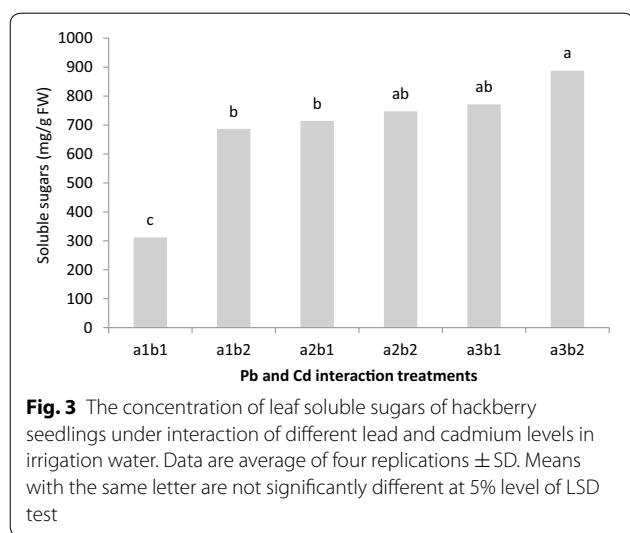
Data are average of 4 replications. For each trait and at each heavy metal levels, means with different letter (a, b or c) are significantly different at 5% level of LSD test

Table 3 Leaf transpiration rate, water conductance, photosynthesis rate and proline concentration of plants under different lead and cadmium treatments

Treatments	mg L ⁻¹	Transpiration rate (mmol H ₂ O/m ² /s)	Water conductance (mole H ₂ O/m ² /s)	Net photosynthesis (μmol CO ₂ /m ² /s)	Leaf proline (μmol/g FW)
Lead levels	0	1.2a	0.024a	1.8a	4.1b
	15	0.95b	0.015b	1.3b	5.5a
	30	0.81b	0.01b	0.97c	5.8a
Cadmium levels	0	1.13a	0.02a	1.5a	4.4b
	5	0.85b	0.012b	1.3a	5.8a

The lead and cadmium levels were applied to pots via irrigation water, 2–3 times per week for 6 months

Data are average of 4 replications. For each trait and at each heavy metal levels, means with different letter (a, b or c) are significantly different at 5% level of LSD test

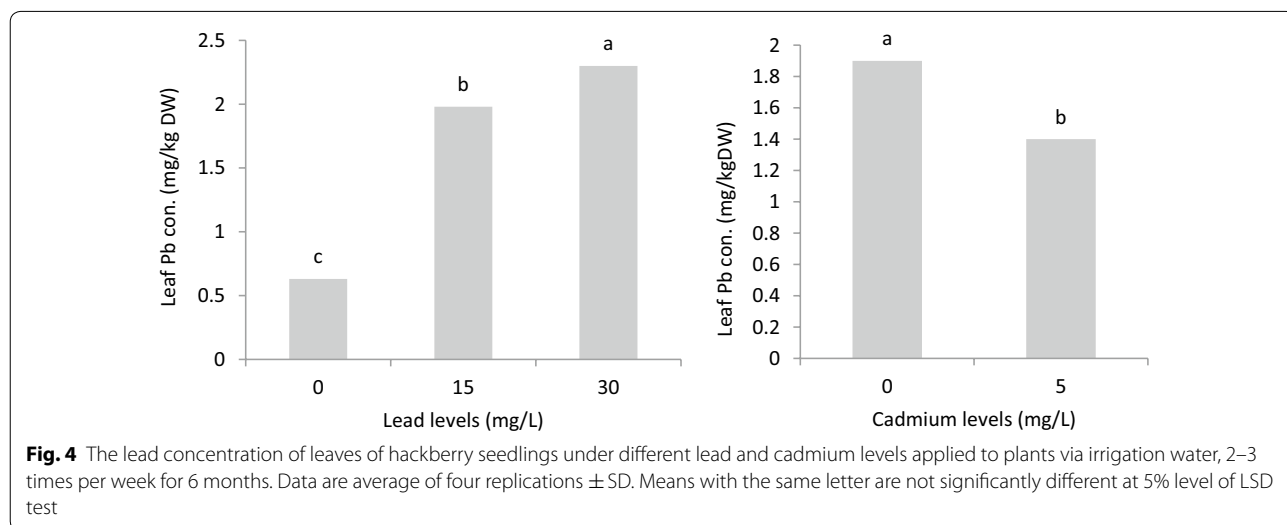


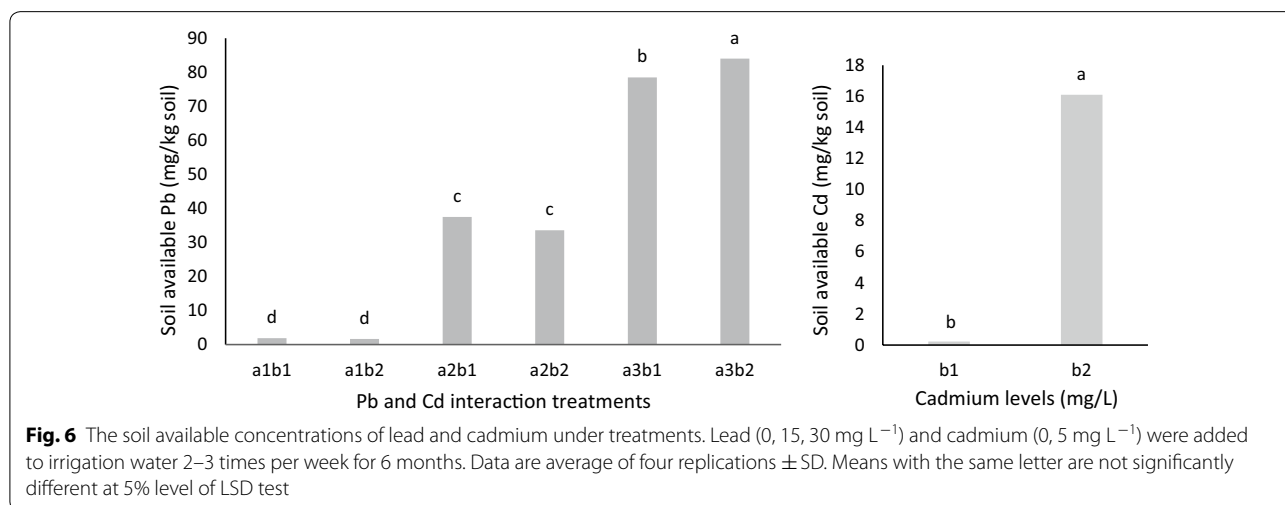
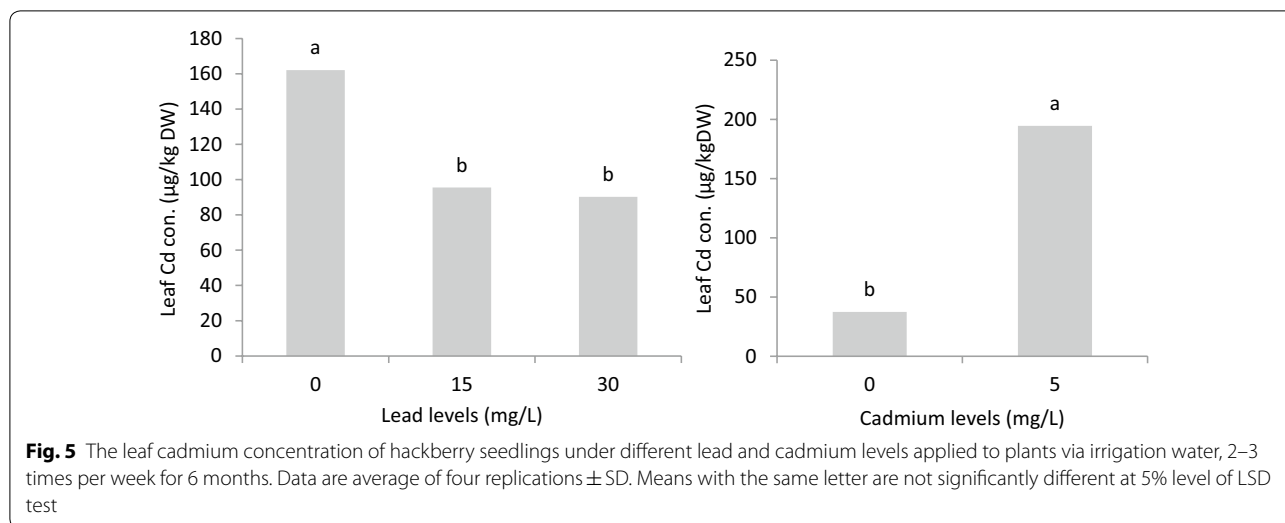
20]. These metabolites can significantly increase the plant general tolerance to stress conditions [8]. Soluble sugars in leaf may have various metabolic roles, the

main to be the osmotic adjustment and the protection that they provide to cell components [16]. Similar roles also are suggested for free amino acids mainly proline in cell, as it may actively involve in Pb and Cd inactivation and complex formation [16, 20].

In this study, the reduction in leaf water conductance and photosynthesis rate was higher than the reduction in leaf transpiration rate under heavy metals applications. Heavy metals can significantly change leaf characteristics including water conductance, photosynthesis and transpirations rates. These leaf traits are among the most important and central compared to many other processes [5]. They are also involved in leaf expansion and plant growth. Photosynthesis is very vulnerable to stress conditions particularly to heavy metals treatment that can disturb different biochemical and metabolic reactions [3, 9]. When heavy metals enter the cell, they interact with SH-groups and inactivate enzymes, and disturb many metabolic processes [16].

Application of heavy metals has been shown to reduce many plant growth parameters including leaf morphophysiological characteristics [3, 16]. Lead and





cadmium are the major heavy metals with toxicity effects on many biological systems [1]. In the present study, high concentrations of lead and cadmium were applied to pot soil via irrigation water; however, lead was used in considerably higher concentration than cadmium. Lower levels of Pb or application of 5 mg L⁻¹ Cd seem to have no significant effect on some growth parameters, despite the total applied amounts via irrigation water during 6 months was very high. When lead and cadmium were applied together, particularly at higher levels, strong adverse effects on growth of hackberry seedlings were observed.

Stress factors particularly heavy metals can have additive, synergic or antagonist effects on plant growth. Additive effects of heavy metals have been reported in many studies [1, 3, 7]. However, in *Jatropha* trees, cadmium was more toxic on plant dry matter than lead, whereas no adverse effect was found on plant dry matter in the

presence of both pollutants. A significant positive effect of low but not high concentrations of cadmium and lead was observed on dry matter of *Jatropha curcas* plants [7]. The lead and cadmium toxicities can have adverse effects on membranes functions, enzyme activity that generally result in harmful oxidative stress [2, 13, 16, 19].

The reduction in hackberry growth was minor under high amounts of applied heavy metals (with relatively high available Pb and Cd in soil), compared to many other studies that showed considerable growth reduction following a single application or limited amounts of heavy metals [1, 12, 15]. Some plant species are quite potent in uptake, translocation and accumulation of heavy metals, known as hyperaccumulator in the process of heavy metal phytoremediation. Most of plant species with enhanced ability for phytoremediation are edible crops. Nevertheless, non-edible plants such as ornamentals are more interesting to be used for

phytoremediation of polluted soils. On the other hand, in many regions application of wastewater for production of vegetable crops have increased the risk of human health; however, if there be a wide national program to use the domestic and industrial wastewater in landscape and for ornamental plants cultivation and maintenance, the maximum beneficial effects are achieved. Cultivation of deep-rooted perennial plants such as hackberry is a good technique for correction of heavy metal-contaminated soils.

Conclusion

In the preset study, high amounts of Pb and Cd were applied to plants (with also high soil available Pb and Cd) during 6 months via irrigation water. The results showed that application of Cd and Pb significantly reduced hackberry growth characteristics; however, the plant growth reduction was minor under relatively high amounts of applied heavy metals compared to many other studies. Many growth parameters including, new shoot growth, leaf area, leaf SPAD value, leaf photosynthesis and transpiration rates were reduced and leaf soluble sugars and proline were increased by lead particularly at higher level of 30 mg L^{-1} , with or without 5 mg L^{-1} cadmium, compared to control plants. The results indicate that European hackberry is a good candidate for cultivation in urban areas and to be irrigated with refined wastewater containing some levels of heavy metals.

Abbreviations

Cd: cadmium; Pb: lead; SPAD: the soil and plant analysis development; EC: electrical conductivity; FC: field capacity.

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Authors' contributions

AR, MK and MS designed the experiment. MH performed the experiments. MH, MS and KSh conducted the laboratory measurements. MH and MS analyzed the data. MS and MH wrote the paper. All authors read and approved the final manuscript.

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All authors listed have contributed significantly to the work and agree to be in the author list.

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Competing interests

The authors declare that they have no competing interests.

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