PHYTOREMEDIATION POTENTIAL OF SEDUM PLUMBIZINCICOLA

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Abstract

A field study was conducted to evaluate the efficacy of sedum plumbizincicola for phytoremediation of contaminated soils in Bulgaria. The experiment was performed on an agricultural field highly contaminated (2540.8 mg/kg Zn, 2429.3 mg/kg Pb and 51.5 mg/kg Cd) by the Non-Ferrous-Metal Works near Plovdiv, Bulgaria. Upon reaching the stage of flowering, the sedum plumbizincicola plants were gathered. The content of heavy metals, micro and macroelements in different parts of sedum plumbizincicola (roots, stems, leaves) was determined by ICP. The chemical composition of root exudates of sedum plumbizincicola was determined by gas chromatography–mass spectrometry. Thirty compounds were identified in the root exudates, of which 6 were saccharides, 9 amino acids, 5 organic acids, 8 sugar acids and alchohols and 2 other compound (hexadecanoic acid, octadecanoic acid). Significant differences were found in the content of the elements in the different parts of sedum plumbizincicola. Pb, Cd, Zn, Cu, Ca and P accumulate in the leaves, while Fe, Mn and Mg accumulate in the root system. Sedum plumbizincicola can be referred to the hyperaccumulator of cadmium and the accumulators of zinc and lead. The high concentration of Cd, Zn and Pb in the leaves and the high translocation (TFZn – 4.21, TFCd - 2.11, TFPb – 2.72) and high bioconcentration factors (BCFCd - 22.68, BCFZn - 2.20) indicate the possibility of sedum plumbizincicola to be used in phytoextraction of heavy metal contaminated soils in Bulgaria.

Keywords: *chemical composition, root exudates, rhizosphere,* sedum *plumbizincicola, polluted soils, phytoremediation*

1. INTRODUCTION

Phytoextraction with technology for cleaning up contaminated soils [1]. Plants with high metal tolerance and the capacity to absorb and move metals in the above-ground parts are key to efficient phytoextraction [2]. Metal hyperaccumulation [3] is commonly associated with species endemic to metal-bearing soils. Four criteria are used to identify hyperaccumulators: (1) the concentrations of heavy metals in the above-ground mass of plants reach the hyperaccumulation threshold (Cd>100 mg/kg; Zn>10,000 mg/kg; Pb>1000 mg/kg); (2) the concentration of elements in the shoots is 10–500 times higher than in normal plants; (3) the concentration of metals in the above-ground mass is greater than that in the roots or translocation factor (TF) (above-ground mass/roots)>1; (4) the concentration of elements in the above-ground mass is higher than in the soil or bioaccumulation factor (BF) (above-ground mass/soil)> 1.

So far, more than 500 species of hyperaccumulator plants have been identified - 450 for Ni, 32 for Cu, 30 for Co, 20 for Se, 14 for Pb, 12 for Zn, 12 for Mn, 5 for As, 2 for Cd [4]. Sedum plumbizincicola (Crassulaceae), genus Sedum, was discovered in 2005 near the city of Zitong (Zn-Pb mining area), northwest of Hangzhou in the western province of Zhejiang, eastern PR China. The species was identified as a Cd-Zn hyperaccumulator in 2007. It is known that the root system of hyperaccumulators regulates the absorption of heavy metals from soils and the rate of accumulation in above-ground parts of plants will depend on the influence of roots on biogeochemical processes in the rhizosphere [5]. Through exudation and absorption, plant roots can lead to changes in the amounts of elements in the soil, pH, redox processes, the formation of organic acid complexes with nutrients and metal chelation [6]. Root exudates are plant metabolites that are released from plant roots to improve nutrient absorption and alleviate the stress response in the environment [7,8]. The chemical composition and amount of root exudates depend on the type of plant, the development stage and the environment [9]. Low-molecular-weight organic-acids (LMWOA), which are released from plant roots, cause increased phytoextraction of heavy metals by stimulating metal mobility and changing nutrient status [10]. Water-soluble root exudates from hyperaccumulator plants increase micronutrient extraction [11,12], and water-insoluble exudates may affect the desorption of metals in the rhizosphere [13]. However, most studies have focused on the role of root exudates and changes in total amount of dissolved organic matter (DOM) or dissolved organic carbon (DOC).There are very few studies related to determining the composition of the root exudates of hyperaccumulators. Knowledge of the composition of root exudates of hyperaccumulators may help clarify the role of root exudates on the mechanisms for heavy metal mobilization or immobilization.

The purpose of this work is to conduct a comparative study, which allows us to determine the quantities and the deposits for accumulation of heavy metals, micro and macroelements in sedum plumbizincicola, the composition of root exudates, as well as the possibilities to use the sedum plumbizincicola for phytoremediation of heavy metal contaminated soils in Bulgaria.

2. MATERIALS AND METHODS

2.1. Materials

The experiment was performed on an agricultural fields contaminated by Zn, Pb and Cd, situated at 0.5 km distance from the source of pollution, the Non-Ferrous Metal Works (NFMW) near Plovdiv, Bulgaria. Rhizosphere soil samples of sedum plumbizincicola and bulk soils were collected. All plants were in their flowering stage during sample collection. Blocks that included the root systems of sedum were excavated and shaken in order to remove loosely attached bulk soil. Any soil that adhered to the root systems was subsequently brushed off and considered to be the rhizosphere. Prior to analysis both the bulk soils and rhizospheres were sieved through 2 mm mesh in order to remove any gravel and plant debris.

Characteristics of bulk and rhizospere soils are shown in Table I. The soils were slightly alkali with moderate content of essential nutrients (P and K) (Table I). The total content of Zn, Pb and Cd in bulk soil is high (2540.8 mg/kg Zn, 2429.3 mg/kg Pb and 51.5 mg/kg Cd, respectively) and exceeds the maximum permissible concentrations (MPC (pH >7.4):120 mg/kg Pb, 3.0 mg/kg Cd, 400 mg/kg Zn).

	Bulk soil		**Rhizosphere soil**	
pH	7.7		7.5	
	Total	DPTA	Total	DPTA
Pb	2429.3	821.7	1610.1	736.65
Cd	51.5	38.1	42.6	22.85
Zn	2540.8	402.1	2040.2	222.2
Cu	190.9	63.4	186.8	72.1
Fe	22042.9	10.5	28286.3	13.7
Mn	823.5	6.4	841.4	5.7
P	731	41.55	613.5	10.8
Ca	25592.6	64.75	24965	215.6
Mg	10141.3	217.4	11903.6	285.6
K	4674.7	337.3	6253.1	232.9

Table 1. Characterisation of the soil and the organic amendments used in the experiment

The test plant was sedum plumbizincicola. At the end of the experiment (upon reaching the stage of flowering), plants were harvested, separated into roots, stems, and leaves and rinsed with deionized water. The plant samples were over-dried for 72 h at 70°C, weighted and ground.

Three plants were used for the collection of root exudates. The roots of sedum were rinsed thoroughly in tap water, and then rinsed three times with deionized water. The plants were placed into bakers with 200 mL of deionized water to collect root exudates for 6 h. Immediately after collection, each sample was successively filtered through a Whatman No.42 filter paper and a 0.45 µm membrane filter to remove root detritus and microbial cell debris. Finally, the root exudates were dried using a vacuum freeze-drying machine.

2.2. Methods

The content of heavy metals, micro and macronutrients in roots, stems and leaves of sedum plumbizincicola was determined by the method of microwave mineralization. The total content of metals in the soil was determined in accordance with ISO 11466 [14]. The mobile forms were extracted by a solution of DTPA [15]. The quantitative measurements were carried out with inductively coupled plasma emission spectrometry (ICP) (Jobin Yvon Emission - JY 38 S, France). Digestion and analytical efficiency of ICP was validated using a standard reference material of apple leaves (SRM 1515, National Institute of Standards and Technology, NIST).

Analysis of the root exudates were carried out using GC-MS analysis. To the dry residue were added 50.0 µl pyridine and 50.0 µl BSTFA (silylating reagent); followed by incubation at 70 ° C for 40 min. and addition of 100 µl of chloroform. The solution was injected into a system consisting of a 7890A gas chromatograph (Agilent Technologies) and a 5975C mass spectral detector (Agilent Technologies). A HP-5ms column was used with parameters: length 30 m, diameter 0.32 mm and film coating thickness 0. 25 µm at the following temperature program: initial temperature 60 ° C, retention 0 min, increase to 300°C by 5 °C/min, retention 10 min; injector and detector temperatures 250°C; helium carrier gas with a flow rate of 1.0 ml /min; mass detector scan range m /z = 50 - 550; injected sample volume 1 µl in flow split mode (split 1:10). Compounds were identified by comparing retention times and relative Kovacs (RI) indices with those of standard substances and mass spectral data from the NIST’08 library (National Institute of Standards and Technology, USA).

3. RESULTS

The results for content of total and DTPA extractable heavy metals, micro and macroelements in bulk soil and rhizosphere soil of sedum plumbizincicola are shown in Table 1. Figure 1 presents the results of the composition of root exudates of sedum plumbizincicola. The results for content of heavy metals, micro and macroelemets in sedum plumbizincicola are shown in Figure 2.

4. DISCUSSION

4.1. Influence of S. plumbizincicola on soil physicochemical properties

Differences in soil physicochemical properties were found in terms of pH, total metal content and the amount of their mobile forms in the bulk soil and rhizosphere soil. A slight decrease in the pH values in the rhizosphere from S. plumbizincicola was observed. Similar results were established by Jiang et al. [16], according to whom there are differences in pH values between 0.24 and 0.45 units in the soils on which hyperaccumulators were grown and in the bulk soils.

The content of Pb, Cd, Zn and P is significantly lower in the rhizosphere, with Fe and K a slight increase is observed, while no significant differences are observed between the values of Cu, Mn and Mg. The amount of mobile forms of Cd and Zn extracted with DTPA in the rhizosphere decreases, while the content of Ca and Mg increases. Our results are in line with the studies of Cieslinski et al. [17], which show that the amounts of microelements in the rhizosphere may differ from those in the bulk soil and may affect their bioavailability. The cultivation of S. plumbizincicola on contaminated soils leads to a significant reduction in the total amounts of Zn and Cd in the soil and extracted with 1 M NH4OAc-extractable Zn and Cd [16].

Soil pH is a major factor that controls the solubility of most heavy metals [18], as roots can alter pH values in the rhizosphere caused by an imbalance in ion absorption and exudation [19]. Lowering the pH of the soil can lead to an increase in the amount of available forms and the absorption of higher amounts of Zn and Cd by plants. However, lowering the pH of the rhizosphere is not always the cause of hyperaccumulation of metals. Hyperaccumulators of the Brassicacea family do not lower the pH of the rhizosphere [5], while S. plumbizincicola leads to acidification of the rhizosphere when grown on soils with a wide range of soil pH, nutrients and metal contamination [16]. Acidification of the rhizosphere of Sedum is associated with the release of organic anions from the roots [20] and greater absorption of Cd [21].

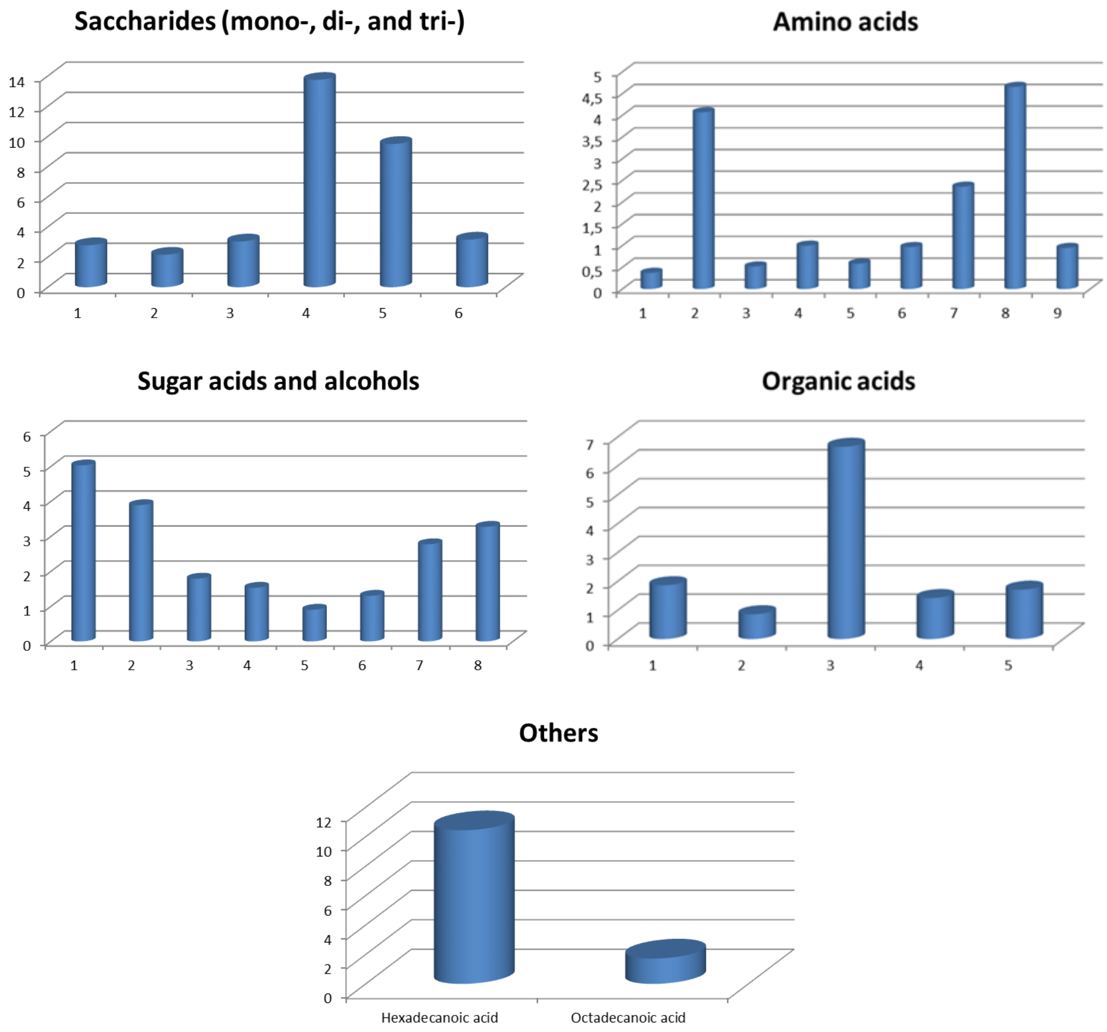
Root exudates have very different compositions, can change rapidly and are influenced by many factors, such as soil structure, plant species, environmental stress, etc. The chromatographic profile shows a complex mixture of components contained in the root exudates of sedum plumbizincicola. Twenty eight compounds were identified in the root exudates, of which 6 were saccharides (xylose methoxyamine, arabinose methoxyamine, fructose methoxyamine isomer, galactose methoxyamine isomer, glucose methoxyamine isomer, sucrose isomer; alpha-D-Glc-(1,2)-beta-D-Fru, melibiose methoxyamine isomer; alpha-D-Gal-(1,6)-D-Glc, 9 amino acids (L-valine, L-leucine, L-isoleucine, L-proline, glycine, serine, L-threonine, L-aspartic acid, pyroglutamic acid, L-glutamic acid, L-phenilalanine, L-Glutamine), 5 organic acids (succinic acid, fumaric acid, malic acid, 4-aminobutyric acid, isocitric acid), 8 sugar acids and alchohols (glycerol, glyceric acid, arabinitol, manitol, sorbitol, galactonic acid, gluconic acid, glucaric acid, galactaric acid, myo-inositol) and 2 others (hexadecanoic acid, octadecanoic acid)(Fig.1). The highest is the content of saccharides (mono-, di-, and tri-), followed by sugar acids and alcohols (20.51), amino acids (15.47), organic acids (12.51) and others (12.16). Main role in the processes of mobilization and translocation of lead, cadmium and zinc in sedum probably have the following compounds L-proline, pyroglutamic acid, L-glutamine, malic acid, glycerol, glyceric acid, myo-inositol, hexadecanoic acid, glucose methoxyamine issomer, sucrose isomer; alpha-D-Glc-(1,2)-beta-D-Fru, and melibiose methoxyamine isomer; alpha-D-Gal-(1,6)-D-Glc.

According to Sun et al.[22] and Yang, et al. [23] organic acids and amino acids play an extremely important role in the processes of accumulation of heavy metals. Organic acids can lower the pH of the rhizosphere, leading to the dissolution of heavy metals associated with insoluble minerals in the soil and increasing their phytoavailability [24,25]. Organic acids have the ability to bind metals, form complexes, and alter their bioavailability. This complexation ability is due to the carboxyl and phenolic hydroxide (OH) groups contained in the organic acids. However, the effect of organic acids on the solubility of heavy metals in soils is ambiguous. On the one hand, organic acids increase the mobility of heavy metals by forming stable metal-ligand complexes or by competing for cation adsorption sites or by reducing the negative electrostatic potential of the soil surface [26]. On the other hand, organic acids could also decrease the mobility of heavy metals by co-adsorbing on soil surfaces and forming soil organic acid–metal bridge (ternary) complexes [27]. It is believed that glyceric acid can activate Pb in the soil.

Amino acids form stable chelates with metal ions through amino and carboxyl groups. Eg. imidazole group of histidine, carboxyl group of glutamine, phenolic ring of tyrosine and thiol group of cysteine, serve as binding sites of metals in proteins [28,29]. Glutamine chelates Zn through the carboxyl group and plays a protective role against Zn toxicity [30,31]. Serine and threonine chelate heavy metals through the hydroxyl group in the side chain. Under cadmium stress, the amount of serine increases in Arabidopsis thaliana. According to Xu et al. [32] L-proline and L-histidine can increase the accumulation of Cd from Solanum nigrum, while according to Luo et al. [33] L-alanine and L-proline have a good effect on activating Pb in the soil in sedum.

Saccharides and polysaccharides have the ability to chelate and adsorb heavy metals, which is mainly due to their high hydrophilicity due to the large number of hydroxyl groups and the high chemical reactivity of these groups [34].

Alcoholic or phenolic hydroxyl groups can form stable complexes by complexation processes and reduce the activity of heavy metal ions [35]. It is known that glycerol can stabilize Pb in the soil.



Saccharides (mono-, di-, and tri-): 1-Arabinose methoxyamine, 2-Fructose methoxyamine isomer, 3 - Galactose methoxyamine isomer, 4- Glucose methoxyamine isomer, 5- Sucrose isomer; alpha-D-Glc-(1,2)-beta-D-Fru, 6-Melibiose methoxyamine isomer; alpha-D-Gal-(1,6)-D-Glc; *Amino acids*: 1-L-Valine, 2-L-Proline, 3-Glycine, 4-Serine, 5-L-Threonine, 6-L-Aspartic acid, 7-Pyroglutamic acid, 8-L-Glutamic acid, 9-L-Phenilalanine; *Sugar acids and alcohols*: 1-Glycerol, 2-Glyceric acid, 3-Manitol, 4-Sorbitol, 5-Gluconic acid, 6- Glucaric acid, 7- Galactaric acid, 8-Myo-inositol; *Organic acids*: 1-Succinic acid, 2-Fumaric acid, 3- Malic acid, 4- 4-Aminobutyric acid, 5- Isocitric acid

Fig. 1. Composition of root exudates of sedum plumbizincicola

4.2. Content of heavy metals, micro and macroelements in sedum plumbizincicola

Significant differences were found in the content of the elements in the different parts of sedum plumbizincicola. Pb, Cd, Zn, Cu, Ca and P accumulate in the leaves, while Fe, Mn and Mg accumulate in the root system.

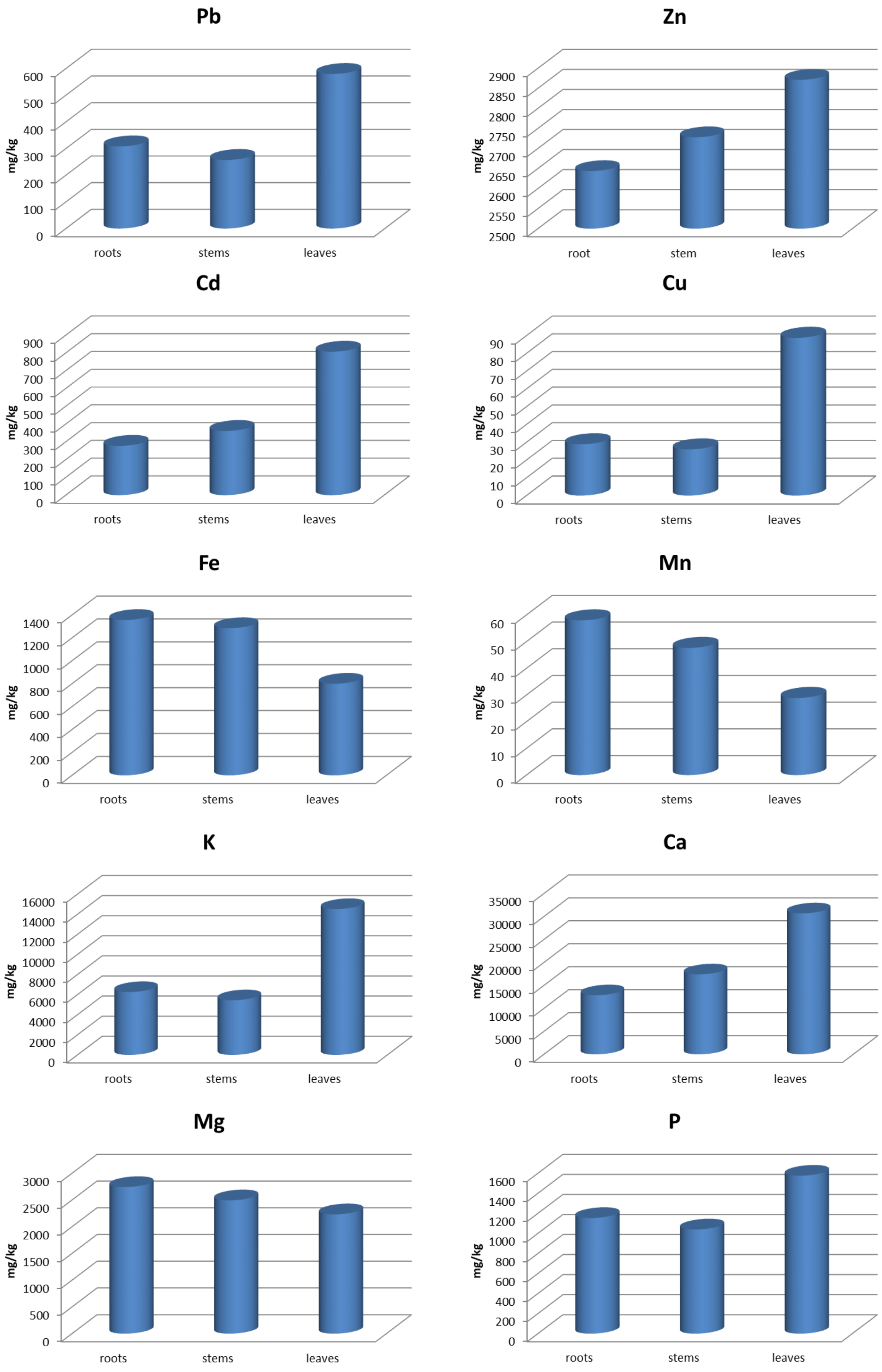


Fig. 2. Content of heavy metals, micro and macronutrients (mg/kg) in sedum plumbizincicola

The content of Pb in the roots reaches up to 308.1 mg/kg, Cd - up to 277.1 mg/kg, Zn - up to 2643.8 mg/kg, Cu - up to 28.9 mg/kg, Fe - up to 1356.5, mg/kg, Mn - up to 57.96 mg/kg, P - up to 1153.18 mg/kg, Ca - up to 12863.1 mg/kg, Mg - up to 2741.8 mg/kg and K - up to 6263.7 mg/kg.

The movement and accumulation of heavy metals, micro- and macro elements in the vegetative organs of sedum plumbizincicola differ significantly. The content of Pb, Cu, P, Fe, Mn, Mg in the stems is lower compared to the root system, while in Cd, K and Ca the opposite trend is observed.

The content of Pb in the stems and leaves reaches up to 257.1 and 579.9 mg/kg, respectively, Cd - up to 361.0 and 806.8 mg/kg, Zn - up to 2728.1 and 2871.4 mg/kg, Cu - up to 25.9 and 88.8 mg/kg, Fe - up to 1285 and 799.8 mg/kg, Mn up to 47.6 and 28.9 mg/kg, P up to 1039.4 and 1575.5 mg/kg, Ca up to 17412.7 and 30697.1 mg/kg, Mg up to 2493.5 and 2231.7 mg/kg, and K - up to 5445.5 and 14522.7 mg/kg.

The movement of Pb from the roots to the above-ground mass in plants is usually low [36,37]. When Pb enters the roots of the plant, it immediately interacts with phosphates, carbonates and bicarbonates contained in high concentrations in the intercellular spaces. As a result of this interaction, Pb precipitates in the form of phosphates or carbonates and does not reach the xylem for translocation [38,39]. However, our results show the significant ability of sedum to move and accumulate Pb in the above-ground mass (leaves), which does not correspond to what was found by Wu et al. [40] that S. plumbizincicola has a low ability to accumulate Pb. A probable reason for this is the higher content of mobile forms in the soil, as well as the influence of metabolites (L-proline, pyroglutamic acid, L-glutamine, malic acid, glycerol, glyceric acid, myo-inositol, hexadecanoic acid, glucose methoxyamine issomer, sucrose isomer; alpha-D-Glc-(1,2)-beta-D-Fru, and melibiose methoxyamine isomer; alpha-D-Gal-(1,6)-D-Glc.) from root exudates that mobilize Pb from the rhizosphere

Our results confirm the ability of sedum to hyperaccumulate Cd in its above-ground mass (>100 mg/kg). The Cd content increases in the following order: roots> stems> leaves, with values being 3 times higher in the leaves, indicating the effective capacity to transport Cd from the stems to the leaves.

The amount of Zn is comparable between the roots, stems and leaves, with the highest reported values for Zn in the leaves. The results show the ability of sedum to accumulate Zn in the above-ground mass, but the values are lower than 10000 mg/kg. The potential ability of S. plumbizincicola to extract Cd and Zn from contaminated soils has been demonstrated in greenhouse and field experiments [40, 41,42] from mine dumps and wastewater. S. plumbizincicola shows different ability to accumulate Cd and Zn, with the highest values reaching 1,470 and 14,600 mg/kg Cd and Zn, which are much higher than 100 mg/kg for Cd and approx./above 10,000 mg/kg for Zn, the levels determining Cd and Zn hyperaccumulator capacity [4].

A probable reason for the lower accumulation of Zn in this study is due to the different soil and climatic conditions between the areas of sedum cultivation. In the province of Zhejiang the soils are sandy, acidic, heavily leached, the annual rainfall varies from 980 to 2000 mm with an average annual temperature of 15–18°C, while the soils in the area of NFMW are alluvial meadow, with annual rainfall 605 mm and average annual temperature of 17°C. The warmest month of the year is July, with an average temperature of 30 °C. In order for phytoextraction to be successful, plants must be tolerant of the soil environment, but also adapted to local climatic conditions [2]. The obtained results confirm that the metal absorption efficiency of S. plumbizincicola is significantly higher in acidic than in alkaline soils [43].

In terms of Cu, K, Ca and P the values between the roots and the stems are close, and again their content in the leaves is the highest. With regard to Fe, higher values were found in the roots and stems and 3 times lower values in the leaves. Similar results were found in Mn. The amount of Mg is comparable between the roots, stems and leaves, with the highest reported Mg values in the roots.

The pH changes in the rhizosphere of plants can be caused by an imbalance in the absorption and exudation of ions. The decrease in the rhizosphere pH may be a reason for an increase in the absorption of soil cations. Sun et al. [44] found that sedum contains very high concentrations of cations, including Ca in its above-ground mass. Plant roots release protons to maintain the balance of ionic charges when they absorb primarily cations [19]. According to Sun et al. [44] the remarkable absorption of cations is probably a driving factor in the acidification of the rhizosphere of Sedum. Plants of the Crassulaceae family are known to be calcitrophic species [45], meaning that they contain large amounts of water-soluble Ca [46]. According to Sayed [47], most members of the Crassulaceae family are plants with crassulacean acid metabolism (CAM). For plants with CAM, calcium plays an important role in counteracting large amounts of carboxylates in vacuoles [48].

4.3. Translocation and bioaccumulation factors

The Translocation Factor (TF) provides information on the ability of plants to digest heavy metals through the roots and to move them to the above-ground mass (leaves). The higher coefficient of translacation (shot/root ratio in the plant) is important for phytoremediation of soils contaminated with heavy metals.The obtained results show that, with respect to Pb, the translocation factor for plants reaches up to 2.72, for Cd up to 4.21 and for Zn up to 2.11.

The effectiveness of phytoremediation is also determined by the bioconcentration factor (BCF). BCF root is a ratio of the content of heavy metals in plant roots to soil content (BCFroots = [Metal] roots/[Metal] soils). In hyperaccumulators, the enrichment factor is higher than 1 and in some cases it may reach 50-100. The obtainned results show that, with respect to Pb, the bioconcentration factor for plants reaches up to 0.34, for Cd up to 22.68, and for Zn up to 2.20. BCF is higher for Cd than for Pb and Zn. Higher values for Cd and Zn are probably a consequence of the greater ability of these elements to accumulate in the above-ground mass than in the roots, which is consistent with the results of Wu et al.[41].

The high concentration of Cd and Zn in the leaves and the high translocation and bioconcentration factors indicate the possibility of sedum plumbizincicola to be used in phytoextraction. Higher root to shoot translocation of these metals indicated that sedum plumbizincicola have vital characteristics to be used for phytoextraction of these metals. Sedum plumbizincicola was specified as a Cd hyperaccumulator; and Zn and Pb accumulators and show potential for phytoextraction of Cd, Zn and Pb in contaminated soils in Bulgaria. Further studies are required to determine the utilization of aboveground mass of sedum plumbizincicola and to enhance the economic value of this plant when growing on industrially polluted soils.

5. CONCLUSIONS

Based on the obtained results, the following conclusions can be made:

1. Sedum plumbizincicola is a plant tolerant to heavy metals and it develops normally when grown on soils contaminated with heavy metals (2540.8 mg/kg Zn, 2429.3 mg/kg Pb and 51.5 mg/kg Cd).
2. Significant differences were found in the content of the elements in the different parts of sedum plumbizincicola. Pb, Cd, Zn, Cu, Ca and P accumulate in the leaves, while Fe, Mn and Mg accumulate in the root system. The Cd content increases in the following order: roots> stems> leaves, with values being 3 times higher in the leaves, indicating the effective capacity to transport Cd from the stems to the leaves. The amount of Zn is comparable between the roots, stems and leaves, with the highest values in the leaves, lower than 10000 mg/kg.
3. Sedum plumbizincicola can be referred to the hyperaccumulator of cadmium and the accumulator of zinc. The high concentration of Cd, Zn and Pb in the leaves and the high translocation factor (TF Zn – 4.21, TF Cd - 2.11, TF Pb – 2.72) and high bioconcentration factor (BCF Cd - 22.68, BCF Zn - 2.20, BCF Pb 0.34) indicate the possibility of sedum plumbizincicola to be used in phytoextraction of heavy metal contaminated soils in Bulgaria.
4. Thirty compounds were identified in the root exudates, of which 6 were saccharides, 9 amino acids, 5 organic acids, 8 sugar acids and alchohols and 2 other compound (hexadecanoic acid, octadecanoic acid). Main role in the processes of mobilization and translocation of lead, cadmium

and zinc in sedum plumbizincicola probably have the following compounds L-proline, pyroglutamic acid, L-glutamine, malic acid, glycerol, glyceric acid, myo-inositol, hexadecanoic acid, glucose methoxyamine issomer, sucrose isomer; alpha-D-Glc-(1,2)-beta-D-Fru, and melibiose methoxyamine isomer; alpha-D-Gal-(1,6)-D-Glc. The highest is the content of saccharides (mono-, di-, and tri-), followed by sugar acids and alcohols (20.51), amino acids (15.47), organic acids (12.51) and others (12.16).

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