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GROWTH AND HEAVY METAL UPTAKE OF MEDICAGO SATIVA L. GROWN IN HEAVY METAL CONTAMINATED CLAY LOAM BROWN FOREST SOIL

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Abstract:

The use of plant for heavy metal-polluted soil rehabilitation is an emerging ecologically sound and safe technique. In vitro and in vivo experiments were conducted to determine the heavy metals impacts on germination, growth and metal uptake by alfalfa biomass. The results of in vitro experiment demonstrated that the rates of seed germination, root and shoot growth were affected by Cd, Cu, Ni and Pb metals at higher concentrations of 80 and 160 µM. The inhibitory decreasing order of metal toxicity on seed germination was Cd > Cu > Ni > Pb > Zn. Meanwhile, the lower concentrations of investigated metals (10 and 20 µM) stimulated the root and shoots length and at 10 and 20 mg/kg concentration levels increase plant biomass. The results showed that alfalfa able to uptake the heavy metals at the applied concentrations ranging from 10 to 160 mg/kg by various degrees. Finally, alfalfa plants demonstrated it's powerful to some extent cleanup the soil environment from heavy metals.

***Keywords*:**

Alfalfa, metal uptake, metal pollution, plant growth and biomass, clay loam brown forest soil

1 INTRODUCTION

Human activity leads to increasing levels of heavy metal contamination. Heavy metals**,** owing to atmospheric and industrial pollution, accumulate in the soil and influence the ecosystem nearby. NICHOLSON *et al*., (2003) concluded that the major sources of soil heavy metals include atmospheric deposition, sewage sludge, livestock manures, inorganic fertilizers and lime, agrochemicals, irrigation water, industrial by-product 'wastes' and composts. JOSHI & LUTHRA (2000) found that the main sources of soil heavy metal pollution are geogenic, mining and smelting, disposal of municipal industrial wastes, use of fertilizers, pesticides and fumes from automobiles. Rapid growth of urbanization as well as transportation and industries are leading to serious environmental hazards (URBAN, 2007). ALVAREZ-AYUSO (2008) mentioned that heavy metal contamination of soils derived from agricultural or industrial activities is one of the major environmental problems in many parts of the world. Among chemical elements and compounds which are regarded as environmental pollutants, toxic metals such as Pb and Cd are the most widely spread and found around the urban agriculture areas (MICO et al., 2006; FITAMO et al., 2007). GUIWEIA et al. (2010) found that the concentration of Cd in the plant shoots, and the activities of catalase and ascorbate peroxidase decreased in plants from polymer-amended soil compared with unamended control. Soil pH affects the speciation and adsorption of heavy metals in soil, determining the mobility, bioavailability and toxicity of the metal.

Free metal ion activities are usually better indices of metal bioavailability and toxicity than are total soluble metal concentrations. As the mass of the water transpired by the plant increases the Cu concentration in both the root and shoot increase (CHENG and ALLEN, 2001). VASILIADOU & DORDAS (2009) found that the Cd level affected the number of leaves and dry matter accumulation, and there were differences among the different cultivars that were used. BAKER and BROOKS (1998) found that some native plant species were able to accumulate unusually high concentrations of potentially phytotoxic elements such as Cd, Cu, Pb, Ni and Zn from metalliferous soils. MA et al. (2009) established that compared with the single factor pollution index (SFPI) of heavy metals calculated for the control site, the average SFPI from the sampling sites decreased in the order of Cr > Cd > Pb > Zn > Ni > Cu. There were notable negative correlations between the integral pollution index of soil heavy metals at all sampling sites and the distances from the railroad. Heavy metals interferes with several metabolic processes, causing toxicity to the plants as exhibited by reduced seed germination, root and shoots growth and phytobiomass, chlorosis, photosynthetic impairing, stunting and finally plant death (ROY et al., 2005). Plant roots participate primarily in the heavy metal cation uptake (LASAT, 2002). Some heavy metals, like Cu and Zn, are essential for plants and animals and others like Cd and Pb are toxic even at low concentrations and have no biological functions. Most metal uptake occurs in the root system, usually *via* absorption, where many mechanisms are available to prevent toxic effects due to the high concentration of metals in the soil and water.

The plant material may be used for non-food purposes; alternatively, it can be ashed followed by recycling of the metals or as disposal in a landfill (ANGEL & LINACRE, 2005). CHHOTU et al. (2008) mentioned that the seed germination, root and shoot growth were affected by Cd, Cu, Ni, Pb and Zn metals at higher concentration of 40 and 50 ppm. However, the lower concentration of heavy metals ranging from 5 to 10 ppm doses were observed to be stimulating the root and shoot length and increase biomass of the alfalfa plant. This study is a part of a comprehensive investigation to monitor heavy metals contained in alfalfa and to assess contamination status, accumulation features.

2 MATERIALS AND METHOD

2.1 Soil sampling, and soil properties

Soil samples were collected from a depth of about 0-20 cm along uncultivated clay loam brown forest soil collected from Gödöllő region, Hungary. Stones and plant residues and other soil impurities were carefully removed from the soil prior to drying process under laboratory condition. The soil samples were screened through 2 mm stainless steel sieve and stored in a plastic bag at room temperature until use. Concentrations of Pb, Zn, Cu, Ni and Cd were measured by atomic absorption spectrophotometer. The soil moisture content was calculated by the weight difference before and after drying at 105°C to a constant weight.

The pH was measured after 30 min of vigorous mixed samples at 1:2.5 (Solid: distilled water ratio). The physico-chemical parameters were measured by standard methods (Table 1).

Table 1. Some chemical properties of the soil used in the experiment

Parameters	*Clay loam brown forest soil, Gödöllő*
$pH_{(H2O)}$	5.33
Humus content %	1.240
Total N content mg kg^{-1}	8.411
NO_3-N, mg kg^{-1}	133.080
NH_4-N, mg kg^{-1}	410.690
Ca, mg kg^{-1}	856.000
Mg, mg kg^{-1}	203.000
Na, mg kg^{-1}	21.000
P_2O_5, mg kg^{-1}	121.310
K_2O, mg kg^{-1}	107.000
Zn, mg kg^{-1}	38.100
Cu, mg kg^{-1}	22.900
Mn, mg kg^{-1}	136.000
Fe, mg kg^{-1}	1187.000
Cd, mg kg^{-1}	0.180
Pb, mg kg^{-1}	15.100
As, mg kg^{-1}	7.400

2.2 Heavy metal salts

Heavy metal solutions used in *in vitro* and *in vivo* experiments were prepared from the following heavy metal salts: Cd as $Cd(NO_3)_2.4H_2O$; Cu as $CuSO_4.5H_2O$; Ni as $Ni(NO_3)_2$; Pb as $Pb(NO_3)_2$, and Zn as $Zn(NO_3)_2.6H_2O$.

2.3 Laboratory experiment

Laboratory experiment was carried out to evaluate the impacts the various concentration levels of heavy metals Cd, Ni, Cu, Pb and Zn on alfalfa (*Medicago sativa* L.) seed germination, root and shoot length. According to VINCENT (1970), alfalfa (*Medicago sativa* L.) seeds were sterilized with 70% ethanol for 30 seconds followed by sterilization with 0.1% mercuric chloride for 5 min. The seeds were thoroughly washed with sterilized distilled water several times to avoid fungal contamination. To measure the effect of heavy metals on seed germination; 10 sterilized seeds were placed in large Petri dishes of 24 cm in diameter with 3 Whatman filter papers and wet with 50 ml equivalent to the following treatment solutions: 0, 10, 20, 40, 80 and 160 µM of each of Cd, Ni, Cu, Pb and Zn. The control Petri dishes were treated by distilled water. Petri dishes were sealed with Parafilm to prevent evaporation. Seeds were incubated at alternating temperatures of 25°C (16 hours) and 18°C (8 hours). The experiment was carried out by three replicates of 10 seeds were used for each treatment. Germination was counted every 3 days for 21 days. Seeds were considered to have germinated when the radical was at least 5 mm long. The germinating seedlings were harvested after 21 days and germination rate (%), shoot and root length were recorded in comparison with heavy metal free control.

2.4 Greenhouse experiment

In vivo, ten seedlings were grown in 2 Kg capacity plastic pots for studying root and shoot growth biomass and metal uptake. Soil moisture content was adjusted to about 45% of water-holding capacity with distilled water. Soil samples were treated by different concentrations: 0, 10, 20, 40, 80 and 160 mg metal/kg soil of each of Cd, Ni, Cu, Pb and Zn. The control Petri dishes were treated by distilled water.

To prevent loss of nutrients and trace elements out of the pots, plastic trays were placed under each pot and the leachate collected was put back in the respective pot. Each treatment of plant consisted of three replicate for statistical purpose. The seedlings were set under photoperiod of 14/10 hrs light/dark cycle and temperatures of 26±2°C during the day and 20±2°C during the night. The average relative humidity was recorded to be 72%. For the metal uptake study, plants were harvested after 8 weeks. The plants were then separated in to root and shoot. The plant samples were washed with distilled water and dried in an oven at 75°C for 2 days and the dry weight of biomass was determined, after which these samples were stored in the paper bags. The samples were considered for analysis of metal content digested with concentrated nitric acid and 30% hydrogen peroxide and then the heavy metal content was determined by an atomic absorption spectrophotometer. The experimentation was layout in a complete randomized block design. Means of three replicates per treatment for each strain were analyzed using ANOVA to determine statistical differences among treatment and LSD at P = 95% was calculated as well as S.D.

3 RESULTS

3.1 Effect of heavy metals on seed germination

The result of *in vitro* study demonstrated a concentration dependent inhibition of the seed germination with regards to metal and alfalfa tolerance (Fig. 1).

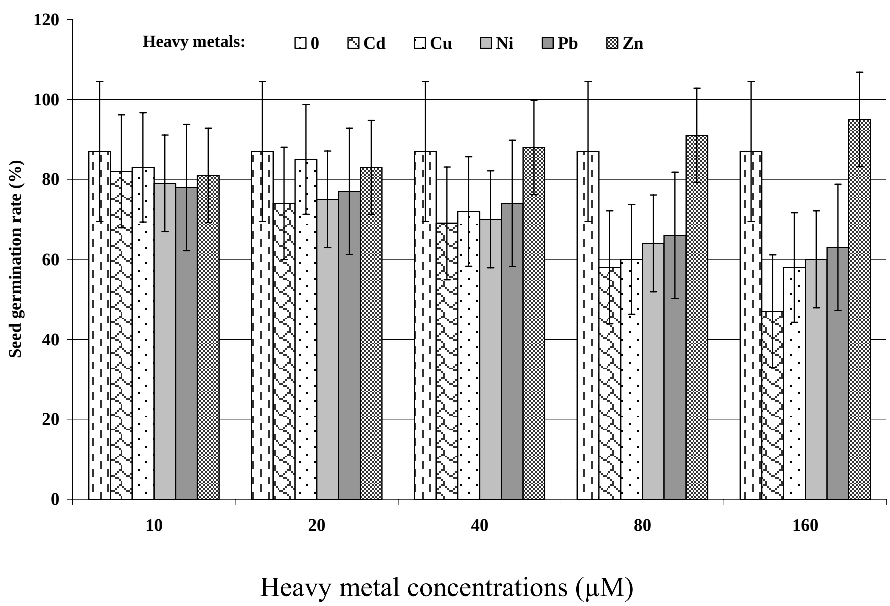


Fig. 1. In vitro effects of heavy metals on alfalfa seed germination after 21 days

The result of this investigation indicated that Cd, Ni and Pb at 10 µM concentration levels had very low toxic effects on seed germination while Cu at the same dose increased seed germination. At the 20 and 40 μM concentrations of Cd, Ni and Pb reduced the seed germination while the seeds were germinated more at 10 and 20 μM levels of Cu. The seed germination inhibited at 80 and 160 μM levels as compared to the control for all the four metals Cd, Ni, Pb and Cu.

Delayed germination was also observed in all cases at higher (80 and 160 μM) concentrations. However, in the same study Zn being the only metal which did not reduce the seed germination. The decreasing order of toxicity for metals on seed germination was Cd > Cu > Ni > Pb >Zn. However, seed germination increased at all Zn concentration levels.

3.2 Effect of heavy metals on root length

The increases in the heavy metal concentration caused root length decrease with stunt growth of roots (Fig. 2). The dose of 10 μM of all investigated heavy metals promoted the root length of the plants as compared to the root growth of the control plants. The heavy metals Cu, Ni, Zn and Pb at 20 μM concentrations further increased the root growth over the control root size. However at the same dose Cd reduced the root length on comparison with the control root elongation.

The metals Cd, Ni, Cu and Pb demonstrated a concentration dependant inhibition of root growth at 40, 80 and 160 μM levels. All Zn concentrations increased the root length than the control root length alfalfa plants. Root toxicity symptom included browning, reduced number of roots hair and growth.

In comparison to the control, plant roots were healthy and normal. The colours of the roots receiving higher heavy metals treatment (80 and 160 μM) except Zn changed gradually over time from creamy white colour to dark brown, an indication of intense suberification. Plants treated at lower concentrations were not significantly affected by the metals. Lateral roots were observed in almost all treated samples of Zn, Cd, Cu, Pb and Ni demonstrated concentration dependant inhibition of root growth at higher concentrations.

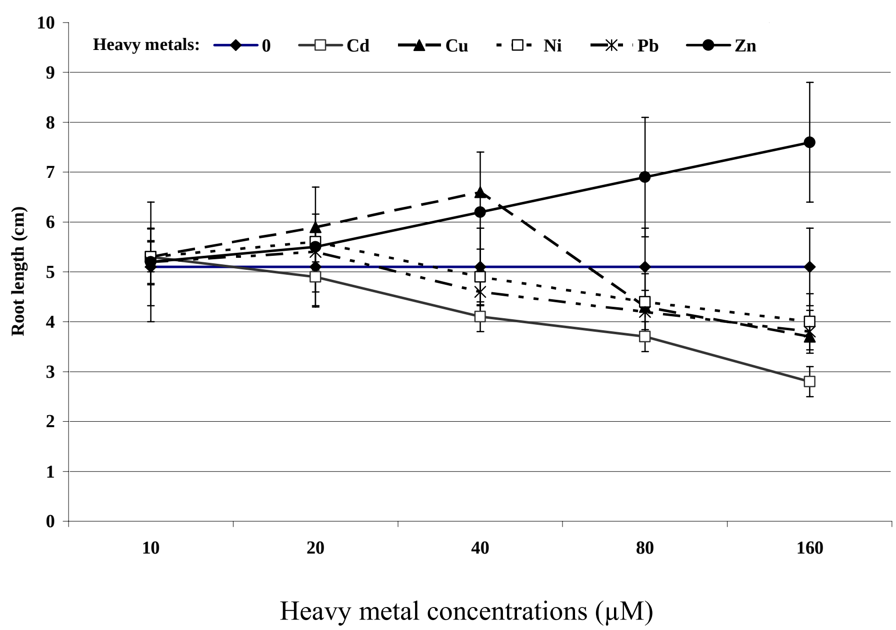
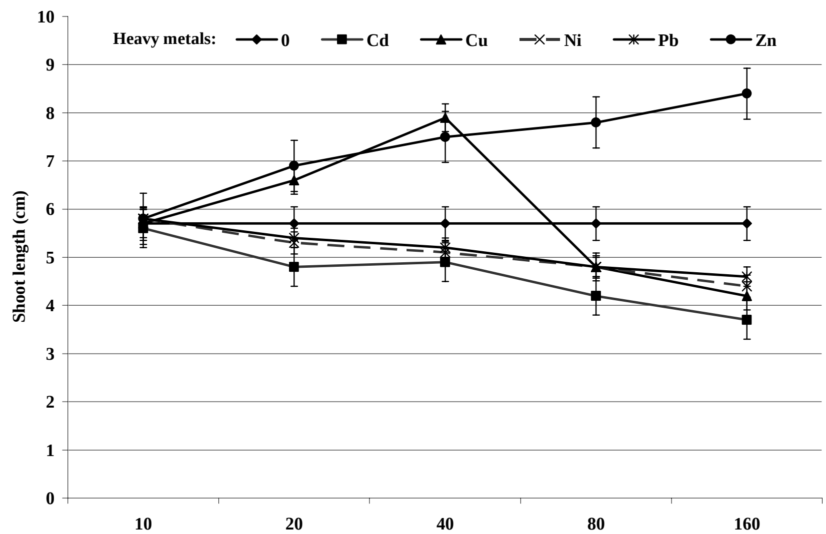


Fig. 2. In vitro effects of heavy metals on alfalfa root length after 21 days germination

3.3 Effect of heavy metals on shoot elongation

The impacts of heavy metals on the shoot elongation are different from their effects on root growth and length (Fig. 3).



Heavy metal concentrations (µM)

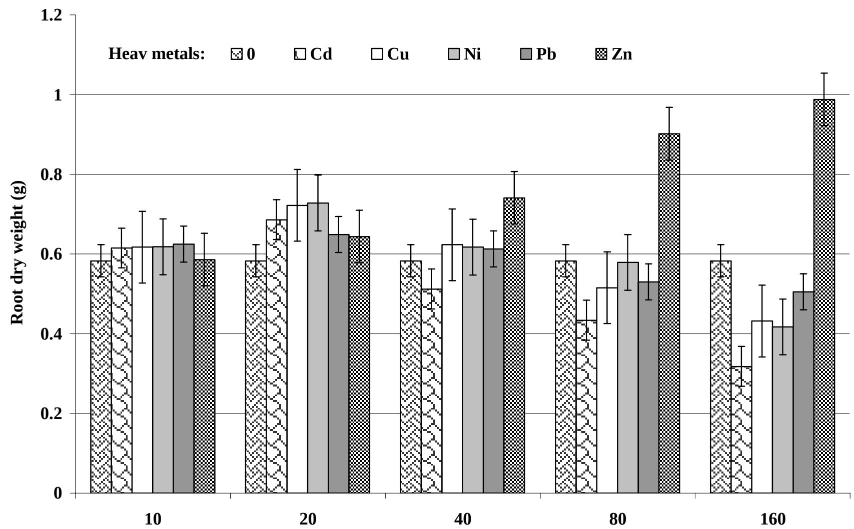
Fig. 3. In vitro effects of heavy metals on alfalfa shoot length after 21 days germination

The shoot length was found slightly reduced than the control alfalfa plants at the 10 µM Cd level. On the other hand, the 10 µM dose of Cu, Pb, Ni and Zn increased the shoot lengths as compared to the control treatment. These results indicate that low concentrations of Cd, Cu, Ni and Pb have micronutrient-like effects on the alfalfa plants and all the plants appeared to be healthy.

The heavy metals Cd, Ni and Pb at 20 and 40 µM doses reduced the shoot growth; however, Cu at the same dose increased the shoot length. When the concentration of these metals was increased to 80 and 160 µM concentrations, the shoot length of the alfalfa plants found a concentration dependant inhibition of shoot growth as compared to the control plants. All plants grown in the soil contaminated with Zn showed increase in the shoot elongation than the plants grown in soil without Zn contamination.

3.4 Effect of heavy metals on plant biomass

The results of *in vivo* experiment indicated that the mean plant biomass (root and shoot dry weights) of alfalfa showed an increasing tendency as the concentrations increased from 10 to 20 for Cd, Cu and Ni. It was found that at 40 mg/kg for plant better grown in soil contaminated by Cu and Ni than in Cd-contaminated soil (Figs. 4a and 4b).

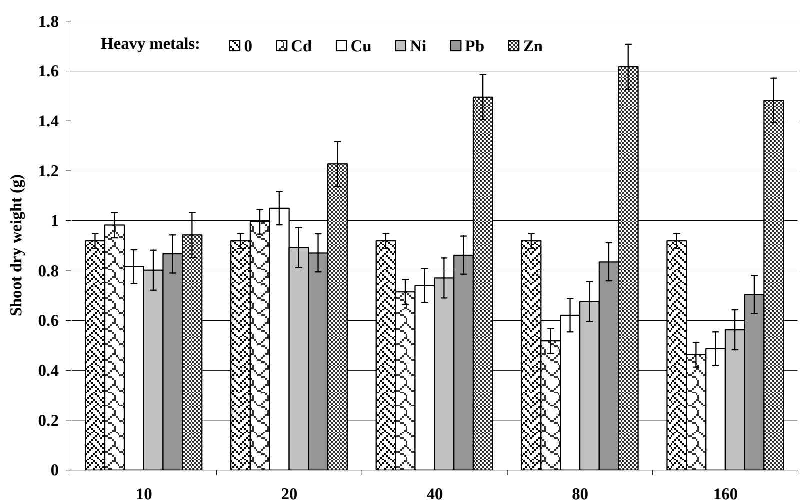


Heavy metal concentrations (mg/kg)

Fig. 4a. In vivo effects of heavy metals on alfalfa root dry weight after 8 weeks plantation in clay loam brown forest soil

The dry weights of root and shoot decreased gradually as the concentration of Cd, Cu and Ni in the soil ecosystem increased to 80 and 160 mg/kg.

The plant dry weights of root and shoot yield affected by the higher concentration levels of Cd caused reduction in the plant biomass. Lead showed low effect on dry weights of root and shoot of the plant.



Heavy metal concentrations (mg/kg)

Fig. 4b. In vivo effects of heavy metals on alfalfa shoot dry weight after 8 weeks plantation in clay loam brown forest soil

3.5 Heavy metal uptake by plant roots and shoots systems

The heavy metals concentration in the plant is affected by many factors such as the metal content supplied in the soil ecosystem and the plant tissue as well as by the interaction between these factors.

The mean uptake of metals Cd, Ni, Pb, Cu and Zn by root (Fig. 5a) and shoot (Fig. 5b) of alfalfa plant increased as the concentrations of these metals in the soil ecosystem increased.

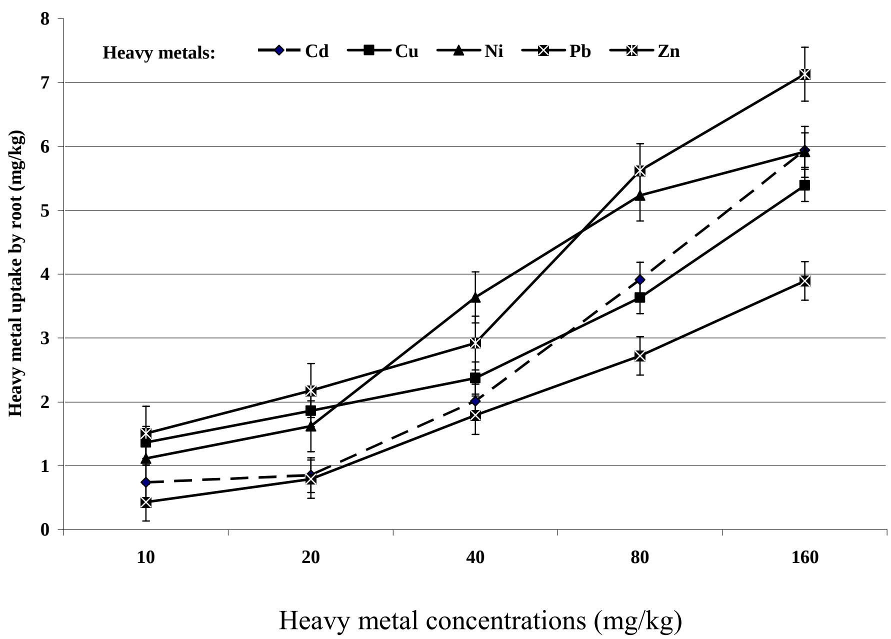
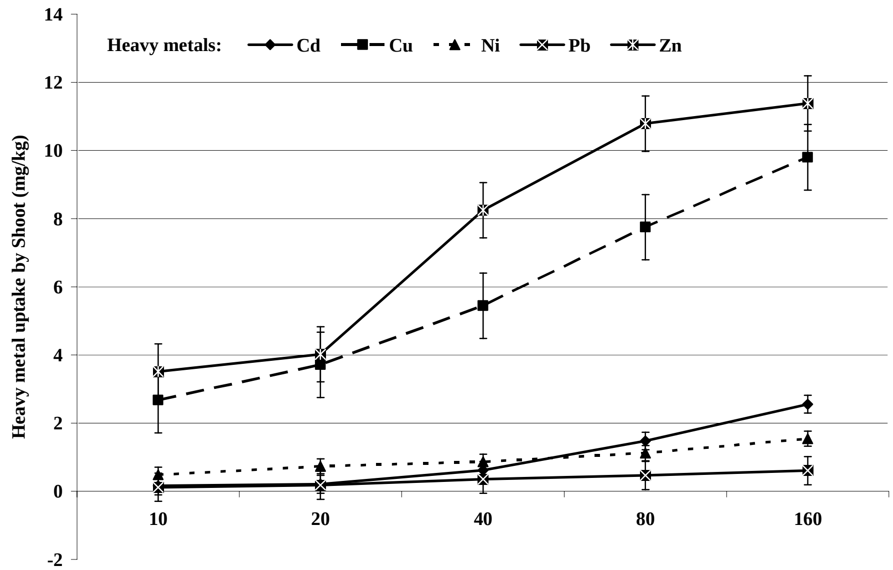


Fig. 5a. Uptakes of heavy metals by alfalfa roots system after 8 weeks plantation in clay loam brown forest soil

The results illustrated that the absorption of heavy metal by root system was directly proportional increased by increasing the concentration of heavy metal. Zinc showed the highest metal uptake by root system, while Pb was the lowest metal absorbed by root system. It was found that the shoot system (Fig. 5b) accumulated Zn and Cu more Cd, Ni and Pb.

In plant, shoot and root were observed to have a characteristic uptake capacity for different metals. The decreasing order of uptake of heavy metals by the alfalfa plants tissues was in the following order: Zn > Cu > Cd > Ni > Pb.



Heavy metal concentrations (mg/kg)

Fig. 5b. Uptakes of heavy metals by alfalfa shoot system after 8 weeks plantation in clay loam brown forest soil

4. DISCUSSION

The spermosphere represents a short-lived, rapidly changing, and microbiologically dynamic zone of soil surrounding a germinating seed. It is analogous to the rhizosphere, being established largely by the carbon compounds released into the soil once the seed begins to hydrate. These seed exudations drive the microbial activities that take place in the spermosphere, many of which can have long-lasting impacts on plant growth and development as well as on plant health (NELSON, 2004).

The results demonstrated that the concentration dependent inhibition of the seed germination. To some extent, our results are in an agreement with PERALTA *et al.*, (2004) who investigated alfalfa plants grown in soil at different growth stages using separate batches of Cr^{6+} at 100 mg/l, and Cd^{2+}, Cu^{2+}, Ni^{2+}, or Zn^{2+} at 500 mg/l. Four days after germination, all metals except Zn^{2+} had lethal effects on the seedlings. After 16 days of germination, Cr^{2+} and Ni^{2+} still had lethal effects on the seedlings and Cd^{2+} and Cu^{2+} destroyed more than 50% of the plant population. While approximately 90% of the plants exposed to Cd^{2+}, Cu^{2+} and Zn^{2+} were able to grow without apparent negative effects 20 days after germination, Cr^{2+} and Ni^{2+} still showed lethal effects. These results demonstrated that the tolerance of alfalfa plants to Cd, Cu and Zn was positively correlated with the age of the plants. Thus, alfalfa seedlings tolerated Zn^{2+} at 500 mg/l at the growth stage of 4 days after germination. Alfalfa plant could be considered potentially feasible to be transplanted in uncontaminated soils where the concentrations of Cd, Cu or Zn are high enough to interfere with alfalfa seed germination.

Increase in the heavy metal concentration in the soil ecosystem caused root length decrease with stunt growth of roots. One of the explanations for roots to be more responsive to toxic metals in environment might be that roots were the specialized absorptive organs so that they were affected earlier and subjected to accumulation of more heavy metals than any of the other organs This could be the main reason that root length was usually used as a measure for determining heavy metal tolerant ability of plant (XIONG, 1998). According to CHAIGNON & HINSINGER (2003), higher concentrations of Cu can inhibit root growth before shoot growth and can accumulate in the roots without any significant increase in its content of the aerial parts. Heavy metals are found to be more toxic for root growth because they accumulate on root and retard cell division and cell elongation. Similar conclusion was drowned by us too as regard to results in Fig. 2. The results indicate that low concentrations of Cd, Cu, Ni and Pb have micronutrient-like impacts on the alfalfa plants. These results agreed with the observation of CHHOTU & FULEKAR (2008). ORMROD et al. (1986) investigated that Ni caused stunted and deformed growth of shoot with symptoms of chlorosis. GYAWALI & LEKHAK (2006) noted that the 11%, 22% and 41% reduction in plant height, respectively, over control. Generally, it was seen that degrees of inhibition of shoot and root growth started from 10 μM concentration. In this respect PERALTA-VIDEA et al. (2004) reported that that Cd affected young plants more than old plants of *P. coccineus*, and Cd applied to the younger plants caused a stronger reduction in growth parameters such as leaf area and fresh weight accumulation and reduce shoot growth by reducing the chlorophyll content and the activity of photosystem I.

The biomass yield affected by the higher concentrations of metals and caused reduction in the plant biomass. Higher heavy metal concentrations can affect physiology; reduced plant growth and dry biomass yield (GRIFFERTY & BARRINGTON, 2000). Authors showed that the increased Zn concentration from 25 to 50 mg/kg had a significantly positive effect on dry biomass yield. The plant biomass may be incinerated either to reduce volume, recover energy, disposed off-using appropriate techniques or recycled to recover valuable metals (ANGEL & LINACRE, 2005). The alfalfa produced greater biomass which result in a higher concentrations uptake of metals was reported by PIVETZ (2001). The phytotoxicity of Cd on growth and dry matter production of a number of cultivated plants have been determined by GONDEK & FILIPEK-MAZUR (2003). Our results are in an agreement with those mentioned above according to the results presented in Figs 4a and 4b.

The mean uptake of Cd, Ni, Pb, Cu and Zn by alfalfa plant systems increased as the concentrations of these metals in the soil ecosystem increased. Alfalfa shoot biomass has demonstrated the ability to bind an appreciable amount of Cu, Ni, Cd, Cr, Pb and Zn from aqueous solutions (TIEMANN et al., 1998). Increased in Pb uptake by alfalfa using EDTA and a plant growth promoter were reported by LOPEZ et al. (2005). The large surface area of roots and their intensive penetration of soil may reduce leaching, runoff and erosion *via* stabilization of soil, offer advantages for metal uptake. Most crop species tend to accumulate Cd at the highest concentrations in the root tissue, followed by leaves, then by seeds or storage organs. Several studies have demonstrated that the metal concentration in the plant tissue is a function of the heavy metals content in the growing environment (CUI et al., 2004). CHENG and ALLEN (2001) found that Cu uptake was linearly related to free Cu^{2+} ion activity and was independent of total Cu concentration in solution. Regarding to the effect of Cu on alfalfa plant growth and biomass, Our investigation showed similar results to WU & HENDERSHOT (2010) who mentioned that The accumulation and toxicity of Cu to pea (*Pisum sativum* L.) roots were investigated. The root uptake of Cu and Ca varied with Ca and H activities. Calcium, H, and Cu competed for root binding with high pH and low Ca favoring more Cu uptake. Root elongation was highly sensitive to root Ca content and correlated better with root-bound Ca and Cu content than with merely dissolved Cu concentrations. A multi-element uptake model was developed to describe Cu and Ca accumulation by treating the pea roots as a collection of three biotic ligands with known site densities and proton-binding constants.

The essential elements (Cu and Zn) are required in low concentrations and hence are known as trace elements or micronutrients, whereas nonessential elements (Cd and Pb) are phytotoxic (GERARD et al., 2000). Zn is relatively mobile in soils and is the most abundant metal in root and shoot of contaminated plants as it is in soils. This metal is necessary as a minor nutrient and it is known that plants have special Zn transporters to absorb this metal (ZHU et al., 1999). The bioavailable of Pb is usually very low due to its strong association with organic matter, Fe-Mn oxides, clays and precipitation as carbonates, hydroxides and phosphates (SHEN et al., 2002). Cadmium also is considered to be mobile in soils but is present in much smaller concentrations than Zn (ZHU et al., 1999). Moreover, many studies have demonstrated that Cd taken up by plants accumulates at higher concentrations in the roots than in the leaves (BOOMINATHAN & DORAN, 2003). In addition, exudation of organic compounds by plant roots, such as organic acids, influence ion solubility and uptake (KLASSEN et al., 2000) through their effects on microbial activity, rhizosphere physical properties and root-growth dynamics (Yang et al., 2005). The higher concentrations heavy metals uptake by alfalfa was reported by REHAB et al. (2002).

5. CONCLUSION

It can be concluded that the low-doses of tested heavy metals applied stimulated the root and shoot elongation of alfalfa plants. At higher concentrations (over 80 μM or 80 mg/kg) of Cd, Cu, Ni and Pb reduced the germination rates and phytobiomass of alfalfa plants, respectively. The study shows that heavy metals were efficiently uptaken by alfalfa plants at all concentrations and the uptake was increased along the increasing concentrations in soil. Finally, alfalfa plants demonstrated it's powerful to some extent cleanup the soil environment from heavy metals.

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