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Phytoremediation of Lead and Copper by Sainfoin (*Onobrychis vicifolia*): Role of Antioxidant Enzymes and Biochemical Biomarkers

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Abstract. This experiment was done in order to evaluate the species ability in absorption and recognizability the plant resistant Sainfoin, (*Onobrychis vicifolia*) to heavy metals lead and copper in 2009. The experimental treatments were arranged as factorial experiment in randomized complete design with four replicates. The first treatment was four lead $Pb(NO_3)_2$ levels 0, 200, 400, 800 and the second treatment was four copper $Cu(SO_4)_2$ levels 0, 150, 300 and 450 mg/kg soil. The results showed significant effects on lead and copper absorption by the sainfoin roots and aerial parts ($P>0.01$). The results also demonstrated that sainfoin had the same ability in lead and copper absorption into root at the highest level of copper and lead alone where 7.68 and 7.34 mg/kg dry weight of these elements were absorbed by roots respectively. In addition, the plant ability in absorbing copper into aerial parts (40.40 mg/kg DW) and was greater than that of lead absorption (15.62 mg/kg DW) . Increasing soil lead and copper concentrations and absorption of the elements, showed a significant increase in the contents of each biomarkers Malondialdehyde (MDA), Dityrosine (D-T) and 8-hydroxy-2-deoxyguanosine (8-2-OH-DG) ($P<0.01$) . The maximum decrease in chlorophyll a, chlorophyll b and total chlorophyll (a+b) contents were observed at the highest level of soil lead (800 mg/kg) and copper (450 mg/kg) levels ($P<0.01$). The activities and functions of three enzymes Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPX) showed a significant increase ($P<0.01$) In general the maximum responses of these enzymes were also observed at the highest level of lead and copper in the soil.

Keywords: Catalase, Dityrosine, 8-hydroxy-2-deoxyguanosine, Glutathione-Peroxidase, Malondialdehyde, Superoxide dismutase.

1. Introduction

Heavy metals are important environmental pollutants present in soils, and toxic levels of some of them (cadmium, copper, lead, etc.) could appear in natural and agricultural areas as a result of anthropogenic activity. Heavy metals are implicated in the generation of oxidative stress in plant cells [1]. ROS (Reactive Oxygen Species) are toxic molecules, include compounds such as superoxide, peroxide, singlet oxygen, and the hydroxyl radical [2,3]. ROS are toxic molecules; unless their concentration is regulated, they can cause protein, membrane, and DNA damage and ultimately cell death [3]. Thus, plants exposed to Heavy metal stress frequently face oxidative stress [6]. Plants possess an antioxidative system to protect themselves against the damage produced by oxygen-derived radical [1]. One technique with potential for reclaiminig these toxic wastes is to make use of heavy metal tolerant plants [9]. Since the species Sainfoin, *Onobrychis vicifolia*, is a perennial plant with deep roots which endures drought and high temperature and is partly tolerant to soil improper conditions, it was supposed in the present study that the species due to having such features might possess ability in absorption of heavy metals lead and copper in contaminated soils and could tolerate these elements. Therefore the ability in absorption of heavy metals lead and copper and also the way to counteract their toxicity were studied. The aims of the present study

were thus to determine the ability of the species organs in absorbing lead and copper, and the changes in antioxidant activity and also the investigation of the changes in the plant cell level.

2. Materials and Methods

The present study was carried out in the research greenhouse of Agricultural and Natural Resources Faculty, Karaj in (2009) . The experimental treatments were arranged as factorial experiment in randomized complete design with four lead $Pb(NO_3)_2$ levels 0, 200, 400 , 800 and four copper $(Cu(SO_4)_2$ levels 0, 150, 300 and 450 mg/kg soil**.** Treatments consisted of 16 pots in each replicated in which lead and copper arranged as factorial with concentrations as described above in 4 replicates on the plant species Sainfoin (*Onobrychis vicifolia)*.

2.1. Heavy metals determination

The washed Plants were separated into roots and shoots, and dried in an oven at 60^{oC} for 48 h, then biomass(DW) was measured. For elemental analysis, the dried plant tissues were ashed in a muffle furnace at $550^{oC}$for 24 h. The ash was digested with a mixture of HNO_3 and $HCLO_4$[5:3 (v/v)], heated on an oven. After cooling, the extracts were diluted and mad up to 25 ml with 1 M HNO_3. Copper and Lead Concentration of the extract was determined by atomic absorption spectrophotometer [5].

2.2. Measurement of the soil available metals:

Dried soil samples were digested with HCl + HNO_3 + $HClO_4$ (3:1:1, w.v.) (Yuan 1988)[10]. Total Cu and other metals were determined by atomic absorption spectrophotometer (Analyst 100, Perkin Elmer, USA), using an acetylene- air flame. Diethylenetriaminepentaacetic acid (DTPA) extractable Cu, Cd, Co, Zn and Pb contents of 10 g soil samples (sample: DTPA, 1:2,w.v.) were determined by atomic absorption spectrophotometer (Page et al. 1982)[11]. The metals in soils were sequentially extracted following the method described by Tessier et al., (1979) [12]. Initially extracted with double-distilled water (2g of soil shaken for 4 h in distilled water of electric conductivity <0.02 µs cm^{-1}, followed by centrifugation during 10 min at 3000 rpm). This step represents the fraction that is water soluble and most easily available to plants and easily leacheable into the groundwater [13].

2.3. Measurement of chlorophyll a, b, a+b:

Chlorophyll a and b assay was done on the basis of the Lichtenthaler (1987) [14] method using the spectrophotometer set.

2.4. Preparation of enzyme extracts:

Leaves from each plant were washed with distilled water and homogenized in 0.16M Tris buffer (pH= 7.5) at 4°C. Then, 0.5 mL of total homogenized solution was used for protein determination by the Lowery *et al.,* (1951) method [15]. Based on the amount of protein per volume of homogenized solution, the following enzymes were assayed in the volume containing a known protein concentration in order to calculate the specific activities of the enzymes. The activity of following enzymes were expressed as specific activity(Umg.$protein^{-1}$).

2.5. Superoxide dismutase (SOD) activity:

The activity was measured based on Misra and Fridovich (1972) [16], in which the activity was measured on the basis of its ability to inhibit free radical chain oxidation in which O^{-}_2 was a chain propagating radical and the autooxidation of epinephrine (0.25 mM) was induced. A SOD standard was used for calibration of activity.

2.6. Catalaze (CAT) activity:

Catalaze activity was measured at 25°C as previously described by Paglia and Valentine (1987) [17], that used hydrogen peroxide as substrate and 1 k of catalaze activity was defined as the rate constant of the first order reaction.

2.7. Glutathion peroxidase (GPX) activity:

The activity was measured by the Paglia and valentine (1987) [17] method in which 0.56M (pH= 7) phosphate buffer, 0.5M EDTA, 1 mM $NaNo_3$, 0.2 mM NADPH were added to the extracted solution. GPX catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized glutathion is immediately converted to the reduced form with the concomitant oxidation of NADPH to NADP. The decrease in absorbance at 340 nm was measured with a spectrophotometer.

2.8. Measurement of Dityrosine:

1.2 grams of fresh tissue material were homogenized with 5 ml of ice-cold 50mM HEPES-KOH, pH 7.2, containing 10 mM EDTA, 2 mM PMSF, 0.1 mM p-chloromercuribenzoic acid, 0.1 mM DL-norleucine and 100 mg polyclar AT. The plan tissue homogenate was centrifuged at 5000 g for 60 min to remove debris. Purification of o,o′_dityrosine in the clear tissue homogenized supernatant fluid was accomplished by preparative HPLC. o,o′_dityrosine was recovered by gradient elution from the C-18 column (Econosil C18, 250mm × 10 mm) [18]. The composition of eluent varied linearly from acetonitrile–water–TFA (1:99:0.02) to acetonitrile–water– TFA (20:80:0.02) over 25 min. The gradient was started 5 min after the injection. A flow rate of 4 ml/min was used. o,o′_dityrosine was analyzed by reversed-phase HPLC with simultaneous UV-detection (280 nm) and fluorescence-detection (ex. 280 nm, em. 410 nm). A phenomenex inertsil ODS2 (150mm × 4.6 mm, 5μm) HPLC column (Bester, Amsterdam, the Netherlands) equipped with a guard column was used for these analyses. A gradient was formed from 10 mM ammonium acetate, adjusted to pH 4.5 with acetic acid, and methanol, starting with 1% methanol and increasing to 10% over 30 min. The flow rate was 0.8 ml/min. A standard dityrosine sample was prepared according to Amado et al., (1984) [19]. Dityrosine was quantified by assuming that it's generation from the reaction of tyrosine with horseradish peroxidase in the presence of H_2O_2 was quantitative (using the extinction coefficient $e_{315} = 4.5\ mM^{-1}\ cm^{-1}$ at pH 7.5) .

2.9. Determination of 8-hydroxy-2-deoxyguanosine (8-OH-2-DG) in urine:

8-hydroxy-2-deoxyguanosine levels in tissue extraction were measured essentially as described previously [20]. Briefly, an automated column switching LCEC method for 8-OH-2-DG is based on the unique selectivity of integral porous carbon column for purines. Samples were injected on to a C8 column and the band containing 8-OH-2-DG was then quantitatively trapped on a carbon column. The selectivity of the carbon column for 8-OH-2-DG allows elimination of interfering peaks by washing the column with a second mobile phase, and then eluting 8-OH-2-DG to an analytical C18 column with an identical mobile phase containing adenosine to displace 8-OH-2-DG. Detection with series colorimetric electrodes provides qualitative certainty for 8-OH-2-DG peak by response ratios.

2.10 Malondialdehyde analysis:

Proteins of tissue homogenate were precipitated with 40% trichloracetic acid (TCA), w/v. The MDA assay was based on the condensation of one molecule malondialdehyde with two molecules of thiobarbituric acid (TBA) in the presence of reduced reagent volumes to increase sensitivity, generating a chromogen with UV absorbance. The TBA + MDA complex was analyzed by HPLC essentially as described by Bird et al, (1983) [21]. Briefly, the HPLC system consisted of a Hewlett + Packard 1050 gradient pump (Avondale, PA) equipped with an automatic injector, a 1050 diode-array absorption detector and a personal computer using Chem Station Software from Hewlett + Packard. Aliquots of the TBA + MDA samples were injected on a 5 mm Supelcosil LC-18 reversed phase column (30 × 4.6 mm). The mobile phase consisted of 15% methanol in double-distilled water degassed by filtering through a 0.5 μm filter (Millipore, Bedford, MA).

The flow rate was 2 ml/min. MDA + TBA standards were prepared using tetraethoxypropane. The absorption spectra of standards and samples were identical with a characteristic peak at 540 nm. Measurements were expressed in terms of malondialdehyde (MDA) normalized to the sample protein

content. Protein content was determined by the method of Bradford, with standard curves prepared using BSA [22].

3. Results

3.1. Copper absorption by Sainfoin organs and the effect of lead on the absorption of the element by the plant:

The analysis of variance showed that the main (lead and copper) and reciprocal effects [lead(0, 200, 400, 800).copper(0, 150, 300, 450)] of the studied treatments were significant ($P<0.01$). Generally the comparison between roots and aerial parts in copper absorption shows that as soil copper content increased , the metal absorption by roots and aerial parts of sainfoin increased ($P<0.01$). At 450 mg kg^{-1} copper in soil copper absorption by the roots increased significantly ($P<0.01$), (7.68 mg/kg dry weight). In addition in comparison with control treatment, at the highest level of copper in soil copper concentration increased (40 mg/g DW) in aerial parts (stem + leaf). In treatments containing copper and lead there was competitive effect on each other. Copper concentration of 300 mg/kg soil and 400 mg/kg lead, there was higher absorption of copper in the roots of the plant. In the presence of lead in the soil and diminishing the copper absorption by roots, the absorption of copper by aerial parts was also limited. With the increase of lead levels in the soil, a reduction in the copper absorption by aerial parts occurred.

3.2. Effect of copper on lead absorption by roots and aerial parts

The comparison of lead absorption by roots and aerial parts show that with the increase in lead in the soil resulted in significant increase in lead absorption by roots and aerial parts at 1% level . Increasing lead levels in the soil led to increase in available lead in the soil . In addition the maximum level of lead in roots was absorbed at 800 mg kg^{-1} lead(7.34mg.g). The highest antagonistic effect of the two elements on lead absorption by roots of Sainfoin were observed at 450 mg copper with 400 mg lead. These results show that copper can diminish lead absorption and compete with lead only at the high levels. Lead absorption by aerial parts increased with increase in lead levels ($P<0.01$). No decreasing effect was observed on lead absorption by aerial parts at different concentrations of copper.

3.3. The effects of lead and copper on chlorophyll (a, b, a + b) contents

With increasing of lead and copper concentration, the amount of chlorophyll a, b and total chllorophyl contents decreased. in addition, the maximum decrease in chlorophyll a, b and total chllorophyl contents was occurred in plants grown on soils containing the maximum level of lead (800 mg kg^{-1}) with the maximum level of copper (450 mg kg^{-1}).

3.4. The effects of lead and copper on biomarkers, Malondialdehyde (MDA), Dityrosine (D-T) and 8-hydroxy-2-deoxyguanosine (8-OH-2-DG)

There was not significant difference between the plants grown on soils with the minimum levels of copper (150 mg kg^{-1}) in MDA content. The maximum changes in lipid peroxidation was in plants grown on soils with the maximum levels of copper (450 mg kg^{-1}) the reverse was correct for the plants grown on soils containing lead, with the use of different levels of lead in the soils, the MDA content was significantly different from the control ($P<0.01$). Maximum content of MDA observed in maximum levels of lead and copper (copper concentration of 450 mg/kg soil and lead with concentration of 800 mg/kg soil). In the case of Dityrosine content it was observed that the changes in Dityrosine content were not significant in plants grown on soils containing lower levels of copper (without the use of lead), but as a result of increase in the soil copper, these changes were significant. An increasing trend was observed in Dityrosine content which occurred in plants grown on soils containing lead alone, although the maximum changes in Dityrosine content of Sainfoin were observed in the maximum concentration of lead (800 mg kg^{-1}) and copper (450 mg kg^{-1}); but it was found that even lower levels of lead can be effective on increase in Dityrosine content. In the case of the 8-hydroxy-2-deoxyguanosine content which is relative to cell nucleus changes, the

maximum changes were observed in plants grown on soils containing both lead and copper elements in the maximum concentrations. The increase in 8-hydroxy-2-deoxyguanosine content was more observable. With comparison between two treatments of lead and copper alone, it was demonstrated that the maximum levels of copper in the soil (450 mg kg^{-1}) increased the content of this feature. These changes were more observable at lead levels of 400 and 800 mg kg^{-1} soil. In general, lead was more effective on all three biomarkers than copper.

3.5. The response of enzymes Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPX) under toxic condition resultant from lead and copper

The activity and function of three enzymes Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPX) in the leaves of studied species in the response to the lead and copper toxicity increased significantly($P<0.01$). The maximum responses of these enzymes were demonstrated at the maximum concentrations of lead (800 mg kg^{-1}) and copper (450 mg kg-1), as the enzymes SOD, CAT and GPX in the leaves of Sainfoin increased at the maximum levels of the two elements by 75.18, 63.87 and 60.82 percent respectively. On the other hand, it can be said that the activity of the enzyme SOD in the leaves of Sainfoin increased three times more than that of the control. The maximum level of used lead in the soil (800 mg kg^{-1}) increased the activity of CAT in the leaves of Sainfoin 1.4 times more than that of the control while under copper toxicity the increase in the activity of this enzyme was 1.2 times more than that of the control at the maximum level of copper (450 mg kg^{-1}), The activity of the enzyme GPX also increased significantly under toxicity resultant from lead and copper, the maximum activity of the enzyme was observed at the maximum levels of lead and copper in the soil which was 1.5 times more than that of normal conditions. In addition, the effect of lead on the activity of the enzyme GPX was more than that of copper.

4. References

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