American-Eurasian J. Agric. & Environ. Sci., 10 (3): 440-449, 2011
ISSN 1818-6769

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

Mahtab Beladi, Davood Habibi, Ali Kashani,
Farzad Paknejad and Tohid Nooralvandi

Agricultural and Natural Resources, Islamic Azad University, Karaj Branch, Iran

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 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.

Key words: Catalase · Dityrosine · 8-Hydroxy-2-deoxyguanosine · Glutathione- peroxidase · Malondialdehyde · Superoxide dismutase

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 an inevitable by-product of aerobic metabolism, being produced during the electron transfer reactions that take place in the mitochondria, chloroplasts and peroxisomes. ROS are toxic molecules; unless their concentration is regulated, they can cause protein, membrane and DNA damage and ultimately cell death [3]. Malondialdehyde (MDA) is the most abundant individual aldehydic lipid breakdown product. It has been stimated that more than 75% of the measured MDA is derived from triunsaturated (trienoic) fatty acids such as α-linoleic acid [3]. The study of Ali and *et al.* 2003, [4] on the effects of lead, nickel and copper on species *salix acmophylla Boiss*, showed that all the three metals reduced total chlorophyll content in concentration dependent manner. The adverse effect tended to increase from Cu<Ni<Pb. Besides reduction in total chlorophyll content was concomitant with increase in MDA level. Dityrosine, as a stable biomarker of ROS mediated protein oxidation is closely correlated with level of oxidative stress [5]. Thus, plants exposed to HM (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]. The importance of antioxidant enzymes is their ability to scavenge

Corresponding Author: Mahtab Beladi, MS Argriculture Research Center, Islamic Azad University, Karaj, Iran.
Tel: +98-09122078806, E-mail: s_mahtab_beladi@yahoo.com.

ROS(Reactive Oxygen Species) and thereby prevent oxidative damage [2]. The antioxidant enzymes are considered to be an important defense system of plants against oxidative stress caused by metals [4]. This system is composed of antioxidant enzymes: Ascorbate peroxidase (APOX), Glutathione reductase (GR), Superoxide dismutase (SOD), Catalase (CAT) and non-enzymatic compounds (Ascorbic acid, Glutathione, Carotenoids, α-tocopherol). There is evidence that in pea plants exposed to Cd^{2+}, the antioxidant system might play a role in detoxification mechanism [1]. Thus, since the plants prossess a number of antioxidant molecules and enzymes that protect them from oxidative damage,the combined action of SOD, POX and CAT is required for protection against the toxic effects of active oxygen species [7]. Researchers have observed that some plants have the ability to grow in sites where soils contain greater than usual amounts of heavy metals or other toxic compounds [8]. 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.

MATERIALS AND METHODS

The present study was carried out in the research greenhouse of Agricultural and Natural Resources Faculty, Karaj in (2009). The relative humidity of the greenhouse was 60% and the minimum and maximum temperatures were 16 and 32°C respectively. 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)*. The experiment was also carried out to study the absorption ability of the plants by measuring lead and copper in different of the plants. In addition the physiological tolerance of species in soils contaminated with lead and copper evaluated by measuring and in addition the physiological tolerance of species in soils contaminated with lead and copper evaluated by measuring of chlorophyll a, b, a+b, enzymes (SOD, CAT, GPX) and biomarkers (MDA, Dityrosine and 8-OH-2-DG)The field soil was sampled from 0-30 cm and then examined to determine soil physical and chemical properties particularly heavy metals lead and copper. The studied soil was loam sandy and EC (electrical conduction) equaled to 5.91 ds m^{-1}; pH was 7.7 and total nitrogen percentage equaled to 0.54% and organic matter content was 0.060%; the phosphorus content was also high as it was 35.4 ppm. The level of lead and copper were 5.5 and 0.82 mg/kg respectively. This content of lead and copper are not toxic for plants. So, the soil was contaminated with concentrations more than the permitted levels. The soil colloids were fragmented and passed through 4 mm sieve. The soil colloids were fragmented and passed through 4 mm sieve. In order to more chelating of the elements with soil colloids and preparing contamination with elements, the treated pots were remained in such a situation for 30 days and after that the cultivation was done. To analyse the effects of the experimental treatments on the plants, sampling was done in early flowering stage and was transported to laboratory.

Greenhouse Experiments: The selected pots were all of the same sizes and 20 cm in upper diameter and 18 cm in lower diameter; the height of the pots were also 20 cm. There were several drainages in the bottom of the pots and several trays were used beneath each pots to prevent the loss of studied elements through leaching. Then the pots were filled with given mass of soil up to a certain height; subsequently, the soil and pots were weighed using a digital scale with a high accuracy (0.01). Each pot was filled with 6000 g soil. After solution spreading the soil to help chelating the elements with soil colloids and preparing contamination with the elements the treated pots remained in such a condition for 30 days and afterwards the cultivation was done.

Heavy Metals Determination: The washed Plants were separated into roots and shoots and dried in an oven at 60°C 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].

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].

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.

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}).

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.

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.

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.

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).

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.

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].

Data Analysis Methods: Statistical analysis of data was done by statistical software of SAS (Release version 9.1). Comparison of means was done through Duncan method at 1%.

RESULTS

According to soil analysis, lead and copper percentages in the soil were below the toxicity levels. Soil pH is 7.7 which shows that the soil is of low affinity to alkaline condition. Lime level in the soil is moderate as consists only 13.5% of the soil. High levels of lime in soils with high amounts of zinc, boron and lead can partly prevent the tension resultant from the elements toxicity. Phosphorus percentage in the soil is high.

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 ($P<0.01$). Table 1 shows the contents of lead and copper in roots and aerial parts (stem + leaf) of Sainfoin. 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.

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 (Table 2). 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

Table 1: Absorption of lead and copper by root and aerial parts (stem + leaf) of Sainfoin, *Onobrychis vicifolia*, (mg/kg) and the interactions between the two elements*.

	Cu pppppppp----															
	Cu 0(mg/kgsoil)				Cu 150(mg/kgsoil)				Cu 300(mg/kgsoil)				Cu 450(mg/kgsoil)			
	Shoot		Root		Shoot		Root		Shoot		Root		Shoot		Root	
Sainfoin	Cu	Pb	Cu	Pb	Cu	Pb	Cu	Pb	Cu	Pb	Cu	Pb	Cu	Pb	Cu	Pb
Pb																
0 (mg kg^{-1} soil)	1.57	0.45	0.65	0.21	11.24	0.58	2.57	0.25	16.38	0.54	4.30	0.23	40.40a	0.51	7.68a	0.16
	±0.25	±0.07	±0.13	±0.043	±2.23	±0.06	±0.21	±0.05	±1.409	±0.15	±0.66	±0.05	±1.56	±0.10	±0.17	±0.02
200(mg kg^{-1} soil)	1.63	3.56	0.49	1.67	7.95	6.13	2.19	2.90	15.70	9.43	3.85b	4.45	28.51b	10.02	6.00	7.30
	±0.09	±0.21	±0.10	±0.06	±0.55	±0.52	±0.40	±0.18	±1.35	±0.31	±0.35	±0.27	±1.82	±0.80	±0.16	±0.35
400(mg kg^{-1} soil)	1.91	11.40	0.85	3.94	9.59	12.72	2.22	4.45	22.53	15.05c	7.02c	5.52	38.62	14.27	6.88	6.31d
	±0.46	±1.49	±0.05	±0.26	±0.47	±0.57	±0.56	±0.34	±2.38	±0.42	±0.81	±0.32	±2.55	±0.37	±0.21	±0.10
800(mg kg^{-1} soil)	1.66	13.87	0.53	7.46	8.78	15.33	2.76	6.58	16.30	15.69e	3.47b	6.07	40.02	15.62	6.40	7.34e
	±0.12	±1.05	±0.03	±0.29	±0.420	±0.16	±0.25	±0.29	±0.82	±0.83	±0.26	±0.47	±1.44	±0.54	±0.39	±0.35

*Data were presented as mean± SD. Data measured at 1% probability level. a: Maximum copper uptake in root and shoot. b: Decrease the effects of lead on copper absorption in the root. c: Reduction effects of lead on copper absorption in the root and Shoot. d:shows the effects of copper on the reduction of lead. e: Maximum absorption of lead &rom the root and shoot.

Table 2: Available lead and copper of soil (mg/kg Soil)*.

	Cu ----							
	0(mg/kgsoil)		150(mg/kgsoil)		300(mg/kgsoil)		450(mg/kgsoil)	
	soil		soil		soil		soil	
Sainfoin	Cu	Pb	Cu	Pb	Cu	Pb	Cu	Pb
Pb								
0 (mg kg^{-1} soil)	15.67 ±2.61	3.36±0.41	45.80±6.25	3.71±0.93	119.55±3.88	3.80±0.74	194.05±19.56	3.88±1.13
200(mg kg^{-1} soil)	12.27±2.14	36.73±23.89	63.52±3.92	48.95±12.71	123.05±8.66	54.75±2.08	176.65±11.66	46.87±8.45
400(mg kg^{-1} soil)	15.37±2.11	101.67±18.35	62.80±6.35	81.10±7.13	134.95±11.70	96.47±6.86	175.52±10.05	107.30 ±10.59
800(mg kg^{-1} soil)	17.82±3.18	219.32±6.94	66.70±5.29	196.77±12.21	125.67±6.36	215.47±12.91	167.90±12.28	209.02 ±24.62

*Data were presented as mean± SD. Data measured at 1% probability level.

Table 3: Antioxidant enzyme activity in Sainfoin leaves plant (U mg^{-1} Protein) and effect of lead and copper on three measured biomarkers MDA, D-T and 8-OH-2-DG in nm/ mg Protein*.

	Cu ----											
	Cu 0 (mg kg^{-1} soil)			Cu 150 (mg kg^{-1} soil)			Cu 300 (mg kg^{-1} soil)			Cu 450 (mg kg^{-1} soil)		
Sainfoin Pb	SOD U.mg protein^{-1}	CAT U.mg protein^{-1}	GPX U.mg protein^{-1}	SOD U.mg protein^{-1}	CAT U.mg protein^{-1}	GPX U.mg protein^{-1}	SOD U.mg protein^{-1}	CAT U.mg protein^{-1}	GPX U.mg protein^{-1}	SOD U.mg protein^{-1}	CAT U.mg protein^{-1}	GPX U.mg protein^{-1}
0 (mg kg^{-1} soil)	968.50 ±18.92	109.25 ±4.85	365.50 ±7.54	999.25 ±54.96	106.75 ±2.98	370 ±14.78	1220.75 ±42.43	121.50 ±6.60	383.75 ±7.13	1442.50 ±44.8	129.75 ±3.86	402.25 ±9.03
200(mg kg^{-1} soil)	1185.75 ±18.51	113.50 ±1.91	379.50 ±6.95	1525 ±42.19	134.37 ±2.97	420.75 ±4.57	1640.75 ±27.1216	144.40 ±4.10	445.25 ±12.68	1464.25 ±594.88	150.32 ±2.24	483.50 ±5.80
400(mg kg^{-1} soil)	1498.50 ±35.06	136.50 ±3.85	420.25 ±4.11	1648 ±37.09	141.65 ±2.75	446.75 ±8.22	2136.25 ±40.28	153.57 ±2.14	491.75 ±12.91	2325.25 ±36.65	161.72 ±3.89	504.50 ±9.71
800(mg kg^{-1} soil)	2058.50 ±58.12	150.57 ±2.10	475.75 ±6.23	2405.75 ±68.05	170.10 ±2.35	518.75 ±3.50	2667.25 ±40.67	183.20 ±2.68	555.75 ±6.07	2934.25a ±38.83	193.150a ±2.16	567.50a ±14.52

	Cu ----											
	Cu 0 (mg kg^{-1} soil)			Cu 150 (mg kg^{-1} soil)			Cu 300 (mg kg^{-1} soil)			Cu 450 (mg kg^{-1} soil)		
Sainfoin Pb	MDA nm mg Protein^{-1}	Dityrosin nm.mg Protein^{-1}	8_OH_DG nm.mg Protein^{-1}	MDA nm mg Protein^{-1}	Dityrosin nm.mg Protein^{-1}	8_OH_DG nm.mg Protein^{-1}	MDA nm mg Protein^{-1}	Dityrosin nm.mg Protein^{-1}	8_OH_DG nm.mg Protein^{-1}	MDA nm mg Protein^{-1}	Dityrosin nm. mg Protein^{-1}	8_OH_DG nm.mg Protein^{-1}
0 (mg kg^{-1} soil)	27.40 ±2.28	35.02 ±2.69	8.65 ±0.54	30.85 ±1.31	32.70 ±1.44	8.48 ±0.46	29.05 ±2.62	32.67 ±2.56	9.35 ±0.19	36.75 ±1.95	41.17 ±2.46	10.62 ±0.17
200(mg kg^{-1} soil)	32.100 ±1.20	33.22 ±2.30	9.24 ±0.13	38.55 ±0.700	41.70 ±1.12	10.77 ±0.52	41.65 ±2.28	45.25 ±0.81	11.88 ±0.35	45.40 ±0.94	47.62 ±2.14	12.66 ±0.16
400(mg kg^{-1} soil)	38.200 ±0.95	39.90 ±1.65	10.83 ±0.41	40.85 ±0.76	43.475 ±0.51	10.65 ±0.10	46.70 ±1.61	52.22 ±0.63	13.99 ±0.25	49.97 ±2.47	58.25 ±0.99	14.70 ±0.36
800(mg kg^{-1} soil)	46.75 ±1.40	48.65 ±0.72	12.45 ±0.25	54.87 ±0.93	61.62 ±1.70	15.65 ±0.23	60.15 ±1.53	72.22 ±1.17	19.12 ±0.63	63.87a ±1.49	77.85a ±1.27	20.27a ±0.994

* Data were presented as mean± SD. Data measured at 1% probability level. a: shows biomarkers highest capacity and also highest enzyme activity.

Table 4: Effect of lead and copper on chlorophyll a, chlorophyll b and total chlorophyll (a + b) , [mg/(g FW)]*.

		Cu											
		Cu 0 (mg kg^{-1} soil)			Cu 150 (mg kg^{-1} soil)			Cu 300 (mg kg^{-1} soil)			Cu 450 (mg kg^{-1} soil)		
Sainfoin	Pb	Chl.T	Chl.a	Chl.b	Chl.T	Chl.a	Chl.b	Chl.T	Chl.a	Ch.b	Chl.T	Chl.a	Chl.b
	0 (mg kg^{-1} soil)	3.7300 ±0.08	1.8375 ±0.06	1.8925 ±0.10	3.6725 ±0.07	1.8025 ±0.04	1.8700 ±0.05	3.5950 ±0.10	1.7225 ±0.14	1.8725 ±0.13	3.2850 ±0.03	1.5850 ±0.07	1.7000 ±0.09
	200(mg kg^{-1} soil)	3.5875 ±0.07	1.7900 ±0.06	1.7975 ±0.02	3.3200 ±0.02	1.6325 ±0.05	1.6875 ±0.04	3.1475 ±0.09	1.5925 ±0.07	1.5550 ±0.10	2.8575 ±0.04	1.4750 ±0.05	1.3825 ±0.08
	400(mg kg^{-1} soil)	3.5400 ±0.04	1.6475 ±0.22	1.8925 ±0.23	3.1700 ±0.04	1.5400 ±0.18	1.6300 ±0.19	2.7000 ±0.08	1.3575 ±0.12	1.3425 ±0.12	2.4175 ±0.06	1.2425 ±0.06	1.1750 ±0.04
	800(mg kg^{-1} soil)	2.7225 ±0.08	1.3850 ±0.08	1.3375 ±0.06	2.6125 ±0.08	1.3500 ±0.06	1.2625 ±0.02	2.4675 ±0.03	1.2375 ±0.02	1.2300 ±0.04	2.2650a ±0.04	1.1400a ±0.03	1.1250a ±0.02

*Data were presented as mean± SD. Data measured at 1% probability level. a: Shows the most reduction capacity of chlorophyll a, b and a+b.

Table 5: Correlation of traits

Traits	SOD	CAT	GPX	MDA	Dityrosine	8-OH-2-DG	Shoot(Pb)	Shoot(Cu)	Chl T
SOD	1								
CAT	0/95185**	1							
GPX	0/95694**	0/97839**	1						
MDA	-0/8770**	-0/8350**	-0/8217**	1					
Dityrosine	-0/8269**	-0/8055**	-0/8052**	0/66459**	1				
8-OH-2-DG	-0/9299**	-0/8964**	-0/8897**	0/89175**	0/93078**	1			
Shoot(Pb)	0/41091*	0/45569**	0/51468**	-0/4303*	-0/4583**	-0/488**	1		
Shoot(Cu)	0/47866**	0/47150**	0/54856**	-0/3619*	-0/4448*	-0/4465*	55610**	1	
Chl T	0/86745**	0/76046**	0/75752**	-0/7301**	-0/7785**	-0/828**	0/3297ns	0/37431**	1

**,*: Significant correlation at 1% and 5% Probability ns: not significant

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.

The Effects of Lead and Copper on Chlorophyll (a, b, a + b) Contents: Table (4) showed that whit 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}).

The Effects of Lead and Copper on Biomarkers, Malondialdehyde (MDA), Dityrosine (D-T) and 8-Hydroxy-2-Deoxyguanosine (8-OH-2-DG): The content of each biomarker, Malondialdehyde (MDA), Dityrosine and 8-hydroxy-2-deoxyguanosine which increase during lipid peroxidation, protein and cell nucleus process respectively, increased in the studied plant due to toxicity resultant from lead and copper (P<0.01). There was not any 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 (Table 3).

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 (Table 3).

DISCUSSION

In the present study the soil was contaminated with two heavy metals lead and copper. Soil analysis prior to contamination with the two elements indicated that there was no signs of toxicity. The high level of phosphorous in the studied soils could increase the cation exchange capacity of soil, as it is suggested that the addition of phosphorous decreases positive charge and thereby increases negative charge or the cation exchange capacity [23]. Although *Onobrychis vicifolia* was more capable of absorbing copper into aerial parts than lead, there was no significant difference in absorbing lead and copper by the roots. Lead transfer into (plants) different organs, of varies depending on species as it has been suggested that the main proportion of absorbed lead by the plant remains in roots, but in previous studies on lead absorption by Miller and Koeppe (1971) [24]. it has been demonstrated that the plants of *Zea mays* accumulate considerable amounts of lead in their leaves. It was found that lead in dicotyledon plants moves through vascular textures is accumulated in farther distinct areas . The main factor of increase, mobility and absorption of two elements lead and copper may be the formation of solute complexes with organic matters. As it was observed in the present study, the fixed and insoluble lead and copper became soluble and accumulated in the root and aerial parts of the studied plant. Various researches have demonstrated that the metal concentration in different parts of the plant is a function of the heavy metal content in the growth environment [8], as with the increase in the lead and copper concentrations in the soil the contents of lead and copper increased in the plant. Each of the elements copper and lead in the present study competed for absorption and extraction. The competition between chemical species for binding in peptide sites depends on factors such as metal chemistry, pH of solution, the nature and property of binding site, the quantity of binding site, the variety of chemical species, metal ions concentration and the selectivity of biomass for forming binds with special species [25]. On the other hand, in the present study soil had little affinity toward alkaline condition, thereby different types of toxic metal ions may have interactions and lead to a range of increasing, multiplying of supplementary and competitive reactions [26]. On the other hand, the antagonistic effects of the two elements are indicative of the probability that the same mechanisms prepare resistance and the possibility of protects its the plant against the tensions resultant from ions toxicity. The increase in activity and function of the enzymes uder tensions resultant from two contaminants lead and copper suggests a defensive system against toxic and detrimental effects of the two metals on the other cell processes of sainfoin, as the activity and function mechanisms of the enzymes prevent more damages of generated free radicals of oxygen as a result of such elements to target organs and thus protect the plant. The direct relationship between lead absorption by the aerial parts(shoot Pb) and the activity and function of three enzymes SOD, CAT and GPX in the same organs suggested the activity of the enzyme in order to prevent more generation of free radicals under lead toxicity. The increase in activity of the enzymes under lead toxicity is a result of changes in

enzyme synthesis, immobility of enzyme inhibitors, or effective molecules which are synthesized under lead toxicity. Thus, since lead increases the formation of oxygen active species in the plant and leads to oxidative tensions in them, there was an increase in particular antioxidant enzymes in such plants, for example, the rice plants which were in a sandy medium containing 0.5 and 1 mmol $Pb(NO_3)_2$ for 20 days, the activity of antioxidant enzymes such as superoxide dismutase, guaiacol peroxidase, ascorbate peroxidase and glutathione reductase increased in the leaves [24]. In addition, *Datura stramonium* and *Chenopodium ambrosioides* elevated their antioxidative enzyme activities in response to cu-toxicity. Besides, there was significant elevation in oxidative damage biomarkers; MDA and dityrosine, when the arial parts of *Datura stramonium* in zone1 (Containing copper) were compared with the same parts of zone 2 (Whitout copper) [5]. In the case of copper toxicity it was found that Cu^{+2} reduces to Cu^{+} in the presence of superoxide (O^{2-}) or reducer factors such as ascorbic acid or GSH, then Cu is able to catalyze the formation processes of hydroxyl radicals (OH^{o}) from hydrogen peroxidase (H_2O_2) on the basis of Haber-Viess reaction. Hydroxyl radical is the most strong oxidative radical in biological systems which is able to react with any biomolecule. By separating hydrogen from a amino containing a carbon, hydroxyl radical can form a kind of protein radical with a carbon in the center, or separate hydrogen from a saturated fatty acid and form a lipid radical which leads to oxidative damages [27]. As demonstrated in the present study these elements prepare cell nucleus, protein and lipid oxidation stress by their function mechanisms, as it was suggested the metal ions may directly interfere with metabolic activities and thereby change the structures of proteins such as enzymes, carrier proteins or regulating proteins due to their strong affinity as ligands to sulfidril and carboxyl groups. These activities are considered as the main reason of toxic effects exerted by the metal [6]. It is concluded, that the increase in the activity of the three studied enzymes were in response to destructive effects of the two metals. Result showed significant negative correlation between antioxidant enzymes activity and biochemical biomarkers. Negative relationship between the capacity of 8-hydroxy-2-deoxyguanosine with three studied enzymes suggested positive effect of antioxidant enzymes in supporting cell from cell nucleus damages. A significant negative correlation at 0.01 and 0.05 probability level was found between lead and copper uptake in shoot and the amount of biochemical biomarkers. This negative correlation is probably due to higher activity of antioxidant enzymes that lower biochemical biomarkers. In addition, the negative correlation between MDA and Dityrosine contents which are tools of detecting lipid and protein peroxidation respectively and the activity of two enzymes SOD and CAT shows that the increase in the two enzymes leads to decrease in lipid and protein oxidation, the correlations also demonstrated that the increase in oxidative stress due to lead and copper on aldehid lipid and protein has led to the increase in the enzyme GPX. The induction of antioxidant enzymes is a part of plant defensive mechanism in response to the increase in the concentrations of oxygen species; by parallel increase in the activity of enzymes interfering with toxification, plants try to counteract the increase in the concentrations of oxygen species which are generated under high toxicity of heavy metals [7]. In fact, plants with high antioxidant activity are more tolerant to oxidative stress [2], Since antioxidants are compounds capable of donate a single electron or hydrogen atom to reduce the opposite compounds [28]. The extent of such tolerance and degree of adaptation is highly variable in which the efficiency and capacity of detoxification mechanisms play an important role. Additionally, a network of sequestration activities and immobilization functions regulate the uptake, distribution and detoxification of excess metal ions in plants [5]. It was also detected in the present study tat the absorption and accumulation of lead and copper in the studied species led to the decrease in chlorophyll content and as was observed lead affected chlorophyll content more than copper, as lead prevented chlorophyll synthesize by preventing the absorption of essential elements Mg and Fe; the photosynthesize apparatus is also destructed due to the limitation of protein N- and S-ligands and the increase in chlorophyllase activity under lead abundance also leads to the increase in destruction of chlorophyll; under this condition chlorophyll a is more effected than chlorophyll b [24]. Therefore, it is probable that the increase in the generation of oxygen active species due to the presence of lead and copper is one of the reasons for the observed decrease in chlorophyll. The decrease in the proportions of chlorophyll a, chlorophyll b and as a result in total chlorophyll (a + b) in comparison with the control treatment, can result from more sensitivity and destruction of chlorophyll under the generated free oxygen due to the presence of lead and copper. It seems that the tension resultant from lead and copper in the studied plant has partly haltered the chlorophyll biosynthesize and biodecomposition. The study of the effects of nickel

chloride and cadmium sulfate on chlorophyll status in aquatic plants such as *Ceratophyllum demersum, Lemna trisulca* and *Myriophyllum spicatum* suggested that the chlorophyll content decreased due to Mg replacement by the heavy metals in the center of porphyrine hoop [29]. In addition, the decrease in total chlorophyll content in this species was accompanied with the increase in malondialdehyde content, this observation is in conformity with the researches by Ali *et al.* (2003) [4]. In the presence of such heavy metals, the toxic effects of lipid peroxidation on chlorophyll content and the decrease in chlorophyll synthesize in different plant species were observed due to toxic metal interactions with group -SH of chlorophyll synthesizing essential enzymes. It is inferred that the decrease in total chlorophyll content is probably due to the interactions of these metals with group -SH of chlorophyll synthesizing enzymes and also to the destruction resultant from lipid peroxidation [4]. We also concluded that the sainfoin (*Onobrychis vicifolia*) possesses more ability in absorbing copper than lead. In addition, the species can activate the antioxidant enzymes SOD, CAT and GPX to counteract with lead and copper, it seems that sainfoin has a great ability in decreasing the toxic effects of these elements by activating the antioxidant system; however the plant may use other mechanisms to decrease the toxic effects which needs more comprehensive investigations.

REFERENCES

1. Groppa, M.D., M.L. Tomaro and M.P. Benarides, 2007. Polyamines and heavy metal stress: the antioxidant behavior of spermine in Cadmium and Copper treated wheat leaves. Biometals, 20: 185-195.
2. Khatun, S., M.B. Ali, E.J. Hahn and K.Y. Paek, 2008. Cooper toxicity in withania somnifera: Growth and antioxidant enzymes responses of in vitro grown plants. Environmental and Experimental Botany, 64: 279-285.
3. Davey, M.W., E. Stals, B. Panis, J. Keulemans, R.L. Swennen, 2005. High-Throughput determination of mabndialdehyde in plant tissues. Analytical Biochemistry, 347: 201-207.
4. Ali, B.M., P. Vajpayee, R.D. Tripathi, U.N. Rai, S.N. Singht and S.P. Singhgh, 2003. Phytoremediation of lead, nickel and copper by *Salix acmophylla boiss*. : Role of Antioxidant Enzymes and Antixidant substances.Bull.Environ.Contam.Toxicol., 70: 462-469.
5. Mashhadi Akbar Boojar. M. and F. Goodarzi, 2007. The Copper tolerance strategies and the role of antioxidative enzymes in three plant species grown on copper mine. Chemosphere, 67: 2138-2147.
6. Sharma, S.S., S. Kaul, A. Metwally, K.C. Goyal, I. Finkemeier and K.J. Dietz, 2004. Cadmium toxicity to barley *(Hordeum vulgar)* as affected by varying Fe nutritional status. Plant Sci., 166: 1287- 1295.
7. Garnczarska, M. and L. Ratajczak, 2000. Metabolic responses of *Lemna minor* to lead ions, II. Induction of antioxidant enzymes in roots. Acta Physiologiae Plantarum, 22: 429-432.
8. Peralta, J.R. , J.L. Gardea-Torresdey, K.J. Tiemann, E. Gomez, S. Arteaga, E. Rascon, J.G. Parsons, 2001. Uptake and effects of five heavy metals on seed Germination and plant Growth in Alfalfa *(Medicago sativa L.)*. Environmental, contamination and Toxicol., 66: 727-734.
9. Sudhakar, C., L. Syamalabai and K. Veeranjaneyulu, 1992. Lead tolerance of certain legume species grown on lead ore tailings. Agriculture, Ecosystems and Environ., 41: 253-261.
10. Yuan, D.W., 1988. Compared study on the pre-treatment methods for measuring soil total copper, zinc, lead, cadmium, nickel and manganese. Agro-Environmental Protection Sci., 7: 34-36.
11. Page, A.L. , R.H. Miler and D.R. Keeney, 1982. Methods of soil analysis, 2. Chemical and microbiological properties. American Society of Agronomy and Soil Science Society of America, Madison, Wisconsin.
12. Tessier, A. P.G.C. Campbell and M. Biosson, 1979. Sequential extractiction procedure for speciation of particular trace metals. Anal. Chem., 51: 844-851.
13. Siebe, C., 1995. Heavy metal availability to plants and soils irrigated with wastewater from Mexico city. Wate sci. Technil., 32: 29-34.
14. Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol., 149: 351-382.
15. Lowry, O., A. Rosebrough and R. Randall, 1951. Protein measurement with folin phenol reagent. J. Biological. Chemistry. 193: 680-685.
16. Misra, H.P. and I. Fridovich, 1972. The generation of superoxide radical during auto oxidation. J. Biol. Chem., 247: 6960-6966.
17. Paglia D.E. and W.N. Valentine, 1987. Studies on the quantitative and qualitative characterization of glutation proxidase. J. Lab. Med., 70: 158-165.

18. Orhanl, H., N.P.E. Vermeulen, C. Tump, H. Zappey and J.H.N. Meerman, 2004. Simultaneous determination of tyrosine, phenylalanine and deoxy guanosine oxcidation products by liguid chromatography - tandem mass spectrometry as non-invasive biomarkers for oxidative damage. J. Chromatography. B., 799: 245-254.
19. Amado, R., R. Aeschbach and H. Neukom, 1984. Dityrosine: in vitro production and characterization. Methods Enzymol., 107: 377-388.
20. Bogdanov, M.B., M.F. Beal, D.R. Meccabe, R.M. Griffin and W.R. Matson, 1999. A carbon column based LCEC approach to routine 8-hydroxy-2-deoxyguanosine measurements in urine and other biological matrices. Free. Rad. Biol. Med., 27: 647-666.
21. Bird, B.R., S.S.O. Hung, M. Hadley and H.H. Draper, 1983. Determination of malonaldehyde in biological materials by high-pressure liquid chromatography. Anal. Biochem., 128: 240-244.
22. Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protin utilizing the principle of protein dye binding. Anal. Biochem., 72: 248-254.
23. Lin, C.J., Liu. L. Liu, T. Zhu, L. Sheng and D. Wang, 2009. Soil amendment application frequency contributes to phytoextraction of Lead by Sunflower at different nutrient levels. Environmental and Experimental Botany, 65: 410-416.
24. Sharma P. and R.S. Dubey, 2005. Lead toxicity in plants. Plant Physiol., 17: 35-52.
25. Gardrea-Torresdey, J.L., G.D. Rosa and J.R. Peralta-Videa, 2004. Use of phytofiltration technologies in the removal of heavy metals: Areviewe. Pure Apple. Chem., 76: 801-813.
26. Taylor, G.J., 1995. Overcoming barriers to understanding the cellular basis of aluminium resistance. Plant and Soil, 171: 89-103.
27. Gaetke, L.M and C.K. Chow, 2003. Copper toxicity oxidative stress and antioxidant nutrients. Toxicol., 189: 197-163.
28. Wong, S.P., L.P. Leong and J.H. Willam Koh, 2006. Antioxidant activities of aqueous extracts of selected plants. Food Chemistry, 99: 775-783.
29. Kupper, H., F. Kiipper and M. Spiller, 1996. Environmental relevance of heavy metal-substituted chlorophylls using the example of water plants. J. Experimental Botany, 47: 259-266.