American-Eurasian J. Agric. & Environ. Sci., 11 (1): 122-128, 2011
ISSN 1818-6769

Copper and Lead Tolerance Strategies in Mustard (*Sinapis arvensis*), Egyptian Clover (*Trifolium alexandrinum*) and Hairy Vetch (*Vicia villosa*): Role of Some Antioxidant Enzymes

Mahya Golshan, Davood Habibi, Seyedeh Mahtab Beladi and Mohammad Javad Maleki

Department of Agronomy, Karaj Branch, Islamic Azad university, Karaj, Iran

Abstract: Nowadays, industrializing of societies, pollution of air and earth to be included as one of main problems of human being. Pollution is risk of farming soils to heavy metals causes problems such as producing of free radicals and oxidative stress in plants.The purpose of this study was investigating the enzymes activity of catalase (CAT) and glutathione peroxidase (GPX) and dityrosine in Mustard(*Sinapis arvensis)*, Egyptian clover(*Trifolium alexandrinum*) and Hairy vetch(*Vicia villosa)* in Research Greenhouse of Islamic Azad University-Karaj Branch in 2009.Above seeds were sown in pot polluted soils with $pb(No_3)_2$ salt in concentrations of 0,200.400,800 mg/kg soil and $Cu(So_4)_2$ salt in concentrations of 0,150,300,450 mg/kg soil in four replicates by using factorial experiment in completely randomized design. Mustard plant due to high absorption of heavy metals tested showed higher catalase activity. Hairy vetch plant produced high glutathion peroxidase to deal with the stress of heavy metals. Egyptian clover plant showed lower ability for producing antioxidant enzymes. Egyptian clover showed protein destruction and as a result, higher dityrosine content compared with the other plants.

Key words: Phytoremediation · Oxidative stress · Antioxidant enzyme · Dityrosine

INTRODUCTION

Environmental pollution takes place from various sources. Human civilization progression and technology development and growing population increasing, have faced communities with air pollution problems that threaten the life of residents of earth and so, environmental protection is considerable by statement in every country. Today, the environmental situation is such as the people of a city or a country are not safe of pollution of another city or country.

Excessive metal concentration in soils pose significant hazard to human, animal and plant health and to the environment in general. Contamination of soils with toxic metals has often resulted from human activities, especially those related to mining, industrial emissions, disposal or leakage of industrial wastes, application of sewage sludge to agricultural soils, manure, fertilizer and pesticide use. Due to the potential toxicity and high persistence of metals, soils polluted with these elements are an environmental problem that requires an effective and affordable and affordable solution [1, 2].

Although traditional technologies for cleaning contaminated soils and waters have proven to be efficient, they are usually expensive, labor intensive and in the case of soil, they produce severe disturbance. More recently, the use of plants in metal extraction(phytoremediation) has appeared as a promising alternative in the removal of heavy metal excess from soil and water [3].

Increased progressively with augmenting concentrations of these metals (Cu, Ni and Pb) in soil [4] Heavy metals, for example Cd, Cr, Cu, Hg, Ni, Pb and Zn can disrupt the physiology and morphology of plants [3] thus, plants exposed to heavy metal stress frequently face oxidative stress [2].

The normal range of copper (Cu) in soil is 2-250 mg kg^{-1} [3]. Excess copper is an efficient generator of ROS (Reactive oxygen species) [5].

Reactive oxygen species (ROS), which include compounds such as superoxide, peroxide, singlet oxygen and the hydroxyl radical [6-8], are an inevitable by-product of aerobic metabolism, being produced during the electron transfer reactions that take place in the mitochondria, chloroplasts and peroxisomes.

Corresponding Author: Mahya Golshan, Department of Agronomy, Karaj Branch, Islamic Azad university, Karaj, Iran.

ROS are toxic molecule [6,9] unless their concentration is regulated, they can cause protein, membrane and DNA damage and ultimately cell death [6,7,9]. Hydrogen atoms located adjacent to unsaturated olefinic bonda are particularly sensitive to oxidative attack, so that the unsaturated lipids of plant membranes are one of the primary targets of such oxidative reactions, although lipid peroxidation also arises due to the activity of lipoxygenases, for example, as part of the response of plants to pathogen invasion and to wounding. The quantification of these primary lipid hydroperoxides is difficult due to their instability and reactivity; thus, the degree of lipid peroxidation is usually estimated by measuring the concentration of secondary breakdown products derived from these initial hydroperoxides [6].

The antioxidant system comprises several enzymes such as superoxide dismutase(SOD), catalase(CAT) and guaiacol peroxidase (G-POD). Superoxide radicals generated converted to H_2O_2 is by the action of SOD and the accumulation of H_2O_2 is prevented by the activities by APX, CAT, C-POD and GPX. Thus, the balance between ROS generation and eradication determines the survival of the system [10].

Antioxidant enzymes can eliminate the active radical oxygen (ROS) which generate in abiotic stress condition. These enzymes protect membranes from destructive effect of ROS and make plants to resist against stress conditions [7,11]. Difference of stress stimulation on antioxidant enzymes activity depend on severity and duration of stress and also species and age of plant [12].

Creating oxidative injury that results in a reduction of plant growth and development. Plants growing on Cu-contaminated environments may develop variety of other defense mechanisms against its toxicity. One of these mechanisms is evoke the antioxidant enzymes induction as a general response to toxic effects of heavy metals [5].

Pb affected soils contain Pb in the range of 400-800 mg Kg^{-1} soil whereas in industrialized areas the level may reach up to 1000 mg $Pb.Kg^{-1}$ soil [10].

Excess Pb causes a number of toxicity symptoms in plants. Pb inhibits photosynthesis, upsets mineral nutrition and water balance, changes hormonal status and affects membrane structure and permeability. Mechanisms of Pb-detoxification include sequestration of Pb in the vacuole, phytochelatin synthesis and binding to glutathione and aminoacids etc. Pb tolerance is associated with the capacity of plants to restrict Pb to the cell walls, synthesis of osmolytes and activation of antioxidant defense system [2].

Plants for confronting against free radical oxygen use their antioxidant defense system, thus antioxidant enzymes activity will increase in order to decline effect of oxidative stress [13].

The purpose of this study is the lead and copper heavy metals effect on production of catalase and glutathion peroxidase and dityrosin biomarker to avoid stress resulting from oxygen free radical production in three species of plants, *Sinapis arvensis* and *Trifolium alexandrinum* and *Vicia villosa*.

MATERIALS AND METHODS

The present study was carried out in the greenhouse experiment of Agricultural and Natural Resources Faculty, Karaj, Iran during 2009. The relative humidity of the greenhouse was 60% and the minimum and maximum temperatures were 16 and 32°C respectively. The experiment was laid out in factorial experiment in completely random design with four lead $Pb(NO_3)_2$ levels 0, 200, 400, 800 and four copper $Cu(SO_4)_2$ levels 0, 150, 300 450 mg/kg soil in 4 replicates on the plant species *Sinapis arvensis*, *Trifolium alexandrinum* and *Vicia villosa*. The experiment was also carried out to study the absorption ability of the plants and in addition the species physiological tolerance in contaminated soils with lead and copper. The field soil was sampled from 0-30 cm and then examined to determine soil physical and chemical properties and also the fertility state and limiting factors particularly heavy metals lead and copper. The soil texture was loamy sand with 5.91 dS/m electrical conductivity and 0.6 % organic carbon and pH= 7.7 and Nitrogen percentage equaled to 0.054% total nitrogen .The selected pots were all of the same size and 20 cm in upper diameter and 18 cm in lower diameter; the heights 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 6 Kg soil. After solution spreading the soil to help chelating the elements with soil cloids and preparing contamination with the elements the treated pots remained in such a condition for 30 days and afterwards the sowing was done.

CU and Pb Content Analysis: 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 °C 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 made up to 25 ml with 1 M HNO_3. Copper and lead concentration of the extract was determined by atomic absorbtion spectrophotometer [2].

Sample Preparation for Enzyme Assay and Protein Measurement: 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 method of Lowery *et al.* [14]. The activity of following enzymes were expressed as specific activity (Activity/mg. protein).

Catalase (CAT) Activity: Catalase activity was measured at 25°C as previously described by Paglia and Valentine [15] that used hydrogen peroxide as substrate and 1 k of catalase activity was defined as the rate constant of the first order reaction [14].

Glutathion Peroxidase (GPX) Activity: The activity was measured by the method of Paglia and valentine [15]in which 0.56M (pH=7) phosphate buffer,, 0.5 M EDTA, 1mM NaN_3, 0.2mM NADPH were added to the extracted solution. GPX catalyses the oxidation of glutathion (GSH) by cumene hydroperoxide. In the presence of glutathion 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 [15].

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) [16] 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 ODS 2 (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). 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 [5.

The data conversion and statistical analysis was done using Excel and SAS software and the means were compared using LSD test at $P<0.05$ confidence level.

RESULT AND DISCUSSION

Table1, showed that the results of variance analysis showed that the main and reciprocal effects of the studied feature were significant ($p<0.01$).

Heavy metals causes the creation of ROS in plants. Results showed Fig.1 and Fig.2 with increasing lead and copper in pots the rate of these elements increased in aerial parts of tested plants. Results showed significant differences between the testing plants in the highest level of lead and copper (lead 800 mg/Kg soil and copper 450 mg/Kg soil).

Table 1: Analysis of variance for measured traits

SOV	df	Mean Square Pb(shoot)	Mean Square Cu(shoot)	MeanSquare CAT	Mean Square GPX	Mean Square Dityrosine
Plant	2	113.01693**	869.68899**	2874435.286**	574813.550**	111165.9266**
Pb	3	4574.70490**	20.37295**	199342.346**	76809.314**	6701.1434**
Plant*Pb	6	15.58713**	6.78262**	51850.126**	13873.616**	626.8581**
Cu	3	26.55408**	24549.41286**	28495.652**	17027.401**	942.6219**
Plant*Cu	6	1.22985^{ns}	201.30886**	4511.357**	670.664**	115.9637**
Pb*Cu	9	16.84300**	11.34409**	762.515**	612.566**	59.9340**
Plant*Pb*Cu	18	5.14817**	9.06255**	584.761**	723.975**	33.4280**
C. V%		9.464298	5.468287	6.306944	4.490726	2.799040

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

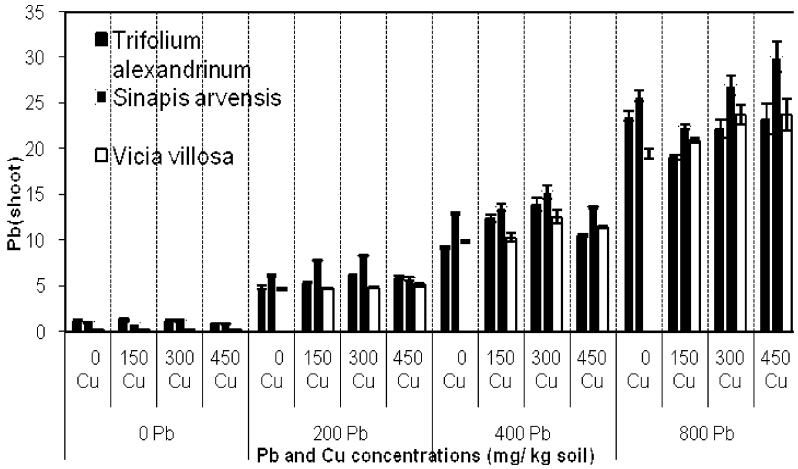

Fig. 1: Lead content in shoot (mg/ g dry weight) and comparing between plants (LSD 0.05)

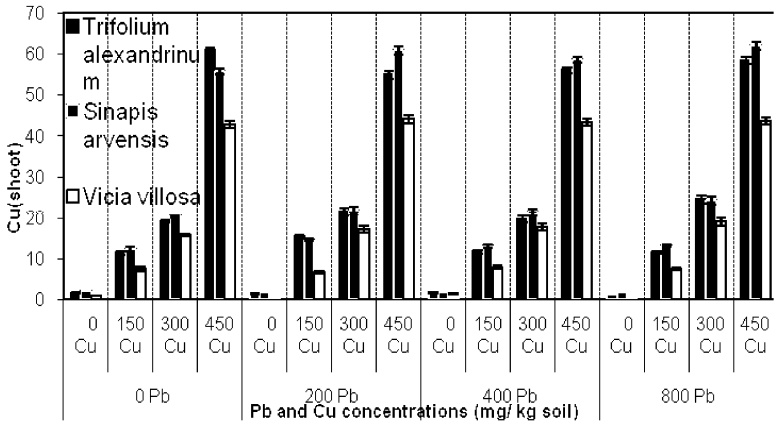

Fig. 2: Copper content in shoot (mg/ g dry weight) and comparing between plants (LSD 0.05)

ROS include the superoxide radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$), all of which affect mainly lipids, proteins, carbohydrates and nucleic acids. To overcome this, cells are equipped with enzymatic and non-enzymatic mechanisms to eliminate or reduce their damaging effects. The importance of antioxidant enzymes is their ability to scavenge ROS and there by prevent oxidative damage. The antioxidant system comprises several enzymes such as superoxide dismutase(SOD), catalase(CAT) and guaiacol peroxidase (G-POD) [10].

Notwithstanding the fate of exudates in the rhizosphere and the nature of reactions involved in phytoextraction and transport of metals by plants being not yet fully understood, it is recognized that they contribute significantly to the accumulation of metals in plants. Chemical compounds likely to occur in the rhizosphere are clearly associated with increase of metals uptake from soil and their translocation to shoots [1].

Results of Gardea- Torresdey *et al.* [3], terrestrial plants such as Indian mustard (*B. juncea*) have been successfully hydroponically cultivated, showing that the absorption of different heavy metals including Zn and Pb can be effectively accomplished and as result of Sudhakar *et al.* [17] the lead content of roots and leaves was significantly increased in all legume species grown on tailings.

As Pb promotes the formation of reactive oxygen species in plants leading to oxidative stress, an increase in the activity of certain antioxidative enzymes has been observed in Pb-treated plants. Activities of several enzymes are reported to be enhanced by Pb treatment. Such apparent enhancement results from changes in enzyme synthesis, immobilization of enzyme inhibitors, or as a result of effect of molecules, which are synthesized under Pb phytotoxicity [2].

Superoxide radicals generated converted to H_2O_2 is by the action of SOD and the accumulation of H_2O_2 is prevented by the activities by APX, CAT, C-POD and GPX. Thus, the balance between ROS generation and eradication determines the survival of the system [10].

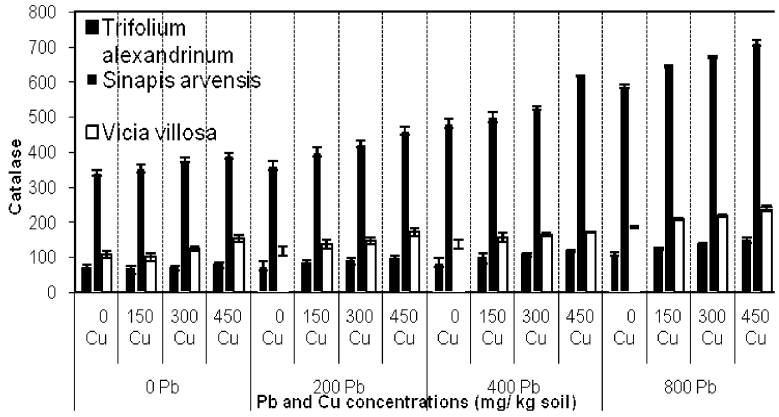

Fig. 3: Catalase enzyme activity and comparing between plants (LSD 0.05)

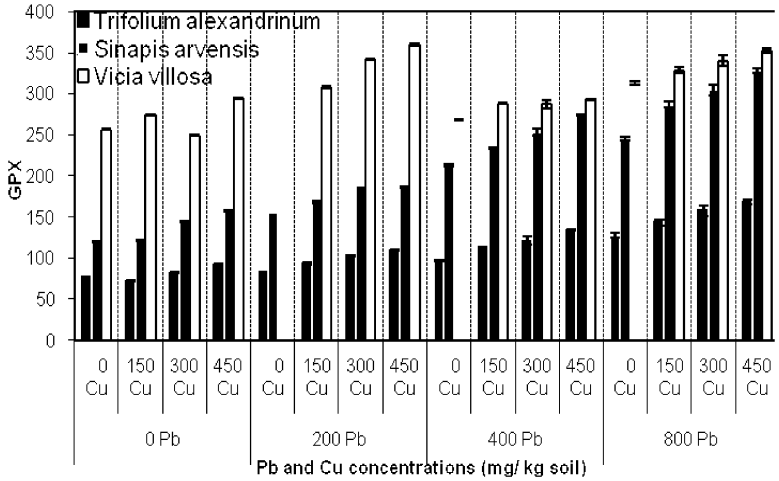

Fig. 4: Glutathion peroxidase activity and comparing between plants (LSD 0.05)

Results showed higher activity of catalase dealing with producing free radical oxygen by stress of heavy metals (Fig.3). *Sinapis arvensis* produce higher catalase activity compared with the other testing plants. Results showed lead and copper stimulated producing higher catalase against ROS production in plant cell. *Sinapis arvensis* compared with the others showed higher catalase activity. Results also showed glutathion peroxidase activity in these plants increased with serving elements more in soil that showed plant stimulation to deal with ROS production in plants.

Glutathion peroxidase protects the membrane lipids from oxidative damage and detoxified the organic peroxides; it can also act on organic hydroperoxides [5].

Vicia villosa showed higher glutathion peroxidase activity in compare with other plants. *Sinapis arvensis* with higher catalase activity and *Vicia villosa* with higher glutathion peroxidase were able to confront heavy metals with their own physiological characteristics.

CAT is important enzyme against oxidative stress, being able to scavenge H_2O_2 [10]

CAT enzyme convert H_2O_2 which are toxic for cells to water and oxygen [7,13,17-19] Similar to GPX, CAT activity also inhibited with increasing concentration of Cu. This decrease may have been due to the response to increased ROS production, which with elapsing time may be inadequate to detoxify high levels of ROS. The impaired antioxidant system may favor accumulation of ROS [10].

Soybean plants grown in culture media containing 20-100 $mg.L^{-1}$ Pb showed increased activity of acid phosphatase, α-amylase and peroxidase in leaves. Increase activity of hydrolytic enzymes as well as of peroxdase in soybean leaves under Pb treatment parallels with the senescence of leaves. As Pb promotes the formation of reactive oxygen species in plants leading to oxidative stress, an increasing in the activity of certain antioxidative enzymes has been observed in Pb-treated

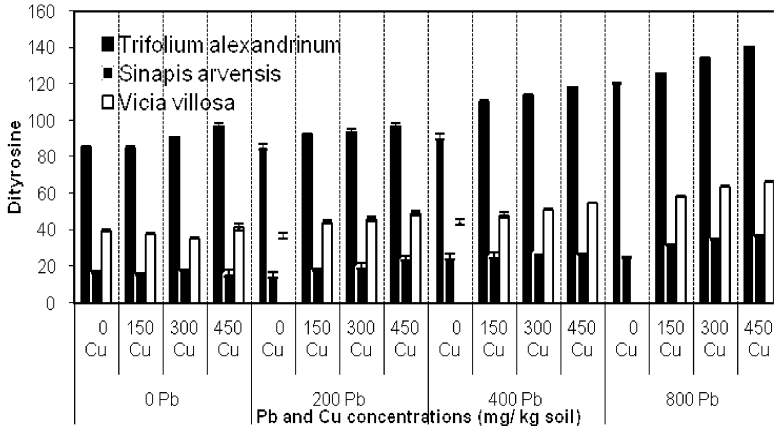

Fig. 5: Dityrosine content and comparing between plants (LSD 0.05)

plants. Rice plants grown for 20 days in sand cultures containing 0.5 mM and 1 mM $Pb(NO_3)_2$ showed increased activities of the antioxidative enzymes superoxide dismutase, guaiacol peroxidase, ascorbate peroxidase and glutathione reductase in roots and leaves [5].

Results of Sharma *et al.* [20], Cd (25 μM) induced a marked increase in catalase activity at most Fe levels except at 250 μM where Fe induced stimulation of catalase did not further increase due to Cd.

According to Boojar and goodarzi [2], antioxidant enzymes activity SOD, CAT, GPX had been increased respectively in leaves and stems and roots dealing with copper stress.

Our results showed fig.5 increasing of dityrosin in all three plants species when lead and copper application increased. Because the toxic intermediates and ROS are short-lived and difficult to measure directly, an alternative approach for oxidative stress monitoring is quantifying their stable end products of oxidative reactions with cellular macromolecules. Dityrosine, as a stable biomarker of ROS mediated protein oxidation is closely correlated with level of oxidative stress. To control the level of ROS and protect the cells they possess a number of low molecular mass antioxidants (ascorbates, glutathione, phenolic compounds, tocopherols) and enzymes scavenging ROS, regenerating the active from of the antioxidants and eliminate or reduce the damages caused by them [5].

According to the Fig.3 and Fig.4 *Vicia villosa* and *sinapis arvensis* have been able to deal with ROS production. *Trifolium alexandrinum* showed the lowest enzymes activity. It means *Trifolium alexandrinum* did not show ability for enzyme activity. Low enzyme activity caused cellular protein destruction in *Trifolium alexandrinum.* Lead and copper stress caused cellular protein destruction in *Trifolium alexandrinum.*

According to the results, *Sinapis arvensis* showed higher ability to absorb heavy metals and enzymes activity. *Trifolium alexandrinum* showed lower resistance against heavy metals stress.

As one of the objectives of this research was investigating of three plant species strategies for resistance mechanisms against lead and copper, we concluded that in pulluted area mustard plant showed higher lead and copper absorbtion in aerial parts of the plant. This plant with producing higher catalase and gluthatione peroxidase activity and lower protein destruction showed a typic characteristic for using this plant for polluted area.

REFERENCES

1. Nascimento, C.W.A.D. and B. Xing, 2006. Phytextraction: A review on enhanced metal availability and plant accumulation. Sci. Agric., 3: 299-311.
2. Sharma, P. and R.S.H. Dubey, 2005. Lead toxicity in plants. Braz. J. Plant Physiol., 17(1): 35-52.
3. Gardea-Torresdey, J.L., J.R. Peralta, G. De La Rosa and J.G. Parsons, 2005. Phytoremediation of heavy metals and study of the metal coordination by X-ray absorption spectroscopy. Coordination Chemistry Reviews, 249: 1797-1810.
4. Ali, M.B., P. Vajpayee, R.D. Tripathi, U.N. Rai, S.N. Singh and S.P. Singh, 2003. Phytoremediation of lead, Nickel and Copper by *Salix acmophylla* Boiss.: Role of Antioxidant Enzymes and Antioxidant Substances. Bull. Environ. Contam. Toxicol., 70: 462-469.

5. MashhadiAkbar Boojar and M.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. Davey, M.W., E. Stals, B. Panis, J. Keulemans and R. L. Swennen, 2005. High throughput determination of malondialdehyde in plant tissues. Analytical Biochemistry, 347: 201-207.
7. Lai, Q.X., Z.Y. Bao, Z.J. Zhu, Q.Q. Qian and B.Z. Mao, 2007. Effects of osmotic stress on antioxidant enzymes activites in leaf discs of P-IPT modified gerbera. J. Zhejiang Univ. Sci., B. 8(7): 458-464.
8. Valentovic, P., M. Luxova, L. Kolarovic and O. Gasparikova, 2006. Effect of osmotic stress on compatible solutes content, membrane stability and water relations in two maize cultivars. Plant Soil Environ., 52(4): 186-191.
9. Badawi, G.H., Y. Yamauchi, E. Shimada, R. Sasaki, N. Kawano, K. Tanaka and K. Tanaka, 2004. Enhanced tolerance to salt stress and water deficit by overexpressing superoxide dismutase in tobacco (*Nicotiana tabacum*) chloroplasts. Plant Sci., 166: 919-928.
10. Khatun, S., M. Babar Ali, E.J. Hahn and K.Y. Paek, 2008. Copper toxicity in *Withania somnifera*: Growth and antioxidant enzymes responses of *in vitro* grown plants. Environmental and Experimental Botany, 64: 279-285.
11. Mohammdkhani, N. and R. Heidari, 2007. Effects of drought stress on protective enzyme activities and lipid peroxidation in two maize cultivars. Pakistan J. Biol. Sci., 10(21): 3835-3840.
12. Pan. Y., L.J. Wn and Z.L. Yu, 2006. Effect of salt and drought stress on antioxidant enzymes activities and SOD isoenzymes of liquorice (*Glycyrrhiza uralensis Fisch*). Plant Growth Regul., 49: 157-165.
13. Arora, A., R.K. Sairam and G.C. Rivastave, 2002. Oxidayive stress and antioxidative system in plants. Current Sci., 82(10,25): 1227-1238.
14. Lowry, O., A. Rosebrough and R. Randall. 1951. Protein measurement with folin phenol reagent. Journal, Biological Chemistry. 193: 680-685.
15. Paglia, D.E. and W.N. Valenyine, 1987. Studies on the quantitative and qualitative characterization of glutathione proxidase. J. Lab. Med., 70: 158-165.
16. 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. Journal of Chromatography B., 799: 245-254.
17. Sudhakar, C., L. Syamalabai and K. Veeranjaneyulu, 1992. Lead torerance of certain legume species grown on lead ore tailings. Agriculture, Ecosystems and Environ., 41: 253-261.
18. Blokhina, O., E. Virolainen and K.V. Fagerstedt, 2002. Antioxidants, oxidative damage and oxygen deprivation stress: a Review. Annals of Botany. 91: 179-194.
19. Liu, J., L.P. Tong, T.W. Shen, J. Li, L. Wu and Z.L. Yu, 2007. Impact of ion implantation on licorice (*Glycyrrhize uralensis Fisch*) growth and antioxidant activity under drought stress. Plasma Science and Technol., 9(3): 301-306.
20. Sharma, SH.S., S. Kaul, A. Metwally, K.C. Goyal, I. Finkemeier and K. J. Dietz, 2004. Cadmium toxicity to barley (*Hordeum vulgare*) as affected by varying Fe nutritional status. Plant Sci., 166: 1287-1295.