

Determination of peroxidase activity, total phenolic and flavonoid compounds due to Lead toxicity in *Medicago sativa* L.¹Ghelich Sima, ¹Zarinkamar Fatemeh, ²Niknam Vahid¹Faculty of biological Sciences, Tarbiat Modares University, Tehran, Iran²Faculty of biology, Tehran University, Tehran, IranGhelich Sima, Zarinkamar Fatemeh, Niknam Vahid; Determination of peroxidase activity, total phenolic and flavonoid compounds due to Lead toxicity in *Medicago sativa* L.**ABSTRACT**

Heavy metal pollution is one of the most important ecological problems in the whole world. Pb is the most common heavy metal contaminant in the environment. In this study the effects of Pb stress (0, 120, 240, 500, 1000 μ M Pb) on 30 days plants of *Medicago sativa* L. were investigated in hydroponic culture. The results indicated that uptake and accumulation of Pb in roots and aerial were increased with increase of Pb concentration in medium. Compared with control plants, increase in the contents of MDA and H₂O₂ were observed in the plants under Pb stress. Moreover, increase in total flavonoids content, and peroxidase activity in roots and shoots of the treated seedlings were observed under Pb stress. Content of total phenolics increased in roots and shoots comparing to than the control.

Key words: Flavonoids, Lead, *Medicago sativa* L., Phenolic compounds, Peroxidase**Introduction**

Heavy metal pollution of air and agricultural soils is one of the most important ecological problems on world scale. Pb is the most common heavy metal contaminant in the environment [20]. Pb contamination has resulted from mining and gasoline activities, Pb containing paints and gasoline. Pb is available to plants from soil and aerosol sources [29]. It is a nonessential element in metabolic processes and may be toxic or lethal to organisms even when absorbed in small amounts [20]. Pb contamination in the plant environments is known to cause highly toxic effects on processes such as depression of seed germination, the disturbance in mitosis induction of leaf chlorosis, toxicity of nucleoli, inhibition of root and shoot growth, reduction in photosynthesis, transpiration, DNA synthesis, and inhibition and activation of enzymatic activities [17,24].

However, there are plant species, which may tolerate toxic amounts of these elements. The identification of metal stress-resistance mechanisms is a fundamental step for understanding the biochemical and molecular mechanisms of stress resistance. Heavy metals induce production and accumulation of reactive oxygen species (ROS) in cells and tissues. ROS accumulation leads to oxidative stress that can damage cellular components, such as DNA, lipids, proteins and sugars [18,26]. ROS homeostasis in plants is tightly regulated by a complex machinery of enzymatic and

non-enzymatic antioxidants. Peroxidase is one of the most antioxidant enzymes and increase the activity of this enzyme were observed in response to Cu, Cd and Al. [3,6,32].

Flavonoids have been suggested to act as antioxidants, protecting plants from oxidative stress [15,23,27,22,31].

One of the mechanisms of metal detoxification, is synthesis and/or exudation of metal chelating agents. Chelating agents, excreted by root apexes to the rhizosphere, chelate with the metal excluding it from the root apex or the ligand-metal complex detoxify metals internally. Organic acids and phenolics are included among these ligands [23]. There have been many reports of induced accumulation of phenolic compounds in plants treated with high concentrations of metals. Antioxidant action of phenolic compounds is due to their high tendency to chelate metals. Phenolics possess hydroxyl and carboxyl groups, able to bind particularly iron and copper [23,18].

Medicago sativa L. (alfalfa) is a flowering plant in Fabaceae family that has several flavonoid compounds in its root and shoot. Previous studies demonstrated that alfalfa plants have the ability to germinate and grow in polluted soil with heavy metals [2,10,11,25,30].

In this study we focused on the uptake and accumulation of Pb in tissues of alfalfa plants and the mechanisms of lead tolerance in this species were investigated.

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Material and methods

Cultivation of plants and Pb treatment:

Medicago sativa L. cv. Hamedani were sterilized with 5% NaClO for 10 min, then rinsed several times with distilled water and germinated on perlite and vermiculite for 7 days. After germination, young seedlings were transferred to 3 L polyethylene containers with 1/2- Hoagland nutrient solution and after one month treated with (0, 120, 240, 500, 1000 μ M Pb). After 10 days, plants were collected. Their shoots and roots were then separated and dried for Pb analysis. And some of them freeze-dried in liquid

nitrogen and placed on -80 freezer for biochemical analysis.

Pb analysis:

Dry plant samples were digested with nitric acid, perchloric acid and sulphoric acid (40:4:1) and impurities were removed by filtration [13]. After digestion, Pb concentrations in plant and soil samples were measured by atomic absorption (SHIMADZU AA- 6709).

Bioconcentration Factor (BCF) and Transfer Factor (TF)

$$\text{Bioconcentration Factor (BCF)} = \frac{\text{Metal content in root tissue}}{\text{Metal content in nutrient solution}}$$

$$\text{Transfer Factor (TF)} = \frac{\text{Metal content in shoot tissue}}{\text{Metal content in root tissue}}$$

Total flavonoid assay:

For measurement of total flavonoid contents, 0.1 g root and shoot were ground in 3 mL of acidified ethanol (99: 1, ethanol: HCl). Samples were then centrifuged at 12000 rpm for 20 min and the supernatant of each sample was gently boiled for 10 minutes in a water bath at 80°C. The absorbance was measured at 270, 300 and 330 nm, and the flavonoid content was calculated using an extinction coefficient of 33000 $\text{M}^{-1}\text{cm}^{-1}$ [12].

Total soluble phenolic compounds assay:

The Folin-Ciocalteu reagent was used to estimate total phenolic content. The extract (60-300 μ l) was diluted with deionized water to 4.8 ml, and 300 μ l of Folin-Ciocalteu reagent was added and shaken. After 8 min, 900 μ l of 20% sodium carbonate solution was added with mixing. The solution was left at 40°C for 30 min before reading the absorbance at 765 nm. Gallic acid (0 - 50 μ g) was used as standard, and the results were reported as mg gallic acid equivalent per gram of fresh weight [35].

Hydrogen peroxide (H_2O_2) content:

Hydrogen peroxide content of root and shoot was measured spectrophotometrically after reaction with potassium iodide (KI). The reaction mixture consisted of 0.5 ml of 0.1% trichloroacetic acid (TCA), root and shoot extract supernatant, 0.5 ml of 100 mM K-phosphate buffer and 2 ml reagent (1M KI, w/v in fresh double-distilled water). The blank probe consisted of 0.1% TCA in the absence of petal extract. After 1 h of reaction in darkness, the

absorbance at 390 nm. The amount of hydrogen peroxide was calculated using a standard curve prepared with known concentration of H_2O_2 [1].

Extraction and assay of peroxidase (PO) activity:

To determine enzyme activities, 0.5 g of root and shoot homogenized with chilled pestle and mortar in 3-ml extract buffer containing 60 mM phosphate buffer (pH 6.1). The homogenate was centrifuged at 12,000g for 15 min in 4°C and the resulting supernatant was used to determine the enzyme activity.

Peroxidase (POD) activities were determined with guaiacol at 470 nm. 500 microliter of the enzyme extract was added to the reaction mixture containing 0.05 ml guaiacol solution and 0.03 ml hydrogen peroxide solution in 3 ml phosphate buffer solution (pH 6.1). Activity of PO was expressed as the increase in absorbance at 470 nm per min per mg protein [9].

Lipid peroxidation:

Lipid peroxidation was estimated in vitro after the formation of malondialdehyde, a by-product of lipid peroxidation that reacts with thiobarbituric acid. Briefly, 0.3 g fresh root and shoot tissues were ground with 5 mL of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 15,000g for 10 min and 0.5 mL of the supernatant was mixed with 2 mL of 0.5% TBA in 20% TCA. The mixture was heated at 90°C for 20 min. After the reaction was stopped, the resultant mixture was centrifuged at 10,000g for 5 min. The absorbance of the supernatant was measured at 532 nm. The values were corrected

for non-specific absorption by subtracting absorbance read at 600 nm. The level of lipid peroxidation was calculated by using the extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$ [9].

Statistical analysis:

The design of all experiments was a complete randomized design and treatments consisted of three replications. Data were evaluated using ANOVA followed by a duncan stest (MsTATC Version 2.1) at $P < 0.05$.

Results:

Fig. 1 shows the concentration of Pb found in root and shoot of plants exposed to the different treatments containing Pb. The uptake and accumulation of Pb in the shoot and roots varied depending on Pb concentrations. Pb content in roots increases with rising Pb concentration in medium and positive linear relation were found between Pb concentration in medium and root tissue concentration during the loading period. Maximum of Pb accumulation observed in $1000 \mu\text{M}$ Pb in. The obtained results indicated the significant difference in the shoot parts. Maximum Pb content in shoot was observed in treatments containing in $1000 \mu\text{M}$ Pb. Lead concentration in roots was higher than that in shoot.

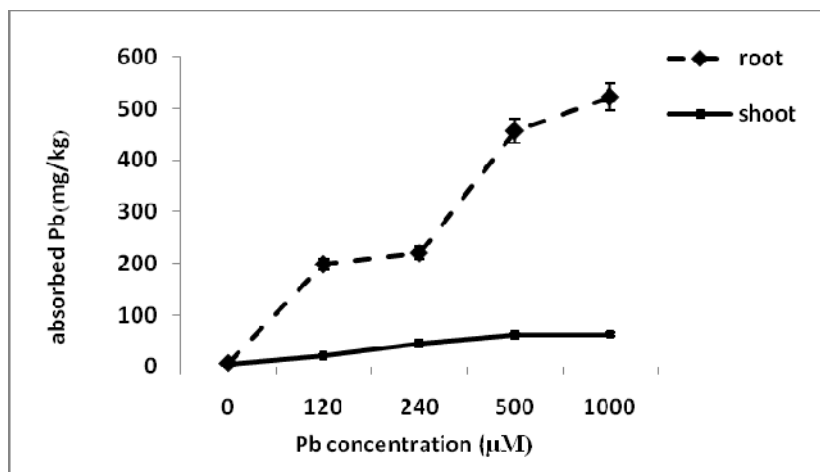


Fig. 1: Pb accumulation in root and shoot.

Table 1 demonstrated the bioconcentration factor and transport factor of lead by *M. sativa* at 120, 240, 500 and $1000 \mu\text{M}$. The bioconcentration factor is found high at $120 \mu\text{M}$ Pb (1.65) and low at $1000 \mu\text{M}$ Pb (0.531). The highest transport factor is found 0.71 at $120 \mu\text{M}$ Pb and the lowest is 0.111 at

$240 \mu\text{M}$ Pb. The potential of plants for phytoremediation is also based on the depletion of metal level from the nutrient solution at various concentrations. The reduction of cadmium concentration in the nutrient solution was attributed to its uptake by the plants.

Table 1: Bioaccumulation factor and transport factor of *M. sativa*.

TF	BCF	Pb concentration in medium (μM)
0.71	1.65	120
0.111	0.917	240
0.203	0.913	500
0.118	0.531	1000

A remarkable increase was observed in flavonoid contents of Pb treated plants, compared to those of the control ones (Figs 2,3).

Total soluble phenols were significantly enhanced by Pb treatment in both leaves and roots (Figure 4). In root, content of total phenols in 120 and $240 \mu\text{M}$ Pb don't have significant differences with controls and in 500 and $1000 \mu\text{M}$ Pb we observed the highest level of these compounds. In shoot all treatments have significant differences with

controls and content of total phenols increased by increase of Pb concentration in medium.

Malondialdehyde(MDA) is a product of lipid peroxidation and a most prominent indicator of oxidative stress in plants exposed to stress conditions. Pb exposure caused a significant increase in the MDA in the roots, which increased with increasing the Pb concentrations (Fig. 5). The significant increase was observed in all treatment than control, and attained a peak at $1000 \mu\text{M}$ Pb.

Figure 6 shows the H₂O₂ content of root and shoot due to Pb treatment. In root of all treatments have significantly difference with control. H₂O₂

content in were increased by the Pb treatments to 240 μM Pb and then decreased. The highest level of H₂O₂ observed in 240 μM Pb.

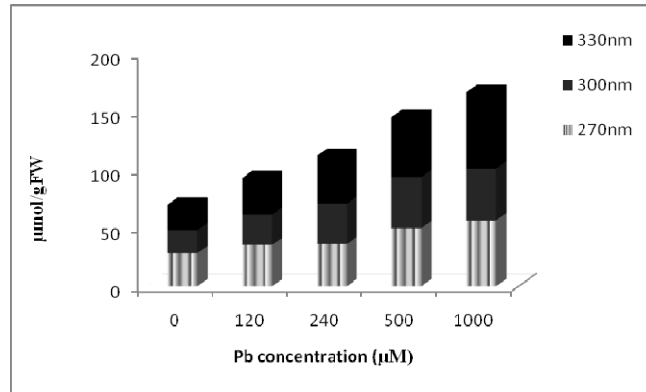


Fig. 2: Flavonoid content of shoot in response to Pb.

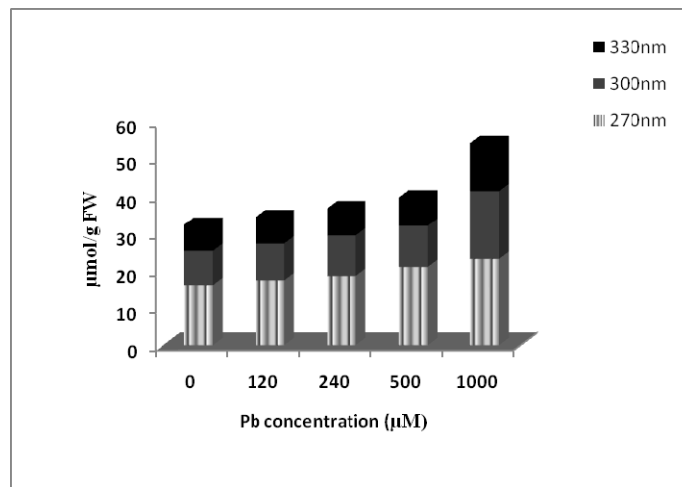


Fig. 3: Flavonoid content of root in response to Pb.

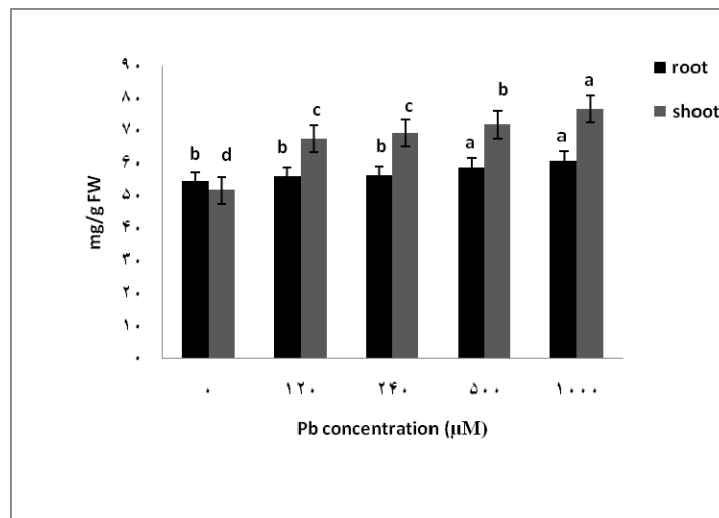


Fig. 4: Total soluble phenolic compounds content in response to Pb.

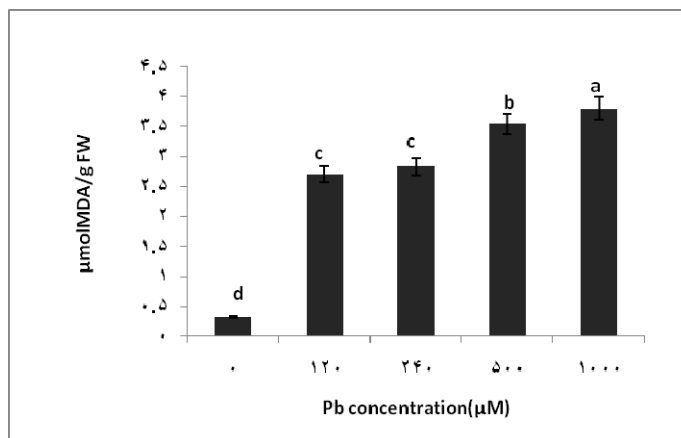


Fig. 5: Root MDA content in response to Pb.

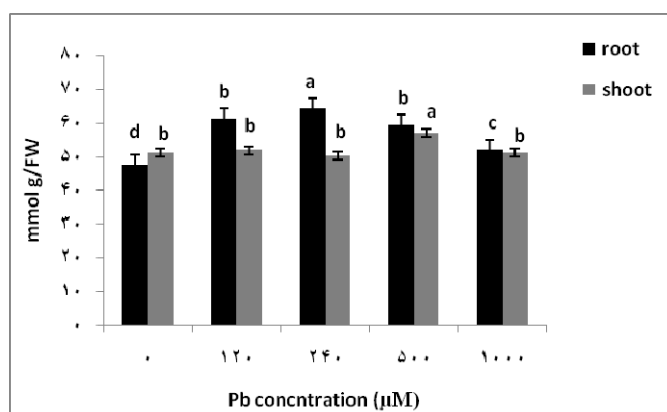


Fig. 6: Hydrogen peroxide content in response to Pb.

Figure 7 indicates the activity of peroxidase in root and shoot in response to Pb. PO activity in were significantly enhanced by the Pb treatments and

tended to increase with the increase in Pb concentration.

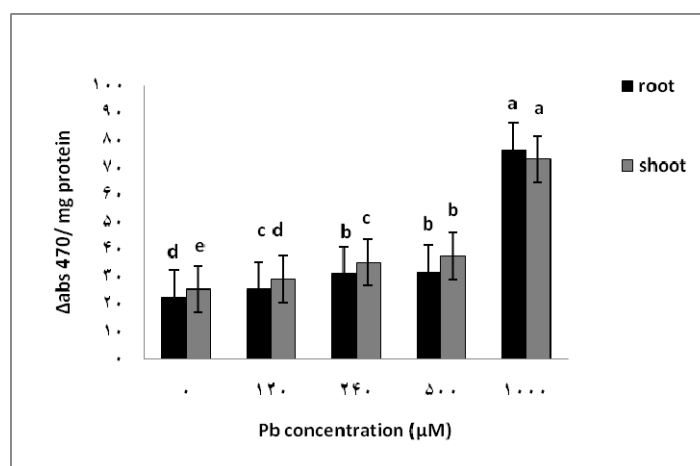


Fig. 7: Peroxidase activity in response to Pb.

Discussion:

As a consequence of industrial development, the environment is polluted with heavy metals. Pb is the most common heavy metal contaminant in the environment [20]. Pb has been shown to accumulate mainly in plant roots, and extremely small amount are transported from root to shoot [17,37]. The great differences between root and shoot concentrations indicate an important control of the internal transport of metals from the root to shoot. Roots of the plant acts as a barrier against heavy metal translocation and this may be a potential tolerance mechanism operating in the roots.

However, our results suggest, that at very high Pb concentrations in culture medium (1000 μ M), Pb transport from roots to shoots increased, which might be due to disruption of plasma membranes by high concentrations of Pb and subsequent reduction of the soil/plant barrier [14]. Also bioconcentration Factor and transfer factor indicated that *M.sativa* plants is not the suitable candidate plant for phytoremediation of Pb.

Heavy Metal Stress induces oxidative stress and ROS production in plants, ROS are known to damage cellular membranes by inducing lipid peroxidation. They also can damage DNA, proteins, lipids and chlorophyll [23,26]. Plants have developed complex systems protecting them from ROS consisting of enzymatic and non-enzymatic antioxidants [23]. Peroxidase is one of the most antioxidant enzymes. In the present study, we found that Pb treatment can increase the PO activity in *M.sativa* plants. The elevated peroxidase activity in Pb-treated plants reflected an increased degree of oxidative stress and damage of the plasma membrane lipids. Enhancement the activity of PO observed in several metal stresses by other researches [9,37].

The lipid peroxidation is the most prominent symptom of oxidative stress. Malondialdehyde (MDA) is a product of lipid peroxidation and a most prominent indicator of oxidative stress in plants exposed to stress conditions [36,37]. In our experiment, high Pb concentrations in growth medium caused a remarkable peroxidation of lipids in root. MDA contents tended to increase with the increase in Pb concentrations in the *M.sativa* roots.

In this study we found a significant increase in the level of H₂O₂ in the Pb-treated plants as compared with the control. H₂O₂ is one of the most popular ROS and its production induced by heavy metal toxicity [23].

All plants produce an amazing diversity of secondary metabolites. One of the most important groups of these metabolites are phenolic compounds. An enhancement of phenylpropanoid metabolism and the amount of phenolic compounds can be observed under different environmental factors and stress conditions [23]. Flavonoids are a family of plant phenolic compounds. Many flavonoid

biosynthetic genes are induced under stress conditions and accordingly, flavonoid levels increase during exposure to abiotic stresses, such as metal toxicity. Flavonoids have been suggested to act as antioxidants, protecting plants from oxidative stress [15]. Flavonoids also may sequester metal ions by chelating and preventing metal-mediated generation of free radicals and, accordingly, may protect the potential biological targets from oxidative stress. Complexes of flavonoids play an important role in limiting metal bioavailability and suppressing metal toxicity [22,23,26,28]. In previous researches, the induction of phenolic compound biosynthesis was observed in wheat in response to nickel toxicity, and in maize in response to aluminium [23]. In our study, content of total soluble phenols and total flavonoids increased in both root and shoot of *M.sativa* plants in response to Pb toxicity.

Conclusions:

As a well-known pollutant, Pb induces oxidative stress in plants. Based on the results increases in H₂O₂ content, peroxidase activity and MDA production indicated the oxidative stress due to Pb toxicity in alfalfa plants. Also enhancement of total phenols and flavonoids in Pb-treated plants indicated the mechanism of Pb tolerance in these plants. Further studies need to understanding the associated mechanisms involved in Pb tolerance in alfalfa plants.

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