Changes of Phytochemical Parameters and Antioxidant Enzymes in *Glycine max* Under Pb Stress

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**A B S T R A C T**

Heavy metals modify growth, morphology and metabolism of plants in several ways. To reveal metal toxicity, the effects of heavy metal Pb on phytochemical parameters of *Glycine max* was determined. So, plants were treated with different concentrations (0, 5, 10 µM) of Pb and the contents of phenolic compounds, total flavonoid, flavonol, antioxidant activity and the activities of enzymatic antioxidants (peroxidase, polyphenol oxidase) as marker for oxidative stress were investigated. This study, factorial experimental design was ordered in a completely randomized block design with three replications. Survey results indicated that the amount of non-enzymatic antioxidant compounds (phenol, flavonoid and flavanol) and antioxidant activity significantly were decreased in all treatments but a non-significant increase was observed in polyphenol oxidase activity, whereas peroxidase was showed a significant decrease. Although, increase in polyphenol oxidase activity is recognized as primary response to metal toxicity for accumulation and tolerance to excess amount of heavy metal, but this increase was not significant. These results show that different response in control and treated plants is due to induction oxidative stress and heavy metal toxicity in plants.

**INTRODUCTION**

Heavy metals are determined as that group of elements that have special weights higher than about 5g/cm³. A number of them (Fe, Co, Mn, Mo, Ni, Cu, Zn) are essential micronutrients and are required for growth and take part in redox reactions, electron transfers and other important metabolic processes in plants. Metals which are considered nonessential (Pb, Cd, Cr, Hg etc.) are potentially extremely toxic for plants [26]. Lead is known to have toxic effects on membrane structure and functions [9]. Lead potentially induces oxidative stress and evidence is storing to support the role of oxidative stress in the pathophysiology of lead toxicity [10].

Recent epidemiological studies have reported that low level lead exposure has a graded association with several disease consequences such as kidney disease, hypertension, peripheral artery disease, neurodegenerative disease and cognitive impairment. Although all these diseases include components of oxidative stress, the relationship of oxidative stress to lead-related disease with low level exposure has been dispraised because studies have been conducted at levels not typically observed in common population [22].

Both abiotic and biotic stresses are known to involve plants to produce reactive oxygen species (ROS) that can cause damage to the tissues or signal the start of physiological defence reactions [7]. Usually, as a result of various biotic and abiotic stresses, increased L-phenylalanine ammonialyase activity and accumulation of many phenolics are observed [30,14]. In abiotic sources, plants produce ROS by primary metabolic processes such as chloroplastic and mitochondrial electron transport [11].

In addition, plants synthesise compounds which are able to reduce the damaging effects of different stresses. The antioxidant properties of phenolics are well-documented [13]. These compounds are present in plants as fundamental or can be synthesised de novo [15]. It is known that the effect of phenolics on growth is a complex process. These compounds may interrere in auxin metabolism, change membrane penetrance, influence respiration and oxidative phosphorylation or protein synthesis [4]. The phenolic compounds such as flavonoids...
are active antioxidants [25] and also cause appeasement of lipid peroxidation [32]. Plants possess enzymatic systems that protect them against $\text{H}_2\text{O}_2$ and other harmful ROS. These include superoxide dismutase, catalase, peroxidase, polyphenol oxidase etc. [33].

Therefore, the present study was to evaluate: the effects of different concentrations of Pb on the phytochemical and activity of enzymatic antioxidants (peroxidase, polyphenol oxidase) on Pb stress.

**MATERIALS AND METHODS**

**Plant materials:**
Soybean seeds (Glycine max var. Williams) were supplied from Oil Seeds Center (Isfahan, Iran). The seeds were saturated with 2-3% Hydrochloric acid solution and were soaked in distilled water for 24 h and then were germinated in pots containing perlite and coco peat (1 seed per pot) and watered with half-strength Hogland nutrient solution. The plants were grown at 25°C temperature, with a 16/8 h day/night photoperiod for 20 days. Seedling at the three-leaf stage treated at different concentrations of Pb(NO$_3$)$_2$ (0, 5, 10 µM). 20 days after treatment, plants were removed and they were washed with distilled water and separated from the aerial parts. Ten grams of each plant powder was extracted in 150 ml of 80% methanol by maceration (72 h). The solvent was removed under the vacuum at temperature below 50°C and the extracts were freeze-dried. Experiments were conducted at Botany lab, Department of Biology, Faculty of Biological Sciences, Islamic Azad University of Falavarjan, Isfahan, Iran.

**Total phenol determination:**
Total phenols were determined by Folin Ciocalteu reagent [29,31]. Different concentrations of each plant extract (10, 20, 40, 60, 80, 100 µl) or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (1 ml, 1:10 diluted with distilled water) and 7% Na$_2$CO$_3$ (1 ml). The mixtures were allowed to stand for 15 min and the total phenols were determined by colorimetry at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 200, 250 mg L$^{-1}$ solutions of gallic acid in methanol : water (50:50, v/v). Total phenol values are expressed in terms of gallic acid equivalent (mg g$^{-1}$ of dry mass), which is a common reference compound.

**Total flavonoid and flavonol determination:**
Aluminum chloride colorimetric method was used for flavonoids determination [21,24]. Each plant extracts (10, 20, 40, 60, 80, 100 µl) in methanol were separately mixed with 1.5 ml of methanol, 1 ml of 2% aluminum chloride, 6 ml of 5% potassium acetate and 2.8 ml of distilled water. It remained at room temperature for 40 min; the absorbance of the reaction mixture was measured at 415 nm for flavonoid assay and then was measured at 440 nm for flavonol assay. The calibration curve was prepared by preparing rutin solutions at concentrations 12.5 to 100 g ml$^{-1}$ in methanol.

**Free radical scavenging activity determination:**
The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extracts [16]. Different concentrations of each extract (10, 20, 40, 60, 80, 100 µl) were added, at an equal volume, to methanolic solution of DPPH (0.004g per 100 ml). After 120 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated for three times. Ascorbic acid were used as standard controls. IC50 values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

**Enzyme extraction and assay:**
The samples, weighing about 50 mg, were homogenized with 2 ml of phosphate buffer pH 6.8 (0.1 M). This portion was centrifuged at 4°C for 10 min at 15,000g in a refrigerated centrifuge. The clear supernatant was taken as the enzyme source.

**Peroxidase Assay:**
2.7 ml sodium phosphate buffer (25 Mmoles), pH 6.8, 100µl gayagol (20 Mi moles), 100µl of H2O2(40 Mmoles), and 100 µl of the enzyme extract. The absorbance of the reaction mixture was measured at 470 nm at time (15, 75 s) for peroxidase assay.

**Polyphenoloxidase Assay:**
2.8 ml sodium phosphate buffer (25 Mmoles), pH 6.8, 100µl pirogalol(10 Mi moles) and 100 µl of the enzyme extract. The absorbancy of the purpurugallin formed was taken at 420 nm at time (40, 100s) for polyphenol oxidase assay (Kar and Mishra, 1976).

**Statistical analysis:**
The experimental design was a split plot in a randomized complete block design with three replications.
The presented data included means of three separate experiments ± SD. In order to analyze the data, SPSS software and ANOVA test were used. Thus, the statistical significance between phytochemical activities values of the extracts was evaluated with a LSD test. P values less than 0.05 were considered to be statistically significant.

Results:
Analysis of data on total phenol showed that Pb poisoning caused significant reduction in polyphenol content compared to non-Pb treatment (P<0.05), so that when Pb concentration was increased, the amount of polyphenol content was declined (Figure 1).

![Fig. 1: Significant reduction of phenol content (concentration and absorbance) of under treatment groups with Pb 5 and 10µM in compared with non-Pb group. Bars are least significant differences where p < 0.05.](image)

Table 1 show the content of total phenols that were measured by Folin Ciocalteu reagent in terms of gallic acid equivalent (standard curve equation: y = 29.85x +0.043, r²= 0.990). The total phenol varied from 38.86 ± 0.05 to 166.83 ± 0.02 mg g⁻¹ in the extract powder. Non-Pb group with total phenol content of 166.83±0.02 mg g⁻¹ had the highest amount among the plants in this study (Figure 2).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean Total phenol ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pb-5µM group</td>
<td>42.88±0.2</td>
</tr>
<tr>
<td>Pb-10µM group</td>
<td>38.86±0.05</td>
</tr>
<tr>
<td>Non-Pb group</td>
<td>166.83±0.02</td>
</tr>
</tbody>
</table>

Each value in the table was obtained by calculating the average of three experiments ± standard deviation.

![Fig. 2: Significant reduction of total phenol(mg/g GA) of under treatment groups with Pb 5 and 10µM in compared with non-Pb group. Bars are least significant differences where p < 0.05.](image)

When lead concentration was increased in a treatment, the flavonoid and flavonol content were significantly decreased compared to control group (P<0.05). So that when Pb concentration was increased, the amount of polyphenol content was declined, but this reduction was not significant (Figure 3,4).

The flavonoid content of the extracts in terms of rutin equivalent (the standard curve equation: y = 0.0603x + 0.0007, r² = 0.985) were between 2.625 ± 0.001 and 5.975 ± 0.028 (Table 2). The flavonoid content in the extracts of under treatment with Pb 5 µM (3.106 ± 0.013 mg g⁻¹) and under treatment with Pb 10 µM (2.625 ± 0.001 mg g⁻¹) were lower than that in the extract of Non-Pb(5.975 ± 0.028 mg g⁻¹) (Figure 5). Table 2 also show the content of total flavonol that were measured in terms of rutin equivalent (standard curve equation: y =
0.0603x + 0.0007, \( r^2 = 0.985 \)). The total flavonol varied from 1.71 ± 0.03 to 3.30 ± 0.01 mg g\(^{-1}\) in the extract powder. Non-Pb group with total flavonol content of 3.30±0.01 mg g\(^{-1}\) had the highest amount among the plants in this study (Figure 6).

Fig. 3: Significant reduction of flavonoid content (concentration and absorbance) of under treatment groups with Pb 5 and 10µM in compared with non-Pb group. Bars are least significant differences where \( p < 0.05 \).

Table 2: Flavonoid content in the studied plant extracts.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean Total Flavonoid ±SD</th>
<th>Mean Total Flavonol ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pb-5µM group</td>
<td>3.106±0.013</td>
<td>2.06±0.02</td>
</tr>
<tr>
<td>Pb-10µM group</td>
<td>2.625±0.001</td>
<td>1.71±0.03</td>
</tr>
<tr>
<td>Non-Pb group</td>
<td>5.975±0.028</td>
<td>3.30±0.01</td>
</tr>
</tbody>
</table>

Each value in the table was obtained by calculating the average of three experiments ± standard deviation.

Fig. 4: Significant reduction of flavonol content (concentration and absorbance) of under treatment groups with Pb 5 and 10µM in compared with non-Pb group. Bars are least significant differences where \( p < 0.05 \).

Fig. 5: Significant reduction of total flavonoid (mg/g RTA) of under treatment groups with Pb 5 and 10µM in compared with non-Pb group. Bars are least significant differences where \( p < 0.05 \).

Figure 7 shows the amount of each extract needed for 50% inhibition (IC50). IC50 of the standard compounds, Ascorbic acid were 58.85 µg/µl. The highest radical scavenging activity was showed by non-Pb group with IC50=61.72 which is nearby of Ascorbic acid. The radical scavenging activity in the plant extracts decreased in the following plants: under treatment groups with Pb 5 and 10µM(Figure 8).

Increasing concentrations of lead, significantly (\( P <0.05 \)) decreased peroxidase enzyme activity (Figure 9), whereas polyphenoloxidase enzyme activity under different lead treatments were increased, but this different were not significant (Figure 10).
Fig. 6: Significant reduction of total flavonol (mg/g RTA) of under treatment groups with Pb 5 and 10µM in compared with non-Pb group. Bars are least significant differences where p < 0.05.

Fig. 7: IC50 (µg/µl−1) values of plant extracts for free radical scavenging activity by DPPH radical. Lower IC50 value indicates higher antioxidant activity.

Fig. 8: Significant increase of antioxidant activity (concentration and absorbance) of under treatment groups with Pb 5 and 10µM in compared with non-Pb group. Bars are least significant differences where p < 0.05.

Fig. 9: Significant reduction of peroxidase enzyme (time and absorbance) of under treatment groups with Pb 5 and 10µM in compared with non-Pb group. Bars are least significant differences where p < 0.05.
**Fig. 10:** Significant increase of polyphenol oxidase enzyme (time and absorbance) of under treatment groups with Pb 5 and 10µM in compared with non-Pb group.

**Discussion:**

According to the results under different treatments of lead in the study, total phenol, flavonoid and flavonol content, antioxidant activity and peroxidase enzyme activity significantly decreased as compared to the control (P<0.05). On the other hand, polyphenoloxidase enzyme activity were increased. This results were similar with results of Yoshimura et al. [35], they reported that high concentrations of heavy metals can decrease activity of antioxidant enzymes.

Heavy metals Cd, Pb, Al, Zn, and Cu induce oxidative stress in plant species [6,20,28]. The most of Pb toxicity in plants is retard growth and alteration in the activity of many key enzymes of various metabolic pathways [23,1]. the mechanisms of ROS production by metals such as Pb, Cd, and Zn ions is less clear, and perhaps mediated, for example, by activation of lipoxygenase or binding to membrane proteins, thus precipitate electron leakages responsible for formation of ROS [27].

The antioxidant enzymes are main cellular defense system against oxidative stress [12,19]. Pb stress in the present study resulted in a high activity of polyphenoloxidase in the plant, the high polyphenoloxidase activity could possibly be the result of both a direct effect of Pb stress in plant tissues and an indirect effect mediated via an increase in levels of O$_2^*$ [6].

The peroxidase regulation as a response of the plant to pollutants can already be used for the phytomonitoring of industrial or thickly urbanised areas. Peroxidases have been shown to be totally sensitive to pollution [34].

Arora et al. [2] show that phenolics (especially flavonoids) are able to alter peroxidation kinetics by changing the lipid packing order. They stabilize membranes by decreasing membrane fluidity and hinder the spread of free radicals and limit peroxidative reaction [2,3].

Antioxidant activity of phenolic compounds is due to their high trend to chelate metals. Phenolics possess hydroxyl and carboxyl groups, able to bind especially heavy metals [17].

Change in soluble phenolics such as mediator in lignin biosynthesis can reflect the typical anatomical change induced by stressors; increase in cell wall tolerance and the creation of physical barriers preventing calls against harmful activity of heavy metals [8]. In recent years there has been a growing interest in antioxidant properties of phenolic compounds.

**Conclusion:**

Heavy metals, especially lead, are a toxic factor limiting crop production. The present study demonstrated that in plants under treatment with Pb, amount of phenol, flavonoid and flavonol compounds and antioxidant activity significantly were decreased but a non-significant increase was observed in polyphenol oxidase activity, whereas peroxidase was showed a significant decrease. These changes were related to many factors including metal concentrations, plant species and plant tissues. It has now become clear that high to moderate doses of lead exposure cause generation of free radicals resulting in oxidative damage to final biomolecules, lipids, proteins and DNA.

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**REFERENCES**


