Effect of Pb toxicity on growth and antioxidant status in *Trigonella foenum graceum*

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**ABSTRACT**

In the present study, the lead phytoaccumulation and its effects on the growth and antioxidant status were determined in *Trigonella foenum graceum*. The phytotoxicity was studied in the germinating seeds under the exposure to increasing concentrations of lead acetate (25, 50, 100, 200 µM). Although there was a decrease in the germination percentage of the seeds to increasing concentrations, normal growth was observed after germination. The plant was capable of tolerating various concentrations of lead revealed by its vigorous growth after the 16th day of treatment. Pb caused significant reductions in the chlorophyll content, protein content and induced changes in the antioxidant enzymes .Pb decreased the activity of SOD and Catalase while the activity of GPx and LPO were induced significantly after Pb exposure. The plant expressed higher tolerance to Pb which is mainly attributed by its normal growth and physiology.

**Keywords**

Phytoaccumulation, Phytotoxicity, Germination.

**Introduction**

Heavy metals are important environmental pollutants and many of them are toxic even at very low concentrations. Lead (Pb) is one of the major heavy metals of the antiquity and has gained considerable importance as a potent environmental pollutant. Lead (Pb) was noted as primary contaminant due to the ability to disperse across environmental multimedia. (Cunningham, S.D. and W.R. Berti, 1993.) (USEPA, 1993). Significant increases in the Pb content of cultivated soils have been observed near industrial areas. Pb tends to accumulate in the surface ground layer and its concentration decreases with soil depth. It is easily taken up by plants from the soil and is accumulated in different organs. Pb is considered a general protoplasmic poison, which is cumulative, slow acting and subtle. Soils contaminated with Pb causes sharp decreases in crop productivity thereby posing a serious problem for agriculture (Freitas et al., 2008).

The major factor limiting the potential for Pb Phytoextraction is low metal bioavailability for plant uptake. To overcome this limitation, synthetic chelators have been proposed to be added to the soil to increase the amount of available Pb (United States Environmental Protection Agency 2000a, b). Once introduced into the soil matrix, Pb is very difficult to remove. The capacity of the soil to adsorb Pb increases with increasing pH, cation exchange capacity, organic carbon content, soil/ water Eh n(redox potential) and phosphate levels (USEPA 1992). Pb hyperaccumulation is particularly rare and restricted only to several species grown in soil or solution (Xiong, 1998; Baker et al., 2000; Sali et al., 2002; Tamura et al., 2005), since Pb prefers to complex with natural organic matter, absorb on clays and oxides, and precipitate as carbonates, hydroxides and phosphates, which limits its solubility in soil (Epstein et al., 1999). The application of certain chelating agents, such as ethylenediaminetetraacetic acid (EDTA), to soils is a way to increase Pb solubility and thus the translocation of Pb from the soils into the plant shoots. This method provides a possibility for an efficient Pb Phytoextraction, although it simultaneously increases the risk of leaching Pb into groundwater (Huang and Cunningham, 1996; Huang et al., 1997; Blaylock et al., 1997; Epstein et al., 1999; Wu et al., 2004).

Strong binding of Pb to the carboxyl groups of carbohydrate in cell walls leads to its diminished transport via apopl ast. An electron microscopic study of root tips from tolerant plants reveals the presence of Pb in the cell wall as well as the cytoplasm. Within the cell, the major part of Pb is sequestered in the vacuole in the form of complexes. This may represent another mechanism of Pb detoxification in plants. Pinocytosis is observed in leaf cells of many plants treated with Pb salt solution. Through pinocytotic vesicles, Pb particles could be discharged into the vacuole (Wierzbica and Antosiewicz, 1993). Accumulation of excess total amino acid in response to Pb can be regarded as an important adaptive response of plants to avoid Pb toxicity( Thanbhorn et al., 2006).

Lead is widely known to be a non-essential element for plants, and can cause adverse effect on the plant’s photosynthesis, chlorophyll synthesis and antioxidant enzymes, resulting in various symptoms of phytotoxicity, such as chlorosis, reduction of biomass, inhibition of root elongation and finally death (Milone et al., 2003). Plant species have developed two basic strategies to resist the toxicity of heavy metals: avoidance and accumulation (Baker, 1987). Most heavy metal-tolerant species have the capability of preventing heavy metal accumulation in their shoots and therefore are called ‘excluders’, while others can take up heavy metals, translocate them into the shoots, and sequester them in non-metabolic-active tissues and organs in less harmful forms (Kupper et al., 2007).

*Trigonella foenum graceum* belongs to the family fabaceae which has been proved to be have different hyperaccumulators. The crop is of significant commercial and nutrient value. In the...
study, we investigated responses of the plant to varied supply levels of Pb including the Pb concentration in plant tissues, antioxidative enzymes, and chlorophyll content in pot culture. The objectives of the present study were to identify the capability investigate the effects of Pb on normal growth and physiology and the mechanisms involved in the detoxification of Pb by Trigonella foenum graceum.

1. Materials and methods
Trigonella foenum graceum seeds were obtained from Tamilnadu Agricultural University, Coimbatore. The seeds were surface sterilized using 0.1% sodium hypochlorite solution and 0.1% mercuric chloride for 10min and rinsed with double distilled water. Seeds were sown in earth pots (30 cm × 25 cm, diameter and depth) containing red soil and farmyard manure in 3:1 proportion. The seeds were randomly placed in the pots. Each pot contained 20 seedlings and made triplicates for the control as well as for different concentrations of lead. They were kept under natural photo radiation with a photoperiod of 12 hours at 200μmol/m²/s and at a temperature of 25 ± 2°C and 40 ± 5% relative humidity. The seeds were initially treated with four different concentrations of lead (lead acetate solutions 0 (control), 25, 50, 100 and 200 μmol/L) in separate pots, till the maximum growth of the crop was observed and the observations were recorded (triplicates). The solutions used for the treatments were 30 ml for each pot and four different chosen concentrations. Totally the solutions were added 8 times during the experimental period. The soil pH was 7.58. The seeds were treated with four different Pb concentrations once every three days with the above solutions. The duration of exposure to the heavy metal was increased to 23 days and the growth was observed.

1.1 Seed germination test
Different concentrations of lead acetate solutions were exposed to the germinating seeds. The number of germinated seeds on the day three and seven after initiation were germination energy and germination percentage respectively. After three days, the shoot length was measured from culms base to the tip of the longest leaf.

Germination index (GI) and vitality index (VI) were calculated using the following equation.

\[ \text{GI} = \Sigma (G/D) \times S \]
\[ \text{VI} = \Sigma (G/D) \times S \]
\[ G_t = \text{germination rate at day} \]
\[ D_t = \text{day t} \]
\[ S = \text{Shoot length} \]

The germination index was calculated according to the method of Ana et al., (2004).

1.2 Estimation of plant growth and biochemical parameters
The plant growth was observed and recorded once every three days. The plant growth parameters including the plant height, the morphology of the leaf and leaf size were measured using an mm scale. The control group was grown normally without the addition of lead acetate. The duration of exposure to the heavy metal was increased to 23 days and the growth was observed.

1.3 Analysis of chlorophyll-α, chlorophyll-β, total chlorophyll, protein
The leaves were washed with distilled water and then ground using mortar and pestle for physiological and biochemical studies. Chlorophyll content in the leaves (1000 mg) of treated and control plants were extracted in 80% chilled acetone and estimated by the method of Arnon (Arnon, 1949) and determined the absorbance of the solution at 645, 663, and 652 nm against the solvent (80%) blank, using spectrophotometer (UV-Vis double beam spectrophotometer 118, Systronics, India). The Chlorophyll content was expressed as mg/g fw (fresh weight).

The Protein content in the leaves (1000 mg) were extracted in with buffers used for enzymes assay, grind well the samples with a pestle and mortar in 5–10 ml of buffer and centrifuge, the supernatant for protein was measured at 660 nm by Lowry’s method (Lowry et al., 1951) using bovine serum albumin (BSA) as the standard protein. The total Protein content was expressed as mg/g fw.

1.4 Effect of Pb on antioxidant status
1.4.1 Enzymic antioxidant system
The plant material (1000mg) was homogenized in 100mM chilled potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% polyvinyl pyrrolidine (w/v) at 4°C. The homogenate was squeezed through four layers of cheese cloth and the extract obtained was centrifuged at 10000 X for 15 mins at 4°C. The supernatant was used to measure the activities of antioxidative enzymes.

The Catalase activity was assayed according to the method of Sinha (Sinha, 1972). The assay mixture contained 0.5 ml of H₂O₂, 1.0 ml of 0.01 M phosphate buffer, pH 7.0 and 0.4 ml of water along with 1.0 ml of the newly prepared leaf homogenate. 0.2 ml of the enzyme was added to initiate the reaction. 2.0 ml of the dichromate/acetic acid reagent was added after 0, 30, 60, 90 seconds of incubation. The colour developed was read at 610 nm. The activity of Catalase was expressed as μmol H₂O₂ oxidised/mg of protein/min.

The activity of Superoxide dismutase was ascertained using the nitrite method of Das et al., (2000). About 100 mg leaves from freshly uprooted plants were extracted using chilled mortar and pestle in 5 ml of 50 mmol/L K-phosphate buffer (pH 7.4) containing 1% (v/v) triton X-100, 10 mM hydroxylamine hydrochloride and 50 μM EDTA. One unit of the enzyme activity was defined as the amount of SOD capable of inhibiting 50% of nitrite formation under the assay conditions. The activity of SOD was expressed as units/ μg protein min.

The activity of Lipid peroxidase was determined as 2-thiobarbituric acid reactive substances (TBARS) by using the standard methods of Okhawa et al., (1979) with minor modifications. For the measurement of the level of lipid peroxidation in plant leaves, the extraction was done in 5 ml of 0.02 M Tris HCl Buffer (pH 7.5). 3 ml of the reaction mixture contained thoroughly mixed TBA-TCA-HCl reagent. The absorbance of chromophore was read at 535 nm. The results are expressed as units of MDA/g of tissue.

Glutathione Peroxidase was assayed according to the method of Rotruck et al., (1973). 4.0 ml of the reaction mixture contained 0.4 M sodium phosphate buffer (pH 7.0), 10 mM sodium azide, 2.5 mM hydrogen peroxide and 4 mM reduced glutathione and a suitable aliquot of enzyme. The increase in the absorbance was read at 412 nm. The activity of GPx was expressed as μg glutathione utilized/min/mg protein.

2.0 Statistical analysis
All the measurements were replicated three times. Average values and the standard deviations (S.D) were calculated by the Microsoft Office Excel 2007 for all the data in this paper. To confirm the variability of the data obtained and validity of results, all the data were subjected for the statistical significance with the package SPSS 11.5 using one way Analysis of Variance.
(ANOVA) and the significance of difference between sample means were calculated. The significant difference was set between treatments as p<0.05 or p<0.01.

**Results and Discussion**

The percentage of germination may reflect the reaction rate of plant seeds to their living environment. Germination index (GI) and vitality index (VI) are two important parameters that reflect the seed quality. From table 1 it can be seen that Germination Index and Vitality Index decreased with the increase of Pb concentrations.

Digitals in the brackets represent the percentage compared to the control (%),
a- Comparison between the control and 25µM
b- Comparison between 25µM and 50 µM
c- Comparison between 50µM and 100 µM
d- Comparison between 100µM and 200 µM
e- Comparison between control and 200 µM
* - Indicates significant difference at p<0.01.

**Physiological and biochemical effects**

**Effect on the plant growth**

In this study, the experimental evidence shows that *T.foenum graceum* was well tolerant to the lead in the soil and grew healthy. The plant showed increased growth response after an exposure of 16 days, the growth response being the increase in the plant height and increase in the leaf area as compared to the control group (Fig. 1). The accumulation of metals in various parts of higher plants is often accompanied by an induction of variety of cellular changes, some of which directly contribute to metal tolerance capacity of plants (Devi and Prasad, 1998; Hall, 2002).

![Fig. 1: The growth pattern and height of T.foenum graceum under different concentrations of lead.](image1)

**Effect on photosynthetic pigments**

Photosynthetic surface area and leaf chlorophyll contents are the main key factors determining the dry matter production in plants. An increase in chlorophyll-a and total chlorophyll content was recorded in leaves of *T.foenum graceum* at 50µM but chlorophyll b showed decreased concentrations. The highest concentrations of chlorophyll a and (a + b) were observed at 100µM Pb, which differed significantly from those at 200µM Pb ($P < 0.05$). However, the concentration of chlorophyll b was not statistically different among all Pb treatments ($P > 0.05$). (Fig. 2).

![Fig. 2. Effects of Pb on the concentrations of chlorophyll a, b and (a + b) of T.foenum graecum. Different letters in the same type of column are significantly different at $P < 0.05$ level. Bars represents (N=3).](image2)

**Effect on protein content**

The protein content in the plant leaves showed significantly increased concentrations in 200 µM lead acetate (Fig. 3). From the experimental evidence it was observed that as the concentration of lead increases the protein content also increases. A steady increase in the total protein content was observed in all the plants grown under lead treatment with different concentrations of lead acetate. Compared to the control, 25µM treated plant had 88% less protein content. Other concentrations of protein are 99%, 103%, 109% protein content for 50 µM, 100 µM and 200µM treated plant respectively. Lead induced increment in the total protein content was observed in other ecotypes during treatment with increasing concentrations of lead (Ejazul Islam et al., 2008).

![Fig. 3. Effect of lead on the protein content](image3)

**Enzymic Antioxidants**

The values are expressed in mean ± SD (n=3). The means are followed by a common letter
1- Inhibition of 50% nitrite formation/min/mg protein
2- 1µM of $H_2O_2$ decomposed/min/mg protein
3- 1 µM of glutathione consumed/min/mg protein
4- nanomoles of MDA/g tissue.

* - the values are significant at 1 % level of significance
# - the values are non significant at 5% level of significance

From the table, it is evident that the activity of Superoxide dismutase and Catalase significantly decreased among the groups. Glutathione peroxidase showed an increase in activity as compared to the control.
The activity of lipid peroxidation also significantly increased as the concentration of lead increased. The activity of SOD and Catalase was found to be declining in *Elsholtzia argyi* during lead treatment (Ejazul Islam et al., 2008).

Catalase is an iron containing enzyme catalyzing the dismutation of \( \text{H}_2\text{O}_2 \) into \( \text{O}_2 \) and \( \text{H}_2\text{O} \). It is a major antioxidant enzyme in curtailing the peroxidative damage in a biological system. The enzyme is found in all aerobic eukaryotes and is important in the removal of \( \text{H}_2\text{O}_2 \) generated in the peroxisomes by oxidases (Redinbaugh et al., 1988) and in the glyoxylate cycle and purine catabolism. Various isofoms are also reported in cytosol and mitochondria. Stress conditions such as salinity, heat, shock or cold causes the depletion of Catalase activity (Marc Dazy et al., 2008). The activity of lipid peroxidation increases significantly in response to the stress due to excess lead. It was concluded that *Trigonella foenum graecum* could tolerate and accumulate high concentrations of lead and it could be regarded as a potential accumulator with respect to the growth pattern observed under increasing concentrations of lead in the soil. The plant in view of its fast and indeterminate growth and high accumulation potential seems to be a suitable candidate to decontaminate landfills moderately contaminated with lead. Longer field experiments are required to verify the real tolerance to lead of *Trigonella foenum graecum*.

### Table 1: Changes of germination index and vitality index at different lead concentrations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>25µM</th>
<th>50 µM</th>
<th>100 µM</th>
<th>200 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germination index</td>
<td>12.56±0.51 (100%)</td>
<td>11.08±0.45 (88.2%)</td>
<td>10.76±0.46 (85.67%)</td>
<td>10.23±0.54 (81.45%)</td>
<td>9.45±0.49 (75.24%)</td>
</tr>
<tr>
<td>Vitality index</td>
<td>50.80±0.23 (100%)</td>
<td>48.21±0.28 (94.90%)</td>
<td>41.01±0.21 (80.73%)</td>
<td>38.65±0.32 (76.08%)</td>
<td>37.33±0.29 (73.48%)</td>
</tr>
</tbody>
</table>

### Table 2: Changes of antioxidative enzymes activities at different lead concentrations

<table>
<thead>
<tr>
<th>Concentration</th>
<th>SOD ( ^\text{a} )</th>
<th>CAT ( ^\text{b} )</th>
<th>GPx ( ^\text{c} )</th>
<th>LPO ( ^\text{d} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.81±0.05a</td>
<td>2.80±0.34a</td>
<td>0.05±0.002a</td>
<td>0.62±0.05a</td>
</tr>
<tr>
<td>25µg</td>
<td>3.05±0.14b</td>
<td>2.52±0.19b</td>
<td>0.19±0.002b</td>
<td>1.03±0.10b</td>
</tr>
<tr>
<td>50µg</td>
<td>2.04±0.06c</td>
<td>1.48±0.19c</td>
<td>0.22±0.004c</td>
<td>1.10±0.09c</td>
</tr>
<tr>
<td>100µg</td>
<td>1.16±0.05d</td>
<td>1.92±0.12d</td>
<td>0.37±0.002d</td>
<td>1.13±0.05d</td>
</tr>
<tr>
<td>200µg</td>
<td>0.16±0.01e</td>
<td>1.90±0.24e</td>
<td>0.46±0.014e</td>
<td>1.32±0.08e</td>
</tr>
</tbody>
</table>

**Conclusion**

Lead is one of the toxic heavy metal and exerts adverse effects on morphology, growth and photosynthetic processes of plants and causes inhibition of enzyme activities, water imbalance, alterations in membrane permeability and disturbs mineral nutrition. Some plants have developed special metal detoxification mechanisms, which allow them to grow in the soils having elevated levels of Lead and other heavy metals (Singh et al., 1997; Sharmaha et al., 2005).

The data presented in the study have demonstrated that the lead treatment in *Trigonella foenum graecum* impairs the chlorophrill, protein and the antioxidant defense system. This suggested that the exposure of the plant to lead resulted in ROS release in the plant cells leading to the induction of various oxidative components under these environmental stresses.

### References


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