

Lead Accumulation and its Effects on Growth and Biochemical Parameters in *Tagetes erecta* L.

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ABSTRACT- *Tagetes erecta* L. was raised in pots containing soil treated with various concentrations of Pb(NO₃)₂ (500, 1000, 1500, 2000 and 2500mg/kg). At maturity plants were separated into root, stem, leaves and inflorescence and lead accumulated in each part was quantified. The effects of lead accumulation on growth was analyzed by the measurement of various growth parameters like root and shoot length, fresh and dry weight of root and shoot and total leaf area per plant. Moreover effect of lead accumulation on biochemical parameters was checked by quantitative estimation of various biochemical parameters like chlorophyll, total protein, free amino acids, total sugar, reducing sugar and starch. Results showed that there is no remarkable negative effect of accumulation of lead on the morphological growth of the plant. Biochemical analysis showed that amount of total protein continuously decreased whereas that of free amino acids continuously increased with increasing concentrations of lead. Amount of chlorophyll, total sugar, reducing sugar and starch contents continuously increased till mid-level i.e., Pb 1500 mg/kg and then continuously decreased at higher concentrations. Results of quantitative estimation of Pb in root, stem, leaves and inflorescence showed that roots accumulated highest amount of Pb followed by stem and leaves, whereas inflorescence contained least amount of Pb.

Key-words- Lead (Pb), Heavy metal, *Tagetes erecta* L., Accumulation, Phytoremediation

INTRODUCTION

Heavy metal pollution is one of the severe problems faced by the world today. As a consequence of the industrial revolution there is an enormous and increasing demand for heavy metals that leads to high anthropogenic emission of heavy metals in the biosphere.^[1] Their accumulation in soil becomes dangerous to all kind of organisms including plants.^[2] These are a unique class of toxicants since they cannot be broken down to non-toxic forms easily. Metals like Pb, Hg, Cd, Ar, and Cr have no biological function and are toxic to life even at very low concentration.^[3]

Lead is a potentially toxic heavy metal with characteristic toxic action and is the main source of environmental pollution. Lead is one of the potentially toxic heavy metal pollutants of the environment with no known biological function and its concentrations are rapidly increased in

agricultural soil.^[4] Naturally, Pb is present in soil, sea water, lakes and rivers. Besides natural sources, exhaust fumes of automobiles, chimneys of factories, mining, effluents from storage battery, smelting of Pb ores, fertilizers, additives in pigments, metal plating and pesticides are also major sources of Pb.^[5] It is also a component of lead batteries, rubber, paints, metal products (steel and brass) and dusts.^[6] Elevated Pb in soils may compromise soil productivity and even a very low concentration can inhibit some vital plant processes, such as photosynthesis, mitosis and water absorption with toxic symptoms of dark leaves, wilting of older leaves, stunted foliage and brown short roots.^[7-8]

MATERIALS AND METHODS

Pot Culture Experiments

The pot culture experiments were conducted in the Botanical garden, Gujarat University, Ahmedabad, India. Plants were grown in 6 groups of pots. Each group contained 21 pots and each pot was filled with 6kg air-dried soil. One group was filled with untreated soil (i.e. control) whereas the other 5 groups were treated with different concentrations of Pb(NO₃)₂ i.e. 500, 1000, 1500, 2000 and 2500mg/kg respectively. Healthy seeds were sown. Plants

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were irrigated regularly. At maturity plants were analyzed for following parameters.

Growth Analysis

At maturity, various morphological parameters such as root length, shoot length, total leaf area and fresh and dry weight of root and shoot per plant were determined for every sample to check the effect of Pb accumulation on above mentioned parameters. Root and shoot length were measured with meter rule. Fresh weight of root and shoot were measured with analytical balance. Plants were dried in hot air oven at 80 °C for 48 hours or until the constant dry weight was attained. The total leaf area was measured by leaf area meter. For five replicates, Average and standard deviation (S.D.) were calculated. Results were expressed as mean±S.D.

Biochemical Analysis

Effects of Pb accumulation on some biochemical parameters such as total and reducing sugar, starch, total protein, free amino acids and chlorophyll were analyzed. The biochemical methods used for estimation of above mentioned parameters are as under. Three replicates were prepared and analyzed from each treatment to analyze each parameter. Results were expressed as mean ± Standard Deviation (S.D.).

Estimation of chlorophyll^[9]

One gram of fresh leaves were ground in a mortar and pestle with 20 ml of 80% acetone. The homogenate was filtered through whatman no. 42 filter paper. The filtrate was saved. The residue was again extracted with 5ml of 80% acetone each time, until it became colourless. All the filtrates were pooled and final volume was made 50ml by adding 80% acetone. This filtrate was utilized for chlorophyll determination. Absorbance was read at 645nm and 663nm in spectrophotometer. The chlorophyll a and chlorophyll b contents were measured by using the formula given by Arnon.^[9]

$$\text{Chlorophyll a (mg/g)} = (12.7 A_{663} - 2.69 A_{645}) / (1000 \times W) \times V$$

$$\text{Chlorophyll b (mg/g)} = (22.9 A_{645} - 4.68 A_{663}) / (1000 \times W) \times V$$

Where, V = Volume of extract (ml), W = fresh weight of the leaf sample (gram)

Estimation of protein^[10]

Fresh tissue weighing 0.5 g was macerated in 10ml cold distilled water followed by 20ml of 10% trichloroacetic acid in mortar and pestle. Homogenate was then centrifuged at 600 rpm for 30 min and the supernatant was discarded. 10 ml of 0.1 N NaOH was added to the pellet and it was centrifuged for 30 min. The supernatant was used for the estimation of protein. One ml of the extract was added with 5 ml of copper reagent 'C' (Reagent C: mixture of reagents A and B in 50:1 ratio; Reagent A: 2% Na₂CO₃ in 0.1 N NaOH; Reagent B: 0.5% CuSO₄ in 1%

sodium potassium tartrate). The tubes were shaken well and incubated for 10 min at room temperature, 0.5 ml of properly diluted Folin-Ciocalteu reagent was added to the solution and mixed thoroughly. The absorbance was read at 660 nm in a spectrophotometer against an appropriate blank. Bovin serum albumin was used as the standard.

Estimation of Total Free Amino Acids^[11]

Sample was prepared by extraction with 80% ethanol. Repeated homogenization and centrifugation were done and supernatants were mixed and used as a sample to determine free amino acids. Total free amino acids were estimated using 2% ninhydrin reagent. This reagent was prepared by mixing the following constituents (A, B and C) in the ratio of 5: 12: 2; (A) 1% ninhydrin in 0.5 M citrate buffer (pH 5.5); (B) pure glycerol; (C) 0.5 M citrate buffer (pH 5.5). To 0.2 ml of extract 3.8 ml of ninhydrin reagent was added. The contents were heated in boiling water bath for 12 min and cooled to room temperature. The optical density of purplish blue coloured resultant solution was measured at 570 nm. Glycine was used as the standard.

Estimation of Total Sugar^[12]

Plant extract was prepared using 80% ethanol. To 1ml of alcoholic aliquot, 1ml 1N H₂SO₄ was added and heated at 49 °C in water bath for 30 minutes for hydrolysis of the mixture. 2-3 drops of methyl red indicator were added followed by addition of 1N NaOH drop wise for neutralization (colour change: pink to yellow). Then 1ml Nelson Somogyi's reagent was added to it and the test tube was kept in boiling water bath for 20 minutes. After cooling off the test tube, 1ml arsenomolybdate was added and final volume was made up to 20ml with distilled water. O.D. was noted at 540nm. Blank was prepared in the same manner. Glucose was used as the standard.

Estimation of Reducing Sugar^[12]

Plant extract was prepared using 80% ethanol. To 1ml of this alcoholic extract, 1ml Nelson Somogyi's reagent was added and kept in boiling waterbath for 20min. After cooling off the test tube, 1ml arsenomolybdate was added and final volume was made up to 20ml with distilled water. Optical density was noted at 540nm. Blank was prepared in the same manner. Glucose was used as standard.

Estimation of Starch^[13]

Sample was prepared by extraction with 80% ethanol. Repeated homogenization and centrifugation were done and residue left at all stages were mixed and used as a sample to determine starch content. The residue was dissolved in 20ml 0.7% KOH and boiled for 40 minutes for gelatinization. It was allowed to cool down and then centrifuged. Supernatant was used for further analysis. 1ml aliquot (Supernatant) was added with 0.5ml 20% acetic acid; 1ml citrate buffer (0.05M, pH 5.0) and 1ml I₂KI and incubated at room temperature for 10minutes. Optical density was measured at 600nm. Blank was prepared in the same manner. Starch was used as standard.

Estimation of Lead (Pb) Content ^[14]

At maturity plants were uprooted from the pots. Plants grown in each concentration were separated into root, stem, leaves and inflorescence and were dried in hot air oven at 80 °C for 48 hours. Each dried plant part of each concentration was powdered thoroughly and used for analysis. 1g dry powder of each sample was weighed into a conical flask and 10ml concentrated HNO₃ was added. The mixture was boiled at a constant temperature for about 45mins. After cooling, 5ml of 70% HClO₄ was added and the mixture was further boiled until the release of dense

white fumes. After cooling, 20ml distilled water was added and heated until a clear solution was obtained. At room temperature, the mixture was filtered through Whatman no. 44 filter paper and transferred quantitatively to a 50ml volumetric flask by adding de-ionized and double-distilled water. Samples were analyzed through Atomic Absorption Spectrophotometer.

STATISTICAL ANALYSIS

One way ANOVA was conducted to compare the means of different treatments at p<0.05 level of significance.

RESULTS AND DISCUSSION

Accumulation of Pb in roots, stem, leaves and inflorescence are depicted in Table 1.

Table 1: Accumulation of Pb in root, stem, leaf and inflorescence of *Tagetes erecta* L.

Pb in Plant Part/ Treatment	Pb in Root mg/kg	Pb in Stem mg/kg	Pb in Leaf mg/kg	Pb in Inflorescence mg/kg
Control	92.75*±7.84	64.07*±5.83	52.57*±4.58	42.08*±3.45
500 mg/kg	1176.16*±52.68	433.80*±37.71	174.06*±15.27	136.16*±10.56
1000mg/kg	1511.12*±74.15	567.44*±50.46	343.08*±24.12	161.60*±12.74
1500mg/kg	1687.93*±69.07	629.73*±43.66	370.32*±22.50	190.75*±16.42
2000mg/kg	1920.84*±81.23	670.52*±62.87	487.71*±31.39	203.66*±19.63
2500mg/kg	1954.35*±90.46	749.55*±70.06	618.28*±53.82	255.08*±23.40

Values are mean±S.D. (n=3), * indicate probability level of significant difference at p<0.05. Replicate (n) = 3

Lead content in plant parts gradually increased by increasing lead concentrations in soil. The results related to the uptake of Pb in this study suggest that roots of *T. erecta* are efficient barriers to Pb translocation to the above ground plant parts. Roots were storing highest amount of Pb followed by stem, leaves and inflorescence respectively. Our results corroborate with the report of higher accumulation of Pb in roots compared to shoots in *Trigonella foenum-graecum* L.^[15] Pb retention in the roots is based on binding of Pb to ion exchangeable sites on the cell wall and extracellular precipitation, mainly in the form of Pb carbonate deposited in the cell wall.^[16] Furthermore, the root cell walls are first target for metals ion in soil solution. The integration of the metal ions in to the cell wall has been noted in several papers.^[17] Once lead has penetrated into the root system, it may accumulate there or may be translocated to aerial plant parts. For most plant species, the majority of absorbed lead is accumulated in the roots, and only a small fraction is translocated to aerial plant parts, as has been reported in *Nicotiana tabacum*,^[18] *Lathyrus sativus*^[19] and *Zea mays*.^[20] There are several reasons why the transport of lead from roots to aerial plant parts is limited. Some of these reasons include immobilization by negatively charged pectins within the cell wall^[21] precipitation of insoluble lead salts in intercellular spaces^[22] accumulation in plasma membranes

^[23] or sequestration in the vacuoles of rhizodermal and cortical cells.^[23] Accumulation potential of *Tagetes erecta* L. was found higher at lower concentrations of Pb whereas at higher concentrations it decreases.

The growth parameters as affected by Pb treatments are shown in Table 2., which shows that different concentrations of Pb did not adversely affect the growth of *T. erecta* in terms of root length, shoot length, fresh and dry weight of root and shoot and leaf area but some minute non significant differences between treatment plants and control plants have been noticed. Although Pb at higher concentrations was found to have reduced level of all the morphological parameters studied. Root length and shoot length continuously decreased with increasing concentration of Pb. Same type of trend has been reported in which lead significantly reduced the root length and shoot length of rice seedlings, and that the degree of inhibition increased with the increase of Pb conc.^[24] Our results are in accordance with the findings of the report in which lead stress negatively affected the fresh and dry weight of shoots and roots with increasing Pb application in *Spinacea oleracea* L.^[25] Root growth is known to be more sensitive than shoot growth to metal toxicity.^[26] Our results also revealed the same trend in which lead treatments have more pronounced effect on fresh and dry weight of root compared to that of shoot.

Table 2: Effect of Pb accumulation on Morphological Parameters

Parameter /Treatment	Root Length (cm)	Shoot Length (cm)	Fresh Weight Root (g)	Fresh weight Shoot(g)	Dry weight Root (g)	Dry Weight Shoot (g)	Leaf Area (cm ²)
Control	13.0*±0.78	31.0*±1.42	3.53*±0.62	11.30*±0.87	0.673*±0.05	2.050*±0.20	87.13*±3.48
500 mg/kg	12.8*±1.24	30.8*±1.10	3.40*±0.44	11.23*±1.66	0.472*±0.02	1.956*±0.43	86.59*±5.01
1000mg/kg	12.8*±1.05	30.8*±0.82	3.34*±0.36	11.08*±0.86	0.455*±0.09	1.954*±0.30	86.37*±5.40
1500mg/kg	12.7*±0.73	30.6*±1.30	3.15*±0.78	10.93*±1.35	0.419*±0.07	1.937*±0.24	86.02*±3.79
2000mg/kg	12.6*±0.57	30.4*±1.05	2.97*±0.48	10.78*±1.24	0.407*±0.05	1.920*±0.41	85.67*±4.07
2500mg/kg	12.4*±1.28	30.0*±0.88	2.81*±0.60	10.48*±1.37	0.373*±0.07	1.900*±0.35	85.21*±4.29

Values are mean±S.D (n=5), * indicate probability level of significant difference at p<0.05. Replicate (n) = 5

The primary effect of Pb toxicity in plants is a rapid inhibition of root growth, probably due to the inhibition of cell division in the root tip.^[27] The decreased shoot and root biomass might be due to interference of Pb with the physiological processes of the plant, as Lead phytotoxicity involves the decrease of enzyme activities, disturbed mineral nutrition, water imbalance, alteration in hormonal status and variation in membrane permeability.^[16] The reduction in root growth by Pb toxicity is most possibly from the result of a non-selective suppression of both cell division and cell elongation of the seedlings.^[27-28]

Table 3 shows the results of changes in biochemical parameters in *T. erecta* due to Pb accumulation. Our results indicate that the exposition of *Tagetes erecta* L. to different concentrations of Pb results in an increase in sugar content at lower concentration whereas at higher concentrations their decrease was observed. Our results corroborate with the findings of increase in soluble sugars at low concentrations of salt stress and decrease at higher

concentrations in *Pisum sativum*.^[29] Same type of results were observed in which soluble sugar content increased at lower concentration whereas decreased at higher concentrations of Pb in *Lemna polyrrhiza* L.^[30] Same type of trend was reported in *Trigonella foenum-graceum* L. (Fenugreek) under the effect of Pb stress.^[15] At higher concentrations of Pb total sugar, reducing sugar and starch content decreased. The negative effect of heavy metals on carbon metabolism is a result of their possible interaction with the reactive center of ribulose biphosphate carboxylase.^[31]

The decrease in protein content as observed at increasing concentrations of Pb in *T. erecta* may be because of (i) enhanced protein degradation process as a result of increased protease activity^[32] which is found to increase under stress conditions (ii) various structural and functional modifications by the denaturation and fragmentation of proteins.^[30]

Table 3: Effect of Pb accumulation on Biochemical Parameters

Parameter /Treatment	Total Protein (mg/g)	Free Amino Acids (mg/g)	Total Sugar (mg/g)	Reducing Sugar (mg/g)	Starch (mg/g)	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total chlorophyll (mg/g)
Control	7.99*±0.75	5.99*±0.34	6.67*±0.52	4.90*±0.45	3.06*±0.19	0.761*±0.02	0.207*±0.01	0.968*±0.03
500 mg/kg	6.60*±0.50	6.49*±0.45	9.56*±1.07	5.49*±0.32	6.16*±0.25	0.923*±0.05	0.320*±0.02	1.243*±0.07
1000mg/kg	5.28*±0.62	7.17*±0.52	10.40*±1.31	5.94*±0.50	7.52*±0.32	0.954*±0.06	0.342*±0.03	1.296*±0.09
1500mg/kg	4.91*±0.25	7.79*±0.38	13.84*±1.65	7.29*±0.38	9.76*±0.78	0.997*±0.08	0.357*±0.01	1.354*±0.09
2000mg/kg	4.43*±0.20	8.13*±0.28	10.65*±1.03	4.95*±0.44	7.58*±0.56	0.917*±0.05	0.321*±0.02	1.238*±0.07
2500mg/kg	4.25*±0.11	9.05*±0.29	7.41*±0.56	3.89*±0.12	5.23*±0.17	0.643*±0.05	0.214*±0.01	0.857*±0.06

Values are mean±S.D (n=3), * indicate probability level of significant difference at p<0.05. Replicate (n) = 3

It is also likely that these heavy metals may have induced lipid peroxidation and fragmentation of proteins due to toxic effects of reactive oxygen species which led to reduced protein content. Decrease in protein content was reported with an increase in lead supply to *Trigonella foenum-graecum* L.^[15] Our studies coincide with the findings in which the same trend of continual decrease of protein content in *Phoenix dactylifera* L. was reported under Pb stress.^[33] In the present investigation content of free amino acids was increasing with increasing Pb concentrations in treated plants. The increase of free amino acid in plant cells could be attributed to the degradation of proteins, substantial increases of proteolysis enzymes such as protease have been reported in many plants exposed to heavy metals which led to reduce total soluble proteins and increase free amino acid.^[34-35]

Several types of pigments are present in plants such as chlorophylls, xanthophylls, carotenoids etc. Among these, chlorophylls are most abundant and important pigments in higher plants. These are responsible for photosynthesis as they capture light. In several cases, heavy metals are known to reduce the productivity by reducing the rate of photosynthesis. Our results suggest that lower concentrations of Pb marginally increased the chlorophyll (chl *a*, chl *b* and total chlorophyll) whereas at higher concentrations of Pb, Chlorophyll (chl *a*, chl *b* and total chlorophyll) started decreasing. Similar trend has been noticed in *Lemna polyrrhiza*.^[30] The decline in chlorophyll content in plants exposed to Pb²⁺ stress is believed to be due to: (a) inhibition of important enzymes, such as δ -aminolevulinic acid dehydratase (ALA dehydratase) and protochlorophyllide reductase^[36] associated with chlorophyll biosynthesis; (b) impairment in the supply of Mg²⁺ and Fe²⁺ required for the synthesis of chlorophylls^[37]; (c) Zn²⁺ deficiency resulting in inhibition of enzymes, such as carbonic anhydrase.^[36] Our results of decrease in chlorophyll content corroborate with the findings of decrease in chlorophyll content with heavy metal stress in *Zea mays* and *Acer rubrum*.^[38]

CONCLUSIONS

The study suggests that due to Pb accumulation in *Tagetes erecta* L., there was a very less negative effect on its growth parameters. Biochemical parameters were affected upto certain extent, but the plant showed quite good capability to accumulate Pb. So *Tagetes erecta* L. can be effectively used for the phytoremediation of the soils contaminated with lead.

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