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Biochemical Responses of Lead in Cowpea (*Vigna unguiculata* (L.) Walp.)

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Abstract: ***Soil contamination with persistent metal Lead (Pb) is a grave concern to be addressed at the global level, since it poses danger of entering in to the human food chain. The present study is an endeavor to assess the biochemical impact of Lead (Pb) on one commonly used vegetable, Cowpea (Vigna unguiculata (L.) Walp). Field studies were conducted from seed to seed to investigate the biochemical responses of cowpea to sole application of different levels of Pb (100µM, 500µM, 1mM, 5mM and 10mM). Applications of Pb caused accumulation Pb in root, stem, leaves and pod. The Pb storage in different regions were in the sequence of root >fruit>stem>leaves. Generally the higher doses of Pb clearly blocked and the lower doses accelerated the uptake and translocation of* Ca, Mg, Fe, Cu, Zn and Mn*. The total protein, total carbohydrate, number of stomata, stomatal index, length and width of stomata were positively affected by the lower levels of Pb (up to 500µM or 1mM and even 5mM of Pb) and negatively affected by its upper level (10mM). However, Pb adversely affected the leaf area in all its treatment.***

Keywords: *Vigna unguiculata;* Bioaccumulation, stomatal index, carbohydrate, Protein, leaf area

1. Introduction

Plants are the target of a wide range of pollutants that vary in concentration, speciation, and toxicity. Among common pollutants that affect plants, Lead (Pb) is one of the most abundant, toxic and frequently encountered pollutant (Shahid et al. 2011; Bharwana et al. 2014). The environmental Pb contamination attained prominence due to constant availability both in past and present by way of mining, smelting, combustion of gasoline, automotive exhaust fumes, effluents from Pb based industries such as paints, paper, pulp, storage batteries, alloys, solder, ceramics, plastics, petrol refining, halogenations, extraction, sulphonation, manufacture of pigments, insulation cables, household wiring, printing inks, glass (Krzesowska 2011) or applications of Pb - contaminated media (sewage, sludge, fertilizers and insecticides) to land (Sammut et al. 2010; Austruy et al. 2014).

Lead pollution results in extremely negative and unfavorable impacts on food production and human health. At the global scale, soil contamination with Pb induces a serious threat of entering into the human food chain. The contamination of the food chain with Pb, Cd, Cu, Ni, and Zn has become unavoidable owing to industrialization and the application of modern technologies (Shahid et al 2013, Venkatachalam, 2017). The uptake of toxic metals by vegetables causes human exposure to environmental pollutants (Niazi et al. 2011).

Lead contaminated soils show a sharp decline in crop productivity. Scientists have done extensive work on the morphological and physiological impact of Pb in plants and evaluated toxic effects on the different aspects such as, productivity and yield (Hussain et al. 2006, Zhang, 2017); growth tolerance index (Wojas et al. 2007); leaf area (Nosalewicz et al. 2008); root, shoot and leaf fresh biomass (Krystofova et al. 2009); inhibition of root elongation (Ghani et al. 2010); plant height (Farooqi et al. 2011); dry biomass (Yasin Ashraf et al. 2016, Azad 2011); induction of leaf chlorosis (Miller et al. 2011)etc. High concentration of Pb eventually may Pb to cell death (Seregin and Ivanov 2001).

Pb influences the overall distribution of nutritional elements within the different organs of the plant. Results from multiple studies demonstrate that nutrient uptake and overall distribution of nutritional elements within the different organs of the plant by plants are significantly affected by the presence of Lead (Gopal and Rizvi 2008; Zhong, 2017). Most of the observed actions of Pb appear to be indirect as a result of mineral imbalance within the tissues.

Pb adversely affects the metabolites content via protein in *Vigna umbellata* (Cheetri *et al*., 2004); carbohydrate in *Phaseolus vulgaris* (Hammed *et al*., 2010) at higher levels.

Stomata play an important role in regulation of plant water balance and gas exchange. Stomatal parameters can be used as a stressful condition signs. Changes can be seen in stomata density and size depending on stress. (Wilkinson and Davies, 2002)
There is insufficient information about the response of cowpea to Pb exposure under field conditions.

The aim of this study is to evaluate the effect of Pb on (1) the accumulation and distribution of Pb and micro and macro nutrients in roots, shoots leaves and fruits. (2) Protein and Carbohydrate content of leaves (3) Leaf area (4) Stomatal variations.

2. Materials and Methods

The experiment was carried out in natural conditions. Soil, sand, and farm yard manure were mixed in the ratio of 4:1:1.5., as per the recommendation by Kerala Agriculture University. Twenty kg of this potting mixture was filled in gunny bag. Different concentrations namely 100µM, 500µM, 1mM, 5mM and 10mM of Lead as Lead nitrate $Pb(NO_3)_2$ were added to the respective gunny bags as basal

dose. Sufficient surface sterilized healthy seeds were sown in each gunny bags. Plants were studied from seed to seed and analyzed for bioaccumulation of elements and various biochemical, physiological and foliar epidermal studies at definite intervals.

Bioaccumulation
Bioaccumulation of Pb, Ca, Mg, Fe, Cu and Zn in root, stem, leaves and pod were analyzed by Atomic Absorption Spectrophotometer (Varian Spectra 220) on 60th day.

Biochemical studies
Total protein and Total carbohydrate: Total protein was determined on 60th day by standard micro–kjeldhal method and estimation of carbohydrate through the method adopted by Shirlaw and Giltchrist (1967).

Physiological studies
Leaf Area: Leaf area was measured by using graph paper on 20th Day.

Foliar epidermal studies
Stomatal distribution on the abaxial (lower) and adaxial (upper) leaf surface was determined using nail polish imprints.
Number of stomata was examined through high power (10 x 40) on 30th and 60th day. The number of stomata was counted from different fields and different leaves of same age. Length and width of stomata were recorded by using calibrated ocular micrometer. Stomatal index was calculated by a methodology used by Meindev and Mansfield (1968).

$$\text{Stomatal index} = \frac{S}{S+E} \times 100$$

S= Number of stomata / unit area.
E= number of epidermal cell / unit area.

Statistical analysis
The data was analyzed statistically using proper statistical tools like Mean, Standard Deviation and ANOVA to make a significant conclusion. The level of statistical significance is represented by * for $p < 0.05$, ** for $p < 0.01$ and NS for not significant.

3. Results

3.1 Bioaccumulation

a) **Lead uptake and accumulation**
Applications of Pb caused an increase in Pb concentration in root, stem, leaves and pod (Fig-.1). The storage was highest in the root. The distribution of Pb within the plant followed the trend root>pod >stem> leaf. Presence of Pb was not at all detected in any plant parts in control, 100µM and 500µM. However, in 10mM, the Pb level increased to 5.17%, 1.63%, 0.97% and 2.01% in root, stem, leaves and pod respectively. At 10mM, root accumulated 3.17 fold more Pb than stem, 5.31 fold more than leaves and 2.57 fold more than pod.

b) Nutrient uptake and accumulation
Lead was found to interfere with the uptake and transport of several micro and macro nutrients like Ca, Mg, Fe, Cu, Zn and Mn. The higher doses of Pb clearly blocked and the lower doses accelerated the uptake and translocation of all these elements in the present studies.

Lead had a stimulatory effect in the accumulation of Ca up to 500µM. Five percentage, 12.43% and 2.98% enhancement was noted in Ca level at 500µM in root, stem, and leaves. Above this concentration inhibitory effect was accounted in this regard (Fig-2). At 10mM, 13.74%, 22.25% and 12.60% reduction was cited in root, stem and leaves respectively. The order of distribution of Ca was stem > leaf > root. At 10mM, stem accumulated 1.63 fold more Ca than root and1.78 fold more than leaves.

The accumulation of Mg in different plant parts was in the order root >stem >leaf. A gradual decrease from 4.16 to 38.29% was noticed in Mg level in root. In stem 10 to 22% increase was noted in its accumulation in concentrations up to 500µM; but 38.29% reduction was enumerated in 10mM. In leaves, Pb intensified the accumulation of Mg as much as 2.86% for 100µM, 7.92% for 500µM, 4.16% for 1mM and yet, 4.01 and 9.51% deduction was noticed in 5 and 10mM respectively (Fig -3).

Lead accelerated the accumulation of Fe up to 500µM in root and leaves and up to 100µM in stem, but blocked Fe accumulation beyond those level (Fig- 4). Twenty two percentage and 5.85% increase was noted in Fe content in root and leaves at 500µM and 1.3% increase in stem at 100µM. The accumulation of Fe gradually declined in various treatments of Pb, thereby, 27.03, 19.54 and 11.05 percentage inhibition was recorded in stem, root and leaves respectively at 10mM. The accumulation of Fe in different plant parts was in the order stem >leaf > root.

The absorption and translocation of Cu differ in different plant parts (Fig-5). In root linear reduction was observed. Accordingly 4.75, 8.34, 14.33, 24.01 and 32.91% deduction was ascertained in 100µM, 500µM, 1mM, 5mM and 10mM respectively. In stem, 10% to 22% increase was detected up to 500µM, then diminishing gradually to 13% in 1mM, 21.98% in 5mM and 29.43% in 10mM. In leaves also Cu content intensified progressively up to 500µM and declined afterwards. Thereby, 17.89% and1.78% increase was recorded in 100µM and 500µM. However 19.88% and 34.80% inhibition were calculated in 5mM and 10mM. The distribution of Cu was root>stem> leaf.

The accumulation of Zn was inhibited by Pb in all treatments in root. A gradual decrease of 14.13 to 34.57% was recognized in various treatments. In 10mM accumulation of Zn lowered 34.57% than the control. In stem, 15.21, 23.88 and 5.05% increase was noted in its accumulation in 100µM, 500µM and 1mM respectively; but 14.25% reduction was recorded in 10mM. In leaves also Pb enhanced the accumulation of Zn as much as 10.54% for 100µM and 22.91% for 500µM; nevertheless 30.33% deduction was noticed in 10mM. The order of distribution of Zn was leaf>stem >root

The amount of Mn in root was raised in 100µM, then it lowered, there by 6.40, 21.12, 24.64 and 28% decrease was

recorded in 500µM, 1mM, 5mM and 10mM respectively. In stem, the accumulation of Mn was found only in 100µM and 500µM, yet no traces of Mn were detected in any other treatments. In leaves, Pb enhanced the accumulation of Mn only in 100µM. Above this concentration storage of Mn was reduced to 5.30% for 500µM, 14.17% for 1mM, 15.45% in 5mM and 25.23% in 10mM. The accumulation of Mn in different plant parts was in the sequence leaf >root> stem (Fig-7)

3.2 Biochemical studies

a) Protein

Total protein content of leaves augmented in lower concentrations of the metal and come down in the higher on 60th day. The lower concentration, 100µM and 500µM expressed 4.72 and 11.65% increase; however 10.59%, 17.85% and 26.32% inhibition were calculated in 1mM, 5mM and 10mM respectively (Fig- 8).

b) Carbohydrate

In cowpea inhibition was observed in the total carbohydrate of leaves in higher concentration, but its level rises in lower concentration on 60th day. Maximum inhibition was seen in 10mM (45.11%) followed by 5mM (19.35%) and 1mM (13.79%). The lower concentration 100µM and 500µM expressed 3.74 and 41.43% increase respectively (Fig- 8).

3.3 Physiological studies

a) Leaf Area

An overall shrinkage in the leaf area with increasing concentration of Pb was measured on 30th day. Leaf area was 486.45mm^2 in control and 111.52mm^2 in 10mM. A prominent compaction by about 17.20, 33.37, 59.62, 75.01 and 77.07% was noted in 100µM, 500µM, 1mM, 5mM and 10mM of Pb subsequently (Fig-9).

3.4 Foliar epidermal studies

a) Number of stomata

On 30th day, an inducing effect was witnessed in treatments up to 1or 5mM and above this it was decreasing in both surfaces. On the adaxial side there were 4 stomata/ field in control and it was increased to 6 in 1mM and reduced to 3 in 10mM (Table-1). On the abaxial side it was 9/field in control and reduced to 8/field in 10mM. It was 12 in 1mM and 13 in 5mM

On 60th day, highest number of stomata per microscopic field was also on the abaxial surfaces. Both sides showed positive effect in treatments up to 500µM or 5mM and above this it was decreasing (Table-.2). On the adaxial surface there were 6 stomata /field in control and it was increased to 7 in 500µM and reduced to 5 in 10mM. On the abaxial surface, 12 stomata per field were recorded in control and it was reduced to 10/field in 10mM. It was 15 in 5mM, which is the maximum number in the abaxial surface.

b) Stomata index

The stomatal indices were found to be increasing up to 1mM or 5mM in both surfaces on both days of observation (Table-1 & 2). Above these concentrations it was decreasing in both cases. On 30th day, in the adaxial surface 13 to 16% increase was noticed in treatments up to 1mM. However, reduction of 14.70% was noted in 10mM. On the abaxial surface the index value increased gradually up to 5mM then decreased. Sixty seven percentage increase was recorded in 5mM, nevertheless 11.61% reduction in 10mM.

On 60th day, up to 14% increase was noticed in various lower treatments; however 6.45% reduction was recorded at10mM in the adaxial side. On the abaxial surface, an average 10% increase was seen in lower treatments, but 7 to 12% reduction in higher treatments of Lead.

c) Length of stomata

The length of stomata on the abaxial surface was greater than that on adaxial surface on 30th day (Table-1). The stomatal length was longer than control up to 5mM of Pb on both surfaces.

On the adaxial surface the average length of stomata in the control was 19.24µm and 14.46% progressive extension was seen up to 5mM. On the abaxial surface the mean length in control was 20.34µm and gradual expansion of 16.58% up to 5mM. However 17.54% and 18.64% shrinkage were seen in 10mM in adaxial and abaxial surface on 30th day.

On 60th day also the stomatal length was found to be extending up to 5mM of Pb on both surfaces Twelve to 18% enhancement in length could be seen in various treatments of Pb in both surfaces. Yet, 6 to 8 % shrinkage was seen in 10mM in both surfaces (Table- 2).

d) Width of Stomata

The stomatal width was also slightly affected by Pb. Its impact was more on its adaxial surface. In both surfaces an increase was noticed in concentration up to 5mM, but it was lesser than control in 10mM (Table- 1 & 2). On 30th day, 3 to 29% increase in width could be seen in various treatments of Pb in both surfaces. But, 3to 9% shrinkage than control were seen in 10mM in both surfaces (Table- 1). On 60th day, 4 to 32 % enhancements in width were viewed in various treatments of Pb in both surfaces. Nevertheless, 7 to 10 % shrinkage were there in 10mM (Table- 2).

4. Discussion

The accumulation of Pb in different regions were in the sequence of root > fruit > stem > leaves in the present studies with cowpea. For most plant species, the majority of absorbed Pb (approximately 95% or more) is accumulated in the roots, and only a small fraction is translocated to aerial plant parts, as has been reported, in tomato (Akinci et al. 2010), *Vicia faba* (Shahid et al. 2011). The up-take of Pb by the root triggered a series of structural alterations with potential functional consequences in the plant.

This is because the roots are the first organ to be in contact with the metal and roots adhere to the soil all the time. Hence, the exposure of roots towards metals in soil is higher; thus increasing the chances of metal accumulation in roots. More over the large surface area of roots due to the root hairs elevate the absorption and absorption of metals and facilitate nutrient uptake (Yap et al. 2010). Roots also function as the site of water and nutrient uptake of plants by

osmosis, therefore all the metals uptake of plant must pass through the roots before reaching the other parts (Clements et al. 2002).The excess metals that are not further transported upwards by the plant will be accumulated in the roots.

Once Pb has penetrated into the root system, it may accumulate there or may be translocated to aerial plant parts. Fruits showed the second highest accumulation of metal due to its property as a storage site.

The higher doses of Pb markedly hampered the uptake and translocation of Ca, Mg, Fe, Cu, Zn and Mn in the present studies. Similar results have been suggested in many reports. Pb physically blocks the uptake of Ca, Mg, K and P in *Zea mays* (Walker et al.1977): decreased the uptake of K, Ca, Mg, Fe and NO3- in *Cucumis sativus* seedlings (Walker et al. 1977); Ca and Mg transport into rice roots (Kim et al. 2002).

In root tips of Norway spruce 40% of the Ca taken up was used in root tips growth. The inhibition of root growth after exposure to Pb may be due to a decrease in Ca in the root tips, leading to a decrease in cell division or cell elongation (Haussling. et al. 1988). The level of Zn, Cu and Mn in shoots of tomato showed significant decreases with increasing Pb applications (Akinci. et al. 2010).

It is also known that Lead exposure decreases the concentration of divalent cations (Zn^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+} and Fe^{2+}) in leaves of *Brassica oleracea* (Sinha et al. 2006), *Vigna unguiculata* (Kopittke et al. 2007) and *Raphanus sativus* (Gopal and Rizvi, 2008). In *Picea abies*, Pb treatment lowered the Mn level of the needles (Sieghardt, 1988). The level of iron in leaves showed decreases with increasing Pb applications. The decrease in leaf chlorophyll concentration caused by heavy metals is generally attributed to an induced Fe deficiency (Wallace et al. 1992). Content of Ca, Mg, Fe, Cu, Zn and Mn in leaves was decreasing by the application of 500µM or1mM Pb and more severe in 10mM of Pb in the present studies (Fig 1-7).
Two mechanisms for decreased uptake of micro and macronutrients under Pb toxicity have been suggested. The first mechanism, termed physical, relies on the size of metal ion radii, whereas the second mechanism, which is a chemical one, relies on the metal induced disorder in the cell metabolism leading to changes in membrane enzyme activities and membrane structure (Lasat, 2002).

However in some cases, stimulation has been reported (Paivoke, 2002). In certain plant species like *Pisum sativum*, elevated nitrogen content was observed in roots at a Pb treatment level of 2mM kg-1 soil which probably occurs due to inhibitory effects of Pb on nitrate reductase activity (Paivoke, 2002). Potassium and magnesium accumulations were even stimulated by Pb.

An increase in total soluble protein content in lower concentrations of heavy metals stress has been reported in several cases (Verma and Dubey, 2003; Mishra et al. 2006). Total content of proteins slightly increased in both aerial parts and roots of sunflower plants treated with 100mM Pb-EDTA (Krystofova, 2009). Singh et al. (2011) reported that protein found to be in the increase in 2.5mg/l Pb as compared to control, while reverse pattern was observed at higher metal concentrations at 3rd day of exposure period in *Hydrilla verticillata*. The increase in total soluble protein content under heavy metal stress may be related to, induced synthesis of stress proteins such as enzymes involved in Krebs cycle, glutathione and phytochelatin biosynthesis and some heat shock proteins (Verma and Dubey 2003; Mishra et al. 2006).

In *Phaseolus vulgaris* growing at 25, 50 and 100ppm of Pb treatment, carbohydrate content was decreased compared to the control and this decrease in total carbohydrate content was observed in the entire experimental period (Hammed et al. 2010).
A general reduction in sugar and starch in particular may presumably be due to the inhibition of Pb on photosynthesis as reported in maize (Sandmann and Bogger 1983). These results are corroborated with finding of Ahmed (1978), who found that treatment of plant with Pb increased respiration rates of its organ and reduced the photosynthetic rates. The negative effect of heavy metals on carbon metabolism is a result of their possible interaction with the reactive centre of ribulose bisphosphate carboxylase (Stiborova *et al.* 1987).

Negative effect was determined the Pb treatments on leaf area in this study. This finding was supported in *Phaseolus mungo* and *Lens culinaris* Azmat et al. 2006); maize (Nosalewicz et al. 2008); *Albizia lebbeck* (Farooqi et al. 2011). Lead induced reduction in leaf area in turn decreases the surface area for photosynthesis and inhibition of chloroplast activity and also the quantum yield of oxygen (Nechushtai et al. 1996). Pb treatment causes growth retardation, which results in a reduced leaf area, the major transpiring organ (Iqbal and Moshtaq, 1987).

Stomatal parameters can be used as signs of stressful condition. Changes can be seen in stomatal features depending on stress. (Wilkinson and Davies 2002). An inducing effect was witnessed in the number of stomata, stomatal index, length and width of stomata in treatments up to 1or 5mM of Pb. But, beyond this level Pb treatment was found inhibitory to all these parameters (Table 1 and 2).

These findings are in agreement with the following results: an increase in the number of stomata per unit area in *Glycine max* (Weryszko-Chmielewska and Hwil, 2005); an increase in number of stomata in the adaxial (upper) leaf surface of *Phaseolus mungo* and *Lens culinaris* (Rafia et al. 2009); slight increase in stomata frequency, Stomata density, Stomata length and width in lower epidermis of pigeon pea leaves (Meerabai et al, 2012); increase in the stomata index values of upper and lower epidermis of *Boerhavia diffusa* (Abdussalam et al, 2013).

According to Kosoburkhov 2004, the photosynthetic activity of plant is governed by factors including stomatal cell size, number of stomata, stomatal conductance and leaf area

5. Conclusion

The storage of Pb was highest in the root. The accumulation of Pb was in the order root>pod >stem>leaf. Beyond 5mM,

Pb negatively affected the uptake and translocation of Ca, Mg, Fe, Cu, Zn and Mn: the content of total protein and carbohydrate in leaves: the number of stomata, stomatal index, length and width of stomata. But, concentration up to 1mM, Pb positively affected all these parameters. An overall shrinkage was noticed in the leaf area due to Pb.

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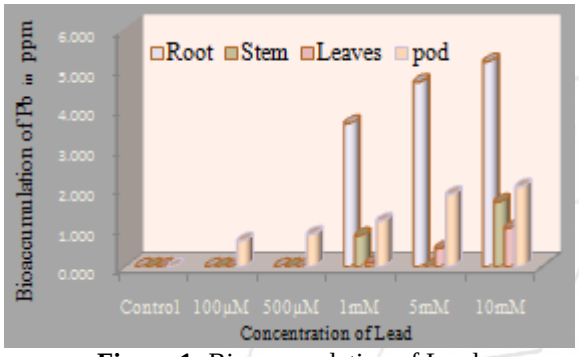

Figure 1: Bioaccumulation of Lead

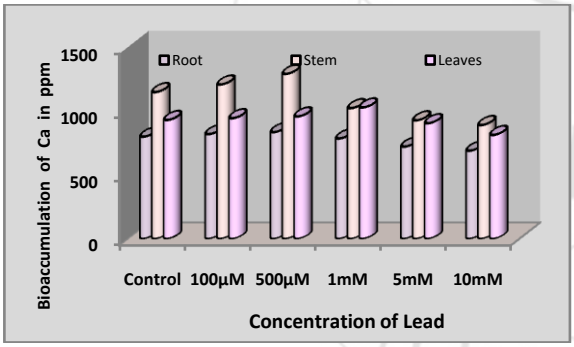

Figure 2: Imact of Lead on the Bioaccumulation of Ca

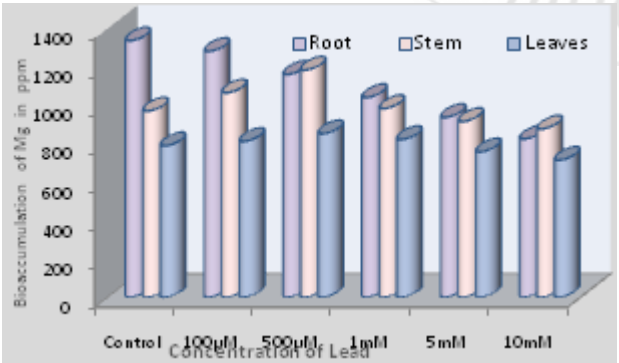

Figure 3: Impact of Lead on the Bioaccumulation of Mg

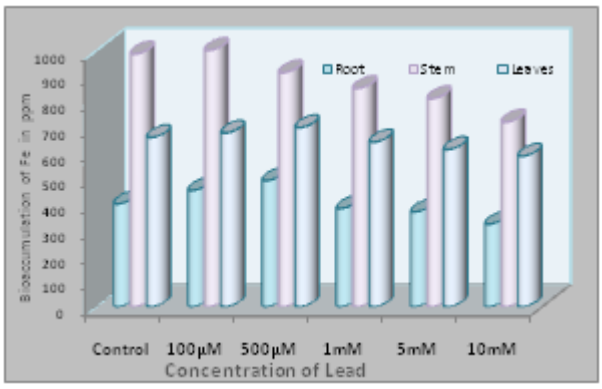

Figure 4: Impact of Lead on the Bioaccumulation of Fe

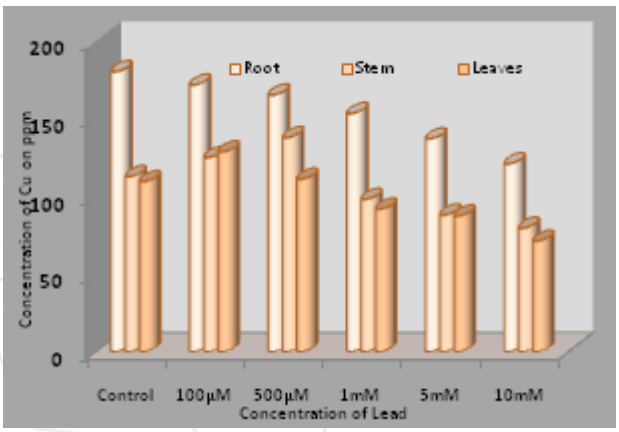

Figure 5: Impact of Lead on the Bioaccumulation of Cu

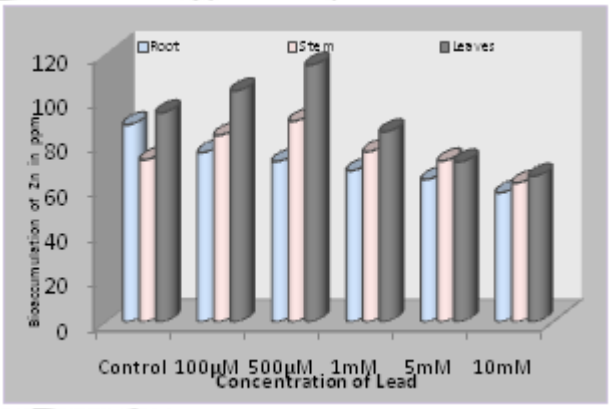

Figure 6: Impact of Lead on the Bioaccumulation of Zn

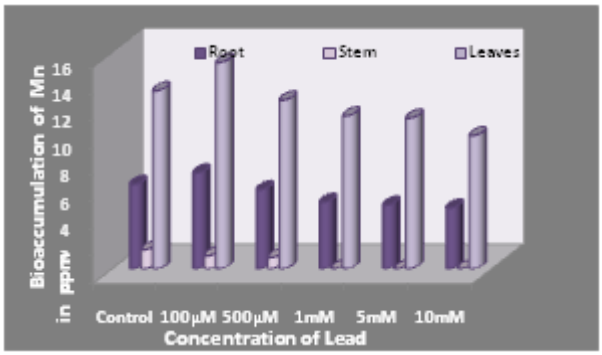

Figure 7: Impact of Lead on the Bioaccumulation of Mn

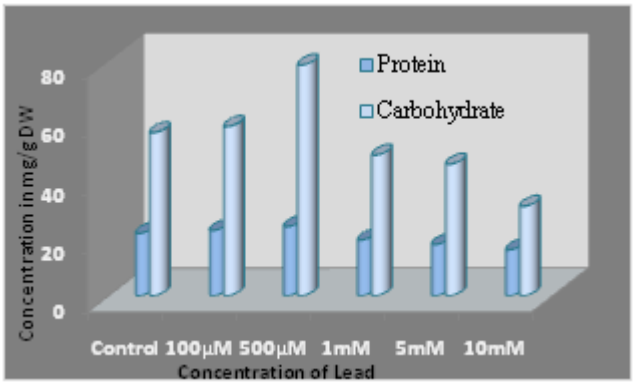

Figure 8: Impact of Lead on the protein and carbohydrate

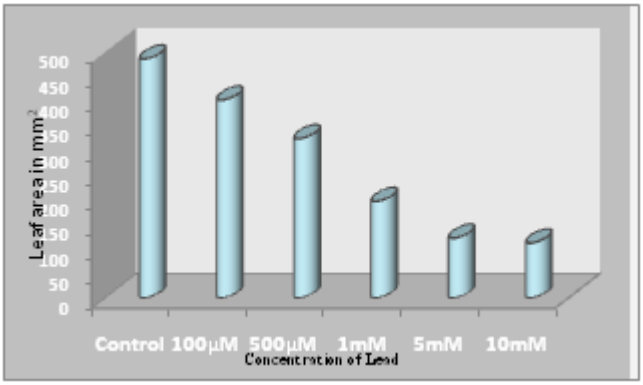

Figure 9: Impact of Lead on Leaf Area

Table 1: Impact of Lead on Stomatal Variations in cowpea on 30th day

Treatment	Abaxial (lower) side				Adaxial (upper) side			
	No. of stomata	Stomatal index	Length (µm)	Width (µm)	No. of stomata	Stomatal index	Length (µm)	Width (µm)
Control	9^a ± 1.27	14.39^a ± 3.51	20.34± 4.67	11.56± 1.5	4^a ± 5.95	12.31^a ± 0.04	19.23± 3.81	11± 1.42
100µM	9^b ± 3.09	16.57^b ± 7.78	21.03± 4.26	11.96± 3.77	5^b ± 3.95	14.33^a ± 0.03	19.38± 2.69	12.37± 4.12
500µM	11^b ± 2.32	21.1^a ± 4.03	22.27± 1.65	12.37± 3.88	6^a ± 5.66	15.47^b ± 0.02	21.03± 4.15	12.78± 4.06
1mM	12^b ± 2.67	23.85^a ± 2.22	23.1± 2.69	13.61± 1.16	5^a ± 7.4^{a}5	13.97^a ± 0.02	21.07± 1.58	13.61± 3.31
5mM	13±3.81	24.01± 4.3	23.7± 2.76	13.79± 1.25	4±1.58	11.6±0.03	22.01± 1.04	14.22± 1.8
10mM	8± 1.65	12.72± 1.58	18.64± 4.3	11.19± 1.4	3±0.84	10.5±0.02	17.54± 1.47	10.07± 2.23
F-value	26.54**	7.32**	11.73**	7.01**	4.11*	3.36*	19.23**	11*

Table 2: Impact of Lead on Stomatal Variations in cowpea on 60th day

Treatment	Abaxial (lower) side				Adaxial (upper) side			
	No. of stomata	Stomatal index	Length (µm)	Width (µm)	No. of stomata	Stomatal index	Length (µm)	Width (µm)
Control	12^a ± 1.49	39.5^a ± 2.28	16.5± 4.95	8.2± 2.34	6^a ± 0.52	34.25^a ± 3.19	21.03± 1.14	11.5± 1.23
100µM	12^a ± 1.49	39.75^b ± 13.92	17.04± 1.64	9.9± 0.3	7^a ± 0.84	34.58^a ± 1.88	22.27± 1.83	12.37± 1.31
500µM	13^a ± 1.19	43.08^a ± 4.01	18.69± 1.85	10.9± 1.25	7^b ± 0.52	36.08^a ± 3	22.68± 1.53	12.37± 1.73
1mM	14^a ± 0.99	43.42^a ± 3.12	19.38± 0.71	10.32± 1.73	6^b ± 1	39.83^b ± 1.99	23.06± 1.41	12.78± 1.69
5mM	15^b ± 4.09	36.58^a ± 4.98	18.15± 2.67	8.6± 0.01	5^a ± 1.07	39.58^b ± 3.15	23.59± 0.48	13.61± 1.18
10mM	10^b ± 1.13	34.67^a ± 4.48	15.4± 2.65	7.6± 1.4	5.08^b ± 0.9	32.04^b ± 3.75	17.54± 2.3	10.96± 0.59
F-value	8.56**	11.46**	16.5*	8.2*	7.94**	9.55**	4.48**	3.57*