Morpho-physiological modulations contribute for enhanced Pb-tolerance of *Vigna unguiculata* L. (Cowpea)

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ABSTRACT *A study was conducted to assess the Lead (Pb) stress tolerance of four crop species Vigna unguiculata L (Cowpea), Vigna radiataL. (Green gram), Vigna mungo L. (Black gram) and Macrotyloma uniflorum Lam. Verdc. (Horse gram). The growth experimentswere conducted in pot cultures and the root growth assay in hydrophonics. The plants were subjected to Pb-stress at 0.5 ppm to 2 ppm concentration (w/v). Morphological analysis of the four legume plants subjected to Pb-stress revealed that the high tolerance to Pb-stress was found in Cowpea plant with higher rooting efficiency, lesser reduction in growth andbiomass. Physiological analysis also supported the morphological studies which revealed better relative water content, enhancedtotal chlorophyll content, higher total free amino acids and proline content along with reduced cell membrane injury, lesser accumulation of free radicals such as superoxide, H_2O_2 content, in cowpea. Hence cowpea showed better Pb-tolerance potential compared to other legumes studied and perhaps be considered for cultivation in poor soils contaminated with Pb and other heavy metals.*

Keywords: *Pb-stress, Cowpea, Physiological analysis, Rooting assay*

Introduction

Man has been meddling with the environment directly or indirectly to create pollution. Among the types of environmental niches affected, agricultural lands are also quite polluted now a days by heavy metal pollutants as result of automobile effluents, industrial effluents etc., Heavy metal is defined to be element which is naturally occurring with high atomic weights and density of 5 times more than water(Tchounwou et al., 2012). Lead is a heavy metal that frequently pollutes various environments and the sources of lead contamination include industrial wastes, fossil fuel combustions, agrochemical usage. This heavy metal could exists as fumes, mists, vapours in the atmosphere and also as a mineral in soil(Sengar et al., 2008). Exposure of plants to heavy metals results in wide ranges of physiological and metabolic alterations(Arif et al., 2016). Changes in antioxidant metabolism of horsegram and bengalgram upon Pb heavy metal stress were studied by (Reddy et al., 2005). Study of 2D proteome of cowpea under the Manganeseheavy metal stress was also done during previous studies (Führs et al., 2008). Hence in the current manuscript, a study was conducted to assess the tolerance ability among the four legume plants *Vigna unguiculata* L (Cowpea), *Vigna radiata* L. (Green gram), *Vigna mungo* L. (Black gram) and *Macrotyloma uniflorum* Lam. Verdc. (Horse gram) to lead heavy metal stress.

Materials and Methods

Legume crop plants used in the study

The four legume species Green gram (*Vigna radiata* L), Black gram (*Vigna mungo* L), Horse gram (*Macrotyloma uniflorum* Lam Verdc) and Cow pea (*Vigna unguiculata* L)

Screening of Four Legume species tolerance to Pb stress through root growth assay

The tolerance of the plants to heavy metals was tested using the Root growth assay(Walley et al., 1974). Tops from 15 day old plant cut just below the transition zone were allowed to root in different concentrations of $Pb(NO_3)_2$ ranging from 0.5 to 2.0 ppm prepared in $Ca(NO_3)_2$. Temperature of 28±2°C; light level of 150 W m^{-2} was maintained for 15 more days. Rooting Data was collected at intervals of 0, 5 and 10 days. Controls were maintained in solution of hydrated $Ca(NO_3)_2$. Triplicates for the each treatment were maintained.

Pb-Stress induction in pot culture

The four legume species seeds were potted in the pots containing 4 kg of air dried red loamy soil and farm yard manure (3:1). Initial Pot water holding capacity of the soil was measured before the plants were potted and the plants were watered only to water holding capacity each day. Since the plants were able to withstand 2ppm of Pb stress in the intial screening, only 2 ppm of Pb stress was induced in the test plants except the controls.

Morphological parameter analysis

Plant growth and Biomass

Plant growth in terms of root and shoot growth was measured. After sampling, the seedlings were separated into roots and shoots and the length of each part was measured using a scale and the results were the average of 3 replicates. The growth of root and shoot was expressed in cm plant^{-1}.The plants were uprooted carefully, washed with deionized water and shoot, root parts were separated and blotted dry with filter paper. The fresh weights of root, shoot were recorded, then the root and shoot material was dried at 80ºC in a hot air-oven for 48 h. The dry mass was recorded. Plant biomass was calculated and expressed in g plant^{-1}.

Physiological parameters

Total Chlorophyll content, Cell membrane integrity and Relative water content

The chlorophyll content was estimated according to the method described by(Hiscox and Israelstam, 1979). Total chlorophyll content was calculated from the formula used by(Arnon, 1949) and expressed as mg g-1 fresh weight.Cell membrane integrity in terms of cell membrane injury was determined in the plants according to (Leopold et al., 1981). The relative water content (RWC) was estimated as reported and calculated from the formula of(Turner, 1981).

Estimation of H_2O_2, Total free Amino acids and Free Proline content

The H_2O_2 content of the leaves and roots were measured according to (Singh et al., 2006)with slight modifications. Fresh plant tissue (500 mg) was homogenized with 2.5 ml of 0.1% (w/v) TCA and the homogenate was centrifuged at 10,000 × g for 15 min. To a 0.5 ml aliquot of the supernatant, 0.5 ml of 100 mM phosphate buffer (pH 7.6) and 1 ml of 1.0 M KI were added. The absorbance was measured at 390 nm by using H_2O_2 standard graph and expressed as µmol g^{-1} of sample.The extraction and estimation of Total free amino acids was done according to the protocol of (Moore and Stein, 1948). The extraction and estimation of free proline was done in both control and Pb-stressed legumes according to the protocol of (Bates et al., 1973).

Results and discussion

Root growth assay results

The results of the root growth assay revealed that these four species showed tolerance or rooting efficiency in Pb-stress. The legume cowpea showed higher tolerance and the least tolerance was observed in case of blackgram (**Figure -1**).Root growth inhibition may also be due to the accumulation of Pb in the meristematic regions (Lane and Martin, 1980).

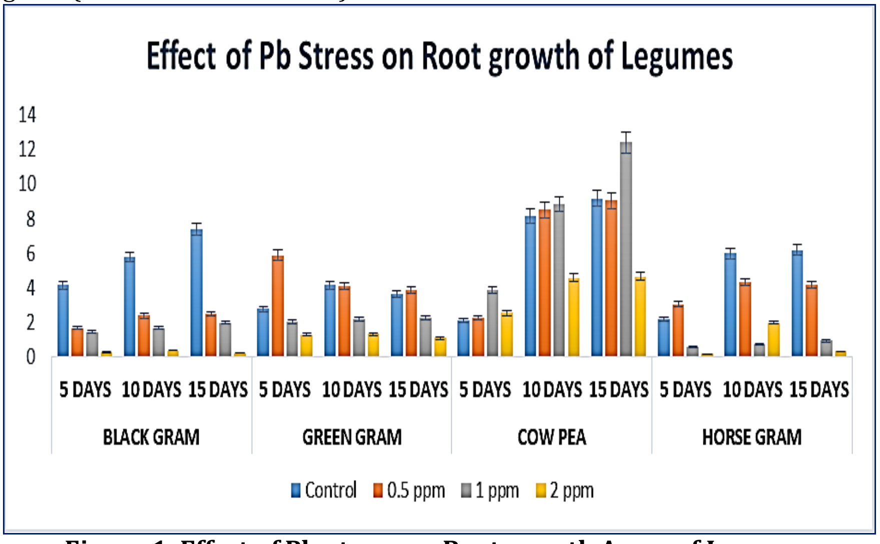

Figure 1: Effect of Pb-stress on Root growth Assay of Legumes

Morphological parameters

Plant growth and Biomass

The growth of the plants in terms of root and shoot length of both control and Pb-treated four legume species were measured on 0th day, 5th and 10th days post Pb-stress and represented in (**Figure – 2**).

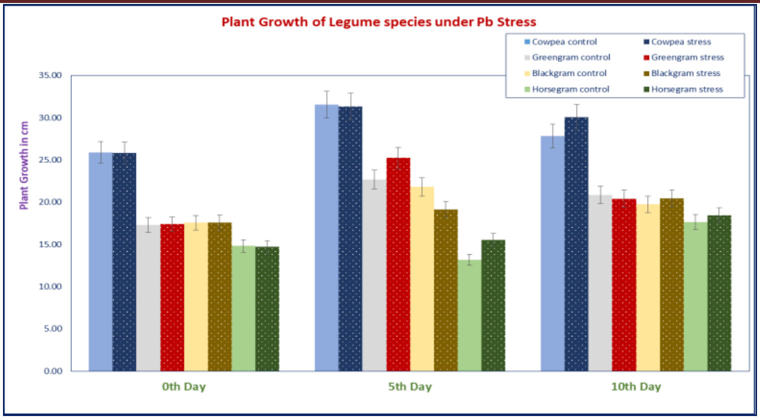

Figure 2: Effect of Pb-stress on Plant growth of Legumes

Cowpea showed 8%, Black gram 3% more and Horsegram 4% more growth compared to respective controls on the 10th day of stress. Contrastingly, Green gram shows 3% less growth compared to controls on the 10th day of stress. Toxic effect of Pb was evident from the reduction in growth and biomass among legume crop plants(**Figure -3**).

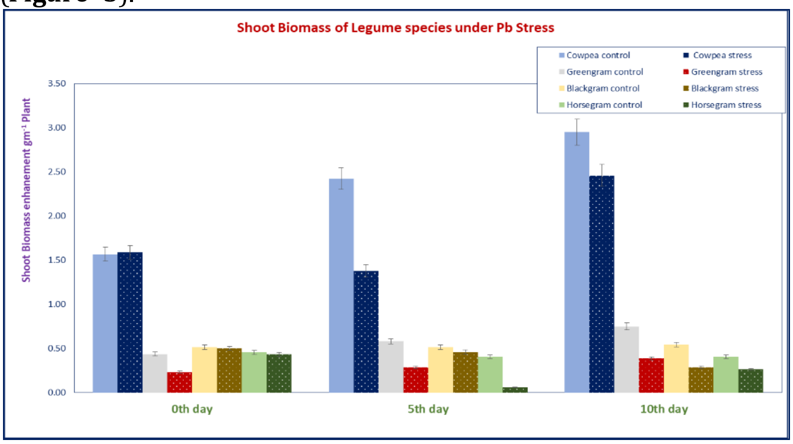

Figure 3: Effect of Pb-stress on Shoot biomass of Legumes

The root biomass and shoot biomass was reduced in Pb-treated black gram plants compared to controls. The shoot biomass reduction was around 48.3% in green gram plants, 47.19% in black gram plants, 35.29% in horse gram plants and 16.6% in cowpea plants on the 10th day of Pb-stress compared to their corresponding controls. The root biomass reduction was around 15.72% in cowpea, 44.56% in green gram, 53.44% in black gram and 79.03% in horse gram on the 10th day of Pb-stress compared to their corresponding controls (**Figure-4**). The effect of heavy metal on seedling growth seems to be different with regards to plant species, cultivars, organs and metabolic processes (Sharma and Dubey, 2005).

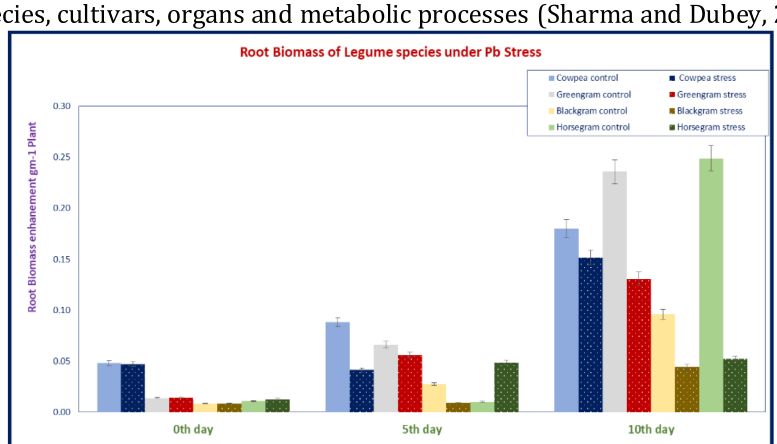

Figure 4: Effect of Pb-stress on Root biomass of Legumes

Physiological parameters

Total Chlorophyll content, Cell membrane integrity and Relative water content

The total chlorophyll content gradually decreased in all legume crop plants with increase in the days of stress. The percentage of chlorophylls were 60.55% in cowpea, 37.61% in green gram, 39.74% in black gram and 67.76% in horse gram plants compared to their respective controls on the 10th day of stress. The most common symptoms caused by Pb toxicity were yellowing of the leaves (chlorosis) and leaf curling was noticed at 10th day of Pb-stress especially in black gram plants (**Figure 5**).

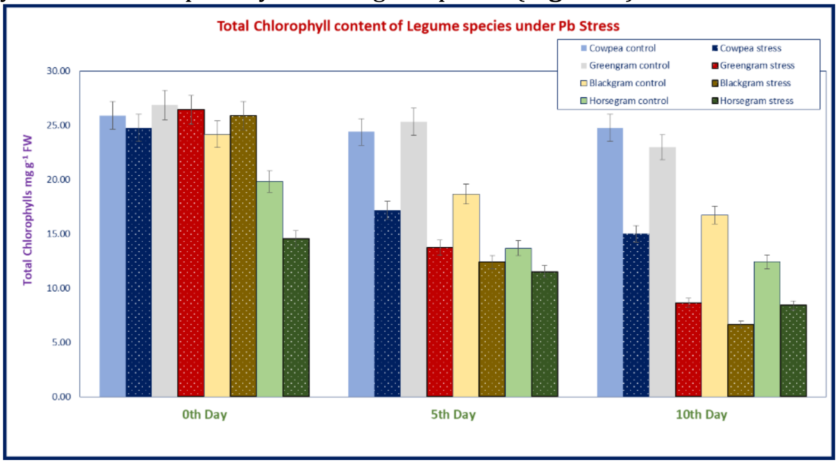

Figure 5: Effect of Pb-stress on Total Chlorophyll content of Legumes

Cell membrane injury significantly increased in all four legume species grown under Pb- stress as number of days of stress progressed. The increase in Cell membrane injury was 6.75% in Cowpea, 18.31% in green gram, 106.09% in black gram and 12.36% in horse gram plants on the 10th day post Pb-stress. In general, the cell membrane injury of all four legume species grown under Pb-stress was observed, but however, the cowpea was able to tolerate cell membrane injury as the least percent of injury was found in the cowpea and highest damage of the cell membrane was identified in case of black gram which is a poor survivor of Pb-stress (**Figure 6**).

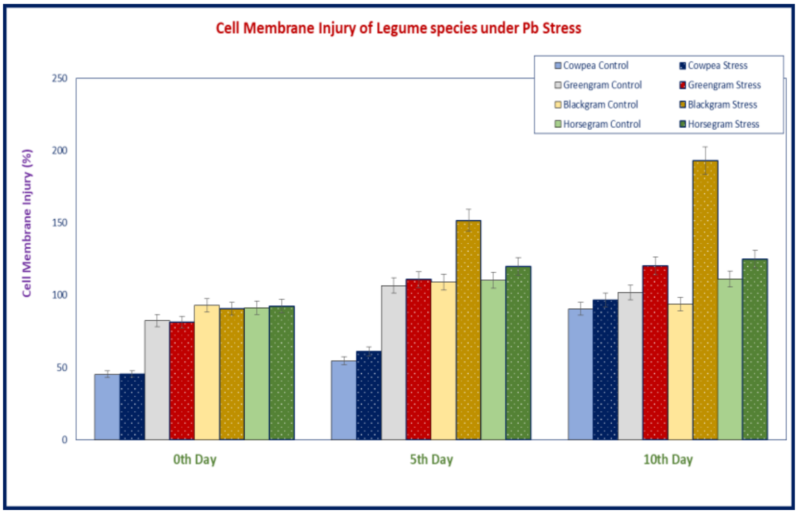

Figure 6: Effect of Pb-stress on Cell Membrane Injury of Legumes

Relative water content significantly decreased in all four legume species grown under Pb-stress as the number of days of stress progressed (**Figure 7**). The relative water content on the 10th day post Pb-stress was 55.15% in cowpea, 46.65% in greengram, 45.98% in blackgram and 54.55% in horse gram. The results suggest that the relative water content decreased in all the four legume species compared to their corresponding controls on 10th day of Pb-stress. The high relative water content was noticed in cowpea and the least percent was found in black gram suggesting that cowpea is a good survivor of Pb-stress while black gram is the poor survivor of Pb-stress damage.

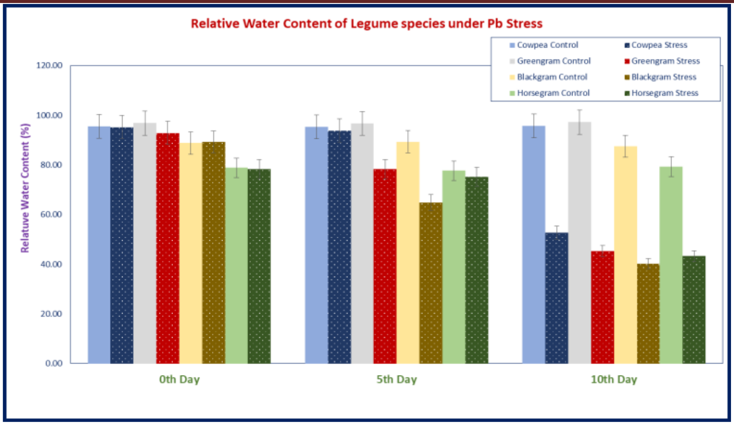

Figure 7: Effect of Pb-stress on Relative Water Content of Legumes

Estimation of H_2O_2, Total free Amino acids and Free Proline content

H_2O_2 in leaves of all legume crop plants was elevated under Pb-stress conditions. The magnitude of elevation was dependent on severity of Pb-stress in plants. However, the increase in H_2O_2content was relatively less in cowpea. The leaf H_2O_2content increased in blackgram (179.23%) compared to their respective controls on the 10th day of Pb-stress (**Figure 8**).

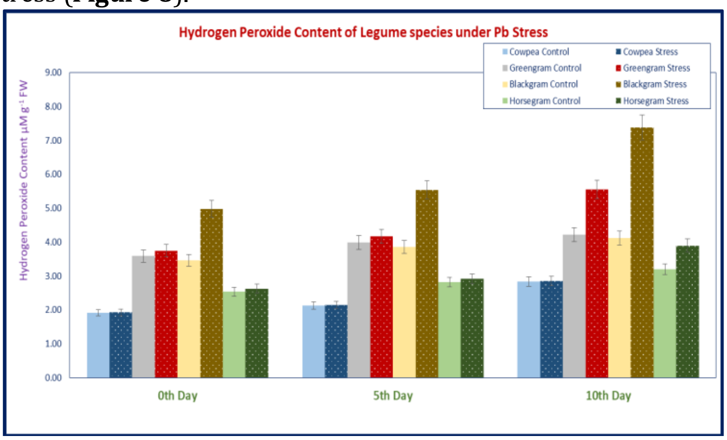

Figure 8: Effect of Pb-stress on Hydrogen Peroxide Content of Legumes

Since the H_2O_2 content was elevated in black gram species, it represents the maximum levels of ROS damage in the black gram compared to its control (100%). Contrastingly, the cowpea plant has reduced levels of H_2O_2 (100.58%) which states that cowpea is tolerant to Pb-stress.**Figure 9** illustrates the effect of Pb concentrations on proline accumulation of leaves four legume crop plants. The accumulation of free proline content increased with days of Pb-stress. However, the increase in proline content was relatively more pronounced in green gram (333.33%) and cowpea (305%). In general, the free proline contents were significantly

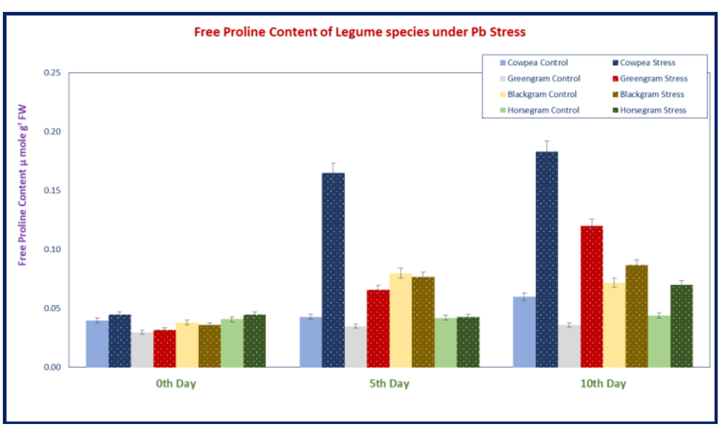

Figure 9: Effect of Pb-stress on Free Proline Content of Legumes

decreased in black gram (12.83%) and moderately decreased in horse gram (159.09%) under Pb-stress.Total Free Aminoacid content in leaves of all legume crop plants was drastically enhanced under Pb-stress conditions possibly due to degradation of proteins. The magnitude of reduction was dependent on severity of Pb-stress in plants. However, the increase of Total Free Aminoacid content was relatively less in cowpea. The leaf Total Free Aminoacid content increased enormously in blackgram (293.25%) compared to their respective controls (100%) on the 10th day of Pb-stress (**Figure 10**). Contrastingly, the cowpea plant has low levels of total free aminoacid content (152.46%) which states that cowpea is relatively more tolerant to Pb-stress.

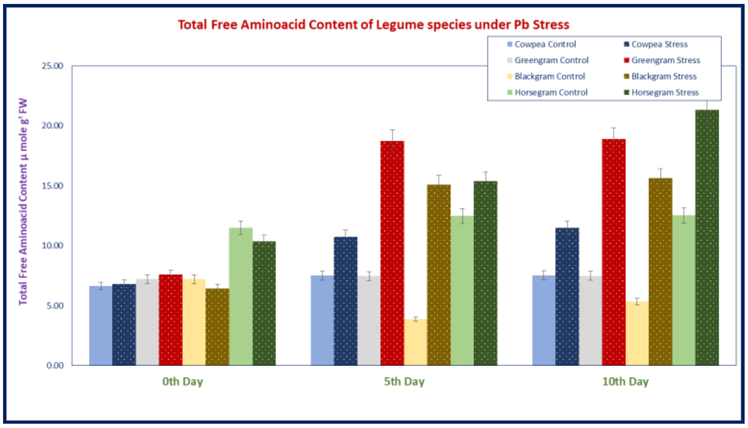

Figure 10: Effect of Pb-stress on Total Free Amino acid Content of Legumes

Conclusions

Based on the analysis and results, cowpea could tolerate Pb-stress and could be used for cultivation in Pb-contaminated soils. It is also noted that cowpea has evolved to up-regulate and down-regulate several proteins for combating Pb-stress.

Acknowledgements

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