Protective role of *Trichoderma logibrachiatum* (WT2) on Lead induced oxidative stress in *Helianthus annus* L.

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Lead (Pb²⁺) is a heavy metal and one of the main environmental pollutants, toxic to plants, animals and humans. Pb²⁺ contaminated soils affect crop productivity. The availability of heavy metals to plants and their toxicity depends on complex rhizospheric reactions involving not only exchange processes between soil and plants but also microbial activities. Here, we evaluated the Pb²⁺ induced oxidative stress in Sunflower, *Helianthus annus* and also determined the protective role played by the plant growth promoting fungus *Trichoderma* sp. against this stress. In this study, six isolates of *Trichoderma* sp. (WT1 to WT6) were screened for the tolerance against different concentrations of Pb²⁺. Then we investigated whether *Trichoderma* sp. (WT2) could be used to combat the Pb²⁺ induced oxidative stress in *Helianthus annus* seedlings. Pot cultures containing 350 and 750 ppm of lead concentrations with and without *Trichoderma* sp. inoculated soil were maintained. Results indicated significant reduction in root and shoot lengths of seedlings grown in Pb²⁺ amended soils after 30 days. The seedling samples were collected in two phases. The levels of SOD, POD and CAT were moderate in 1st phase. The seedlings exposed to Pb²⁺, plants grown in uninoculated soil were found to show decreased activities of antioxidant marker enzymes. We observed that *Trichoderma* inoculation significantly elevated these enzyme levels compared to that of seedlings exposed to Pb²⁺. The development of stress-tolerant plant-fungus associations may be a promising strategy for mycoremediation and soil amelioration measures.

**Keywords:** Bioremediation, Heavy metal, Mycoremediation, Pb²⁺, Plant stress, Soil Pollution, Sunflower

Plants face both biotic and abiotic challenges posed by environment as well as humans. While environmental factors broadly includes attack by pathogens, lack of optimum temperature, humidity, rainfall, nutrients, etc., non-judicious use of pesticides, agrochemicals and mobilization of heavy metals into the biosphere constitute human activity.

Heavy metals impose many deleterious effects such as inhibition of photosynthesis, reduction in cell division, lowers nutrient uptake and germination percentage¹,², browning of root tips³ phytotoxicity and senescence. High levels of Cd, Pb and Zn in soil inhibit plant metabolic functions; result in retarded growth and cause senescence⁴,⁵. Lead (Pb²⁺) inhibits enzyme activity at cellular level by reacting with their sulphydrl groups⁴. Excess of Ni²⁺ in soil causes various physiological alterations and diverse toxicity symptoms such as chlorosis and necrosis in different plant species⁶,⁷.

Bioaccumulation and biomagnifications stands in between plants and human beings which drives toxic heavy metals from soil to human physiological system through plants. Vegetables take up metals by absorbing them from contaminated soils, as well as from deposits on parts of the vegetables exposed to the air from polluted environments⁸. Maize (*Zea mays*) has been reported to accumulate chromium, particularly in roots, and suggested to be a potential crop for phyto remediation of Cr-contaminated soils⁹. Lakra *et al.*⁹, have reported impact of multiple stress including Cd and Pb stress in Indian mustard. In Groundnut, lead content of seed (edible part) has been reported to be well above the maximum permissible limit for food stuffs.¹⁰. Findings on content of heavy metals (Zn, Cd, Pb, Fe, Cu, Cr, Hg, Co, Ni and Mn) in food crops grown in oil exploration areas indicated potential health hazards faced by the indigenous population who feed on these crops¹¹. This heavy metal is known cause many ill effects to human beings. Especially the young and developing brains of the children are most vulnerable to lead exposure¹². Lead (Pb²⁺) can also lead to brain dysfunction in adults¹³.
Microbes are known to play an inevitable role in environmental cleanup, phytoremediation, in particular. Agricultural and industrial importance of *Trichoderma* is highlighted by many researchers in the past. The unique nature of this fungus, being tolerant to recalcitrant pollutants including harmful agrochemicals, heavy metals and poly aromatic hydrocarbons is well documented. Biomass of *Trichoderma* along with calcium alginate beads has been reported to enhance biosorption of heavy metal chromium. IR spectral analysis of *Trichoderma viride* cell wall revealed the presence of hydroxyl groups and amide groups that play vital role in biosorption of heavy metals.

In this context, interactions of soil-plant root-microbes which play important roles in regulating heavy metal movement from soil to the edible parts of crops is of great concern. The present study examines the protective role played by edible parts of crops is of great concern. The present study examines the protective role played by *Trichoderma* spp. in combating heavy metal stress faced by Sunflower *Helianthus annus* (L.).

**Materials and Methods**

**Collection and maintenance of fungal species**

Six different fungal species belonging to the genus *Trichoderma* were obtained from Laboratory of Pathology, Citrus Research Station, YSR Horticultural University, Tirupati, Andhra Pradesh, India. The samples were transferred to PDA (Potato Dextrose Agar), sterile media for *in vitro* testing. The samples were kept in the refrigerator at 4°C, after the fungal biomass was reached to maximal growth. These colonies are suitable for transferring to new media and were used in our experiments as resource.

**Screening and identification of metal tolerant fungus**

To test the heavy metals resistance pattern, the heavy metal Pb, used as Lead nitrate was added to PDA at different concentrations ranging 50-1000 ppm (50 ppm intervals) and pregrown fungal discs of 9 mm diameter were inoculated and incubated at 25°C for one wk. The minimum inhibitory concentration (MIC) of the heavy metals was designated as the highest concentration at which the organism was able to tolerate within 72 h. Based on the growth on agar plate, the most tolerable isolate is selected. DNA was isolated from this *Trichoderma* sp. and was identified by 18S rDNA (ITS1-ITS4 region) sequencing. PCR amplified product was sequenced by automated Sequencer at Eurofin genomics, Bangalore, India. Related sequences were searched using Basic Local Alignment Search Tool (BLAST) programme from the Gen-Bank database (http://www.ncbi.nlm.nih.gov/blast/). The multiple sequence alignment and pair wise alignment were made using BioEdit version 5.09. The neighborhood-joining bootstrap tree was created using CLUSTAL W 1.6 matrix by the CLUSTAL X programme ver. 1.81.

**Preparation of the liquid media and mass multiplication of fungus**

After proper growth of fungus on the solid media, liquid media were prepared with the following composition (g/L): potato extract 25, dextrose 20, tetracycline 0.25 (to prevent bacteria growth). The pH 5.1 ± 0.2 was maintained using Lactic acid and KOH (3%). About 9 mm of fungal disc was inoculated in autoclaved and cooled media. After incubation for 14 days, fungal mat was filtered and weighed.

**Pot experimentation**

A red sandy loam soil was collected from agricultural field around Tirupati, dried for 72 h, powdered to pass through 2 mm sieve, and spiked with lead nitrate of Analytical grade (Qualigens). Seeds of *H. annus* crop were collected from Agricultural Research Station of Andhra Pradesh located at Tirupati, Andhra Pradesh India.

Detailed study was carried out on *H. annus* seedlings in the experimental farm of Citrus Research Station, Tirupati. Six kg of soil was used in each pot having dimension 1×1×1 ft. The soil in each pot was homogenized with lead nitrate solution and left for 2 days for stabilization as per experimental need. Six pots were prepared and each treatment was replicated three times. The treatments were allotted to the individual pots as follows:

- T1, (control) pot with 3 kg of soil only; T2 & T3, 350 and 750 ppm Pb solution prepared using ddH2O and mixed with 3 kg of soil, respectively; T4, with 100 g of *T. logibrachiatum* (WT2) mat mixed thoroughly with 3 kg of soil; and T5 & T6, 100 mL of 350 and 750 ppm Pb solution + 100 g of *T. logibrachiatum* (WT2) mat mixed thoroughly with 3 kg of soil, respectively. Each treatment had three replicates.

Seeds of test species were sown in the pots at equal distances. Six seedlings were maintained in each pot after 1 wk of sowing. Experimental potted plants were grown under net house covered with transparent polythene sheet to protect them from rain water leaching, but kept open to air and ambient temperatures. The experiments were designed to...
harvest the plants two times at 15 days interval i.e., on 15th (phase 1) and 30th day (phase 2) of plant age for further analysis.

**Assay of antioxidant enzymes**

About 1 g of plant material was homogenized in 50 mM phosphate buffer (pH 7.0) containing 1% insoluble polyvinylpyrrolidone (w/v) at 4°C with mortar and pestle (0.1 g FW/mL buffer), filtered through four layers of cheese cloth and centrifuged at 15000 ×g for 10 min. The supernatant obtained was designated as crude enzyme extract and was used for various antioxidant enzyme assays.

Measurement of super oxide dismutase (SOD) activity was assayed by the following protocol\(^\text{23}\). About 3 mL of reaction mixture containing 0.1 mL methionine (200 mM), 0.01 mL nitro blue tetrazolium (NBT, 2.25 M), 0.1 mM EDTA 3 mM, 1.5 mL of potassium buffer (100 mM), 1 mM distilled water and 0.05 mL enzyme extract were poured in a test tube, and the reaction began by adding 0.1 mL of riboflavin (60 mM) under fluorescent lamps (two 15 watts for 15 min). Reaction stopped by changing the light and covering tubes with black cloth. Absorbance recorded at 560 nm and the activity of the enzyme reported as units per mg protein.

Measurement of peroxidase (POD) activity was done by taking 3 mL of the reaction mixture containing 2.5 mL sodium phosphate buffer (0.05 mM and pH 7), 30 µg leaf protein and 20 µL of guaiacol (200 mM). Then, reaction mixture added to cuvette and before assay 10 µL of hydrogen peroxide (30%) as the electron acceptor was added to the reaction mixture. The reaction mixture without hydrogen peroxide was used as a control to calibrate the spectrophotometer to zero. The absorbance was measured at 475 nm for 60 s at 25°C and enzyme activity was presented as mg protein absorption per minute\(^\text{24}\).

Catalase activity was assayed by adding 1.5 mL of reaction mixture containing 30 µL of water, 50 µL of buffer Tris-HCl (1 M and pH 8), 5 mM EDTA, 900 µL of hydrogen peroxide (10 mM) to 20 µL supernatant. Absorption was recorded at 240 nm by spectrophotometer for 60 s. Catalase activity was measured as absorbance per minute per mg protein\(^\text{25}\).

**Native gel electrophoresis and isoenzyme staining**

Polyacrylamide gel electrophoresis (PAGE) for superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) isoenzymes assay was performed with 7% (w/v) polyacrylamide gel as described by Laemmli\(^\text{26}\). SOD electrophoresis proteins were separated by native PAGE using 5% stacking and 10% running gels with a buffer consisting of 0.025M Tris and 0.192 M glycine (pH 8.3) at 100 V for 3.5 h. The total amount of protein applied per lane was 20 µg. After electrophoresis, the gels were incubated with 1 mM KCN and 30% H2O2 followed by incubation with a reaction mixture (0.1 M EDTA, 0.098 mM NBT, 0.030 mM riboflavin and 2 mM N,N,N,N-tetramethylethylendiamine in K phosphate buffer, pH 7.8) for 30 min in the dark. The gels were washed in distilled water and visualized with regular light.

**Detection of POD and CAT isoenzymes**

For POD isoenzymes, the gels were rinsed in water and the gel was stained in a solution containing 0.06% (v/v) H2O2, 0.1% (w/v) benzidine and 0.1% (v/v) acetic acid at room temperature till the brown colour\(^\text{27}\). For CAT isoenzymes, gels were incubated in 0.01% H2O2 for 10 min and developed in a 2% (m/v) FeCl3 and 2% K3Fe(CN)6 (m/v) solution for 10 min until the colourless bands were appeared\(^\text{28}\).

**Results**

**Screening of fungal isolates for tolerance to heavy metals**

Six *Trichoderma* isolates were screened for their tolerance to 50, 100, 150 up to 1000 ppm of Pb\(^{2+}\). Data indicated decrease in number of isolates tolerant to Pb\(^{2+}\) at higher concentration of Pb\(^{2+}\). Out of six isolates, WT2 isolate obtained highest mycelia growth at 750 ppm within 3 days incubation. The sequence of this isolate was deposited in NCBI, USA and Accession number was obtained for *Trichoderma logibrachiatum* (EF552704). The same isolate was used for further experiments.

*H. annus* seedlings were tested for growth under varying Pb\(^{2+}\) levels. It was observed that visible symptoms like stunted growth started at 350 ppm. At 750 ppm heavy metal stress indications like necrotic spots on leaves, browning of stem and drooping of plant occurred after 30 days. Blackening of roots and death of plants was noticed at further higher concentrations. Hence, 350 and 750 ppm of Pb\(^{2+}\) concentrations were chosen in further experiments. Also, *T. logibrachiatum*, the best isolate in the present study showed tolerance up to 750 ppm of Pb\(^{2+}\).

**Measurement of root and shoot lengths**

In the first phase, there was no significant difference in shoot and root lengths observed in all treatments. However, *Trichoderma* inoculation found
to enhance root and shoot growth. Where as in second phase, seedlings raised in Pb$^{+2}$ containing soils showed significant decrease in root and shoot lengths when compared to control treatment (Fig. 1). However, combined treatment with Trichoderma and Pb$^{+2}$ showed a significant increase in root and shoot lengths when compared with Pb$^{+2}$ induced seedlings.

**Enzyme activity**

When H. annus seedlings were raised under 350 and 750 ppm of lead with and without fungal associations, elevated levels of SOD activity was recorded during first phase sampling. However, no significant difference was observed between control and Trichoderma inoculated treatments. POD levels estimated after 15 days showed increased enzyme level in seedlings subjected to 350 ppm Pb$^{+2}$, whereas all other treatments showed lower or equal levels compared to control. Catalase was well induced in all treatments compared to control. Maximum increase of all the three antioxidant enzyme activities were noticed in seedlings raised at 350 ppm of Pb$^{+2}$ amended soil after 15 days of growth period.

Investigation on antioxidant enzyme (SOD, POD and CAT) activities after 30 days resulted in significant decline in case of seedlings grown in Pb$^{+2}$ contaminated soils compared to respective control plants (Fig. 2). Trichoderma significantly reversed the Pb$^{+2}$ induced effects. Sunflower seedlings raised in soils containing both Trichoderma and Pb$^{+2}$ were found to exhibit higher antioxidant enzyme levels than seedlings grown under 350 and 750 ppm of Pb$^{+2}$.

Electrophoresis analysis of SOD, POD and CAT isoforms revealed staining intensities of samples under different treatments correlated with their quantitative changes Fig. 3.

**Discussion**

Microorganisms are ubiquitous that exists in varied environments, which are also known to tolerate stress imposed by heavy metals and survive successfully. This can be stated as adaptation, an important mechanism for their survival despite presence of soil contaminants. In our present study, we observed that out of 6 Trichoderma isolates, (WT2) T. logibrachiatium could tolerate and adapt to
a maximum level (750 ppm) of Pb\(^{+2}\) amended medium. Similarly, screening of metal tolerance activity of various fungal isolates was carried by different researchers\(^{30}\). Vargas-Gracia et al.\(^{31}\) revealed that dead cells were less efficient at biosorbing heavy metals than living cells. Research on biosorption and accumulation was carried using Trichoderma\(^{32}\), Penicillium and Fusarium\(^{33}\). In certain bacteria, lead resistance can be due to precipitation of lead phosphate within the cells\(^{34}\).

Lead is a toxic heavy metal that accumulates in soils thus contaminating groundwater. It is also able to enter food web. Fertilizers are also known to carry traces of lead which can contaminate food\(^{35}\). As plants must adapt (or die) to the conditions where they grow, the presence of heavy metals can induce oxidative stress and the activation of several defense factors in the plants\(^{36}\). High Pb\(^{+2}\) concentration was known induce oxidative stress by increasing the production of ROS in plants\(^{37}\). Hence, in the present study, antioxidant enzymes and growth of seedlings were evaluated for measuring heavy metal stress in H. annus seedlings. We investigated the combinatorial effect of lead and Trichoderma on growth of sunflower plants. Further, the levels of all the three quantified antioxidative enzymes were found upregulated compared to their respective controls in the first phase. It suggests that various treatments induced antioxidative enzymes in seedlings and are able actively eliminate reactive oxygen species.

It is evident from the results that in the second phase the enzyme activities were inhibited in case of seedlings grown in only Pb\(^{+2}\) contaminated soils. This decrease was inversely proportional to concentration of Pb\(^{+2}\) in soil. Combined reports of many research works states that the levels of antioxidant enzymes in plants were decreased at elevated range of heavy metal exposure\(^{38}\). Possible explanations for the decrease in SOD activity under metal stress may be linked to inactivation of enzyme by excess production of ROS and unspecific enzyme degradation\(^{39}\) or the binding of nonessential heavy metals to the active center of the enzyme\(^{40}\). The sulfhydryl groups that play vital role in enzyme activity may be impaired by lead\(^{41}\). Several enzymes have metal cofactors so there could be a link between these enzymes expression and metal availability\(^{42}\).

It can be noticed from our present study that sunflower plants are relieved from lead stress by Trichoderma inoculation. In the second phase, we observed that in spite of growing in lead polluted environment, plants possessing Trichoderma in their rhizosphere exhibited elevated antioxidative scavenging activity. It helps in eliminating toxic ROS to maintain plant health. In this situation, Trichoderma could have rescued H. annus seedlings by influencing bioavailability of lead. This is in agreement with Abou-Shanab et al.\(^{43}\) who have earlier reported that microbes release chelators or bring about redox changes in response to metal. Glomalin is a glycoprotein that sequesters metals like Cd, Cu and Zn and reduce their availability, thus reducing risk of toxicity to other soil microorganisms and plants growing in the immediate vicinity\(^{44}\).

Microorganisms removes heavy metals from the contaminated soils adopting various mechanisms\(^{15,45-47}\) viz.: (i) biosorption or metal sorption to cell surface by physiochemical mechanisms; (ii) bioleaching i.e., heavy metal mobilization through excretion of organic acids or methylation reactions; (iii) biomineralization—heavy metal immobilization through the formation of insoluble sulfides or polymeric complexes; (iv) intracellular accumulation; (v) enzyme-catalyzed transformation-redox reactions;
and (vi) chelation of heavy metals by siderophores. On the basis of energetic requirements, biosorption or metal sorption to cell surface by physiochemical mechanisms appears to be the most common mechanisms.

Thus, it can be concluded that *Trichoderma* can be deployed to protect plant health, growth and quality of plant products raised in soils with contaminants. The present research also provides scope to correct the past mistakes of humankind made by leaving heavy metal pollutants into the environment.

References
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