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In vitro accumulation of lead nitrate in safflower seedling and its impact on plant protein

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Abstract

The changes in the morphology, biochemical, antioxidant enzyme activity and protein profile in Safflower were investigated. The plant was treated with different concentrations (100 and 150µM) of $Pb(NO_3)_2$ to find the effect of heavy metal stress in *Carthamus tinctorius* and changes in growth and oxidative stress in one-week and two-week-old plant were examined. The present study has revealed that the presence of heavy metal stress in the plant seedlings causes significant effect in morphological characters, with a decrease in the leaf/root color, size, length and number of leaf/root as compared to control. Similarly, the carbohydrate content was decreased in both leaf and root explants while pigments chlorophyll (a+b) decreased in leaf explants. Proline and polyphenolic compounds were increased compared to control which indicates the excellent antioxidative ingredient to protect damage induced by free radicals. The protein profile of safflower (*Carthamus tinctorius*) strongly influenced by severe heavy metal stress (100µM and 150µM). It was analyzed through SDS-PAGE and it was found that the protein concentration decreased with increase in metal treatment.

Keywords: Antioxidants, Biomass, Lead nitrate, Protein, Safflower.

Introduction

Safflower (*Carthamus tinctorius*L.) highly branched, herbaceous plant is a member of family Asteraceae**,** animportant oilseed crop of semi-arid regions and cultivated mainly for its seeds. Carthamus (synonym of the Arabic word *quartum*, or *gurtum*) refers to the colour of the dye extracted from safflower flowers/petals. Beside the ability to take up essential nutrients, plants are able to absorb and accumulate other metals, even those with unknown metabolic function. The presence of heavy metals (HM) in excess amounts is a global problem, threatening the health of vegetation, wild life and humans (Heckathorn et al., 2004). Heavy metal pollution in air, water and agricultural soil is of major ecological concern due to its impact on human health through the food chain and its high persistence in the environment (Valko et al., 2005). Plants exposed to stressing agents such as drought, salinity, excess of heavy metals, air pollutants or pathogens have developed strategic defense mechanism that vary between species and the nature of stressing agent. Lead is an omnipresent toxic metal and is detectable in practically all phases of the inert environment and in all biological systems. Disruption of tissue oxidant/ antioxidant balance, alteration of lipid metabolism, and substitution for zinc in various zinc-mediated processes are some of the metabolic repercussions of lead toxicity (Ahamed et al., 2007). Lead and calcium compete for the same binding sites on a large family of ion - binding proteins composed of calmodulin and related proteins. Calmodulin and related proteins. Calmodulin serves as sensor for the concentration of calcium within cells. Lead acts by displacing calcium ions bound to calmodulin. Lead impairs normal calcium homeostasis and uptake by calcium membrane channels and substitutes for calcium in calcium sodium at pumps. By displacing zinc finger protein or zinc-binding sites in receptors channels (Lidsky and Schneider, 2003).

Hypothetically, the strength of a toxic effect of all trace metals depends principally on the absorption, concentration, and persistence of the eventual toxicant at its location of action. The final toxicant is the metal species that reacts with the endogenous target molecule such as receptors, enzymes, DNA, proteins, or lipid or critically alters the biological environment, producing structural and functional changes that results in toxic damage. Lead (Pb) is one of the most abundant, ubiquitous toxic elements posing a critical concern to human and environmental health. It can cause multiple directs and indirect effects on plant growth and metabolism, along with visible symptoms including stunted growth and small leaves, as well as leading to membrane disorganization and reduced photosynthesis (Sharma & Dubey, 2005; Ahmad et al., 2008). In addition, it is generally accepted that toxic levels of heavy metals can affects a variety of physiological processes in plants. One of the major consequences is the production of large quantities of reactive oxygen species (ROS), which can cause damage to proteins, lipids and DNA (Schutzendubel & Polle, 2002). Therefore ROS production and removal must be efficiently controlled. To minimize the damaging effects of ROS, the plants cells posses evolved non-enzymatic and enzymatic antioxidatives defence mechanism. The latter mainly include superoxide dismutase (SOD), peroxidise (POD) and catalase (CAT) (Miller et al., 2008). Lead usually enters plants by similar pathway as micro or macronutrients. However the metabolic pathways underwent by this element within plant cells are not fully understood (Patra et al., 2004). Although lead is not an element for plants, it gets easily absorbed and accumulated in different plant pars.

Table 1. HPLC quantification of polyphenolic compounds from the alcoholic extract of leaf and root (Both control and metal stressed) of *C. tinctorius*

Plant material	Time	Peak area	Compound	Amount (mg/ml)
Leaf control	3.517	2373647	Ferulic acid	0.003
	2.175	49770	Quercetin dehydrate	0.0003
	4.125	634063	o-Coumaric acid	0.2175
	6.667	307489	Kaemferol	0.024
7th day leaf stress	3.517	5661841	Ferulic acid	0.009
	2.133	154758	Quercetin dehydrate	0.0009
	4.117	1074418	o-Coumaric acid	0.3685
	6.667	757041	Kaemferol	0.061
14th day leaf stress	3.500	3085135	Ferulic acid	0.0048
	2.183	58860	Quercetin dehydrate	0.0003
	4.117	634247	o-Coumaric acid	0.2176
Root control	3.525	236449	Ferulic acid	0.0003
	2.142	20138	Quercetin dehydrate	0.00012
7th day root stress	4.108	87582	o-Coumaric acid	0.030
	3.483	558334	Sinnapic acid	0.00089
14th day root stress	3.500	224595	Ferulic acid	0.0003
	4.117	65542	o-Coumaric acid	0.022
	2.108	22284	Quercetin dehydrate	0.0001

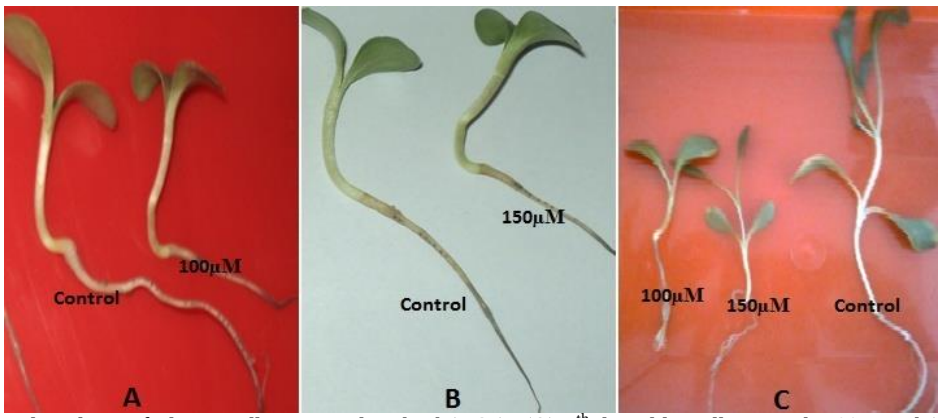

Fig 1. Morphological analysis of plant seedling treated with $Pb(NO_3)_2$. (A) 7th day old seedlings with 100µM $Pb(NO_3)_2$ and control, (B) 7th day old seedlings with 150µM $Pb(NO_3)_2$ and control, (C) 14th day old seedlings with 100µM and 150µM $Pb(NO_3)_2$ and control.

Uptake of Pb in plants is regulated by pH, particle size and cation exchange capacity of the soil as well as by root exudation other physio-chemical parameters. Early studies showed that lead can inhibited seedlings growth and decreased the biomass, as well as induced the changes of SOD, POD and CAT activity in some plant species (Verma and Dubey, 2003; Xiong et al., 2006; Quereshi et al., 2007; Gao et al., 2009). These results were important to understand that Lead (Pb) treated Plant showed increase level of lipid peroxidation as evidenced from the increased malondialdehyde content coupled with the increase in the activities of superoxide dismutase (SOD), catalase (CAT), peroxidise (POD), glutathione reductase (GR), glutathione S-transferase (GST) compared to control (untreated) plants. Lead stress caused significant changes in the activity of antioxidative enzymes. The effect of lead was found to be concentration dependent. It can cause multiple direct and indirect effects on plant growth and metabolism, along with visible symptoms including stunted growth and small leaves, as well as leading to membrane disorganization and reduced photosynthesis (Sharma and Dubey, 2005; Ahmad et al., 2008). Proteins are compounds of fundamental importance for all function of cell. Protein variation is an essential part of plant response to abiotic and biotic stress as well as for adaptation to environmental conditions (Vierstra, 1993). The aim of this study was to evaluate the response of safflower variety-HUS-305 after the treatment with lead nitrate for the analysis of the effects of this metal on the rate of germination capacity, morphological, biochemical, and molecular level protein and antioxidative enzyme activity involved in the system. Consequently to determine the safflower plants have evolved exclusive mechanisms to compact with environmental stress.

Results

Morphological analysis of the plant showed affect in germination percentage with the various concentrations of lead nitrate and age of explants compared with the control one. The result in relation to the effect of various concentrations of lead on growth performance measured in term the morphological changes (colour, size) these includes colour of leaf, which is light in stressed seedlings as compared to controlled. Colour of roots was also dull in stressed seedlings. Size of leaves was small and less in number in treated plants as compare to control (Fig. 1). Biochemical study of plant materials (leaves, roots) extracts of 2.5N HCl, 3% aqueous sulphosalisylic acid and acetone gives the affordable information by showing the presence of carbohydrates, chlorophyll, carotenoids and proline in the sample at both the concentrations of metal stress. The carbohydrate content was decreased in the stressed seedlings. Soluble carbohydrate contents in plants decreased with increasing concentration of heavy metals. Results showed that carbohydrate amount in safflower leaf is more as compare to roots but it has been find out that in concentration (100 µm and 150 µm) of lead nitrate the carbohydrate content was decreased in both leaf and root explants of 7^{th} days old seedling as compare to control. But in 14^{th}days old seedlings, carbohydrate concentration was decreased more in roots as compare to leaf of stressed plant in 150 µm concentration than control (Fig. 2). Significant difference among 7^{th} and 14^{th} day leaf explants of seedlings in two concentrations of lead nitrate was observed during the analysis of pigment. It is clear that chlorophyll and carotenoid contents were decreased in both days stressed plants as compare to control but simultaneously it has also been observed that chlorophyll a, b content were decreased more in 14^{th} day leaf explants in 100 µm concentration of lead nitrate as compare to 150µm Pb $(NO_3)_2$ (Fig. 3) while carotenoid contents were decreased more in 14^{th} day leaf explants in 150 µm concentration of lead nitrate (Fig. 4). Increase in the osmoprotectant proline content was directly proportional to the heavy metal i.e.-lead nitrate concentration. But it has been observed that proline contents was more in 7^{th} day old seedlings in both 100,150 µm concentration of $Pb(NO_3)_2$ as compare to14days old seedlings. Proline content was greater in roots than in leaves (Fig. 5). The phytochemical analysis conducted on safflower extract revealed the presence of flavonoids, alkaloids and saponins etc. Flavonoid content was decreased in the stressed seedlings in both concentration of lead nitrate. Further it was observed that 14^{th} day seedlings contains less amount of the flavonoid (2.36) than 7^{th} day seedlings (4.5). As the time of metal stress increased, the flavonoid contents decreased. Flavonoids found to be more in leaves (1.86) than in roots (0.41) (Fig. 6). The total phenol content of the 7^{th} day old methanolic leaf extract (control) was 8.19 mg gallic equivalent/g of fresh leaf extract and methanolic root extract (control) was 4.13mg gallic equivalent/g of fresh root extract respectively with reference to standard curve, while phenol content was increased (11.89 mg gallic equivalent/g of fresh leaf extract) and (6.13 mg gallic equivalent/g of fresh root extract) of 100 µm concentration of Pb $(NO_3)_2$ and (9.00 mg gallic equivalent/g of fresh leaf extract) and (8.38mg gallic equivalent/g of fresh root extract)of 150 µm concentration of Pb $(NO_3)_2$. Similarly the total phenol content of the 14^{th} day old methanolic leaf extract (control) was 9.47 mg gallic equivalent/g of fresh leaf extract and methanolic root extract (control) was 4.63mg gallic equivalent/g of fresh root extract respectively with reference to standard curve, further phenol content was increased (17.83mg gallic equivalent/g of fresh leaf extract) and 10.63mg gallic equivalent/g of fresh root extract) of 100 µm concentration of Pb $(NO_3)_2$ and (10.51 mg gallic equivalent/g of fresh leaf extract) and (5.51mg gallic equivalent/g of fresh root extract) of 150 µm concentration of Pb $(NO_3)_2$ (Fig. 7). Conclusively it may explain that stressed leaf explants contain more polyphenols as compare to leaf (control) and root explants (control/stressed). HPLC studies revealed the presence of phenolic acids in the alcoholic extracts of the plant material (Table 1). A numbers of peaks were detected, some of which could be identified in the presence of rare standards. In the safflower (control) ferulic acid, O-coumeric acid, kaemferol and quercetin dehydrates are commonly present in explants (leaf) while sinnapic acid, ferulic acid, and quercetin dehydrates are identified only in root explants. Same compounds were in higher concentrations present in 7^{th} day leaf explants when the plant was exposed in 100 µm concentration of Pb $(NO_3)_2$ .While it has been observed that kaemferol was absent in 14^{th} day treated leaf explants. Similarly in 7^{th} and 14^{th} day root explants (stressed) has shown extra peak of compound O-coumeric acid that is absent is root (control). The antioxidant activity was maximum in control (60.79%) while the same has been reduced when the plants are exposed in various concentrations of lead nitrates i.e., 31.49% in leaf stress at $100\mu MPb(NO_3)_2$while 20.26% in leaf stress at $150\mu MPb(NO_3)_2$. Time and concentration of metal toxicity showed that as the concentration increased from $100\mu MPb(NO_3)_2$ to $150\mu MPb$ $(NO_3)_2$ and seedlings are exposed from 7^{th} day up to 14^{th} day, results explained that 14^{th} days leaves in 150µm Pb $(NO_3)_2$ concentration showed too much reduction in antioxidative property (20.26%) as compare to stressed roots (39.72%) in 14^{th} day treated plants in 150µm Pb $(NO_3)_2$ concentration. Similar result was

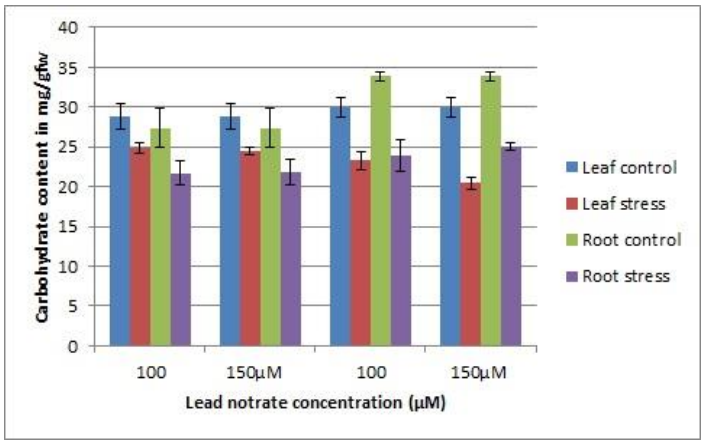

Fig 2. Carbohydrate content in leaf and root at 7th and 14th day of $Pb(NO_3)_2$ treatment with 100µM and 150µM.

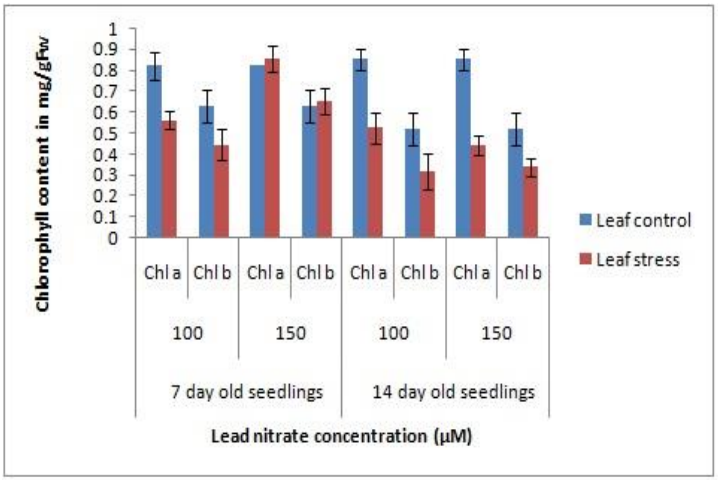

Fig 3. Chlorophyll content in leaves at 7th and 14th day of $Pb(NO_3)_2$ treatment with 100µM and 150µM in mg/gfw .

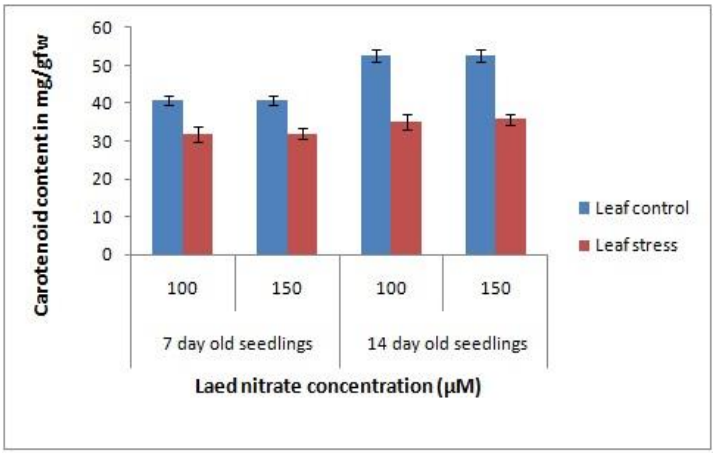

Fig 4. Carotenoid content in leaves at 7th and 14th day of $Pb(NO_3)_2$ treatment with 100µM and 150µM in mg/gfw.

obtained for 100µm $Pb(NO_3)_2$for both 7th and 14th day seedlings. This explains that free radical scavenging activity has been reduced when the plant is exposed in lead contaminated soil. The enzymes SOD and peroxidase were increased in the leaves/roots of the plant when they are exposed in heavy metal lead contamination as compare to control. With increases in the concentration of lead, catalase activity was corresponding decreased (Fig 8). It was observed during experiments that protein contents were decreased as the metal concentration was increased in the treatment. Toxicity of lead altered the protein content of leaves/roots. There was a decrease of 4.8% and 14.8% as compare to control in 7th and 14th day leaf explants treated with 100µm Pb $(NO_3)_2$ concentrations respectively. While 5.0% and 18.44% protein was reduced in the same day leaf explants exposed under 150 µm Pb $(NO_3)_2$concentrations.Similarly the protein contents were also decreased in root explants (7th and 14th day treated plants under 100-150 µm Pb $(NO_3)_2$ concentrations) as compare to control roots (Fig. 9). The SDS-PAGE pattern of polypeptides extracted from various days (7th and 14th days) leaf/root of safflower at different concentrations i.e. 100/150µm Pb $(NO_3)_2$ were analyzedusing 12% SDS gel showed that the polypeptide decrease as the concentration of metal increases compared with control. It is clear that there are few more polypeptides in between 29-205kDa are present in 14th day leaf control that are also decrease in stressed leaf explants of the same day in 100µm concentration of Pb $(NO_3)_2$ and too much detoriation of protein was observed in stressed leaves (Fig. 10, Lane 4) treated under 150µm concentrationof Pb $(NO_3)_2$.

Discussion

The results of the present study confirm that metal stress can causes a reduction in vegetative growth and decrease in total plant productivity. That might be due to inhibition of cell division, cell elongation or combination of both under metal stress. Biomass of leaves was higher at lower concentration of stress that was gradually declined towards higher concentration this is similar to earlier studies that low concentration of metal stress increased the plant dry matter and yield activity (Jayakumar and Vijayarengan, 2008). Increased metal stress has adversely affected the photosynthesis by altering the chlorophyll content. Production of biomass is affected that depends on accumulation of carbon products through photosynthesis (Panda et al., 2010). The effects of lead on seedlings growth seems to be different with regards to plant species, cultivars, organs and the metabolic processes (Sharma and Dubey, 2005). Morphological studies had shown that minimum growth response occurred in the plant when the lead concentration was increased in the soil than standard one. There was gradual decrease in the plant height, root and shoot length at the different increasing concentration of the lead. Similar observations have been observed on *Triticum sativum* and *Lens esculanta* (Mesmar and Jaber1991). In the present study, the exposure of lead significantly affected different parameters of safflower such as carbohydrates, chlorophyll (a+b) contents, carotenoid content, proline, total polyphenols, flavonoids, antioxidants and total protein contents. Decrease in all above factors was observed except proline. This may be because proline a non-essential amino acid is synthesized in the living organism whenever it is subjected to stress high/low temperature, high salinity or heavy metal concentration.

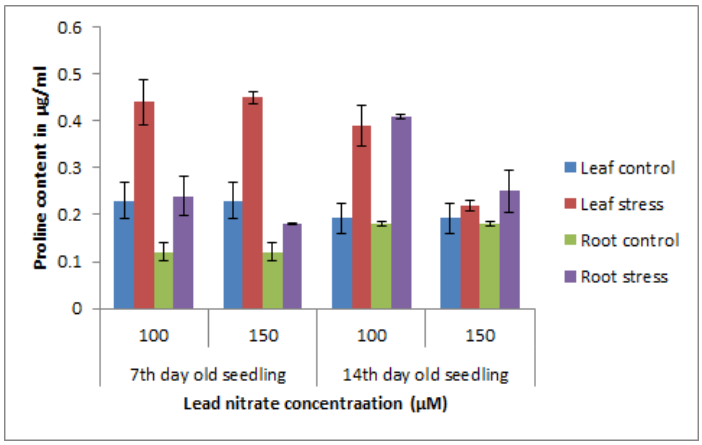

Fig 5. Proline content in leaf and roots at 7th and 14th day of $Pb(NO_3)_2$ treatment with 100µM and 150µM.

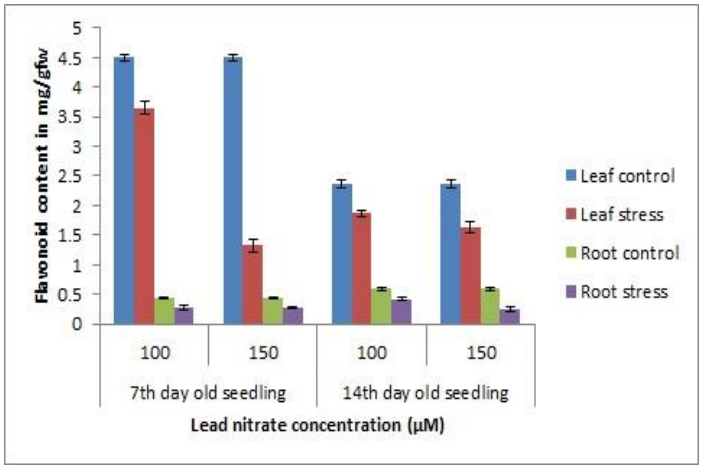

Fig 6. Flavonoid content in leaf and root at 7th and 14th day of $Pb(NO_3)_2$ treatment with 100µM and 150µM.

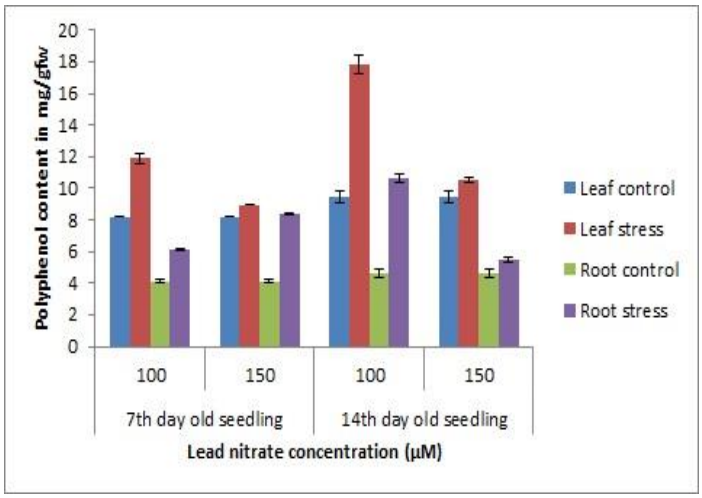

Fig 7. Polyphenol content in leaf and root at 7th and 14th day of $Pb(NO_3)_2$ treatment with 100µM and 150µM

Proline accumulation is a common metabolic response of higher plants and has been the subject of numerous reviews (Stewart and Larher, 1980: Samaras et al, 1995; Taylor, 1996; Rohdes et al, 1999). Proline may also function as a hydroxyl radical scavenger (Smirnoff and Cumbes, 1989). Also proline content is proved to be essential for stress tolerance because of its active role in osmotic adjustment, protection of the enzyme structure, stabilization of membranes and defence against hydroxyl radicals (Silva-Ortega et al.2008).The decline in the levels of these chlorophyll pigments had shown the metal interference with pigment metabolism. Similar observations were made (Mukherji and Maitra1976) in rice where lead toxicity resulted in lowering the chlorophyll a/b ratio. Lead was found to inhibit δ amino levulinic acid dehydratase activity in mung bean resulting in a decrease in chlorophyll. Lead also distorts the membrane structure of chloroplasts, which ultimately leads to decrease in chlorophyll content. Flavonoids serve as health promoting compound as a results of its anion radicals (Hausteen 1983). From the above results it is clear that increasing polyphenols in stressed leaf explants known to support bioactive activities and thus responsible for the anti-oxidant activities of this plant. Flavanoids have been shown to exhibit their actions through effects on membrane permeability, and by inhibition of membrane-bound enzymes such as the ATPase and phospholipase A2 (Li et al., 2003), and this property may explain the mechanisms of antioxidative action of *C. tinctorius*. With increase in the concentrations and time of the treatment of heavy metals, there was increase the anti-oxidative enzyme activity SOD and peroxidase while decrease in Catalase. From the results it has been cleared that lead nitrates does not impose any kind of oxidative stress because the activity of antioxidant enzymes was higher. An increase in peroxidase activity probably represents an induced protective reaction against damage by free radicals. The result of DPPH scavenging activity assay in this study indicates that the plant was potentially active. This suggests that the plant extract contain compounds that are capable of donating hydrogen to a free radical in order to remove odd electron which is responsible for radical's reactivity. Plants with antioxidant activities have been reported to possess free radical scavenging activity (Das and Pereira 1990). Free radicals are known as major contributors to several clinical disorders such as diabetes mellitus, cancer, liver diseases, renal failure and degenerative diseases as a result of deficient natural antioxidant defense mechanism (Parr and Bolwell 2000). All parts of Safflower have been reported for antioxidant property and edible and beneficial to human health (Hiramatsu et al., 2009). One of the major effects of the heavy metals on plants is reported to be a decrease in the protein content by hindering protein synthesis. Lead, zinc and cadmium in *Hordeumvulgare* plant (Stiborova et al., 1986a), copper and lead in *Zea mays* (Stiborova et al., 1986b), lead and cadmium in *Lemna minor* (Mohan and Hosetti, 1997) have been reported to decrease protein contents. Synthesis of stress protein under salt stress in safflower (Bhima) has been reported (Patil, 2011).

Material and methods

Plant material

Carthamus tinctorius L. variety HUS 305 (Indian safflower) was used to evaluate the physiological and biochemical activities under stress at an early seedling stage. *Carthamus tinctorius*L.

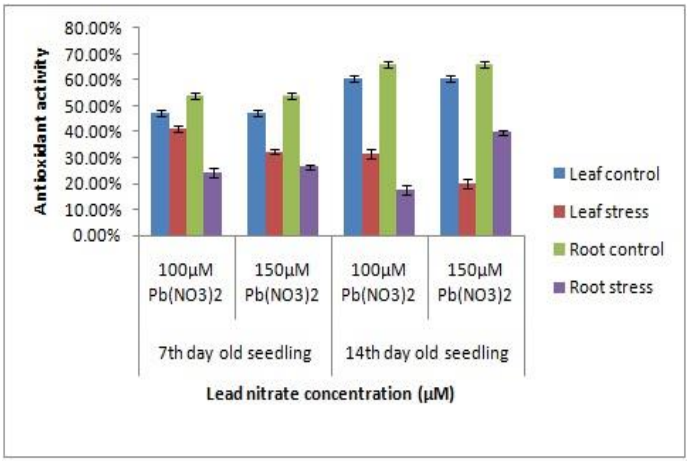

Fig 8. Antioxidant activity of methanolic extract of *Carthamus tinctorius* of 7th and 14th day ofof$Pb(NO_3)_2$ treatment with 100µM and 150µM concentration with control.

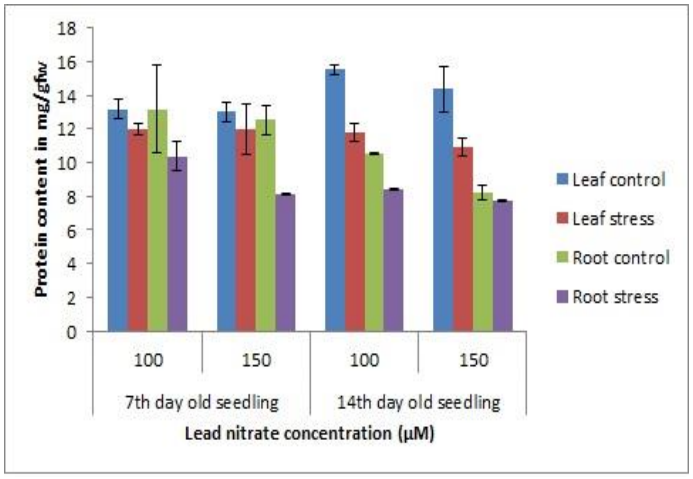

Fig 9. Protein content in 7th and 14th day seedlings (control and stressed) of both the concentrations 100 and 150µM $Pb(NO_3)_2$.

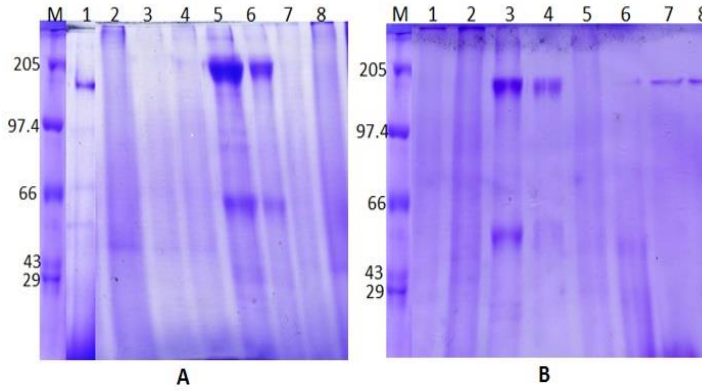

Fig 10. SDS – Page bands of proteins obtained from control and 100µM $Pb(NO_3)_2$ treated seedlings (A) and 150µM $Pb(NO_3)_2$treated seedlings (B) at 7th and 14th day. M is for marker and series 1-8 represents 7th day leaf control, 7th day Leaf stress, 7th day root control, 7th day root stress, 14th day leaf control, 14th day Leaf stress, 14th day root control, 14th day root stress.

variety HUS 305 was used for the study as it widely adapted high yielding variety and its seeds were obtained from the project coordinating unit (Safflower) Solapur, Maharashtra state, India. Initially, seeds were treated through surface disinfectant with 70% ethanol for 1min., then transferred to 2% Hypochlorite solution for 10min. finally rinsed with distilled water for 5 times. Seeds were germinated in culture tubes in a growth chamber at 25±2°C, less than 24 hours light. Seedlings of comparable size were transferred to each of 20 plastic beakers (250ml) containing Hoagland solution. Plants of half of the beakers (50plants) were exposed to 100µM concentration of Pb $(NO_3)_2$ and other half to 150µM Pb $(NO3)_2$. . Control and two weeks old seedlings of all the concentration of lead were collected for further analysis of the study.

Growth parameters

Control (both days), 7th and 14th days old seedlings of both the concentrations of lead nitrate i.e-100µm and 150µm were collected for further analysis of our study. Leaf and root explants extract of 7th and 14th days old safflower's seedlings treated with various concentration of lead nitrate (metal stress) along with its control were used in the present study to investigate their comparative changes at the morphological, biochemical, phytochemical, antioxidant properties and protein level.

Biochemical analysis

Fresh leaves and roots were used for the estimation of soluble sugars. Analysis of sugar was done by Anthrone reagent. For this leaves three week old seedlings was treated with the different concentrations of cadmium chloride and carbohydrate was estimated by Anthrone reagent. The quantitative estimation of chlorophyll (a + b) was done using modified Arnon method (1949). For this, Fresh leaves of control and stressed plants were homogenized in cold 100% 2 and centrifuged to collect the supernatant and made up to 5mL. The absorbance was recorded at 645nm, 663nm and 740nm against 80% acetone as blank and chlorophyll (a + b) was determined as per Arnon method. Proline estimation was done using ninhydrin (Bates et al., 1973).

Phytochemical analysis

Total flavonoid was estimated by using modified aluminium chloride colorimetric method (chang et al., 2002).The control and treated leaves were excised from seedlings and extract was prepared by homogenizing 1g of fresh leaves in 10mL methanol with mortar and pestle. The homogenate was then centrifuged at 9000rpm for 25min to obtain a clear supernatant and aliquots were mixed with 95% ethanol, 10% aluminium chloride, 1M potassium acetate and distilled water and the reaction was incubated at room temperature for 30 minutes. The absorbance was measured at 415nm against blank.The quantitative estimation of polyphenols was done by homogenizing 5g of fresh leaves in 10mL 75% methanol in mortar and pestle and centrifuged to obtain a clear supernatant. Aliquots of supernatant were mixed with distilled water and Folin's reagent and reaction mixture was incubated for 5 minutes and 25% sodium bicarbonate was added. Absorbance was measured at 725nm and standard curve was obtained using various various concentrations of Gallic acid (Modnicki et al., 2009).

Antioxidant enzymes extraction and assay

The control and treated leaves were excised from the seedlings (0.1g) and homogenized with mortar and pestle at 4^0C in extraction buffer (50 mM phosphate buffer, pH 7.0). the homogenate was then centrifuged at 15000 rpm for 25 minutes. The homogenized was used as the crude extract for the catalase (CAT) enzyme activity (Beers Jr and Sizer, 1952). The CAT activity was determined spectrophotometrically by following the decline in A_{240} as H_2O_2. Superoxide dismutase activity was estimated by it measuring the inhibition of photochemical reduction of NBT at 560nm (Schickler and Capsi 1999).

Protein profiling by SDS-PAGE

Protein profiling in the normal and cadmium treated plant samples were analyzed by SDS PAGE. The samples of protein were subjected to PAGE as described in (Laemmli, 1970).

Statistical analysis

All the experiments were repeated twice with three replicates (n=3) and data presented are mean ± S.E.

Conclusion

Thus the present study indicates that the plants undertake many adaptive mechanisms for their survival under metal stress which includes morphological as well as biochemical characters. It could be thus concluded that biochemical tolerance to lead toxicity is related to the capacity of plant to activation of antioxidant defense system, higher values of total phenolics and accumulation of proline an universal protectant of various stress, may be used for Phytoextraction.

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