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Responses of *Ricinus communis* L. (Castor bean, phytoremediation crop) seedlings to lead (Pb) toxicity in hydroponics

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ABSRACT

Lead (Pb) is not essential for metabolism of organisms. Lead toxicity to nervous system in human is well established. It is released into the environment through various technogenic and geogenic sources. Soils are often co-contaminated with potentially toxic metals like lead, cadmium etc. and petroleum and chlorinated hydrocarbons etc. Organics can be degraded to less toxic forms by bioremediation strategies, while inorganics such as Pb, cannot be degraded. Phytoremediation is one of the effective strategies to achieve natural attenuation.

Ricinus communis L. (Castor bean, Euphorbiaceae) is a potential candidate for environmental cleanup and revegetation of contaminated lands. Published literature acknowledges its outstanding remediation functions. Additionally, its environmental sustainable aspects and circular economics are attracting researchers in the field of agriculture and environmental sciences.

This paper investigates the responses of castor bean seedlings to Pb-toxicity in hydroponics, which offers unique clues for understanding toxicity and tolerance manifestations.

1. Introduction

Lead (Pb) is released into the environment through techno-and geogenic processes. According to the Agency for Toxic Substance and Disease Registry (ATSDR), lead is the second in the list of ‘‘Top twenty hazardous substances’’. Lead is a naturally occurring heavy metal as the earth’s crust is rich in lead (Anonymous 2011, Kabata-Pendias 2011). The major sources of Pb in the environment are shown in Fig. 1.

Lead is a highly toxic element for plants at all concentrations and has no metabolic significance. Lead exposure of plants shows various toxic symptoms such as growth reduction, chlorosis, reduced stomatal conductance, altered metabolism, inhibit photo-synthesis etc. (Ashraf & Tang 2017, Kumar & Prasad 2015, Sharma & Dubey 2005).

The way to move forward in phytoremediation is to identify plants with desirable and unique features for phytoremediation as shown in figure 2. To achieve this, toxicity bioassays in various model experimental systems such as a) plant tissue cultures b) plants in hydroponic culture and c) plants cultivated in pots in green house or in the field is a necessary step. Scientific information gleaned from these different experimental setups would be extrapolated to phytoremediation research. Each of these approaches and experimental set-ups has their own advantages and limitations. However, scientific knowledge acquired in the above model experiments in the last three decades has advanced the field of phytoremediation which is a proven technology today and it is accepted by regulatory agencies and scientific community globally (Figure 3 & 4) (Doran 2009).

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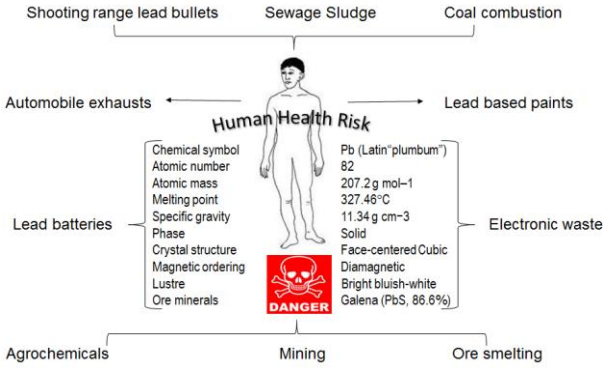

Figure 1
Lead sources in the environment available to plants.

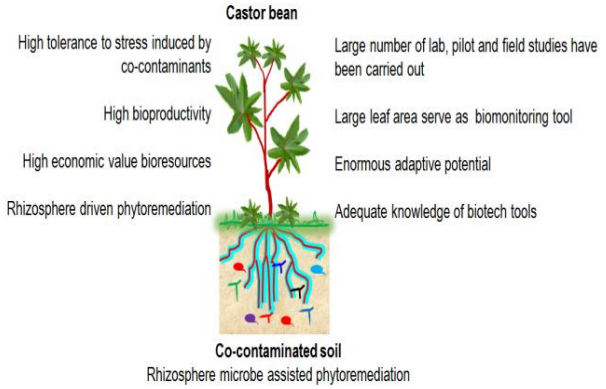

Figure 2
A schematic sketch showing the unique featuresthat are of *R. communis* (castor bean) as a phytoremediation crop

(Adhikari & Kumar 2012, Ananthi & Manikandan 2013, Bauddh et al 2015, Berman et al 2011, Bosiacki et al 2013, De Souza et al 2012, Deligiannis et al 2009, Gonzalez-chavez et al 2015, Goyal et al 2014, Huagang et al 2011, Jumat et al 2010, Kiran & Prasad 2016, 2017, Kumar & Prasad 2015, Li et al 2011, Ogunniyi 2006, Pal et al 2013, Pandey 2013, Romeiro et al 2006, Sailaja 2008, Sharma & Dubey 2005, Tang et al 2015,Wu et al 2012, Yi et al 2014, 2016)

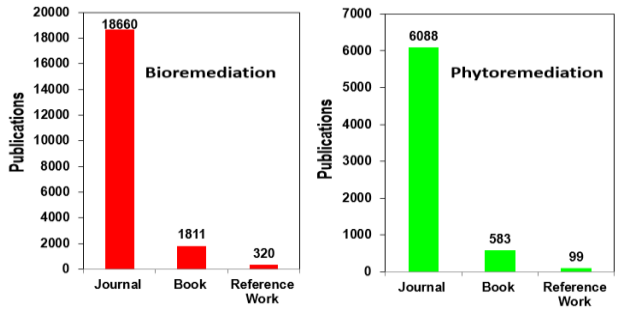

Figure 3
Classification of article content *i.e.* Journal, book and reference work. Keywords used = Bioremediation, Phytoremediation. Data source: www.sciencedirect.com

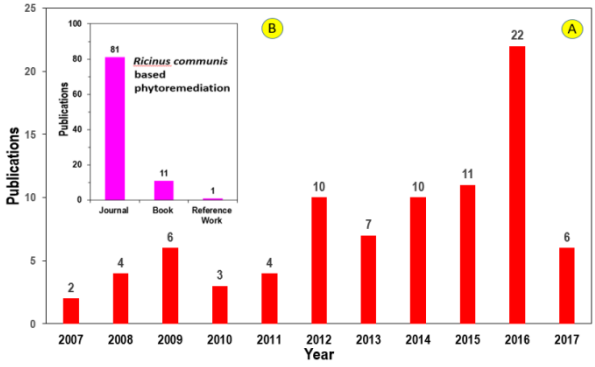

Figure 4
A) Number of papers published on *Ricinus communis* based phytoremediation. Keyword used = *Ricinus communis*, Phytoremediation B) Classification of article content *i.e.* Journal, book and reference work. Data source: www.sciencedirect.com

2. Material and Methods

Castor bean (*Ricinus communis* L) a member of the family Euphorbiaceae. *Ricinus communis* L. has been selected because of the following properties: (i) it was found to grow luxuriantly in the Pb contaminated sites, (ii) the plant produces high biomass in industrial and polluted urban areas without exhibiting any morphological changes and toxic symptoms and (iii) from literature it is known that it has the ability to accumulate potentially toxic metals. Thus, hydroponic experiments will be helpful in understanding the key mechanism (Hadi et al 2015, Zhi-Xin et al 2007).

Plant material

Seeds of castor bean variety DCS-108 were obtained from IIOR (Indian Institute of Oil Research), Hyderabad.

Seed germination and seedling culture

The castor seeds were surface sterilized with 4% sodium hypochlorite and the seedlings were germinated in petri dishes and kept in the dark for 2 to 3 days. Uniform seedlings of the same size were transferred to modified Hoagland's media in plant growth chamber at 16/8 h day/night and at 25˚C (Hoagland & Arnon 1950). Phosphate and sulphate were replaced by chloride and nitrate and pH of the modified solution was maintained at 5 to avoid the precipitation. The nutrient media was replaced every 3 days to provide a fresh dose of nutrient elements and to avoid algal growth (Figure 5 & 6).

Pb treatment: dose responses

The rational for selecting various Pb concentration and treatment duration for the experimentation was based on preliminary bioassay with respect to Pb toxicity. After growing the castor seedling for one month in Hoagland's media, plants of uniform height were selected and treated with Pb (NO_3) at different concentrations in modified Hoagland's media for 10 days.

Quantification of Pb accumulation in plant

Metal treated plant roots were washed thoroughly with 0.1M HNO_3 to remove metals adhered on to the root surface. Roots, stems and leaves were excised and oven dried at 80˚C for 3 days. Dried plant material of 0.1g was acid digested with HNO_3 in a Microwave digester and analyzed for Pb content using Atomic Absorption Spectrophoto-meter (Perkin Elmer A400).

*Estimation of Chlorophyll, Protein, Proline and Lipid peroxidation (MDA***)**

Chlorophyll was determined in the acetone extract (80% v/v) (Arnon 1949) at 663 and 645 nm and the concentration was expressed as mg chlorophyll per g fresh weight. Protein estimation was done according to Lowry et al. (1951). Free proline was measured by following the method of Bates et al. (1973). Lipid peroxidation in leaves was determined as a function of malondialdehyde (MDA) with slight modifications (Heath & Packer 1968).

Elemental analysis by energy dispersive X-rays spectroscopy (EDS)

Energy–dispersive X-ray spectroscopy is an analytical technique used for the elemental/chemical analysis of specimen. Treated samples (200 µM and 400 µM) along with control are made into fine powder, mounted on aluminium stubs, coated with gold-palladium. The elemental analysis was done with EDS (Oxford instruments) coupled with field emission scanning electron microscope (FESEM, Ultra 55-carl Zeiss). The EDS analysis was carried out at an operating voltage of 20 KV and working distance of 8.5 mm. With the help of INCA software, X-ray emission based spectral peaks were analyzed.

Anthocyanin content

Fresh leaves 1g were uniformly homogenised in 3 ml of extraction mixture (2.37 ml methanol, 0.6 ml water, 0.03 ml HCl). The crushed material was then centrifuged at 5000 g for 15 min. The supernatant absorbance was taken at 530 and 657 nm using UV-VIS spectrophotometer (Mancinelli 1984).

Absorbance (A) = Ab_{530}- ($0.25 \times Ab_{657}$)

Anthocyanin content = $(A \times Mol.wt \times DF \times 1000) / \varepsilon$

X-ray diffraction analysis

X-ray Diffraction (XRD) analysis was carried out by using Siefert Model SF 60 XRD system. Fine root powder of control, 200 and 400 µM were analyzed at typical scanning angles of $2\theta = 20 – 600$.

Detection of cell death

To determine the changes in viability of cells after Pb treatment, 0.1g of freshly harvested roots were stained with 0.25% (w/v) aqueous solution of Evans blue for 15 min (Baker & Mock 1994). After washing with milliQ water for 30 min, roots were excised and soaked with 3 ml of N,N-dimethylformaide for 1 hour at room temperature. The absorbance of released Evans blue was measured at 600 nm.

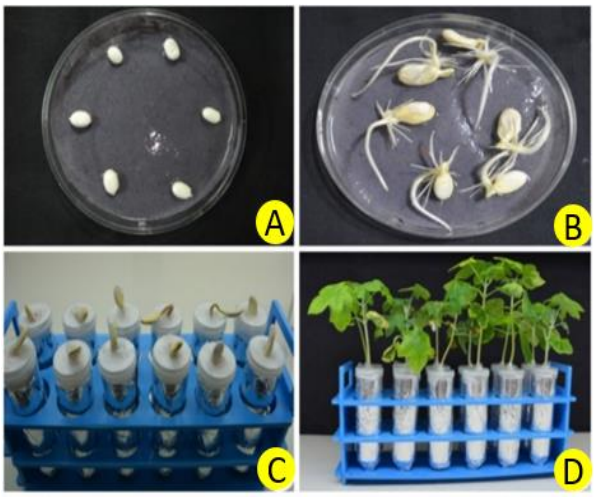

Figure 5
(A&B) Castor bean seed germination in petriplates; (C&D), Hydroponics set up in modified Hoagland's solution

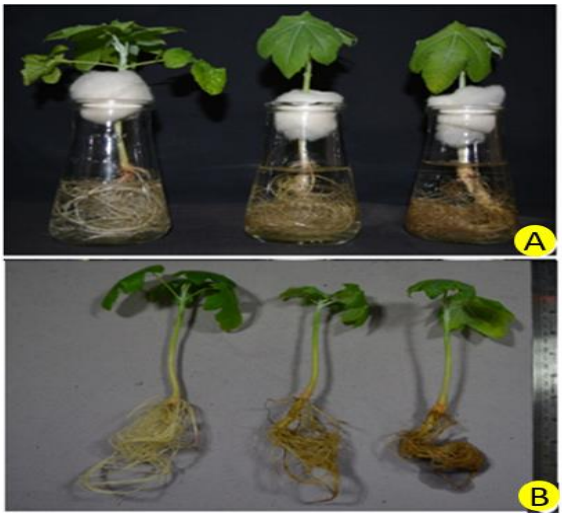

Figure 6
(A&B) Visual changes in root architecture of one-month old castor seedlings exposed to Pb after 10 days treatment

Estimation of H_2O_2 in root tissue

Fresh roots 0.1g were homogenized in an ice bath with 5 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 rpm for 15 min at 4˚C and 0.5 mL of the supernatant was added to 0.5 mL of 10mM potassium phosphate buffer (pH 7.0) and 1 ml of 1M potassium iodide. H_2O_2 concentration was estimated at 390nm on the absorbance of a stan-

dard curve and was expressed nmol g $^{-1}$ FW (Velikova et al. 2000).

3. Results and Discussion

Pb treatment at different concentration caused different levels of phytotoxicity, which includes chlorosis, visible damage of leaf and reduction in growth. Based on the visible observation and previous literature reported (Kiran and Prasad 2017), Pb concentration of 200 and 400 µM were selected for treatment. Most of selected Pb concentration was showing toxic symptoms at 10 days of treatment. The flasks without Pb (NO_3) were kept as control.

Lead accumulation in plant

Accumulation of metal in plant tissue was calculated on dry weight basis. Upon increasing the concentration, Pb accumulation in plant parts increased significantly as compared to control. Roots are the main accumulation site as they absorbed much higher quantities (19.53±0.11 mg g^{-1} dry weight (DW) than stems (0.38±0.003 mg g^{-1} DW) while in leaves, Pb accumulation was (0.05±0.003 mg g^{-1} DW) (Table 1). Lower concentration of Pb in stems and below detectable limits in leaves confirmed the decreased translocation of Pb within the plant. Upon increasing Pb concentration plant growth is reduced (Tandy et al. 2005) and some toxic symptoms like chlorosis, yellowing across the lamina, leaf fall and darkening of roots was observed (Tanhan et al. 2007).

Table 1

Lead accumulation in roots, stem and leaves of castor plants grown for 10 days at different doses of Pb(NO_3).

Concentration of Pb taken up by the plant (mg g^{-1}DW)			
Pb treatment	Leaf	Shoot	Root
Control	0	0	0
200 µM	0.050±0.003	0.076±0.003	16.67±0.04
400 µM	0.055±0.003	0.386±0.003	19.53±0.11

Lead induced changes in chlorophyll, protein, proline and lipid peroxidation

Contents of Chl a, b and total chlorophyll were reduced about 50% in 200 µM and about 30% in 400 µM when compared to the control upon exposure of the treatment period. It was suggested that heavy metals interfere with chlorophyll biosynthesis by substitution of central Mg^{2+} ion (Sengar et al. 2008). Protein levels were significantly decreased about 80% in both 200 and 400 µM Pb treatment when compared to control. The dose dependent increase in protein content of Pb treated roots was observed due to inhibition of protein synthesis or protein oxidation (Aravind and Prasad 2003). Proline content was increased at 200 µM Pb, but slightly greater at 400 µM when compared to control. İt is suggested that aminoacids like proline helps the plant to combat non-enzymatically against free radicals produced by lead (Sharmila and Saradhi 2002). MDA estimation, an indicator of lipid peroxidation, showed that the MDA concentrations were ignificantly increased than control after Pb treatment. MDA concentration in roots of castor plants were elevated after 10 days due to Pb toxicity and the magnitude of elevation ranged from 50 folds at 200 and 400 µM of Pb more than control respectively. (Pourraut et al 20011a). Reference be made to Kiran and Prasad (2017) for details..

Element analysis by Energy Dispersion Spectroscopy (EDS)

Results of EDS give the atomic or chemical characteristics of analysed tissue. As a first barrier to metal toxicity, most plants accumulate metals and nutrients in the roots and restrict its transport to aerial parts. Microanalysis of elements was performed at the same site in 0, 200 and 400 µM of Pb treated root samples. Elements such as oxygen (O), potassium (K), magnesium (Mg), chlorine (Cl), calcium (Ca), copper (Cu) and lead (Pb) were detected in root tissue (Table 2 & Figure 7). In analysed tissue, O and K were contributed as major elements. X-ray microanalysis of untreated samples showed high spectral peaks for all elements, except Mg and Cu. Copper was not detected in treated plants. Qualitative percentage composition analysis revealed that the percentage of all elements was decreased except element O, which was significantly increased upon increased concentration of metal within the roots. Elemental microanalysis helps us to understand the composition of elements within the tissue deposits (Nagata 2004; Shillito et al. 2009)

Table 2

Analysis of atomic percentage of elements by energy dispersive spectroscopy (EDS) in roots of castor plants treated with 0, 200 and 400 µM of Pb for 10 days.

Atomic % of elements in roots of castor plants			
Element	Control	200 µM Pb	400 µM Pb
O	88.33	90.24	93.29
K	5.29	3.65	1.68
Mg	2.95	1.16	0.64
Cl	2.17	1.63	1.32
Ca	1.07	2.07	0.95
Cu	0.19	ND	ND
Pb	ND	1.26	2.12

ND- Not Detecable

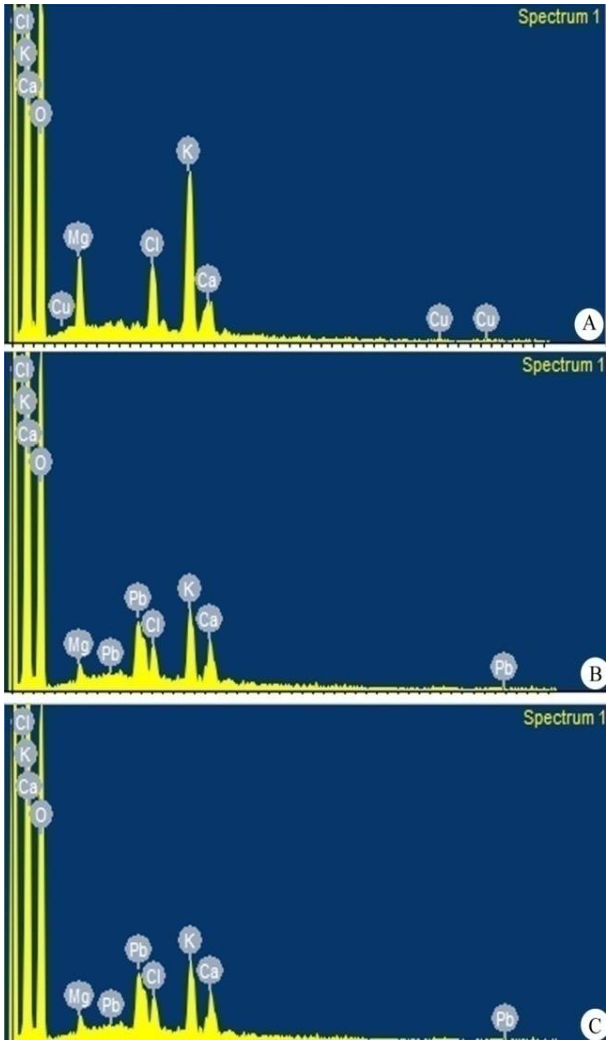

Figure 7
Energy dispersive X-ray spectral line profile of the root powder of castor: control (A), 200 µM Pb (B) and 400 µM Pb(C). EDS micrograph showed the elemental atomic percentage of the imaged area for the sample using FESEM/EDS.

Lead induced changes in anthocyanin concentration

Anthocyanin content was increased one fold in 200 µM and 0.5 fold at 400 µM when compared to the control after 10 days of treatment period (Figure 8). Under Pb stress and at lower concentration, anthocyanin shows effective strategy against ROS generation (Kumar and Prasad 2015). Anthocyanin plays putative role in scavange free radicals but also have ability to bind heavy metal ions, biosynthesized through the phenylpropanoid pathways. It is suggested that phenylalanine ammonia lyase (PAL), a key enzyme in flavanoids synthesis is targetted by of heavy metal imposed stress causing the inhibition of anthocyanin biosynthesis (Dube et al. 1933).

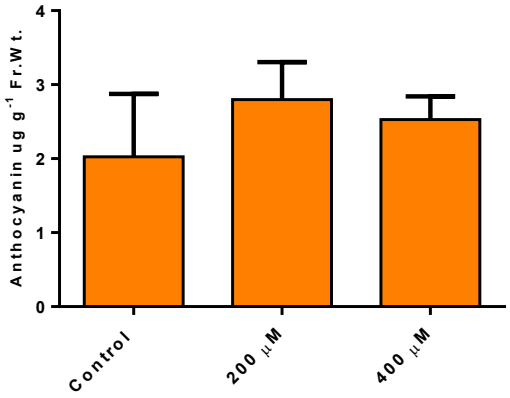

Figure 8
Anthocyanin content in roots of castor seedlings after 10 days of treatment.

X-ray Diffraction (XRD) analysis

Sharp intensity XRD peaks have been observed at typical scanning angles of 2θ = 20 - 600. The Sharp peaks present in the figure indicated the crystalline nature of the material. In addition, several other low intensity peaks corresponding to other crystalline phases of carbons have also been observed (Figure 9). After binding to Pb, the porous structures of the carbon adsorbents increased. These causes high intensity XRD peaks. Hence crystalline phases should have been increased (Jeyakumar and Chandrasekaran 2013).

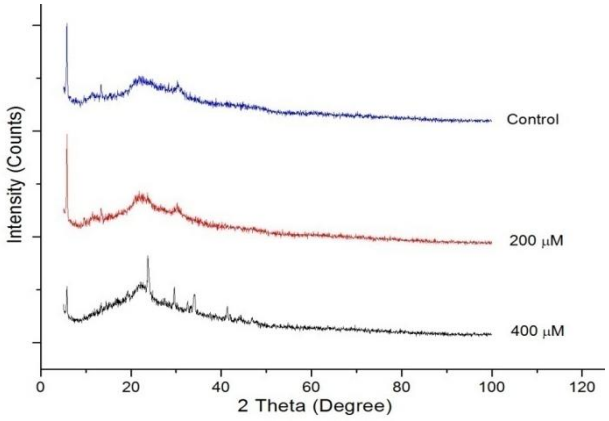

Figure 9
XRD pattern peaks of Control, 200 and 400 µM after 10 days of Pb treatment.

Lead induced cell death

Lead induced oxidative damage in castor seedlings was quantitatively confirmed by staining with Evans blue. Lead addition resulted in higher accumulation of Evans blue (0.01 folds) in 200 µM and (0.03 folds) in 400 µM compared to control (Figure 10). This could be

possible due to decline in nutrients and loss of plasma membrane integrity and membrane damage induced by lead which leads to disruption of cell wall at the elongation zone (Haung et al. 2008).

Hydrogen peroxide (H_2O_2) estimation

H_2O_2 production increased after 10 days of treatment with respect to control (Figure 11). Our data revealed that Pb toxicity promoted oxidative stress with enhanced production of H_2O_2. It is believed that an increased production of free radicals in a cell is due to disturbance of electron transport chain in membranes (Malecka et. al 2009)

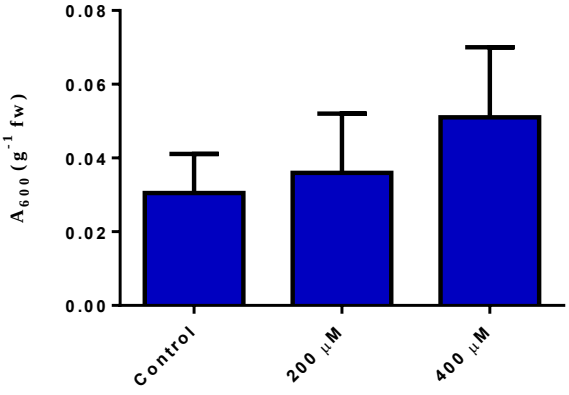

Figure 10
Cell death by Evans blue uptake and

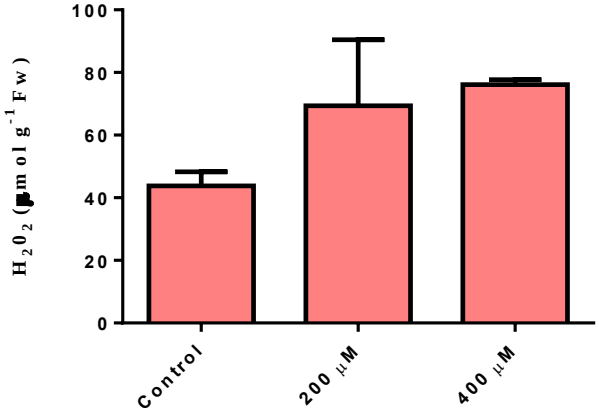

Figure 11
H_2O_2 estimation in roots of castor seedlings after 10 days of Pb treatment.

Conclusions

The results of the present study revealed that roots are the major sites for metal accumulation and Pb accumulation in the tissue is concentration dependent. Roots accumulated more Pb than stem and below detectable level in leaves. This suggests Pb immobilization in root or decreased translocation from root to shoot which represents attractive strategy for defence mechanism. The decrease in element atomic % and increase cell death, H_2O_2 production showed that analyzed Pb concentration have potential to cause oxidative damage in roots. Increased anthocyanin showed adaptive mechanism in Pb treated plants. These findings confirm the toxicity and tolerance strategies of *Ricinus communis* under Pb stress. Future research is required on the microlocalization and detoxification mechanism of Pb to improve our understanding. This kind of information would be useful for the development of suitable remediation strategies (Ashraf & Tang 2017)

4. Acknowledgements

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