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BIOCHEMICAL AND PHYSIOLOGICAL ANALYSIS OF ZINC TOLERANCE IN *Jatropha curcas*

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Growth parameters

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ABSTRACT

Jatropha curcas L., widely recognized as a viable option for production of bio diesel, has been assessed for its ability to withstand stress induced by supra-optimal zinc concentrations. In the present study plants were exposed to varying Zinc (Zn) concentrations (0, 500, 1000, 1500 and 2000 mg/kg), and and different growth, physiological and biochemical parameters were studied**.** It was reported that up to 1500 mg/kg Zn, no significant effects on most of the growth parameters of the plants could be seen. However at 2000 mg/kg Zn, a clear retardation of growth was visible, which was apparently reflected by the physiological as well as biochemical parameters. These effects were more profound in the aerial parts of the plant. Atomic Absorption Spectra (AAS) profiles suggested that Zn got mainly accumulated in the roots after absorption from the soil. Osmotic adjustments indicated significantly increased accumulation of proline, phenols and reducing sugars with increasing concentration of Zn as compared to the control. Membrane damage was not observed up to 1000 mg/kg concentration. Jatropha, owing to its tolerance to supra-optimal Zn concentrations is, thus, a suitable candidate for phytoremediation of Zn from contaminated soils along with cultivation for biofuel production.

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1 Introduction

Heavy metals (HMs), frequently referred to lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), cadmium (Cd), copper (Cu), mercury (Hg) and nickel (Ni) occur naturally in the soils. Beyond a certain concentration, these heavy metals are toxic. In recent years, their concentrations in soil have become a concern worldwide (Rascio & Navari-Izzo, 2011; Villiers et al., 2011). Zn is a necessary cofactor for many biological reactions, known to limit oxidative degradation of auxin and is necessary to maintain membrane integrity (Tsonev & Lidon, 2012). Zinc concentration in soils lesser than 125 ppm is considered optimum for the growth of plants (Hussain et al., 2010). Plants growing in such edaphic environments display Zn concentrations in the range 0.02-0.04 mg g^{-1} dry weight (Tsonev & Lidon, 2012). Higher concentrations of Zn in soil, however, have direct effects on the growth and yields of the plants (Chibuike & Obiora, 2014), and thus adversely affect the agriculture.

The general symptoms are stunting of shoot, curling and rolling of young leaves, death of leaf tips **(**Rout & Das, 2003) and chlorosis (Rout & Das, 2003). Due to Zn toxicity, the activity of proteins present in the plasma membrane and especially the activity of SH groups gets affected which causes damage to membrane stability. As soon as heavy metals pass through the plasma membrane, they can immediately interact with all metabolic processes (Rout & Das, 2003). To avoid Zn toxicity in plants, the excess quantities of Zn shall be cleaned up from the soil. Among several methods available for such clean up, phytoremediation is catching attention in recent years, as plants survive for longer durations and have potential to permanently fix the pollutants. Plants have many cellular mechanisms involved in the detoxification of heavy metals and thus tolerance to metal stress. These include the binding of metals to cell wall and extracellular exudates, reduced uptake or efflux pumping of metals at the plasma membrane, chelation of metals in the cytosol by peptides such as phytochelatins, repair of stress-damaged proteins and the compartmentation of metals in the vacuole by tonoplast located transporters (Hall, 2002). However, this necessitates that plants being used for phyto-remediation should be non-edible and can grow effectively at the polluted sites (Nanda & Abraham, 2011).

In view of the above, we have assessed the potential of jatropha, which is also being projected as a promising bio fuel crop, to survive and thrive under condition of higher soil concentrations of Zn. It is a small tree that has naturalized in most parts of the world and grows in a variety of agro-climatic areas. Many studies show the potential of *J. curcas* to recover and reclaim heavy metal contaminated soil (Yadav et al., 2009).

2 Materials & Methods

2.1 Plant material and Zn concentrations

Mature, healthy and current harvest seeds of *J. curcas* strain DARL-2 were soaked overnight in 0.1% (w/v) Bavistin, washed several times under running tap before sterilizing with 70% (v/v) ethanol and followed by three washes of sterile water. Thereafter, seeds were allowed to germinate on moist filter papers in Petri dishes. After germination, seedlings of uniform size were selected and transplanted into pots containing autoclaved mixture of sand and soil in 1:1 ratio. $ZnSO_4.5H_2O$ solution was added in the pots to obtain the Zn^{+2} concentrations of 0 (control), 500, 1000, 1500, 2000 mg/kg of soils. Experiment was conducted with three replicates each, and replication had five pots having three plants each. Both control and treated pots were irrigated at regular interval.

The Zn concentration in soil and in different parts of the plant (root, stem and leaf) was estimated using Atomic Absorption Spectrometer (M Series 650294v129, Thermo Electron Corporation, USA) fitted with an air-acetylene burner, expressed as mg/g dry weight of the sample.

2.2 Growth parameters

Root length, shoot length, total number of leaves, fresh weight and dry weight of root and stem were recorded for each treatment after 4 months.

2.3 Physiological parameters

Total chlorophyll (a + b) and carotenoids were determined from fresh leaf (100 mg FW) according to Arnon (1949). The leaf material was ground in a pre-chilled mortar in acetone (80% v/v). After homogenization, the mixture was filtered and the volume was adjusted to 10 ml with cold acetone. The absorbance of the extract was measured at 645, 663, and 470 nm using a spectrophotometer (UV-Vis Dual Beam, Labomed inc.) and the pigments content were calculated. The chlorophyll stability indices (CSI) were determined using the formula:

Total chlorophyll content in stressed leaves / total chlorophyll content in control leaves X 100

The leaf relative water content (RWC) was determined according to Patade et al. (2011). Fresh weight (FW) of the leaf was recorded immediately after plucking from the plant. After 24 h of saturation with deionized water the turgid weight (TW) was recorded. Dry weight (DW) was recorded after drying the leaves for 48 hrs in the hot air oven at 70^{o}C. The RWC was calculated as:

RWC (%) = [(FW-DW)/ (TW-DW)] X100

Reducing sugar was estimated as described by Miller (1959). About 100 mg leaf sample was homogenized in 3ml of 80% ethanol. The homogenate was centrifuged at 6000 g for 10 min at 48^{o}C and the supernatant was mixed with equal volume of 3, 5-dinitro-salicylic acid (DNSA) reagent. Distilled water was

vortexing and the tubes were placed in a boiling water bath for 10 min after which they were cooled on ice. The absorbance was measured at 540 nm and the reducing sugars content (mg g^{-1}FW) was calculated based on standard curve with glucose as standard.

Leaf pieces (~1.0 cm^2) after washing with distilled water were transferred to glass culture tubes containing 20 ml distilled water and incubated for 24 h with intermittent shaking. Electric conductivity was recorded using EC meter (WTW, Germany). EC_1 was recorded after 24 hrs of incubation of the leaf. Tubes were capped and then autoclaved at 121^oC for 20 min. to completely kill the tissues and release all electrolytes. EC_2 was recorded after cooling the solution to room temperature. Membrane damage rate (MDR) was calculated using the formula (Lutts et al., 1995):

MDR (%) = (EC_1 / EC_2) X 100

Proline content was determined according to Bates et al. (1973). 200 mg of leaf was homogenized in aqueous sulfosalicylic acid (3% w/v). The filtered homogenate was reacted with equal volume each of acid ninhydrin and acetic acid for 1 h at 100°C in a water bath. The reaction mixture was extracted with toluene and the absorbance was recorded at 520 nm using toluene as a blank. Proline concentration (µg g^{-1} FW) was determined from a standard curve using L-proline as a standard.

Lipid peroxidation was determined according to the method of Heath & Packer (1968). 100 mg of leaf was homogenized in 1.5 ml of 0.25% Thiobarbituric acid (TBA) in 10% Trichloroacetic acid (TCA). The mixture was heated at 95^oC for 30 min. and then cooled in ice, it was then centrifuged at 10000 g for 10 min. Absorbance of the supernatant was read at 532 nm and 600 nm, keeping 0.25% TBA in 10% TCA as blank. MDA content was calculated according to its extinction coefficient of 155 $mM^{-1}cm^{-1}$.

Total phenolic content was estimated according to Folin-Cioalteu method as described by Ainsworth & Gillespie (2007). The leaf tissue was ground to a fine powder using liquid nitrogen. 2 ml of 95% (v/v) ice cold methanol was then added to the ground tissue and incubated for 48 h at room temperature in dark. It was then centrifuged at 13000 g for 5 min. Supernatant (100 µl) was taken and mixed with 200 µl of 10% (v/v) F-C reagent to which, 800 µl of 700 mM Na_2CO_3 was added and again incubated at room temperature for 2 h. Absorbance was recorded at 765 nm. Total phenolic content was calculated based on standard curve with gallic acid as standard and expressed as mM μM^{-1} gallic acid equivalent.

2.4 Antioxidant enzyme assays

CAT activity was measured in a reaction mixture (1.0 ml) containing 50 mM phosphate buffer (pH 7.0) and 15 mM H_2O_2 initiated by adding 50 µl enzyme extract and the activity was determined by monitoring decrease in absorbance at 240 nm (E = 39.4$mM^{-1}cm^{-1}$) for 2 min. at intervals of 15 sec, as a result of H_2O_2 decomposition. The slope of the rate assay (ΔA) was used to determine the enzyme activity, which was expressed as µmol.mg $protein^{-1}min^{-1}$.

APX activity was determined according to Nakano & Asada (1981). The reaction mixture (2.0 ml) contained 50 mM phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM H_2O_2 and 0.1 mM EDTA. The reaction was started by adding 100 µl of crude enzyme. The H_2O_2 dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm (E = 2.8 Mm^{-1} cm^{-1}). APX activity was measured in terms of µmol.mg $protein^{-1}min^{-1}$.

GPX activity was determined according to Kar & Feierabend (1984). The reaction mixture (1.0 ml) contained 50 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 10 mM guiacol and 10 mM H_2O_2. Oxidation of guiacol was monitored by measuring the increase in absorbance at 470 nm (E = 26.6 $Mm^{-1}cm^{-1}$) for 1 min at interval of 15 s after addition of 50 µl of crude enzyme. GPX activity was measured in terms of µmol of tetraguaicol formed mg $protein^{-1}min^{-1}$.

2.5 Statistical Analysis

Mean, standard error and statistical significance of mean values for different parameters were determined. Analysis of variance (ANOVA) for all the variables was performed using Cropstat for Windows (7.2.2007.2 module, IRRI, Phillipines).

3 Results

3.1 Growth responses

Compared to the control, the growth parameters were not significantly (p>0.05) affected up to 1500 mg/kg Zn (Table 1) as seen by non-significant differences in various growth parameters (root length, shoot length, fresh and dry weight of the root and fresh and dry weight of the stem). However, at 2000 mg/kg Zn all the growth parameters were significantly (p<0.05) reduced except the root length as compared to the control (Table 1).

3.2 Zn accumulation in different plant parts

A significant amount of Zn was detected in *J. curcas* plants grown at different concentrations of metal. Accumulation was maximum in the roots, i.e., 8.93 mg/g DW followed by the stem 3.61 mg/g DW and leaves 0.79 mg/g DW (Figure 1). About nine folds higher Zn level was detected in the roots at 2000 mg/kg Zn as compared to the control. Similarly the level of Zn in the stem and leaves of plants at 2000 mg/kg Zn was 11.5 folds and 1.27 folds respectively in relation to the control.

Table 1 Effect of different concentrations of Zn on growth parameters of *J. curcas*. Standard error of three treatment means (SE) and LSD values are given in the last row.

Zn conc. (mg/kg soil)	Root length (cm)	Shoot length (cm)	Total leaves	Fresh weight stem (g)	Dry weight stem (g)	Fresh weight Root (g)	Dry weight Root (g)
0	8.233	21.767	9.000	3.625	2.493	0.270	0.056
500	8.483	22.333	8.33	3.785	2.690	0.467*	0.072*
1000	7.050	20.833	9.00	3.699	2.462	0.330*	0.063*
1500	5.267	20.733	10.67*	3.277	2.570	0.234	0.053
2000	4.583	14.600*	8.00	1.778*	1.398*	0.161*	0.024*
SE	0.778	0.293	0.24	0.128	0.093	0.013	0.001
5% LSD	2.295	0.956	0.77	0.418	0.303	0.039	0.005

The values marked with asterisk (*) are significantly different from control at $P \leq 0.05$, as determined using Least Significant Difference (LSD) test.

3.3 Zn concentration in soil before planting and after harvesting of *J. curcas*

The results of soil analysis showed that the percent uptake of Zn increased significantly ($p<0.05$) at different concentrations as compared to the control (Table 2). At 2000 mg/kg Zn concentration, the percentage uptake of Zn from soil increased by 6.68 folds as compared to the control.

3.4 Chlorophyll and carotenoid contents

The total chlorophyll and carotenoides contents, and the chlorophyll stability index was increased significantly ($p<0.05$) at 1000 mg/kg Zn as compared to the control but at lower (500 mg/kg) and higher (≥ 1500 mg/kg) concentrations of Zn, no difference in the total chlorophyll content and the chlorophyll stability index were observed (Table 3).

3.5 Osmotic adjustments

In response to different concentrations of Zn, RWC of the leaf was not significantly ($p>0.05$) changed as compared to the control, but the accumulation of reducing sugars has significantly ($p<0.05$) increased in relation to the control. Significantly ($p<0.05$) higher content of total phenol (1.44 folds) and proline (2 folds) was observed at 2000 mg/kg Zn as compared to the control (Figure 2a&b).

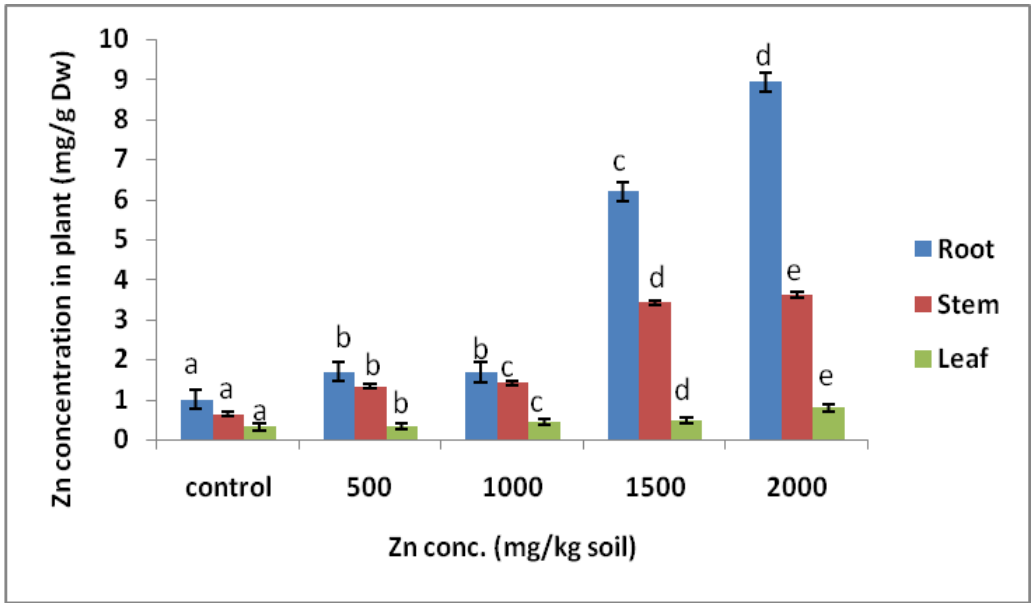

Figure 1 Accumulation of Zn in plant parts exposed to different concentrations of Zn. Different letters indicate significant differences at p>0.05, as determined using Least Significant Difference (LSD) test. Error bars indicate SE of three treatment means.

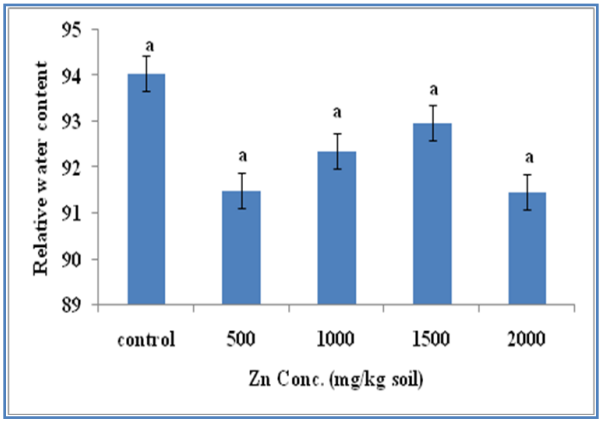

Figure 2a Effects of different concentrations of Zn on RWC measured in leaves of *J. curcas*. Different letters indicate significant differences at p>0.05, as determined using Least Significant Difference (LSD) test. Error bars indicate SE of three treatment means.

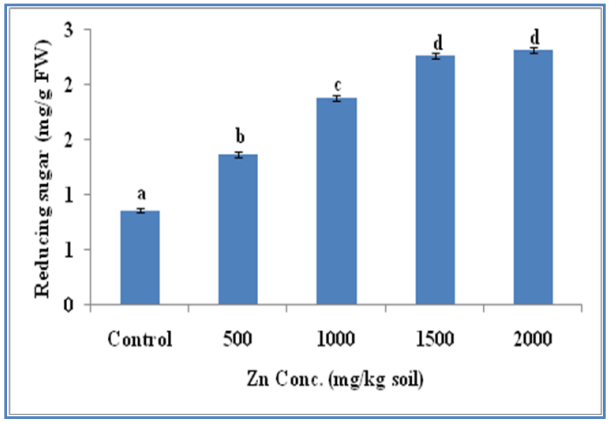

Figure 2b Effects of different concentrations of Zn on reducing sugar measured in leaves of *J. curcas*. Different letters indicate significant differences at p>0.05, as determined using Least Significant Difference (LSD) test. Error bars indicate SE of three treatment means.

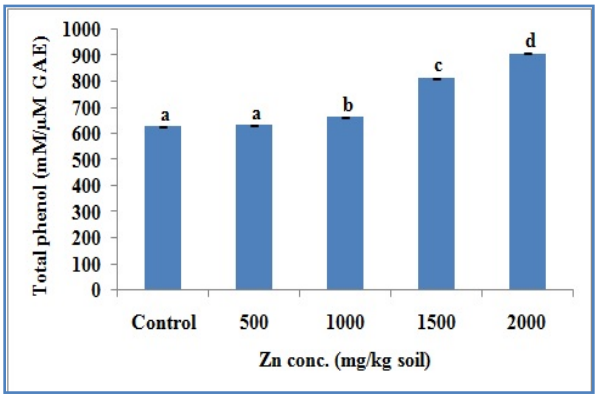

Figure 2c Effects of different concentrations of Zn on total phenol measured in leaves of *J. curcas*. Different letters indicate significant differences at p>0.05, as determined using Least Significant Difference (LSD) test. Error bars indicate SE of three treatment means.

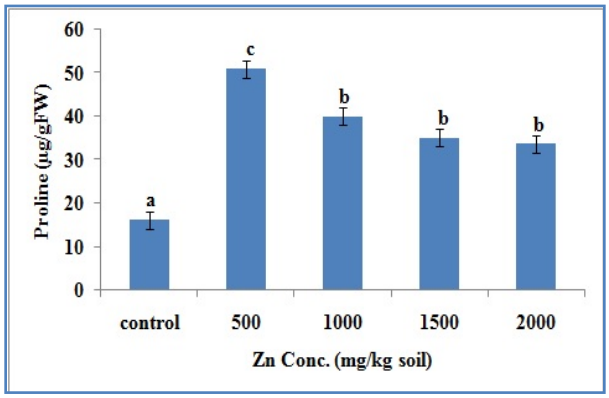

Figure 2d Effects of different concentrations of Zn on proline content measured in leaves of *J. curcas*. Different letters indicate significant differences at p>0.05, as determined using Least Significant Difference (LSD) test. Error bars indicate SE of three treatment means.

3.6 Lipid peroxidation and Membrane damage rate

Higher concentrations of Zn has affected the membrane properties which is revealed by significantly (p<0.05) increased (1.31 folds) amount of MDA content at 2000 mg/kg Zn as compared to the control (Figure 3a). However, the electrical conductivity of the leaves was not significantly (p>0.05) changed in response to higher Zn concentrations as compared to the control (Figure 3b).

3.7 Antioxidant enzyme activities

The activities of the antioxidant enzymes (CAT, APX and GPX) were significantly (p<0.05) increased in response to higher concentrations of Zn as compared to the control (Figure 4a-c). In relation to the control, significantly ($p \leq 0.05$) higher CAT activity was observed in all the treatments and the highest increase of 2.8 folds was observed at 1000 mg/kg Zn. The APX activity was significantly ($p \leq 0.05$) increased up-to 1500 mg/kg Zn (2.3 folds) but at 2000 mg/kg Zn the APX activity was decreased in relation to the control. GPX activity was also significantly ($p \leq 0.05$) increased in all the treatments in relation to the control and the highest increase of 3.4 folds was observed at 2000 mg/kg Zn.

Table 2 Analysis of the soil sample used for growing *J. curcas* exposed to different concentrations of Zn.

Initial Zn conc. in soil (µg/g)	Final Zn conc. in soil (µg/g)	% Zn uptake by plant from soil
(Control) 12	10.33	13.89
500	55.00*	89.00*
1000	103.00*	89.70*
1500	112.00*	92.53*
2000	144.00*	92.80*
SE	0.82	1.00
LSD p≤0.05	0.58	0.71

The values marked with asterisk (*) are significantly different from control at P ≤ 0.05, as determined using Least Significant Difference (LSD) test.

Table 3 Effect of different concentrations of Zn on pigment content in *J. curcas.* Standard error of three treatment means (SE) and LSD values are given in the last row.

Zn conc. (mg/kg soil)	Chl (a + b) (mg/gFW)	Chlorophyll stability index (CSI)	Carotenoids (mg/gFW)
0	8.40	100	415.56
500	9.16	109.06	442.47
1000	14.22*	169.33*	633.56*
1500	9. 01	107.28	483.21
2000	7.99	95.06	354.70
SE	0.49	5.99	18.39
LSD	1.61	19.55	59.97

The values marked with asterisk (*) are significantly different from control at P ≤ 0.05, as determined using Least Significant Difference (LSD) test.

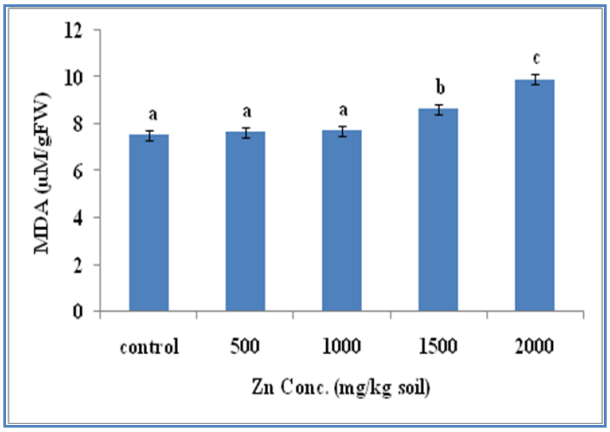

Figure 3a Effects of different concentrations of Zn on MDA measured in leaves of *J. curcas*. Different letters indicate significant differences at p>0.05, as determined using Least Significant Difference (LSD) test. Error bars indicate SE of three treatment means.

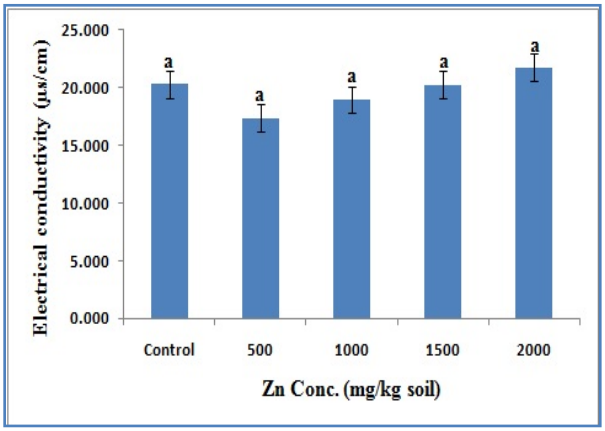

Figure 3b Effects of different concentrations of Zn on EC measured in leaves of *J. curcas*. Different letters indicate significant differences at p>0.05, as determined using Least Significant Difference (LSD) test. Error bars indicate SE of three treatment means.

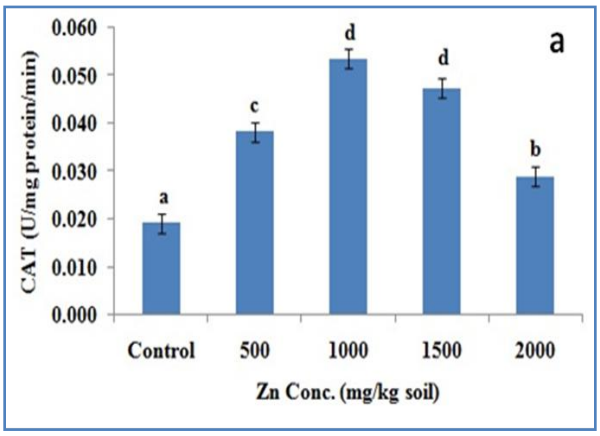

Figure 4a Effects of different concentrations of Zn on CAT activity measured in leaves of *J. curcas*. Different letters indicate significant differences at p>0.05, as determined using Least Significant Difference (LSD) test. Error bars indicate SE of three treatment means.

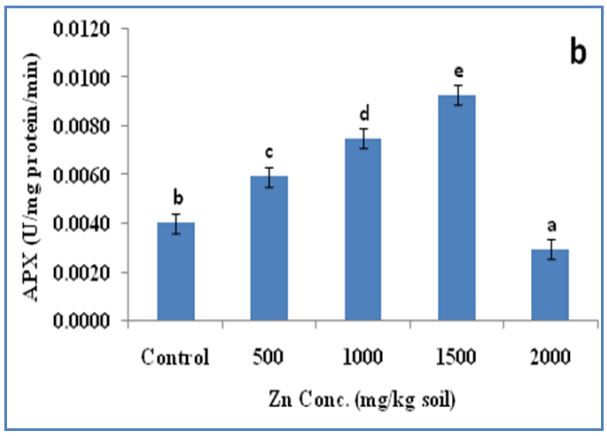

Figure 4b Effects of different concentrations of Zn on APX activity measured in leaves of *J. curcas*. Different letters indicate significant differences at p>0.05, as determined using Least Significant Difference (LSD) test. Error bars indicate SE of three treatment means.

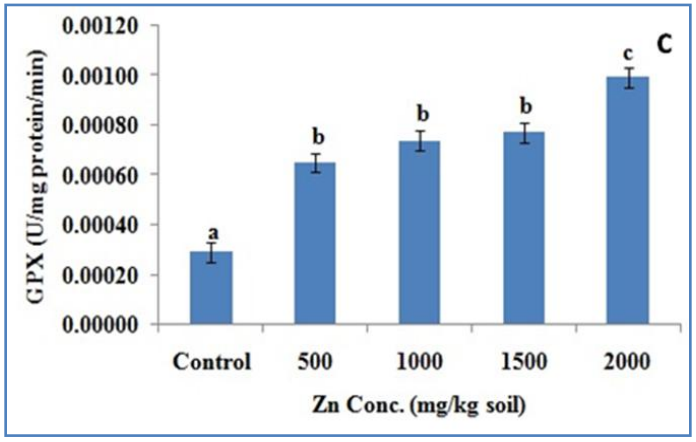

Figure 4c Effects of different concentrations of Zn on GPX activity measured in leaves of *J. curcas*. Different letters indicate significant differences at p>0.05, as determined using Least Significant Difference (LSD) test. Error bars indicate SE of three treatment means.

Discussion

Despite being an essential micronutrient, the threshold of toxicity due to Zn varies among plant species (Tsonev & Lidon, 2012). *J. curcas*, has remarkable ability to withstand elevated levels of Zn concentration, often accumulating excess concentrations within the cells. It was reported that shoot length, total number of leaves, fresh weight of stem, fresh weight of root, dry weight of stem and dry weight of root were not affected up to 1500 mg/kg Zn. At 2000 mg/kg Zn, however, a significant decrease in the growth parameters was observed, though no significant reductions occurred in the root length. Growth inhibition is a general phenomenon associated with most of heavy metals (Luo et al., 2010) and there are reports which show that higher Zn concentrations results in biomass decline and inhibition of cell elongation and division (Tsonev & Lidon, 2012).

Zn acts as a structural and catalytic component of proteins, enzymes and as a co-factor for normal development of pigment biosynthesis which could be the reason behind increased chlorophyll and carotenoid contents at 1000 mg/kg Zn as compared to the control. Chlorophyll pigments are present in the chloroplasts of leaves and it has been found that under stress the amount of chloroplast increases for maintaining the photosynthesis in plants. Increased content of photosynthetic pigments was also observed by other workers (Jamil et al.,

2007; Pinhiero et al., 2008; Rahdari et al., 2012) under different abiotic stresses.

The RWC in *Jatropha* plants grown at different concentrations of Zn was not significantly affected as compared to the control, indicating ability of *Jatropha* to osmotically adjust to higher concentrations (upto 2000 mg/kg) of Zn in soil. Osmotic adjustment was also inferred in terms of levels of accumulation of reducing sugars, phenols and proline in leaf tissues. The accumulation of reducing sugars could be a result of starch degradation, and helps in adjusting water potential in the cytosol, i.e., intracellular osmotic adjustment. In case of accumulation of heavy metals, altered water potential would be instrumental in adjusting to higher concentrations of accumulated ions in the vacuole, and would protect integrity of cellular membranes (Naghavi, 2014).

Proline too is a well documented osmolyte involved in abiotic stress tolerance including heavy metal stress (Chandra et al., 2012; Corcuera et al., 2012; Diaz et al., 2014; Pandey & Gupta, 2015). Elevated levels of proline during stress conditions could be a result of increased catabolism of the phenolic compounds (Hamid et al., 2010). Importantly, free proline chelates the metal ions, forming non-toxic metal-proline complexes, thereby protecting cellular structures, and metabolism thereof (Patel et al., 2013). Results of present study indicated that increase in total phenolic content at higher concentrations of Zn as compared to the control. Presumably, the oxidative effects of metal ions and metalloids are prevented by the antioxidant activity of phenolics that allows them to scavenge free ions due to their redox properties, thereby showing elevated levels of proline (Hamid et al., 2010). The breakdown of phenolics is triggered by the enzymes and a trailing cascading reactions, what we commonly also refer to as participation of 'antioxidant enzymes', which would involve hydrogen donors and quenchers of reactive oxygen species (ROS). H_2O_2 is an important ROS that disrupts the functions of the cell. CAT, APX and GPX are important enzymes that regulate the levels of H_2O_2 (Hosseini & Poorakbar, 2013). We have found an increased activity of CAT, APX and GPX enzymes at different concentrations of Zn as compared to control suggesting higher abilities of *Jatropha* to withstand oxidative stress generated by Zn.

From the above discussion, it is inferred that *J. curcas* can remove a significant amount of Zn from the soil and roots are the primary sink for accumulation of the metal, causing almost no damage to the plant growth.

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Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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