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In Vitro Studies on Zinc, Copper and Cadmium Accumulation Potential of *Jatropha curcas* L. Seedlings

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ABSTRACT

Germination rate, growth, accumulation capacity and tissue-wise distribution pattern of metals in *Jatropha curcas* L. when exposed to increasing concentrations of heavy metals ranging from 100-700 µM Zn and 50-200 µM of Cu and Cd were evaluated under aseptic *in vitro* conditions. Growth of seedlings was found significantly affected rather than germination frequency. There was significant decline in the shoot heights with increasing concentrations of Cd and Cu as compared to Zn, exhibiting differential metal specific tolerance levels. Accumulation of metal increased concomitantly with increasing exposure level of all the metals studied. The pattern of metal accumulation was in the order of root > stem > leaf for all three metals. Among the metals, the accumulation pattern was Cd > Cu > Zn, Cd > Zn > Cu and Zn > Cd > Cu for root, stem and leaf samples, respectively. The Translocation Factor values with respect to leaves and stem tissues in combination were mostly <1 in all concentrations studied, except in Cu control, Zn 300 and 500 µM, where it was slightly higher, suggesting that metal accumulated mostly in plant roots. Biological Accumulation Coefficient values showed that jatropha could accumulate Cd more efficiently than Cu and Zn. This study helps in understanding optimal growth and accumulation performance of *J. curcas* in different soil contamination levels of Cu, Cd and Zn and would be useful for a value added approach for eco-restoration of specific metal-contaminated sites.

Keywords: tolerance, metal accumulation, Translocation Factor, Biological Accumulation Coefficient, phytoremediation
Abbreviations: Cd, cadmium; **Cu**, copper; **DW**, dry weight; **Zn**, zinc

INTRODUCTION

Heavy metal contamination of soils is a major environmental problem. A large number of sites worldwide are contaminated due to mining, energy production, and agricultural activities. These metals are non-biodegradable and hence they persist for long periods in aquatic as well terrestrial environment. They may get mobilized through soils to reach groundwater which may enter biogeochemical cycle leading to bio-magnification. Copper (Cu) is an essential micronutrient for plants as it is a component of several proteins and enzymes involved in various metabolic pathways but it can be toxic when present in amounts higher than its permissible limits in soil. At concentrations above normal levels, Cu inhibits growth and also interferes with the important cellular processes such as photosynthesis and respiration (Yruela 2005). Zinc (Zn) and cadmium (Cd) contamination is primarily caused by their release into the environment from industrial activities such as mining and smelting of metalliferous ores, electroplating, fertilizers and pesticides (Raskin and Ensley 2000). Both Zn and Cd often combine with other elements at hazardous waste sites to form compounds of chlorides, oxides and sulfides (ATSDR 1994, 1999). These contaminants remain persistent in the soil by binding to soil particles. Excessive Zn and Cd levels in soil cause adverse effect on human health and disruption of natural ecosystems.

Conventional remediation methods of heavy metal contaminated sites are expensive and environmentally destructive. Technologies based on environmental friendly and low-cost processes would be more appropriate. Phytoremediation is an emerging technology that aims to extract or inactivate heavy metals from soils (Salt *et al*. 1998). The new remediation technology is competitive, and may be superior to existing conventional technologies at sites where phytoremediation is applicable (Prasad and Freitas 2003). The heavy metals are not retained in the roots but are translocated to the shoots and get accumulated in above ground organs, especially leaves, at concentrations 100-1000 fold higher than those found in non-hyperaccumulating species (Rascio and Navari-Izzo 2011). These plants are then harvested and disposed off as hazardous waste or incinerated for metal recovery. Excessive amount of heavy metals may adversely affect plant propagation and growth. The success of phytoremediation depends upon selection of plant species that maximize the contaminant removal (Ebbs and Kochian 1998; Raskin and Ensley 2000). The response of different plant species to particular metal varies markedly (Baker *et al*. 2000).

Jatropha curcas is a drought-resistant shrub or tree which belongs to the Euphorbiaceae family. It is a multipurpose tree of Mexico and Central American origin with a long history of cultivation in America, Africa and Asia. The seeds of jatropha contain 27-40% oil that can be processed to produce high-quality biodiesel fuel usable in a standard biodiesel engine. Previous researchers carried out metal tolerance studies of *J. curcas* in soil as substrate without any amendments (Mangkoedihardjo and Surahmaida 2008) as well as with various amendments such as biosludge, biofertiliser, fly ash, dairy sludge and limestone along with their effects on accumulation of metals. (Juwarkar *et al.* 2008; Jamil *et al.* 2009; Yadav *et al.* 2009; Ghavri and Singh 2010; Yadav *et al.* 2010; Wu *et al.* 2011). However, in soil studies, there are many other factors such as microflora of the soil affecting the metal accumulation and tolerance. On the contrary, tissue culture technique is more convenient requiring relatively shorter period of time for evaluating metal accumulation and tolerance in plants, with no interference of external biotic factors. Using *in vitro* techniques with uniform and regulated aseptic growth conditions, the

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effect of different metals on plant growth and accumulation potential can be studied precisely. This technique can also be used for screening of plants that can tolerate and accumulate high amount of metals in their tissues. The aim of the present study is to evaluate Zn, Cu and Cd tolerance and accumulation capacity of *J. curcas in vitro* for value added and optimized remediation approach of such metal-contaminated soils.

MATERIALS AND METHODS

Surface sterilization

Seeds of *J. curcas* were purchased from a local supplier. These were washed in running tap water for 30 min followed by treatment with 3-4 drops of detergent and 1% Bavistin for 45 min on shaker. Thereafter the seeds were treated with 4% savlon for 10 min. Jatropha seeds were then surface sterilized with sodium hypochlorite solution (approximately 4% (w/v) available chlorine) for 30 min in a laminar clean airflow cabinet. After each treatment seeds were thoroughly washed with deionized water. The testa and white papery covering was removed aseptically to reduce the contamination.

In vitro metal treatments and culture conditions

Aliquots of filter sterilized solution of $ZnSO_4 \cdot 7H_2O$, $CdCl_2$, $CuSO_4 \cdot 5H_2O$ were added aseptically to the molten medium to attain final concentration of 100 µM, 300 µM, 500 µM, 700 µM for Zn and 50 µM, 100 µM, 150 µM, 200 µM for Cd and Cu. Medium without metal solution was used as control. The media were distributed in cotton plugged culture tubes. Surface-sterilized seeds were then cultured on sterile agar-gelled deionized water (pH 5.8) supplemented with various concentrations of Zn, Cd and Cu. For each treatment 20 seeds were cultured. Cultures were incubated in dark for 3-4 days for radical emergence. Germinated seeds were transferred to 16-h photoperiod of 32 µM of photons m^{-2} s^{-1} at 25±2°C. At the end of 15 days seedlings were taken out from test tube and washed with deionized water to remove adhering agar.

Growth measurement and metal content analysis

Shoot height was noted. Leaves stem and roots of the seedlings were separated. They were collected in glass vials and fresh weight was taken. These vials were then kept in oven at 100°C and weighed intermittently till the constant weights were attained. The dried samples were ground to fine powder with mortar and pestle. The dry tissue powder (150 mg) for leaf and stem samples was taken in Borosil vials. Since the roots displayed reduced growth in metal treatments, sampling tissue amount for metal quantification was greatly reduced. Therefore, all the available quantity of the dry tissue powder was used for metal estimation.

For metal estimation, the method of Singh *et al*. (2006) was followed. The powder was digested with 3 ml nitric acid and 1 ml perchloric acid. The volume of the digested sample was made to 10 ml with deionized water. The Cd, Cu and Zn accumulation was estimated by Atomic Absorption Spectrophotometer (Perkin Elmer 1100B).

The experiment was repeated in triplicate. The values were expressed as mean ± standard deviation (SD). The data was subjected to one-way ANOVA (Analysis of Variance) at a significance level of $P < 0.05$. Individual comparisons of treatments were made with controls and significantly different values were determined using a student's *t*-test at $P < 0.05$.

Translocation of metals from roots to shoots was determined by Translocation Factor (TF) which was calculated by [Concentration of the metal in shoot/Concentration of the metal in root] (Bu-Olayan and Thomas 2009). However, in our study the concentration of metal in the shoots was derived by cumulative metal accumulation values in leaves and stem tissues. Metal accumulation in *J. curcas* was evaluated by calculating Biological Accumulation Coefficient (BAC). Alloway (1990) calculated BAC by [Concentration of metal in shoot/Concentration of metal in soil]. However, in the present study BAC was calculated by:

Concentration of the metal in shoot/Concentration of the metal in medium.

RESULTS

Plant morphology

The effect of heavy metals on *J. curcas* varied with the type of metal and its concentration in the medium. In the seedlings exposed to Zn, the lamina was elongated and the stem became thicker than control seedlings at 300, 500 and 700 µM. Leaves were curled at 150 and 200 µM after exposure to both Cd and Cu. Root length and number were reduced at higher metal concentrations in all the metal-containing media. In both metals, Cd and Cu there was no root development at all at 150 and 200 µM while for Zn, root growth was affected at ≥ 300 µM. At 300 and 500 µM very minute roots were observed while at 700 µM there was no root growth at all. Browning was observed at the tip of roots of seedlings growing in Cd and Cu at highest concentrations.

Seed germination frequency and growth

Seed germination is the first parameter to be studied when estimating phytoremediation potential. The frequency of seed germination ranged between 91 and 100%, 93 and 98% and 98 and 100% in Cd, Cu and Zn treatments, respectively, compared to the control in which germination ranged between 95 and 100%. This shows that seed germination was not significantly affected due to metal stress.

Shoot and root growth of *J. curcas* was severely affected by Cd and Cu more than by Zn, particularly at higher concentrations (**Table 1**). As the concentration of Cd and Cu increased in the medium, growth was significantly retarded (**Fig. 1**). At the end of 15 days of incubation, shoot height decreased by 3-4 cm in all the metal treatments at the highest concentrations.

Metal accumulation

There was a linear increase in metal accumulation as the concentration of all three metals in the medium increased. The amount of metal accumulation varied in different seedling tissues (**Fig. 2**). Cd was not detected in control plants. Metal accumulation was highest in roots followed by stem and then leaf for all three metals studied. Roots accumu-

Table 1 Effect of Zn, Cu and Cd on germination frequency and seedling growth in *J. curcas*.

Concentration (µM)	Germination frequency (%) Mean ± SD	Shoot height (cm) Mean ± SD
Zn		
0	100 ± 2.89	9.45 ± 1.07
100	98.33 ± 0.0	9.39 ± 1.31
300	100 ± 0.0	9.33 ± 0.77
500	100 ± 0.0	8.17 ± 0.32
700	100 ± 0.0	6.24 ± 0.68*
ANOVA	NS	$P < 0.01$
Cu		
0	96.67 ± 2.89	9.58 ± 1.36
50	96.67 ± 2.89	7.78 ± 0.67
100	96.67 ± 11.55	7.52 ± 0.79
150	93.33 ± 0.0	7.19 ± 0.52*
200	93.33 ± 0.0	6.43 ± 0.77*
ANOVA	NS	$P < 0.05$
Cd		
0	95.00 ± 5	6.42 ± 1.64
50	91.67 ± 10.41	4.05 ± 0.53
100	96.67 ± 5.77	3.37 ± 0.58*
150	100 ± 0.0	3.13 ± 0.65*
200	100 ± 0.0	2.3 ± 0.65*
ANOVA	NS	$P < 0.01$

*indicates values significantly different at $P < 0.05$

Fig. 1 Effect of Zn (A), Cu (B) and Cd (C) on seedling growth in *J. curcas*.

lated the most Cd (3727.12 µg/g DW) relative to Cu (1105.8 µg/g DW) and Zn (1198.98 µg/g DW). In the stem, the accumulation pattern followed the order Cd > Zn > Cu while for leaves it was Zn > Cd > Cu.

TF and BAC values are shown in **Tables 2** and **3**. TF values were higher after exposure to Zn than Cd and Cu. With respect to Zn accumulation in leaf and stem tissues, the TF values were higher for all the tested concentrations than that of control plants. TF values of Cu in both leaf and stem tissues were higher in the control than in all the remaining treatment levels. The TF value of Cu in control leaves was 0.65, higher than that in control stem (TF = 0.52). BAC values did not differ significantly in leaf and stem samples of Cu and Cd treatments. In the leaf, stem and root following Zn treatment as well as in the root tissues of Cu and Cd treatments, a colinear decrease in BAC values was observed with increasing concentration of each metal in the media. Among the tissues, BAC values were highest in root tissues than in leaf and stem tissues. Amongst the various metal treatments, BAC values were higher in Cd than in Cu and Zn treatments in all tissues (**Table 3**).

DISCUSSION

Our study reveals that the presence of Zn, Cu and Cd in medium has no adverse effect on the germination of jatropha seeds. Similarly, in a previous study, seed germination in peanut was not affected (Kumar *et al*. 2008) when seeds were exposed to an increasing concentration of Cd. As evident from shoot and root growth, the present experiment shows that *J. curcas* seedlings could tolerate up to 700 µM of Zn, 200 µM of Cu and 150 µM of Cd. These results show the differential metal tolerance of jatropha with regard to the metal type. In a particular plant species the level of tolerance towards different metals vary (Kumar *et al*. 2009).

In our study we found that *J. curcas* leaves accumulated more Zn than Cd or Cu. Higher accumulation of Zn in leaves was similarly observed in red beet, field pumpkin, chicory, common bean, barley, maize, white cabbage, and alfalfa (Sekara *et al*. 2005). Seedling growth was not affected at 100, 300 or 500 µM but it was greatly affected at 700 µM. Roots absorb a metal from the substrate and then transport it to the leaves via the stem. Leaves act as a sink and retain metal ions. Among the three metals studied, Zn was less toxic to *J. curcas* than Cd and Cu, possibly because Zn is a co-factor of several enzymes (Das and Maiti 2007).

In soil, Cu can be a stress factor by causing physiological responses that can decrease the vigour of plants and inhibit plant growth (Ouzounidou 1994). However, Cu is required by biological systems as a structural and catalytic

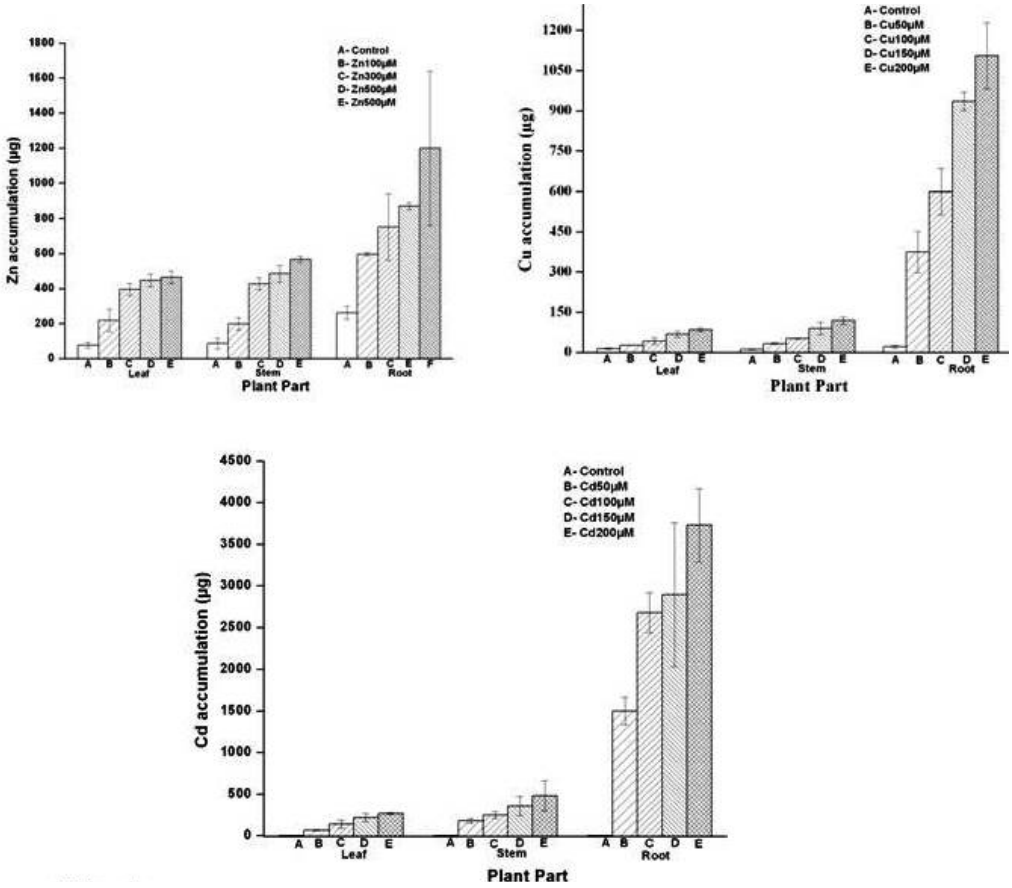

Fig. 2 Accumulation of Zn, Cu and Cd in different parts of the *J. curcas* seedlings.

Table 2 Translocation Factor (TF) for Zn, Cu and Cd in leaves and stems.

Concentration (µM)	TF value	Concentration (µM)	TF value
Zn leaf		**Cu stem**	
Control	0.29	Control	0.52
Zn100	0.36	Cu50	0.08
Zn300	0.52	Cu100	0.08
Zn500	0.51	Cu150	0.09
Zn700	0.38	Cu200	0.10
Zn stem		**Cd leaf**	
Control	0.33	Control	0.0
Zn100	0.33	Cd50	0.04
Zn300	0.56	Cd100	0.05
Zn500	0.55	Cd150	0.07
Zn700	0.47	Cd200	0.07
Cu leaf		**Cd stem**	
Control	0.65	Control	0.0
Cu50	0.07	Cd50	0.11
Cu100	0.07	Cd100	0.09
Cu150	0.07	Cd150	0.12
Cu200	0.07	Cd200	0.12

Table 3 Biological Accumulation Coefficient (BAC) for Zn, Cu and Cd in leaves, stems and roots.

Concentration (µM)	BAC value	Concentration (µM)	BAC value
Zn leaf		**Cu root**	
Control	-	Control	-
Zn100	0.018	Cu50	0.077
Zn300	0.011	Cu100	0.062
Zn500	0.007	Cu150	0.064
Zn700	0.005	Cu200	0.057
Zn stem		**Cd leaf**	
Control	-	Control	-
Zn100	0.017	Cd50	0.016
Zn300	0.012	Cd100	0.018
Zn500	0.008	Cd150	0.019
Zn700	0.007	Cd200	0.018
Zn root		**Cd stem**	
Control	-	Control	-
Zn100	0.051	Cd50	0.048
Zn300	0.021	Cd100	0.033
Zn500	0.015	Cd150	0.032
Zn700	0.014	Cd200	0.032
Cu leaf		**Cd root**	
Control	-	Control	-
Cu50	0.005	Cd50	0.408
Cu100	0.004	Cd100	0.364
Cu150	0.004	Cd150	0.262
Cu200	0.004	Cd200	0.254
Cu stem			
Control	-		
Cu50	0.006		
Cu100	0.005		
Cu150	0.006		
Cu200	0.006		

enzyme component. In the present experiment, at 150 and 200 µM of Cu, leaf size was reduced and there was no root development at 200 µM. Reduction in leaf size was also observed in *Vigna radiata* when exposed to high (100-250 mg/kg) Cu concentrations (Manivasagaperumal *et al*. 2011). This reduction in leaf and root development could be due to the reduction in cell division, toxic effect of heavy metals on photosynthesis, respiration and protein synthesis. In the present study, less Cu accumulated in stem and leaves of *J. curcas* than Cd and Zn.

Our experiments show that Cd was more toxic than Cu and Zn. Seedling growth was reduced as the concentration of the metal in the medium increased. Leaf development was also affected in the metal-containing media. Leaf curling and browning was observed. A brown colored deposition was observed at the tip of the root at 150 and 200 µM. Such browning was also observed in peanut seedlings exposed to Cd (Kumar *et al*. 2008). Roots absorbed maximum amount of Cd. This could be because roots are in direct contact with the medium hence more Cd ions are available for absorption, adsorption or uptake. Metal content in all the tissues increased as the concentrations of metals in the medium increased. Likewise, an increase in metal concentration with increasing concentration of metal in medium was also observed in peanut and *Pongamia pinnata* (L.) seedlings (Kumar *et al*. 2008, 2009). The present experiments demonstrate that *J. curcas* can absorb, accumulate and translocate heavy metals into the shoot. This is a prerequisite for plants to be used for phytoremediation. Jatropha seeds are non-edible while the plants can absorb adequate amounts of heavy metals. Therefore, this plant species can be used for phytoremediation of heavy metals. There are some reports on heavy metal accumulation in plant tissues of oil producing plants. Angelova *et al.* (2004) studied metal accumulation and distribution in oil crops and claimed that oil plants were not suitable for growing in industrially polluted regions till the stage of commercial ripeness, because the seeds of sunflower, sesame and peanuts could not be used either directly for food or for processing. From the oil crops, rapeseed could be used as a potential crop for cleaning the soil of heavy metals in industrially polluted regions, if it were used mainly for fodder, technical purposes, and biodiesel. In another study carried out by Zheljazkov *et al.* (2006) on essential oil crops it was observed that there was no detectable amount of Cd, Cu, or Pb in the oils of any of the three species (peppermint, basil, and dill). Hence it was confirmed that these can be grown in soils enriched with Cd, Pb, and Cu medium without risk for metal transfer into the oils, and without significant alteration of essential oil composition that may impair marketability.

Mangkoedihardjo and Surahmaida (2008) reported that Cd accumulation was more than that of lead (Pb) in 100% Pb and Cd-polluted soils and also at various concentrations of Pb and Cd. Juwarkar *et al.* (2008) studied the effect of biosludge and biofertilizer addition on arsenic and chromium accumulation at 0, 25, 50, 100, 250, 500 mg/kg in soil and Zn accumulation at 0, 1000, 2000, 3000 and 4000 mg/kg and asserted that the addition of biosludge and biofertilizer reduced metal uptake while the metal was stabilized in the soil. Similar experiments were carried out by Yadav *et al.* (2009, 2010) wherein the effect of dairy sludge and biofertilizer on accumulation of arsenic, chromium and zinc, and the effect of biosludge and biofertilizer on accumulation of chromium at 0, 25, 50, 100, 250 mg/kg was studied; they concluded that the metal was stabilized in the soil causing a reduction in the uptake by plant tissues. Jamil *et al*. (2009) studied the effect of EDTA in soil on metal accumulation and observed that the pattern of metal accumulation was of the order of root > stem > leaf tissue. Further, they reported that metal accumulation increased with the addition of EDTA. Ghavri *et al.* (2010) reported that stem tissues of plants grown in wasteland soil accumulated two fold, while leaves accumulated 2-4 fold higher Fe when compared to accumulation in garden soil. The accumulation pattern was similar in root, stem and leaves in soil amended with 40% cowdung or sand. Wu *et al.* (2011) studied metal (Al, Zn, Cu, Pb and Cd) accumulation of jatropha grown on metal-contaminated soil as well as the effect of limestone addition. They observed that the accumulation pattern was in the order of root > stem > leaf. The addition of limestone decreased the phytoavailability of soil metals.

A TF value greater than 1 indicates that plants translocate heavy metals effectively from roots to shoots (Baker and Brooks 1989). Sun *et al*. (2009) carried out studies on *Bidens pilosa* in which the TF and Bioaccumulation factor values were more than 1. These standards were used for the validation of *B. pilosa* as a hyperaccumulator. In another study carried out by Yu and Zhou (2009) it was found that the TF of Cd in *Mirabilis jalapa* reached the maximum at

different tested Cd levels when phosphorus was added at 100 mg/kg concentration. In the present study, with respect to individual leaf and stem tissues, none of the tissues showed a TF value greater than 1. This shows that the plant mostly accumulates metal in the root tissues. However, when the cumulative TF values of the leaf and stem tissues were considered it was found that they were slightly higher than 1 in Cu control, 300 and 500 µM Zn, indicating better root to shoot translocation of metal. There might be phytostabilization of metal in the roots at most of the concentrations studied. Similar results were obtained in sunflower where the TF was less than 1 in all treatments (Zadeh *et al*. 2008). The rate and extent of translocation within plants depends on the metal and plant species. Li *et al.* (2007) carried out studies in a restored manganese mineland with different plants and used BAC values for determining the accumulation potential of different metals. Maiti and Jaiswal (2008) reported highest BAC values of root and shoot for *Typha latifolia* for all the metals (Mn, Zn, Cu, Pb, and Ni) found in the natural vegetation growing on fly ash lagoons. The study indicated that metals accumulated by the plants growing in the fly ash lagoons were largely retained in roots, as shown by Translocation Factor values <1. BAC values of all the metals were highest in roots followed by stem and leaf tissues possibly because the roots are in direct contact with the medium facilitating efficient accumulation of metal.

CONCLUSIONS

Effect of different heavy metals on seed germination rate, growth and accumulation of *J. curcas* was carried out in this study. Cd and Cu were found to be more toxic to *J. curcas* as compared to Zn in terms of seed germination, shoot and root growth. The study reveals that roots of *J. curcas* can accumulate highest amount of all the metals as compared to leaf and stem. TF values of lesser than 1 in most of the metal treatments implies that, while *J. curcas* is not a hyperaccumulator but it can be recommended for effective stabilization of metals in roots. The present study pertaining to the germination frequencies and the Zn, Cu and Cd metal accumulation characteristics of *J. curcas* particularly in the early seedling growth stage enables the use of this plant for eco-restoration as well as phytoremediation along with the additional benefit of value-added bio-diesel production in such metal contaminated sites.

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