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In Vitro Studies on Chromium and Copper Accumulation Potential of *Pongamia pinnata* (L.) Pierre Seedlings

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ABSTRACT

Pongamia pinnata (L.) Pierre is an oil-producing tree species. The potential of seed-derived pongamia oil as biodiesel has been identified but its potential for phytoremediation of contaminated sites and for phytoextraction of heavy metals remains unexplored. The objective of the present study was to determine the effect of chromium (Cr[VI]) and copper (Cu) on growth and metal uptake in different parts of *P. pinnata* seedlings grown *in vitro* in medium containing Cr or Cu. Pongamia seeds were cultured in MS medium supplemented with various concentrations of Cr (0-800 μM) and Cu (0-400 μM). After 6 weeks of incubation shoot height and root length of the seedlings were noted. The results demonstrated that growth of pongamia seedlings exposed to Cr(VI) concentrations ranging from 0 to 800 μM were not affected whereas Cu (0-400 μM) affected the root growth. Metal analysis carried out by atomic absorption spectroscopy demonstrated maximum accumulation of Cr in seed coat followed by root, leaves and cotyledons. In Cu the pattern was different. Cu content was optimum in seed coat followed by leaf, root and cotyledons. Least metal content was detected in stem in both treatments either in chromium or copper. High metal content in the seed coats demonstrates its ability to selectively absorb metal from the medium and retain it. This property of the seed coat may be exploited for selective absorption of toxic metals from liquid waste.

Keywords: absorption, AAS, pongamia, phytoremediation

Abbreviations: AAS, atomic absorption spectrophotometer; **Cr**, chromium; **Cu**, copper; **DW**, dry weight; **FW**, fresh weight

INTRODUCTION

Heavy metals form the main group of inorganic contaminants and recovery of sites contaminated with such compounds is one of the major challenges for environmental institutions. Heavy metals are released into the environment by mining, smelting, tanning industries, electroplating, manufacturing, agriculture and waste disposal technologies (Shanker *et al*. 2005a; Yruela 2005). These, when enter into the soil, have definite adverse effects on plants and animals and thus ultimately on human health. Chromium (Cr) is highly toxic to plants and is detrimental to their growth and development. Copper (Cu) is an essential redox active transition metal that is involved in many physiological processes in plants because it can exist in multiple oxidation states *in vivo* (Yruela 2005). At concentrations above those required for optimal growth, Cu interferes with important cellular processes such as photosynthesis and respiration and inhibits plant growth (Yruela 2005). In view of the seriousness of metal pollution, considerable efforts are being directed towards development of new, more cost-effective technologies to minimize soil contamination.

Despite of the potential, progress in the field of phytoremediation towards developing transgenic phytoremediator plant species is rather slow. This can be attributed to the lack of our understanding of complex interactions in the soil and indigenous mechanisms in the plants that allow metal translocation, accumulation and removal from a site (Shah and Nongkynrih 2007). Thus, for this technology, the first prerequisite is to identify plants with potential to absorb and regulate toxic metals or to survive on land with pollutants. Metal hyperaccumulator plants are relatively rare, often occurring in remote areas geographically and threatened by devastation from mining activities (Shah and Nongkynrih 2007). Hyperaccumulator accumulate appreciable quantities of metal in their tissue regardless of the concentration of metal in the soil, as long as the metal in question is present (Prasad and Freitas 2003). A *Holoptelia integrifolia* tree growing on a manganese mine dump was identified for its ability to accumulate a high level of manganese whereas two other species growing in the same location did not demonstrate a similar characteristic (Raju *et al*. 2008). Several studies have been conducted to evaluate the effects of different heavy metal concentrations on living plants. Some plants which grow on metaliferrous soils have developed the ability to accumulate massive amounts of the indigenous metals in their tissues without exhibiting symptoms of toxicity (Prasad and Freitas 2003) and the ability of a plant to hyperaccumulate any one metal may infer some ability to accumulate other metals (Prasad and Freitas 2003). There are a few reports on different tree species, including *Salix* (willow), *Betula* (birch), *Poplus* (poplar), *Alnus* (alder) and *Acer* (sycamore) (Pulford and Watson 2003; Pulford and Dickinson 2006) and on *H. integrifolia* (Raju *et al*. 2008) on different metals. However, these studies were carried out in field conditions. In the field, plants grow in a complex environment containing organic and inorganic components in addition to soil microbes. *In vitro* techniques offer the potential to grow plant tissues in media formulated to study the effect of specific metals singly. For optimization of biomass production and phytoextraction, it is important to know if the metals of interest are primarily concentrated in roots, wood, bark or leaves. This is equally important for the selection of the most appropriate technology for processing metal-enriched plant material after harvest (Unterbrunner *et al*. 2007).

Growing trees have been suggested (Rosselli *et al*. 2003) as a low cost, sustainable and ecologically sound solution to the remediation of heavy metal contaminated land. *Pongamia pinnata* (L.) Pierre is a medium-sized, fast-growing evergreen tree species. Seeds of pongamia contain 30-35% of oil, which has potential as raw material for

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production of biodiesel (Vivek and Gupta 2004). This tree can thrive in a wide range of agroclimatic conditions and serves as a rich source of flavonoids and oil for industrial applications. Oil and tissue extracts of *P. pinnata* are known for their antifungal and antibacterial activity (Meera *et al.* 2003). Role of the pongamia seed coat in microbial infection and its influence on germination *in vitro* has been demonstrated (Sujatha and Hazra 2006). However, little is known about their capacity to tolerate and accumulate metal(s). To test the tolerance of this plant against heavy metals and to evaluate its ability to accumulate and translocate heavy metals to aerial tissues we studied seedlings cultured in the presence of Cr and Cu. As a rich source of tree-borne oilseeds, *P. pinnata* is a species of choice for waste utilization and value addition to waste land.

MATERIALS AND METHODS

Pods of pongamia were collected from plants growing locally. Seeds extracted from the pods were washed with tap water followed by treatment with liquid detergent for 10 min. These were surface sterilized by treating with 1% (w/v) Bavistin (BASF India Ltd.) for 1 h on a shaker followed by treatment with 4% (v/v) aqueous solution of Savlon (antiseptic liquid preparation containing 3% cetrimide and 1.5% chlorhexidine gluconate, Johnson and Johnson, Mumbai, India) for 5 min and thereafter with 0.1% mercuric chloride (w/v) for 8 min. These were washed thoroughly with sterile distilled water to eliminate the adhering mercuric chloride prior to culturing in agar-gelled MS basal medium supplemented with 1 mg/L benzyl adenine (Sigma Chemical USA) and 2% sucrose (w/v) (Sujatha and Hazra 2007). The pH of the media was adjusted to 5.8 prior to autoclaving.

After autoclaving, 100, 200, 400, 600 and 800 µl of filter-sterilized solution of $CuSO_4$ (24.9 g L^{-1}) or $K_2Cr_2O_7$ (29.4 g L^{-1}) were added aseptically in the 200 ml molten medium. The final concentrations of Cu in the media were 50, 100, 200, 300 and 400 µM, whereas for Cr the concentrations were 100, 200, 400, 600 and 800 µM, respectively. Cultures were incubated in a 16 h photoperiod (32 µE m^{-2} s^{-1}) at 25 ± 2°C. The experiments were repeated 4 times with 10 replicates in each. Emergence of radicals in the seeds was scored for germination. Germination of the seeds was asynchronous and the plant growth was slow till 4 weeks. Therefore the cultures were harvested after 6 weeks and the shoot height and root length of the seedlings were noted to determine the morphological changes. Whenever necessary data were transformed and analyzed by one way ANOVA and the means separated by Fisher's LSD test at $P \leq 0.05$. All values are means of four independent experiments.

Chromium or Cu accumulated in the seed coat, cotyledons, root, stem and leaves of seedlings were determined using atomic absorption spectroscopy (Singh *et al.* 2006). Metal analyses were repeated three times. Tissues of two seedlings from each concentration were pooled for each analysis. Plant samples were thoroughly washed with deionized water to remove adhering medium and weighed. These were dried in an oven at 90–100°C till constant weight was achieved. The data on the DW^{-g} FW was calculated to determine the change in mass due to accumulation of metal in each organ. The dried samples were ground into fine powder and stored. Powdered samples (50–300 mg) were digested in HNO_3: $HClO_4$, 3:1 mixture for metal estimation using AAS. The Cu or Cr content of each organ was determined using AAS.

RESULTS AND DISCUSSION

Seed germination is the first physiological process affected by metal stress. Thus the ability of a seed to germinate in medium containing Cr would be indicative of its level of tolerance to this metal (Peralta *et al.* 2001). In our study, germination of seeds in media with and without Cr was asynchronous. Some of the cultures which developed bacterial contamination around the seeds in contact of medium were scored to determine the germination frequency only but were avoided for scoring the shoot and root lengths. Germination was hypogeal and the seeds remained in contact of medium throughout the culture period (**Fig. 1A**).

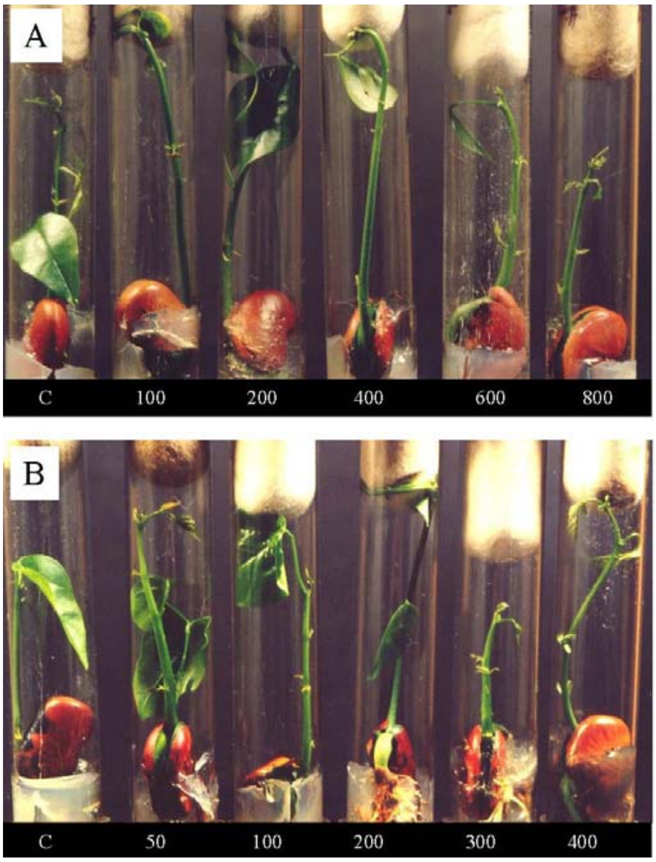

Fig. 1 Effect of chromium (A) and copper (B) on *Pongamia* seed germination and seedling growth after 6 weeks. Concentrations of metals are in µM.

Table 1 Effect of Cr and Cu on pongamia seed germination and seedling growth.

	Germination frequency Mean ± SD (10 d)	**Shoot height (cm) Mean ± SD (6 weeks)**	**Root length (cm) Mean ± SD (6 weeks)**
Cr $^{+6}$ µM			
0	71 ± 15.3 (28)	6.2 ± 1.7 (19)	4.2 ± 1.2 (23)
100	87 ± 9.5 (35)	6.8 ± 1.0 (24)	3.4 ± 0.1 (29)
200	69 ± 16.4 (27)	8.8 ± 3.6 (14)	3.9 ± 2.3 (22)
400	62 ± 22.1 (25)	7.0 ± 2.0 (17)	4.3 ± 0.8 (24)
600	62 ± 31.6 (24)	6.4 ± 2.7 (12)	4.9 ± 0.8 (21)
800	67 ± 15.6 (27)	5.3 ± 0.9 (14)	4.2 ± 1.3 (24)
ANOVA	ns	ns	ns
Cu^{+2}			
0	53 ± 14 (18)	5.8 ± 3.2 (11)	3.3 ± 1.2 (17)
50	64 ± 28 (25)	6.5 ± 1.9 (18)	3.8 ± 0.9 (23)
100	62 ± 15 (25)	6.0 ± 0.6 (17)	3.4 ± 0.8 (22)
200	62 ± 19 (22)	7.2 ± 1.9 (12)	2.9 ± 0.8 (21)
300	67 ± 21 (26)	4.0 ± 3.3 (11)	2.0 ± 0.4 (16)
400	76 ± 11 (23)	6.8 ± 0.6 (11)	1.8 ± 0.4 (22)
ANOVA	ns	ns	$P < 0.05$

The values in parentheses indicate the number of replicates.

The frequencies of germination in seeds ranged between 62 and 87% (**Table 1**). In medium without metal the germination frequency was 71%. It was optimum (87%) in medium with 100 µM Cr. With an increase in Cr concentration there was no significant decrease in the frequency of response. A decrease in germination frequency in the presence of Cr was reported for *Echinochloa colona* at 200 µM, *Phaseolus vulgaris* at 500 ppm (i.e. 9.61 mM; Shanker *et al.* 2005a) and *Medicago sativa* at 40 ppm (i.e. 769 µM; Peralta *et al.* 2001). The mean heights of the pongamia seedling shoots in Cr ranged between 5.3 cm to 8.8 cm (**Table 1**). In medium devoid of Cr the height was 6.2 cm. There

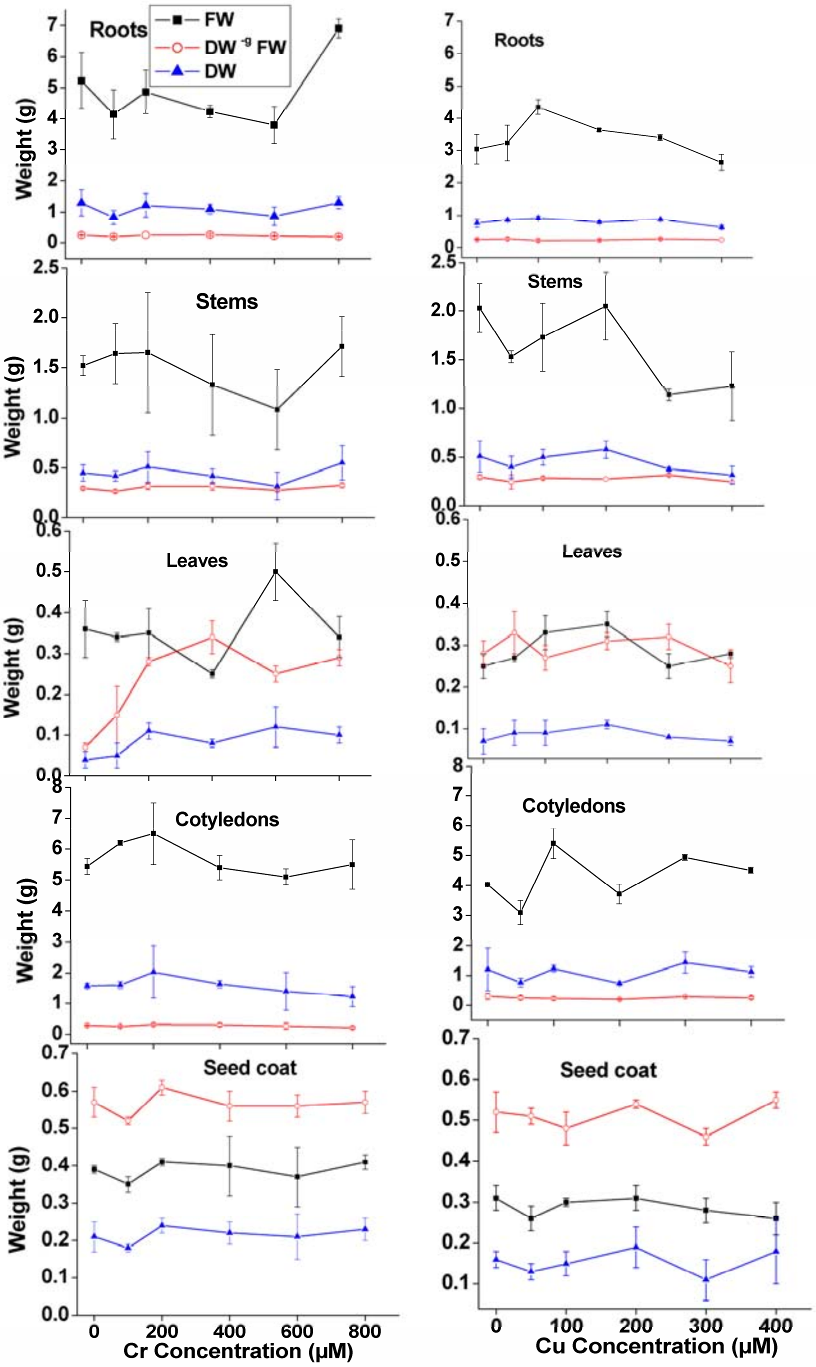

Fig. 2 Effect of various concentrations of chromium and copper on fresh weight (FW), dry weight (DW) and DW $^{-g}$ FW in the different parts of *Pongamia* seedlings.

was no significant difference in the shoot or root lengths of the plants cultured for 6 week in media containing various concentrations of Cr. In an *ex vitro* study inclusion of Cr (VI) at 5 ppm (96 µM) and 10 ppm (192 µM) in the growth medium caused a decrease in growth rate of the primary root and demonstrated strong inhibition in the shoot growth in maize, tomato and cauliflower (Sanità Di Toppi *et al*. 2002). Concentrations of Cr (VI) greater than 200 µM were toxic to plants as revealed both by arrested growth of roots and shoots of *Arabidopsis thaliana* using an *in vitro* system (Castro *et al*. 2007). Similarly in an *in vitro* study in tumbleweed Cr (III) suppressed root growth at 20 ppm (384 µM) (Gardea-Torresdey *et al*. 2005). The germination and growth of *P. pinnata* was not affected at 0-800 µM of Cr tested. The seedlings appeared healthy and green with opened leaves in all the media with and without Cr (**Fig. 1A**) after 6 weeks of culture.

The frequencies of seed germination in copper (50-400 µM)-containing media ranged between 53 and 76% (**Table 1**). The pattern of germination frequency in Cu was different from the pattern noted in Cr. Germination frequency was not affected due to presence of Cu in medium. A similar, non-significant effect on germination was noticed by Street *et al.* (2007) in *Bowiea volubilis* and *Merwilla natalensis* due to the presence of different concentrations (1, 2, 5, 10, 20 and 50 mg L^{-1}) of Cu. However, they reported a decrease in germination frequency of *Eucomis autumnalis* due to presence of Cu at similar concentrations. Seed germination was not affected in *Elsholtzia haichowensis*, *E. aypriani* and *E. ciliata* but shoot length was significantly affected at 50 and 100 µM of Cu (Xia and Shen 2007). In our study there was no change in the mean height of seedlings in the presence of Cu at the concentrations tested although a significant reduction in root elongation indicated adverse effects of Cu stress (**Table 1**) in the roots. The leaves of the seedlings remained partially unfurled (**Fig. 1B**).

The data on the fresh weight (FW) (**Fig. 2**) and Cr content (**Fig. 3**) in different parts of the seedlings reveal inter-

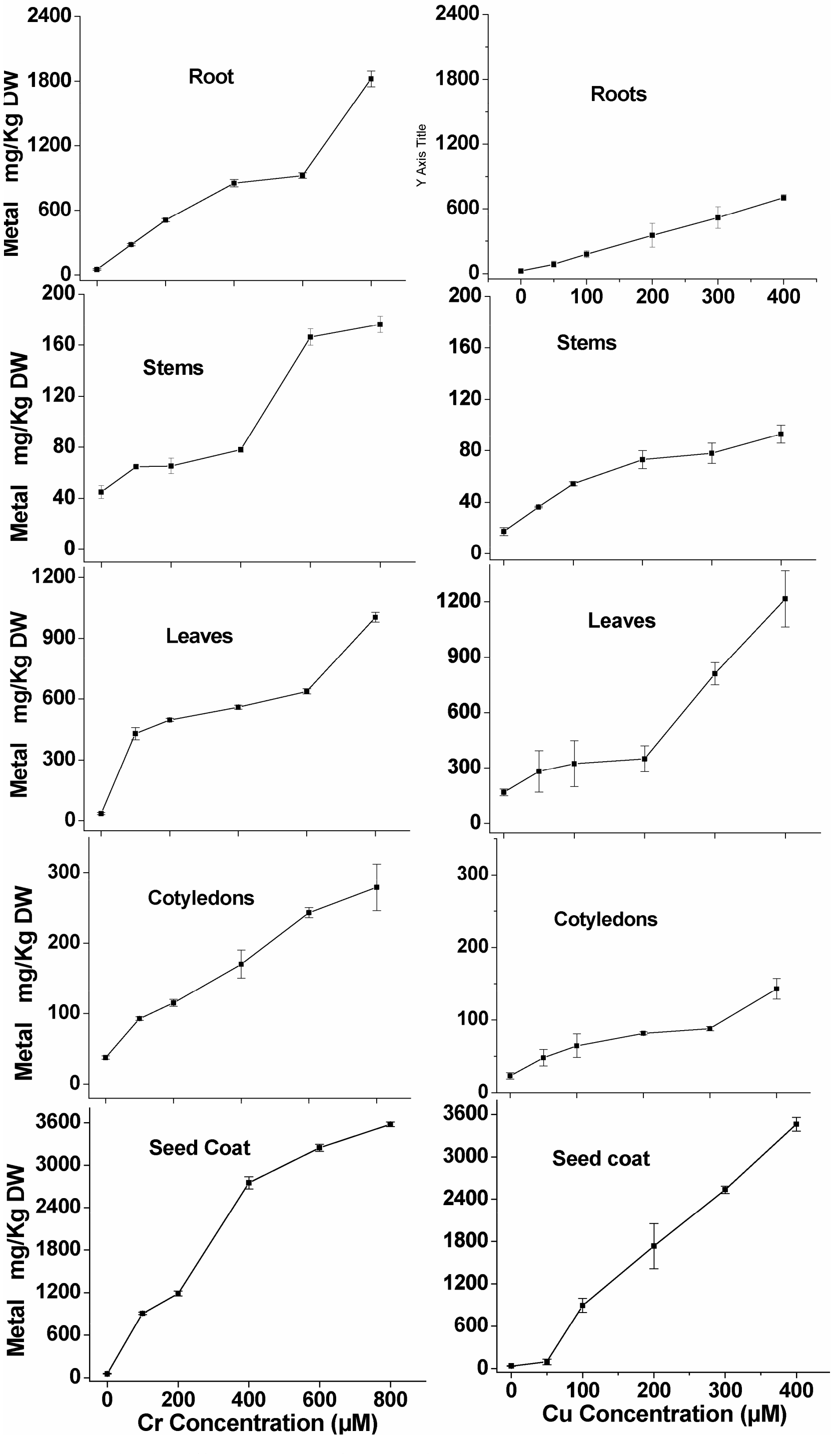

Fig. 3 Chromium and copper accumulation in different organs of *Pongamia pinnata* seedlings.

esting observations. The alterations in FW in the organs of the seedlings were not significant at any point of growth due to the presence of Cr. This is also evident from the non significant data on shoot heights and root lengths (**Table 1**) of the seedlings at various concentrations of Cr. This indicates that the seedlings tolerated 100–800 μM Cr and maintained growth and differentiation of all the organs similar to the control. For roots, stems and cotyledons the DW^{-g} FW remained unaltered indicating non accumulation of metals in these organs. On the contrary, in the leaves and seed coat there was a dramatic increase in DW^{-g} FW indicating possible accumulation of Cr in these two organs. In the seed coat the DW^{-g} FW was optimum.

Chromium detected in the organs of plant growing in medium devoid of Cr (**Fig. 3**) cannot be explained and may be attributed to the background reading noted in the blank experiments. In this study, the background reading is treated as a uniform factor for all estimations. There was an increase in chromium content in all parts of seedling with increasing concentration of chromium in medium. Highest amount of Cr (56–3578 mg Kg^{-1} DW) was detected in the seed coat followed by roots, leaves, cotyledons and stems (**Fig. 3**). Chromium content was low (37.5–279 mg Kg^{-1} DW) in the cotyledons although it was adjacent and close to the seed coat, which had highest amount of Cr.

On germination the roots of seedlings came into direct contact with the medium. This resulted in absorption of the metal from the medium and transport to the leaves through the stem vasculature. Roots have been shown to accumulate more chromium as compared to stem and leaves in cauliflower and *Genipa mericana* (Chatterjee and Chatterjee 2000; Barbosa *et al.* 2007). Compared to the control there was a sharp increase in Cr content in both root (88.5–706.0 mg Kg^{-1} DW) and leaves (282.0–1214.0 mg Kg^{-1} DW) whereas it was much less (36.5–93 mg Kg^{-1} DW) in the stem. This is possibly due to accrual of the metal in root upto the optimum level and thereafter transfer of the metal from medium to leaf via root and stem and deposition in the leaf. The leaves act as sink and retain the metal ions. A high concentration of Cr in the root at the basal end and in leaves at the terminal end with low concentration of the metal in the intermediate organ (stem) is intriguing and needs further examination. Previous studies have suggested that Cr is normally retained in the plant root in the form of Cr (III) (Han *et al* 2004; Montes-Holguin *et al* 2006; Mangabeira *et al* 2006). Although all three organs are connected through the vasculature, the low Cr content in the stem is possibly due to restricted distribution of the metal in the vascular tissue of the stem, thus leaving the cortical tissue unaffected. HRI-SIMS analysis revealed that the transport of chromium is restricted to the vascular system of roots, stems and leaves in *Lycopersicum esculentum* (Mangabeira *et al.* 2006). The unaffected elongation of the stem in the seedlings (**Table 1**) in presence of different concentrations of Cr supports this hypothesis. With accumulation of Cr in the leaves the leaf opening was retarded but not restricted (**Fig. 1A**). Leaf fall was not noticed in any of these cultures.

On germination the cotyledons were partially exposed to the medium as the coat was still attached. Thus low metal content detected in the cotyledon is possibly due to limited uptake of metal during absorption of moisture from the medium containing the metal or due to absorption/adherence of the metal in the seed coat.

In our study, there was significant accumulation of Cr in leaves as compared to stem. However, in a study with temperate trees including *Betula pendula* and *Salix* spp. grown on contaminated sites in the field, Pulford *et al.* (2001) demonstrated that Cr was less available in aerial parts of the plant. This was further confirmed in hydroponic systems in the glass house. In a pot culture experiment Shanker *et al.* (2005b) noticed poor uptake of Cr in *Albizia amara*, *Casuarina equisetifolia*, *Tectona grandis* and *Leucaena leucocephala* seedling roots. The uptake of Cr could be increased by amendment of the potting mixture with citric acid. Poor translocations of Cr to aerial parts make these trees poor choice for phytoremediation of Cr-contaminated sites (Shanker *et al.* 2005a). In contrast to the pot culture studies, the present experiment was conduced in test tubes under more controlled condition. As $K_2Cr_2O_7$ was uniformly dissolved in the medium, it was more bioavailable to the seedlings under study. On estimation of Cr it was observed that significant amount of the metal was transferred to leaves (**Fig. 3**). It needs to be tested if the failure of the plants to uptake Cr in the earlier studies (Pulford *et al.* 2001) was due to non-availability of the metal. *P. pinnata* seedlings grown *in vitro* could uptake the metal and transfer it to other organs. It is evident (**Fig. 1A, Table 1**) that germination of the pongamia seeds and elongation of the seedlings were not affected in the concentrations and exposures of Cr tested. Growing this plant for longer periods in the contaminated sites will confirm if this tree can be considered for phytoremediation of Cr-contaminated sites.

Compared to control there was no significant difference in fresh weight of seedling parts due to copper stress (**Fig. 2**) although there was significant increase in copper content in all the parts of the seedlings. Seed coat accumulated maximum amount of Cu and the stem had the least. Copper content in different parts of seedlings increased with increment in Cu in medium (**Fig. 3**). Roots have been known to accumulate more Cu as compared to stem and leaves in sunflower and Elsholtzia (Lin *et al.* 2003; Peng *et al.* 2007). But in our study Cu accumulation was more in leaves. Similarly *Datura stramonium* was found to accumulate more Cu in leaves as compared to roots (Boojar and Goodarzi 2007). Through the root, Cu is translocated to the other organs and deposited in the leaves, which did not open fully. The normal elongation of the stem and low Cu content (93.0 ± 7.00 mg Kg^{-1} DW) in 400 μM Cu containing medium, supports the hypotheses that the metal transport through stem remains restricted to the vasculature and do not affect the other cell types of the stem.

The optimum ability of this plant to accumulate and tolerate Cr needs to be tested. In medium with 400 μM Cu, the elongation of the roots was affected adversely when the roots of these seedlings had 706.0 ± 26.4 mg of Cu Kg^{-1} of DW. However, its elongation remained unaffected when the Cr content in this organ was 1823 ± 73 mg Kg^{-1} of DW (**Fig. 3**) in medium with 800 μM of Cr. These results indicate that in a particular plant species the level of tolerance towards different metals, vary. Selection of plants either under natural conditions of environmental pollution or *in vitro* may result in the selection of plants tolerant to toxic metal ions (Bojarczuk 2004). Thus, data generated *in vitro* on tolerance of metal by various species will be useful for selection of species for specific metal contaminated site. *In vitro* techniques have been used successfully in isolation of somaclonal variants to improve the potential of Indian mustard (*Brassica juncea* L.) to extract and accumulate toxic metals (Nehneva *et al* 2007). This technique is also used in development of transgenics for phytoremediation of metal contaminated sites (Hoewyk *et al* 2005).

The present experiment demonstrate that: (1) *P. pinnata* seedlings can tolerate 100-800 μM of Cr in growth medium and 50-400 μM of Cu. (2) Increased Cr and Cu content in the roots and leaves of pongamia seedlings suggests that this plant has a mechanism for absorption of these metals by the roots and translocate these to upper parts. This character of a plant is a prerequisite for phytoremediation, phytoextraction and phytomining. (3) The levels and mechanisms of tolerance against these two metals differ. Cu is more toxic compared to chromium. (4) Low metal content in the stem with unaffected shoot elongation, indicate restricted accumulation of the metals in the stem. (5) High metal content in the seed coat and low metal content in the attached cotyledon, confirms the protective role of the coat against toxic metals. (6) High metal content in the seed coats also demonstrates its ability to absorb metal from medium. This characteristic of the seed coat to hold significant amount of metal is an important phenomenon and may be exploited further for selective absorption of toxic metals from liquid waste. The natural ability of this plant to produce vegetable oil as raw material for biodiesel, tolerance towards Cr and Cu, and translocation of metal to aerial parts is suggestive of its suitability as a plant of choice for phytoremediation, phytoextraction and phytomining.

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