Studies on metal tolerance in *Arachis hypogaea*
3.1 INTRODUCTION

Peanut, or groundnut (Arachis hypogaea), is a species in the legume family Fabaceae, native to South America, Mexico and Central America. It is an annual herbaceous plant growing to 30 to 50 cm (1 to 1½ ft) tall. The leaves are opposite, pinnate with four leaflets (two opposite pairs; no terminal leaflet), each leaflet 1 to 7 cm long and 1 to 3 cm broad. The flower is typical pea flower in shape, 2 to 4 cm (¼ to 1½ in) across, yellow with reddish veining. After pollination, the fruit develops into a legume 3 to 7 cm (1 to 2 in) long containing 1 to 4 seeds, which forces its way underground to mature (http://en.wikipedia.org/wiki/Peanut).

Peanut flowers are located in the axils of the leaves and are never at the same node as vegetative branches, although very short internodes on some plants may make it appear that they are. The first flower appears from 4 to 6 weeks after planting. Each flower is subtended by two bracts; the lower, on an axis of the inflorescence and the upper in the axil of the lower bract. The flower contains five petals: a standard, two wings, and two petals fused to form a keel. The flower has 10 stamens, two of which are usually not fully developed. The pistil consists of an ovary, style, and stigma. Anthesis and pollination usually occur at sunrise with pollination taking place within the closed keel of the flower (http://www.lanra.uga.edu/peanut/knowledgebase/).

Peanut (Arachis hypogaea L.) is one of the principal economic crops of the world, ranking 13th among food crops. Asia is the largest producer, followed by Africa, North, Central and South America. In India, over 81% of its total produce is utilized for oil extraction, 12% utilized as seed, 6% for domestic use and 1% for export. Peanut, which is also known as groundnut and monkeynut, is a rich source of energy because of its high oil and protein content. The peanut plant is unusual because it flowers above ground and pods containing one to four seeds are produced underground. Its seeds are rich source of edible oils and contain 40 -50% fat, 20 - 50 % protein, and 10 to 20 % carbohydrate. The seeds are nutritious and contain vitamin E, niacin, folacin, calcium, phosphorus, magnesium, zinc, iron, riboflavin, thiamine, potassium etc. Peanuts, peanut oil and peanut protein meals constitute an important segment of world trade in oilseeds and products. It is the fifth most important oilseed in the world. Peanut is used for different purposes: food (raw, roasted or boiled,
Studies on metal tolerance in peanut cooking oil), animal feed (pressings, seeds, green material, straw), and industrial raw material.

Peanut (*Arachis hypogaea* L.) is a unique leguminous plant for its characteristic behavior to produce the pods underground in direct contact with soil. This large seeded oilseed crop is cultivated extensively for production of edible oil and protein rich grains. Suitability of cultivating this crop for other purposes like phytoremediation, phytomining, production of biodegradable plastic, production of biodiesel etc. has not been explored. Oilseed crops of the family *Brassicaceae* are identified as hyper accumulators and have been studied significantly for phytoremediation of metal contaminated sites (Prasad and Freitas, 2003) and for production of biopolymers (Houmiel et al., 1999). Literature on the effect of toxic metals on peanut plant is scanty. From analysis of plants growing in presence of Cd under field and pot conditions it was shown that significant differences exist between cultivars of peanut in terms of Cd accumulation in kernels (Bell et al., 1997). Comparing Cd accumulation by peanuts and other grain legumes grown in field and glass house condition it was speculated that peanuts may be accessing Cd from deeper layers in the soil profile. These authors also reported that peanut shoots contained concentrations of Cd four to five times higher than in kernels and half of the Cd in kernel contained in the testa. Other researchers demonstrated that Cd enters the peanut kernel mainly through direct uptake by root hairs on the pods (Bledsoe et al., 1949). Whereas other researchers have shown that Cd uptake by peanut is via the main root system with direct pod uptake contributing less than 5% of the total Cd in the kernel (McLaughlin et al., 2000).

In recent years, great stride has been made over various techniques of remediation of contaminated soils. However, in many developing countries, it is not practiced in farmland as the high cost and slow process of remediation often succumb to the high demand to produce foodstuff. In a recent study, a novel alternative strategy to reduce the risk of soil contaminants entering the human food chain without fallowing the land has been proposed: Pollution-safe cultivars (PSCs), that is, the cultivars in which edible parts accumulate certain pollutant at low enough level for safe consumption when grown in contaminated soil, were screened and explored among the cultivars of a major staple crop-paddy rice (*Oryza sativa* L.) when they
were grown experimentally in Cd contaminated soil (Yu et al., 2006). The concept of PSC is grounded on the basis of prior studies, which have shown that the uptake and accumulation of metal pollutants by plants can help in selection of suitable cultivar.

It appears that peanut has the double advantage for absorption of metal from soil through roots and directly through the pods. Therefore, it is of interest to understand biochemical changes that the peanut plants adopt against oxidative stress induced by accumulated metal ions. The primary objective of this study is to assess the effects of Cr, Cu and Cd in developing peanut seedling in vitro. The study was designed to investigate; (i) the impact of Cr, Cu and Cd on seedling growth, lipid peroxidation and differential enzymatic responses of plant to understand the role of these enzymes in providing protection to the plant against Cr, Cu and Cd and (ii) effect of these metals on structural morphology of the plant.

3.2 EXPERIMENTAL PROTOCOL

Plant materials and growth conditions

Peanut (Arachis hypogaea L.) seeds, procured from the local market were washed with detergent for 10 min followed by repeated washing with deionized water. Thereafter the seeds were treated with 4% savlon (v/v) (liquid antiseptic, Johnson and Johnson, India) for 12 min. On removing savlon with repeated washing with sterile water, the seeds were disinfected by 0.1% HgCl$_2$ (w/v) treatment for 10 min. Adhering HgCl$_2$ was eliminated by rinsing the seeds with sterile deionized water. The culture medium was agar gelled MS basal medium supplemented with 2% sucrose (w/v). The pH of the media was adjusted to 5.8 prior to autoclaving. Stock solution (100 mM) of potassium dichromate (K$_2$Cr$_2$O$_7$), copper sulfate (CuSO$_4$) and cadmium chloride (CdCl$_2$) were prepared and filter sterilized. Suitable aliquots of filter sterilized solution of CdCl$_2$ and CuSO$_4$ were added aseptically to attain final concentrations of 50 µM, 100 µM, 200 µM and 300 µM metal. However, concentration of Cr was 50 µM, 100 µM, 200 µM 300 µM, 500 µM and 1000 µM. Medium without metal was used as control. The media were distributed in cotton plugged culture tubes (20 ml/tube). The testa of seeds was removed aseptically and the seeds were cultured in with and without metals. After incubating the cultures for
3-4 days in dark for radicle emergence, the seedlings were transferred in 16 h photoperiod of 32 µE m$^{-2}$ s$^{-1}$ at 25 ± 2 °C. Germination and development of seedlings took 10 days.

**Effect of Cr, Cu, Cd on peanut seedling**

Seedlings were harvested after 4 weeks for quantification of Cr, Cu and Cd content in the leaves, stems and roots, enzyme assays and for determination of lipid peroxidation product level (TBARS). The morphologies of the plants were recorded prior to harvesting. Parameters including germination frequency, shoot height, root lengths were noted. Shoot height was measured from cotyledon node to shoot tip. Experiments were repeated 3 times using 10 replicates.

**Histological studies**

Peanut seedlings growing in 100 µM of each metal for 4 weeks were used for histological studies. Stem and leaf tissues were taken after 30 d of incubation whereas root tissue was taken after 10 d, 20 d and 30 d of incubation. Upper most leaflets were subjected to histological studies. These were cut into small (approx 3 x 4 mm) pieces and were fixed in FAA (formaldehyde: glacial acetic acid: alcohol, 5:5:90, v/v) for 48 h at room temperature. The procedure followed for sample preparation is described in Chapter 2.

**Absorption of metals by pods and distribution of metal in seed, testa and shell**

The unique character of this plant to produce the pods underground in direct contact with soil has never been exploited for phytoremediation. To use this crop, or the pods, or the peels of the seeds for selective absorption of metals from the contaminated sites there is need to determine the ability of the different parts of the pods to absorb the metals. Therefore the experiments were conducted to study absorption of metal in seedpod.

The selected pods were washed thoroughly with deionized distilled water to remove the adhering mud. These were then sun dried to remove moisture. The weights of the individual dry pods were checked intermittently till it reached the
constant weight. Drying the seeds in the oven at higher temperatures was avoided to minimize degradation of lipids and proteins in the seeds. Ten tubes were kept for each treatment i.e. control, 50, 100, 200, and 300 µM of Cr, Cu and Cd. The washed, dried pods were taken individually and soaked in 10 ml solution of each metal of above concentrations for 24 and 48 hours at room temperature. After incubation, the adhering moisture was wiped and the samples were dried in the oven until constant weight was reached. Shell, testa and seeds were separated from the pods and treated separately for the estimations. Samples were grinded to obtain fine powder. These powders were used for metal estimation using AAS technique described in Chapter 2.

**Preparation of tissue extract for Catalase and Guaiacol peroxidase activity**

Tissue samples (0.75 g) were homogenized in chilled extraction buffer composed of 50 mM sodium phosphate buffer pH 7.0, 0.5 % Triton X-100, 0.1 mM EDTA and 1 % polyvinylpyrrolidone at 4°C. Homogenate was filtered through the folds of muslin cloth and centrifuged at 12,000 g for 16 min at 4°C. The activity of SOD, CAT, and GPX was estimated in supernatant. The total protein content was estimated following the method of Lowry et al. (1951) using bovine serum albumin (Sigma) as standard protein.

**Lipid peroxidation and enzyme activity**

Procedure followed for total enzyme activity measurement and lipid peroxidation is described in Chapter 2.

**Metal estimation**

On harvesting the seedlings after 4 weeks, the root, stem and leaves were separated. Plant roots were thoroughly washed with deionized water to remove adhering medium and weighed. Drying the roots on filter paper eliminated adhering moisture. Tissues of three seedlings from each concentration were pooled for each analysis and weighed (FW). These were dried in oven at 95°C until constant weight was reached. The detailed procedure followed for metal estimation is described in Chapter 2.
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**Data analysis**

The results are the mean ± Sd of three repeats. All data were subjected to one-way ANOVA analysis. Comparison from control and between means of different treatments was done by Duncan’s multiple range tests (metal content, SOD, CAT, GPX activity and TBARS content).

### 3.3 RESULTS AND DISCUSSION

#### Effect of Cr, Cu and Cd on seed germination

Seed germination is the first physiological process affected by metal stress. Thus, the ability of a seed to germinate in medium containing metals would be indicative of its level of tolerance to these metals. The germination frequency was ranged from 32% to 70% in medium with or without Cr (Table 3.1). In media with and without Cu it ranged in 81% to 87% (Table 3.2). However the frequency was between 89 to 96% in media with and without Cd (Table 3.3).

**Table 3.1** Effect of Chromium on peanut (SB-11) seedling growth after 4 weeks. Values are mean ± Sd (n=3)

<table>
<thead>
<tr>
<th>Conc. of Cr</th>
<th>Germination frequency Mean ± Sd</th>
<th>Shoot height (cm) Mean ± Sd</th>
<th>Root length (cm) Mean ± Sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>70 ± 5.00</td>
<td>9.29 ± 1.4</td>
<td>6.59 ± 1.01</td>
</tr>
<tr>
<td>50 μM</td>
<td>50 ± 13.33</td>
<td>8.55 ± 0.86</td>
<td>5.85 ± 0.25</td>
</tr>
<tr>
<td>100 μM</td>
<td>58 ± 18.9</td>
<td>8.63 ± 3.51</td>
<td>4.73 ± 1.49</td>
</tr>
<tr>
<td>200 μM</td>
<td>35 ± 5.00</td>
<td>9.34 ± 1.23</td>
<td>4.41 ± 0.95</td>
</tr>
<tr>
<td>300 μM</td>
<td>38 ± 7.64</td>
<td>10.21 ± 2.53</td>
<td>3.83 ± 0.66</td>
</tr>
<tr>
<td>500 μM</td>
<td>38 ± 5.64</td>
<td>6.22 ± 0.28</td>
<td>1.15 ± 0.01</td>
</tr>
<tr>
<td>1000 μM</td>
<td>32 ± 2.64</td>
<td>3.35 ± 0.07</td>
<td>0.86 ± 0.06</td>
</tr>
</tbody>
</table>

Anova       Sig 1%     Sig 1%     Sig 1%

From the tables it is apparent that seed germination was inhibited significantly in presence of either Cr or Cu. Growth of the seedlings retarded at higher
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concentration of these metals in medium. In Cr shoot, root differentiation and elongation remained unaffected till the concentration of 300 µM (Table 3.1, Fig. 3.1 A, B) but was suppressed at 500 µM and 1000 µM (6.2 cm and 3.3 cm). The shoot growth suppression was more pronounced at higher concentration. Similarly, root growth was decreased with increasing concentration of Cr in medium.

![Figure 3.1: Effect of chromium on peanut seedling after 4 weeks. Treatments are in µM.](image)

Decrease in root growth at higher concentration of Cr could be due to inhibition of cell division and elongation of cells. Our data supports the earlier observation of Cr effect on plant growth (Gardea-Torresdey et al., 2005; Castro et al., 2007). The size of *Salsola kali* plants (shoot and root) grown in 20 mg L$^{-1}$ Cr for 15 d was significantly smaller than the control plant (Gardea-Torresdey et al., 2005).
Higher concentration of Cr VI (400 and 800 µM) were toxic to *A. thaliana* plant as revealed by decreased seed germination and arrested growth of roots and shoots (Castro et al., 2007).

In peanut, shoot elongation was suppressed with increasing concentration of Cu in medium (Table 3.2, Fig. 3.2) and root elongation was affected severely. Copper is incorporated in the tissue culture medium in trace concentrations as this metal serve as cofactor for some of the enzymes involved in plant growth metabolism. However at higher concentrations, this metal is toxic and affects the growth adversely. Exposure of 15 d in higher concentration of Cu (200 and 350 µM) in *Lycopersicon esculentum* plant, significantly reduced the root elongation as compared to control (Martins and Mourato, 2006).

**Table 3.2** Effect of Copper on peanut (SB-11) seedling growth after 4 weeks. Values are mean ± Sd (n=3)

<table>
<thead>
<tr>
<th>Conc. of Cu (µM)</th>
<th>Germination frequency Mean ± Sd</th>
<th>Shoot height (cm) Mean ± Sd</th>
<th>Root length (cm) Mean ± Sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>87 ± 3.30</td>
<td>10 ± 1.20</td>
<td>6.6 ± 0.61</td>
</tr>
<tr>
<td>50 µM</td>
<td>82 ± 2.16</td>
<td>11 ± 0.28</td>
<td>6.4 ± 0.28</td>
</tr>
<tr>
<td>100 µM</td>
<td>82 ± 2.06</td>
<td>9.9 ± 0.37</td>
<td>5.4 ± 0.46</td>
</tr>
<tr>
<td>200 µM</td>
<td>82 ± 2.21</td>
<td>7.5 ± 1.30</td>
<td>3.0 ± 0.66</td>
</tr>
<tr>
<td>300 µM</td>
<td>81 ± 1.50</td>
<td>6.7 ± 1.18</td>
<td>1.6 ± 0.29</td>
</tr>
<tr>
<td>Anova</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sig 5%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sig 1%</td>
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</tr>
</tbody>
</table>

Suppression of plant growth has been reported due to presence of Cu (Yruela, 2005). Ouzounidou et al. (1995) showed that excess Cu (80 µM), after 15 d of treatment in maize roots affected root elongation significantly. Cuypers et al. (2000) studied the effect of excess Cu on *Phaseolus vulgaris* and found that the leaf area was significantly reduced but the effect on shoot growth was less pronounced. *In vitro* study in *Withania somnifera*, showed significant reduction in shoot length and root elongation at 100 and 200 µM of Cu concentration when grown for 30 d (Khatun et al., 2008). Cu-induced inhibition in root growth of rice seedlings can be due to cell
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wall stiffening, and this can be an explanation to the Cu-excess effect on roots influencing plant growth (Chen et al., 2000).

![Figure 3.2: Effect of copper on peanut seedling after 4 week. Treatments are in µM.](image)

Similarly Cd also demonstrated adverse effect on peanut seedling growth by affecting both shoot and root elongation (Table 3.3, Fig. 3.3). Cd can interfere with morphogenesis, by inhibiting cell division and cell enlargement (Dalla Vecchia et al., 2005). Germination of peanut seeds at high frequency in medium containing Cd suggests that this metal does not affect the process of germination at the concentrations tested. However, the growth of the seedlings was retarded. The seedlings appear healthy and green in all concentrations of Cd tested. Retarded growth of each part of peanut seedling confirms adverse effect of Cd on plant differentiation. This effect was more obvious on differentiation of the lateral roots. Exposure of 2 week in Cd at various concentrations (10, 20 40 ppm) inhibited the seed germination and seedling growth of *Medicago sativa* (Peralta et al., 2001).

Cd exhibited toxicity to seed germination and seedling growth at various concentration (0.2-0.8 mM) in *A. thaliana* when it was exposed for 6 d (Li et al., 2005). A significant decrease in germination and root elongation occurred at all the levels (0.5-3 mM) of Cd stress, ranging from 13% at 0.5 mM Cd to 27% at 3.0 mM Cd, as compared to the control plant in *Sorghum bicolor* (Kuriakose and Prasad, 2008).
Table 3.3 Effect of Cadmium on peanut (SB-11) seedling after 4 weeks. Values are mean ± Sd (n=3)

<table>
<thead>
<tr>
<th>Conc. of Cd (μM)</th>
<th>Germination frequency Mean ± Sd</th>
<th>Shoot height (cm) Mean ± Sd</th>
<th>Root length (cm) Mean ± Sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>89 ± 3.82</td>
<td>7.25 ± 2.40</td>
<td>6.84 ± 1.06</td>
</tr>
<tr>
<td>50</td>
<td>90 ± 2.42</td>
<td>6.56 ± 1.73</td>
<td>6.07 ± 0.86</td>
</tr>
<tr>
<td>100</td>
<td>96 ± 5.47</td>
<td>6.26 ± 2.09</td>
<td>5.85 ± 0.62</td>
</tr>
<tr>
<td>200</td>
<td>92 ± 5.09</td>
<td>5.26 ± 1.71</td>
<td>4.70 ± 0.71</td>
</tr>
<tr>
<td>300</td>
<td>93 ± 5.71</td>
<td>2.85 ± 1.03</td>
<td>2.59 ± 1.29</td>
</tr>
<tr>
<td>Anova</td>
<td>NS</td>
<td>Sig 5 %</td>
<td>Sig 1%</td>
</tr>
</tbody>
</table>

At the highest concentrations of Cd and Cu, the roots became dark and dehydrated and formation of lateral roots and root hairs was inhibited. From the data, it is apparent that Cr is more inhibitory for peanut seed germination while Cd restrain the seedling growth more followed by Cu and Cr.

Fig 3.3: Effect of cadmium on peanut seedling after 4 weeks. Treatments are in μM.

In the seeds, during germination proteins and starch are hydrolysed, to provide amino acids and sugars to the developing embryo axes. Under Cr (10, 50 mM)
exposure of 21 d in *Phaseolus vulgaris* in pot culture a decrease in both α and β-amylase has been reported (Zeid, 2001). Similarly decrease in α and β-amylase activity was noticed in sorghum due to Cd treatment (Kuriakose and Prasad, 2008). These enzymes are important for germination as the decreased activities of these enzymes may suppress germination due to impaired supply of sugar to the embryo axes. The inhibitory effect of these metals was obvious on differentiation of the lateral roots in peanut seedlings (Fig. 3.1, Fig. 3.2, Fig. 3.3).

A common theme in animal stress physiology is the ‘fight-or-flight’ response an acute response that enables animals to defend themselves or flee away from a stressful situation. Plants being sessile organisms have developed an extensive array of defensive responses. Many plants redirect their growth when exposed to stress. It is hypothesized (Potters et al., 2007) that such morphogenic responses are part of a general acclimation strategy that constitutes the ‘flight’ response of plants. Most heavy metals are phytotoxic affecting growth and development. However, chronic exposure also induces a specific SIMR (Stress induced morphogenic response) phenotype, characterized by an inhibition of root elongation, and enhanced formation of lateral roots. In peanut seedlings, stress induced by Cd and Cu and Cr at higher concentrations, caused root thickening, decrease in root diameter and inhibition of root and shoot elongation (Fig. 3.1, 3.2 and 3.3). In higher plant (review Horst, 1995) and *A. thaliana* (Pasternak et al., 2005) it is demonstrated that inhibition of organ elongation is due to lack of cellular elongation. Ouzounidou et al. (1992) reported that metal affects ultrastructure of merismatic cells altering the ribosomal RNA biosynthesis, thus affecting plant growth. However, Cd had no effect on the morphology of the Cd tolerant plant *Phragmites australis* (Ederli et al., 2004). This indicates that SIMRs are not caused by exposure to heavy metals *per se* but by the degree of stress. In peanut, Cr upto 300 μM was found to induce more lateral roots by inhibiting elongation of main root (Fig. 3.1 A). The number of roots formed in Cr treated *Triticum aestivum* increased by 13 and 25% in plants exposed to 250 or 500 μg/ml Cr-salts, respectively, but decreased by 61% at 1000 μg/ml CrCl₃ (Hasnain and Sabri, 1997). In our study there was decrease in root length at higher concentrations (500 and 1000 μM) of Cr in medium (Fig 3.1 B). Plants have evolved a large variety of distinct morphological adaptations to limit exposure to unfavourable
environmental conditions. One example is the dwarf architecture (cushions, tussocks) that is found in plants from alpine or arctic environments. The functionality of such architecture is linked to the capability to create a favourable microclimate, a climate in which the shoot apical meristem in particular is relatively well protected, enabling rapid resumption of growth when conditions become favourable. It is attractive to interpret SIMRs in terms of such protective responses, aimed at evading stress exposure (Potters et al., 2007).

**Effect of chromium, copper and cadmium on metal accumulation, lipid peroxidation and antioxidative enzyme activity**

**Chromium**

The Cr content in root was between 30-758 mg/Kg DW, in stems 14-200 mg/Kg DW and in leaves 13-138 mg/Kg DW (Fig. 3.4 A). The peanut seedling accumulated metal in dose dependent manner. The peanut seedling might have maintained its normal growth of shoot because of less accumulation of Cr at lower concentrations (50-300 µM) in stem (Table 3.1). In case of Cr, the accumulation pattern was interesting. The levels of Cr in peanut seedling treated with 50 and 100 µM were in the order as follows: roots>leaves>stems and roots>stems>leaves at 200-500 µM of Cr (Fig. 3.4 A). Peanut seedling growth suppression at 500 µM might be due to sharp increase in Cr accumulation at 500 µM. After exposure of the seedlings to Cr, the level of Cr in the tissues increased with increasing Cr concentration in the medium. The supply of Cr stimulated the accumulation of Cr mainly in roots but also to a less extent in stems and leaves. Root accumulated optimum Cr followed by stem and leaves at higher concentration of Cr. The effects of Cr on TBARS concentration are presented in Fig. 3.4 B. Compared to control root, TBARS concentration was not increased significantly upto 200 µM, but was increased significantly at higher concentration of Cr (300 and 500 µM) indicating a rise in lipid peroxidation with the increasing concentration of Cr in medium. There was no significant change in TBARS content in leaf tissue due to Cr treatment. But in stem significant increase in TBARS content was noted at highest concentration of Cr (500 µM). Highest value of TBARS was noted in root treated with 500 µM Cr.
The activities of antioxidant enzymes (SOD, CAT and GPX) in peanut seedling are presented in Fig. 3.4 (C, D, E) when the seedlings were exposed to different concentration of Cr. In roots SOD activity was optimum at 100 µM and in the other concentrations, activity was significantly less than control. Whereas in stem, SOD activity was optimum at 500 µM and low activity was noticed at 200 µM. The presence of Cr in medium markedly reduced SOD activity in leaves after the treatment for 4 wk, and the inhibition increased with increasing Cr concentration (Fig. 3.4 C). There was no significant change in CAT activity in peanut root, when the seedlings were exposed to various concentration of Cr. The role of CAT in Cr induced toxicity in roots seems to be limited in the present study.

Fig 3.4: Effect of various concentrations of Cr on (A) metal accumulation, (B) lipid peroxidation (C, D, E) antioxidative enzyme activities on peanut seedling after 4 wk of incubation (means ± Sd). Means with common letter(s) are not significantly different at P< 0.05 according to Duncan multiple range test (n=3).
In stem and leaves the CAT activity was significantly reduced at higher concentration of Cr (200, 300 and 500 µM). It was observed that CAT activity was higher in leaves in comparison to root and stems (Fig. 3.4 D). In peanut roots GPX activity was not affected. In stems, GPX activity decreased significantly at higher concentrations (300 and 500 µM) of Cr in medium (Fig. 3.4 E). In case of leaves, GPX activity remained unaffected upto 300 µM but at 500 µM there was a sharp reduction in GPX activity as compared to control.

**Copper**

The seedling of peanut were used for the present study to understand their ability to accumulate Cu content and physiological changes. The accumulation of Cu in roots, stem and leaves of peanut seedling varied depending on different Cu concentrations used. Cu concentration varies in roots between 9- 942 mg/Kg DW in stems 14-65 mg/Kg and in leaves 28-176 mg/Kg DW (Fig. 3.5 A). The Cu content in roots, stem and leaves of peanut seedling increased significantly with increasing concentration of Cu in medium. The roots of plant exposed to 300 µM accumulated large amount of Cu and the Cu level was approximately 104 times higher than that of control. The Cu content in roots of plant treated with 50, 100 and 200 µM were about 6.6, 19 and 63 times higher than that of control respectively.

Like metal content TBARS content was also estimated in different organs of peanut seedling exposed to various Cu concentrations. Unlike in Cr treatment there were no significant changes in lipid peroxidation in roots, stems and leaves of peanut seedlings exposed to Cu (Fig. 3.5 B). The effect on antioxidative enzyme in different organs of peanut seedling exposed to various Cu concentrations for 4 wk can be seen in Fig. 3.5 (C, D, E). Superoxide dismutase activity reduced significantly at higher concentrations (200 and 300 µM) in roots and stems. However, in leaves SOD activity was maximum at 100 µM of Cu and in the rest of concentration activity was virtually identical to control.
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Fig 3.5: Effect of various concentrations of Cu on (A) metal accumulation, (B) lipid peroxidation (C, D, E) antioxidative enzyme activities on peanut seedling after 4 wk of incubation (means ± Sd). Means with common letter(s) are not significantly different at P< 0.05 according to Duncan multiple range test (n=3).

At higher concentration of Cu (200 and 300 µM), CAT activities was diminished significantly in roots and leaves of peanut seedling. However, in stem CAT activity was maximum at 50 µM of Cu and in rest of concentration activity remained unaltered. This pattern of CAT activity in stem can be correlated with the least Cu content in peanut stem as compared to root and leaves. Guaiacol peroxidase
activities were significantly decreased in all three organs of peanut seedling with increasing concentration of Cu in medium.

**Cadmium**

The Cd accumulation in root was (0-1001 mg/Kg DW), stem (0-314 mg/Kg DW) and in leaves it was 0-137 mg/Kg DW (Fig. 3.6 A). No Cd was detected in control plants. There was parallel increase in Cd concentration in each of these parts indicating absorption of Cd from medium and distribution to all the parts of the plant. The amount of Cd absorbed by each of these organs increased with increase in concentration of Cd in medium. There was significant metal accumulation in all three parts of seedling.

The TBARS were determined in the seedling parts after 4 week of culture in control medium. The level of TBARs increased in all three parts of the peanut seedlings developed in presence of Cd (Fig. 3.6 B). This indicates presence of increased lipid peroxides in all these parts at all the concentration of Cd tested. The level of TBARS content was increased significantly at 300 µM in all the three part of seedling as compared to control. The TBARS content was highest in the stem (27 nmol g\(^{-1}\) FW) followed by root (24.3 nmol g\(^{-1}\) FW) and the leaves (19 nmol g\(^{-1}\) FW) at 300 µM.

In case of Cd treated seedlings there was significant decrease in SOD enzyme activity in roots. Effect on SOD enzyme activity due to Cd stress can be seen in Fig. 3.6 C. The SOD (102 U/mg) activity was optimum in control whereas in 300 µM it was very less (28 U/mg). However, in stems and leaves SOD activity decreased at 50 µM and at other concentrations there was slight increase in enzyme activity, although the increase was always less than control seedling.

Pattern similar to SOD, was also noticed in CAT activity in different organs of peanut seedling. In root, CAT decreased significantly and in stem and leaves after a transient decrease in activity at 50 µM there was continuous increase in CAT activity. But CAT activity was always less than control (Fig. 3.6 D). CAT activity was higher in leaf (21-49 U/mg) as compared to stem (6-13 U/mg) and root (6-10 U/mg). There was continuous significant decrease in GPX activity in all the three organs of peanut seedling due to Cd stress (Fig. 3.6 E).
Fig 3.6: Effect of various concentrations of Cd on (A) metal accumulation, (B) lipid peroxidation (C, D, E) antioxidative enzyme activities on peanut seedling after 4 wk of incubation (means ± Sd). Means with common letter(s) are not significantly different at P< 0.05 according to Duncan multiple range test (n=3).

GPX activity decreases from 6.4 to 3.1 U/mg (root), 4 to 1.7 U/mg (stem) and 4.4 to 1.6 U/mg (leaves) in control and 300 μM respectively. GPX activity decreased significantly with increasing concentration of Cd in medium.
Discussion

Plants have the potential to uptake metals from the contaminated soil and tolerate certain levels of heavy metals that would be toxic to any other known organisms. Plant roots has been known to accumulate more Cr metal (Shanker et al., 2005a; Wenshanke, 2007; Sunil Kumar et al., 2008b) as compared to stems and leaves. Low accumulation of Cr metal in stem can explain the unaltered change in shoot height of peanut seedling. It must be noted that Cr is a toxic and nonessential element to plants and hence the plants may not possess any specific mechanism of transport of Cr. The Cr(VI) is transported by active mechanism by using carriers of essential anions such as sulfate (Cervantes et al., 2001). Fe, S and P are known to compete with Cr for transport binding (Wallace et al., 1976). At higher concentration of 500 μM of Cr there was sharp increase in accumulation in root. But once the Cr metal was inside in plant roots it was able to transfer the Cr to stems and leaves efficiently. Huge accumulation of Cu in peanut root at higher concentrations might have induced excess stress, which contributed to reduced root length. Peanut root accumulated highest amount of Cu and transfer of metal from roots to above ground part was less. This could be a strategy of this plant to tolerate Cu toxicity, so that normal functions of photosynthesis could be carried out in leaves. Highest amount of accumulated Cu in roots as compared to amount of Cr in roots can be due to presence of specific transporter for Cu as it is an essential element for plant growth (Yruela, 2005). The primary site of Cd accumulation was the root followed by stem and leaves. The amount of Cd transferred into the leaf was less in all the concentrations tested. This could be due to high accumulation in root and slow transfer of metal to the leaves. As the plant differentiates, heavy metal cations are absorbed by the roots and transferred to stem and the leaves. Presumably, after the emergence of the radicals and their differentiation, the roots are directly exposed to Cd. Thus, the Cd concentration in this organ increased with the concentration of the Cd ion in the medium and is much higher as compared to stem and leaves. Up to a supply of 200 μM the Cd concentration increased in the stem and leaves with increasing concentration of Cd in the medium. However, in 300 μM, both in stem and leaves the Cd concentration increased drastically. At the concentration of 300 μM the cell growth is further inhibited due to increased accumulation of Cd. This was reflected in
the reduced stem elongation (Table 3.3) and increased Cd content (Fig. 3.6 A) in the stem. With reduced Cd translocation from the stem and ongoing slow cellular growth in the leaves, the Cd concentration appears lowest in this organ and a gradation in Cd concentration was maintained between the organs. At this concentration the leaf size was reduced. Reduced root growth, chlorosis and leaf rolls are the main and easily visible symptoms of cadmium toxicity in plants (Benavides et al., 2005; Wojcik et al., 2006; Van Belleghem et al., 2007). In an attempt to study the translocation of Cd in the rice plant, it has been demonstrated that 91-100% of the Cd in the grains was deposited from the phloem. This confirmed the contribution of the phloem in Cd transport to rice grains (Tanaka et al., 2007). Extensive work has been done by several researchers to understand the mechanisms of metal accumulation and tolerance in plants and has been reviewed (Polle et al., 2003), but the complex network of interactions of hormone and nutrient factors in abiotic stress tolerance is not fully understood.

Stress induced production of highly cytotoxic species of oxygen (ROS) can seriously disrupt normal metabolism through oxidative damage to cellular components. One of such damaging effect of ROS is the peroxidation of membrane lipids (Gajewska et al., 2006). This process may severely affect the functional and structural integrity of biological membranes, resulting in increased plasma membrane permeability leading to leakage of potassium ions and other solutes (Cakmak, 2000). The increased accumulation of lipid peroxides is indicative of enhanced production of ROS (Gratao et al., 2005). In this study lipid peroxides was quantified by estimating the thiobarbituric acid reacting substances (TBARS). TBARS is the final product of peroxidation of membrane lipids and accumulates when the plants are subjected to oxidative stresses. TBARS content was unaltered or slightly changed till 300 μM of Cr treatment. However, at 500 μM there was sharp increase in TBARS content in roots and leaves indicating the Cr induced oxidative stress at this concentration in peanut seedling. Cu induced oxidative stress was not sufficient to induce lipid peroxidation in peanut seedling. Increase in lipid peroxidation in plants due to Cu stress has been reported in plants (Peng et al., 2006; Guoa et al., Khatun et al., 2008). Increase in TBARS, following Cd exposure have been observed in several plant species (Benavides et al., 2005; Gomes-Junior et al., 2006), with cell membranes
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being severely affected by peroxidation leading to irreversible damage. The variation in lipid peroxides in different parts of the same seedling in similar medium with same concentration of metals and identical culture conditions, suggest existence of alternative mechanisms of metal tolerance in different organs of the plant.

The induction of a particular group of enzyme activities is considered to play an important role in the cellular defence strategy against oxidative stress, caused by toxic metal concentrations (Sarita and Rohit, 2006). SOD, CAT, and GPX are among the major antioxidant enzymes involved in scavenging ROS. Differential responses of antioxidant enzymes were observed in roots, stems and leaves of peanut seedling after exposure to different concentration of Cr, Cu and Cd in medium.

Chromium has been known to affect several physiological activities and produce severe stress reaction which can affect enzyme activities. The enzyme activities parameter are most sensitive one in evaluating the effect of stress on plant system. Increase at low concentration, and inhibition of enzyme activities at higher concentration of Cr has been reviewed by Shanker et al. (2005a). This response to chromium can vary among plant species and among different tissue (Shanker et al., 2004; Pandey, 2005; Yu 2007). In roots, inspite of higher accumulation of Cr, CAT and GPX activity remains unaltered, these enzymes protects the roots from oxidative stress induced by excess Cr. This is also supported from root length of seedling (Table 3.1) where it remains unaffected till 300 μM. In our study, in stem SOD enzyme play the major role to fight the stress induced by Cr. In leaves where the accumulation of metal was more as compared to stem at higher concentrations (300- 500 μM) of Cr, this might have led to inhibition of the enzyme activities. Reduction in SOD activity at higher concentration in roots suggests interaction of Cr with this enzyme. In stem, upto 300 μM Cr treatment SOD, GPX and CAT activities were similar to control or slightly reduced. But at 500 μM sharp increase in SOD or large drop in GPX and CAT was noticed. This can be directly correlated with low metal accumulation upto 300 μM (30-94 mg/Kg DW) and at 500 μM (200 mg/Kg DW almost double) of Cr treatment in stem. This might explain the unaltered shoot length of peanut seedling upto 300 μM of Cr treatment. So from the data it is apparent that there is direct correlation between metal treatment, seedling shoot growth and antioxidative
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enzymes activities. In stem and leaves CAT activity reduced significantly at higher concentration of Cr. CAT is an iron–porphyry biomolecule. The decreased activity of CAT indicated that Cr is either interacting with iron in metabolic pool or affecting the availability of active form of iron (Sharma et al., 2003).

Cu ions act as cofactors in many enzymes such as Cu/Zn superoxide dismutase (SOD), cytochrome c oxidase, amino oxidase, laccase, plastocyanin and polyphenol oxidase. At the cellular level, Cu also plays an essential role in signaling of transcription and protein trafficking machinery, oxidative phosphorylation and iron mobilization (Yruela, 2005). Thus, plants require Cu as an essential micronutrient for normal growth and development; when this ion is not available plants develop specific deficiency symptoms, most of which affect young leaves and reproductive organs. The redox properties that make Cu an essential element also contributes to its inherent toxicity. Thus, at high concentrations, Cu can become extremely toxic causing symptoms such as chlorosis and necrosis, stunting, leaf discoloration and inhibition of root growth (Assche and Clijsters, 1990; Marschner, 1995) and altered enzyme antioxidative enzymes activities.

It has been confirmed in many studies that when copper is in excess, it can promote and stimulate generation of Fenton-type reactive oxygen species leading to increase in antioxidant enzyme activities as a defense system (Weckx and Clijsters, 1996; Rama Devi and Prasad, 1998; Ducic and Polle, 2005). This response to excess copper can vary among plant species, age, duration of treatment and among different tissue (Lombardi and Sebastiani, 2005; Martins, 2006; Chamshedhine et al., 2008). Many studies have illustrated the inhibition effect on antioxidative enzymes by excess copper (Luna et al., 1994; Maribel and Satoshi, 1998; Boojar, 2007). CAT is another important enzyme against oxidative stress, being able to scavenge H$_2$O$_2$, which is majorly produced by SOD. CAT activity in the present study decreased in all the organs. CAT is sensitive to O$_2^{-1}$ radicals and thus their increasing level due to decreased activity of SOD under metal stress may result in inactivation of enzyme (Cakmak, 2000). Efficient functioning of SOD blocks O$_2$– driven cell damage (Cakmak, 2000) by converting it to H$_2$O$_2$, which is then reduced to water and molecular oxygen by the action of enzymes APX, GPX and CAT working at different locations in the cell. Interestingly in peanut root, it was noticed that at lower
concentration (50 μM) of Cu activities of all the three enzymes were identical to control but at higher concentration (200 and 300 μM) there was sharp reduction in antioxidative enzyme activities (Fig. 3.5 C, D, E). Similar pattern was noticed in GPX activity in stem and leaves. This pattern of the antioxidative enzymes system at 50 μM Cu may imply that the tolerance mechanism involves a system that reduces the formation of or removes free radicals, preventing the production of $O_2^-$ and, therefore, reducing the requirement to activate the antioxidative enzymes. But at higher concentration this system breaks down and antioxidative enzyme activities decreases sharply. Similar effect was noticed in Elsholtzia splendens leaves due to Cu treatment (Peng et al., 2006). This could be due to low Cu accumulation at 50 μM and highest metal accumulation at 200 and 300 μM of applied Cu in peanut seedling.

It is apparent from the data that this huge accumulation of Cu in roots at 200 and 300 μM leads to sharp inhibition in antioxidative enzyme activities and might have resulted in reduced root growth (Table 3.2). This observation points to the fact that peanut seedlings possibly respond differently towards the lipid peroxidation and antioxidant enzymes activity in Cu induced stress. There are reports concerning Cu induced activation of antioxidant system in plants (Ratkevicius et al., 2003). In facts, plants with enhanced activities of antioxidative enzymes have been shown to be tolerant to oxidative stress (Mittler et al., 2004). The inhibition of SOD activity was higher at higher Cu or Cd concentration. This indicates that inhibition of SOD fail to scavenge $O_2^-$ to protect plant from cellular damage.

Plants have evolved both enzymatic and non-enzymatic mechanisms for ROS scavenging (Singh et al., 2006). The reduction of the SOD enzyme activity observed in peanut seedling (Fig. 3.6 C) at different Cd concentration is probably due to enhanced level of $H_2O_2$ and its derivative ROS as observed in Cd treated Phaseolus vulgaris (Someshkaraiah et al., 1992) and Helianthus annuus (Gallego et al., 1996). Our results have indicated a decline in catalase activity in roots, stem and leaves of Cd treated peanut seedling, which may be due to inhibition of enzyme synthesis or change in assembly of enzyme subunits (Ogawa et al., 1997). Reduction in CAT activity in the peanut after 4 weeks culture could be due to inhibition of the enzyme caused by increased accumulation of Cd. Previous studies have shown that Cd
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reduces the contents of some nutrients, such as magnesium, calcium, or iron (Azevedo et al., 2005). As iron is a constituent or cofactor of most antioxidative enzymes (Ranieri et al., 2003), reduction in iron level will cause reduction in CAT activity. There was continuous significant decrease in GPX activity in all the three organs of peanut seedling due to Cd stress. Both increase and decrease in GPX activity has been reported in plants exposed to Cd as reviewed by Gratao et al. (2005). Excess accumulation of Cd in roots, stems and leaves, might have induced oxidative stress which caused the inhibition or decrease in activity of effective quenchers of ROS. The role of peroxidases as stress enzymes (Gasper et al., 1991) in plants has been widely accepted and it has been shown that the peroxidase activity can be used as a potential biomarker for sub-lethal metal toxicity in examined plant species (Radotic et al., 2000). The enzymes analyzed in this work and others including peroxidase and SOD, have been examined in wide range of plant species subjected to the growth in the presence of Cd and considerable disparities in the responses has been recorded. These variation has ranged from increase, through no change, to decrease which are probably due to variation in plant species, tissue or organ, metal, metal concentration and length of exposure (Chaoui et al., 1997; Dixit et al., 2001; Azevedo et al., 2005; Patel et al., 2005; Demirevska-Kepova et al., 2006; Singh et al., 2006; Scebba et al., 2006; Lin et al., 2007; Dinakar et al., 2008; Sunil Kumar et al., 2008a). Decrease in the antioxidative enzymes activity suggest the oxidative stress induced by Cd in peanut seedling. In our study with peanut there was inhibition of various antioxidative enzymes activities at higher concentration of all the three metals used, except in stem where there was stimulation of SOD due to Cr (500 μM) treatment. It is apparent that peanut was more tolerant towards Cr as compared to Cu and Cd.

**Histological studies**

There are many experimental data on the effect of Cr, Cu or Cd on young seedling plant treated with heavy metals after seed germination (Rauser and Meuwly, 1995; Ouzounidou et al., 1995; Quartacci et al., 2001; Maksymiec and Krupa, 2006; Van Belleghem et al., 2007; Castro et al., 2007; Kovačík et al., 2008; Rai and Mehrotra, 2008). However, under field conditions, plants are exposed to the effect of heavy metals as soon as the seed comes into contact with the soil solution. Therefore,
we found it important to examine the effect of Cr, Cu and Cd added already to the medium used for germination of seeds. The Cr, Cu and Cd were steadily present in medium during the entire experiment. The effect of environment stress on plant is determined by responses of individual cells in which the integrity of structure and function is affected (Ouzounidou et al., 1995). The strategies by which plant cells or organs respond to heavy metal have attracted considerable attention (Ouzounidou et al., 1995; Van Belleghem et al., 2007; Sahi et al., 2007; Singh et al., 2007). However, the mechanisms by which heavy metals affect and damage plants at the cellular and organ levels of organization is still poorly understood. It has been well documented that roots accumulate optimum metal and restrict the movement of metal to shoot and leaves because root is first organ which comes in contact with soil or medium. Hence, the role of roots is very important as they may act as a place for deposition and inactivation of the metal. The importance of understanding how cell damage, and its converse, survival, are determined is considerable (Ouzounidou et al., 1995). Studies of architecture show how different cells respond and the nature of the damage caused to the cell.

Histology study was carried out to study the anatomical changes induced by these three metals. As noticed earlier a differential morphological response was seen in peanut seedling in root induced by these metals (Fig 3.1, 3.2 and 3.3). Peanut seedlings growing at 100 µM were taken for histological studies. Root tissues were taken at different time of exposure (10 d, 20 d and 30 d). Effects of these metals on root after 10 d can be seen in Fig. 3.7. The basic structure of root is typical of leguminous crop plants with an epidermis formed by small cells, a broad cortical zone of parenchyma cells arranged radially in its inner part and alternately in its peripheral region, and a broad central cylinder with tetrarch organization of the vascular system (Fig. 3.7 A, B). In case of Cr treatment a different type of response was noticed. After 10 d of Cr treatment the shape of outer cortical cells was different as compared to normal oval shape in control seedling root cells. (Fig. 3.7 C). There was granular deposition in outer region of endodermal cells and in pericycle regions of Cr treated cells. In Cr treatment there was increase in numbers of pericycle cell layers. There was stimulation of premature secondary growth of xylem in Cr treated root as compared to control cells (Fig. 3.7 D). There was increase in number of pith cells.
Copper was found to induce severe changes in vascular bundles of roots. Further as compared to control roots whereas the stele was in tetrarch condition there was a lack of complete differentiation and pith formation in response to Cu treatment (Fig. 3.7 F). Similar effect was reported in As treated roots in *Phaseolus aureus* (Singh et al., 2007). Cortical region was broader and cortical cells were loosely arranged as compared to control cortex. There was no lateral root formation in Cu treated cells. In case of Cd treated seedling slight thickening in cortical cell wall and reduction in number of cells was noticed (Fig. 3.7 G, H). This may be due to accumulation of Cd in intercellular spaces internal cortex and cell wall of root. *Phragmites australis* root cortical cells has been shown to accumulate Cd (Ederli et al., 2004). Van Belleghem et al. (2007) showed the accumulation of Cd in intercellular spaces of *Arabidopsis thaliana* root cortical cells. In xylem and phloem cells shrinkage and granular deposition was observed. After 10 d of incubation, it is apparent from the data that Cu was more toxic for peanut root growth as compared to Cd and Cr.
Fig 3.7 Light micrographs of transverse section of root of control (A, B) and metal treated (C-H) after 10 d of incubation. Normal cell layer in control root (A, B). Change in outer cortical cell shape, increase in number of pericycle cell layer in Cr treated cells (C, D). Broader cortical region (E), differentiation of steler region and pith cells was affected in Cu treated root (F). Thickening and granular deposition in phloem and xylem cells in Cd treated root cells (G, H). c-cortex, e-epidermis, en- endodermis, p-phloem, pe- pericycle layer, x-xylem.
After 20 d of Cr treatment the number of lateral roots was more as compared to number of lateral roots in control root cells. In case of Cr treatment cambium layer was more pronounced between xylem and phloem as compared to control cambium layer (Fig. 3.8 C). In Cr treated seedling root there was reduction in number of pith cells (Fig. 3.8 D). Secondary growth in root cells was noticed in metal treated as well as control cells. After 20 d in Cu stressed root, stele region has been differentiated. Cu caused damage to cortical cells (Fig. 3.8 E, F). After 20 d it seems xylem, phloem and pith cells started to differentiate although there was shrinkage in xylem and phloem cells as compared to normal cells. Pith was having less number of cells as compared to normal control root pith cells. After 20 d in Cd treated seedling distortion of xylem and pith cells was noticed as compared to normal control plant. Number of pith cells was decreased as compared to normal cells. Interestingly, phloem cells number was decreased and it seems that these cells were forced to be localized in very small area as compared to control phloem cells which were well developed and distributed in between xylem cells (Fig. 3.8 G, H).
Fig 3.8 Light micrographs of transverse section of root of control (A, B) and metal treated (C-H) after 20 d of incubation. Normal cell layer in control root (A, B). Loosening of pith cells in Cr treated cells (C, D). Distorted cortical region (E), shrinkage in phloem, xylem cells and reduced pith cells Cu treated root (F). Distorted cortical, phloem cells in Cd treated root cells (G, H). ca -cambium c- cortex, e-epidermis, p-phloem, x-xylem.
After 30 d of Cr treatment outer cortical cells in some regions were hexagonal shaped as compared to normal oval shaped cells in control roots (Fig. 3.9 A). The roots of control seedling showed internally the xylem surrounded by cambial region, with four to six layers of cells (Fig. 3.9 A). The pericycle, endodermis surrounded the phloem outside the cambium. However in roots of plants exposed to Cr there was slight proliferation and intensification of cambial cells. However, similar effect was observed in radish seedling root when challenged with Cd (Vitoria et al., 2003). Proliferation and intensification was also observed in pericycle cell layer in Cr treated roots of peanut seedlings. Interestingly, the pith cells, which were in small number after 20 d, now the number of pith cells was, increased as compared to normal control root pith cells. Increase in root pith cell due to Cr treatment has been reported in *Scirpus lacustris* (Suseela et al., 2002). It has been reported in plants that Cr enters the plant system and is accumulated in high amounts in root system (Shanker et al., 2005; Wenshanke, 2007; Sunil Kumar et al., 2008b) although Cr is transported to shoot and leaves. The increased cell proliferation in pericycle and cambial region of root exposed to Cr may be a strategy which could lead to an increase in water uptake and transport. The increase in pericycle cell layer produces the more lateral roots which might be helping the peanut to absorb more water and nutrient from the medium. Therefore helping the peanut seedling to fight Cr induced stress. Since it has been suggested that pericycle opposite to xylem contribute to origin of lateral roots in peanut (Yarbrough et al., 1949). However the Cr at concentration of 200 µM in *A. thaliana* root arrested the cell division and cell elongation (Castro et al., 2007). Alterations of plant growth and stimulation of lateral root and root hair by Cr might occur through a mechanism, similar to that of mineral nutrient that affects that root architecture of *Arabidopsis thaliana* plant according to their abundance in environments (Lopez Bucio et al., 2003; Castro et al., 2007). After 30 d of incubation there was complete distortions of xylem and phloem cells in Cu stressed plant as compared to normal cells in control root (Fig. 3.9 E, F). In Cu exposure distribution of vascular bundles was disturbed. There was distortion in secondary xylem. From the Fig. 3.9 F it is clear that some ray of primary xylem cells coming from pith invade the phloem cells and breaking it into two patches as compared to normal one patch of phloem cells in control root cells. Xylem and phloem cells are involved in transport of
Studies on metal tolerance in peanut food and minerals. Accumulation of Cu in xylem or phloem may disturb these cells. Disturbance of vascular bundle might be due to Cu accumulation in these cells. Cu accumulation has been noticed in xylem cells of *Azolla* and *Sesbania drummondii* roots (Sela et al., 1988; Sahi et al., 2007). Cortical cells were loosely arranged as compared to control. This might be due to accumulation of Cu in these cells. Cu accumulation has been noticed in *Sesbania drummondii* root cortical cells (Sahi et al., 2007). Ouzounidou et al. (1995) showed vacuolation in outer cortex and central cylinder of maize roots due to Cu toxicity. The author reported the disruption of plastid membrane and release of starch grains from amyloplast due to Cu toxicity in maize root xylem cells. The result of our study is consistent with the hypothesis that Cu inhibits root growth and alters root architecture. The occurrence of disintegration in cortical and vascular bundle cells suggest that peanut root cell do not respond uniformly to stressful conditions and suggest development of resistant strategy to Cu toxicity.
Fig 3.9 Light micrographs of transverse section of root of control (A, B) and metal treated (C-H) after 30 d of incubation. Normal cell layer in control root (A, B), pronounced cambium layer and increased in number of pith cells due to Cr treatment (C, D). Distorted cortical region (E), and divided phloem in two parts, in Cu treated root (F). Ruptured epidermal cells, increased intercellular space in cortical region and distorted vascular bundles in Cd treated root cells (G, H). ca - cambium c- cortex, e-epidermis, p-phloem, x-xylem.
After 30 d cortical cells of control seedling were normal and well compact with intact epidermis (Fig. 3.9 A). In Cd treated seedling rupture of epidermis, loosely arranged middle cortical cells and increased intercellular space was noticed (Fig 3.9 G). Due to Cd treatment some granular deposition was noticed in pericycle cell layer. There was increase in number of phloem cells as compared to normal cells. Growth of secondary xylem cells was retarded (Fig 3.9 H). Pith cell size increased in Cd treated cells. However Vitoria et al. (2003-2004) reported the proliferation of cambial cell in radish seedling after treating with 500 µM of Cd for 24 hr. At higher concentration (1000 µM) of Cd, they reported the disintegration of epidermal and external cortical cell layers and loss of pressure potential in the cortical cells. Therefore leading to formation of conspicuous intercellular air spaces. In our study with peanut seedling, at lower concentration (100 µM) of Cd this effect was noticed. An alternation in root differentiation has been reported in plant subjected to Cd treatment (Schutzendubel et al., 2001; Vitoria et al., 2003/4; Wojcik et al., 2005; Belleghem et al., 2007). Schutzendubel et al. (2001) reported lignin deposition in Pinus silvestris plant root. Wojcik et al. (2005) reported the damage in cortical, epidermal and pericycle cell layer of maize root. Belleghem et al. (2007) reported the substantial damage to cytoplasm of cortical, pericycle and vascular cylinder cells when A. thaliana was exposed to Cd (50 µM). In peanut seedling we observed alternation in root cortex and vasculature in plants exposed to Cd, leading to toxicity in peanut root. At higher concentration of Cd (100, 200 and 300 µM) and Cu (200 and 300 µM) treatment a black deposition was noticed in peanut roots (Fig. 3.2, 3.3). This black deposition of unknown material is possibly composed of epidermal, cortical dead and decomposed cells (Vitoria et al., 2003/4). In Arabidopsis halleri the cell wall of epidermal cells accumulated the Cd and Zn ions (Kupper et al., 2000). Belleghem et al. (2007) reported that Cd was bound with phosphate in A. thaliana root epidermal and cortical cells suggesting that such an accumulation of Cd may be due to precipitation of Cd phosphate in root cells. A similar process might be responsible for presence of unknown black deposition observed in peanut roots. Complexation of Cd is tissue and age dependent. Ku”pper et al. (2004) showed in young and mature tissues (leaves, petioles, and stems), a higher percentage of Cd was bound by sulfur (S) ligands (e.g. phytochelatins) than in senescent tissues where oxygen (S) ligand was involved in
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*Thlaspi caerulescens*. This may indicate that young tissues require strong ligands for metal detoxification in addition to the detoxification by sequestration in the epidermal vacuoles.

Effect of metals on stem anatomy can be seen in Fig. 3.10 (C-H). The stem of control untreated seedling exhibited well-developed pith cells, rings of fully established xylem and phloem surrounded by intact cortical and epidermal cell. The vascular bundles are endarch and vary from 20 to 40 (Fig 3.10 A). Six of them are larger than the rest. A typical large bundle consists of an inner xylem portion, a transverse cambium zone, a band of phloem and an outer cap of phloem fibers (Yarbrough, 1957b). In case of Cr treated stem, breakage of pith and cortex cells was noticed (Fig. 3.10 C, D). Cr stimulated the pith cell formation in peanut root. In peanut seedling, breakdown of pith cells has been seen in old primary roots and is of regular occurrence in this plant (Yarbrough, 1949). In old seedling, therefore the entire hypocotyledonary axis is hollow. The collapse of outer region is a very striking feature of aging hypocotyl. But in peanut stem treated with Cr breakage of cortical and pith cells suggest that by unknown mechanism Cr might have induced this effect prematurely. Whereas in Cu and Cd vascular bundles were more affected where shrinkage of phloem and xylem cells was observed (Fig. 3.10 F, H). In Cd treated stems there was distortion of outer cortex cells as compared to normal cortical cells in control stem. Centrad to each bundles lie 1-3 large cells and few cells present in pith were usually filled with stainable substance usually phlobaphene a tannin derivative (Yarbrough, 1957b) were observed in vascular bundles of control as well as metal treated stem.
Fig 3.10. Light micrographs of transverse section of stem of control (A, B) and metal treated (C-H) after 30 d of incubation. Normal cell layer in control stem (A, B). Rupture of cortex and pith due to Cr treatment (C, D). Shrinkage in xylem and phloem cell in Cu treated stem (E, F). Shrinkage in xylem and phloem cell, distortion of cortical cells in Cd treated stem (G, H). c-cortex, e-epidermis, p-phloem, x-xylem.
In peanut leaves the palisade layer, which lies directly below the epidermis, is composed of two to four cells loosely stacked in columnar fashion (Fig. 3.11 A). A prominent and unusual feature of the leaflet in transverse section is the single layer of large thin walled cells contiguous to the lower epidermis. Prominent in this layer are certain cells twice as long as the chlorenchyma and much wider (Fig. 3.11 A) water storage parenchyma cells (Pallas, 1980). Large intercellular spaces are characteristics of this tissue. A fan-shaped mass of xylem and a narrow arc of phloem constitute the central part of the midrib in the peanut (Yarbrough, 1957a). In leaves obvious effect was seen in palisade layer, where granular deposition was noticed in Cu treated leaves followed by less deposition in Cd and Cr treated leaves (Fig. 3.11 C). In Cr treatment number of xylem cells was increased (Fig. 3.11 B). The simulation of cell division in pericycle layer, cambium layer in root and in vascular bundles of leaves tissue could be a strategy to survive with Cr induced stress in peanut seedling. Due to Cu treatment, thickening of vascular bundle cells were observed (Fig. 3.11 C). This could be due to Cu accumulation in vascular bundle since leaf xylem cells of *S. drummondii* has been shown to accumulate Cu (Sahi et al., 2007). Peanut leaves treated with Cd showed increased in xylem cell size as compared to control leaves (Fig. 3.11 D). In Cu treatment number of water storage cells was less as compared to control leaves. This subepidermal layer of large water storing parenchyma cells may help in compensating water loss in any development of leaf water stress (Pallas, 1980). Due to Cu stress there could be disturbance in water storing parenchymatous cell leading to accumulation of granular deposition in palisade layer.
Fig 3.11. Light micrographs of transverse section of leaf of control (A) and metal treated (B-D) after 30 d of incubation. Normal cells in control leaf (A) Number of xylem cells increased in Cr (B) treated peanut leaf. Granular deposition in palisade layers and decrease in water storage cells in palisade layer in Cu treated leaf (C) Granular deposition in palisade layers in Cd treated leaf (D) e-epidermis, Pal-palisade layer, Vb-vascular bundles, WSP- water storage parenchyma cells.

Apart from physiological and morphological changes, metal accumulation also result in structural changes in leaves, stems and roots. A differential alteration in tissue differentiation was noticed in peanut seedling due to different metal treatments. This ‘mosaic’ response enables the tissues root, stem or leaves as whole, to better survive the stress conditions. The occurrence of well-preserved cell components or cells within same tissues indicates that there is no uniform response to metal stress-induced effects. The ability of some cells to accumulate metal and then die, would allow other cells to maintain non damaging concentration of metal and hence continue to function (Ouzounidou et al., 1995).

Taken altogether it seems all the three metals showed differential effect on root anatomy after different time of duration. In copper treated cells after 10 d of incubation, after the emergence of root radicle, there was enough stress to block the
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differentiation of vascular bundles. After 20 d seedling might have tried to cope with the stress induced by copper, so differentiation of vascular bundle was noticed. However, due to exposure of further 10 d of the copper to seedling might have disrupted the phloem cells and caused the disturbance of xylem cells. This may have leads to interruption in normal functioning of vascular bundles. Therefore, the peanut seedling might have transferred less amount of Cu metal to upper parts of seedling. This is also evident by shrinkage of vascular bundles in stem tissue. Copper induced the granular deposition in palisade layer; this layer has maximum number of chloroplast. So copper must have affected the photosynthetic apparatus of this layer causing the granular deposition. This may explain the inhibitory effect of Cu on antioxidant enzymes of leaves as compared to other two metals (Cr and Cd). In case of Cd it seems that it affected mostly cortex cells and xylem cells of root and stem tissue. Whereas chromium was able to induce more number of lateral roots as compared to other two metals.

Absorbsion / adsorbtion of metal in various parts of peanut pod soaked in K$_2$Cr$_2$O$_7$, CuSO$_4$ and CdCl$_2$ solution for 24 and 48 hrs

There are reports (Bell et al., 1997; Angelova et al., 2004; Dinakar et al., 2008; Sunil Kumar et al., 2008a) describing the effect of metals on growth of peanut plants. However the unique character of this plant to produce the pods underground in direct contact with soil have never been exploited for phytoremediation. To use this crop, or the pods, or the peels of the seeds for selective absorption of metals from the contaminated sites there is need to determine the ability of the different parts of the pods to absorb the metals. In the initial part of this chapter we discussed the information gathered on the sensitivity of this crop towards Cr, Cu and Cd toxicity.

The present experiment was carried out to determine the ability of the different parts of peanut pods (Fig. 3.12) to absorb Cr, Cu and Cd metal on soaking in corresponding metal solution for 24 and 48 hours. To assess this character the plant has to be grown in metal containing soil till maturity in pot culture by mixing the metal salt in the potting mixture or by cultivating the crop till maturity in metal contaminated site. Both these methods are labour, energy, cost and land intensive and
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involve agricultural practices. Secondly for pot culture method one needs the infrastructure to handle those toxic metals in the green house under controlled conditions. Pot culture methods have the additional disadvantage of disposing the huge amount of earth (metal contaminated potting mixture) after the experiment is over. Growing the crop in the contaminated land will involve the cultivation practices from the germination till maturation of the crop (4-5 months). Moreover the crop has to be protected from grazing and from using it for edible purposes. Keeping these in view, this study was carried out to determine the absorption behavior of shell, testa and seed of the podded seeds using a simple experimental procedure under nonsterile condition. To the best of our knowledge, this experimental design has never been used earlier.

**Cr absorption / adsorption**

From the Table 3.4 it is clear that there was increase in chromium metal content in Shell, testa, seed and after 24 hr and 48 hr of soaking in Cr solution. Presence of Cr in the parts of the pods under the control condition cannot be explained. The pods were procured from the seed shops. The possibility of application of some Cr salt for preservation of seed cannot be ruled out.

**Table 3.4:** Cr accumulation in various parts of peanut pods in 24 and 48 hrs. Values presented are means ± Sd (n=3)

<table>
<thead>
<tr>
<th>Conc. of Cr</th>
<th>Shell mg/Kg DW Mean ± Sd 24h</th>
<th>Shell mg/Kg DW Mean ± Sd 48h</th>
<th>Testa mg/Kg DW Mean ± Sd 24h</th>
<th>Testa mg/Kg DW Mean ± Sd 48h</th>
<th>Seed mg/Kg DW Mean ± Sd 24h</th>
<th>Seed mg/Kg DW Mean ± Sd 48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18 ± 0.7</td>
<td>25 ± 2.8</td>
<td>10 ± 0.7</td>
<td>14 ± 1.4</td>
<td>7.0 ± 1.4</td>
<td>8.0 ± 1.4</td>
</tr>
<tr>
<td>50 μM</td>
<td>78 ± 12</td>
<td>108 ± 15</td>
<td>28 ± 2.8</td>
<td>56 ± 4.2</td>
<td>15 ± 2.1</td>
<td>21 ± 6.4</td>
</tr>
<tr>
<td>100 μM</td>
<td>106 ± 3.5</td>
<td>133 ± 3.0</td>
<td>57 ± 19</td>
<td>78 ± 9.1</td>
<td>22 ± 5.0</td>
<td>27 ± 1.4</td>
</tr>
<tr>
<td>200 μM</td>
<td>109 ± 4.2</td>
<td>166 ± 9.1</td>
<td>69 ± 12</td>
<td>81 ± 7.7</td>
<td>31 ± 4.2</td>
<td>35 ± 0.8</td>
</tr>
<tr>
<td>300 μM</td>
<td>125 ± 5.6</td>
<td>189 ± 5.0</td>
<td>76 ± 14</td>
<td>102 ± 19</td>
<td>39 ± 6.3</td>
<td>48 ± 6.3</td>
</tr>
<tr>
<td>ANOVA</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
<td>5%</td>
<td>5%</td>
<td>5%</td>
</tr>
</tbody>
</table>
Cr content was maximum in the shell followed by testa and seed. In testa and seed there was gradual increase in Cr with increase in concentration and time duration. However in case of shell there was significant increase in chromium metal content with increase in chromium in solution and with extended exposure of 24 hr to metal.

**Cu absorption/adsorption**

From the Table 3.5 it is clear that there was increase in copper metal content in shell, testa and seed after 24 hr and 48 hr of soaking in Cu solution. Copper absorption increased significantly with the increasing concentration of Cu and also with extended time of 24 hr.

**Table 3.5:** Cu accumulation in various parts of peanut pods in 24 and 48 hrs. Values presented are means ± Sd (n=3)

<table>
<thead>
<tr>
<th>Conc. of Cu</th>
<th>Shell mg /Kg DW Mean ± Sd 24h</th>
<th>Shell mg /Kg DW Mean ± Sd 48h</th>
<th>Testa mg /Kg DW Mean ± Sd 24h</th>
<th>Testa mg /Kg DW Mean ± Sd 48h</th>
<th>Seed mg /Kg DW Mean ± Sd 24h</th>
<th>Seed mg /Kg DW Mean ± Sd 48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11 ± 1.4</td>
<td>15 ± 2.1</td>
<td>6.9 ± 0.1</td>
<td>8.0 ± 0.7</td>
<td>1.3 ± 0.0</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>50 μM</td>
<td>42 ± 5.6</td>
<td>53 ± 4.2</td>
<td>20 ± 2.8</td>
<td>38 ± 2.1</td>
<td>11 ± 3.5</td>
<td>14 ± 2.8</td>
</tr>
<tr>
<td>100 μM</td>
<td>104 ± 6.3</td>
<td>122 ± 5.6</td>
<td>46 ± 1.4</td>
<td>64 ± 1.4</td>
<td>13 ± 1.4</td>
<td>20 ± 2.9</td>
</tr>
<tr>
<td>200 μM</td>
<td>246 ± 9.1</td>
<td>263 ± 4.2</td>
<td>106 ± 27</td>
<td>129 ± 14</td>
<td>21 ± 4.2</td>
<td>27 ± 1.4</td>
</tr>
<tr>
<td>300 μM</td>
<td>306 ± 28</td>
<td>406 ± 19</td>
<td>135 ± 31</td>
<td>209 ± 36</td>
<td>23 ± 2.1</td>
<td>30 ± 2.1</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Sig 1%</td>
<td>Sig 1%</td>
<td>Sig 1%</td>
<td>Sig 1%</td>
<td>Sig 1%</td>
<td>Sig 1%</td>
</tr>
</tbody>
</table>

**Cd absorption / adsorption**

Shell covering the testa and seed accumulated highest amount of metal followed by testa and seed. We assume that in 24 h the seeds did not absorb any metal as it is protected by the pods and the testa. However after the extended incubation in the concentration of 100μM and above the metal was detected in the seeds too. This suggests that there is no stringent barrier between the seed and rest of the parts of the pod and possibly the metal enters the different parts by passive diffusion when the concentration of metal is high in medium.
**Table 3.6:** Cd accumulation in various parts of peanut pods in 24 and 48 hrs. Values presented are means ± Sd (n=3)

<table>
<thead>
<tr>
<th>Conc. of Cd</th>
<th>Shell (mg /Kg DW Mean ± Sd 24h)</th>
<th>Testa (mg /Kg DW Mean ± Sd 24h)</th>
<th>Testa (mg /Kg DW Mean ± Sd 48h)</th>
<th>Seed (mg /Kg DW Mean ± Sd 24h)</th>
<th>Seed (mg /Kg DW Mean ± Sd 48h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>50 μM</td>
<td>425 ± 5.8</td>
<td>7.0 ± 2.3</td>
<td>24 ± 2.1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>100 μM</td>
<td>517 ± 4.2</td>
<td>17 ± 2.1</td>
<td>28 ± 7.0</td>
<td>ND</td>
<td>15 ± 0.7</td>
</tr>
<tr>
<td>200 μM</td>
<td>713 ± 6.6</td>
<td>27 ± 4.3</td>
<td>106 ± 29</td>
<td>ND</td>
<td>28 ± 1.0</td>
</tr>
<tr>
<td>300 μM</td>
<td>910 ± 14</td>
<td>57 ± 18.3</td>
<td>182 ± 23</td>
<td>ND</td>
<td>46 ± 1.4</td>
</tr>
<tr>
<td>ANOVA</td>
<td>1%</td>
<td>5%</td>
<td>1%</td>
<td>ND</td>
<td>5%</td>
</tr>
</tbody>
</table>

ND* (Not detected).

**Fig 3.12** Different parts of peanut seedpod

Shell of the seed showed high metal accumulation ability. It was observed that in case of Cd treatment most of metal was absorbed in shell and lesser amount was transferred to testa and seed as compared to Cr or Cu. The results showed that shell played the role of a selective filter for heavy metal towards the seed and depended upon the type of element. This suggests that the shells can possibly be used effectively in absorption of metal from contaminated effluents. The peanut shells being highly porous, possibly provides extensive surface area for the metal to bind. This results in absorption and accumulation of high amount of metal in the shell. However the high Cd in shell could be due to either absorption only or may be due to both adsorption and absorption.
3.4 CONCLUSIONS

The present study demonstrated that: peanut seedling can tolerate 50-300 μM concentration of Cr in growth medium. Higher concentration of Cr (500 μM) was toxic for peanut seedling growth. This was also evident in lipid peroxidation and antioxidative enzyme activity values. In peanut seedling upto 300 μM of Cr, either the activities remain unaltered or slightly affected. But at higher concentration (500 μM) of Cr either sharp increase or decrease in enzyme activities was noticed. Similarly in peanut treated with Cr, morphoanatomy showed an increased number in pericycle and cambium cell layer in root cells and peanut leaf showed increase in vascular bundle cells. These changes might have helped peanut seedling to combat Cr induced stress.

Peanut seedling growth was severely reduced by Cd and Cu treatment as also evident by significant decrease in antioxidative enzyme activities (in root, stem and leaves) as compared to control. In morphoanatomy of peanut treated with Cu or Cd, there was reduced differentiation in steler region of root cells, shrinkage in vascular bundles of stem cells. These results confirmed the toxic effects of Cu and Cd on peanut seedling growth.

Peanut seedling was more tolerant to Cr as compared to Cd or Cu. Peanut root accumulated the highest amount of metal as compared to the stem or leaves. Differential accumulation of Cr, Cu and Cd in different organs of peanut suggest that this plant has a different mechanism for absorption of these metals by roots and transport these to upper parts. Alteration in lipid peroxidation and antioxidative enzymes activities suggest the oxidative stress is induced in peanut seedling. High metal content in shell of peanut seedpod demonstrate its ability to absorb metal from solution. This ability of peanut seedpod shell may be exploited to develop biodegradable matrices for selective absorption of toxic metals form liquid waste.