STUDIES ON METAL TOLERANCE IN PLANTS

BY

SUNIL KUMAR

A THESIS

SUBMITTED TO THE **UNIVERSITY OF PUNE**

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN **BIOTECHNOLOGY**

RESEARCH GUIDE
Dr. SULEKHA HAZRA

PLANT TISSUE CULTURE DIVISION
NATIONAL CHEMICAL LABORATORY
PUNE – 411 008, INDIA.

December, 2008

Dedicated to My Parents…

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Acknowledgements

First of all I would like to give the full credit of my thesis to my parents and family. This thesis is the result of five years of work whereby I have been accompanied and supported by many people. It is a pleasant aspect that I have now the opportunity to express my gratitude for all of them.

First and foremost with a deep sense of gratitude and indebtedness, I thank my mentor, ***Dr. Sulekha Hazra*** *for her invaluable guidance, constructive criticism, encouragement and keen interest. My experience with her has inculcated in me qualities that will always remain ingrained in my heart, mind and soul. Her scientific, methodical, focused and positive approach combined with exceptional zeal and dynamism are some of the qualities which I admire most and I often yearn for them. Her constructive criticism and frequent discussions inspired me to go ahead in difficult times and cope up with every little problem during my Ph.D. I learned to believe in my future, my work and myself. Thank you Mam for making me a better person, guiding me and improving my silliest and minutest mistakes.*

I am extremely grateful to the previous Head of PTC Division, National Chemical Laboratory ***Dr. S.K. Rawal,*** *and current Head* ***Dr. B.M. Khan*** *for the support and help extended during my PhD work. I also thank all the faculty members* ***Dr. Subhada Thengane****, Dr.* ***D.K. Kulkarni****, Mr.* ***Dhage, Dr. D.C. Agrawal*** *of division for all their help and valuable suggestions from time to time.*

I take this opportunity to thank Prof. ***R.J.Thengane****, Department of Botany, University of Pune, who directed me to the field of Plant Tissue Culture in NCL.*

This research has been supported by grant from The ***Council of Scientific and Industrial Research (CSIR),*** *New Delhi who awarded me Junior and Senior Research Fellowship for this work. The financial support from CSIR for fellowship and research grant provided in the network program P24-COR0008 and NWP0019, on phytoremediation is gratefully acknowledged. I would like to thank* ***Dr. Sivaram****, Director,* ***National Chemical Laboratory****, for allowing me to submit my work in the form of a thesis.*

I could not have managed at any step without the help and sincere efforts of ***Dr. Urmil J. Mehta,*** *Her friendly attitude and soothing words of solace always helped me to relieve my tensions. Thank you Madam for being there always!!!!*

My life in the lab was smooth because of my colleagues during my PhD tenure. My heart felt thanks for all the help and suggestions rendered by my lab mates in the lab. My research life would not have been enjoyable and exciting without the company of my dear lab mates Sujatha Raman, Shweta Singh, Bhuban Mohan panda, Raju D, Poonam and Suchi. I am very much thankful to all the students of PTC division for their support and cooperation.

Special thanks to my dear friends Arshad Khan, Dilip Depan, Sarvesh Soni, Sampa Sarkar and Kiran Pawar. I shall always cherish the wonderful company of my dear friends, who have always been there

for me thru thick and thin. I truly believe that without your support it would have been impossible to accomplish the goal! I won't forget the moments we have spent together.

The main credit of my thesis goes to my dear parents whose unending love, encouragement and sacrifice has made me achieve this milestone today. To my father who has always encouraged me to fulfill my dreams and to my Mother for her perseverance, encouragement and support. I am also fortunate to have a very caring grandmother. To my dearest brothers Anil, Rahul, Chetan, Deepak, Vishal and my sisters Anita and Kajal. Their cheerful and loving nature made me forget my tensions at all times.

To my uncles Mr. Ramniwas, Rameshwer, Ramsharan and Aunties and family for the blessings that they have showered upon me at every step. I have been blessed with extremely loving and caring father and mother in law. I am grateful for the love and support they have given to me always.

Life would not have been complete without my soulmate "Dr. Sushma Singh" who sparkled my life with her love and affection. She has been extremely understanding, patient and a constant bastion of strength and perseverance. I am extremely lucky to have a companion like her.

Last but not least I thank God Almighty for giving me the strength and courage at every step of life.

SUNIL KUMAR

Date :
Place: Pune

CERTIFICATE OF THE GUIDE

CERTIFIED that the work incorporated in the thesis entitled **"Studies on Metal Tolerance in Plants"** submitted by Mr. Sunil Kumar was carried out by the candidate under my supervision at the Plant Tissue Culture Division, National Chemical Laboratory, Pune, India. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

(Dr. Sulekha Hazra)
Guide

Place: Pune

Date:

DECLARATION BY THE CANDIDATE

I declare that the thesis entitled **"Studies on Metal Tolerance in Plants"** submitted by me for the degree of Doctor of Philosophy is the record of work carried out by me under the guidance of **Dr. Sulekha Hazra** and has not formed the basis for the award of any degree, diploma, associateship, fellowship, titles in this or any other university or other institute of higher learning.

I further declare that the material obtained from other sources has been duly acknowledged in the thesis.

December, 2008

Sunil Kumar

Key to abbreviations

ANOVA	Analysis of variance
APX	Ascorbate peroxidase
AdS	Adenine Sulphate
BA	6-Benzyl amino purine
BSA	Bovine serum albumin
°C	Degree Celsius
CAT	Catalase
Cd	Cadmium
Cu	Copper
Cr	Chromium
d	Day
DPX-4 mountant	[189-(2-chloro-N-(4-methoxy-1,3,5-triazin-2-yl amino carbonyl) benzene sulphanamide)]
EDTA	Ethylenediaminetetraacetic acid
EC	Enzyme Commission
GCS	Glutamine cysteine synthetase
GPX	Guaiacol peroxidase
GSH	Reduced glutathione
GR	Glutathione Reductase
h	Hour
$HgCl_2$	Mercuric Chloride
H_2O_2	Hydrogen peroxide
MS	Murashige and Skoog medium (1962)
MDA	Malondialdehyde
NBT	Nitro blue tetrazolium
$OH^{\cdot}$	Hydroxyl radical

PVP	Polyvinyl pyrrolidone
POX	Peroxidase
ROS	Reactive oxygen species
Sd	Standard deviation
SH	Schenk and Hildebrandt
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reacting substance
TBA	Tertiary butyl alcohol (2-methyl propan-2-ol)
v/v	Volume/volume (concentration)
w/v	Weight/ volume (concentration)
wk	Week
2,4-D	2,4-dichlorophenoxy acetic acid
DW	Dry Weight
FW	Fresh Weight
AAS	Atomic Absorption Spectroscopy

ABSTRACT

Some metals at lower concentration are essential for cells but all metals are toxic at higher concentrations. Heavy metal pollution of soil and water caused by mining, burning of fossils fuels, smelting of metalliferous ores, and agriculture waste, is a major environment problem and exposure to these metals can be toxic to living cells. Use of plants for decontamination/minimization of heavy metal pollutants has attracted attention because of the problems associated with pollutant removal using conventional methods such as soil replacement, solidification, electro-kinetic extraction and washing strategies. Phytoremediation is a cost effective emerging technology based on the use of green plants to clean up the polluted sites and is accepted publicly. Research efforts made towards understanding the mechanism of metal tolerance has generated a great deal of information but it remains ill understood. Thus, there is scope for research to understand the mechanism of metal tolerance by various plant species and to identify the site and form of metal accumulation within plant system. This thesis entitled "**Studies on Metal Tolerance in Plants**" was designed to study the metal stress on different plant species including a herb (Peanut), a shrub (Jojoba) and a tree (Pongamia).

Peanut (*Arachis hypogaea* L.) is a unique leguminous plant for its characteristic behavior to produce the pods underground in direct contact with soil. It has the double advantage for absorption of Cd from soil through roots and directly through the shells. Jojoba (*Simmondsia chinensis*) is an industrial crop – its seed wax is used in the cosmetic industry, as a lubricant, etc. The crop has considerable potential for cultivation in arid and semi-arid regions. *In vitro* nodal segments of jojoba respond to salinity in a similar way as the whole plant, so plant tissue culture technique could be used for preselection and evaluation of metal tolerance in this species.

Trees are ideal for remediation of heavy metals. They can withstand and accumulate higher concentration of pollutants owing to their large biomass and size. These can reach

a huge area and great depths for their extensive rooting. Trees prevent erosion, and the spread of the contaminant, because of their perennial presence. *Pongamia pinnata* (L.) Pierre is a medium sized, fast growing evergreen tree species. Its seed oil is a potential source of raw material in production of biodiesel. This tree can thrive in wide range of agroclimatic conditions and serve as rich source of flavonoids and oil for industrial applications. Pongamia was selected as a model system to study the effect of metal stress.

Objectives of the study:

"**Studies on metal tolerance in plants**" was taken up with the following objectives.

1. To study the influence of chromium, copper and cadmium induced stress on peanut seedlings cultured *in vitro*.
2. To study the influence of chromium, copper and cadmium on shoot cultures of jojoba (shrub).
3. To study effect of chromium, copper and cadmium on Pongamia (tree) seed germination, seedling growth and distribution of metal in different parts of plant.

The thesis is divided into five chapters followed by summary and list of references.

CHAPTER 1: General Introduction

This chapter covers the literature on metal tolerance and toxicity in plants. It includes the role of different antioxidative enzymes including superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (GPX), in stress induced by the metals. The importance and significance of the selected plants like peanut, jojoba, pongamia are emphasized. The significance and objectives of the study is included in this chapter.

CHAPTER 2: Materials and Methods

Materials and methods for tissue culture, metal analysis and biochemical analysis implemented during the course of work are described in this chapter. Methods specific to individual experiments have been dealt with in respective chapters.

CHAPTER 3*:* Effect of metals on *Arachis hypogaea*

This chapter describes the effect of different metals (Cr, Cu and Cd) on germination, seedling growth and metal accumulation in different organs of peanut after 4 weeks. Germination frequency was affected significantly in case of Cr and Cu. Cd was less effective. Seedling growth was severely affected by Cd followed by Cu and Cr. Metal content in different organs and enzymatic activities were assayed after 4 weeks of culture. Differential response was noted in metal accumulation, lipid peroxidation and antioxidative enzymes activities including superoxide dismutase, catalase and guaiacol peroxidase, in different organs of peanut seedling. Copper was more toxic to these antioxidative enzymes followed by Cd and Cr. Histological studies were conducted to study the changes in cellular distribution and morphoanatoamy of root, stem and leaves confirming the adverse effect of these metals.

CHAPTER 4: Effect of metals on *in vitro* shoot cultures of Jojoba

Established shoot cultures of jojoba were used for this experiment. Shoot cultures were exposed to different concentration of Cr, Cu and Cd. Metal accumulation in stem and leaves was determined using Atomic Absorption Spectroscopy. Activity of enzymes including superoxide dismutase, catalase and guaiacol peroxidase and lipid peroxidation product level was estimated after 1 d, 7 d and 14 d of exposure. Differential response in enzyme activity and lipid peroxidation was noted. Cu was more inhibitory for these antioxidative enzymes followed by Cd and Cr. Jojoba was more tolerant towards Cr.

CHAPTER 5: Effect of metals on *Pongamia pinnata*

This chapter describes the effect of different metals (Cr, Cu and Cd) at various concentrations on *Pongamia* seed germination and seedling growth. Parameters including germination frequency, seedling growth and metal accumulation in different organs was tested. Germination frequency was not affected in any of the metals at the concentrations tested. Cd was more inhibitory for seedling growth followed by Cu and Cr. Chromium

used at higher concentration (600-800 μM) was not inhibitory for pongamia seedlings. Cu accumulation in pongamia seedling was optimum in seed coat followed by leaves, root, cotyledons and stem. In Cd and Cr seed coat accumulated optimum amount of metal followed by root, leaves, cotyledons and stem. Seed coat accumulated highest amount of metal as compared to other organs.

SUMMARY

The main findings of this research work conducted on different plant species are summarized in this section.

CHAPTER I

Introduction and Review of Literature

INTRODUCTION

Effect of metals on plants

About three quarters of the elements in the periodic table are metals. Nevertheless only about eight metals are essential for plant life. The other metals are non-essential or even toxic to plants. Year long research uncovered increasingly ingenious systems by which plants endeavor efficient handling of these different metals. These systems include tactics such as selective uptake, metal sequestration, detoxification and a range of protection mechanisms to obtain a well-balanced metal concentration in plants. Although chief pieces in this puzzle of metal management by plant were already known, the complexity of the research remains a challenge for plant biologist.

Tolerance to heavy metal in plants may be defined as **the ability to survive in a soil that is toxic to other plants and is manifested by an interaction between a genotype and its environment** (McNair et al., 2000). The term tolerance is however, more widely used in the literature to include changes that may occur experimentally in the sensitive response to heavy metals.

The toxicity of metals is determined by their physical and chemical properties and bioavailability of the elements (Duffus, 2002). Some heavy metals, such as Cu, Zn, Mn, Mo are essential nutrients for plant, necessary for healthy growth and development. They act as catalytic or structural components of proteins. Higher concentrations of these micronutrients or small amount of non-essential elements such as Cd, Cr, Pb, Sn, and Hg can be toxic for plant life.

Changes in the environment force plants to adapt to surrounding conditions (Etherington, 1988). Industrialization has made the environment even more complex for plant survival by adding numerous pollutants into the atmosphere (Oleksyn and Innes, 2000) and soil (Fernandez and Henriques, 1991). Mining of metals, phosphate containing fertilizer, soil enrichment by silt and human industry, have created piles of metal enriched waste (Reichman, 2002; Juwarkar et al., 2007). These metals can infiltrate in the soil, be carried by rain or be dissipated by the wind, resulting in metal contaminated sites.

Excessive amount of heavy metals adversely affect plant growth and development. Presence of elevated levels of heavy metal ions trigger wide range of cellular responses including changes in gene expression, synthesis of metal detoxifying peptides, lipid peroxidation, inhibition of root elongation, induction of callose synthesis, disturbance of calcium homeostasis, the functioning of calcium channels and the membrane potential. The availability of heavy metals to plants and thus their toxicity depends upon complex rhizospheric reactions, involving not only exchange process between soil and plants but also microbial activities. Uptake of metals can be either passive, driven by a concentration gradient across the membrane or substrate specific or energy dependent (Williams et al., 2000).

Chromium

Chromium (Cr) is the 24th element of the Periodic table. It exists in a series of oxidation states with a valency -2 to +6; the most important stable states are 0 (elemental metal), +3 (trivalent) and +6 hexavalent. Chromium was first discovered in the Siberian red lead ore (crocoite) in 1798 by the French chemist Vauquelin. In contrast to other toxic trace metals like cadmium, lead, mercury and aluminum, Cr has received little attention from plant scientists. Its complex electronic chemistry has been a major hurdle in unraveling its toxicity mechanism in plants (Shanker et al., 2005a). The impact of Cr contamination in the physiology of plants depends on the metal speciation, which is responsible for its mobilization, subsequent uptake and resultant toxicity in the plant system. Chromium and its compounds have multifarious industrial uses. They are extensively employed in leather processing and finishing (Nriagu, 1988), in the production of refractory steel, drilling muds, electroplating cleaning agents, catalytic manufacture and in the production of chromic acid and speciality chemicals. Hexavalent Cr compounds are used in industry for metal plating, cooling tower water treatment, hide tanning and, until recently, wood preservation. These anthropogenic activities have led to the widespread contamination that Cr shows in the environment and have increased its bioavailability and biomobility. The leather industry is the major cause for the high influx of Cr to the biosphere, accounting for 40% of the total industrial use (Barnhart, 1997).

Chromium is a toxic, nonessential element to plants; hence, they do not possess specific mechanisms for its uptake. Therefore, the uptake of this heavy metal is through carriers used for the uptake of essential metals for plant metabolism. The toxic effects of Cr are primarily dependent on the metal speciation, which determines its uptake, translocation and accumulation. The pathway of Cr (VI) transport is an active mechanism involving carriers of essential anions such as sulfate (Cervantes et al., 2001). Fe, S and P are known also to compete with Cr for carrier binding (Wallace et al., 1976).

Uptake of excess Cr can lead to deleterious effect on plant growth. Cr can cause reduced germination (Peralta et al., 2001; Shanker, 2003) inhibit root growth, reduce leaf number, leaf area, biomass (Shanker, 2003; Juwarkar et al., 2008), reduce shoot growth (Mei et al., 2002; Kumar et al., 2008), cause alteration in uptake of N, P, K, Zn, Cu, B (Khan et al., 2000). Chromium induced metabolic disturbance such as inhibition of photosynthesis, inhibition of electron transfer chain (Shanker et al., 2003), disturbance in water relations (Davies et al., 2002) has been reported. Increased production of metabolites (e.g., glutathione, ascorbic acid) as a direct response to Cr stress has been noted (Shanker, 2003). Alteration in ROS (Reactive Oxygen Species) scavenging enzyme activities, increased lipid peroxidation due to excess Cr has been observed in plants (Shanker et al., 2005a).

Copper

Copper is the 29th element of the Periodic table. The most stable oxidation state is +2 oxidation state. Copper (Cu) is an essential redox-active transition metal for plants. The average content of Cu in plant tissue is 10 μg g^{-1} dry weight (Baker and Senef, 1995). It is an important component of several enzymes, many of which are involved in electron transfer chain in mitochondria and chloroplast e.g. plastocyanin and cytochrome oxidase. The 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. Within the plant cell, Cu is required in at least six locations: the cytosol, the endoplasmic reticulum, the mitochondrial inner membrane, the chloroplast stroma, the thylakoid lumen and the apoplast. The chelating capacity of the cytosol is so high that effectively no free Cu

ions are present in the cell (Finney and O'Halloran, 2003). Toxic levels of Cu occur naturally in some soils whereas others may contain high levels of Cu as a result of the anthropogenic release of heavy metals into the environment through mining, smelting, manufacturing, agriculture and waste disposal technologies.

Excess Cu concentrations are toxic to plant cells. Thus its uptake and turnover has to be tightly regulated (Yruela, 2005). Most likely Cu enters the cytosol via specific transporters of copper transporter protein family (COPT) (Sancenon et al., 2003). The COPT transporters belong to the evolutionary conserved Cu transporter family (CRT), characterized by three transmembrane domains and a high methionine content (Pilon et al., 2006). The latter is thought to play a role in Cu translocation; however the mechanism of CRT action has to be unraveled.

Excess Cu can cause disorders in plant growth and development by adversely affecting important physiological processes in plants. Plants grown in the presence of high levels of Cu normally show reduced biomass and chlorosis symptoms (Simova-Stoilova et al., 2002). A lower content of chlorophyll and alterations of chloroplast structure and thylakoid membrane composition was found in leaves under such growth conditions (Ciscato et al., 1997; Quartacci et al., 2000). Cu toxicity is related to the binding of Cu to sulfhydryl groups of the plasma membrane (Yruela, 2005). In the cell environment Cu can exist in two oxidation states, Cu^{2+} and Cu^{+}. Thus Cu is a redox active metal and while it binds to proteins, it is used in essential electron transfer reactions. It is well known that transition metals like Cu catalyze the formation of hydroxyl radicals ($OH^{\bullet}$) from the non-enzymatic chemical reaction between superoxide ($O_2^{\bullet -}$) and H_2O_2 (Haber-Weiss reaction). Hence, the presence of excess Cu can cause oxidative stress in plants and subsequently increase the antioxidant responses due to increased production of highly toxic oxygen free radicals. Accordingly, it was observed that excess Cu in plants led to oxidative stress inducing changes in the enzyme activity and content of some components of the antioxidative pathways i.e., ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), superoxide dismutases (SODs), guiacol peroxidase (De Vos et al., 1992; Gupta et al., 1999; Wang et al., 2004).

Cadmium

Cadmium is the 48th element and a member of group 12 in the Periodic table of elements. The most common oxidation number of cadmium (Cd) is +2. Cadmium is a non-essential element that negatively affects plant growth and development. It is released into the environment by power stations, heating systems, metal-working industries or urban traffic. It is widely used in electroplating, pigments, plastic stabilizers and nickel-cadmium batteries (Sanitá di Toppi and Gabrielli, 1999). It is recognized as a significant pollutant due to its high toxicity and large solubility in water (Pinto et al., 2004).

The degree to which higher plants are able to take up Cd depends on its concentration in the soil and its bioavailability, modulated by the presence of organic matter, pH, redox potential, temperature and concentrations of other elements. In particular, the uptake of Cd ions seems to be in competition for the same transmembrane carrier with nutrients, such as K, Ca, Mg, Fe, Mn, Cu, Zn and Ni (Benavides et al., 2005). In some plant species, it can decrease up to 50% in dry matter production (Almeida et al., 2007). It is easily absorbed and translocated to different plant parts (Almeida et al., 2007) and trigger a sequence of reactions leading to: inhibition (Schützendübel and Polle, 2002a) or growth reduction of the aerial part and the root system (Mendelssohn et al., 2001; Kuriakose and Prasad, 2008); induction of phytochelatin production (Cobbett and Goldsbrough, 2002); interference in chlorophyll biosynthesis and activity of enzymes, such as peroxidase, ascorbate peroxidase, catalase, glutathione synthetase, glutathione reductase, dehydroascorbate reductase, superoxide dismutase, guaiacol peroxidase, monodehydro ascorbate reductase (Vassilev et al., 2002); induction of apoptotic bodies and oligonucleosomal DNA fragments (Almeida et al., 2007); induction of oxidative stress (Almeida et al., 2007); stimulation of secondary metabolism, lignification and, finally, cellular death (Schützendübel and Polle, 2002b).

Plant stress

Defining stress in plants is complicated, since no fixed stress points can be set. Elstner and Oßwald (1994) distinguished seven classes that can cause stress to plants: light, radiation, temperature, hydrations, chemical factors such as salts, heavy metals,

pH, air pollutants, mechanical factors such as wind, fire, cutting, pressure and biological influences such as insects, infections, competition. Within these classes the stress factors can be separated into biotic and abiotic stress factors. In a broad sense, **plant stress can be defined as "any unfavorable condition or substance that affects or blocks a plant's metabolism, growth or development"** (Lichtenthaler, 1996).

Metal toxicity, homeostasis and detoxification

Toxicity comprises inactivation of biomolecules by either blocking essential functional groups or by displacement of essential metal ions (Goyer, 1997). In addition auto oxidation of redox active metals and production of ROS by Fenton reaction causes cellular injury. Other aspects of metal toxicity are probably related to increased production of ROS by enzymatic mechanism (Reichman, 2002). Various systems assist to the regulation of metal homeostasis and tolerance in plants: metals can be chelated and sequestered to the vacuole by metal binding peptides or can be chelated by organic acid and amino acids. Furthermore, metal responsive changes in various membrane transport system (Hall, 2002; Hall and Williams, 2003) and in gene expression pattern has been reported (Jonak et al., 2004). Potential mechanism of metal detoxification and tolerance in higher plant has been given in Fig. 1.1 as suggested by Hall (2002).

At present neither the different toxicity behavior with respect to different metals, nor the mechanisms leading to the plant defense response have been completely elucidated. There is still discussion in the literature over the possible mechanisms of metal toxicity and metal tolerance. This probably reflects the general lack of understanding of metal toxicity and also the complex nature of plant response to metal toxicity.

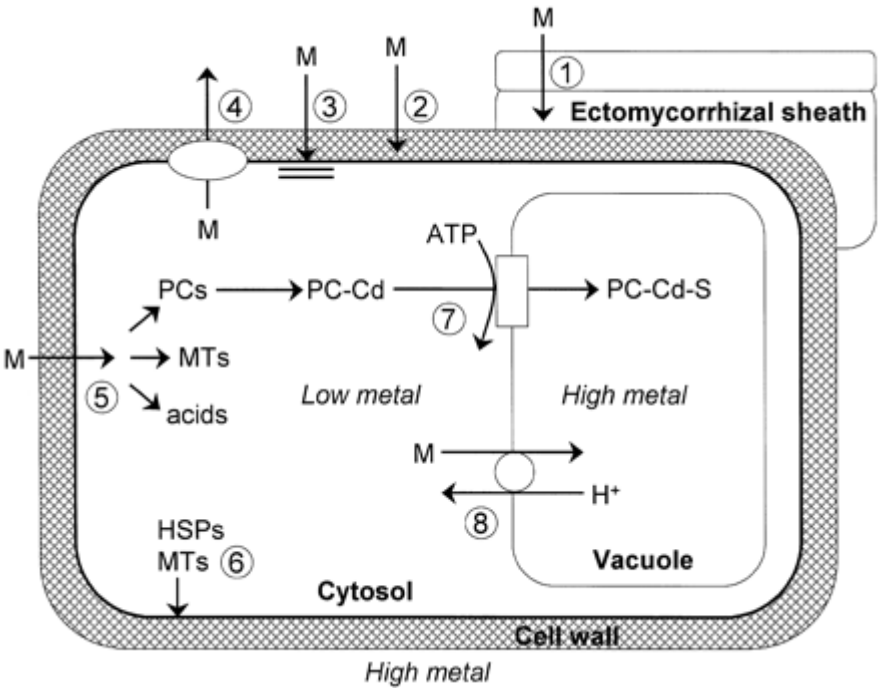

Fig 1.1 Summary of potential cellular mechanisms available for metal detoxification and tolerance in higher plants. 1. Restriction of metal movement to roots by mycorrhizas. 2. Binding to cell wall and root exudates. 3. Reduced influx across plasma membrane. 4. Active efflux into apoplast. 5. Chelation in cytosol by various ligands. 6. Repair and protection of plasma membrane under stress conditions. 7. Transport of PC-Cd complex into the vacuole. 8. Transport and accumulation of metals in vacuole (Hall, 2002).

Phytochelatins (PC) and Metallothioneins (MC)

The PCs and MTs play important role in metal chelation and sequestration. These are different classes of cysteine rich, heavy metal binding proteins. Metallothioneins are small gene encoded polypeptides, whereas phytochelatins are enzymatically synthesized.

Phytochelatins

Phytochelatins (PC) are among the best characterized metal binding complexes. The term covers a whole family of cysteine rich metal binding peptides with a general structure of repetitions of the dipeptide γ-glutamyl -cysteinyl followed by a terminal glycine. It is catalyzed by PC synthase (PCS), a constitutive enzyme requiring post-translational activation by heavy metals and/or metalloids, in particular Cd, Ag, Pb, Cu, Hg, Zn, Sn, As and Au both *in vivo* and *in vitro* (Chen et al., 1997; Wojcik and Tukiendorf, 2005). This suggests the role for PC in metal tolerance in plants. Based on the data of Vatamaniuk et al. (2000) a model of how an enzyme might be activated by such a wide range of metals was proposed. In short, after entering the cell Cd will be bound to glutathione to from a glutathione thiolate. This glutathione thiolate can directly act as substrate for PC biosynthesis. PC synthesis would simply be terminated as a result of exhaustion of substrate, thus leading to exhaustion of the Cd-glutathione complex. *In vitro* experiments have shown that a series of metal-sensitive plant enzymes can tolerate a 10- to 1000-fold concentration of Cd in the form of a PC complex than as free radical ion (Kneer and Zenk, 1992). Over expression of the PCS gene *AtPCS1* in *Arabidopsis thaliana* did not enhance the tolerance to Cd, on the contrary hypersensitivity for Cd and Zn, but not for Cu, was observed (Lee et al., 2003). Taken together the role of PCs in metal tolerance seems not yet completely elucidated and thus the detoxifying effect of PCs may actually be a part of complex mechanism.

Metallothioneins

Metallothioneins (MTs) are ubiquitous low molecular mass cysteine (cys)-rich proteins, that bind metal ions in metal-thiolate clusters identified in mature embryos of wheat plants as early cys-labeled protein (Hamer, 1986). Plant MTs are subdivided in four categories, based on the arrangement of cysteine residues. It is thought that the differences in structure and number of central amino acids dictates which metal the MT gives tolerance to or detoxifies (Garcia-Hernandez et al., 1998). It has been shown that MTs are expressed ubiquitously and conserved in plants, pointing to an important role in the plant metal interaction. Plants overexpressing the yeast MT genes show an improved heavy metal tolerance (Hasegawa et al., 1997). On the other

hand, plant MT genes increase the heavy metal tolerance of MTs deficient yeast (Thomas et al., 2003).

Organic and Amino Acids

Organic acids and amino acids are suggestive potential ligands for chelation, owing to the capacity of metal ions to react with S, N and O. Citrate, malate, and oxalate have been implicated in a range of processes, including differential metal tolerance, metal transport through xylem and vacuolar metal sequestration (Shah and Nongkynrih, 2007). Citric acid has been hypothesized to be a major Cd^{2+} ligand at low Cd^{2+} concentrations (Wagner, 1993). This acid has been shown to form complexes with Ni^{2+} in Ni-accumulating plants (Sagner et al., 1998) and contribute to Zn accumulation and tolerance (Goldbold et al., 1984). Oven et al. (2002) reported an increase in the level of citric acid upon exposure to cobalt ions. Similarly malate is proposed as a cytosolic Zn chelator in Zn tolerant plants (Shah and Nongkynrih, 2007). Salicylic acid is also reported to have a role in maintenance of ionic homeostasis in *Medicago sativa* seedlings treated with Cd or Fe (Dražio et al., 2006). The role of histidine (His) in metal chelation was also studied and the His content of the xylem sap on exposure to Ni in Ni-hyperaccumulator *Alyssum lesbiacum* was reported to increase 36-fold (Kramer et al., 1996). Supplying His to a non-accumulating species greatly increased both its Ni tolerance and the capacity to transport Ni to the shoot. However, the His response seem to be a species specific mechanism of Ni tolerance as it was not observed in another Ni-hyperaccumulator, *Thlaspi geosingenses* (Persans et al., 1999). Recently, Nakazawa and coworkers (2004) selected and characterized nickel-tolerant cells from tobacco and suggested the correlation of Ni tolerance to the concentration of His in these cells.

Reactive oxygen species (ROS)

In many stress situations the plant reacts to the perceived stress by an elevated production of ROS (Dat et al., 2000). The production and role as central signaling component of these ROS has been studied intensively in plants under biotic stress conditions (Wojtasjek, 1997; Able et al., 2000; Able et al., 2003). Elevated ROS levels were demonstrated after ultraviolet B irradiation (Makerness et al., 2001), cold

stress (Prasad et al., 1994), elevated level of excitation energy (Karpinski et al., 1999), wounding (Orozco-Cardenas and Ryan, 1999) and Ozone (Schraudner et al., 1998).

An optimal supply of CO_2 determines the availability of NADP to leaves via the Calvin cycle for the electron transport chain. NADP receives the electrons from ferredoxin (via ferredoxin-NADP reductase) in the electron transport chain during photosynthetic light reaction and this results in generation of molecular oxygen. Abiotic stress factors that inhibit Calvin cycle function (i.e. CO_2 fixation and NADPH consumption) aggravate the situation. For instance, salt-stress induced stomatal closure limits CO_2 availability, while at the same time light-driven electron transport proceeds at high rates. Light absorption by leaves, then exceeds the demand for photosynthesis and the excess excitation energy leads to an overreduction of the electron transport chain. In molecular terms, an imbalance between the consumption of reductant (NADPH), in assimilation (during carbon fixation), and the need of the electron transport chain for the regenerated electron acceptor at the photosystem I (P1) site (NADP) can lead to the transfer of electrons to alternative acceptors (Foyer and Noctor, 2000; Tausz et al., 2004).

The formation of ROS is thus, initiated by the univalent reduction of O_2, or by the transfer of excess excitation energy to O_2. The transfer of electrons—one, two or three electrons—leads to the generation of superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) or a hydroxyl radical ($HO^{\bullet}$) respectively (Mittler, 2002). Reduction of molecular O_2 proceeds through different steps, thus generating several O_2 radical species (Hippeli et al., 1999). The reaction chain requires initiation at the first step whereas subsequent steps are exothermic and can occur spontaneously, either catalyzed or not.

$$O_2 \longrightarrow (H)O_2^{\bullet -} \longrightarrow H_2O_2 \longrightarrow OH^{\bullet} + H_2O \longrightarrow 2\,H_2O$$

$$O_2^{\bullet -} + Fe^{3+} \longrightarrow Fe^{2+} + O_2$$

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^{-} + OH^{\bullet}$$

$$\text{Overall:} \quad H_2O_2 + O^{\bullet -}_2 \longrightarrow OH^{-} + OH^{\bullet} + O_2$$

The last species generated by this series of reductions is the hydroxyl radical ($OH^{\cdot}$). It has a very strong potential and a half-life of less than 1 ms (Dat et al., 2000). As a result, it has a very high affinity for biological molecules at its site of production, reacting at almost diffusion-controlled rates ($K > 10^9\ M^{-1}\ s^{-1}$). Under optimal growth conditions the production of ROS in cells is estimated at a constant rate of 240 µM $O_2^{\cdot -}$ and a steady state level of 0.5 µM H_2O_2. However, under stress conditions ROS formation can accelerate to 720 µM $O_2^{\cdot -}$ and steady state level of 5-15 µM H_2O_2 have been measured (Polle, 2001).

Despite the fact that for many years, ROS have been considered to be damaging molecule, it is now recognized that ROS especially H_2O_2 have a role as indicators of oxidative stress and in cellular signaling pathways. This topic has been reviewed (Dat et al., 2000; Foyer and Noctor, 2005; Pitzschke and Hirt, 2006; del Rio et al., 2006) by several authors.

Lipid peroxidation

Lipid hydroperoxides are non-radical intermediates derived from unsaturated fatty acids, phospholipids, glycolipids, cholesterol esters and cholesterol itself. Their formation occurs in enzymatic or non-enzymatic reactions involving ROS which are responsible for toxic effects in the body via various tissue damages. These ROS include among others hydroxyl radicals, lipid oxyl or peroxyl radicals, singlet oxygen, and peroxinitrite formed from nitrogen oxide (NO), all these groups of atoms behave as a unit and are now named "free radical". These chemical forms are defined as, **species capable of independent existence that contains one or more unpaired electrons (those which occupy an atomic or molecular orbital by themselves)**. They are formed either by the loss of a single electron from a non-radical or by the gain of a single electron by a non-radical. They can easily be formed when a covalent bond is broken if one electron from each of the shared pair remains with each atom, this mechanism is known as **homolytic fission**. In water, this process generates the most reactive species, hydroxyl radical $OH^{\cdot}$. Chemists know well that combustion that at high temperature is able to rupture C-C, C-H or C-O bonds is a free-radical process. The opposite of this mechanism is the **heterolytic fission** in which, after a covalent

break, one atom receives both electrons (this gives a negative charge) while the other remains with a positive charge. Abiotic stresses including heavy metals result in molecular damage to plant cells either directly or indirectly (Cuypers et al., 2002; Zhang et al., 2005). Protonation of radical of O_2 can produce the hydroperoxyl radical (HO_2^-, H_2O_2), which can convert fatty acids to toxic lipid peroxides, destroying biological membranes (Zhang et al., 2005). Malondaldehyde (MDA) formation is used as the general indicator of the extent of lipid peroxidation resulting from oxidative stress.

An efficient system to regulate ROS

Superoxide dismutase (SOD **EC 1.15.1.1**) is an important antioxidant enzyme and is the first line of defence against oxidative stress in plants. SOD causes dismutation of superoxide radicals at almost diffusion-limited rates to produce H_2O_2 (Salin, 1987). SODs are categorized into three main groups on the basis of the metal cofactor (Jithesh et al., 2006): (i) Cu/Zn SOD: These enzymes have copper and zinc as their cofactors and are localized in plants mainly in the cytosol and chloroplasts. (ii) Mn SOD: Mn SOD possesses manganese as its cofactor and is localized in mitochondria and also in the peroxisomes. (iii) Fe SOD: Fe SODs are found predominantly in chloroplasts in plants and are absent in animals (Alscher et al., 2002).

Catalase (CAT EC 1.11.1.6) is haem-containing tetrameric enzymes involved in the removal of H_2O_2. Plant catalase are involved in photorespiratory functions (Jithesh et al., 2006), scavenging of H_2O_2 during β-oxidation of fatty acids in germinating seeds (Jithesh et al., 2006) and also during other abiotic stress conditions (Willekens et al., 1997). Catalases are present in peroxisomes, glyoxysomes, and related organelles where H_2O_2 generating enzymes, such as glycolate oxidase, are found. There are three main isoforms: CAT1, CAT2, and CAT3 (Dat et al., 2000). CAT1 is peroxisomal and involved in scavenging photorespiratory H_2O_2; CAT2 is preferentially expressed in the vascular tissue, while CAT3 has a role in glyoxysomal processes (Willekens et al., 1997). Although they are involved in H_2O_2 scavenging, their turnover being

continuous, their steady-state level can be rapidly lowered under any stress conditions in which translation is inhibited or degradation enhanced (Dat et al., 2000).

Peroxidases scavenge H_2O_2 by reducing it to H_2O in the presence of a reducing compound. The reducing substrates render the difference between ascorbate peroxidase (APX) and glutathione peroxidase. Peroxidases have a higher affinity to H_2O_2 than CAT and they are found throughout cell, e.g. in the chloroplast, mitochondria and the cytoplasm. Peroxidase can be regulated either at mRNA, protein or at enzyme activity level under numerous stresses. One of these peroxidases, guaiacol peroxidase (**GPX EC 1.11.1.7**), utilizes aromatic electron donors such as guaiacol and pyrogallol as substrate but only oxidizes ascorbate at a rate of approximately 1% that of guaiacol (Gratao et al., 2008).

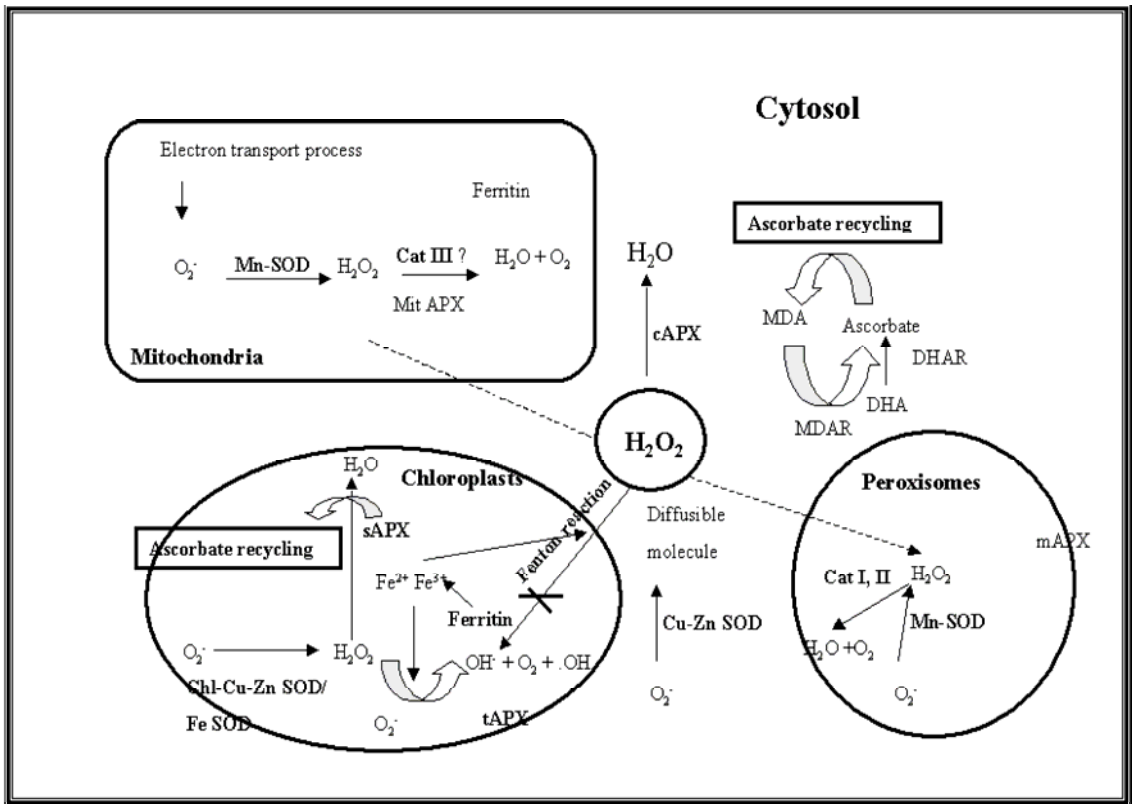

Fig 1.2 Antioxidant network in different organelles of plant cell (Jithesh et al., 2006).

Variations in activities of SOD, GR (glutathione reductase), APOX (Ascorbate peroxidase), POD and CAT was noted with Cd concentration and plant species

Benavides et al. (2005). In *Helianthus annuus* leaves, Cd decreased GSH content, and decreased or increased the activity of the antioxidative enzymes SOD, CAT, APOX, and GR depending on Cd concentration, the organ used and the age of the plants. In *Phaseolus vulgaris* roots and leaves, 5 mM Cd enhanced activities of the peroxidases GPOX and APOX, and raised lipid peroxidation (Chaoui et al., 1997). In two species of *Alyssum*, GR activity increased at 0.02 mM Cd but decreased at 0.05 mM Cd (Schickler and Caspi, 1999). Cadmium produces oxidative stress by generating free radicals and reactive oxygen species (ROS). These species react with lipids, proteins, pigments, nucleic acids, and cause lipid peroxidation and membrane damage thereby affecting cell viability (Gratao et al., 2005). It has been confirmed in many studies that when Cu is in excess, it promotes and stimulates generation of Fenton-type reactive oxygen species leading to increase in antioxidant enzyme activities as a defense system (Weckx and Clijesters, 1996; Rama Devi and Prasad, 1998; Ducic and Polle, 2005). This response to excess Cu vary among plant species and among different tissue (Lombardi and Sebastiani, 2005; Martins, 2006). Antioxidative enzymes affected due to Cr, Cd or Cu stress has been listed in Tables (1.1, 1.2 and 1.3).

Table 1.1 Antioxidant enzymes modified in different plant species exposed to variable chromium concentration

Chromium conc.	**Exposure time**	**Plant species**	**Antioxidant enzymes modified**	**References**
1- 1000 µM	24 d	*Triticum aestivum*	CAT	Sharma et al., 1995
500 µM	56 d	*Brasssica oleracea*	CAT,GPX	Chatterjee and Chatterjee, 2000
20,200 µM	7 d	*Pisum sativum*	SOD,NADH dehydrogenase	Dixit et al., 2002
24,48,96,192 µM	4 d	*Vigna radiata*	CAT,GPX,SOD	Samantary, 2002
0,0.1,1,10,100 mM	8 d	*Triticum aestivum*	SOD,CAT,GPX	Panda et al., 2003
10,20,500,100 µM	6,24,48,72 h	*Oscimum tenuiflorum*	SOD,CAT,GPX,APX	Rai et al., 2004
50, 100 µM	10 d	*Sorghum bicolor*	CAT,APX, GR,SOD	Shanker and Pathmanabhan, 2004
50 µM	2-120 h	*Vigna radiate*	CAT,SOD,APX,GR	Shanker et al., 2004
0.2,2, 20 µM	1-15 d	*Brassica juncea*	CAT,APX, GR,SOD	Pandey et al., 2005
10,40,80,160 µM	48, 96, 144 h	*Pistia stratiotes*	CAT,APX,SOD	Sinha et al., 2005
0,10,100,1000 (µM)	24,48 h	*Polytrichum commune*	CAP,GPX,SOD,GR	Chaudhari and Panda, 2005
2 mg/L	20,40,60, 80,100 h	*Convolvulus arvensis*	CAT	Montes Holguin et al., 2006
0,50,100 µM	24/48 h	*Rice*	SOD,GPX,GR	Panda, 2007
1- 12.5 mg/L	192 h	*Hybrid willows*	CAT,GPX,SOD	Yu et al., 2007
0,5,10,25,50, 100,200 mg/lit	7 d	*Glycine max*	CAT,POX,	Ganesh et al., 2008
0.6 µM-50 mM	14 d	*Fontinalis antipyretica*	SOD,CAT,GPX,APX,GR,	Dazy et al., 2008
1,10,100 µM	20 d	*Amaranthus viridis*	CAT,GPX,SOD	Liu et al., 2008

Table 1.2 Antioxidant enzymes modified in different plant species exposed to variable copper concentration

Copper conc.	**Exposure time**	**Plant species**	**Antioxidant enzymes modified**	**References**
0-100 µM	24 h	*Avena sativa*	CAT,GPX,SOD	Luna et al., 1994
630 µM	20-220 h	*Phaseolus vulgaris*	CAT,APX,SOD	Weckx and Clijsters 1996
50 µM	7 d	*Lycopersicon esculentum*	CAT,APX,GPX	Mazhoudi et al., 1997
2, 4 µM	24 h	*Ceratophyllum demersum*	CAT,APX,SOD	Rama Devi and Prasad, 1998
0.25-10 µM	0-96 h	*Lemna minor*	CAT,GPX,GR,GST	Teisseire and Guy, 2000
50 µM	11 d	*Triticum durum*	NADPH oxidase, Lipoxygenase	Quartacci et al., 2001
50,100,200 µM	3 d	*Scenedesmus bijugatus*	GSH-POD, GST, GCS	Nagalakshmi and Prasad, 2001
0.5 – 25 µM	7 d	*Holcus lanatus*	SOD	Hartley et al., 2001
500-10000 mg/Kg	60 d	*Salix acmophylla*	GPX,SOD	Ali et al., 2003
0-300 µM	7 d	*Arabidopsis thaliana*	CAT,GPX,SOD	Drazkiewicz et al., 2004
10 µM	2-50 h	*Phaeodactylum tricornutum*	CAT,SOD, GR	Morelli and Scarano, 2004
15,150,1500 µM	5 d	*Hordeum vulgare*	CAT,APX,GPX,SOD	Demirevska-Kepova et al., 2004
10,50,100 µM	10,20 d	*Prunus cerasifera*	CAT,SOD	Lombardi and Sebastiani, 2005
20,100 µM	4 d	*Pisum sativum*	CAT,APX,GPX,SOD	Chaoui and Ferjani, 2005
0.1,1,100 µM	25,50 d	*Morus alba*	CAT,APX,GPX,GR	Tewari et al., 2006
100 µM	1,5,15 h	*Arabidopsis thaliana*	CAT,APX,SOD	Maksymiec and Krupa, 2006
25,50,100, 500 µM	12 d	*Elsholtzia splendens*	CAT,APX,GPX,SOD	Peng et al., 2006
5,10,25,50 µM	20,40 d	*Panax ginseng*	CAT,APX,GPX,GR,SOD	Ali et al., 2006
5, 50 µM	7 d	*Arabidopsis thaliana*	SOD	Drążkiewicz et al., 2007
10,100 µM	35 d	*Hordeum vulgare*	GPX,SOD	Guoa et al., 2007
10,25,50,100, 200 µM	30 d	*Withania somnifera*	CAT,GPX,GR,SOD	Khatun et al., 2008
25 µM	20,40,60, 80,100 h	*Solanum lycopersicon*	CAT,GPX,APX,GR	Chamseddine et al., 2008
3,60,120 µM	1,3,7 d	*Matricaria chamomilla*	CAT,GPX,GR	Kováčik and Bačkor, 2008
0.32,10,100, 300 µM	6 d	*Elsholtzia haichowensis*	APX,GPX,SOD	Zhang et al., 2008

Table 1.3 Antioxidant enzymes modified in different plant species exposed to variable cadmium concentration

Cadmium conc.	**Exposure time**	**Plant species**	**Antioxidant enzymes modified**	**References**
500 µM	12 h	*Healianthus anus*	CAT,SOD,APX,GR,DHAR	Gallego et al., 1996
4 , 40 µM	7 d	*Pisum sativum*	CAT,SOD,APX,GPX	Dixit et al., 2001
5 , 50 µM	48 h	*Glycine max*	CAT,SOD,APOX	Balestrasse et al., 2001
100, 500 µM	20 d	*Oryza sativa*	CAT,SOD,GPX	Shah et al., 2001
2000,5000 µM	0-96 h	*Saccharum officinarum*	CAT,SOD,GR	Fornazier et al., 2002
50 µM	21 d	*Phragmites australis*	CAT,SOD,APX,GR	Iannelli et al., 2002
5, 50 µM	48 h	*Populus canescens*	CAT,SOD,AP,GR,MDAR	Schutzendubel and Polle, 2002a
5 µM	10 d	*Pisum sativum*	CAT,APX,GPX	Metwally et al., 2003
1, 10 µM	10 d	*Triticum durum*	CAT,SOD,APX,GPX	Milone et al., 2003
300, 500 µM	21 d	*Arabidopsis thaliana*	CAT,SOD,APX,GPX,GR	Cho and Seo, 2004
100, 500 µM	20 d	*Oryza sativa*	CAT,SOD,APX,GPX,GR	Hsu and Kao, 2004
2 µM	0-72 h	*Phaseolus vulgaris*	GPX,APX,GR	Smeets et al., 2005
1,2,5,7,10 mM	6,12,36,72,192 h	*Allium sativum*	CAT,SOD, GPX	Zhang et al., 2005
1,5,10,25,50, 100 µM	1,2,4,7 d	*Bacopa monnieri*	CAT, SOD,APX,GPX,GR	Mishra et al., 2006
0.1,1,5,10 µM	7,14,21,33 d	*Lycopersicon esculentum*	SOD, GPX	Dong et al., 2006
50 µM	14 d	*Pisum sativum*	SOD,CAT	Romero-Puertas et al., 2007
5,16,37 µM	10 d	*Bechmeria nivea*	APX,GR	Liu et al., 2007
1, 10 µM	7 d	*Arabidopsis thaliana*	CAT,APX,SOD	Semane et al., 2007
27,54 µM	4 d	*Vicia faba*	CAT,GPX,SOD	Lin et al., 2007
25,50,100 µM	10,15, 20,25 d	*Arachis hypogaea*	CAT,GPX,SOD, GR,APX	Dinakar et al., 2008
50,100,200 300 µM	14,28 d	*Arachis hypogaea*	CAT,GPX	Sunil Kumar et al., 2008
100, 200 µM	72 h	*Nicotiana tabacum*	CAT,GPX,GR,GST	Gratao et al., 2008
25 µM	20,40,60,80,10 0 h	*Solanum lycopersicon*	CAT,GPX,APX,GR	Chamseddine et al., 2008
3,60,120 µM	1,3,7 d	*Matricaria chamomilla*	CAT, GPX, GR	Kováčik and Bačkor, 2008

Plant tissue culture

Plant tissue culture is a technique, often used for rapid production of many genetically identical plants using relatively small amounts of space, supplies and time. Plant tissue culture relies on the the ability of the plant cells to regenerate whole plant (totipotency). It is the science and art of growing isolated plant cells, tissues, organs or whole plants on semisolid or in liquid synthetic nutrient media under controlled environment. Plant tissue culture techniques have vast potential for various applications.

Plant tissue culture technique can be used in study of metal tolerance of a plant by exposing it in culture media containing known quantities of the specific metal. Plants identified using this method can then be tested for phytoremediation of polluted land degraded due to metal toxicity. This technique also offers the potential to study the effect of metal on cells, callus, isolated organs and whole plants. *In vitro* systems have been used for studying metal induced oxidative stress (Batková et al., 2008). In strawberry culture, addition of silver nitrate inhibited ethylene production and increased contents of chlorophyll and soluble protein and activities of SOD, POX and CAT (Qin et al., 2005). The addition of calcium was found important for induction of somatic embryogenesis in *Eucalyptus urophylla* connected with increased contents of proteins and sugars and POX activity (Arruda et al., 2000). In peach rootstock culture, iron deficiency caused a reduction of chlorophyll and carotenoid content and CAT and SOD activities (Lombardi et al., 2003). On the contrary, *Prunus cerasifera* grown *in vitro* tolerated Cu up to 50 μM concentration. Its ability to tolerate this rather high Cu concentration was partially due to induction of SOD and CAT gene expression and in consequence increased SOD and CAT activities (Lombardi and Sebastiani, 2005).

A rapid increase in CAT activity was detected (14-fold higher after 15 d) in *Saccharum officinarum* calli grown under 0.5 or 1 mM $CdCl_2$ while SOD activity did not exhibit any major variation (Fornazier et al., 2002). In sunflower calli, low concentration (5 μM) of Cd increased CAT and POX activities, but higher concentrations (50 and 500 μM) decreased the activities of both enzymes. However, in calli which were able to survive at 50 μM Cd for 6 months, CAT and POX activities were higher than in control calli (Azevedo et

al., 2005). In coffee cell suspension cultures, 0.5 mM Cd induced lipid peroxidation, increased activities of CAT, GR and SOD while decreased APX activity (Gomes et al., 2006).

In cell cultures of *Sesbania drummondii* contents of antioxidants and activities of antioxidative enzymes increased after addition of Hg up to 4 µM concentration and then severely declined at 50 µM Hg (Israr and Sahi, 2006). Exposure of suspension culture of *Panax ginseng* to 50 µM Cu resulted in strong growth inhibition and oxidative stress (accumulation of H_2O_2, MDA, increased lipoxygenase activity). Ascorbate and glutathione were oxidized to dehydroascorbate and glutathiondisulfide. SOD activity was increased (mainly due to induction of FeSOD) while CAT and POX activities were inhibited (Ali et al., 2006). In suspension cultures of *Coffea arabica*, addition of $NiCl_2$ into medium induced rapid accumulation of Ni in cells and increase in activities of SOD, CAT, APX, POX and GR (Gomes et al., 2006). Lipid peroxidation and alterations in antioxidative enzymes were the main responses of coffee cell suspension on application of selenite (Gomes et al., 2007). Indian ginseng plant (*Withania somnifera*) treated with different concentrations of Cu for 30 d *in vitro* showed decrease in SOD and CAT activity whereas GPX activity was increased (Khatun et al., 2008). Activity of SOD, CAT, GPX and APX was increased in *Nicotiana tabacum* cell suspension culture, after exposure to Cd or Ni upto 72 h (Gratao et al., 2008). Peanut seedling culture was used in study of Cd toxicity (Sunil Kumar et al., 2008 a). Increases in GPX and lipid peroxidation were noted in different organs (root, stem and leaves) of peanut after 4 wk of incubation. Influence of Cr and Cu on developing *Pongamia pinnata in vitro* was also studied (Sunil Kumar et al., 2008b). It was observed that Cu was more toxic for pongamia seedling germination and growth as compared to Cr metal.

Significance of the present research program

Research efforts made towards understanding the mechanism of metal tolerance has generated a great deal of information but it remains ill understood. Thus, there is scope for research to understand the mechanism of metal tolerance by various plant species and to identify the site and form of metal accumulation within plant system. This **research program** entitled "**Studies on Metal Tolerance in Plants**"

was designed to study the metal stress on different plant species in culture. The species used for the study include a herb (Peanut), a shrub (Jojoba) and a tree (Pongamia). Plant Tissue Culture techniques are used as a tool in this study to grow the plants in presence of the metals tested. The metals tested include Cr, Cu and Cd.

Plants identified till date for phytoremediation are generally annual herbs, with a low or null economic value and very little biomass. One of the goals in the present investigation was to combine phytoremediation with crops of commercial interest or high biomass producers. Phytoremediation of contaminated sites with plants of economic value will not only remediate the sites but also add value to it. Therefore peanut, jojoba and pongamia whose products are of commercial interest were tested for Cr, Cu and Cd tolerance.

Peanut *(Arachis hypogaea* L.)

Scientifically termed as *Arachis hypogaea,* peanut is a large seeded legume, largely cultivated around the world as source of edible oil and proteins. The unique property of peanut crop is that, after fertilization there is peg formation leading the zygote under the soil where the seeds develop inside the shell. This character of peanut makes it different from other legumes.

The potential of this plant in phytoremediation is not yet explored. The peanut pod contains 4 parts namely;

i) the outermost semi woody shell
ii) the testa
iii) the seed (Dicotyledonous)
iv) the embryo axis

The outermost covering to the peanut fruit is the shell. It is the part which comes in direct contact with the soil and hence contaminating metals, etc, if present. Inside the shell there is testa covering the dicotyledonous seed. This is a thin covering on the seed. The embryo axis lies between the cotyledons. The seed is protected by the testa and the shell. Due to this arrangement of the shell, testa and seed, the seed is protected inside. It is the shells, which come in direct contact of the soil. Peanut have never been assessed for its ability to withstand metal stress or for the purpose of phytoremediation although the pods grow in direct contact of the soil. In the present

study, the behavior of peanut seedlings is studied *in vitro* in presence of Cr, Cd and Cu.

Jojoba (*Simmondsia chinensis*)

Jojoba is an herbaceous plant, which grows in arid and semi-arid regions. It is an industrial crop. Its seed wax is used in the cosmetic industry and as a lubricant. There is evidence that *in vitro* nodal segments of jojoba respond to salinity in a similar way as the whole plant (Roussos and Pontikis, 2007). The objective of using jojoba as a model in this study was to unravel, for the first time, the effect of metal stress on shoot culture, metal accumulation capacity in different parts of the shoots, lipid peroxidation and antioxidative response.

Pongamia (*Pongamia pinnata* (L.) Pierre)

Trees are ideal in the remediation of heavy metals as they can withstand and accumulate higher concentration of pollutants owing to their large biomass and size. These can reach a huge area and great depths for their extensive rooting and can stabilize an area. They prevent erosion, and the spread of the contaminant, because of their perennial presence.

Pongamia pinnata (L.) Pierre is a medium sized, fast growing evergreen tree species. Its seed oil is a potential source of raw material in production of biodiesel. It can thrive in wide range of agroclimatic conditions and serve as a rich source of flavonoids and oil for industrial applications. Pongamia seedlings were used as a model system to study the effect of metal toxicity in a tree species.

Objective of the present investigation:

"Studies on metal tolerance in plants" was taken up with the following objectives.

1. To study the influence of chromium, copper and cadmium induced stress on peanut (herb) seedlings cultured *in vitro*.
2. To study the influence of chromium, copper and cadmium on shoot culture of jojoba (shrub).
3. To study effect of chromium, copper and cadmium on pongamia (tree) seed germination, seedling growth and distribution of metal in different parts of plant.

CHAPTER 2

Materials and Methods

INTRODUCTION

This chapter describes the materials, general techniques routinely practiced in plant tissue culture and methods used for enzyme activity measurement, lipid peroxidation estimation and metal estimation. The materials and methods, specific to particular experiment, are dealt in details in respective chapters.

A. Materials

Most of the consumables and chemicals were procured from various local suppliers.

***Glasswares and Plasticwares*:**

Test tubes (25x150 mm), conical flasks (250 ml capacity), pipettes (0.1, 0.2, 1, 2, 5, 10 ml capacity) and measuring cylinders (25 ml, 100 ml, 1000 ml capacity) of Borosil, India were used for culturing the tissues and for preparation of media. Autoclavable, screw cap bottles (100, 250 & 500 ml) for storing stock solutions were procured from Qualigens, India. Glassware used for histological studies were coupling jar (60 ml capacity), slides (Blue Star, India) and cover slips (Micro-Aid, India).

Plasticwares including sterile disposable plastic petriplates of 55 and 85 mm diameter were procured from Tarson, Pune. Klin wrap, used for sealing the petriplates. Micropipette of different precision measurements (1000 µl, 200 µl, 100 µl, 20 µl, 10 µl and 2 µl) and microtips were procured from Gilson and Tarson respectively.

***Chemicals*:**

Chemicals used for surface sterilization procedures were Bavistin® (BASF, India), Savlon (Johnson and Johnson Limited, USA) and Mercuric chloride (Qualigens Fine Chemicals, India).

Inorganic salts and vitamins used for preparation of culture media and for other experiments were of Analar grade (BDH, Hi-Media and Qualigens Fine Chemicals, India). Sucrose, Agar agar (bacteriological grade), used as gelling agent in the semisolid culture medium was procured from Hi-Media (India).

Chemical used for enzyme extraction and enzyme assay including sodium dihydrogen phosphate, disodium hydrogen phosphate, triton-x-100, polyvinylchloride were procured from Himedia. Hydrogen peroxide was procured from Qualigens Fine Chemicals, India and guaiacol, methionine, nitobluetetrazolium, EDTA, riboflavin were procured from Sigma (U.S.A.).

Chemicals including trichloroacetic acid and thiobarbituric acid were obtained from Sigma (U.S.A.) for lipid peroxidation estimation. Chemical used for metal estimation including perchloric acid and nitric acids were procured from Qualigen fine chemicals, India.

For histological studies formaldehyde solution, glacial acetic acid and xylene were procured from Qualigens Fine Chemicals, India. Ethanol, 2-methyl propan-2-ol (tert butyl alcohol), iron alum was from S.D. fine chemicals, India. Paraffin wax (m.p. 58-60°C) was from (E. Merck, India Ltd.); Haematoxylin and Eosin stain was from Hi-Media Laboratories Pvt. Ltd., Bombay, and DPX-4 mountant [189-(2-chloro-N-(4-methoxy-1,3,5-triazin-2-yl amino carbonyl) benzene sulphanamide)] was from BDH, India.

Equipments:

The major equipments used include,

***pH meter (Thermo Orion A+)*:**

pH is the negative logarithm of hydrogen ion concentration. The measurement of pH in pH meter (Thermo Orion) is based on ion exchange in between hydrated layers formed on glass surface. Change in ion exchange results in emf or voltage difference causing current flow. The current intensity gives the value of pH. The pH meter consists of two electrodes a calomel electrode and a glass electrode. The glass electrode contains a silver chloride and 0.1 N hydrochloric acid. Its tip is covered by a special glass surface, which allows only H^+ ions to pass through it. The zero of the dial is first set by mechanical means. Then the knob for temperature compensation is adjusted for the temperature of the solution. This is essential since equilibrium constant of a reaction does vary with temperature. As a next step, the electrodes are dipped in to a standard buffer solution (This is the solution whose $[H^+]$ is accurately known] of known pH. The dial read this value. The electrodes are now removed,

washed well with distilled water, and dipped into the unknown solution. The dial reads the pH value of the solution.

***Electronic Balance (Contech)*:**

A manual top loading balance was used for quick weighing and for analytical purposes. This is a single pan balance of capacity 100-200 gm, sensitivity 0.1 mg operating on 230 V 50 H_2 AC mains. Precision of ± 0.005 g, weighing range 0-1, 200 g, digital read out was used for making stock solutions of growth regulators and for other fine weighing.

***Autoclave*:**

The autoclave (Nat Steel Equipment Private Limited, Mumbai, India) was used for sterilization of media, glasswares, water, dissecting instruments, etc. and also for decontamination of contaminated cultures. It is based on the application of steam under pressure. High pressure provides high boiling point than normal temperature that contains more heat content, which causes coagulation of proteins and cell death. It has a thermostat with a double-jacketed steam chamber. Thus the temperature and pressure can be maintained. The culture media are autoclaved at 121ºC and 15 lb/inch² pressure for 20 minutes. Glass wares and other materials are autoclaved for 1h.

***Laminar airflow ultra clean unit* (Micro-Filt, Pune, India):**

It is a bench on which all aseptic manipulations are carried out. In laminar with the help of an air pump, air is passed through the HEPA filters. The pore size of the filter is 0.22 microns. The fan fitted in this unit pushes the air through the filter at high pressure from one side creating positive pressure inside the chamber. So the entry of any contaminant is prohibited from the open side of the bench. The instrument is fitted with UV tubes in addition to the fluorescent tubes. Before using the instrument UV is put on for 15 to 20 minutes and bench is to be cleaned with 96% alcohol swab to eliminate microorganisms. The laminar used for present studies was a horizontal laminar type. Here airflow is in horizontal direction. It is the most common type used in tissue culture labs.

***Atomic Absorption Spectrophotometer* 1100B (Perkin Elmer, USA):**

Atomic absorption spectroscopy (AAS) determines the presence of metals in liquid samples. In their elemental form, metals absorb ultraviolet light when excited by heat. Each metal has a characteristic wavelength that will be absorbed. The AAS instrument looks for a particular metal by focusing a beam of UV light at a specific wavelength through a flame and into a detector. The instrument measures the change in intensity. The determination of element is based on atomic absorption. When light emitted by the lamp passes from monochromator, it becomes plane-polarized light. The intensity of flame and lamplight produces a resultant intensity. When element absorbs light there is a decrease in intensity and this is detected as a flash by photomultiplier. Flash and light intensity decrease is directly proportional to concentration of the element. A computer data system converts the change in intensity into an absorbance. The sensitivity of the instrument used in this study is 2 ppm. Initially standard sample is passed to determine the original wave absorbed. Then the sample is passed and concentration is determined.

***Centrifuge*:**

Centrifuge is an instrument which is based upon the principle of centrifugation separation process which works on an applied centrifugal field, such as the relative molecular mass, shape and density. The basis of centrifugal techniques is to exert a larger force than does the earth's gravitational field, thus increasing the rate at which the particles sediment. Particles that differ in density, shape or size can be separated because they sediment at different rates in centrifugal field, each particle sedimenting at a rate that is directly proportional to the applied centrifugal field. Centrifuge was used for isolation of tissue extract used for enzymes activity estimation and protein estimation.

Other instruments used in the course of the present study include Magnetic stirrer (Remi, India), Steamer (Ultradent, India), Temperature controlled oven (Pathak Electricals, India), Light microscope (Carl-Zeiss Jena), Microtome (Reichert Jung), Camera (Nikon/Zeiss), membrane filter sterilizing unit (Laxbro, Pune) and Pipetman (Gilson/Tarson) were used. With the exception of pipetman, microtome, microscopes

and camera, all other equipments used in the course of this study are fabricated by different companies in India.

B. Methods

***Preparation of glassware and instruments*:**

Glassware used in our studies was cleaned by boiling in saturated solution of sodium bicarbonate for 1 h followed by washing in tap water. These were then immersed in 30% nitric acid solution for 30 min and were washed thoroughly with tap water. After rinsing with double distilled water these were allowed to dry on a draining rack.

Tubes and flasks were plugged with absorbent cotton (Safe Surgical Industries, Beawar, India). All dissecting instruments were either wrapped singly or were put in closed aluminum cans for sterilization by autoclaving. Ordinary grade filter paper pieces of approximately 10x20 cm were kept in stack alternatively with brown paper pieces of similar size. These were packed in autoclavable plastic bags with 20-25 pieces in each bag and autoclaved. Dissection and transfer of explants were carried out on these papers under aseptic conditions and disposed after use. Pipet tips used for aseptic addition by micropipets were arranged in cases meant for their size, wrapped with brown paper and autoclaved. Sterilization of the glassware and instruments was carried out by autoclaving at 121°C for 1 h in 15 lbs/(inch)^2.

***Preparation of media*:**

Success of a tissue culture protocol depends on the appropriate composition of the medium. Several basal formulations like Murashige's and skoog media, Schenk and Hildebrandt media. Concentrations of the macro and microelements, salts and organic constituents of the MS (Murashige and Skoog, 1962), modified SH (Schenk and Hildebrandt, 1972) basal medium are listed below. Stock solutions of the media ingredients were prepared by dissolving weighed amounts of these salts as given below in distilled water. Appropriate aliquots of these solutions were mixed to prepare the media.

COMPOSITION OF MURASHIGE AND SKOOG'S BASAL MEDIUM

(Composition of the stock solutions)

MS MAJOR (1000 ml) 20X

KNO_3	38.16 grams
NH_4NO_3	33.16 grams
$CaCl_2.2H_2O$	8.96 grams
$MgSO_4.7H_2O$	7.56 grams
KH_2PO_4	3.56 grams

MS MINOR (500 ml) 100X

H_3BO_3	310 mg
$MnSO_4$	1115 mg
$ZnSO_4$	430 mg
Na_2MoO_4	12.5 mg
$CoCl_2.6H_2O$	1.25 mg
KI	41.5 mg
$CuSO_4.5H_2O$	1.25 mg

VITAMINS (500 ml) 100X

Nicotine amide	25 mg
Glycine	100 mg
Thiamine	5 mg
Pyridoxine	25 mg

INOSITOL 100X

5 grams of Inositol was dissolved in 500 ml of distilled water.

Fe-EDTA (500 ml) 100X

$FeSO_4$	1.390 grams
EDTA	1.865 grams

COMPOSITION OF SCHENK AND HILDEBRANDT (SH) MEDIA

(**Composition of the stock solutions)**

SH MAJOR (500 ml) 20X

KNO_3	25 grams
$CaCl_2.2H_2O$	2.0 grams
$MgSO_4.7H_2O$	4.0 grams
$NH_4H_2PO_4$	3.0 grams

SH MINOR (100 ml) 100X

H_3BO_3	50 mg
$MnSO_4$	100 mg
$ZnSO_4$	10 mg
Na_2MoO_4	1 mg
$CoCl_2.6H_2O$	1 mg
KI	10 mg
$CuSO_4.5H_2O$	2 mg

SH VITAMINS (100 ml) 100X

Nicotine amide	5 mg
Thiamine	50 mg
Pyridoxine	5 mg

INOSITOL 100X

10 g of Inositol was dissolved in 100 ml of distilled water.

Fe-EDTA (100 ml) 100X

$FeSO_4$	150 mg
Na_2 – EDTA	200 mg

Stock solutions of growth regulators (GR) were prepared by adding few drops of solvent in the required amount of growth regulator to dissolve. After dissolution, the required concentration was made by the addition of double distilled water and stored in refrigerator in sterilized bottles.

For media preparation a calculated amount of aliquots were added from these stock solutions. Unless mentioned, pH of all the media was adjusted to 5.6-5.8 using 1N NaOH or 1N HCl after mixing all the constituents except the gelling agent. The volume was made up with double distilled water. Gelling agent (agar agar) was then added and heated on water bath or steamed for the agar to melt. Molten medium was dispersed into sterile culture tubes (20 ml of media), flasks (100 ml of media) or bottles (80 ml of media) after thorough mixing. Semisolid medium containing agar was used in most of the studies unless otherwise mentioned. All the culture media were autoclaved for 20 min. at 121°C and 15 lbs/(inch)^2.

***Culture conditions*:**

Cultures were incubated in light in culture room adjusted at 25±2°C with 16 h photoperiod at 32 μE $m^{-2}s^{-1}$ light intensity.

***Histological Techniques*:**

Sections were prepared for histological studies following the methods described by Sharma and Sharma, 1980. The tissues were cut into small pieces (approx 3 x 4 mm) and were fixed in FAA (formaldehyde: glacial acetic acid: alcohol, 5:5:90, v/v) for 48 h at room temperature. Tissues were dehydrated using graded concentrations of tertiary butyl alcohol and embedded in paraffin wax (mp 58-60°C). Serial sections of 10 μM were cut using a rotary microtome (Reichert-Jung 2050, Germany). Sections were double stained with haematoxylin-eosin and mounted with DPX (Loba Chemie, Mumbai, India) for studies under microscope.

***Filter sterilization*:**

The solutions and liquid used in the experiments was filter sterilized by passing through membrane filters. All the particles, microorganisms and viruses which are bigger than the pore diameter of the filter used (0.22 μm) are removed. The

greatest advantage of this method is that thermolabile substances like vitamins, amino acids, hormones viz., GA3, zeatin, Abscisic acid (plant growth regulators) can be sterilized unchanged. In present investigation $K_2Cr_2O_7$, $CuSO_4$ and $CdCl_2$ solution were filter sterilized.

***Lipid Peroxidation*:**

The reaction of lipid peroxides with thiobarbituric acid (TBA) has been widely adopted as a sensitive assay method for lipid peroxidation in animal tissues (Ohkawa et al., 1979). In the present investigation, the level of lipid peroxidation products and thiobarbituric reacting substances (TBARS) in different tissues was estimated following the modified method used in studies on arsenic tolerance in ferns (Srivastava et al., 2005). Approximately 0.250 g of frozen plant tissue samples was cut into small pieces, homogenized with the addition of 2.5 ml of 5% trichloroacetic acid, and centrifuged at 10000 g for 15 min at room temperature. Equal volumes of supernatant and 0.5% thiobarbituric acid in 20% trichloroacetic acid were added in a new tube and incubated at 96 °C for 25 min. The tubes were transferred into an ice bath and then centrifuged at 8000 g for 5 min. The absorbance of the resulting supernatant was recorded at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The concentration of lipid peroxides, were quantified and expressed as total TBARS in terms of μmole g^{-1} FW using an extinction coefficient of 155 mM^{-1} cm^{-1}.

***Activities of antioxidant enzymes*:**

The activity of Superoxide dismutase (SOD) was assayed by the method of Beauchamp and Fridovich (1971) by measuring its ability to inhibit the photochemical reduction of nitrobluetetrazolium (NBT). The 3 ml reaction mixture contained 50 mM phosphate buffer (pH 7.8), 15 mM methionine, 87 μM NBT, 2.4 μM riboflavin, 0.1 mM EDTA and 100 μL of enzyme extract. The test tubes were shaken and were illuminated with 15 W fluorescent lamp. The absorbance was taken at 560 nm. An illuminated blank without protein gave the maximum reduction of NBT, and therefore, the maximum absorbance at 560 nm. SOD activity is presented as absorbance of blank minus absorbance of sample, giving the total inhibition,

calculated per microgram protein. The activity of SOD was expressed as units mg^{-1} protein. One unit of activity is the amount of protein required to inhibit 50 % initial reduction of NBT under light.

The catalase (CAT) activity was measured by the method of Aebi (1984). The assay system comprised of 50 mM sodium phosphate (pH 7), 20 mM H_2O_2, and 100 µL of enzyme extract in the final volume of 3 ml. Decrease in the absorbance was taken at 240 nm. The molar extinction coefficient of H_2O_2 at 240 nm was taken as 0.04 cm^2 μmol^{-1}. Enzyme activity was expressed as units mg^{-1} protein.

Guaiacol peroxidase (GPX) activity was measured by the method of Chance and Maehly (1955). The reaction was initiated by addition of H_2O_2 and change in optical density at 470 nm was measured at intervals of 10 s for 2 min. Activity was calculated using the extinction coefficient 26.6 mM^{-1} cm^{-1} for the oxidized tetraguaiacol polymer. Enzyme activity was expressed as units mg^{-1} protein.

Metal estimation

After harvesting the seedling, seedling were separated into roots, stems and leaves, after particular time of incubation for different experiments. These tissues were dried in oven at 80^oC till constant weight was reached. The dried plant samples were ground to fine powder with pestle and mortar. Weighed amount of powder was used for Cr, Cu and Cd estimation. These were digested with 3 ml of nitric acid and 1 ml of 70% perchloric acid on a hot plate under the hood. Digested sample solution was made to 10 ml volume with deionized water. Metal content in these samples was determined using Atomic Absorption Spectroscopy (Perkin Elmer 1100B). Metal content was calculated in mg per Kg of dry weight of tissue.

CHAPTER 3

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 (¾ 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,

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 ($K_2Cr_2O_7$), 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.

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)

Conc. of Cr	**Germination frequency Mean ± Sd**	**Shoot height (cm) Mean ± Sd**	**Root length (cm) Mean ± Sd**
Control	70 ± 5.00	9.29 ± 1.4	6.59 ± 1.01
50 μM	50 ± 13.33	8.55 ± 0.86	5.85 ± 0.25
100 μM	58 ± 18.9	8.63 ± 3.51	4.73 ± 1.49
200 μM	35 ± 5.00	9.34 ± 1.23	4.41 ± 0.95
300 μM	38 ± 7.64	10.21 ± 2.53	3.83 ± 0.66
500 μM	38 ± 5.64	6.22 ± 0.28	1.15 ± 0.01
1000 μM	32 ± 2.64	3.35 ± .07	0.86 ± 0.06
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

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.

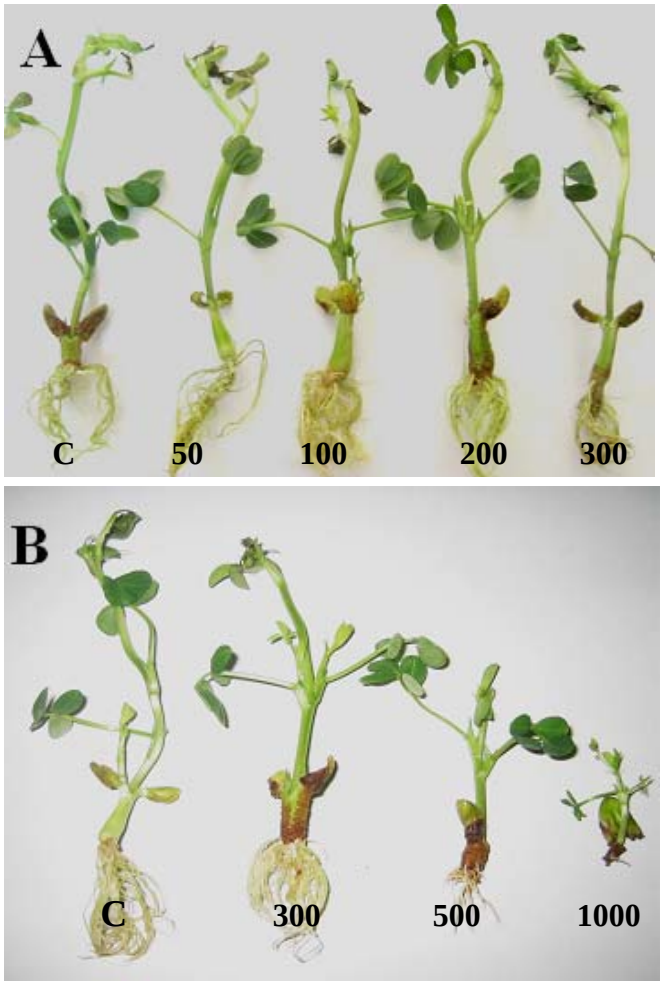

Fig 3.1: Effect of chromium on peanut seedling after 4 weeks. Treatments are in µM.

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)

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

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

wall stiffening, and this can be an explanation to the Cu-excess effect on roots influencing plant growth (Chen et al., 2000).

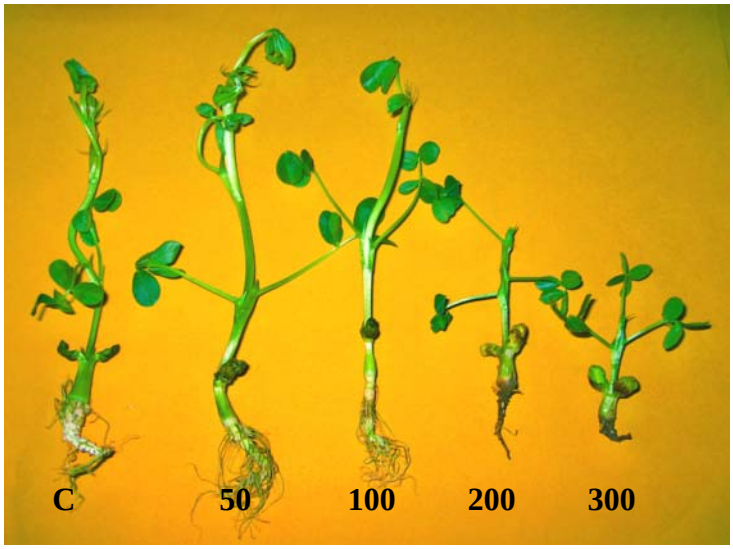

Fig 3.2: Effect of copper on peanut seedling after 4 week. Treatments are in µM.

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)

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

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 $CrCl_3$ (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.

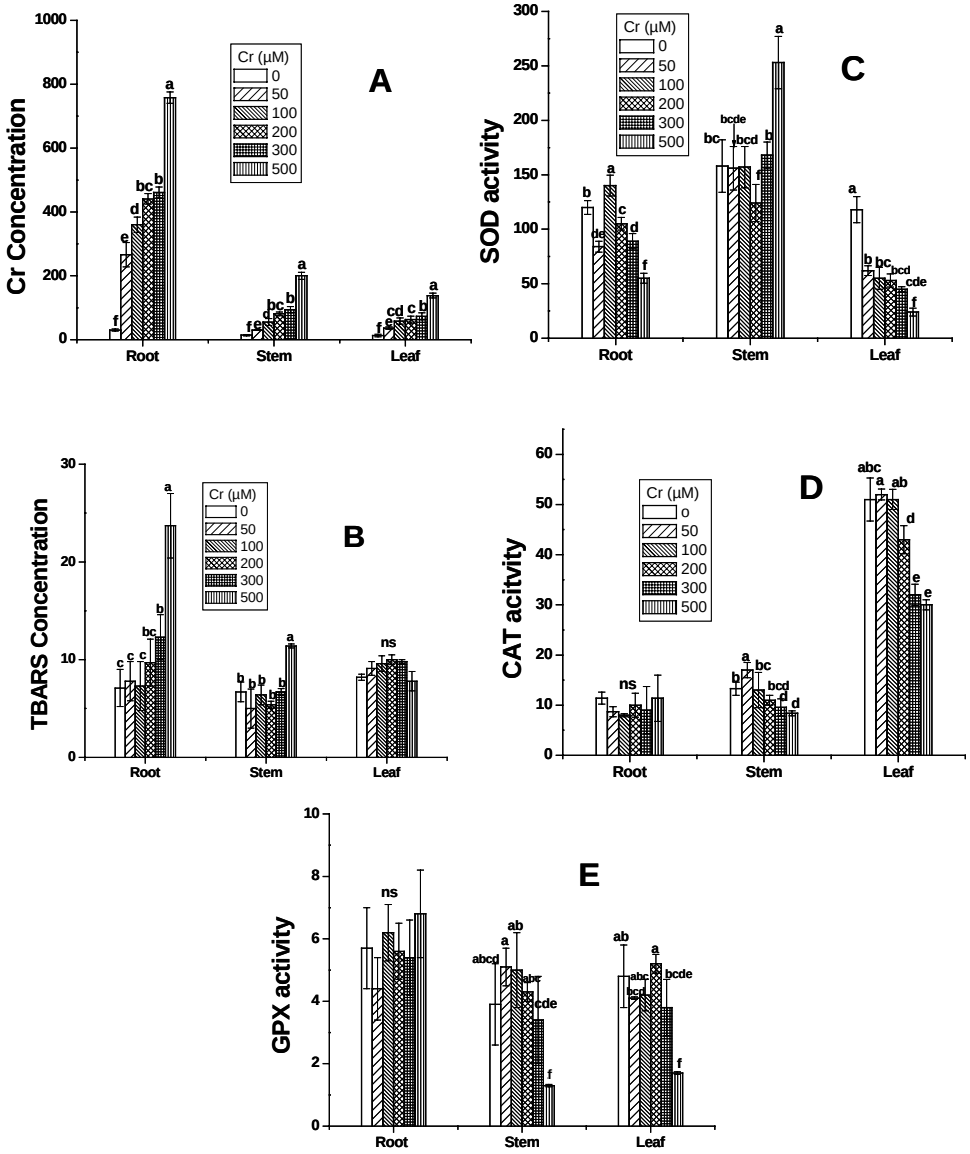

Fig 3.4: Effect of various concentrations of Cr on (A) metal accumulation, (B) lipid peroxidation (C, D, E) antioxidative enzyme activities on peanut seedling after 4 wk of incubation (means ± Sd). Means with common letter(s) are not significantly different at $P< 0.05$ according to Duncan multiple range 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.

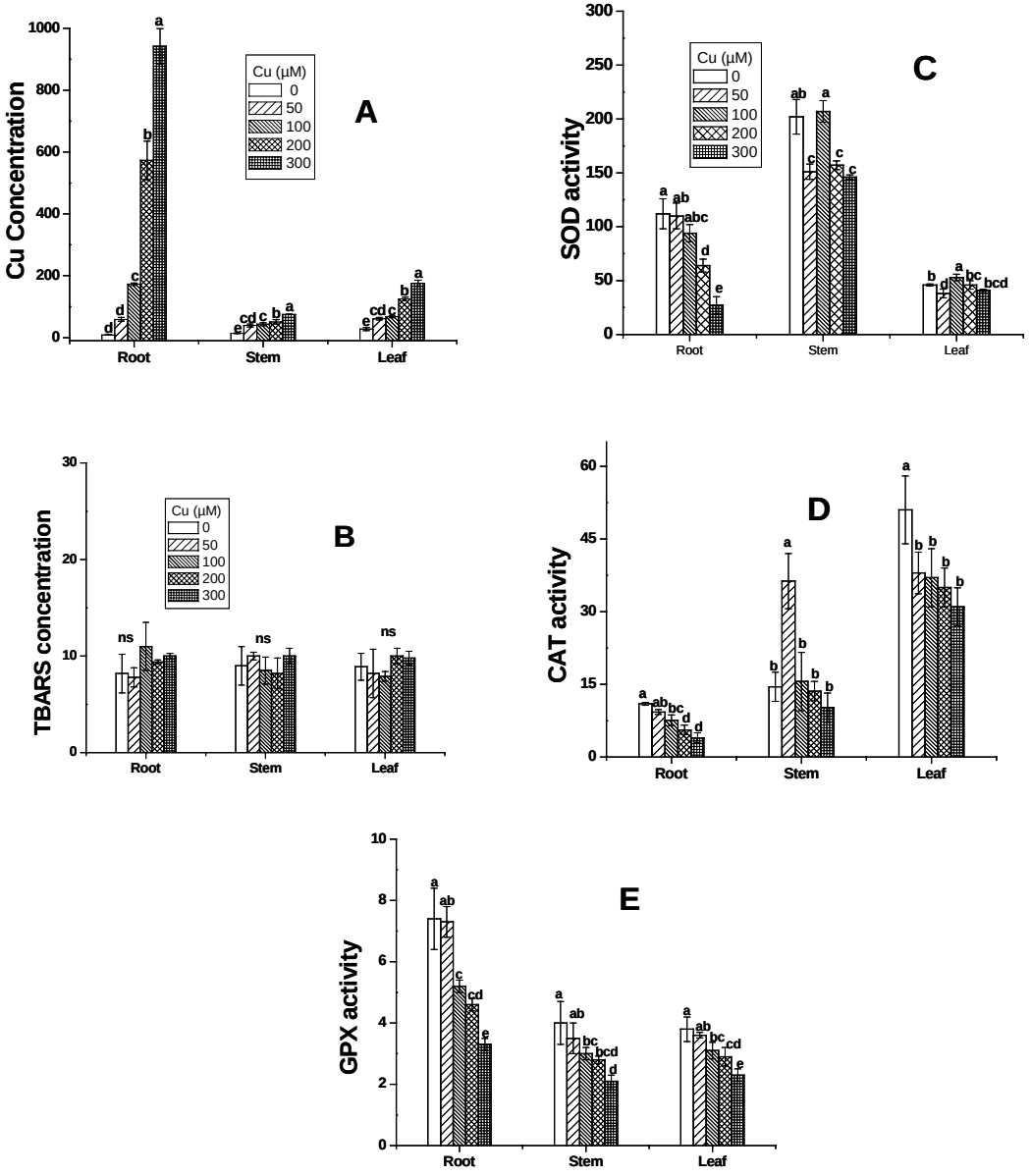

Fig 3.5: Effect of various concentrations of Cu on (A) metal accumulation, (B) lipid peroxidation (C, D, E) antioxidative enzyme activities on peanut seedling after 4 wk of incubation (means ± Sd). Means with common letter(s) are not significantly different at $P< 0.05$ according to Duncan multiple range 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 nmolg^{-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).

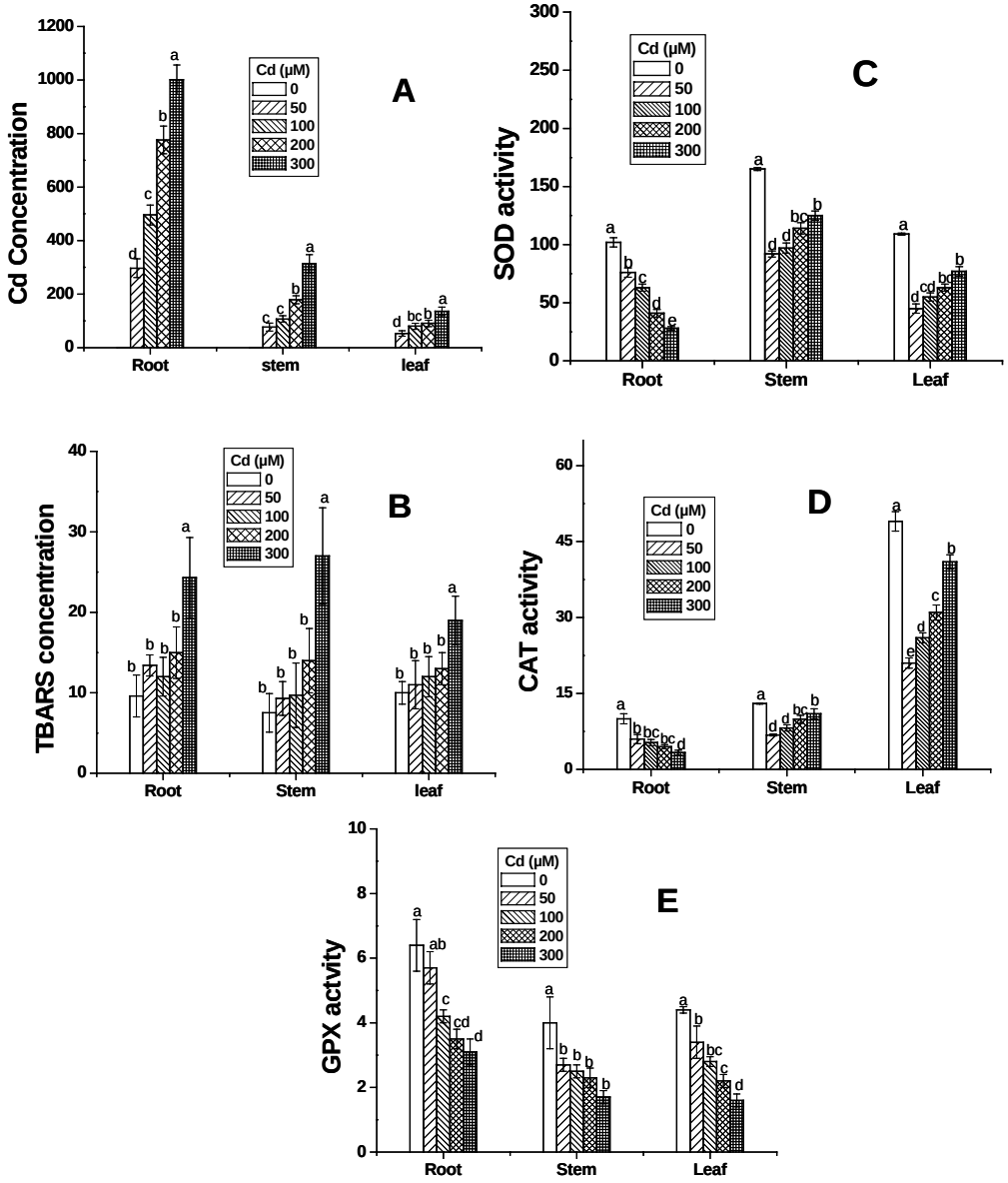

Fig 3.6: Effect of various concentrations of Cd on (A) metal accumulation, (B) lipid peroxidation (C, D, E) antioxidative enzyme activities on peanut seedling after 4 wk of incubation (means ± Sd). Means with common letter(s) are not significantly different at P< 0.05 according to Duncan multiple range 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

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

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 Clijesters, 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; Chamseddine 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_2O_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_2O_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 $O_2^{\bullet-}$ 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 $O_2^{\cdot-}$ 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* (Somashekaraiah 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

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; Kova´cˇik 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.

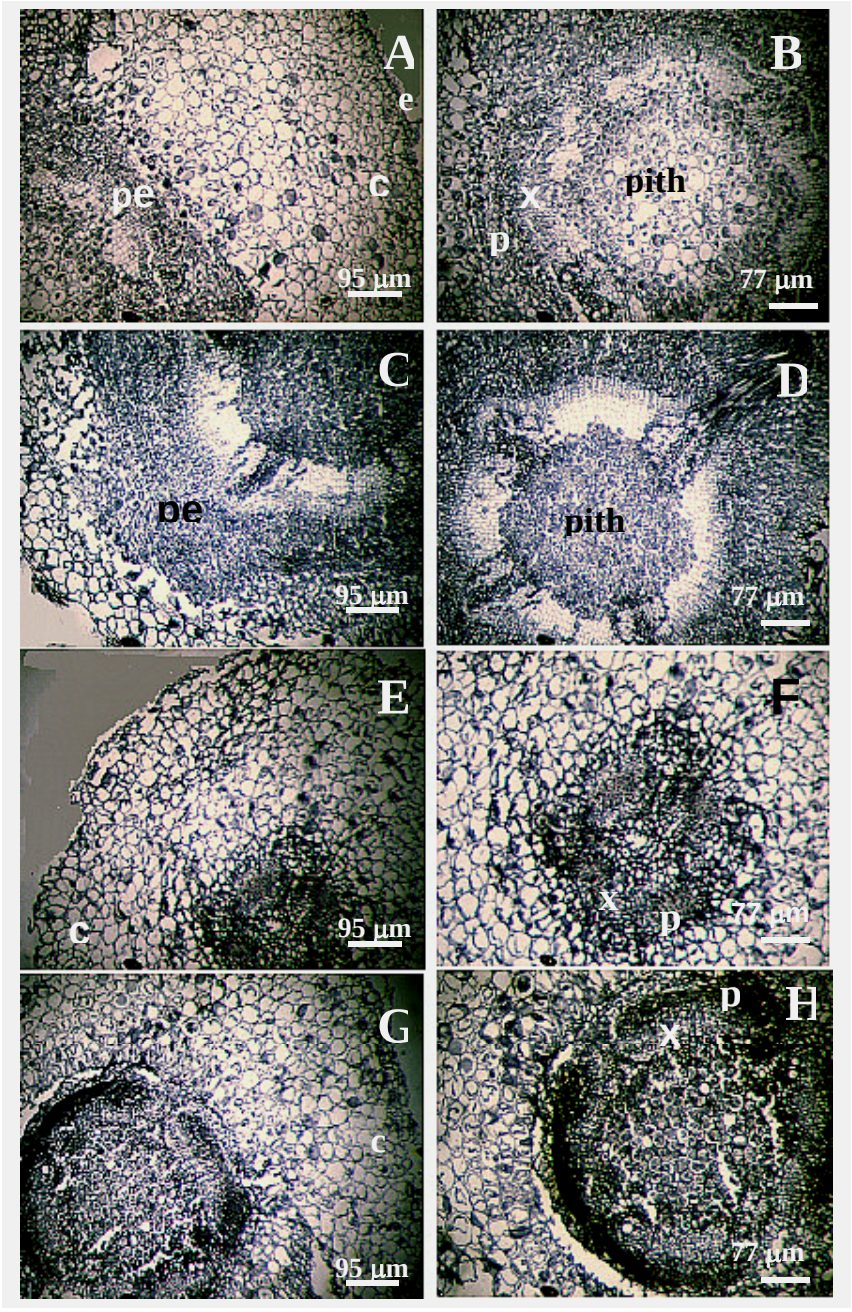

Fig 3.7 Light micrographs of transverse section of root of control (A, B) and metal treated (C-H) after 10 d of incubation. Normal cell layer in control root (A, B). Change in outer cortical cell shape, increase in number of pericycle cell layer in Cr treated cells (C, D). Broader cortical region (E), 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).

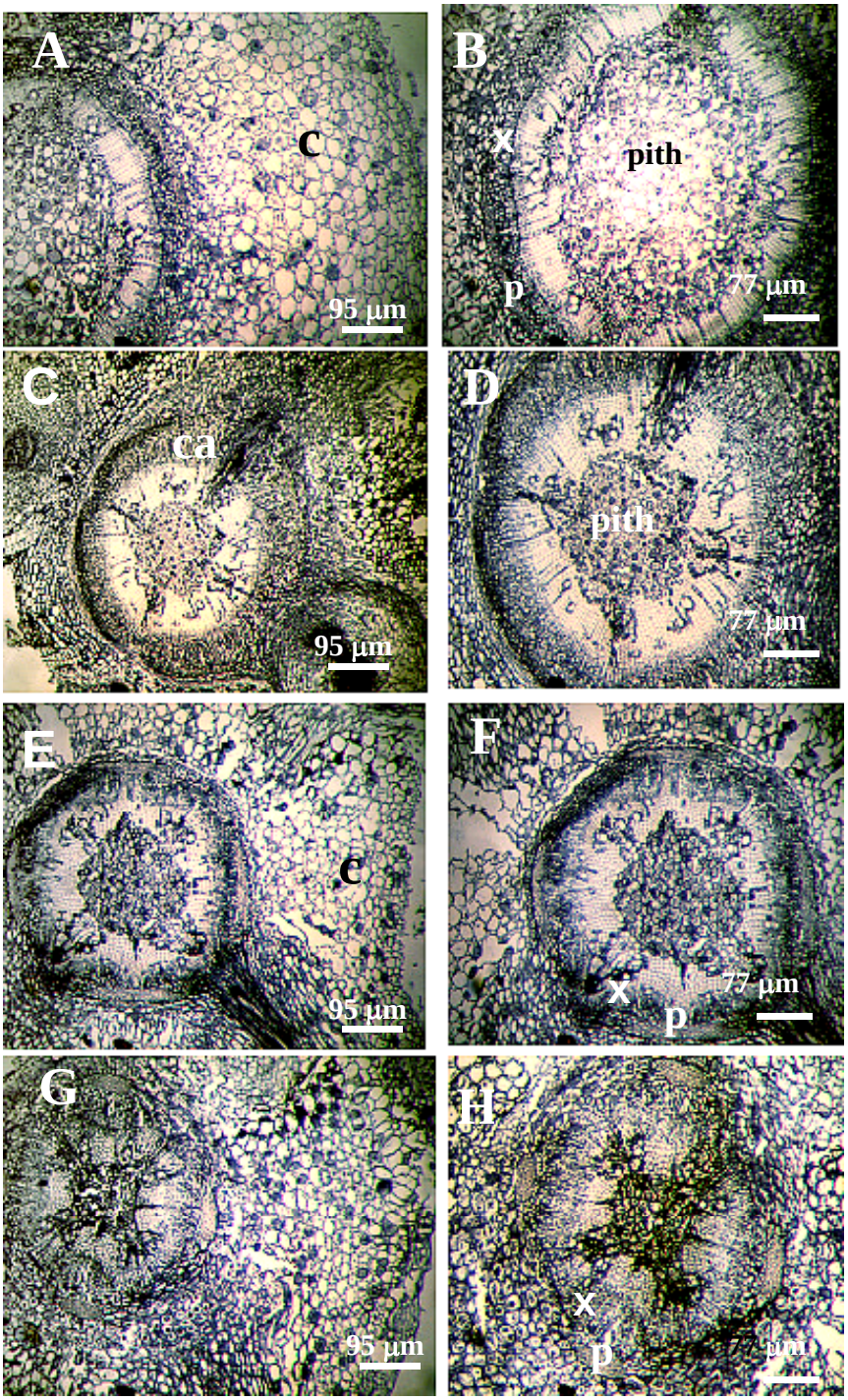

Fig 3.8 Light micrographs of transverse section of root of control (A, B) and metal treated (C-H) after 20 d of incubation. Normal cell layer in control root (A, B). Loosening of pith cells in Cr treated cells (C, D). Distorted cortical region (E), shrinkage in phloem, xylem cells and reduced pith cells 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 compare 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

food and minerals. Accmulation of Cu in xylem or phloem may disturb these cells. Disturbance of vascular bundle might be due to Cu accumulation in these cell. 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.

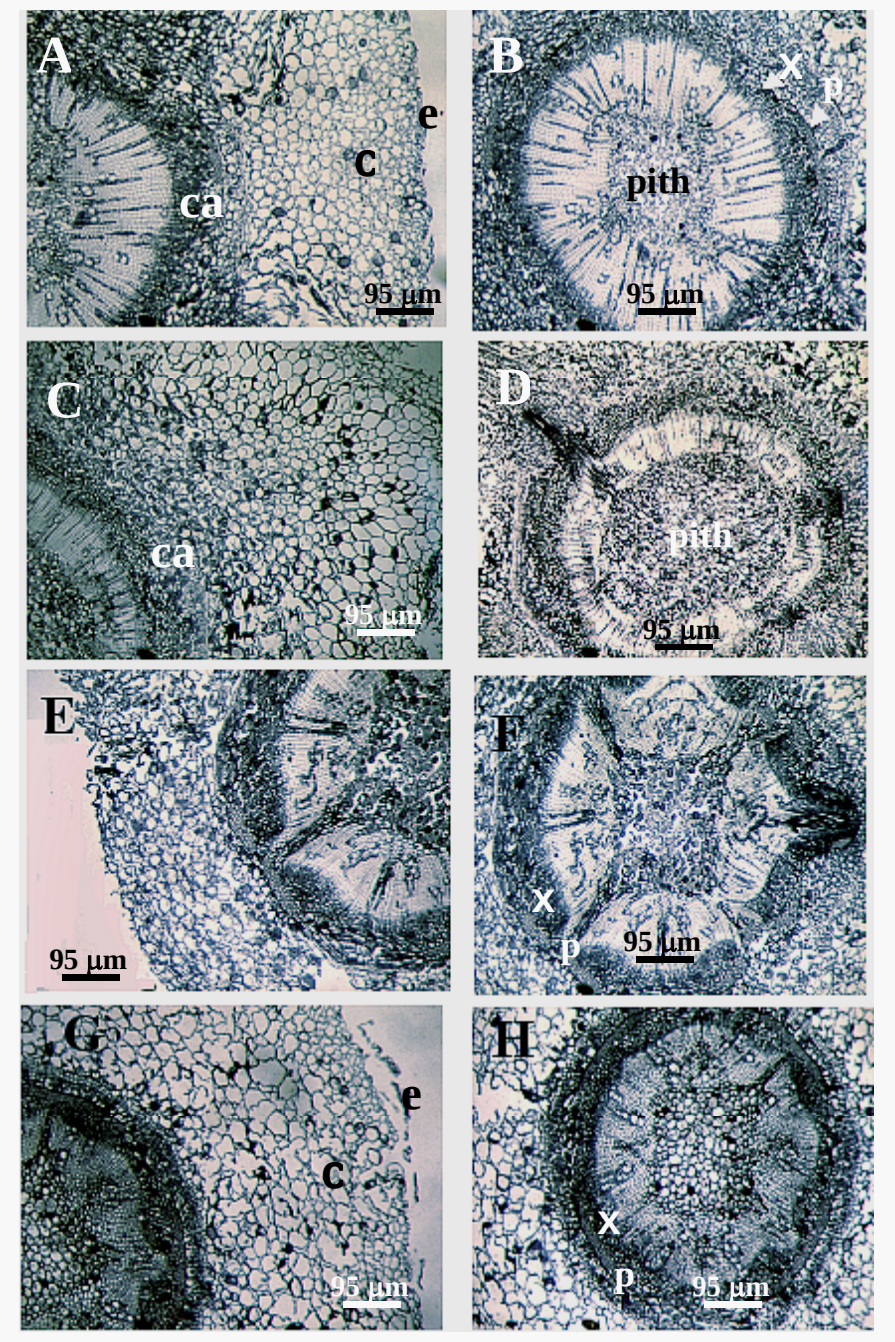

Fig 3.9 Light micrographs of transverse section of root of control (A, B) and metal treated (C-H) after 30 d of incubation. Normal cell layer in control root (A, B). pronounced cambium layer and increased in number of pith cells due to Cr treatment (C, D). Distorted cortical region (E), and divided phloem 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

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.

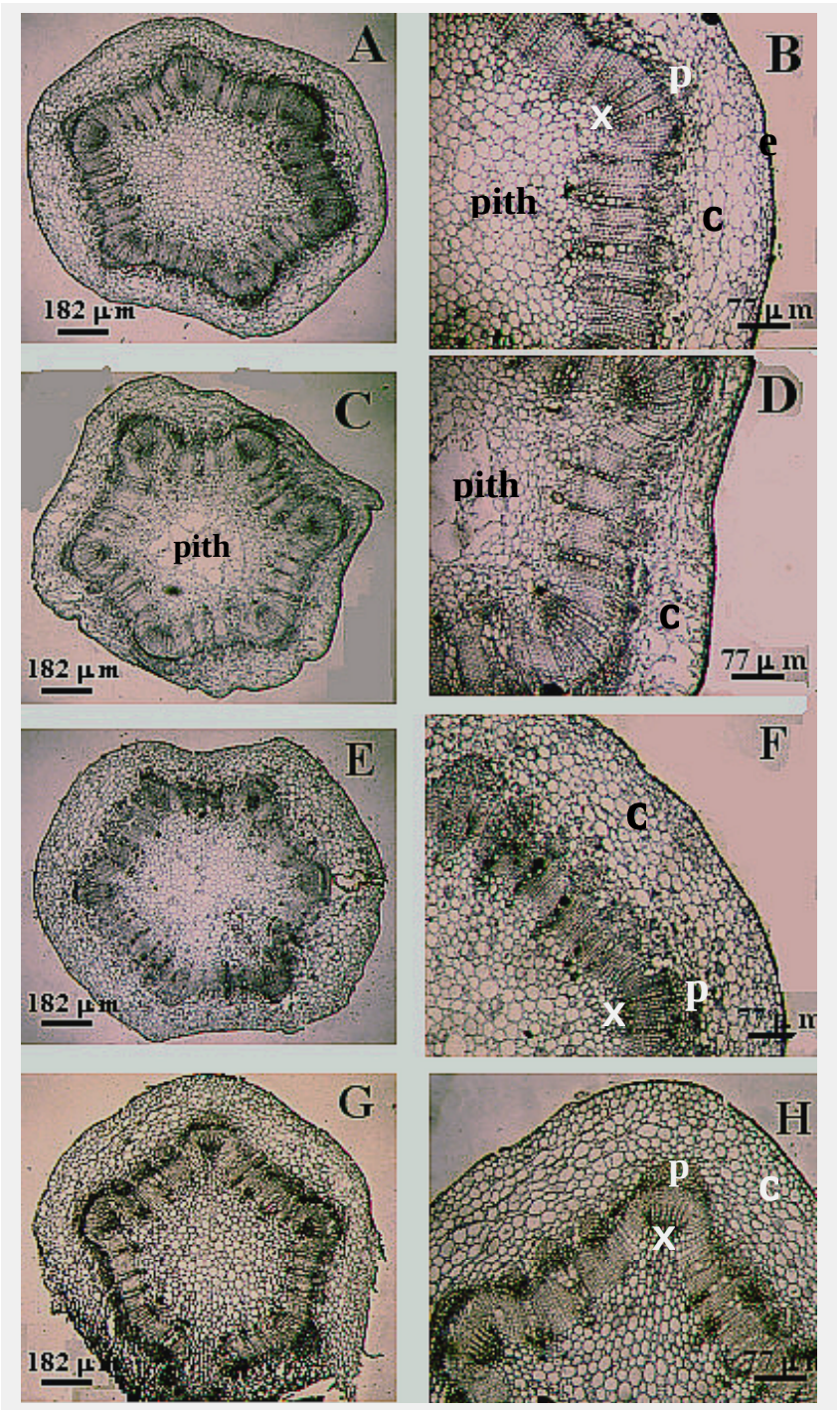

Fig 3.10. **Light micrographs of transverse section of stem of control (A, B) and metal treated (C-H) after 30 d of incubation.** Normal cell layer in control stem (A, B). Rupture of cortex and pith due to Cr treatment (C, D). Shrinkage in xylem and phloem cell in Cu treated stem (E, F). Shrinkage in 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.

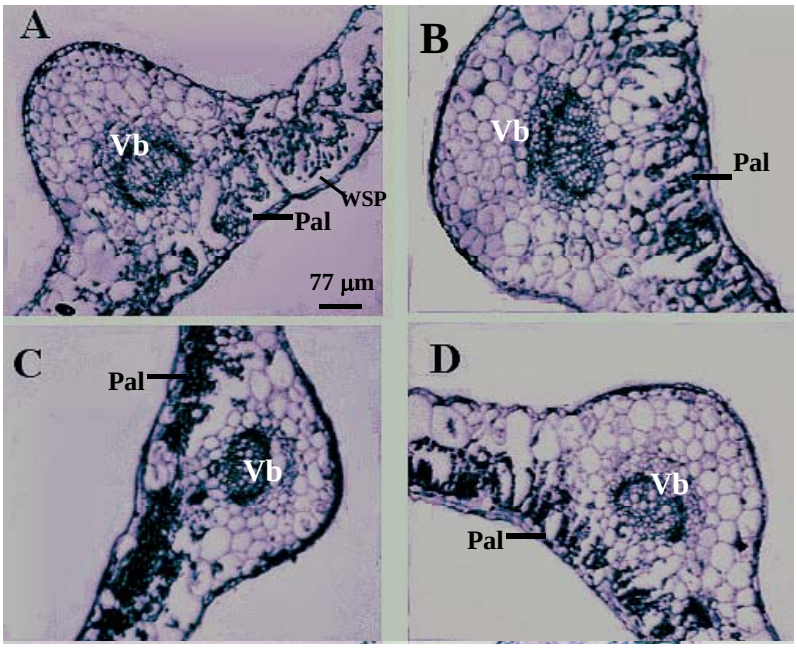

Fig 3.11. **Light micrographs of transverse section of leaf of control (A) and metal treated (B-D) after 30 d of incubation.** Normal cells in control leaf (A) Number of xylem cells increased in Cr (B) treated peanut leaf. Granular deposition in palisade layers and decrease in water storage cells in 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

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 $K_2Cr_2O_7$ $CuSO_4$ 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

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)

Conc. of Cr	**Shell mg /Kg DW Mean ± Sd 24h**	**Shell mg /Kg DW Mean ± Sd 48h**	**Testa mg /Kg DW Mean ± Sd 24h**	**Testa mg /Kg DW Mean ± Sd 48h**	**Seed mg /Kg DW Mean ± Sd 24h**	**Seed mg /Kg DW Mean ± Sd 48h**
Control	18 ± 0.7	25 ± 2.8	10 ± 0.7	14 ± 1.4	7.0 ± 1.4	8.0 ± 1.4
50 µM	78 ± 12	108 ± 15	28 ± 2.8	56 ± 4.2	15 ± 2.1	21 ± 6.4
100 µM	106 ± 3.5	133 ± 3.0	57 ± 19	78 ± 9.1	22 ± 5.0	27 ± 1.4
200 µM	109 ± 4.2	166 ± 9.1	69 ± 12	81 ± 7.7	31 ± 4.2	35 ± 0.8
300 µM	125 ± 5.6	189 ± 5.0	76 ± 14	102 ± 19	39 ± 6.3	48 ± 6.3
ANOVA	1%	1%	1%	5%	5%	5%

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)

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

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)

Conc. of Cd	Shell mg /Kg DW Mean ± Sd 24h	Shell mg /Kg DW Mean ± Sd 48h	Testa mg /Kg DW Mean ± Sd 24h	Testa mg /Kg DW Mean ± Sd 48h	Seed mg /Kg DW Mean ± Sd 24h	Seed mg /Kg DW Mean ± Sd 48h
Control	ND	ND	ND	ND	ND	ND
50 µM	425 ± 5.8	582 ± 8.2	7.0 ± 2.3	24 ± 2.1	ND	ND
100 µM	517 ± 4.2	796 ± 14	17 ± 2.1	28 ± 7.0	ND	15 ± 0.7
200 µM	713 ± 6.6	943 ± 4.	27 ± 4.3	106 ± 29	ND	28 ± 1.0
300 µM	910 ± 14	966 ± 7.1	57 ± 18.3	182 ± 23	ND	46 ± 1.4
ANOVA	1%	5%	5%	1%	ND	5%

ND* (Not detected).

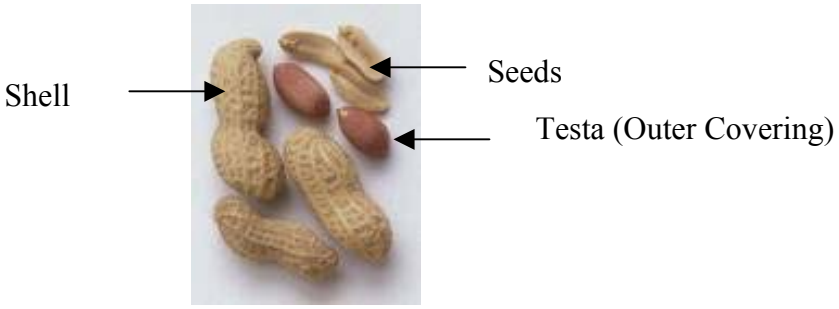

Fig 3.12 Different parts of peanut seedpod

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.

CHAPTER 4

Studies on metal tolerance in Simmondsia chinensis

4.1 INTRODUCTION

Jojoba belongs to family buxaceae with genus *Simmondsia* and species *chinensis*. Jojoba is a woody evergreen shrub whose leaves are leathery, thick, grayish green in color, and elliptical in shape, reaching up to 1 1/2" in length. Distinctively, these leaves are vertically oriented, an adaptation which lessens sun impact at summer high noon, while exposing each leaf to the less extreme temperatures of morning and evening. Jojoba plants are dioecious female and male flowers occur on separate plants (http://www.saguaro-juniper.com/i_and_i/trees&shrubs/jojoba/jojoba.html). These are pale-greenish in color, occurring in a dense cluster. Blossoming times for Jojoba are extremely variable (from December to July), depending on weather variations. Jojoba nuts contain a liquid wax, which is highly resistant to spoilage and stable under high temperatures. As such, it is very high quality oil, used for industry and also in cosmetics. This shrub may reach quite large sizes, occasionally more than 7 feet tall. Jojoba is now cultivated commercially in Argentina, Australia, Chile, Egypt, India, Israel, Mexico, Peru, South Africa and the USA. Jojoba is being examined for its potential as a crop in many countries around the world with climate and soil conditions similar to those of its native habitat.

Heavy metals have become one of the main abiotic stress agents for living organisms because of their increased use in the developing fields of industry leading to high bioaccumulation and toxicity (Kova´cˇik and Backer, 2008). Chromium (Cr), copper (Cu) and cadmium (Cd) are the major concern for environment health. Copper is an essential micronutrient for plant growth but at concentrations above those required for optimal growth, it is toxic for plant.

Heavy metal toxicity induces the production of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen (Arvind and Prasad, 2003) and may result in significant damage to cellular constituents. Proteins and membrane lipids are especially prone to attack by free radicals and are considered as indicator of oxidative stress in plants. Oxidation of membrane lipids results in the increased concentration of thiobarbituric acid reacting substances (TBARS). TBARS, a product of lipid peroxidation has been seen to be greatly accumulated after heavy metal exposure (Gratao et al., 2005; Chamseddine et al., 2008). Defense system of the plant to

reactive oxygen species constitutes the enzymes like superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), glutathione reductase and antioxidative compounds such as glutathione, carotenoids etc. (Mishra et al., 2006). These enzymes have been shown to be affected by exposure to Cr, Cu and Cd (Gratao et al., 2005; Panda and Choudhary, 2005). SOD is responsible for dismutation of O_2^- to form H_2O_2 and O_2, whereas CAT, GPX, APX are enzymes that catalyze the conversion of H_2O_2 to water and O_2 (Gratao et al., 2005). Information focused on the relationship between heavy metals and oxidative stress in plants is now available in more recent years. However a general conclusion is awaited about the critical toxic metal concentrations in environment.

Plants identified for phytoremediation are generally annual herbs, with a low or null economic value and very little biomass (Arru et al., 2004). Phytoremediation with crops of commercial interest or high biomass producers has been emphasized (Bona et al., 2007). Jojoba (*Simmondsia chinensis*) is an industrial crop – its seed wax is used in the cosmetic industry and as a lubricant (Roussos and Pontikis, 2007) with considerable potential in arid and semi-arid regions. Research on water status and photosynthesis rates has shown that jojoba has ability to withstand relatively high levels of salinity (Roussos and Pontikis, 2007). There is evidence that nodal segments of jojoba *in vitro* respond to salinity in a similar way as the whole plant, therefore *in vitro* culture could be used for preselection and evaluation of salt (Roussos and Pontikis, 2007) and hence for metal toxicity. Nevertheless, little is known about the mechanisms involved, which could contribute much in screening for metal tolerance during the early stages of development. Screening of germplasm in contaminated fields could be slow, cost and labour intensive. It is suggested that *in vitro* screening of organs or propagules would be an efficient, simple alternative and promising method (Mills and Benzioni, 1992) for studies on tolerance in plants. *In vitro* culture has been used in investigating responses of plants to toxic metal ions (Bozarckzuk, 2004). With the use of *in vitro* techniques, these researchers have selected plants tolerant to metal ions, including aluminum, copper, manganese and nickel. Numerous *in vitro* experiments have focused on the effects of high concentrations of heavy metals on the regeneration of plants tolerant or sensitive to industrial pollution (El-Aref and Hamada, 1998; Ramgareeb, 1999). Selection of plants under natural conditions of environmental pollution or *in vitro* may result in the selection

of clones tolerant to toxic metal ions (Gori et al., 1998; Barnabas et al., 2000). *In vitro* selection of plants tolerant to toxic ions contained in the soil may lead to production of plants that are better adapted to environmental pollution and can enable better management of degraded soil (e.g. industrial areas and highways).

Single node explant of jojoba has been used in study of the role of polyamines in adaptation mechanism of jojoba under saline conditions (Roussos and Pontikis, 2007). Such *in vitro* system would also be valuable material for research on mechanisms of metal tolerance in plants sensitive to toxic metal ions. In the present investigation, jojoba shoot explants were cultured in media containing various concentrations of Cr, Cu and Cd. Effect of these metals on jojoba tissues under culture was studied by estimating the SOD, CAT, GPX and lipid peroxidation.

4.2 EXPERIMENTAL PROTOCOL

In vitro culture and metal treatment

Jojoba culture, maintained in modified Schenk and Hildebrandt (SH) medium (Chaturvedi and Sharma, 1989) were used for this experiment. The media were supplemented with 3 % (m/v) sucrose as carbon source. The pH of the media was adjusted to 5.8 and gelled with 0.7 % agar. Stock solution (100 mM) of potassium dichromate ($K_2Cr_2O_7$, copper sulfate ($CuSO_4$) and cadmium chloride ($CdCl_2$) were prepared and filter sterilized. After autoclaving the media, suitable aliquot from stock solution was added to medium to attain the final concentration of 50, 100, 200 and 300 μM of Cr, Cu and Cd respectively. After addition of metal salts, the media were stirred thoroughly before distribution in culture tubes. Twenty ml medium was poured into each tube.

Jojoba shoots were cultured in test tubes containing medium supplemented with individual heavy metal salts like $K_2Cr_2O_7$, $CuSO_4$ and $CdCl_2$. Medium without metal was used as control. Two explants were cultured in each test tube. Cultures were incubated at 25 ± 2º C under 16 hr photoperiod in 32 μmole $m^{-2}s^{-1}$ light intensity. The experiment was repeated thrice with twenty replicates in each treatment.

Enzyme activities and lipid peroxidation under different stress conditions and exposure were determined in leaves isolated from shoots. These estimation were carried

out after 1, 7 and 14d of culture. Metal estimation was carried out in stem and leaves after 14d of incubation All of these analysis were repeated thrice.

Lipid peroxidation and enzyme activity

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

Gravimetric analysis

On harvesting the shoot culture after 14 days, stem and leaves were separated and were used for metal estimation. Base of the shoot cultures were thoroughly washed with deionized water to remove adhering medium. Drying the shoots on filter paper eliminated adhering moisture. Tissues of four cultures from each concentration were pooled for each analysis and weighed (Fresh weight-FW). These were dried in oven at 80 ^{o}C till constant weight was reached and dry weight was noted. The dry weights per gram of fresh weight (dw/fw) were determined.

Metal estimation

The dried plant samples were ground to fine powder with pestle and mortar. Weighed amount of powder was used for Cr, Cu and Cd estimation. The samples were digested in HNO_3: $HClO_4$, 3:1 mixture and Cr, Cu and Cd concentration were determined by AAS (Sunil Kumar et al., 2008a).

Data analysis

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

4.3 RESULTS AND DISCUSSION

Jojoba (*Simmondsia chinensis*) is a slow growing, dioecious plant, which grows in arid region under marginal conditions. This plant has deep root system and has the ability to thrive in unfavorable conditions. The ability of this plant to tolerate toxic metal has never been tested systematically. In the present study we used shoot culture of female plant of this species. *In vitro* cultures are good models for studying effect of different metals from their deficiency to toxicity (Baťková et al., 2008). *In vitro* system (nodal explants of *in vitro* shoots) have been used in study of heavy metals stress in- *Bacopa monniera* and - *Holarrhena antidysenterica* (Ali et al., 1998; Agarwal and Sharma, 2006) and shoot cultures of jojoba have been used in study of salt stress (Roussos and Pontikis, 2007). Plant tissue culture provides an excellent tool to assess the natural tolerance of plants for various metal salts and to study the mechanism of metal tolerance.

Antioxidative enzymes are important tools, which protects plants from stress. SOD causes dismutation of superoxide radicals at almost diffusion limited rates to produce H_2O_2 (Salin, 1987). It plays an important part in determining the concentration of O_2^{-1} and H_2O_2 in plants and hence performs a key role in the defence mechanism against free-radical toxicity (Bowler et al., 1992). However, function of catalase and peroxidase is to catalyze the removal of H_2O_2.

Table 4.1 Metal (Cr, Cu and Cd) accumulation in different parts of jojoba after 14 days of incubation. Values presented are means ± Sd (n=3)

Metal Supply (μM)	**Metal accumulation (Stem) mg/kg DW**		
	Cr	**Cu**	**Cd**
0	21 ± 3.53	27 ± 12	0
50	80 ± 7.77	189 ± 15	81 ± 1.41
100	140 ± 14.1	212 ± 7.8	270 ± 1.36
200	147 ± 3.53	957 ± 6.3	343 ± 19.0
300	168 ± 3.53	959 ± 24	528 ± 18.3
ANOVA	Sig 1%	Sig 1%	Sig 1%
	Leaves mg/kg DW		
0	20 ± 0.70	21 ± 1.1	0
50	111 ± 7.07	141 ± 23	75 ± 6.36
100	167 ± 12.7	148 ± 29	118 ± 2.82
200	171 ± 6.36	518 ± 21	241 ± 15.5
300	282 ± 11.3	776 ± 9.8	299 ± 7.71
ANOVA	Sig 1%	Sig 1%	Sig 1%

Cr Effect

Accumulation of Cr in jojoba shoot culture was less as compared to Cu and Cd (Table 4.1). Cr accumulation was more in leaves than in stem. It increased significantly in both stem and leaves with increasing concentration of Cr in medium (Table 4.1). The pattern of antioxidant enzyme activity in Cr exposed leaves varied with concentration and exposure. In presence of Cr, stimulation of SOD activity was noticed at 50 and 100 μM after 1d in Cr metal (Fig. 4.1 A). It remained unaffected at higher concentrations (200 and 300 μM). After 7d exposure activity of SOD increased at 100 μM, and reduced in 300 μM. After 14d, SOD activity at 50, 100 μM and 300 μM of Cr was significantly less than control. In Cr treatment, CAT activity did not alter significantly after 1d exposure. However, after 7d exposure CAT activity was significantly elevated at 50 μM (Fig. 4.1 B). At end of 14 day there was significant increase in CAT activity at all concentration of Cr tested. It is apparent that jojoba leaves responded by elevation in CAT activity with extended incubation and increased concentration of Cr. In GPX activity there was no significant change after 1d of exposure in Cr (Fig. 4.1 C), whereas it increased

significantly after 7d of exposure in 50, 100, and 200 µM. There was an overall decrease in lipid peroxidation product level (Fig. 4.1 D) with extension of culture period and the changes in these products in the leaves with concentration and incubation period were not significant.

From the data it appears that, jojoba leaf responded to Cr stress, and the activity of the antioxidative enzymes increased. Similar response was noted in *O. tenuiflorum* leaves when exposed to Cr containing media (Rai et al., 2004). Similar increase in SOD and GPX

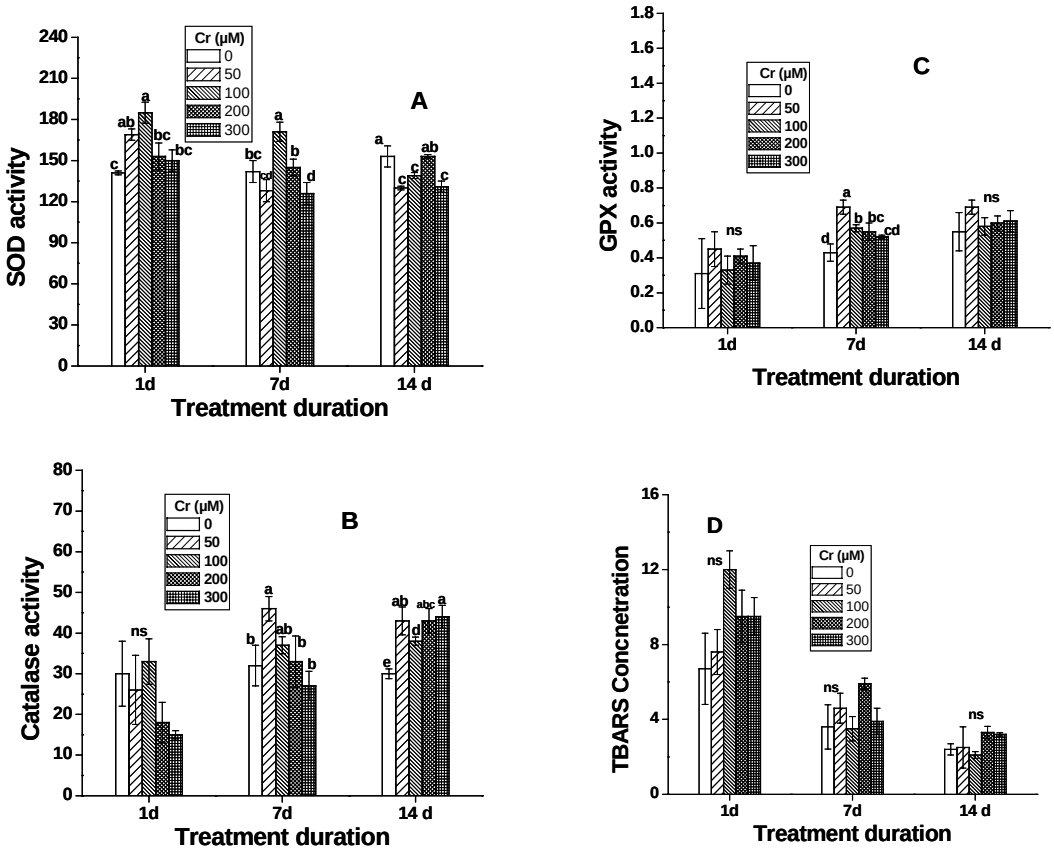

Fig 4.1 Effect of chromium on enzyme (A) superoxide dismutase (B) Catalase (C) guaiacol peroxidase and (D) lipid peroxidation in jojoba leaves. Means with common letter(s) are not significantly different at P< 0.05 according to Duncan multiple range test (n=3).

enzyme activity has been reported in *Amaranthus viridis* and *Glycine max* (Liu et al., 2008; Ganesh et al., 2008). The combined action of SOD, CAT and GPX is critical in

mitigating the effect of oxidative stress as enhanced production of ROS can lead to membrane destabilization and can be cytotoxic. In absence of any adopted protective mechanism, cell structure and function can be severely damaged. At the end of experiment decreased activity of SOD leads to accumulation of superoxide ion, but increased activity of CAT and GPX can degrade the accumulated hydrogen peroxide thereby helping to fight the oxidative stress induced by Cr stress in jojoba leaves.

Cu Effect

Accumulation of Cu in jojoba shoot was more as compared to Cd or Cr treatment (Table 4.1). Cu accumulation was more in stem as compared to leaves. It increased significantly in both stem and leaves with increasing concentration of Cu in medium (Table 4.1). In Cu treated shoots, after exposure of 1d SOD activity was not affected significantly. With increasing exposure of time for 7d SOD activity decreased significantly with increasing concentration of Cu in the medium (Fig. 4.2 A). Extended incubation of 14d did not cause further diminution in SOD activity and was maintained till 14d.

In Cu, CAT activity remains unaffected after exposure of 1d and increased significantly after 7d in 50 μM and decreased in higher concentrations. It was minimum in 300 μM (Fig. 4.2 B). Guaiacol peroxidase activity in leaves was decreased significantly after 1d (Fig. 4.2 C) in Cu containing medium. The changes in GPX activity was not significant statistically when estimated after 7d. However after 14d the GPX activity was inhibited significantly in all concentrations of Cu. Cu was more inhibitory for GPX activity as compared to Cr and Cd. In Cu, there was no change in lipid peroxidation after 1d of exposure but after 7 and 14d there was significant increase in TBARS accumulation at 200 and 300 μM respectively (Fig. 4.2 D).

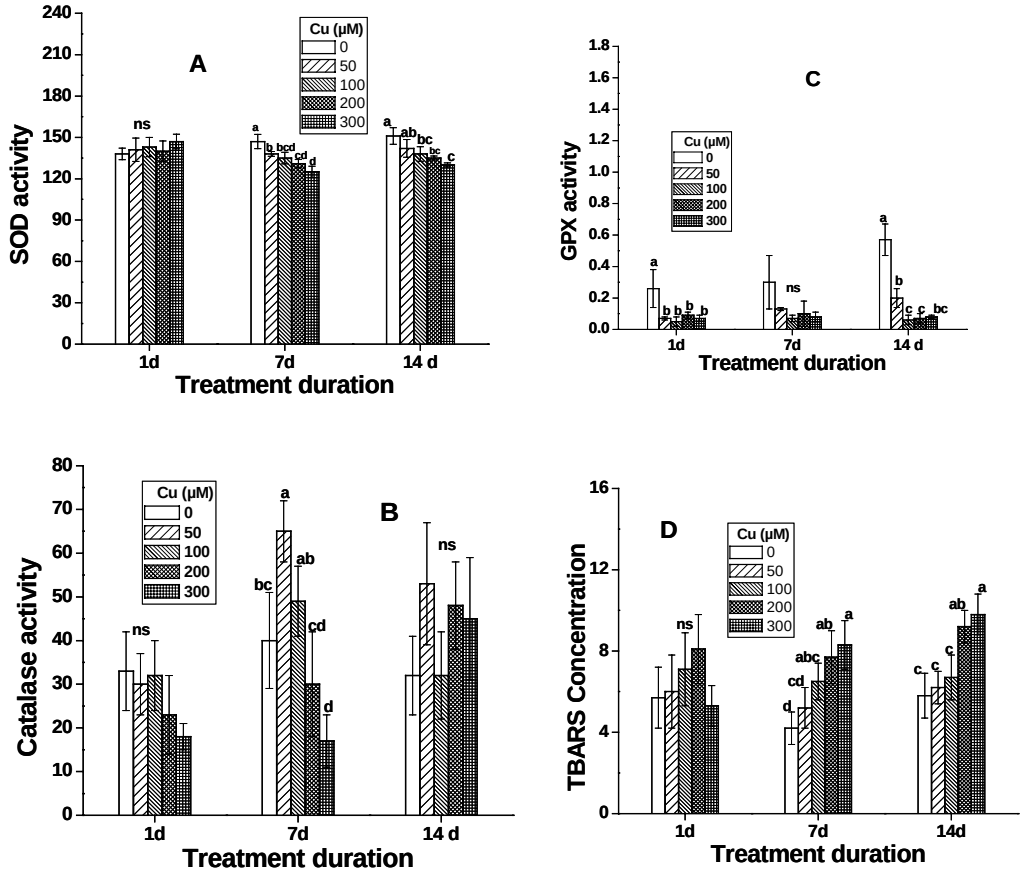

Fig 4.2 Effect of copper on enzyme (A) superoxide dismutase (B) Catalase (C) guaiacol peroxidase and (D) lipid peroxidation in jojoba leaves. Means with common letter(s) are not significantly different at P< 0.05 according to Duncan multiple range test (n=3).

In Cu treatment CAT was more active as compared to activity of SOD and GPX. At the end of experiment, activity of SOD and GPX in Cu treated leaves was less than control. The Cu mediated decrease in SOD and GPX activity may possibly be the result of either a direct effect of Cu ions on the gene for SOD and GPX or it may be indirect effect, mediated via an increase in $O_2^{-\bullet}$ radicals (Khatun et al., 2008). Khatun et al. (2008) reported the decrease in activity of SOD and CAT in *Withania somnifera* due to Cu stress. Increase in SOD and CAT activity was observed in peach root stock grown at 100 µM of Cu (Lombardi and Sebastiani, 2005). Increase in SOD and GPX has been reported in *A. thaliana* due to Cu stress (Drazkiewicz et al., 2004).

Cd effect

Accumulation of metal increased significantly in both stem and leaves with increasing concentration of Cd in medium. Stem accumulated more metal as compared to leaves (Table 4.1). Changes in SOD activity was noted with concentration and exposure (Fig. 4.3 A). After 1 and 7d of exposure in cadmium SOD activity was significantly reduced as compared to control. Optimum suppression of SOD activity in leaves was observed when shoot cultures were exposed to 300 μM for 7d (Fig. 4.3 A). In the higher concentrations (200 and 300 μM), SOD activity was always less than control in all the treatment durations. It was noted that the SOD activity which was reduced after 7d in 50 μM concentration, revived, and was similar to control on extended exposure. The SOD activity in leaves revived in 300 μM too, but was still less than control after 14d. It is interesting that inhibition of SOD activity was more pronounced at all time point, at higher concentration (200 and 300 μM) in Cd treatment as compared to Cr treatment. Reduction in SOD activity in jojoba leaves subjected to higher concentration of Cd may result from deficiency of metal essential for catalytic action of this enzyme. SOD is a metalloenzyme containing Fe, Cu/Zn or Mn in its prosthetic group and high concentration of Cd have been shown to decrease Fe, Mn and Zn (Gussarsson et al., 1994). Decreased SOD activity due to Cd treatment has been reported in tomato leaves (Chamseddine et al., 2008). There was no significant change in CAT activity in the leaves of the shoots exposed for 1d in Cd (Fig. 4.3 B). However, the activity of this enzyme decreased significantly at higher concentrations (200 and 300 μM) in leaves of the shoots exposed for 7d and remained low upto 14d. Optimum inhibition of CAT activity was noted in leaves of the jojoba shoot culture, exposed for 14d in 300 μM Cd. The GPX activity in control medium tend to increase in jojoba shoots with exposure. In contrast to SOD and CAT, there was significant increase in GPX activity in the leaves after the exposure of the shoots for 1d at higher concentrations (200 and 300 μM) of Cd (Fig. 4.3 C).

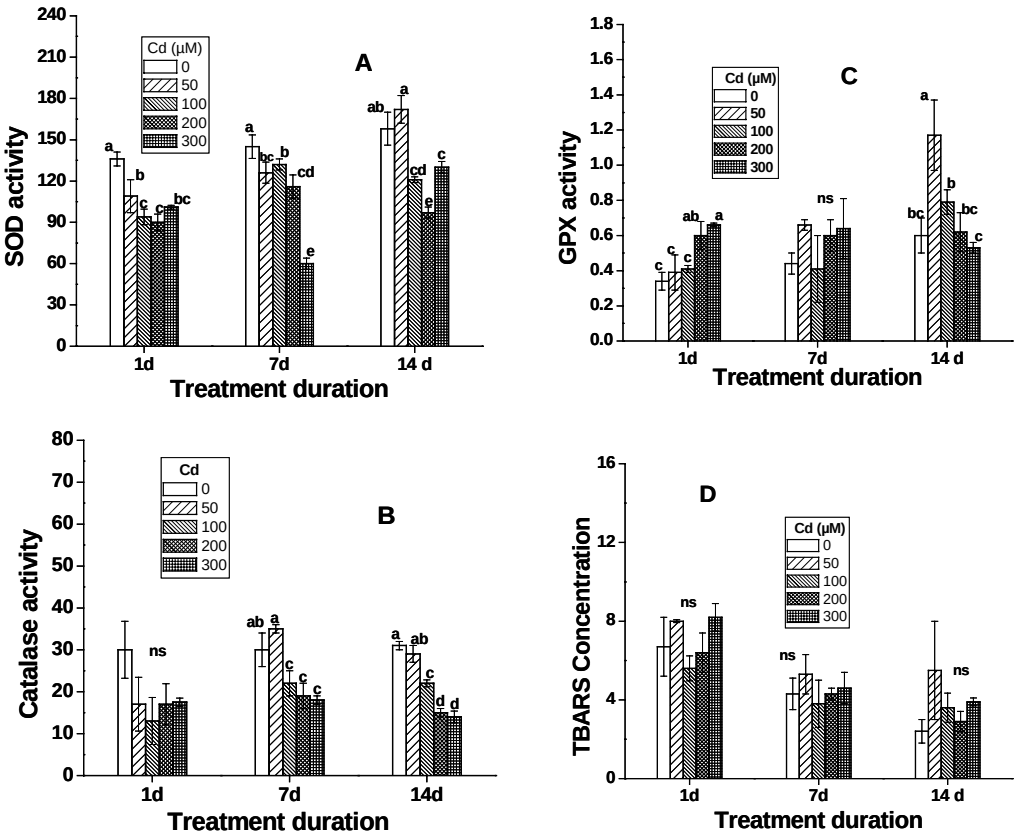

Fig 4.3 Effect of cadmium on enzyme (A) superoxide dismutase (B) Catalase (C) guaiacol peroxidase and (D) lipid peroxidation in jojoba leaves. Means with common letter(s) are not significantly different at P< 0.05 according to Duncan multiple range test (n=3).

Thereafter, there was no further increase in the activity of GPX after 7 or 14 days in these concentrations of Cd but in 50 µM the enzyme activity tend to increase with exposure and was significantly high after 14d in Cd. This increase in activity at this concentration could be due to direct interaction or indirect activation of GPX enzyme by Cd ions. There was no change in lipid peroxidation in any concentration or exposure in Cd (Fig. 4.3 D). Compared to the 1d culture the lipid peroxidation product levels were reduced with extended culture in media without or with Cd although data were not significant statistically.

From the results of this experiment it is apparent that in presence of Cd, the increase in Cd accumulation in the leaves was associated with decrease in SOD and CAT

activities and TBARS content whereas GPX activity increased. The decrease in SOD and CAT activities could be due to the stress imposed on the shoots. Increase in GPX activity suggest activation of the intrinsic defence tool to resist Cd induced oxidative damage.

Decrease in SOD and CAT activities has been noted in leaves of *Miscanthus sinensis* when it was challenged with Cd (Scebba et al., 2006). This suppression of CAT activity by Cd might be due to increased accumulation of superoxide ion as activity of SOD was diminished. CAT is sensitive to O_2^{-1} radicals and thus their increasing level due to decreased activity of SOD under stress may result in inactivation of enzymes (Cakmak, 2000). However, despite the inhibition of CAT activity, accumulation of its substrate, H_2O_2 might not occur since it could be utilized in GPX catalyzed reaction.

Literature on comparisons of effect of different heavy metal on SOD, CAT and GPX are scarce. Differential antioxidative enzyme response was noticed in jojoba leaves due to stress induced by the metals tested. Increase in CAT and GPX activity has been observed in *Matricaria chamomilla* leaves, when exposed to Cd or Cu for different time periods (Kova´cˇik and Backer, 2008). Similar increase in CAT and GPX activity has been observed in tomato leaves, exposed to Cd or Cu for different time periods (Chamseddine et al., 2008). In jojoba leaves inhibition of SOD activity by Cd, Cu and elevation by Cr indicate different chemical properties of these elements, therefore different responses evoked by metal treatment In jojoba leaves Cd at higher concentration (200 and 300 μM) was found to be more inhibitory for SOD activity as compared to Cr and Cu in every time point. Interestingly, CAT activity was not affected after 1d exposure in any of the metal tested. Chromium was more stimulatory for catalase activity as compared to Cd and Cu.

Lipid molecules are very sensitive to the reactive oxygen species (ROS) generated under oxidative stress conditions and the presence of lipid peroxides is generally accepted as an indicator of stress (Becana et al., 1998). Excess level of metal ions particularly redox metals have been reported to induce the production of ROS in plants. In jojoba leaves unaltered TBARS content in Cd and Cr may indicate that oxidative stress was not sufficient to alter membrane composition. In our experiments, copper was the strongest inductors of lipid peroxidation (Fig. 4.2 D) in jojoba leaves. The main site of attack by

metal in a plant cell is usually the cell membrane and may result in significant damage to cellular component (De Vos et al., 1991, Vangronsveld and Clijsters, 1994).

Table 4.2. Effect of Cr, Cu and Cd on DW g^{-1} FW in different parts of jojoba shoots after 14 days of incubation. Values presented are means ± Sd (n=3).

Conc. of metal used	**Cr**	**Cu**	**Cd**
	Stem Dw/fw (g) mean ± Sd	**Stem Dw/fw (g) mean ± Sd**	**Stem Dw/fw (g) mean ± Sd**
Control	0.23 ± 0.01	0.17 ± 0.01	0.22 ± 0.01
50 µM	0.24 ± 0.02	0.29 ± 0.03	0.32 ± 0.02
100 µM	0.23 ± 0.02	0.25 ± 0.01	0.26 ± 0.05
200 µM	0.27 ± 0.04	0.22 ± 0.03	0.27 ± 0.05
300 µM	0.24 ± 0.01	0.20 ± 0.01	0.22 ± 0.02
ANOVA	**NS**	**Sig 1%**	**NS**
	Leaves Dw/fw (g)	**Leaves Dw/fw (g)**	**Leaves Dw/fw (g)**
Control	0.15 ± 0.01	0.18 ± 0.01	0.22 ± 0.06
50 µM	0.20 ± 0.01	0.20 ± 0.02	0.25 ± 0.02
100 µM	0.19 ± 0.03	0.21 ± 0.02	0.23 ± 0.01
200 µM	0.22 ± 0.01	0.20 ± 0.01	0.25 ± 0.13
300 µM	0.21 ± 0.01	0.17 ± 0.03	0.23 ± 0.11
ANOVA	**Sig 1%**	**NS**	**NS**

Jojoba shoot cultures were grown in various concentrations of Cr, Cu and Cd (Fig. 4.4) for 14d. Accumulation of metal was estimated in stem and leaf of Jojoba culture after 14d of exposure. The DW g^{-1} FW were determined to assess the increase in mass due to accumulation of the metal in each organs (Table 4.2). Jojoba shoots showed differential accumulation of three metals. Leaves accumulated more Cr metal as compared to stem. There was parallel increase in DW g^{-1} FW in leaves suggesting the possible accumulation of Cr metal in leaves. Accumulation of metal was significantly increased in both stem and leaves with increasing concentration of chromium in medium. In case of Cd and Cu stem was found to accumulate more metal as compared to Cr metal. Jojoba shoots accumulated maximum copper followed by Cd and Cr. This is also evident by the increase in DW g^{-1} FW of Cu treated shoots as compared to control shoots.

Maximum accumulation of Cu in stem may be due to the reason that without root, vasculature of shoot is directly exposed to medium, where specific transporter for Cu (Yruela, 2005) may have absorbed the highest amount of metal. The non-significant change in DW g^{-1} FW of stem (Cd,) and leaves (Cd, Cu) could be due to Cd and Cu induced cytotoxicity in these organs. The reduced activity of antioxidative enzymes in leaves (Cd, Cu) can be correlated with cytotoxicity of these metals. The plants can adopt different strategies/mechanisms to tolerate heavy metal in the soil/growing media. There are different mechanisms such as exclusion, reduced heavy metal uptake, inclusion (sequestering and compartmentalizing metals in organs and organelles) and phytochelating binding (Baker, 1981; Hall, 2002; Lombardi and Sebastiani, 2005). Our data suggests that jojoba shoot culture might adopt reduced metal uptake for Cr at the concentrations used. But once Cr was inside the jojoba shoot it was able to transfer the metal to leaves efficiently. In relation to higher TBARS content at the end of experiment in Cu treated jojoba shoots stronger negative effect on reduced homeostasis can be assumed, which can be correlated with maximum accumulation of copper in leaves as compared to Cd or Cr. Cu induced more oxidative stress evidenced by increased lipid peroxidation product level in leaves. It can be correlated with increased Cu accumulation in leaves. An increase in the intracellular concentration of copper produces the reactive hydroxyl radical (OH·) by reaction of superoxide radicals with H_2O_2 through a metal-catalyzed Haber-Weiss reaction thereby causing the increased lipid peroxidation product level in jojoba leaves.

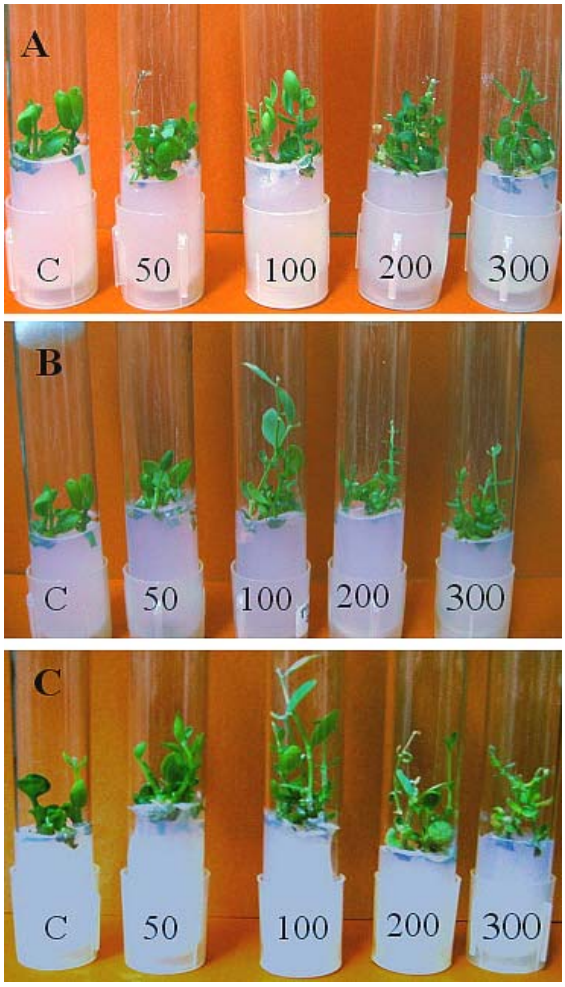

Fig 4.4 Jojoba shoot cultures in various concentrations of Cr (A), Cu (B) and Cd (C) after 14d of exposure. Treatments are in μM.

4.4 CONCLUSIONS

In our study jojoba was found to be more tolerant towards Cr followed by Cd and Cu. Higher level of enzymatic activities, unaltered TBARS content and reduced metal uptake suggests the reason for tolerating higher level of Cr in jojoba. In Cd treatment GPX and in Cu treatment CAT was more active to fight the oxidative stress induced by these metals. Without roots the jojoba shoots showed differential effect on metal accumulation, lipid peroxidation and antioxidative enzymes, which suggest the different mechanism of tolerance in jojoba shoot culture for these metals. Variation in enzyme

activities may be due to different chemical properties of these metals leading to differential interaction with various enzymes. Difference in jojoba response to Cr, Cu and Cd can be explained by a divergence in some other heavy metal defence mechanisms. Therefore it confirms that such *in vitro* system could be used for studying the mechanism of stress conditions. Metal toxicity obviously altered the enzymatic activities involved in oxidative stress metabolism. Higher concentration of Cu caused oxidative damage as evidenced by increased lipid peroxidation. Due to Cu treatment, GPX activity was severely affected at all time points, as compared to Cd or Cr treatments. Our findings add new comparative information on antioxidative response of plant under heavy metal stress. The result indicates that the early oxidative stress induced by all the three metals was less pronounced, however at the end of experiment oxidative stress was more pronounced with copper as compared to cadmium and chromium. This is evident by increased lipid peroxidation induced by copper. In conclusion this study demonstrates that *in vitro* system could be a useful approach to study biochemical and physiological alterations induced by Cd, Cr or Cu in jojoba shoot cultures. Jojoba shoots elevated SOD, CAT and GPX activities in order to mitigate Cr stress damages. Thus, this jojoba shoots can be considered quite tolerant to chromium.

CHAPTER 5

Studies on metal toxicity in Pongamia pinnata

5.1 INTRODUCTION

Pongamia (Indian Beech) is a genus having one species *Pongamia pinnata* L. [*Pongamia glabra*, Vent.; *Derris indica*, Lamk.]. It belongs to the family Leguminosae and sub-family *Papilionaceae*.

Pongamia, popularly known as Karanja, is an important shade tree of India. The Pongam tree is considered to be native to India (Western Ghats). It is also found in China, Florida, Malaysia, Seychelles, Philippines and Australia (Daniel, 1997). Pongam is capable of growing under a wide range of agroclimatic conditions and is common around coastal areas, riverbanks, tidal forests and roadsides. It is medium sized glabrous, fast growing, evergreen tree, attains generally 8–18 ft height (Anonymous, Wealth of India, 1998). The trunk is short with drooping branches. Bark is greyish brown covered with tubercles. It has imparipinnate leaves with 5-7 ovate or elliptical leaflets. Presence of viral galls in leaves is a frequent incident. It has flowers, which are pinkish to white. Different stages of pod formation are seen in the fruit. The pod is thick, woody, compressed, and elliptic to obliquely oblong with a short curved beak. Seeds usually one or two, are thick, reniform, broad wrinkled with reddish brown leathery testa. The seeds are said to be viable for a year. Natural reproduction is through seed or by root suckers. The germplasm (2n=22) is reported to tolerate drought, frost, heat, limestone, salinity, sand and shade (Sujatha, 2007).

The tree starts bearing seeds at the age of 4-7 years (Sujatha, 2007). The harvest season extends in general from November- December to May-June. The yield of the seed is said to range from 9-90 kg per tree, indicating a yield potential of 900-9000 kg/seed/hectare. The seeds are mainly valued for the oil obtained from them that has many industrial and medicinal applications.

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, agricultural and waste disposal technologies (Shanker et al., 2005a; Yruela, 2005). These when enter into the

soil have definite adverse effects on plant and animals and thus ultimately on human health. Chromium is highly toxic to plants and is detrimental to their growth and development. Copper 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 the important cellular processes such as photosynthesis and respiration and inhibits plant growth (Yruela, 2005). Cadmium is not an essential element for plant metabolism. Cadmium is added to soil from the metal working industries, waste, and urban traffic, cement factories and as a byproduct of phosphate fertilizers (Pal et al., 2006). It can cause many toxic symptoms, such as inhibition of growth and photosynthesis, activation or inhibition of enzymes, disturbances in plant water relationships, ion metabolism and formation of free radicals (Pal et al., 2006). These two metals (Cd and Cu) are of great importance as environment pollutant because they are very commonly found in industrially contaminated soil and wastewater and sewage sludge (Salt et al., 1998). In view of the seriousness of metal pollution, considerable efforts are being directed towards development of new, more cost effective technologies to minimize contamination in soil.

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 their concentration of metal in the soil, as long as the metal in question is present (Prasad and Freitas, 2003). A *Holoptelia integrefolia* tree growing on manganese mine dump is identified for its ability to accumulate increased amount of manganese whereas two other species growing in the same location do not demonstrate

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 hyperaccmulate any one metal may infer some ability to accumulate other metals (Prasad and Freitas, 2003). However many of described hyperaccumulators lack characteristics important for phytoextraction: perennial habit, high biomass production, extensive root mass and high transpiration rate. The woody plants have been mentioned attributes and therefore the woody plants are target for application in remediation, e.g. poplar trees have already been used for cleaning up of Se (Pilon-Smits et al., 1998). Phytoremediator woody species, with (i) high biomass production, (ii) deep root system, (iii) high growth rate, (iv) high capacity to grow in soils with low nutrient availability and (v) high capacity to allocate metals in the trunk, can be an alternative for the recovery of degraded soils due to excess metallic elements. Phytoremediation using woody species is ecological and economically viable due to the low cost of implantation, promoting soil stabilization that limits the expansion of metallic contaminants (Almeida et al., 2007).

There are a few reports on different tree species, including *Salix* (willow), *Betula* (birch), *Poplus* (poplar), *Alnus* (alder), *Acer* (sycamore) (Rev; Pulford and Watson, 2003; Pulford and Dickinson, 2006) and on *Holoptelia integrifolia* (Raju et al., 2008) on different metals. Different studies have been done on willow and poplar species in field conditions to evaluate their potential for removal of metal contaminants in field (Eltrop et al., 1991; Greger and Landberg, 1999; Robinson et al., 2000; Granel et al., 2002; Klang-Westin and Eriksson, 2003; Keller et al., 2003; Rosselli et al., 2003; Laureysens et al., 2005; Meers et al., 2005). Overall, the findings suggest that several willow and poplar species tend to accumulate Cd and Zn. However, these studies were carried out in field conditions. However, 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. In other tree species such as *Albizia amara, Casuarina equisetifolia, Tectona grandis* and *Leucaena lueococephala* experiments were carried out in pot culture (Shanker et al., 2005b) in which *A. amara* was found to accumulate maximum chromium as compared to other species. 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 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). The first objective of this study was to determine the metal tolerance of *P. pinnata*. The second objective was to document the uptake and translocation patterns of the metals. Knowledge of the pattern of metal uptake and sequestration is important for environment projects, where metal translocation into above ground shoots or foliage, as opposed to roots, is essential for harvesting. As a rich source of tree-borne oilseeds, *P. pinnata* is a species of choice for waste utilization and value addition to waste land.

5.2 EXPERIMENTAL PROTOCOL

Plant materials and growth conditions

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 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). The pH of the media was adjusted to 5.8 prior to autoclaving.

After autoclaving, 100 µl, 200 µl, 400 µl, 600 and 800 µl of filter-sterilized solution of $K_2Cr_2O_7$ (29.4 g L^{-1}), $CuSO_4$ (24.9 g L^{-1}) or $CdCl_2$ (18.3 g L^{-1}) were added aseptically in the 200 ml molten medium. The final concentrations of Cu and Cd 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 16 h photoperiod 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. Shoot height and root length of the sterile seedlings were noted after 6 week to determine the morphological changes.

Metal Estimation

Chromium, Cu or Cd accumulated in the seed coat, cotyledons, root, stem and leaves of seedlings were determined using AAS. 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, dried on filter paper and weighed. These were dried in oven at 90–100°C till constant weight was achieved. These were ground into fine powder and stored. Powdered

samples 50–300 mg was digested in HNO_3: $HClO_4$, 3: 1 mixture for metal estimation using AAS. Metal content of each organ was determined using AAS.

Data analysis

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 at least three independent experiments.

5.3 RESULTS AND DISCUSSION

Plant tissue culture technique has been used extensively to study the effect of various biotic and abiotic factors on plant cells and tissues. While results obtained on soil grown specimens, certainly reflect real world conditions much better than *in vitro* systems. But they are expensive and time consuming and results in terms of metal accumulation and tolerance may be soil specific and therefore difficult to compare. Field-testing requires two or three years at least to determine response of the tree species. To some extent, these problems may be overcome by use of *in vitro* screenings. *In vitro* selection can enable differentiation between species and that the relative performance can broadly correspond to those observed in field. In the present studies this technique is applied to study the effect of Cr, Cu and Cd on developing pongamia seedlings. To explore the full potential of this tree in remediation of and value addition to the wasteland there is need to study the response of this plant in presence of polluting substances and the distribution of the metals in different organs.

Plant growth and development are essential processes of life and propagation of the species. They are continuous and mainly depend on external resources present in the environment. Growth is chiefly expressed as a function of genotype and environment, which consists of external growth factors and internal growth factors. Presence of metal in the external environment can lead to changes in the growth and development pattern of the plant (Shankar et al., 2005b). The effect of these metals (Cr, Cu and Cd) on pongamia growth has been discussed in the following section.

Effect of Cr on seed germination

Seed is a stage in the plant life cycle that is well protected against various stresses. However, soon after imbibition and subsequent vegetative developmental processes, they become stress sensitive in general. Seed germination is the first physiological process affected by metal stress. Thus the ability of a seed to germinate in medium containing metal would be indicative of its level of tolerance to metal (Peralta et al., 2001). Germination of seeds in media with and without Cr was asynchronous and some of the seeds germinated after 2 week of culture. Some of the cultures which developed bacterial contamination around the seeds in contact of medium were scored to determine the germination frequency 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. 5.1 A). There was no morphological change in the seedlings and the asynchronous growth was prevalent after incubation for 4 week. Assuming that the growth of the seedlings may synchronize with longer incubation, or the symptoms of metal stress may appear on extended culture of the seedlings, these cultures were incubated for an extended period of 2 week before the morphological observations were noted. The frequencies of germination in seeds ranged between 62–87% (Table 5.1). In medium without the metal, germination frequency was 71%. It was optimum (87%) in medium with 100 μM Cr. With increase in Cr concentration there was no significant decrease in the frequency of response.

Table 5.1 Effect of Cr on pongamia seed germination and seedling growth.

Cr^{6+} (µM)	**Germination frequency Mean ± Sd (10 d)**	**Shoot Ht Mean ± Sd cm (6 wks)**	**Root length Mean ± Sd cm (6 wks)**
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

*The figure in parenthesis indicate number of replicates

Decrease in germination frequency in presence of Cr are reported in *Echinochloa colona* at 200 µM, *Phaseolus vulgaris* at 500 ppm (i.e. 9.61 mM) (Shanker et al., 2005a) and in *Medicago sativa* at 40 ppm (i.e. 769 µM) (Peralta et al., 2001). Reductions of 32–57% in sugarcane bud germination were observed with 20 and 80 ppm Cr, respectively (Jain et al., 2000). The mean heights of the Pongamia seedling shoots in Cr ranged between 5.3 cm to 8.8 cm (Table 5.1). In medium devoid of Cr the height was 6.2 cm. There 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 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 *A. thaliana* using *in vitro* system (Castro et al., 2007). Similarly in an *in vitro* study in tumbleweed Cr (III) suppressed the root growth at 20 ppm (384 µM) (Gardea-Torresdey et al., 2005). The germination and growth of *P. pinnata* was not affected in the concentrations (0-800 µM) of Cr tested. The seedlings appear healthy and green with opened leaves in all the media with and without Cr (Fig. 5.1 A) after 6 week of culture.

This is in contrast to the earlier above reports describing decrease in plant growth in presence of Cr at the concentrations tested with pongamia.

Effect of Cu on seed germination

The frequency of seed germination in copper (50-400 μM) containing media ranged between 53–76% (Table 5.2). The pattern of germination frequency in Cu containing medium was different from the pattern noted in Cr containing medium. Although the germination frequency tends to increase with increase in metal ion in the medium, but the data was not significant statistically. Minimum frequency was noted in medium devoid of Cu. Similar nonsignificant effect on germination was noticed by Street et al., (2007) in *Bowiea volubilis* and *Merwilla natalensis* due to presence of different concentration of Cu (1, 2, 5, 10, 20 and 50 mg L^{-1}). However, they reported decrease in germination frequency of *Eucomis autumnalis* due to presence of copper at similar concentration. Seed germination was not affected in *Elsholtzia haichowensis, E. aypriani* and *E. ciliata* but shoot length was affected significantly at 50 and 100 μmol/L of Cu supply (Xia and Shen, 2007). However in our study there was no change in mean heights of seedlings in presence of Cu at the concentrations tested although a significant reduction in root elongation indicated adverse effects of copper stress (Table 5.2) in the roots. Lower concentration (50 μM) of Cu was stimulatory for pongamia seedling growth. Such stimulatory effect of low concentration of Cu on seed germination and seedling growth has been noticed in *Elsholtzia haichowensis* (Lou et al., 2004). The leaves of the seedlings remained partially unfurled (Fig. 5.1 B). The seedling growth of pongamia, especially the root growth was much more sensitive to Cu than seed germination.

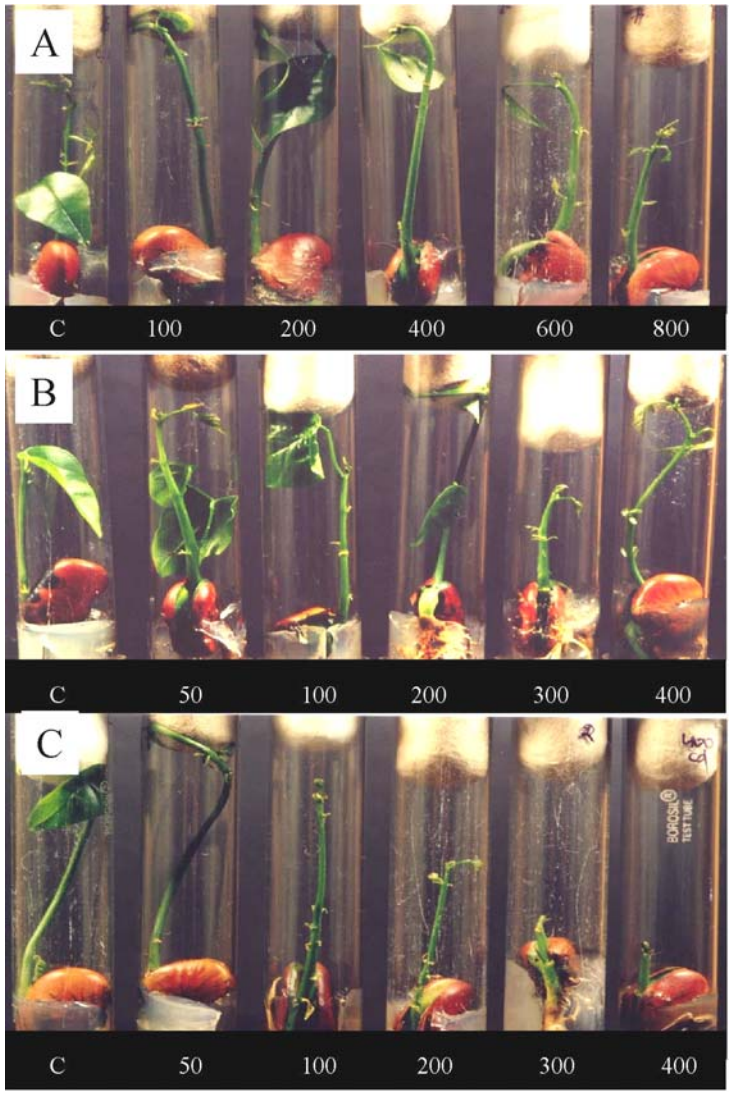

Fig 5.1 Effect of Cr (A) and Cu (B) Cd (C) on Pongamia seed germination and seedling growth after 6 weeks. Treatments are in μM.

The inhibitory action of Cu in root growth may be due to a reduction in cell division and retardation of normal root cell growth (Ouzounidou, 1994).

Table 5.2 Effect of $CuSO_4$ on pongamia seeds germination.

Cu^{2+} (µM)	Germination frequency Mean ± sd (10 days)	Shoot Ht Mean ± sd cm (6 wks)	Root length Mean ± sd cm (6 wks)
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	Sig 5%

*The figure in parenthesis indicate number of replicates

Some of the metals including Mn (100 µmol L^{-1}), Zn (30 µmol L^{-1}), Co (0.1 µmol L^{-1}), Mo (1 µmol L^{-1}), B (100 µmol L^{-1}), and Cu (0.1 µmol L^{-1}) are incorporated in the tissue culture medium in trace amounts as they participate in several enzymatic reactions as co-factors. Additional amount of Cu (50–400 µM) supplemented in the medium did not have any adverse effect on germination or shoot growth although it affected the root growth adversely.

Effect of Cd on seed germination

The frequency of seed germination in cadmium (50-400 µM) containing media ranged between 50–79% (Table 5.3). The germination frequency was suppressed at higher concentration of Cd as compared to control although data was not significant statistically. Cd has been shown to inhibit seed germination in various plant species (Peralta et al., 2001; Street et al., 2007; Li et al., 2005; Kuriakose and Prasad, 2008). Cd was found to affect seedling growth at the concentrations tested. The reduced germination of seeds under Cd stress is due to depressive effect of Cd on the activity of hydrolyzing enzymes like acid phosphatases, proteases and amylases and on the subsequent transport of sugars to the embryo axes as depicted in sorghum (Kuriakose and Prasad, 2008). Studies have shown that seed coat the main barrier to metals prevents

contamination of embryos until it is violated by the radicle. Our results support the idea that tissues covering embryo play a role in selective penetration of heavy metals in seeds. This was first suggested by the fact that seeds still germinated in high concentration of (400 µM) of Cd and Cu. But subsequently seedling growth was severely affected. A standard protocol to calculate indices of plant resistance involves measurement of the length of the main root in control and metal treatment (Baker, 1993). Root elongation and shoot elongation both were suppressed significantly (Table 5.3) due to Cd induced stress. Similar effects of Cd were seen in peanut seedling when treated with Cd (Sunil Kumar et al., 2008a). Roots represent the first barrier to the selective accumulation of ions present in the solution. The presence of Cd in the growth medium decreased the root growth of seedlings. Cell growth and consequently plant growth are severely inhibited by Cd toxicity (Prasad, 1995). In our study root growth and shoot growth was affected severely due to Cd toxicity (Fig. 5.1 C). Pongamia seedling growth was severely affected by Cd followed by Cu and Cr.

Table 5.3 Effect of $CdCl_2$ on pongamia seeds germination.

Cd^{2+} (µM)	**Germination frequency Mean ± Sd (10 d)**	**Shoot Ht Mean ± Sd cm (6 wks)**	**Root length Mean ± Sd cm (6 wks)**
0	79 ± 18.9 (31)*	6.12 ± 1.94 (19)	3.98 ± 1.14 (31)
50	86 ± 15.4 (34)	7.62 ± 1.54 (23)	3.41 ± 0.71 (33)
100	72 ± 21.8 (28)	5.00 ± 1.58 (20)	2.25 ± 0.66 (27)
200	79 ± 07.6 (31)	2.25 ± 2.72 (22)	1.16 ± 0.27 (30)
300	76 ± 22.5 (27)	1.75 ± 1.01(15)	0.82 ± 0.33 (26)
400	50 ± 10.0 (15)	0.73 ± 0.10(10)	0.79 ± 0.54 (13)
Anova	NS	Sig 1%	Sig 1%

*The figure in parenthesis indicate number of replicates

Chromium accumulation in pongamia

The data on the FW (Fig. 5.2) and Cr content (Fig. 5.3) in different parts of the seedlings reveal interesting observations. Compared to control there was no significant alterations in FW in any of the organs of the seedlings at any point of growth due to the presence of Cr. This is also evident from the unaltered shoot heights and root lengths (Table 5.1) of pongamia seedlings in various concentrations of Cr. This indicates that the seedlings tolerated 100-800 μM of Cr and maintained growth and differentiation of all the organs. The DW g^{-1} FW were determined to assess the increase in mass due to accumulation of the metal in cells/organs. For roots, stems and cotyledons the DW g^{-1} FW remained unaltered indicating non-accumulation of the metal in these organs. On the contrary in the leaves and the seed coat there was dramatic increase in DW g^{-1} FW indicating possible accumulation of Cr metal in these two organs. In seed coat the DW g^{-1} FW was optimum.

Metal content in different parts of seedling including seed coat, cotyledons, root, shoot and leaves was estimated. Presence of Cr detected in the organs of the control plant growing in medium devoid of Cr (Fig. 5.3) 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 the 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}) was detected in seed coat followed by root, leaves, cotyledons and stem (Fig. 5.3). The Cr content was low (37.5 – 279 mg Kg^{-1}) in the cotyledons although it was bonded to the seed coat, which shows highest amount of Cr content. Possibly the seed coat allowed selective absorption of moisture leading to imbibition of water in cotyledons and embryo axes to trigger germination. As a result the germination process which depends on the mobility of the storage products in the cotyledons remained unaffected.

On germination the roots of the seedlings came in direct contact of the medium. This resulted in absorption of the metal from medium and transport to the leaves through

the vasculature of stem. Roots have been known to accumulate more Cr (Chatterjee and Chatterjee, 2000; Barbosa et al., 2007). Compared to the control there was sharp increase in Cr content in both root (88.5–706 mg Kg^{-1} DW) and leaves (282-1214 mg Kg^{-1} DW) whereas it was very less (36-93 mg Kg^{-1} DW) in the stem. This is possibly due to accrual of the metal in the root upto the threshold level and thereafter transfer of the metal from medium to leaf via root and stem and deposition in the leaf. The leaves act as the sink and retain the metal ions, as it cannot transfer it to any other organ. The high concentration of Cr in the root at the basal end and leaves at the terminal end with low concentration of the metal in the intermediate organ (stem) is intriguing. Previous investigation 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). This phenomenon needs to be studied more closely to understand the mechanism of Cr transport that is active in pongamia and which helps in maintaining the low concentration of the metal in the stem. It may be presumed that due to the loss of moisture during transpiration, Cr is transported to the leaves with the moisture taken up by the seedling from the medium. 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 leaving the cortical tissue unaffected. The unaltered elongation of the stem in the seedlings (Table 5.1) in presence of different concentrations of Cr supports this hypothesis. High resolution imaging secondary ion mass spectrometry (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). With accumulation of Cr in the leaves the leaf opening was retarded but not restricted (Fig. 5.1 A). Leaf fall was not noticed in any of these cultures. In leaves chromium was considered to be complexed with oxalate ligands. Organic acid such as oxalic acid has been reported as potential chelators of Cr (Mangabeira et al., 2006).

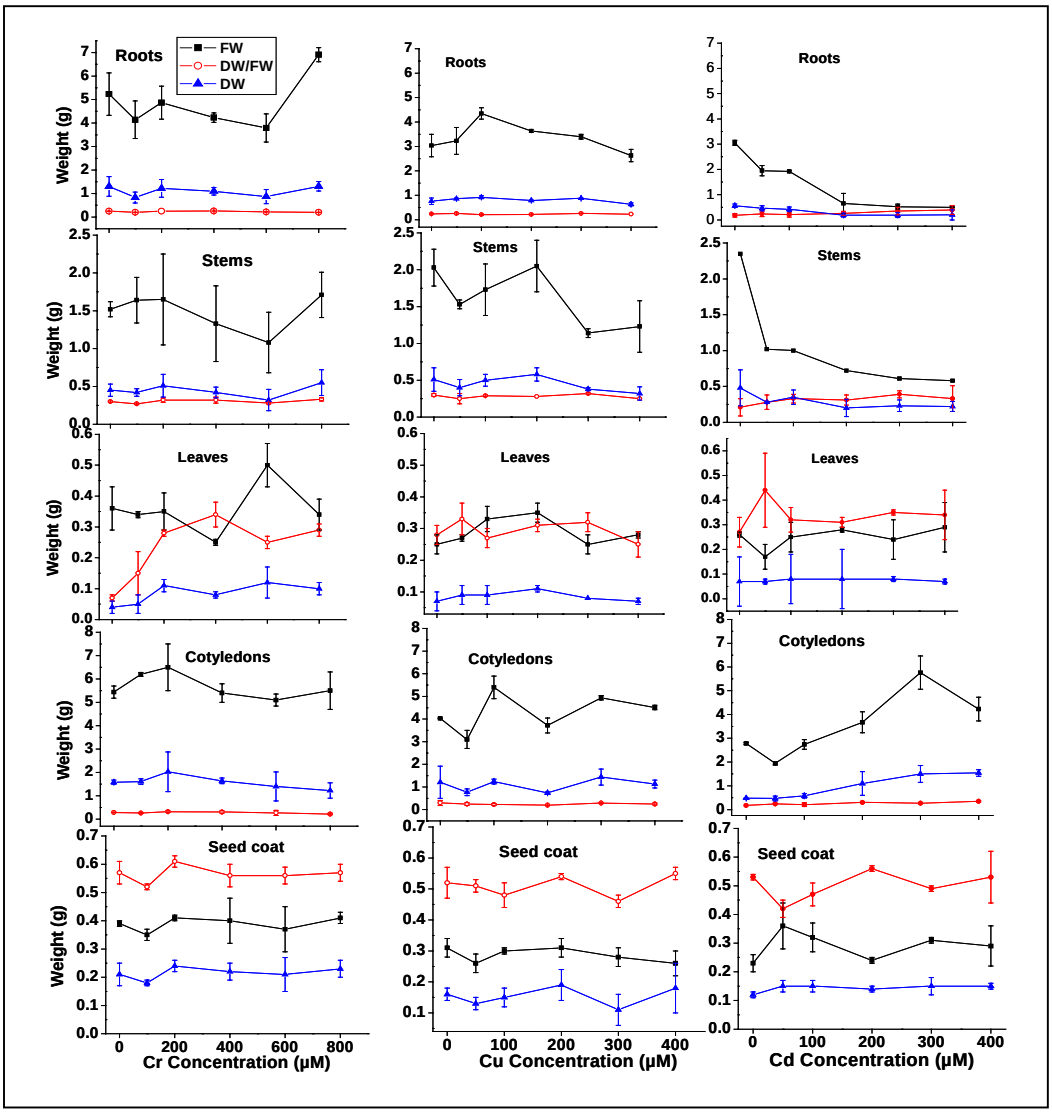

Fig 5.2 Effect of various concentrations of Cr, Cu and Cd on FW, DW and DW g^{-1} FW in the different parts of Pongamia seedlings. Values presented are means ± Sd (n=3).

Low Cr content in the cotyledons and high Cr content in the seed coat bonded to it (Fig. 5.3) suggest selective absorption of metal by the seed coat. Presumably, the seed coat absorbs the metal with moisture and selectively permits movement of water toward the cotyledons. This lead to mobilization of the storage products in cotyledons resulting

in unaltered germination frequency (Table 5.1) in presence of the toxic metal in medium. On germination the cotyledons were only 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 while in contact of the medium during and after germination.

In our study, there was significant accumulation of chromium 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 Shankar 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 chromium to aerial parts make these trees poor choice for phytoremediation of chromium contaminated sites (Shanker et al., 2005a). In contrast to these 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. 5.3) of *Pongamia pinnata*. 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. *Pongamia pinnata* seedlings grown *in vitro* could uptake the metal and transfer it to the other organs. It is evident from the data (Fig. 5.1 A, Table 5.1) 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 used for phytomining of Cr and for phytoremediation of Cr contaminated sites. Cultivation of this tree at these sites will not only minimize the Cr from the sites but also be a source of raw materials for production of biodiesel and Cr.

Copper accumulation in pongamia

Copper is required by biological systems as a structural and catalytic enzyme component and in the soil Cu^{2+} can be a stress factor by causing physiological responses that can decrease the vigour of the plants and inhibit plant growth (Lin et al., 2003). There was no significant difference in fresh weight of different organ of pongamia seedling due to copper stress (Fig. 5.2) although there was significant increase in copper content in different parts of seedling as compared to control. The result from this investigation indicated that *P. pinnata* has the potential ability to absorb and accumulate Cu from the medium. Like Cr pongamia seedling was able to translocate Cu to above ground parts of the plant. Seed coat accumulated maximum amount of Cu and the stem had the least. Copper content in different parts of seedlings was increased with increase in Cu in the medium. The roots of pongamia had 706 ± 26.4 mg of Cu Kg^{-1} of DW tissue as compared to 1823 ± 73 mg of Cr $Kg^{-1}$of DW (Fig. 5.3). In spite of lower Cu content, root elongation was affected adversely. However, the amount of Cu was more (1214 ± 152 mg Kg^{-1} DW) in the leaves compared to Cr (1004 ±24 mg Kg^{-1} DW). Root has been known to accumulate more Cu as compared to stem and leaves (Lin et al., 2003; Peng and Yang, 2007). The plant accumulated the Cu in roots less than the leaves of the seedling. Similarly *Datura stramonium* was found to accumulate more Cu in leaves as compared to roots (Boojar and Goodarzi, 2007). The mechanisms of Cu absorption and accumulation were different among various plant species. Chatterjee and Chatterjee, (2000) reported when cauliflower was exposed to Cu, a major portion of the Cu remained in the roots and only a little amount was translocated to the tops. Some plant root can accumulate elevated concentrations of Cu and prevent translocation of metals to the growing parts of the plant (Utriainen et al., 1997). In our study most of Cu uptake by *P. pinnata* seedlings was accumulated in the leaves (Fig. 5.3). Ouzounidou et al. (1994) observed that at high external Cu concentrations, the copper sensitive plants contained more copper in above ground part than the Cu tolerant plants. Similarly Lou et al. (2004) reported that most of Cu uptake by *E. haichowensis* (which is a Cu tolerant plant) was accumulated in roots as compared to above ground parts of the plant. This suggests that,

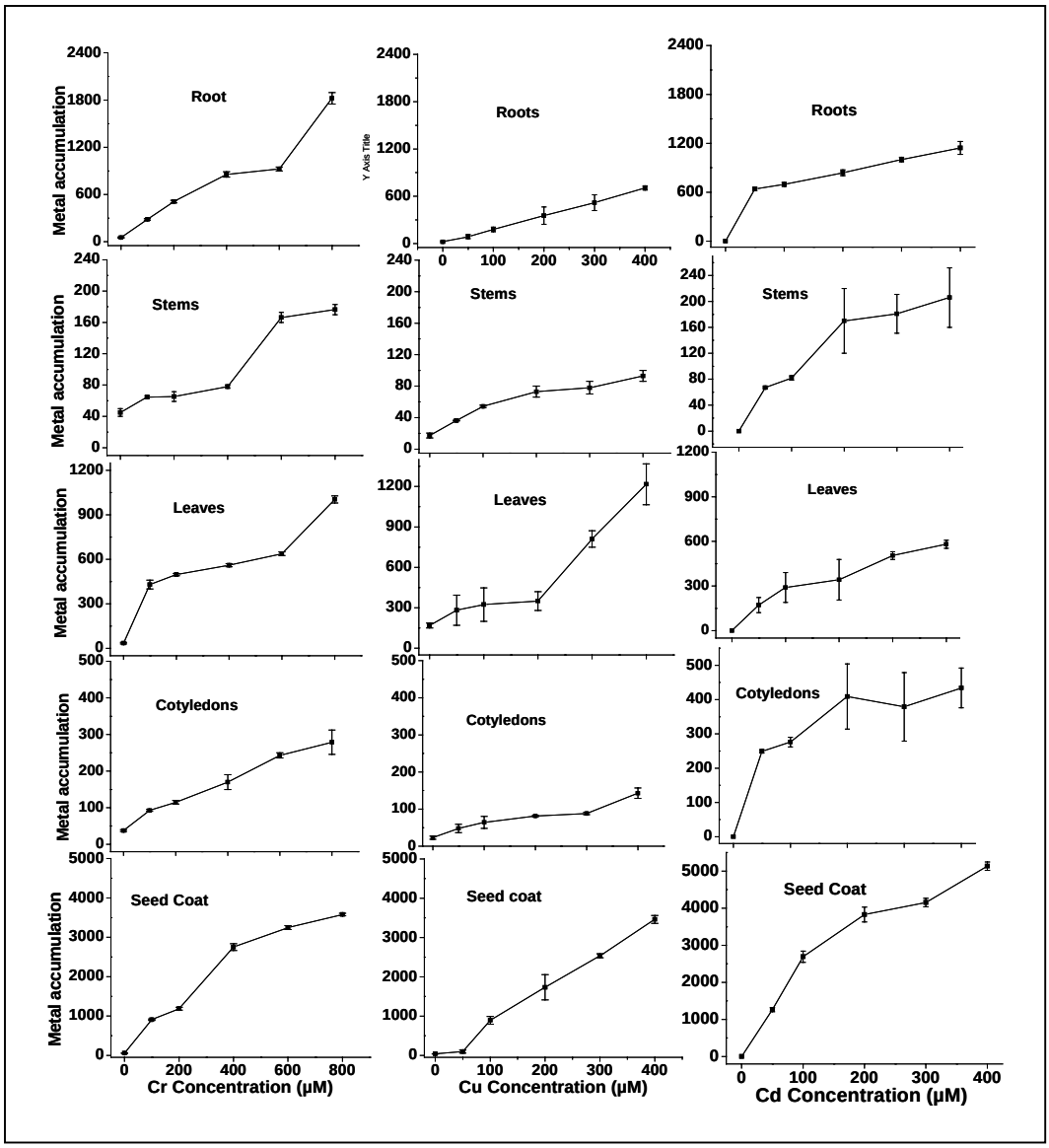

Fig 5.3 Chromium, copper and cadmium accumulation in different organs of *Pongamia pinnata*. Values presented are means ± Sd (n=3).

sensitive plant transport more Cu to above ground part than tolerant plants. It is possible that pongamia transported the Cu to leaves to protect the roots from excessive Cu stress. On crossing the threshold level at the root, Cu is translocated to the other organs and

deposited in the leaves which did not open fully. The unaltered elongation of the stem with low Cu content (93 ± 7.00 mg Kg^{-1} DW) supports the hypotheses that the metal transport through stem remains restricted to the vasculature.

Cadmium accumulation in pongamia

There was significant decrease in fresh weight (Fig. 5.2) of root and shoot due to Cd stress in plant. This is also evident from the suppression of shoot heights and root lengths (Table 5.3) of pongamia seedlings in various concentrations of Cd. There was increase in DW/FW ratio for root, stem and leaves as compared to control plant, although data were not significant statistically. Like other two metal pongamia seedling was able to translocate Cd to above ground parts of the plant. Seed coat accumulated maximum amount of Cd and the stem had the least. Cadmium content in different parts of seedlings was increased with increase in Cd in the medium. The roots of pongamia accumulated (1143 ± 80.0 mg of Cd $Kg^{-1}$of DW) cadmium less than chromium (1823 ± 73 mg of Cr $Kg^{-1}$of DW) but accumulation was more as compared to Cu (706.0 ± 26.4 mg of Cu Kg^{-1} of DW) stressed plant (Fig. 5.3). Interestingly the accumulation of cadmium in leaves (581 ± 26.0 mg of Cd $Kg^{-1}$of DW) as compared to the amount of Cu (1214.0 ± 152 mg Kg^{-1} DW) and Cr (1004 ±24.0 mg Kg^{-1} DW) was less. Seed coat showed optimum accumulation of cadmium (Fig. 5.3) similar to Cr and Cu as compared to other organs. Study of metal uptake showed that Cu has higher mobility in pongamia tissue and accumulated in leaf in amount much higher than Cr or Cd. But Cd and Cr were preferentially retained in roots (Fig. 5.3). However Kovacik and Backor (2008) showed that Cd was transferred in leaves and Cu accumulated in roots of *Matricaia chamomill.* Roots are known to accumulate more Cd followed by stems and leaves (Benavides et al., 2005; Garcia et al., 2006; Zhu et al., 2007). But in our study leaves accumulated high Cd as compared to stem. It was interesting to note that accumulation of metal in shoots of pongamia was least in all the metal tested in which accumulation of Cd in pongamia shoot was high as compared to Cr and Cu. This may support the known fact that Cd root to shoot transport is most likely driven by transpiration stream (Salt et al., 1995).

Accumulation of high Cd in stem in comparison to other two metals can explain the decreased length of shoots. High metal content in cotyledons could be explained, as it was in contact of medium so this tissue could have absorbed/ adsorbed metal from the medium.

Seed is a stage in the plant life cycle that is well protected against various stresses. However, soon after imbibition and subsequent vegetative developmental processes, they become stress sensitive in general. Therefore, seeds are thought to carefully monitor such external parameters as light, temperature and nutrient in order to maintain the protective state until external conditions become favorable for following developmental processes (Li et al., 2005). Maximum accumulation of metal in seed coat of pongamia therefore may have resulted in the unaltered germination frequency. It was observed that cadmium was more toxic for pongamia seedling growth followed by Cu and Cr.

The biggest advantage of using plants for cleaning the environment is the utilization of their inherent agronomic traits and benefits of plants like high biomass, extensive root systems, ability to withstand environmental stress, etc. (Bizily et al., 1999). Plant facilitated bioremediation is aesthetically pleasing and makes the environment green and clean. As the entire process is solar energy driven, no artificial source of energy is required to drive the bioremediation process, making it cost-effective and environmental friendly (Bizily et al., 1999). Plants offer a permanent, *in situ* non-intrusive, self sustaining method of removal of soil contaminant. Planting vegetation on a contaminated site also reduces erosion by wind and water. Phytoextraction enables to reclaim and recycle precious metals and other useful materials from the soil making the process economically beneficial for investors (Moffat, 1995). In addition, plants used in bioremediation do not disturb the topsoil, thus conserving its utility (Sykes et al., 1999).

5.4 CONCLUSIONS

In a phytoremediation program for soil contaminated by heavy metals, the plants having ability to tolerate increased amount of metals needs to be chosen. The present experiments demonstrate that: (1) *Pongamia pinnata* seedling can tolerate 100-800 μM concentration of Cr in growth medium and 50-400 μM concentration of Cu. (2) Differential accumulation of Cr, Cu and Cd content in the roots and leaves of pongamia seedlings suggests that this plant has a different mechanism for absorption of these metals by the roots and transport 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 three metals, differ. Cd was more toxic as compared to copper and chromium. (4) Low metal content in the stem with unaffected shoot elongation, indicate restricted or no accumulation of the metals in the stem. (5) High metal content in the seed coat and low metal content in the cotyledon bonded to it, 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 to develop biodegradable matrices 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.

Plant behavior in metal contaminated soils could differ from that in solution/medium, so species performance in the field should be evaluated. Other factors affecting the growth of field plants, include:

1. Spatial contamination of soil is heterogeneous, but root tend to proliferate in less contaminated regions. This decreases the metal uptake and translocation into the plant (Kuzovkina et al., 2004). In this research, plant roots were in contact with a homogeneous medium.

2. Mycorrhizae can protect plant roots in heavy metal polluted soils and probably decrease the translocation of metal. It is known that willows benefit from vesicular-arbuscular endomycorrhizal as well as ectomycorrhizal associations (Lodge, 1989). There is evidence that *Betula* tolerance to Zn may depend on an ectomycorrhizal association that limits plant tissue concentration of this metal because of metal adsorption to the surface of hyphae (Denny and Wilkins, 1987).

Summary

SUMMARY

Nature, a precious gift given to human by God, is gradually degraded by a serious problem of environmental pollution. Effluents waste released by industries is a major source of causing pollution and has been found to contain a number of pollutants, which are toxic for plant growth at higher levels. Heavy metals in soil are released from various anthropogenic sources, such as phosphate fertilizers, tanning industries and wastewater sludges. The toxic metals at higher concentration have created stressed environment. Chromium (Cr), copper (Cu) and cadmium (Cd) are important heavy metals and these are considered to cause serious problem to environment and exposure to these metals can be toxic to living cells.

Phytoremediation is a cost effective emerging technology based on the use of green plants to clean up the polluted sites and is accepted publicly. Research efforts made towards understanding the mechanism of metal tolerance has generated a great deal of information but it remains ill understood. Thus, there is scope for research to understand the mechanism of metal tolerance by various plant species and to identify the site and form of metal accumulation within plant system. This thesis entitled "**Studies on Metal Tolerance in Plants**" was designed to study the metal stress on different plant species including a herb (Peanut), a shrub (Jojoba) and a tree (Pongamia).

The present study was designed with the following objectives:

1. To study the influence of chromium, copper and cadmium induced stress on peanut (herb) seedlings cultured *in vitro*.
2. To study the influence of chromium, copper and cadmium on shoot culture of jojoba (shrub).
3. To study effect of chromium, copper and cadmium on pongamia (tree) seed germination, seedling growth and distribution of metal in different parts of plant.

In plants exposed to high metal concentrations, mechanisms to counteract the oxidative burst are crucial for its survival. Peanut (*Arachis hypogaea* L.) is a unique

leguminous plant for its characteristic behavior to produce the pods underground in direct contact with soil. It has the double advantage for absorption of Cd from soil through roots and directly through the shells. To investigate the temporal sequence of physiological reactions of peanut seedlings (*Arachis hypogaea* L.) to Cr, Cu and Cd exposure, seeds were cultured in increasing concentrations of these metals ranging from 50-1000 μM for Cr and 50-300 μM for Cu and Cd. Germination frequency was scored and the distribution of metal in root, stem and leaves were determined after 4 weeks of culture. Lipid peroxidation and activities of antioxidative enzymes including superoxide dismutase (EC 1.15.1.1), catalase (CAT; EC 1.11.1.6) and guaiacol peroxidase (GPX; EC 1.11.1.7) were estimated in these three parts of the plant.

Germination of peanut seedling was reduced significantly due to Cr and Cd treatment. Peanut seedling growth was suppressed at higher concentrations of these two metals. Peanut root absorbed the highest amount of metal followed by stem and leaves. Lipid peroxidation product level remained unaltered in all the three organs of peanut seedling treated with Cu. In case of Cr treatment at higher concentration (500 μM), lipid peroxidation product level increased significantly in root and stem. In leaves it remained unchanged. Peanut seedling showed differential response in antioxidative enzymes towards Cr and Cu induced stress. In peanut seedling upto 300 μM of Cr, either the enzyme activities remain unaltered or slightly affected. But at higher concentration (500 μM) of Cr either sharp increase or decrease in enzyme activities was noticed. 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. Similar pattern was noticed in GPX activity in stem and leaves. These activity patterns of the antioxidative enzymes system at 50 μM of Cu, may imply that the tolerance mechanism involves a system that reduces the formation of or removes free radicals, preventing the production of $O_2^{\bullet -}$ and, therefore, reducing the requirement to activate the antioxidative enzymes. But at higher concentration this system breaks down and antioxidative enzyme

activity decreases sharply. (**"Response of developing peanut seedling towards chromium and copper" manuscript under preparation**).

Germination of seedling was not affected but the growth of seedling was severely suppressed with increase in concentrations of $CdCl_2$. Pattern of Cd distribution in three organs varied with concentration of metal in medium. Increased lipid peroxidation was detected in all the three parts of the developing seedling with increase in metal accumulation (**Sunil Kumar et al., 2008, Journal of plant Nutrition and Soil Science 171 (3), 440-447**). Superoxide dismutase, CAT and GPX activity varied in the three parts of the seedlings with concentration of Cd.

Histology study was carried out to study the anatomical changes induced by these three metals. Peanut seedling 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). However, stem and leaves were taken after 30 d of exposure. Since root is the first organ to come in contact with the medium, we found it important to examine the effect of Cd, Cu and Cr added already to the medium used for germination of seeds. The Cd, Cu and Cr were steadily present in medium during the entire experiment. Due to Cr treatment, peanut responded by 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. However, Cu was inhibitory for development of steler region of peanut root. Similarly, Cd inhibited the differentiation of vascular bundles of peanut root. Chromium was more toxic to peanut stem as compared to Cu and Cd. In peanut leaf due to Cu treatment, granular deposition was noticed in palisade parenchyma cell layers. The results of the present study generate data on the effect of Cr, Cu and Cd stress in peanut seedlings and demonstrate the advantages of tissue culture model in study of the complex network of interactions of various factors in abiotic stress tolerance in plants.

Jojoba (*Simmmondsia chinensis*) is an industrial crop – its seed wax is used in the cosmetic industry and as a lubricant with considerable potential in arid and semi-arid regions. Research on water status and photosynthesis rates has shown that jojoba has

ability to withstand relatively high levels of salinity. There is evidence that nodal segments of jojoba *in vitro* respond to salinity in a similar way as the whole plant, therefore *in vitro* culture could be used for preselection and evaluation of salt and hence for metal toxicity. Established shoot cultures of jojoba were used for this experiment. Shoot cultures were exposed to different concentration of Cu, Cr and Cd (0-300 μM). Activity of enzymes including superoxide dismutase, catalase and guaiacol peroxidase and lipid peroxidation product level was estimated in jojoba leaves after 1d, 7d and 14d of exposure. Differential response in enzyme activity and lipid peroxidation was noted. Jojoba was more tolerant towards Cr as compared to Cd or Cu. Metal estimation was carried out after 14d of incubation. Jojoba shoots accumulated maximum copper followed by Cd and Cr. Cu induced more oxidative stress evidenced by increased lipid peroxidation product level in jojoba leaves. Cu was more inhibitory for GPX activity as compared to Cd or Cr (**Differential response of Jojoba culture (*Simmmondsia chinensis*) to heavy metals (Cr, Cu and Cd) manuscript under preparation)**.

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), copper (Cu) and Cd on growth and metal uptake in different parts of *Pongamia pinnata* seedlings grown *in vitro* in medium containing Cr, Cu or Cd. Pongamia seeds were cultured in MS medium supplemented with various concentrations of Cr (0-800 μM), Cu and Cd (0-400 μM). After 6 weeks of incubation shoot height and root length of the seedlings were noted. Germination of pongamia seeds was not affected by any of the metal tested. The results demonstrated that growth of pongamia seedlings exposed to Cr(VI) with concentrations ranging from 0 to 800 μM were not affected whereas Cu (0-400 μM) affected the root growth. Cadmium was inhibitory for both shoot and root elongation. 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. In Cd, accumulation of metal was optimum in seed coat followed by root, leaves and cotyledons. Least metal content was detected in stem in all treatments either in Cr, Cu or Cd. 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 (**"*In vitro* studies on chromium and copper accumulation potential of *Pongamia pinnata* (L.) Pierre seedling" Sunil Kumar et al., Bioremediation Biodiversity and Bioavailability, 2008- In press**)

Majority of the reports available on studies on metal tolerance or stress has been conducted in field or pot cultures. These systems are not fully defined as the soil is composed of several organic and inorganic components and also microbes. The effect demonstrated by a plant system cultured *in vitro* is more specific and the data is interpreted with reference to the control plants cultured under identical conditions. The data generated from this study not only demonstrate the effect of metal stress on different plant species (peanut, jojoba and pongamia) in culture but also confirms the suitability of the plant tissue culture techniques as an useful tool for studies on metal stress.

Bibliography

Able AJ, Guest DL, Sutherland MW (2000) Hydrogen peroxide yield during the incompatible interaction of tobacco suspension cells inoculated with *Phytophtora nicotianae*. Plant Physiol. 124, 899-910.

Able AJ, Sutherland MW, Guest DL (2003) Production of reactive oxygen species during non specific elicitation, non host resistance and filed resistance expression in cultured tabacco cells. Funct. Plant Biol. 30, 91-99.

Aebi H (1984) Catalase *in vitro*, In: Colowickand, S.P., Kaplan, and N.O.: Methods in Enzymology. New York Academic Press, London, Toronto, Montreal Sydney, Tokyo, Sao Paulo, 105, 121-126.

Agarwal V, Sharma K (2006) Phytotoxic effects of Cu, Zn, Cd and Pb on *in vitro* regeneration and concomitant protein changes in *Holarrhena antidysenterica*. Biol. Plant. 50, 307-310.

Ali G, Srivastava PS, Iqbal M (1998) Effect of cadmium and copper on growth of *Bacopa monniera* regenerants. Biol. Plant. 41, 635-639.

Ali BM, Hahn EJ, Paek KY (2006) Copper-induced changes in the growth, oxidative metabolism, and saponin production in suspension culture roots of *Panax ginseng* in bioreactors. Plant Cell Rep. 25, 1122-1132.

Almeida AA, Valle RR, Mielke MS, Gomes FP (2007) Tolerance and prospection of phytoremediator woody species of Cd, Pb, Cu and Cr. Braz. J. Plant Physiol. 19, 83-98.

Alscher RA, Erturk N, Heath (2002) Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J. Exp. Bot. 53, 1331–1341.

Angelova V, Lvanova R, Ivanov K (2004) Heavy metal accumulation and distribution in Oil crops. Communications in Soil Science and Plant Analysis 35, 2551-2566.
Anonymous (1988) Wealth of India: Raw Materials V3 (206-211) CSIR Publication, New Delhi.

Anonymous (1988) Wealth of India: Raw Materials V3 (206-211) CSIR Publication, New Delhi.

Aravind P, Prasad MNV (2003) Zinc alleviates cadmium-induced oxidative stress in *Ceratophyllum demersum* L.—a free floating freshwater macrophyte. Plant Physiol. Biochem. 41, 391–397.

Arru L, Rognoni S, Baroncini, Bonatti PM, Perata P (2004) Copper localization in *Cannabis sativa* L. grown in a copper rich solution. Euphytica 140, 33-38.

Assche VF, Clijsters H (1990) Effects of metals on enzyme activity in plants. Plant Cell Environ. 13, 195-206.

Azevedo H, Gomes C, Pinto G, Santos C (2005) Cadmium effects in sunflower membrane permeability and changes in catalase and peroxidase activity in leaves and calluses. J. Plant Nutr. 28, 2233-2241.

Baker AJM (1981) Accumulators and excluders. Strategies in the response of plants to heavy metals. J. Plant Nutr. 3, 643–654.

Baker AJM (1993) Cadmium sensitivity and constitutive resistance. In: Methods in ComparativePlant Ecology, pp. 211–213 (Hendry, G.A.F. and Grime, J.P., Eds.). University of Sheffield, Sheffield, UK.

Baker DE, Senef JP (1995) Copper. In: Alloway BJ (ed), Heavy metals in soils, pp.179-205. Blackie Academic and Professional, London.

Balestrasse KB, Gardey L, Gallego SM, Tomaro ML (2001) Response of antioxidant defence system in soybean nodules and roots subjected to cadmium stress. Aust. J. Plant Physiol. 28, 497-504.

Barbosa RMT, Almeida AF, Mielke MS, Loguercio LL, Pedro AO, Mangabeira PAO, Gomes FP (2007) A physiological analysis of *Genipa mericana* L.: A potential phytoremediator tree for chromium polluted watersheds. Environ. Exp. Bot. 61, 264-271.

Barnabas B, Kovacs G, Hegedus A, Erdei S, Horvath G (2000) Regeneration of doubled haploid plants from *in vitro* selected microspores to improve aluminium tolerance in wheat. J. Plant Physiol. 156, 217-222.

Barnhart J (1997) Occurrences, uses, and properties of chromium. Regul. Toxicol. Pharm. 26, S3–S7.

Baťková P, Pospíšilová J, Synková H (2008) Production of reactive oxygen species and development of antioxidative systems during *in vitro* growth and *ex vitro* transfer. Biol. Plant. 52, 413-422.

Beauchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44, 276–287.

Becana M, Moran JF, Iturbe-Ormaetxea I (1998) Iron-dependent oxygen free radical generation in plants subjected to environmental stress: toxicity and antioxidant protection. Plant and Soil 201, 137–147.

Bell MJ, McLaughlin MJ, Wright GC, Cruickshank A (1997) Inter- and intra- specific variation in accumulation of cadmium by peanut, soybean, and navy bean. Aust. J. Agr. Res. 48, 1151-1160.

Benavides MP, Gallego SM, Tomaro ML (2005) Cadmium toxicity in plants. Braz. J. Plant Physiol. 17, 21-34.

Bizily SP, Rugh CL, Summers AO, Meagher RB (1999) Phytoremediation of methylmercury pollution: merB expression in *Arabidopsis thaliana* confers resistance to organomercurials. Proc. Nat. Acad. Sci. USA 96, 6808- 6813.

Bledsoe RW, Comar CL, Harris HC (1949) Absorption of radioactive calcium by peanut fruits. Science 109, 329–330.

Bojarczuk K (2004) Effect of Toxic Metals on the Development of Poplar (*Populus tremul* (*Populus tremula* L. × *P. alba* L.) Cultured *in vitro*. Polish J. Eviron. Studies 13, 115-120.

Bona E, Marsano F, Cavaletto M, Berta G (2007) Proteomic characterization of copper stress response in *Cannabis sativa* roots. Proteomics 7, 1121-1130.

Boojar MMK, Goodarzi F (2007) The copper tolerance strategies and the role of antioxidative enzymes in three plant species grown on copper mine. Chemosphere 67, 2138-2147.

Bowler C, Van Montagu M, Inz´e D (1992) Superoxide dismutase and stress tolerance. Ann. Rev. Plant Physiol. Mol. Biol. 43, 83–116.

Cakmak I (2000) Possible roles of zinc in protecting plant cells from damage by reactive oxygen species. New Phytol. 146, 185–205.

Castro RO, Trujillo MM, Bucio JL, Cervantes C, Dubrovsky J (2007) Effects of dichromate on growth and root system architecture of *Arabidopsis thaliana* seedlings. Plant Sci. 172, 684-691.

Cervantes C, Garcia JC, Devars S, Corona FG, Tavera HL, Torres-guzman JC (2001) Interactions of chromium with micro-organisms and plants. FEMS Microbiol. Rev. 25, 335–347.

Chamseddine M, Wided BA, Guy H, Marie-Edith C, Fatma J (2008) Cadmium and copper induction of oxidative stress and antioxidative response in tomato (*Solanum lycopersicon*) leaves. Plant Growth Regul. (In Press).

Chance, BA, Maehly C (1955) Assay of catalase and peroxidase, in Colowick, S.P., Kaplan.O.: Methods in Enzymology. New York Academy Press, 764- 775.

Chaoui A, EI Ferjani E (2005) Effects of cadmium and copper on antioxidant capacities, lignification and auxin degradation in leaves of pea (*Pisum sativum* L.) seedlings. C.R. Biol. 328, 23-31.

Chaoui A, Mazhoudi S, Ghorbal MH, Ferjani EEL (1997) Cadmium and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities in bean (*Phaseolus vulgaris* L.) Plant Sci.127, 139-147.

Chatterjee J, Chatterjee C (2000) Phytotoxicity of cobalt, chromium and copper in cauliflower. Environ. Pollut. 109, 69–74.

Chaturvedi HC, Sharma M (1989) *In vitro* production of cloned plants of jojoba (*Simmondsia chinensis*) through shoot proliferation in long-term culture. Plant Sci. 63, 199-207.

Chaudhary S, Panda SK (2005) Toxic effect, oxidative stress and ultrastructual changes in moss *Taxitheelium nepalense* (Schwegr) broth, under lead and chromium toxicity. Water, Air and Soil Pollut. 167, 73-90.

Chen J, Zhao J, Goldsbrough PB (1997) Characterization of phytochelatin-synthase from tomato. Physiol. Plant. 101, 165-172.

Chen LM, Lin CC, Kao CH (2000) Cu toxicity in rice seedlings: Changes in antioxidative enzyme activities, H_2O_2 level and cell wall peroxidase activity in roots. Bot. Bull. Acad. Sinica 41, 99–103.

Cho U, Seo N (2004) Oxidative stress in *Arabidopsis thaliana* exposed to cadmium is due to hydrogen peroxide accumulation. Plant Sci. 168, 13-120.

Ciscato M, Valcke R, Van LK, Clijsters H, Navari-Izzo F (1997) Effects of *in vivo* copper treatment on the photosynthetic apparatus of two *Triticum durum* cultivars with different stress sensitivity. Physiol. Plant. 100, 901-908.

Cobbett C, Goldsbrough P (2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annu. Rev. Plant Biol. 53, 159-182.

Cuypers A, Vangronsveld J, Clijsters H (2000) Biphasic effect of Cu on the ascorbate-gluthathione pathway in primary leaves of *Phaseolus vulgaris* seedlings during the early stages of metal assimilation. Physiol. Plant. 110, 512–517.

Cuypers A, Vangronsveld J, Clijsters H (2002) Peroxidases in roots and primary leaves of *Phaseolus vulgaris* copper and zinc phototoxicity: a comparison. J. Plant Physiol. 189, 869–876.

Dalla Vecchia F, Rocca NL, Moro I, De Faveri S, Andreoli C, Rascio N (2005) Morphogenetic, ultrastructual and physiological damages suffered by submerged leaves of *Elodea canadensis* exposed to cadmium. Plant Sci. 168, 329-338.

Daniel JN (1997) *Pongamia pinnata*- a nitrogen fixing tree for oilseed. NFT Highlights site: *http://www*.Winrock.org/forestry/factpub/FACTSH/P_pinnata.html.

Dat J, Vandenebeele S, Varanova E, Van Montagu M, Inzé D, Van Breusegem F (2000) Dual action of the active oxygen species during plant stress responses. Cell. Mol. Life Sci. 57, 779-795.

Davies FT, Puryear JD, Newton RJ, Egilla JN, Grossi JAS (2002) Mycorrhizal fungi increase chromium uptake by sunflower plants: influence on tissue mineral concentration, growth, and gas exchange. J. Plant Nutr. 25, 2389–2407.

Dazy M, Béraud E, Cotelle S, Meux E, Masfaraud JF, Férard JF (2008) Antioxidant enzyme activities as affected by trivalent and hexavalent chromium species in *Fontinalis antipyretica* Hedw. Chemosphere 73, 281–290.

De Vos CHR, Schat H, De Wall MAM, Vooijs R, Ernst WHO (1991) Increased resistance to copper induced damage of the roots cell plasmalemma in copper tolerant *Silene cucubalus*. Physiol. Plant.82, 523–528.

De Vos CHR, Vonk MJ, Voojis R, Schat H (1992) Glutathione depletion due to copper-induced phytochelatin synthesis causes oxidative stress in *Silene cucubalus.* Plant Physiol. 98, 853-858.

Del Rio L, Sandalio LM, Corpas FJ, Palma JM, Barroso JB (2006) Reactive oxygen species and reactive nitrogen species in Peroxisomes, production, scavenging and role in cell signaling. Plant Physiol. 141, 330-335.

Demirevska-Kepova K, Simova-Stoilova L, Stoyanova ZP, Feller U (2006) Cadmium stress in Barley: growth, leaf pigment and protein composition and detoxification of reactive oxygen species. J. Plant Nutr. 29, 451-468.

Denny HJ, Wilkins DA (1987) Zinc tolerance in *Betula* spp. New Phytol. 106, 545-553.

Dinakar N, Nagajyothi PC, Suresh S, Udaykiran Y, Damodharam T (2008) Phytotoxicity of cadmium on protein, proline and antioxidant enzyme activities in growing *Arachis hypogaea* L. seedlings. J. Environ. Sci. 20, 199-206.

Dixit V, Pandey V, Shyam R (2001) Differential antioxidative responses to Cd in roots and leaves of Pea (*Pisum sativum* L.cv.Azad). J. Exp. Bot. 52, 1101-1109.

Dixit V, Pandey V, Shyam R (2002) Chromium ions inactivate electron transport and enhance superoxide generation *in vivo* in pea (*Pisum sativum* L.cv. Azad) root mitochondria. Plant Cell Environ. 25, 687–690.

Dong J, Wu F, Zhang G (2006) Influence of cadmium on antioxidant capacity and four microelement concentrations in tomato seedlings (*Lycopersicon sculentum*). Chemosphere 64, 1659-1666.

Dražic G, Mihalovic N, Lojic M (2006) Cadmium concentration in *Medicago sativa* seedlings treated with salicylic acid. Biol. Plant 50, 239-244.

Drazkiewicz M, Skórzynska-Polit E, Krupa Z (2004) Copper-induced oxidative stress and antioxidant defence in *Arabidopsis thaliana.* Biometals 17, 379-387.

Drążkiewicz M, Skórzyńska-Polit E, Krupa Z (2007) The redox state and activity of superoxide dismutase classes in *Arabidopsis thaliana* under cadmium or copper stress. Chemosphere 67, 188-193.

Ducic T, Polle A (2005) Transport and detoxification of manganese and copper in plants. Braz. J. Plant Physiol. 17, 103-112.

Duffus JH (2002) 'Heavy metals a meaningless term?'. Pure Appl. Chem. 74, 793-807.

Ederli L, Reale L, Ferranti F, Pasqualini S (2004) Responses induced by high concentration of cadmium in *Phragmites australis* roots. Physiol. Plant. 121, 66–74.

El-Aref HM, Hamada AM (1998) Genotypic differences and alterations of protein patterns of tomato explants under copper stress. Biol. Plant. 41, 555-564.

Elstner EF, and Oßwald WF (1994) Mechanism of oxygen activation during plant stress. Proc. Royal Soc. Edinberg 102B, 131-154.

Eltrop L, Brown G, Joachim O, Brinkmann K (1991) Lead tolerance of Betula and Salix in the mining area of Mechernich/Germany. Plant and Soil 131, 275-285.

Etherinton JR (1988) The aims and development of plant ecology. Environment and plant ecology, 2nd ed. Manchester; John Wiley and Sons, Ltd pp. 1-4.

Fernandez JC and Henriques FS (1991) Biochemical, physiological and structural effects of excess copper in plants. The Bot. Rev. 57, 246-273.

Finney LA, O'Halloran TV (2003) Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. Science 300, 931-936.

Fornazier RF, Ferreira RR, Vitória AP, Molina SMG, Lea PJ, Azevedo RA (2002) Effects of cadmium on antioxidant enzyme activities in sugar cane. Biol. Plant. 45, 91-97.

Foyer CH, Noctor G (2005) Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. The Plant Cell 17, 1866-1875.

Foyer HC, Noctor G (2000) Oxygen processing in photosynthesis: regulation and signaling. New Phytol. 146, 359–388.

Gallego SM, Benavides MP, Tomaro ML (1996) Effect of heavy metal ion excess on sunflower leaves: evidence for involvement of oxidative stress. Plant Sci. 121, 151-159.

Gajewska E, Shaba M, Andrzejewska R (2006) Nickel induced inhibition of wheat root growth is related to hydrogen peroxide production but not to lipid peroxidation. Plant Growth Regul. 49, 95- 103.

Ganesh KS, Baskaran L, Rajasekaran S, Sumathi K, Chidambaram ALA, Sundaramoorthy P (2008) Chromium stress induced alterations in biochemical and enzyme metabolism in aquatic and terrestrial plants. Coll. Surf. B: Biointer. 63, 159–163.

Garcia JS, Gratao PL, Azevedo RA, Arruda MAZ (2006) Metal contamination effects on sunflower (*Helianthus annuus* L.) growth and protein expression in leaves during development. J. Agric. Food Chem. 54, 8623-8630.

Garcia-Hernandez M, Angus Murphy, Lincoln T (1998) Metallothioneins 1 and 2 have distinct but overlapping expression Patterns in *Arabidopsis thaliana*. Plant Physiol. 118, 387-397.

Gardea-Torresdey JL, de la Rosa G, Peralta-Videa JR, Montes M, Cruz-Jimenez G, Cano-Aguilera I (2005) Differential uptake and transport of trivalent and hexavalent chromium by tumbleweed (*Salsola kali*). Arch. Environ. Contam. Toxicol. 48, 225–232.

Gasper T, Penel C, Hagege D, Greppin H (1991) Peroxidase in plant growth, differentiation and development processes. In: J. Laborzewsky, H. Greppin, C. Penel and T. Gaspar, Editors, Biochemical, Molecular and Physiological Aspects of Plant Peroxidases, University M. Curie, Sklodowska pp. 249–280.

Goldbold DL, Horst WJ, Collins JC, Thumann DA, Marschner H (1984) Accumulation of zinc and organic acids in the roots of zinc-tolerant and non-tolerant ecotypes of *Deschampia caespitosa*. J. Plant Physiol. 116, 59-69.

Gomes-Junior RA, Moldes CA, Delite FS, Pompeu GB, Gratão PL, Mazzafera P, Lea PJ, Azevedo RA (2006) Antioxidant metabolism of coffee cell suspension cultures in response to cadmium. Chemosphere 65, 1330-1337.

Gomes RA Jr, Gratão PL, Gaziola SA, Mazzafera P, Lea PJ, Azevedo RA (2007) Selenium-induced oxidative stress in coffee cell suspension cultures. Funct. Plant Biol. 34, 449-456.

Gori P, Schiff S, Santandrea G, Bennici A (1998) Response of *in vitro* cultures of *Nicotiana tabacum* L. to copper stress and selection of plants from Cu-tolerant callus. Plant Cell Tiss. Org. Cult. 53, 161-169.

Goyer RA (1997) Toxic and essential metal interaction. Annu. Rev. Nutr. 17, 37-50.

Granel T, Robinson B, Mills T, Clothier B, Green S, Fung L (2002) Cadmium accumulation by willow clones used for conservation, stock fodder and hytoremediation. Aust. J. Soil Res. 40, 1331-1337.

Gratão PL, Pompeu GB, Capaldi FR, Vitorello VA, Lea PJ, Azevedo RA (2008) Antioxidant response of *Nicotiana tabacum* cv. Bright Yellow 2 cells to cadmium and nickel stress. Plant Cell Tiss. Org. Cult. 94, 73-83.

Gratao PL, Polle A, Lea PJ, Azavedo RA (2005) Making the life of heavy metal stressed plants a little easier. Funct. Plant Biol. 32, 481-494.

Greger M, Landberg T (1999) Use of willow in phytoextraction. Internat. J. Phytoremed.1, 115-123.

Guoa TR, Zhang GP, Zhang YH (2007) Physiological changes in barley plants under combined toxicity of aluminum, copper and cadmium. Coll. Surf. B: Biointer. 57, 182–188.

Gupta M, Cuypers A, Vangronsveld J, Clijsters H (1999) Copper affects the enzymes of the ascorbate-glutathione cycle and its related metabolites in the roots of *Phaseolus vulgaris*. Physiol. Plant 106, 262-267.

Gussarsson M (1994) Cd- induced alterations in nutrients composition and growth in *Betula pendula* seedlings: the significance of fine roots as primary target for Cd toxicity. J. Plant Nutr. 17, 2151–2163.

Hall JL (2002) Cellular mechanisms for heavy metal detoxification and tolerance. J. Exp. Bot. 53, 1-11.

Hall JL, Williams LE (2003) Transition metal transporters in plants. J. Exp. Bot. 54, 2601-2613.

Hamer DH (1986) Metallothioneins. Annu. Rev. Biochem. 55, 913-951.

Han FX, Sridhar BBM, Monts DL, Su Y (2004) Phytoavailability and toxicity of trivalent and hexavalent chromium to *Brassica juncea.* New Phytol.162, 489-499.

Hartley-Whitaker J, Ainsworth G, Meharg AA (2001) Copper- and arsenate-induced oxidative stress in *Holcus lanatus* L. clones with differential sensitivity. Plant Cell and Environ. 24, 713-722.

Hasegawa I, Terada E, Sunairi M, Wakita H, Shinmzchi F, Noguchi A, Nakajima M, Yazaki J (1997) Genetic improvement of heavy metal tolerance in plants by transfer of the yeast metallothioneins gene (Cup1). Plant and Soil 196, 277-281.

Hasnain S, Sabri AN (1997) Growth stimulation of *Triticum aestivum* seedlings under Cr-stresses by non-rhizospheric pseudomonad strains. Environ. Pollut. 97, 265–273.

Hippeli S, Heiser I, Elstner EF (1999) Activated oxygen and free oxygen radicals in pathology: new insights and analogies between animals and plants. Plant Physiol. Biochem. 37, 167–178.

Horst WJ (1995) The role of the apoplast in aluminium toxicity and resistance of higher plants. *Z.* Pflanzenernahr. Bodenkd. 158, 419–428.

Houmiel KLS, Slater D, Broyles L, Casagrande S, Colburn K, Gonzalez TA, Mitsky SE, Reiser D, Shah NB, Taylor M, Tran HE, Valentin KJ, Gruys KJ (1999) Poly (beta-hydroxybutyrate) production in oilseed leukoplasts of *Brassica napus*. Planta 209, 547–550.

Hsu YT, Kao CH (2004) Cadmium toxicity is reduced by nitric oxide in rice leaves. Plant Growth Regul. 42, 227-238.

http://www.lanra.uga.edu/peanut/knowledgebase/

http://en.wikipedia.org/wiki/Peanut

http://www.saguaro-juniper.com/i_and_i/trees&shrubs/jojoba/jojoba.html

Iannelli MA, Pietrini F, Fiore L, Petrilli L, Massacci A (2002) Antioxidant response to cadmium in *Phragmites australis* plants. Plant Physiol. Biochem. 40, 977–982.

Israr M, Sahi SV (2006) Antioxidative responses to mercury in the cell cultures of *Sesbania drummondii*. Plant Physiol. Biochem. 44, 590-594.

Jain R, Srivastava S, Madan VK, Jain R (2000) Influence of chromium on growth and cell division of sugarcane. Ind. J. Plant Physiol. 5, 228–231.

Jithesh MN, Prashanth SR, Sivaprakash KR, Parida AK (2006) Antioxidative response mechanisms in halophytes: their role in stress defence. J. Gen. 85, 237-254.

Jonak C, Nakagami H, Hirt H (2004) Heavy metal stress, activation of distinct Mitogen-Activated Protein Kinase Pathways by Copper and Cadmium. Plant Physiol. 136, 3276-3283.

Juwarkar AA, Nair A, Dubey KV, Singh SK, Devotta S (2007) Biosurfactant technology for remediation of cadmium and lead contaminated soils. Chemosphere 68, 1996-2002.

Juwarkar AA, Kumar SY, Kumar P, Kumar SS (2008) Effect of biosludge and biofertilizer amendment on growth of *Jatropha curcas* in heavy metal contaminated soils. Environ. Monit.Assess. 145, 7-15.

Karpinski S, Reynolds H, Karpinska B, Wingsle G, Creissen G, Mullineaux P (1999) Systemic Signaling and Acclimation in Response to Excess Excitation Energy in *Arabidopsis.* Science 284, 654-657.

Keller C, Hammer D, Kayser A, Richner A, Brodbeck W, Sennhauser M (2003) Root development and heavy metal phytoextraction efficiency: comparison of different plant species in the field. Plant and Soil 249, 67-81.

Khan AG, Kuek C, Chaudhry TM, Khoo CS, Hayes WJ (2000) Role of plants, mycorrhizae and phytochelators in heavy metal contaminated land remediation. Chemosphere 41, 197–207.

Khatun S, Babar Ali M, Hahn EJ, Paek KY (2008) Copper toxicity in *Withania somnifera*: Growth and antioxidant enzymes responses of *in vitro* grown plants. Environ. Exp. Bot. (In press).

Klang-Westin E, Eriksson J (2003) Potential of Salix as phytoextractor for Cd on moderately contaminated soils. Plant and Soil 249, 127-137.

Kneer R, Zenk MH (1992) Phytochelatins protect plant enzymes from heavy metal poisoning. Phytochemistry 31, 2663-2667.

Kova´cˇik J and Backer M (2008) Oxidative status of *Matricaria chamomilla* plants related to cadmium and copper uptake. Exotoxciology 17, 471-479.

Kova´cˇik J, Bacˇ kor M, Kadukova J (2008) Physiological responses of *Matricaria chamomilla* to cadmium and copper excess. Environ. Toxicol. 23, 123-130.

Kramer U, Cotter-Howells JD, Charnock JN, Baker AJM, Smith AC (1996) Free histidine as a metal chelator in plants that accumulate nickel. Nature 379, 635-638.

Ku¨ pper H, Mijovilovich A, Meyer-Klaucke W, Kroneck PMH (2004) Tissue- and Age-Dependent Differences in the Complexation of Cadmium and Zinc in the Cadmium/Zinc Hyperaccumulator *Thlaspi caerulescens* (Ganges Ecotype) Revealed by X-Ray Absorption Spectroscopy. Plant Physiol. 134, 748-757.

Kumar GP, Yadav SK, Thawale PR, Singh SK, Juwarkar AA (2008) Growth of *Jatropha curcas* on heavy metal contaminated soil amended with industrial wastes and Azotobacter – A greenhouse study. Bioresource Technol. 99, 2078-2082.

Kupper H, Lombi E, Zhao FJ, Mcgrath SP (2000) Cellular compartmentation of cadmium and zinc in relation to other elements in the hyperaccumulator *Arabidopsis halleri*. Planta 212, 75-84.

Kuriakose SV, Prasad MNV (2008) Cadmium stress affects seed germination and seedling growth in *Sorghum bicolor* (L.) Moench by changing the activities of hydrolyzing enzymes. Plant Growth Regul. 54, 143–156.

Kuzovkina YA, Knee M, Quigley MF (2004) Cadmium and copper uptake and translocation in five willow (Salix L.) species. Internat. J. Phytoremed. 6, 269-287.

Laureysens I, De Temmerman L, Hastir T, Van Gysel M, Ceulemans R (2005) Clonal variation in heavy metal accumulation and biomass production in a poplar coppice culture. II. Vertical distribution and phytoextraction potential. Environ. Pollut. 133, 541-551.

Lee S, Petros D, Moon JS, Ho TS, Goldsbrough PB, Korban S (2003) Higher levels of ectopic expression of *Arabidopsis thaliana* phytochelatin synthesase do not led to increased cadmium tolerance and accumulation. Plant Physiol. Biochem. 41, 903-910.

Li W, Khan MA, Shinjiro YS, Kamiya Y (2005) Effects of heavy metals on seed germination and early seedling growth of *Arabidopsis thaliana.* Plant Growth Regul. 46, 45–50.

Lichtenthaler HK (1996) An introduction to the stress concept in plants. J. Plant Physiol. 148, 4-14.

Lin AJ, Zhang XH, Chen MM, Cao Q (2007) Oxidative stress and DNA damages induced by cadmium accumulation. J. Environ. Sci. 19, 596-602.

Lin J, Jiang W, Liu D (2003) Accumulation of copper by roots, hypocotyls, cotyledons and leaves of sunflower (*Helianthus annuus* L.). Bioresource Technol. 86, 151-155.

Liu D, Zou J, Wanh M, Wusheng J (2008) Hexavalent chromium uptake and its effects on mineral uptake, antioxidant defence system and photosynthesis in *Amaranthus viridis* L. Bioresource Technol. 99, 2628-2636.

Liu Y, Wang X, Zeng G, Qu D, Gu J, Zhou M, Chai L (2007) Cadmium-induced oxidative stress and response of the ascorbate–glutathione cycle in *Bechmeria nivea* (L.) Gaud. Chemosphere 69, 99-107.

Lodge DJ (1989) The influence of soil moisture and flodding on formation of VA-endo and ectomycorrhizae in *Populus* and *Salix*. Plant and Soil 117, 243-253.

Lombardi L, Sebastiani L, Vitagliano C (2003). Physiological, biochemical, and molecular effects of *in vitro* induced iron deficiency in peach rootstock Mr.S2/5. J. Plant Nutr. 26, 2149-2163.

Lombardi L, Sebastiani L (2005) Copper toxicity in *Prunus cerasifera*: growth and antioxidant enzymes responses of *in vitro* grown plants. Plant Sci. 168, 797-802.

López-Bucio J, Cruz-Ramírez A, Herrera-Estrella H (2003) The role of nutrient availability in regulating root architecture. Curr. Opin. Plant Biol. 6, 280–287.

Lou L-q, Shen Z-g, Li X-g (2004) The copper tolerance mechanisms of *Elsholtzia haichowensis*, a plant from copper-enriched soils. Environ. Exp. Bot. 51, 111–120.

Lowry OH, Rosenberg NJ, Farr AL, Randall RJ (1951) Protein measurement with folin phenol reagent. J. Biol. Chem. 193, 265-275.

Luna CM, González CA, Trippi VS (1994) Oxidative Damage Caused by an Excess of Copper in Oat Leaves. Plant Cell Physiol. 35, 11-15.

Mackerness AS, John CF, Jordan B, Thomas B (2001) Early signaling components in ultraviolet-B responses: distinct roles for different reactive oxygen species and nitric oxide. FEBS Lett. 489, 237-242.

Maksymiec M, Krupa Z (2006) The effects of short-term exposition to Cd, excess Cu ions and jasmonate on oxidative stress appearing in *Arabidopsis thaliana*. Environ. Exp. Bot. 57, 187–194.

Mangabeira PA, Gavrilov KL, Furtado de Almeida A, Oliveira AH, Severo MI, Rosa TS, da Costa Silva D, Labejof L, Escaig F, Levi-Setti R, Mielke MS, Loustalot FG, Galle P (2006) Chromium localization in plant tissues of *Lycopersicum esculentum* Mill using ICP-MS and ion microscopy (SIMS). App. Surf. Sci. 252, 3488-3501.

Maribel LDT, Satoshi (1998) Antioxidant responses of rice seedlings to salinity stress. Plant Sci. 135, 1–9.

Marschner H (1995) Mineral nutrition of higher plants. Academic Press, London.

Martins LL, Mourato MP (2006) Effect of excess copper on tomato plants: growth parameters, enzyme activities, chlorophyll, and mineral content. J. Plant Nutrit. 29, 2179–2198.

Mazhoudi S, Chaoui A, Ghorbal MH, Ferjani EE (1997) Response of antioxidant enzymes to excess copper in tomato (*Lycopersicon esculentum*, Mill.). Plant Sci. 127, 129-137.

McLaughlin MJ, Bell MJ, Wright GC, Cozens GD (2000) Uptake and partitioning of cadmium by cultivars of peanut (*Arachis hypogaea* L.). Plant and Soil 222, 51-58.

McNair MR, Tilstone GH, Smith SS (2000) The genetics of metal tolerance and accumulation in higher plants. - In: Terry, N., Bañuelos, G. (ed.): Phytoremediation of Contaminated Soil and Water. Pp. 235-250. Lewis Publishers, Boca Raton.

Meera B, Kumar S, Kalidhar SB (2003) A review of the chemistry and biological activity of *Pongamia pinnata.* J. Med. Aro. Plant Sci. 25, 441-465.

Meers E, Lamsal S, Vervaeke P, Hopgood M, Lust N, Tack FMG (2005) Availability of heavy metals for uptake by *Salix viminalis* on a moderately contaminated dredged sediment disposal site. Environ. Pollut. 137, 354-364.

Mei B, Puryear JD, Newton RJ (2002) Assessment of Cr tolerance and accumulation in selected plant species. Plant and Soil 247, 223– 231.

Mendelssohn IA, McKee KL, Kong T (2001) A comparison of physiological indicators of sublethal cadmium stress in wetland plants. Environ. Exp. Bot. 46, 263-275.

Metwally A, Safronova VI, Belimov AA, Dietz KJ (2004) Genotypic variation of the response to cadmium toxicity in *Pisum sativum* L. J. Exp. Bot. 56, 167-178.

Mills D, Benzioni A (1992) Effect of NaCl salinity on growth and development of jojoba clones: II. Nodal segments *in vitro*. J. Plant Physiol. 139, 737–741.

Milone MT, Sgherri C, Clijters H, Navari-Izzo F (2003) Antioxidative responses of wheat treated with realistic concentrations of cadmium. Environ. Exp. Bot. 50, 265-273.

Mishra S, Srivastava S, Tripathi RD, Govindarajan R, Kuriakose SV, Prasad MNV (2006) Phytochelatin synthesis and response of antioxidants during cadmium stress in *Bacopa monnieri* L. Plant Physiol. Biochem. 44, 25-37.

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7, 405–410.

Mittler R, Vanderauwera S, Gollery M, Breusegem FV (2004) Abiotic stress series. Reactive oxygen gene network of plants. Trends Plant Sci. 9, 490-498.

Moffat S (1995) Plants proving their worth in toxic metal cleanup. Science 269, 302-303.

Montes-Holguin MO, Peralta-Videa JR, Meitzner G, Martinez-Martinez A, de la Rosa G, Castillo-Michel HA, Gardea-Torresdey JL (2006) Biochemical and spectroscopic studies of the response of *Convolvulus arvensis* L. to chromium(III) and chromium(VI) stress. Environ. Toxicol. Chem. 25, 220-226.

Morelli E, Scarano G (2004) Copper-induced changes of non-protein thiols and antioxidant enzymes in the marine microalga *Phaeodactylum tricornutum*. Plant Sci. 167, 289-296.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant 15, 473-497.

Nagalakshmi N, Prasad MNV (2001) Responses of glutathione cycle enzymes and glutathione metabolism to copper stress in *Scenedesmus bijugatus*. Plant Sci. 160, 291-299.

Nakazawa R, Kameda Y, Ito T, Ogita Y, Michihata R, Takenaga H (2004) Selection and characterization of nickel tolerant tobacco cells. Biol. Plant. 48, 497-502.

Nriagu JO (1988) Production and uses of chromium. Chromium in natural and human environment. New York, USA7 John Wiley and Sons; pp. 81– 105.

Ogawa K, Kanematsu S, Asada K (1997) Generation of superoxide anion and localization of Cu–Zn superoxide dismutase in the vascular tissue of spinach hypocotyls their association with lignification. Plant Cell Physiol. 38, 1118–1126.

Okhawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxide in animal tissues by Thiobarbituric acid reaction. Anal. Biochem. 95, 351-358.

Oleksyn J and Innes JL (2000) Air pollution and forest in heavily industrialized regions- an introduction. In Forest dynamics in heavily polluted regions, 1st ed., (Innes JL and Oleksyn J eds.) Oxon: CABI Publishing 1-7.

Orozco-Cardenas M, Ryan CA (1999). Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. PNAS 96, 6553-6557.

Ouzounidou G (1994) Copper-induced changes on growth, metal content and photosynthetic function of *Alyssum montanum* L. plants. Environ. Exp. Bot. 34, 165–172.

Ouzounidou G, Ciamporova M, Moustakas M, Karataglis S (1995) Responses of maize (*Zea mays* l.) plants to copper stress—I, growth, mineral content and ultrastructure of roots. Environ. Exp. Bot. 35, 167-176.

Ouzounidou G, Elefteriou EP, Karataglis S (1992) Ecophysiological and ultra structural effects of copper in *Thlaspi ochroleucum* (Cruciferae). Can. J. Bot. 70, 158-169.

Oven M, Grill E, Golan-Goldhirsh A, Kutcxhan TM, Zenk MH (2002) Increase in free cysteine and citric acid in plant cells exposed to cobalt ions. Phytochemistry 60, 467-474.

Pal M, Horvath E, Janda T, Paldi E, Szalal G (2006) Physiological changes and defense mechanism induced by Cd stress in maize. J. Plant Nutr. Soil Sci. 169, 239-246.

Pallas JE Jr (1980) An apparent anomaly in peanut leaf conductance. Plant Physiol. 65, 848-851.

Panda SK (2007) Chromium mediated oxidative stress in developing rice seedling. J. Plant Physiol. 164, 1419-1428.

Panda SK, Choudhary I, Khan MH (2003) Heavy metal phytotoxicity induces lipid peroxidation and affect antioxidant in wheat leaves. Biol. Plant. 46, 289-294.

Panda SK, Choudhury S (2005) Chromium stress in plants. Braz. J. Plant Physiol. 17, 95-102.

Pandey V, Dixit V, Shyam R (2005) Antioxidative responses in relation to growth of mustard (*Brassica juncea* cv. Pusa Jaikisan) plants exposed to hexavalent chromium. Chemosphere 61, 40-47.

Pasternak T, Rudas V, Potters G, Jansen MAK (2005) Morphogenic effects of abiotic stress: reorientation of growth in *Arabidopsis thaliana* seedlings. Environ. Exp. Bot. 53, 299–314.

Patel MJ, Patel JN, Subramanian RB (2005) Effect of cadmium on growth and the activity of H_2O_2 scavenging enzymes in *Colocassia esculentum*. Plant and Soil 273, 183-188.

Peng HY, Yang XE, Yang MJ, Tian SK (2006) Responses of antioxidant enzyme system to Copper toxicity and copper detoxification in the Leaves of *Elsholtzia splendens*. J. Plant Nutrit. 29, 1619–1635.

Peng HY, Yang XE (2007) Characteristics of copper and lead uptake and accumulation by two species of *Elsholtzia*. Bull. Environ. Contam. Toxicol. 78, 152-157.

Peralta JR, Gardea Torresdey JL, Tiemann KJ, Gomez E, Arteaga S, Rascon E (2001) Uptake and effects of five heavy metals on seed germination and plant growth in alfalfa (*Medicago sativa* L.) Bull. Environ. Contam. Toxicol. 66, 727–734.

Persans MW, Yan X, Patnoe JM, Kramer U (1999) Molecular dissection of the role of histidine in hyperaccumulation in *Thlaspi geosingense*. Plant Physiol. 121, 1117-1126.

Pilon M, Abdel-Ghaney S, Cohu C, Gagolin K, Ye H (2006) Copper factor delivery in plant cells. Curr. Opin. Plant Biol. 9, 256-263.

Pilon-Smits EAH, de Souza MP, Lytle CM, Shang C, Lugo T, Terry N (1998) Selenium volatilization and assimilation by hybrid poplar (*Poplus tremula* x *alba*). J. Exp. Bot. 49, 1889-1892.

Pinto E, Sigaud-Kutner TCS, Leitão MAS, Okamoto OK, Morse D, Colepicolo P (2003) Heavy metal-induced oxidative stress in algae. J. Phycol. 39, 1008-1018.

Pinto AP, Mota AM, de Varennes A, Pinto FC (2004) Influence of organic matter on the uptake of cadmium, zinc, copper and iron by sorghum plants. Sci. Tot. Environ. 326, 239–247.

Pitzschke A, Hirt H (2006) Mitogen activated protein kinases and reactive oxygen species signaling in plant. Plant Physiol. 141, 351-356.

Polle A (2001) Dissecting the superoxide dismutase-ascorbate-glutathione-pathway in chloroplasts by metabolic modeling. Computer simulations as a step towards flux analysis. Plant Physiol. 126, 445–462.

Polle A, Schutzendubel A (2003) Heavy metal signaling in plants: linking cellular and organismic responses, in Hirt, H., Shinozaki K: Plant responses to abiotic stress. From Topics in current Genetics, Vol.4, Springer Verlag, Berlin, Heidelberg, pp. 187-215.

Pollution of agricultural land due to waste disposal from tannery industries. (http://www.aciar.gov.au/project/LWR1/1993/022).

Potters G, Pasternak TP, Guisez Y, Palme KJ, Jansen MAK (2007) Stress-induced morphogenic responses: growing out of trouble? Trends Plant Sci. 12, 98-105.

Prasad MNV (1995) Cadmium toxicity and tolerance in vascular plants. Environ. Exp. Bot. 35, 525–545.

Prasad MNV, Freitas HM (2003) Metal hyperaccumulation in plants - Biodiversity prospecting for phytoremediation technology. Electronic J. Biotechnol. 6, 285-305.

Prasad TK, Anderson MD, Martin BA, Stewart CR (1994) Evidence for Chilling-Induced Oxidative Stress in Maize Seedlings and a Regulatory Role for Hydrogen Peroxide. The Plant Cell 6, 65-74.

Pulford ID, Dickinson NM (2006) "Phytoremediation Technologies Using Trees" in: Prasad MNV, Sajwan KS, Ravi Naidu (eds) Trace elements in the environment: Biogeochemistry, Biotechnology and Bioremediation. CRC Press. Boca Raton. 726 pp. Taylor and Francis Group.

Pulford ID, Watson C (2003) Phytoremediation of heavy metals –contaminated land by tree- a review. Environ. Internat. 29, 529-540.

Pulford ID, Watson C, McGregor SD (2001) Uptake of chromium by trees: prospects for phytoremediation. Environ. Geochem. Health 23, 307– 311.

Qin YH, Zhang SL, Zhang LX, Zhu DY, Syed A (2005) Response of *in vitro* strawberry to silver nitrate (AgNO3). HortScience 40, 747-751.

Quartacci MF, Cosi E, Naveri-Izzo F (2001) Lipids and NADPH dependent superoxide production in plasma membrane vesicles from roots of wheat grown under copper deficiency or excess. J. Exp.Bot. 52, 77-84.

Quartacci MF, Pinzino C, Sgherri CLM, Dalla Vecchia F, Navari-Izzo F (2000) Growth in excess copper induces changes in the lipid composition and fluidity of PSII-enriched membranes in wheat. Physiol. Plant. 108, 87-93.

Rai V, Mehrotra S (2008) Chromium-induced changes in ultramorphology and secondary metabolites of *Phyllanthus amarus* Schum & Thonn. – an hepatoprotective plant. Environ. Monitor. Assess. 1-9 (In Press).

Rai V, Vajpayee P, Singh SN, Mehrotra S (2004) Effect of chromium accumulation on photosynthetic pigments, oxidative stress defense system, nitrate reduction, proline level and eugenol content of *Ocimum tenuiflorum* L. Plant Sci.167, 1159-1169.

Radotic K, Ducic T, Mutavdzic D (2000) Changes in peroxidase activity and isoenzymes in spruce needles after exposure to different concentrations of cadmium. Environ. Expt. Bot. 44, 105–113.

Raju D, Kumar S, Mehta UJ, Hazra S (2008) Differential accumulation of Manganese in three mature tree species (Holoptelia, Cassia, Neem) growing on a mine dump. Curr. Sci. 94, 639-643.

Rama Devi S, Prasad MNV (1998) Copper toxicity in *Ceratophyllum demersum* L. (Coontail), a free floating macrophyte: Response of antioxidant enzymes and antioxidants. Plant Sci. 138, 157-165.

Ramgareeb S, Watt MP, Marsh C, Cooke JA (1999) Assessment of Al^{3+} availability in callus culture media for screening tolerant genotypes of *Cynodon dacctylon*. Plant Cell, Tiss. Org. Cult. 56, 65-68.

Ranieri A, Baldan B, Castagna A, Sebastiani L, Soldatini G (2003) H_2O_2 accumulation in sunflower leaves as a consequence of iron deprivation. J. Plant Nutr. 26, 2187-2196.

Ratkevicius N, Correa JA, Moenne A (2003) Accumulation, synthesis of ascorbate and activation of ascorbate peroxidase in *Enteromorpha compressa* (L.) Grev (Chlorophyta) from heavy metal-enriched environments in northern Chile. Plant cell Environ. 26, 1599-1608.

Rauser WE, Meuwly P (1995) Retention of cadmium in roots of maize seedlings. Plant Physiol. 109, 195-202.

Reichman SM (2002) The response of plants to metal toxicity: a review focusing on copper, manganese and zinc. The Australian minerals and energy environment foundation 14, 1-54.

Robinson BH, Mills TM, Petit D, Fung LE, Green S, Clothier B (2000) Natural and induced cadmium-accumulation in poplar and willow: implications for phytoremediation. Plant and Soil 227, 301-306.

Romero-Puertas MC, Corpas FJ, Rodríguez-Serrano M, Gómez M, Río LA, Sandalio LM (2007) Differential expression and regulation of antioxidative enzymes by cadmium in pea plants. J. Plant Physiol. 164, 1346-1357.

Rosselli W, Keller C, Boschi K (2003) Phytoextraction capacity of trees growing on a metal contaminated soil. Plant and Soil 256, 265-272.

Roussos AP, Pontikis AC (2007) Changes of free, soluble conjugated and bound polyamine titers of jojoba explants under sodium chloride salinity *in vitro*. J. Plant Physiol. 164, 895-903.

Rout GR, Samantaray S, Das P (1999) Chromium, nickel and zinc tolerance in *Leucaena leucocephala* (K8). Silvae Genet. 48, 151–157.

Sagner S, Kneer R, Warner T, Cosson JP, Deus-Neumann B, Zenk MH (1998) Hyperaccumulation, complexation, distribution of nickel in *Sibertia acuminata*. Phytochemistry 47, 339-347.

Sahi SV, Israr M, Srivastava AK, Gardea-Torresdey JL, Parsons JG (2007) Accumulation, speciation and cellular localization of copper in *Sesbania drummondii*. Chemosphere 67, 2257-2266.

Salin ML (1988) Toxic oxygen species and protective systems of the chloroplasts. Physiol. Plant. 72, 681-689.

Salt DE, Prince RC, Pickering IJ, Raskin I (1995) Mechanisms of cadmium mobility and accumulation in Indian mustard. Plant Physiol. 109, 1427–1433.

Salt DE, Smith RD, Raskin I (1998) Phytoremediation. Annu. Rev. Plant Physiol. Mol. Biol. 49, 643–668.

Samantaray S, Rout GR, Das P (1999) Studies on differential tolerance of mungbean cultivars to metalliferous minewastes. Agribiol. Res. 52, 193– 201.

Samantaray S, Rout GR, Das P (2001) Induction, selection and characterization of Cr and Ni-tolerant cell lines of *Echinochloa colona* (L.) Link *in vitro*. J. Plant Physiol. 158, 1281-1290.

Samantary S (2002) Biochemical responses of Cr-tolerant and Cr–sensitive mung bean cultivars grown on varying levels of chromium. Chemosphere 47, 1065-1072.

Sancenon V, Puig S, Mira H, Thiele DJ, Penarrubia L (2003) Identification of a copper transporter family in *Arabidopsis thalliana*. Plant Mol. Biol. 51, 577-587.

Sanità di Toppi L, Fossati F, Musetti R, Mikerezi I, Favali MA (2002) Effect of hexavalent chromium on maize, tomato and cauliflower plants. J. Plant Nutr. 25, 701-717.

Sanitá di Toppi L, Gabbrielli R (1999) Response to cadmium in higher plants. Environ. Exp. Bot. 41, 105-130.

Sarita S, Rohit S (2006) Effect of iron on lipid peroxidation, and enzymatic and non-enzymatic antioxidants and bacoside-A content in medicinal plant *Bacopa monnieri* L. Chemosphere 62, 1340-1350.

Scebba F, Arduin I, Ercoli L, Sebastiani L (2006) Cadmium effects on growth and antioxidant enzymes activities in *Miscanthus sinensis*. Biol. Plant. 50, 688-692.

Schenk RU, Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. 50, 199-204.

Schickler H, Caspi H (1999) Response of antioxidant enzymes to nickel and cadmium stress in hyperaccumulator plants of the genus *Alyssum.* Physiol. Plant. 105, 39-44.

Schraudner M, Moeder W, Wiese C, Van Camp W, Inzé D, Langebartels C, Sandermann H Jr (1998) Ozone-induced oxidative burst in the ozone biomonitor plant, tobacco. Plant J. 16, 235–245.

Schutzendubel A, Polle A (2001) Cadmium induced changes in antioxidative systems, hydrogen peroxide content and differentiation in Scot pine roots. Plant Physiol. 127, 887-898.

Schutzendubel A, Polle A (2002a) Cadmium and H_2O_2-induced oxidative stress in *Populus canescens* roots. Plant Physiol. Biochem. 40, 577-584.

Schützendübel A, Polle A (2002b) Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. J. Exp. Bot. 53, 1351-1365.

Sela M, Tel-OR E, Fritz E, Hutterman A (1988) Localization and toxic effects of cadmium, copper and uranium in *Azolla*. Plant Physiol. 88, 30-36.

Semane B, Cuypers A, Smeets K, Belleghem FV, Horemans N, Schat H, Vangronsveld J (2007) Cadmium responses in *Arabidopsis thaliana*: glutathione metabolism and antioxidative defence system. Physiol. Plant.129, 519-528.

Sen AK, Mondal NG, Mandal S (1994) Toxic effects of chromium (VI) on the plant *Salvinia natans* L. Environ. Ecol. 12, 279–283.

Shah K, Kumar RG, Verma S, Dubey RS (2001) Effect of cadmium on lipid peroxidation, superoxide anion generation and activities of antioxidant enzymes in growing rice seedling. Plant Sci. 161, 1135-1144.

Shah K, Nongkynrih JM (2007) Metal hyperaccumulation and bioremediation. Biol. Plant. 51, 618-634.

Shanker AK (2003) Physiological, biochemical and molecular aspects of chromium toxicity and tolerance in selected crops and tree species. PhD Thesis, Tamil Nadu Agricultural University, Coimbatore, India.

Shanker1 AK, PathmanabhanG (2004) Speciation dependant antioxidative response in roots and leaves of sorghum (*Sorghum bicolor* (L.) Moench cv CO 27) under Cr (III) and Cr (VI) stress. Plant and Soil, 265, 141–151.

Shanker AK, Djanaguiraman M, Sudhagar R, Chandrashekar CN, Pathmanabhan G (2004) Differential antioxidative response of ascorbate glutathione pathway enzymes and metabolites to chromium speciation stress in green gram (*Vigna radiata* (L.) R.Wilczek. cv CO 4) roots. Plant Sci. 166, 1035-1043.

Shanker KA, Cervantes C, Loza-Tavera H, Avudainayagam S (2005a) Chromium toxicity in plants. Environ. Internat. 31, 739–753.

Shanker AK, Ravichandran V, Pathmanabhan G (2005b) Phytoaccumulation of chromium by some multi purpose tree seedlings. Agroforestry Syst. 64, 83–87.

Sharma AK, Sharma A (1980) Chromosome Techniques-Theory and Practice, Butterworths, London.

Sharma DC, Chatterjee, Sharma CP (1995) Chromium accumulation and its effect on wheat (*Triticum aestivum* L. cv.HD 2204) metabolism. Plant Sci. 111, 145-151.

Sharma DC, Sharma CP (1996) Chromium uptake and toxicity effects on growth and metabolic activities in wheat, *Triticum aestivum* L. cv. UP 2003. Ind. J. Exp. Biol. 34, 689– 691.

Sharma DC, Sharma CP, Tripathi RD (2003) Phytotoxic lesions of chromium in maize. Chemosphere 51, 63–68.

Simova-Stoilova L, Stoyanova Z, Demirevska-Kepova K, Smilova E (2002) Effect of Cu and Mn toxicity on growth parameters and photosynthetic pigments of young barley plants. Compt. Rend. Acad. Bulg. Sci. 55, 83-88.

Singh S, Eapen S, D'souza SF (2006) Cd accumulation and its influence on lipid peroxidation and antioxidative systems in an aquatic plant *Bacopa monnieri.* L. Chemosphere 62, 233-246.

Singh HP, Batish DR, Kohli RK, Arora K (2007) Arsenic-induced root growth inhibition in mung bean (*Phaseolus aureus* Roxb.) is due to oxidative stress resulting from enhanced lipid peroxidation. Plant Growth Regul. 53, 65-73.

Sinha S, Saxena R, Singh S (2005) Chromium induced lipid peroxidation in the plants of *Pistia stratiotes* L.: role of antioxidants and antioxidant enzymes. Chemosphere 58, 595-604.

Smeets H, Cuypers A, Lambrechts A, Semane B, Hoet P, Laere AV, Vangronsveld Somashekaraiah BV, Padmaja K, Prasad ARK (1992) Phytotoxicity of cadmium ions on germinating seedlings of mung bean (*Phaseolus vulgaris*): involvement of lipid peroxides in chlorophyll degradation. Physiol. Plant. 85, 85–89.

Smeets H, Cuypers A, Lambrechts A, Semane B, Hoet P, Laere AV, Vangronsveld J (2005) Induction of oxidative stress and antioxidative mechanisms in *Phaseolus vulgaris* after Cd application. Plant Physiol. Biochem. 43, 437-444.

Somashekaraiah BV, Padmaja K, Prasad ARK (1992).Phytotoxicity of cadmium ions on germinating seedlings of mung bean (*Phaseolus vulgaris*): involvement of lipid peroxides in chlorophyll degradation. Physiol. Plant. 85, 85–89.

Srivastava M, Lena Q Ma, Singh N, Singh S (2005) Antioxidant responses of hyper-accumulator and sensitive fern species to arsenic. J. Exp. Bot. 56, 1335-1342.

Street RA, Kulkarni MG, Stirk WA, Southway C, Vanstaden G (2007) Toxicity of metal elements on germination and seedling growth of widely used medicinal plants belonging to Hyacinthaceae. Bull. Environ. Contam. Toxicol. 79, 371-376.

Sujatha K (2007) Tissue culture studies and molecular characterization of pongamia (*Pongamia pinnata* (L.) Pierre), an oil producing tree legume. A thesis submitted to University of Pune.

Sujatha K, Hazra S (2006) *In vitro* regeneration of *Pongamia pinnata* Pierre. J. Plant Biotechnol. 23, 263-270.

Sunil Kumar, Mehta UJ, Hazra S (2008a) Accumulation of Cd in growing peanut (*Arachis hypogaea* L.) seedlings and its effect on lipid peroxidation, and on the antioxidative enzymes Catalase and Guaiacol peroxidase. J. Plant Nutrit. and Soil Sci. 171, 440-447.

Sunil Kumar**,** Mehta UJ, Hazra S (2008b) In *vitro* study on Chromium and Copper Accumulation Potential of *Pongamia pinnata* (L.) Pierre seedlings. Bioremediation, Biodiversity and Bioavailability (In Press).

Suseela MR, Sinha S, Singh S, Saxena R (2002) Accumulation of Chromium and Scanning Electron Microscopic Studies in *Scirpus lacustris* L. Treated with Metal and Tannery Effluent. Bull. Environ. Contam. Toxicol. 68, 540–548.

Sykes M, Yang V, Blankenburg J, Abu Bakr S (1999) Biotechnology: working with nature to improve forest resources and products. Int. Environ. Conf. 29, 631-637.

Takemura T, Hanagata N, Dubinsky Z, Karube I (2002) Molecular characterization and response to salt stress of mRNAs encoding cytosolic Cu/Zn superoxide dismutase and catalase from *Bruguiera gymnorrhiza.* Trees 16, 94–99.

Tanaka K, Fujimaki S, Fujiwara T, Yoneyama T, Hayashi H (2007) Quantitative estimation of the contribution of the phloem in cadmium transport to grains in rice plants (*Oryza sativa* L.). Soil Sci. Plant Nutr. 53, 72-77.

Tausz M, Sircelj H, Grill D (2004) The glutathione system as a stress marker in plant ecophysiology: is a stress-response concept valid? J. Exp. Bot. 55, 1955–1962.

Teisseire H, Guy V (2000) Copper-induced changes in antioxidant enzymes activities in fronds of duckweed (*Lemna minor*). Plant Sci. 153, 65-72.

Tewari RK, Kumar P, Sharam PN (2006) Antioxidant responses to enhanced generation of superoxide anion radical and hydrogen peroxide in copper stressed mulberry plant. Planta 223, 1145-1153.

Thomas JC, Davies EC, Malick FK, Endreszl C, Willaims Cr, Abbas M, Petrella S, Swisher K, Perron M, Edwards R, Ostenkowski P, Urbanczyk N, Wiesend WN, Murray KS (2003) Yeast metallothioneins in transgenic tobacco promotes copper uptake from contaminated soils. Biotech. Prog. 19, 273-280.

Unterbrunner R, Puschenreiter M, Sommer P, Wieshammer G, Tlustoš P, Zupan M, Wenzel WW (2007) Heavy metal accumulation in trees growing on contaminated sites in Central Europe. Environ. Pollution 148, 107-114.

Utriainen MA, Kärenlampi LV, Kärenlampi SO, Schat H (1997) Differential tolerance to copper and zinc of micropropagated birches tested in hydroponics. New Phytol. 137, 543– 549.

Van Belleghem F, Cuypers A, Semane B, Smeets K, Vangronsveld K, d'Haen J, Valcke R (2007) Subcellular localization of cadmium in roots and leaves of *Arabidopsis thaliana*. New Phytol. 173, 495-508.

Vangronsveld J, Clijsters H (1994) Toxic effects of metals. In: Farago, M.E. (Ed.), Plant and the Chemical Elements—Biochemistry, Uptake, Tolerance and Toxicity. VCH, New York, pp. 149–177.

Vassilev A, Vangronsveld J, Yordanov I (2002) Cadmium phytoextraction: present state, biological backgrounds and research needs. Bulg. J. Plant Physiol. 28, 68-95.

Vatamaniuk OK, Mari S, Lu YP, Rea PA (2000) Mechanism of heavy metal ion activation of phytochelatin synthease-blocked thiols are sufficient for PC synthase catalyzed trasnpeptidation of glutathione and related peptides. J. Biol. Chem. 275, 31451-31459.

Vitoria AP, Rodriguez APM, Cunha M, Lea PJ, Azevedo, RA (2003/4) Structural changes in radish seedlings exposed to cadmium. Biol. Plant. 47, 561-568.

Vivek X, Gupta AK (2004) Biodiesel production from Karanja oil. J. Scient. Indust. Res. 63, 39-47.

Wagner GJ (1993) Accumulation of cadmium in crop plants and its consequences to human health. Adv. Agron. 51, 173-212.

Wallace A, Soufi SM, Cha JW, Romney EM (1976) Some effects of chromium toxicity on bush bean plants grown in soil. Plant and Soil 44, 471– 473.

Wang H, Shan X-q, Wen B, Zhang S, Wang Z-j (2004) Responses of antioxidative enzymes to accumulation of copper in a copper hyperaccumulator of *Commoelina communis*. Arch. Environ. Contam. Toxicol. 47, 185-192.

Weckx JEJ, Clijsters HMM (1996) Oxidative damage and defense mechanisms in primary leaves of *Phaseolus vulgaris* as a result of root assimilation of toxic amounts of copper. Physiol. Plant. 96, 506-512.

Willekens H, Inz´e D, Van Montagu M, Van Camp W (1995) Catalase in plants. Mol. Breed. 1, 207–228.

Willekens H, Chamnongpol S, Davey M, Schraudner M, Langebartels C, Van Montagu M (1997) Catalase is a sink for H_2O_2 and is indispensable for stress defence in C3 plants. EMBO J. 16, 4806–4816.

Williams LE, Pittmans JK and Hall J (2000) Emerging mechanism for heavy metal transport in plants. Bioch. Et. Biophys. Acta 1465, 104-126.

Wojcik M, Tukiendorf A (2005) Cadmium uptake, localization and detoxification in *Zea mays*. Biol. Plant. 49, 237-245.

Wojcik M, Skorzyska- Polit E, Tukiendorf A (2006) Organic acid accumulation and antioxidants enzyme activities in *Thalspi caerulescens* under Zn and Cd stress. Plant Growth Regul. 48, 145-155.

Wojtaszek P (1997) 'Oxidative burst: an early plant response to pathogen infection' Biochem. J. 322, 681-692.

Xia Y, Shen G (2007) Comparative studies of copper tolerance and uptake by three plant species of the genus *Elsholtzia*. Bull. Environ. Contam. Toxicol. 79, 53-57.

Yarbrough JA (1949) *Arachis hypogaea*. The seedling, its cotyleons, hypocotyl and roots. Am. J. Bot. 36, 758-772.

Yarbrough JA (1957a) *Arachis hypogaea*. The seedling, its epicotyl and foliar organs. Am. J. Bot. 44, 19-30.

Yarbrough JA (1957b). *Arachis hypogaea*. The form and structure of the stem. Am. J. Bot. 44, 31-36.

Yruela I (2005) Copper in Plants. Braz. J. Plant Physiol. 17, 145-156.

Yu H, Wang JL, Fang W, Yuan JG, Yang ZY (2006) Cadmium accumulation in different rice cultivars and screening for pollution-safe cultivars of rice. Sci. Total Environ. 370, 302-309.

Yu XZ, Gu JD, Huang SZ (2007) Hexavalent chromium induced stress and metabolic responses in hybrid willows. Exotoxicology 16, 299-309.

Zeid IM (2001) Responses of *Phaseolus vulgaris* to chromium and cobalt treatment. Biol. Plant. 44, 111-115.

Zhang H, Jiang Y, He Z, Ma M (2005) Cadmium accumulation and oxidative burst in garlic (*Allium sativum*). J. Plant Physiol.162, 977-984.

Zhang H, Xia Y, Wang G, Shen Z (2008) Excess copper induces accumulation of hydrogen peroxide and increases lipid peroxidation and total activity of copper–zinc superoxide dismutase in roots of *Elsholtzia haichowensis*. Planta 227, 465-475.

Zhu Y, Yu H, Wang J, Fang W, Yuan J, Yang Z (2007) Heavy metal accumulations of 24 Asparagus bean cultivars grown in soil contaminated with Cd alone and with multiple metals (Cd, Pb and Zn). J. Agric. Food Chem. 55, 1045-1052.

Author's Publication

RESEARCH WORK PUBLISHED OR COMMUNICATED

1. Sunil Kumar, Urmil J. Mehta and Sulekha Hazra (2008). Accumulation of Cd in growing peanut (*Arachis hypogaea* L.) seedlings and its effect on lipid peroxidation, and on the antioxidative enzymes Catalase and Guaiacol peroxidase. J. of Plant Nutrition and Soil Science 171 (3), 440-447.

2. Sunil Kumar, Urmil J. Mehta and Sulekha Hazra (2008). *In vitro* study on Chromium and Copper Accumulation Potential of *Pongamia pinnata* (L.) Pierre seedlings. Bioremediation, Biodiversity and Bioavailability (In Press).

3. Raju D., **Sunil Kumar,** Urmil J. Mehta and Sulekha Hazra (2008). Differential accumulation of Manganese in three mature tree species (Holoptelia, Cassia, Neem) growing on a mine dump. Current Science 94 (5), 639-643.

4. Sunil Kumar, Urmil J. Mehta and Sulekha Hazra (2004). "Application of *in vitro* techniques to identify the potential of *Albizzia lebbeck* and *Simmondsia chinensis* in removing contaminant metal". Proceedings of the International Symposium on "Soil and Groundwater Contamination, Risk Assessment and Remedial Measures" pp 225 – 234, NGRI, Hyderabad, India.

5. Sunil Kumar and Sulekha Hazra (2005)**.** Tissue culture approach to understand Chromium accumulation in *Pongamia pinnata*. Plant Biotechnology: New Frontiers. Proceeding of National Symposium pp. 321-327, CIMAP Lucknow, India.

PRESENTATIONS AT NATIONAL/ INTERNATIONAL SEMINARS

1. **Sunil Kumar** and Sulekha Hazra. "Influence of Cu on shoot cultures of Jojoba". A paper presented at National Seminar on New Trends in Biotechnology. DBT Co-Sponsored Symposium organized by Dhempe college of Arts and Science in Collaboration with NIO, Goa during 11th and 12th January, 2007.

2. Actively participated in UGC sponsored **National workshop** in **"Techniques in Cell Biology"** held at Kolkata University, Kolkata during 30th March –5th April 2007.

3. **Sunil Kumar** and Sulekha Hazra (2006)**.** Tissue culture approach to understand Chromium accumulation in *Pongamia pinnata*. A poster presented on National Science Day at National Chemical Laboratory, Pune.

4. **Sunil Kumar**, Urmil J. Mehta and Sulekha Hazra (2004). "Application of *in vitro* techniques to identify the potential of *Albizzia lebbeck* and *Simmondsia chinensis* in removing contaminant metal". A paper presented at International Conference on "Soil and Groundwater Contamination, Risk Assessment and Remedial Measures" held at NGRI Hyderabad during 8th-11th December, 2004.

5. Raju D., **Sunil Kumar,** Urmil J. Mehta and Sulekha Hazra. Accumulation of Mn in three tree species growing on Manganese Mine Dump. A poster presented at International Conference on " Toxic Exposure Related Biomarker" held at NEERI Nagpur during 10th-11th January, 2008.

Accumulation of cadmium in growing peanut (*Arachis hypogaea* L.) seedlings—Its effect on lipid peroxidation and on the antioxidative enzymes catalase and guaiacol peroxidase

Sunil Kumar[1], Urmil J. Mehta[1], and **Sulekha Hazra[1]***

[1] Plant Tissue Culture Division, National Chemical Laboratory, Pune-411008, India

Abstract

In plants exposed to high metal concentrations, mechanisms to counteract the oxidative burst are crucial for its survival. To investigate the temporal sequence of physiological reactions of peanut seedlings (*Arachis hypogaea* L.) to cadmium exposure, seeds were cultured in increasing concentrations of $CdCl_2$, ranging from 50 to 300 μM. Germination frequency was scored, and the distributions of Cd in root, stem, and leaves were determined after 2 and 4 weeks of culture. Lipid peroxidation and activities of antioxidative enzymes including catalase (CAT; EC 1.11.1.6) and guaiacol peroxidase (GPX; EC 1.11.1.7) were estimated in these three parts of the plant. Germination of seedlings was not affected, but the growth of seedlings was severely suppressed with increasing concentrations of $CdCl_2$ and incubation period. Pattern of Cd distribution in the three organs varied with concentration and period of exposure to Cd. Increased lipid peroxidation was detected in all parts of the developing seedlings with increasing metal accumulation. Catalase and guaiacol peroxidase activity varied in the three parts of the seedlings with concentration of Cd and incubation period. Guaiacol peroxidase activity appears to be more active in scavenging the reactive oxygen species in developing peanut seedlings. The results of the present experiment demonstrate the advantages of a tissue-culture model system in studying the complex network of interactions of various factors in stress tolerance.

Key words: abiotic stress / heavy metal / stress tolerance / TBARS / thiobarbituric acid / tissue culture

Accepted January 2, 2008

1 Introduction

Cadmium is recognized as a metal posing threat to agricultural food quality due to its mobility in the soil–plant system (*Pal* et al., 2006). It is added to soil from the metal-working industries, waste incinerators, urban traffic, cement factories, and as a byproduct of phosphate fertilizers (*Pal* et al., 2006). In areas with low anthropogenic activity, Cd can be released as a result of rock-mineralization processes. It enters into cells due to its similar chemical and physical characteristics to plant nutrients, using Ca channels or Fe, Mn, or Zn transporters (*Wojcik* et al., 2006). It can cause many toxic symptoms, such as inhibition of growth and photosynthesis, activation or inhibition of enzymes, disturbances in plant–water relationships, ion metabolism, and formation of free radicals (*Pal* et al., 2006).

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 hyperaccumulators and have been studied significantly for phytoremediation of metal-contaminated sites (*Prasad* et al., 2003) and for the 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 the presence of Cd under field and pot conditions, it was shown that significant differences exist between peanut cultivars in terms of Cd accumulation in kernels (*Bell* et al., 1997). 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 was contained in the testa. Other investigations demonstrated that Ca enters the peanut kernel mainly through direct uptake by root hairs on the pods (*Bledsoe* et al., 1949). To the contrary, other researchers showed that Cd uptake by peanut occurs *via* the main root system with direct pod uptake contributing less than 5% to the total Cd accumulation in the kernel (*McLaughlin* et al., 2000).

With respect to phytoremediation, it appears that peanut has the double advantage by absorbing Cd from soil through roots and directly through the pods. However, this species is not a hyperaccumulator. Genetic transformation of peanut using *Agrobacterium*-mediated transformation and by direct DNA-delivery methods are standardized by several researchers (*Cheng* et al., 1996; *Joshi* et al., 2005), and transgenics

* Correspondence: Dr. S. Hazra; e-mail: s.hazra@ncl.res.in

of peanut carrying organic- (*Joshi* et al., 2005) and inorganic-mercury (*Yang* et al., 2003)–tolerant genes have been developed. Present research was conducted to study the effect of $CdCl_2$ on developing seedlings of peanut *in vitro* keeping in view that a thorough understanding of the biochemical detoxification strategies, which this species adopted against oxidative stress induced by metal ions, is needed to design the approach to manipulate heavy-metal accumulation.

Cadmium produces oxidative stress by generating free radicals and reactive oxygen species (ROS). These species react with lipids, proteins, pigments, nucleic acids, and cause lipid peroxidation and membrane damage thereby affecting cell viability (*Gratao* et al., 2005). Increased production of toxic oxygen derivatives is considered a common feature of stress conditions. Some of the pathways that are intimately related with stress in plant cells are linked to two antioxidative enzymes, peroxidase (EC.1.11.1.7), and catalase (EC 1.11.1.6) (*Gratao* et al., 2005).

In the present research, a plant tissue–culture technique was used as a tool to study the effect of Cd on peanut seed germination and growth. The experiment was designed to investigate (1) extent of Cd-induced stress in seedling germination and growth, (2) distribution of metal in different parts of the seedlings developed in the presence of Cd, (3) effect of $CdCl_2$ on lipid peroxidation in different organs, and (4) differential antioxidative response in root, leaves, and stems of the plant.

2 Materials and methods

2.1 Plant material and growth conditions

Peanut (*Arachis hypogaea* L.) seeds, cultivar JL-24, 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, these 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 testa of these seeds was removed aseptically, and the seeds were cultured in media with and without Cd. The culture medium was agar-gelled MS basal medium supplemented with 2% sucrose (w/v) and the pH adjusted to 5.8 prior to autoclaving. Aliquots of filter-sterilized solution of $CdCl_2$ were added aseptically to attain final concentrations of 50 μM, 100 μM, 200 μM, and 300 μM $CdCl_2$. Medium without $CdCl_2$ was used as control. The media were distributed in cotton-plugged culture tubes (20 mL per tube). After incubating the cultures for 3–4 d in dark for radical emergence, the seedlings were transferred in 16 h photoperiod of 32 μmol photons m^{-2} s^{-1} at 25±2°C.

Germination and development of seedlings took 10 days. To obtain sufficient amount of tissue for estimations, observations were recorded and enzyme activities were determined after 2 weeks. Subsequently, to assess the effects of longer exposure, the studies were repeated after 4 weeks. Parameters including germination frequency, shoot height, and root length were noted. Shoot height was measured from cotyledon node to shoot tip. Experiments were repeated five times using ten replicates. Seedlings were harvested after 2 and 4 weeks for quantification of Cd concentration and enzyme assays.

2.2 Estimation of Cd concentration in various parts of seedlings

On harvesting the seedlings after 2 and 4 weeks, the root, stem, and leaves were separated. Plant roots were thoroughly washed with deionized water to remove adhering medium and weighed after eliminating adhering moisture using filter paper. Tissues of three seedlings from each concentration were pooled for each analysis after 2 and 4 weeks and weighed (fresh weight, FW). These were dried in an oven at 95°C to constant weight. The dry weights (DW) per gram of FW were determined prior to using the dry tissues for Cd estimations. The dried plant samples were ground to fine powder with pestle and mortar. The samples were digested in HNO_3-to-$HClO_4$ 3:1 mixture, and Cd concentrations were determined by AAS. The estimations were repeated four times pooling tissues from three seedlings for each estimation.

2.3 Lipid peroxidation

The level of lipid peroxidation products, thiobarbituric reacting substances (TBARS), in peanut seedling tissues was estimated following the modified method used in studies on arsenic tolerance in ferns (*Srivastava* et al., 2005). Approximately 0.25 g of frozen plant-tissue samples were used for each estimation. The concentration of lipid peroxides was quantified and expressed as total TBARS in terms of nmol $(g\ FW)^{-1}$ using an extinction coefficient of 155 $mM^{-1}\ cm^{-1}$. Level of lipid peroxidation in terms of TBARs was assessed in the seedlings cultured for 2 and 4 weeks in media with and without Cd. The experiment was repeated five times with five replicates in each concentration of Cd.

2.4 Determination of catalase and guaiacol peroxidase activity

Tissue samples (0.25 g) were homogenized in chilled extraction buffer containing 50 mM sodium phosphate buffer pH 7.0, 0.5% Triton X-100, and 1% polyvinylpyrrolidone. This was filtered through the folds of muslin cloth. After the homogenate was centrifuged at 10,000 *g* for 20 min at 4°C, the supernatant was used for enzyme assay. Catalase (CAT, EC 1.11.1.6) activity was determined using the method described earlier (*Aebi*, 1984). The activity was assayed following the decrease in absorbance due to H_2O_2 decomposition at 240 nm for 30 s. One unit of activity for CAT was defined as the calculated consumption of 1 μM H_2O_2 min^{-1} $(g\ FW)^{-1}$. The experiment was repeated four times with four replicates. Activity of guaiacol peroxidase (GPX, EC 1.11.1.7) was measured by the method of *Chance* and *Maehly* (1955). The reaction was initiated by addition of H_2O_2, and change in optical density at 470 nm was measured at intervals of 10 s for

2 min. Activity was calculated using the extinction coefficient 26.6 $mM^{-1}\,cm^{-1}$ for the oxidized tetraguaiacol polymer. One unit of peroxidase activity was defined as the calculated consumption of 1 μM H_2O_2 min^{-1} $(g\ FW)^{-1}$. Experiment was repeated four times with four replications.

2.5 Data analysis

The results are the mean ± SD of replications. All data were subjected to one-way ANOVA analysis, using at least the 5% level of significance. When necessary, log-transformation was employed to maintain homogeneity of variance, which was evaluated using plots of studentized residues.

3 Results and discussion

3.1 Effect on seed germination and seedling growth

The concentrations of Cd used in the present experiment on peanut seedlings were derived from the results of previous experiments on the Cd tolerance of peanut callus (*Chakravarty* and *Srivastava,* 1994). These researchers recorded the mitotic abnormalities in the dividing cells and synthesis of Cd-binding proteins. In the present study, radicle emergence was considered as germination. The frequency of germination scored after 2 weeks varied from 80% to 92% (Tab. 1) in medium with and without Cd. On extended incubation for 2 weeks, the frequency of response increased and ranged between 89% and 93%. Although not significant, a trend of increasing germination frequency was noted with increasing concentration of Cd and with extended incubation time.

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 (Fig. 1). In control medium, the shoot height was 5.77 cm (Tab. 1) after 2 weeks. It reached the height of 7.25 cm on extended incubation of 2 weeks. In Cd-containing media, the shoot and root lengths were reduced compared to control seedlings in 2 and 4 weeks of culture. The increase in shoot and root length during the extended incubation for another 2 weeks in the presence of Cd was slower, suggesting that the elongation process is retarded upon longer exposure. The seedlings appeared healthy and green in all concentrations of Cd tested. The leaf size was reduced in 300 μM as compared to the control seedlings. The root differentiated in all media, but the elongation after 2 weeks was reduced in the presence of Cd. The roots of the seedlings were bushy due to development of lateral roots both in control medium and in lower concentrations (50 and 100 μM) of Cd.

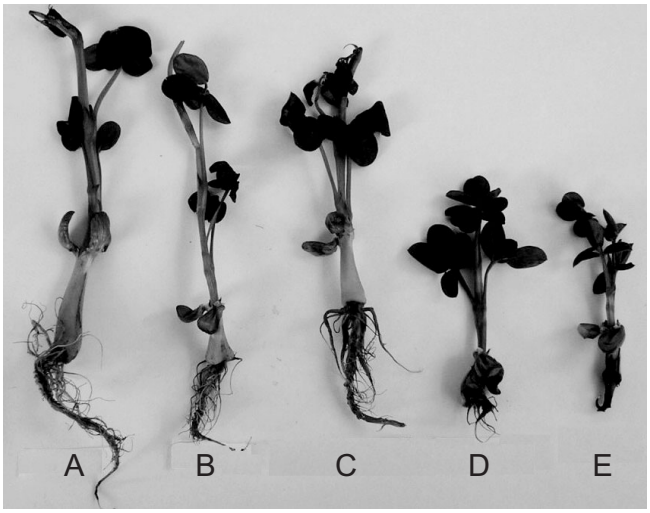

Figure 1: Effect of Cd on *Arachis hypogaea*–seedling growth after 2 weeks of culture in control medium (A) and in medium containing 50, 100, 200, 300 μM of Cd (B, C, D, E, respectively).

Retarded growth of each part of the peanut seedling confirms adverse effect of Cd on plant differentiation. This effect was more obvious on differentiation of the lateral roots. In some of the seedlings cultured with 100–300 μM Cd, a black deposition was noted at the origin of the lateral root. Such deposition of unknown material has been reported in the root of radish exposed to Cd (*Vitoria* et al., 2003). It was presumed that this unknown material is composed of epidermal and cortical dead and decomposed cells, which accumulated externally on the root surface.

Table 1: Effect of $CdCl_2$ on seed germination after 2 and 4 weeks (means ± SD).

Concentration of $CdCl_2$ (μM)	**Germination frequency**		**Shoot height** (cm)		**Root length** (cm)	
	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
Control	80 ± 15.8	89 ± 3.82	5.77 ± 0.81	7.25 ± 2.40	4.79 ± 0.51	6.84 ± 1.06
50 μM	83 ± 19.2	90 ± 2.42	3.07 ± 0.68	6.56 ± 1.73	3.73 ± 0.29	6.07 ± 0.86
100 μM	86 ± 11.4	96 ± 5.47	3.67 ± 0.90	6.26 ± 2.09	4.03 ± 0.07	5.85 ± 0.63
200 μM	90 ± 8.36	92 ± 5.09	2.09 ± 0.70	5.26 ± 1.71	2.06 ± 0.64	4.70 ± 0.71
300 μM	92 ± 4.47	93 ± 5.71	1.48 ± 0.35	2.85 ± 1.03	1.13 ± 0.15	2.59 ± 1.29
ANOVA	ns	ns	$p < 0.01$	$p < 0.05$	$p < 0.01$	$p < 0.01$

3.2 Effect on biomass and distribution of Cd in different parts of seedling

There was a parallel increase in Cd concentrations in each of these parts indicating absorption of Cd from medium and distribution to all plant parts. The amount of Cd absorbed by each of these organs increased with increasing concentration of Cd in the medium. No Cd was detected in control plants. In the initial 2 weeks, Cd accumulation in each part of the seedling followed a definite pattern (Fig. 2A–C). The primary site of Cd accumulation was the root followed by stem and leaves. Roots are known to accumulate more Cd as compared to stems and leaves (*Benavides* et al., 2005; *Garcia* et al., 2006; *Zhu* et al., 2007). The amount of Cd transferred into leaves was less in all the concentrations tested. The pattern of Cd accumulation in the initial period and the later period of incubation varied in the organs. Analyzing the data on Cd concentration in different parts of the seedlings in 2 and 4 weeks (Fig. 2A–C) indicates that Cd absorption by the root and thereafter transportation to the stem was rapid during the first 2 weeks. In later weeks, the fraction of Cd accumulating in roots was reduced (Fig. 2A–C). On the contrary, in the stem in the initial part of the culture period, Cd accumulation was less. In the later 2 weeks, up to 200 μM, the Cd accumulation was lower. However, at higher concentration and longer incubation, Cd concentration was high in this organ. This could be due to high accumulation and slow transfer of metal to the leaves. In the leaves, Cd accumulation was significantly higher in the later period compared to the initial 2 weeks.

Presumably, from emergence of the radicles and their differentiation thereafter, 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 both after 2 and 4 weeks as compared to stem or leaves. As the plant differentiates, heavy-metal cations are absorbed by the roots and transferred to the stem and leaves. In the stem and leaves, up to a supply of 200 μM, the concentration of Cd increased with its concentration in the medium. However, in 300 μM, both in stem and leaves the Cd concentration increased drastically in the last 2 weeks. Possibly the capacity of the stem to accommodate more metal increased with its slow cellular growth in the lower concentrations of Cd, reflecting as reduced Cd concentration in the stem when expressed in relation to DW. 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 (Tab. 1) and increased Cd concentration (Fig. 2B) 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. Upon exposure to 300 μM Cd, accumulation in both stem and leaves was much higher than during the first 2 weeks. Shoot elongation was markedly reduced at this concentration (Tab. 1) after 4 weeks. At this concentration, the leaf size was reduced, too. Reduced root growth, chlorosis, and leaf rolls are the main and easily visible symptoms of Cd toxicity in plants (*Benavides* et al., 2005; *Wojcik* et al., 2006; *Van Belleghem* et al., 2007). As the increase in biomass in this concentration was less in both stem and leaves, there is a drastic relative

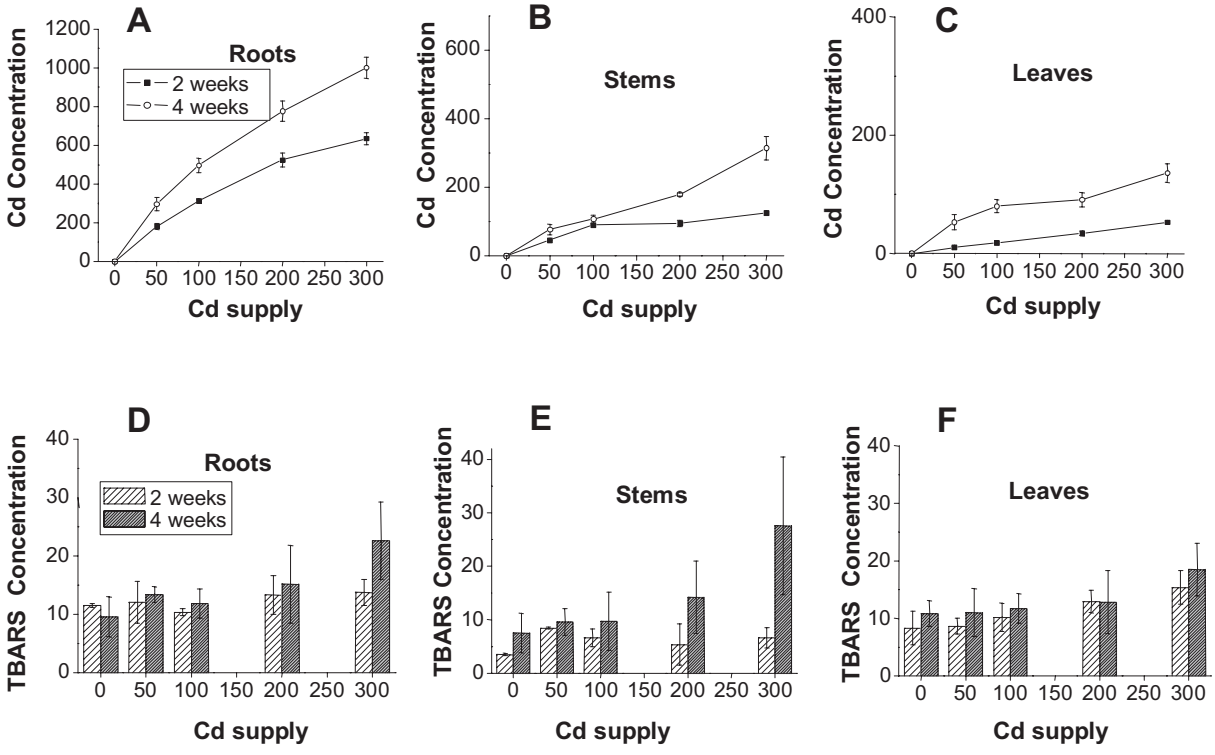

Figure 2: Cadmium accumulation in roots (A), stems (B), and leaves (C) of *Arachis hypogaea* seedlings and its effect on level of TBARS (D, E, F) as indicator of lipid peroxidation at different concentrations and exposure of Cd (means ± SD).

increase in Cd concentration in both organs. In an attempt to quantify the translocation of Cd in rice, it has been demonstrated that 91%–100% of the Cd in grains was imported *via* the phloem, confirming the importance of the phloem in Cd transport (*Tanaka* et al., 2007). In peanut, compared to the amount of Cd accumulated in the leaves in the first 2 weeks, the amount was much higher in the second part of culture, suggesting the involvement of the stem vasculature in transport of Cd to the leaves. Extensive work has been done by several researchers to understand the mechanisms of metal accumulation and tolerance in plants and has been reviewed recently (*Polle* and *Schützendübel*, 2003), but the complex network of interactions of hormone and nutrient factors in abiotic stress tolerance is not fully understood.

3.3 Lipid peroxidation

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 effects 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 were quantified by estimating the thiobarbituric acid reacting substances (TBARS).

The TBARS were determined in the seedling parts after 2 week of culture in control medium. The TBARS concentration was highest in roots (11.53 nmol [g FW]$^{-1}$) followed by leaves (8.33 nmol [g FW]$^{-1}$) and stems (3.51 nmol [g FW]$^{-1}$). In the roots of control plants (Fig. 2D), which were in direct contact with the medium, the TBARS concentration during the last 2 weeks of incubation did not change, whereas in stems and leaves, it was significantly higher (Fig. 2 E and F) after 4 weeks of incubation. The lipid peroxides in roots, stems, and leaves in control plants after 2 weeks and its increase in stem and leaves on extended incubation could be due to the stress encountered by these organs of the plants grown *in vitro* (*Cassells* and *Curry*, 2001). It is not clear, whether the unaltered TBARS in the roots of 2- and 4-week cultures is due to the continuous contact of this organ with the medium, allowing for the diffusion of TBARS out of the root tissue.

The level of TBARS increased in all three parts of the peanut seedlings (Fig. 2D–F) developed in the presence of Cd. Increases of TBARS following Cd exposure have been observed in several plant species (*Benavides* et al., 2005; *Gomes-Junior* et al., 2006), with cell membranes being severely affected by peroxidation leading to irreversible damage. In the roots of the plants cultured in the presence of Cd, the TBARS increased gradually with time and concentration reaching the highest level (22.62 nmol [g FW]$^{-1}$) after 4 weeks in 300 μM Cd. The pattern of TBARS accumulation varied in the stem. In 2 weeks, it increased in all the concentrations compared to the control, but the data do not show a definite pattern. At 4 weeks, the lipid peroxides in stems increased with the Cd concentration up to 200 μM Cd (14.18 nmol [g FW]$^{-1}$), and a dramatic increase (27.58 nmol [g FW]$^{-1}$) was noted in 300 μM Cd. In the leaves, lipid peroxidation increased with concentration and period of incubation, but at 300 μM Cd, TBARS concentration was not as high (18.48 nmol [g FW]$^{-1}$) as in the stem. The variation in lipid peroxides in different parts of the same seedling in similar medium and identical culture conditions suggest the existence of alternative mechanisms of Cd tolerance in different organs of the plant. The morphological, gravimetric, and lipid peroxidation studies suggest marked Cd stress–induced changes in the cultures grown for 4 weeks in the presence of 300 μM Cd.

3.4 Effect of cadmium on CAT and GPX

Oxygen–free radical–mediated oxidative stress has been implicated as one of the underlying agents causing tissue injury following exposure of plants to stress conditions (*Dixit* et al., 2001). To protect from such injuries, plants are equipped with various constitutive and inducible antioxidants (*Dong* et al., 2006). Excessive levels of H_2O_2 could be minimized by the activities of oxygen radical–detoxifying enzymes like CAT and GPX (*Gratao* et al., 2005). Thus, often the activities of these enzymes are used as markers to determine stress in plants. In the present study, accumulation of Cd and increased lipid peroxidation were noted in peanut seedlings cultured in various concentrations of Cd.

We noted CAT activity in all the organs even in the absence of the toxic metal in the medium. Activity of CAT increased in roots after 2 weeks of culture with increasing concentration of Cd in the medium (Fig. 3A). The activity was highest in the roots in the seedlings developed in 300 μM Cd. In the 4-week cultures, the CAT activity was reduced in this organ in all the concentrations of Cd. *Patel* et al. (2005) showed an increase in CAT activity in *Colocassia esculentum* exposed to Cd. Our data on Cd concentration in roots (Fig. 2A) showed a rapid accumulation of Cd in the roots within 2 weeks of culture. Reduction in CAT activity in the roots after 4 weeks of culture could be due to inhibition of the enzyme caused by increased accumulation of Cd. Previous studies have shown that Cd reduces the concentration of some nutrients, such as Mg, Ca, or Fe (*Azevedo* et al., 2005). As Fe is a constituent or cofactor of most antioxidative enzymes (*Ranieri* et al., 2003), reduction in Fe level may cause reduction in CAT activity.

In stems, the pattern of CAT activity was similar to that observed in roots (Fig. 3B). During the first 2 weeks, it increased in 100, 200, and 300 μM Cd and dropped on extended incubation. *Skorzynska-Polit* et al. (2003) observed reduced CAT activity in *Arabidopsis thaliana* exposed to Cd. However, data generated from peanut stem tissue on CAT activity were not significant. The amount of Cd detected in this organ was low (Fig. 2B). Presumably, the amount of Cd accumulated in this organ was not high enough to induce effective stress. Alternately, Cd inhibited CAT indirectly by reducing one or more inorganic nutrients.

In leaves, the Cd concentration after 2 weeks was further reduced compared to the stem (Fig. 2C). Activity of CAT

remained unchanged in leaves with respect to concentration of Cd after 2 and 4 weeks except in the presence of 200 μM Cd after 2 weeks. The initial increase in CAT activity in leaves of these cultures dropped back to control level with increasing concentration or period of incubation. The Cd concentration of the leaves was 34 ± 4.0 mg kg^{-1} in plants cultured with 200 μM Cd. Significant amounts (136 ± 16 mg Cd kg^{-1}) were detected in leaves of plants cultured for 4 weeks in 300 μM Cd. Similar to the CAT activity in the roots after 2 and 4 weeks in 300 μM Cd, the CAT activity in leaves dropped to the control level after extended incubation in the presence Cd. This phenomenon remains unexplained with the present state of knowledge as the majority of the cellular processes induced by toxic-metal stress is complex and depends on several environmental, chemical, and biological factors. Cadmium was found to produce oxidative stress, but in contrast to other heavy metals such as Cu, it does not seem to act directly on the production of reactive oxygen species *via* Fenton and/or Haber Weiss reactions (*Salin*, 1988). On the other hand, Cd ions can inhibit or stimulate the activity of several antioxidative enzymes (*Gratao* et al., 2005). Some of the processes interfered by the presence of Cd in leaves include interaction with water balance causing damages in the photosynthetic apparatus (*Gratao* et al., 2005), and active inhibition of stomatal opening by some mechanism which needs to be explored. *Singh* et al. (2006) demonstrated a decrease in CAT activity both in roots and leaves of *Bacopa monnieri. Scebba* et al. (2006) depicted different patterns of CAT activity in roots and leaves in *Miscanthus sinensis.* The CAT activity detected in organs of the peanut plants cultured under control conditions could be due to processes induced by growing cultures under simulated condition (*Cassells* and *Curry*, 2001).

Like lipid peroxidation and CAT activity, GPX activity was detected in all plant parts under controlled condition without Cd (Fig. 3D–F). The activity was highest in the roots followed by leaves and stems. Increased GPX activity was detected in roots and stems of the seedlings exposed to Cd indicating triggering of the ROS-scavenging activity. At higher concentrations (200–300 μM), it increased in both 2- and 4-weeks cultures. In roots, the activity reached 24 μmol min^{-1} g^{-1} after 2 weeks incubation in the presence of 300 μM Cd. It increased dramatically and reached 45 μmol min^{-1} g^{-1} on extended incubation of the cultures for additional 2 weeks. In agreement, GPX activity was found to increase in *A. thaliana, Colocasia,* and tomato plants exposed to Cd (*Skorzyska-Polit* et al., 2003; *Patel* et al., 2005; *Dong* et al., 2006). A similar pattern of activity was noted in the stem, in which the GPX activity in 300 μM Cd increased from 7 μmol min^{-1} g^{-1} after 2 weeks to 21 μmol min^{-1} g^{-1} after 4 weeks. In the stem, the GPX activity increased linearly with increasing concentration of Cd and incubation period. Both increases and decreases in GPX activity have been reported in plants exposed to Cd as reviewed by *Gratao* et al. (2005). In the leaves, the GPX activity did not increase with increasing concentrations of Cd, but it increased with increase in incubation time in the presence or absence of Cd. Coffee-suspension culture did not show a clear response in GPX activity (*Gomes-Junior* et al., 2006). However, *Demirevska-Kepova* et al. (2006) reported the increase in GPX activity in barley leaves. In leaves, the activity was higher in the cultures incubated for 4 weeks, irrespective of the Cd concentration used. This observation points to the fact that the peanut leaves possibly respond differently towards lipid peroxidation and GPX activity in Cd-induced stress. The GPX activity detected in the control plants also indicates the stress in the plants during culture.

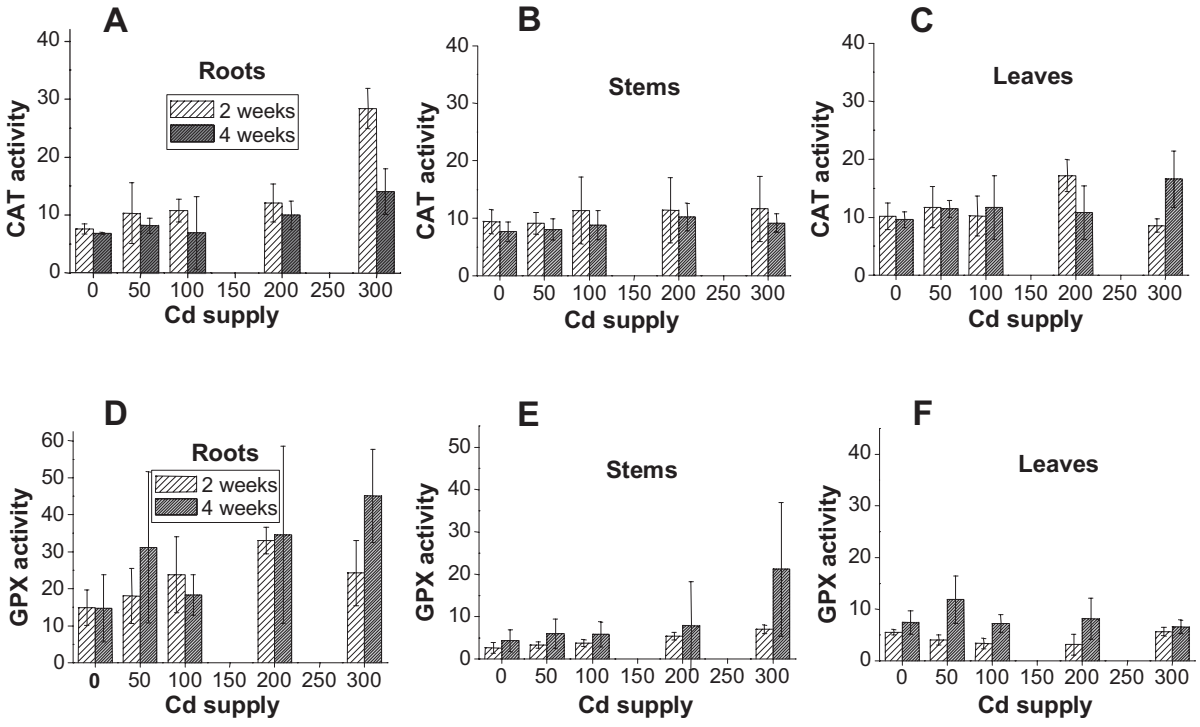

Figure 3: Activities of the antioxidant enzymes CAT (A, B, C) and GPX (D, E, F) in roots, stems, and leaves of *Arachis hypogaea* seedlings cultured in control and Cd-containing medium (means ± SD).

The majority of studies on metal tolerance or stress have been conducted in field or pot cultures. These systems are not fully defined as the soil is composed of several organic and inorganic components and also microbes. The effect demonstrated by a plant system cultured *in vitro* is more specific, and the data are interpreted with reference to the control plants cultured under identical conditions. The data generated from this study not only demonstrate the effect of Cd stress on peanut seedlings in culture, but also confirm the suitability of the plant tissue–culture technique as a useful tool for studies on heavy-metal stress.

From the results, it appears that up to the concentration of 300 μM Cd, germination of seedlings is not affected, but growth is retarded. After incubation for 4 weeks in Cd-containing medium, there was a gradient in metal content in the three organs with roots followed by stem and leaves. This could be due to rapid absorption of metal by the roots and gradual transport to the stem and leaves. Lipid peroxidation is triggered in the plants in culture without metal stress, indicating that the seedlings encounters stress due to the artificial culture conditions. However, it may be assumed that this stress is uniform in all the cultures including controls and stressed plants. This study also shows that peanut seedlings under stress are more responsive towards lipid peroxidation. For scavenging ROS in peanut plants under stress conditions, GPX is more active than CAT.

Acknowledgments

We acknowledge *C.S.I.R. India*, for Research Fellowship awarded to Sunil Kumar and for the research grant provided in the *network program (P24-COR0008) on phytoremediation*.

References

Aebi, H. (1984): Catalase in vitro, in Colowick, S. P., Kaplan, N. O.: Methods in Enzymology. New York Academic Press, London, Toronto, Montreal Sydney, Tokyo, Sao Paulo, pp. 121–126.

Azevedo, H., Gomes, C., Pinto, G., Santos, C. (2005): Cadmium effects in sunflower membrane permeability and changes in catalase and peroxidase activity in leaves and calluses. *J. Plant Nutr.* 28, 2233–2241.

Bell, M. J., McLaughlin, M. J., Wright, G. C., Cruickshank, A. (1997): Inter- and intra-specific variation in accumulation of cadmium by peanut, soybean, and navy bean. *Aust. J. Agr. Res.* 48, 1151–1160.

Benavides, M. P., Gallego, S. M., Tomaro, M. L. (2005): Cadmium toxicity in plants. *Braz. J. Plant Physiol.* 17, 21–34.

Bledsoe, R. W., Comar, C. L., Harris, H. C. (1949): Absorption of radioactive calcium by peanut fruits. *Science* 109, 329–330.

Cakmak, I. (2000): Tansely review no. 111. Possible role of zinc in protecting plant cell from damage by reactive oxygen species. *New Phytol.* 146, 185–205.

Cassells, A. C., Curry, R. F. (2001): Oxidative stress and physiological, epigenetic and genetic variability in plant tissue culture: implications for micro propagators and genetic engineers. *Plant Cell Tiss. Org. Cult.* 64, 145–157.

Chakravarty, B., Srivastava, S. (1994): Responses to cadmium toxicity during *in vitro* growth in *Arachis hypogaea*. *Bull. Environ. Contam. Toxicol.* 52, 749–755.

Chance, B. A., Maehly, C. (1955): Assay of catalase and peroxidase, in Colowick, S. P., Kaplan, O.: Methods in Enzymology. New York Academy Press, London, Toronto, Montreal Sydney, Tokyo, Sao Paulo, pp. 764–775.

Cheng, M., Jarnet, R. L., Li, Z., Demski, J. W. (1996): Production of fertile transgenic peanut (*Arachis hypogaea* L.) plants using *Agrobacterium tumefaciens. Plant Cell Rep.* 15, 653–657.

Demirevska-Kepova, K., Simova-Stoilova, L., Stoyanova, Z. P., Feller, U. (2006): Cadmium stress in Barley: growth, leaf pigment and protein composition and detoxification of reactive oxygen species. *J. Plant Nutr.* 29, 451–468.

Dixit, V., Pandey, V., Shyam, R. (2001): Differential antioxidative responses to Cd in roots and leaves of Pea (*Pisum sativum* L. cv. Azad). *J. Exp. Bot.* 52, 1101–1109.

Dong, J., Wu, F., Zhang, G. (2006): Influence of cadmium on antioxidant capacity and four microelement concentrations in tomato seedlings (*Lycopersicon esculentum*). *Chemosphere* 64, 1659–1666.

Gajewska, E., Shaba, M., Andrzejewska, R. (2006): Nickel induced inhibition of wheat root growth is related to hydrogen peroxide production but not to lipid peroxidation. *Plant Growth Regul.* 49, 95–103.

Garcia, J. S., Gratao, P. L., Azevedo, R. A., Arruda, M. A. Z. (2006): Metal contamination effects on sunflower (*Helianthus annuus* L.) growth and protein expression in leaves during development. *J. Agric. Food Chem.* 54, 8623–8630.

Gomes-Junior, R. A., Moldes, C. A., Delite, F. S., Pompeu, G. B., Gratão, P. L., Mazzafera, P., Lea, P. J., Azevedo, R. A. (2006): Antioxidant metabolism of coffee cell suspension cultures in response to cadmium. *Chemosphere* 65, 1330–1337.

Gratao, P. L., Polle, A., Lea, P. J., Azevedo, R. A. (2005): Making the life of heavy metal stressed plant a little easier. *Funct. Plant Biol.* 32, 481–494.

Houmiel, K. L., Slater, S., Broyles, D., Casagrande, L., Colburn, S., Gonzalez, K., Mitsky, T. A., Reiser, S. E., Shah, D., Taylor, N. B., Tran, M., Valentin, H. E., Gruys, K. J. (1999): Poly (beta-hydroxybutyrate) production in oilseed leukoplasts of *Brassica napus*. *Planta* 209, 547–550.

Joshi, M., Chen, N., Fleming, G., Hazra, S., Ye, C., Nairn, C. J., Yang, H., Ozias-Akins, P. (2005): Use of green fluorescent protein as a non-destructive marker for peanut genetic transformation. *In Vitro Cell. Dev. Biol.* 41, 437–445.

McLaughlin, M. J., Bell, M. J., Wright, G. C., Cozens, G. D. (2000): Uptake and partitioning of cadmium by cultivars of peanut (*Arachis hypogaea* L.). *Plant Soil* 222, 51–58.

Pal, M., Horvath, E., Janda, T., Paldi, E., Szalai, G. (2006): Physiological changes and defense mechanism induced by Cd stress in maize. *J. Plant Nutr. Soil Sci.* 169, 239–246.

Patel, M. J., Patel, J. N., Subramanian, R. B. (2005): Effect of cadmium on growth and activity of H_2O_2 scavenging enzymes in *Colocassia esculentum. Plant Soil* 273, 183–188.

Polle, A., Schützendübel, A. (2003): Heavy metal signaling in plants: linking cellular and organismic responses, in Hirt, H., Shinozaki, K.: Plant responses to abiotic stress. From Topics in current Genetics, Vol. 4. Springer Verlag, Berlin, Heidelberg, pp. 187–215.

Prasad, M. N. V., Freitas, H. M. de O. (2003): Metal hyper accumulation in plants – biodiversity prospecting for phytoremediation technology. *Electronic J. Biotechnol.* 6, 285–321.

Ranieri, A., Baldan, B., Castagna, A., Sebastiani, L., Soldatini, G. (2003): H_2O_2 accumulation in sunflower leaves as a consequence of iron deprivation. *J. Plant Nutr.* 26, 2187–2196.

Salin, M. L. (1988): Toxic oxygen species and protective systems of the chloroplasts. *Physiol. Plant.* 72, 681–689.

Scebba, F., Arduini, I., Ercoli, L., Sebastiani, L. (2006): Cadmium effects on growth and antioxidant enzymes activities in *Miscanthus sinensis*. *Biol. Plant.* 50, 688–692.

Singh, S., Eapen, S., D'souza, S. F. (2006): Cd accumulation and its influence on lipid peroxidation and antioxidative systems in an aquatic plant *Bacopa monnieri*. L. *Chemosphere* 62, 233–246.

Skorzynska-Polit, E., Drazkiewicz, M., Krupa, Z. (2003): The activity of the antioxidant system in cadmium treated *Arabidopsis thaliana*. *Biol. Plant.* 47, 71–78.

Srivastava, M., Lena, Q., Ma, Singh, N., Singh, S. (2005): Antioxidant responses of hyper- accumulator and sensitive fern species to arsenic. *J. Exp. Bot.* 56, 1335–1342.

Tanaka, K., Fujimaki, S., Fujiwara, T., Yoneyama, T., Hayashi, H. (2007): Quantitative estimation of the contribution of the phloem in cadmium transport to grains in rice plants (*Oryza sativa* L.). *Soil Sci. Plant Nutr.* 53, 72–77.

Wojcik, M., Skorzynska-Polit, E., Tukiendorf, A. (2006): Organic acids accumulation and antioxidant enzyme activities in *Thlaspi caerulescens* under Zn and Cd stress. *Plant Growth Regul.* 48, 145–155.

Van Belleghem, F., Cuypers, A., Semane, B., Smeets, K., Vangronsveld, K., d'Haen, J., Valcke, R. (2007): Subcellular localization of cadmium in roots and leaves of *Arabidopsis thaliana*. *New Phytol.* 173, 495–508.

Vitoria, A. P., Rodriguez, A. P. M., Cunha, M., Lea, P. J., Azevedo, R. A. (2003): Structural changes in radish seedlings exposed to cadmium. *Biol. Plant.* 47, 561–568.

Yang, H., Naian, J., Ozias-Akins, P. (2003): Transformation of peanut using using a modified bacterial mercuric ion reductase gene driven by an actin promotor from *Arabidopsis thaliana*. *J. Plant Physiol.* 160, 945–952.

Zhu, Y., Yu, H., Wang, J., Fang, W., Yuan, J., Yang, Z. (2007): Heavy metal accumulations of 24 Asparagus bean cultivars grown in soil contaminated with Cd alone and with multiple metals (Cd, Pb and Zn). *J. Agric. Food Chem.* 55, 1045–1052.

ing the extent of intra- and inter-specific genetic diversity existing in *N. tabacum* and *N. rustica*. The species and genus-specific AFLP markers identified in this study would be useful in introgression breeding programmes of tobacco.

1. Goodspeed, T. H., The genus *Nicotiana*, Chronica Bot Wallham, Mass, USA, 1954, p. 536.
2. Narayan, R. K., Nuclear DNA changes, genome differentiation and evolution in *Nicotiana* (Solanaceae). *Plant Syst. Evol*., 1987, **157**, 161–180.
3. Anon., Information bulletin on crop improvement, Central Tobacco Research Institute, Rajahmundry, 2003.
4. Singh, K. D., Narishmha Rao, C. V., Prabhu, S. R. and Subba Rao, R., Factors influencing TSNA in Indian tobacco. Paper presented at the CORESTA Congress, New Orleans, USA, 22–27 September 2002.
5. Clegg, M. T., In *Plant Population Genetics, Breeding and Genetic Resource* (eds Brown A. H. D. *et al.*), Sinauer Associate Inc, Sunderland, MA, USA, 1997, pp. 98–115.
6. Bogani, P., Lio, P., Intrieri, M. C. and Buiatti, M. A., Physiological and molecular analysis of genus *Nicotiana*. *Mol. Phylogenet. Evol*., 1997, **7**, 62–70.
7. Rufty, R. C., Yi, H. Y. and Wernsman, E. A., RAPD markers linked to wild fire resistance in tobacco identified through bulked-segregant analysis. In Bulletin CORESTA, Montréal, Abstr. AP 63, 1997, p. 50.
8. Yi, H. Y., Rufty, R. C. and Wernsman, E. A., Mapping root-knot nematode resistance gene (*RK*) in tobacco with RAPD markers. *Plant Dis*., 1998, **82**, 319–322.
9. Del Piano, L. *et al*., Genetic variability in *Nicotiana tabacum* and *Nicotiana* species as revealed by RAPD procedure. *Beitr. Tabakforsch. Int*., 2000, **19**, 191–195.
10. Doyle, J. J. and Doyle, J. L., Isolation of plant DNA from fresh tissue. *Focus*, 1990, **12**, 13–15.
11. Vos, P. *et al.*, AFLP; a new technique for DNA fingerprinting. *Nucleic Acids Res*., 1995, **23**, 4407–4414.
12. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, 1989.
13. Rohlf, F. J., NTSYS-pc: Numerical taxonomy and multivariate analysis system, version 2.02. Exter Software, Setauket, New York, 1998.
14. Jaccard, P., Nouvelles recherches Sur la distribution florale Bull Soc vaeed. *Sci. Nat*., 1908, **44**, 223–270.
15. Botstein, D., White, R. L., Skolnick, M. and Davis, R. W., Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum. Genet*., 1980, **32**, 314–331.
16. Ren, N. and Timko, M. P., AFLP analysis of genetic polymorphism and evolutionary relationships among cultivated and wild *Nicotiana* species. *Genome*, 2002, **44**, 559–571.
17. Law, J. R., Donini, P., Koebner, R. M. D. and Reeves, J. C., DNA profiling and plant variety registration. The statistical assessment of distinctiveness in wheat using AFLP. *Euphytica*, 1998, **102**, 335–342.
18. Breyen, P., Rombaut, D., Van, A., Van, M. and Gerrats, T., AFLP analysis of genetic diversity within and between *Arabidopsis thaliana* ecotypes. *Mol. Gen. Genet*., 1999, **261**, 627–634.

ACKNOWLEDGEMENTS. The work was carried out at the National Research Centre on Plant Biotechnology (NRCPB), Indian Agricultural Research Institute, New Delhi, under the National Agricultural Technology Project of the Indian Council of Agricultural Research, New Delhi. We thank Dr K. R. Koundal, Director, NRCPB, for providing facilities and the Director, Central Tobacco Research Institute Rajahmundry, for providing experimental material.

Received 3 September 2007; revised accepted 28 December 2007

Differential accumulation of manganese in three mature tree species (Holoptelia, Cassia, Neem) growing on a mine dump

D. Raju, Sunil Kumar, Urmil J. Mehta and Sulekha Hazra*

Plant Tissue Culture Division, National Chemical Laboratory, Pune 411 008, India

Three trees, including *Cassia siamea* (Cassia), *Azadirachta indica* (Neem), *Holoptelia integrifolia* (Holoptelia) belonging to three different families were identified from a manganese mine tailing dump. Manganese content in dump soil and in the stem, green leaves and dry, fallen leaves of the plants was determined. Values were compared with similar samples collected from normal vegetation. Under control condition, manganese content was highest in Cassia. Distribution of metal in samples collected from the dump site revealed that Holoptelia has a special ability to accumulate high amounts of manganese under stress condition followed by Cassia and Neem. There is no literature on metal accumulation in Holoptelia. Mechanism of manganese sequestration in Holoptelia is different from the other two trees growing in the same soil.

Keywords: *Azadirachta indica*, *Cassia siamea*, *Holoptelia integrifolia*, hyperaccumulator, manganese, mine dump.

MANGANESE (Mn) is a trace element found in varying amounts in all tissues and is among the mostly used elements in the industry. It is an essential micronutrient and activator for enzymes involved in tricarboxylic acid cycle. However, Mn is toxic when in excess and consequently it represents an important factor in environment contamination and causes various phytotoxic effects[1].

Phytoremediation is an environmental clean-up strategy in which selected green plants are employed to remove, contain or render environmentally toxic contaminants harmless. This is an emerging biotechnological application and operates on the principles of biogeochemical cycling[2]. This remediation approach is attracting attention from various governments as a cost-effective and environment-friendly green technique to clean-up heavy metal polluted soil using hyperaccumulators[3]. The generation of scientific information on heavy metal-accumulating plants is so extensive that in the last decade a commercial industry has been developed for the application of phytoextraction to restore heavy metal-contaminated sites[4].

Most experimental studies of heavy metal tolerance confirm that populations growing in metal-contaminated

*For correspondence. (e-mail: s.hazra@ncl.res.in)

habitats are different from those growing in clean sites of the same species by possessing genetically based tolerance[5]. Some plants that grow on naturally metal-contaminated soils may adapt and develop to survive and accumulate greater concentration of heavy metals in their shoots than other plant species[6]. High accumulation of manganese uptake in roots, stem and leaves of *Phytolaccia acinosa* populations has been shown[7].

Over the past 10 years, woody plants have been shown to be excellent candidates for phytoremediation, due to rapid growth, high biomass, profuse root apparatus and low impact on the food chain and human health[8,9]. Majority of such work concerns accumulation capacity and biomass production of woody plants as a response to high concentration of pollutants[10]. Phytoremediation using trees provides a potential opportunity to extract or stabilize metals. It involves the use of trees that readily transport targeted metals from soil to plant organs, which allows removal of metal by harvesting from the plant. This process takes longer time but helps in the greening of the land and in reducing pollution[11]. However, there is need to identify trees having the ability to uptake and translocate the metal to the aerial parts. The ability of five woody species, including *Alnus*, *Fraxinus*, *Sorbus*, *Salix* and *Betula* from polluted soil to their above-ground tissues was studied[12]. In a recent study[13], interaction of calcium, with copper and cadmium accumulation in roots and stem of Norway spruce (*Picea abies* L.) has been demonstrated. However, little is known about uptake, accumulation and detoxification of heavy metals, especially about Mn in woody plants[14].

In the present investigation, three naturally growing tree species, including *Cassia siamea* (Cassia), *Azadirachta indica* (Neem) and *Holoptelia integrifolia* (Holoptelia) were identified on a manganese mine dump. An experiment was conducted to generate information on Mn accumulation and distribution in these trees. The data were compared with those generated from samples collected from the trees growing in natural vegetation in contamination-free soil. The dry, fallen leaves of the respective trees were collected from the ground under the trees and were analysed for Mn content.

The manganese mine is located in Gumgaon, Maharashtra. We identified three plants, including Holoptelia, Cassia and Neem growing naturally in the mine dump where sorting of the ore is being carried out (Figures 1 and 2). This was the only Holoptelia growing in the mining area, whereas several plants of Cassia and Neem could be seen growing naturally on the dump. The three plants identified for our study are within an area of approximately 400 sq. m. Leaves and stems (woody twigs) of these trees were collected for metal analysis. The dry leaves from the ground under these trees were also analysed. Similar samples were collected from the trees growing locally in normal soil for control. Soil samples collected from the dump and the normal vegetation were assessed for Mn content. The colour of the soil collected from the normal vegetation and from the dump site was compared.

Dust accumulated on the surface of the leaves and twigs was removed by washing thoroughly with tap water. This was followed by thorough washing of the samples with deionized water. The samples were then dried on a filter paper to eliminate adhering moisture from the surface. The same process was repeated for the control leaves and stem of all three species. Fresh leaves and stem of Holoptelia, Cassia and Neem were taken (1–3 g) in pre-weighed glass beakers of 50 ml volume. Fresh weights of the tissues were determined from the difference in weights. Samples were dried in a oven at 100°C and weighed intermittently until constant weight. Dry weight (DW) per gram of fresh weight (FW) was determined. Dried plant materials were ground in mortar and pestle to a fine powder for Mn analysis.

Manganese estimation was carried out following the method of Shraddha *et al.*[15]. In brief, the dried powder

Figure 1. Trees growing on the manganese mine dump. ***a***, *Holoptelia integrifolia*; ***b***, *Cassia siamea*.

Figure 2. Trees growing on the manganese mine dump. ***a***, *Azadirachta indica*.

(150 mg) of plant tissues was taken in Borosil vials. These were digested with 3 ml of nitric acid and 1 ml of 70% perchloric acid on a hot plate under the hood. Digested sample solution was made to 10 ml volume with deionized water. Mn content in these samples was determined using Atomic Absorption Spectroscopy (Perkin Elmer 1100B). Mn content was calculated in μg per g of dry tissue.

The pH of the soil samples was determined following the method of Sparks *et al.*[16]. In brief, samples collected from the dump site and local vegetation were air-dried at room temperature. Soil samples were taken in glass beakers and after adding distilled water were kept on a shaker for 1 h. On removing from the shaker, these were kept stationary for a period of 1 h for the suspended particles to settle down. The pH of the solution was measured using a pH meter (Model 420A, ORION).

Manganese content in the soil sample was determined using the method described in *Lab Procedures*[17]. Moisture-free samples were ground with mortar and pestle to make a fine powder and the powder was sieved. In brief, 1 g of sieved soil was taken in a test tube and 4 ml of each extracting solution (0.05 N HCl and 0.025 N H_2SO_4) was added and kept on shaker for 15 min. The solution was filtered through Whatman No. 42 filter paper and the volume was made up to 10 ml with extracting solutions. Mn content was determined by Atomic Absorption Spectroscopy.

The aim of the present study was to characterize some of the tree species that naturally colonized in Mn mine tailings. The objective was to assess these plants for their ability to uptake and accumulate Mn in the different organs. The tree species, including Holoptelia, Cassia and Neem, do not appear in the list of the known hyperaccumulators. The Mn hyperaccumulators (>10,000 μg per g) are from the families Apocynaceae, Celastraceae, Clusiaceae, Myrtaceae and Proteaceae[18–20]. In addition, mention may be made of *Eleutherococcus* (formerly *Acanthopanax*) *sciadophylloides* (Araliaceae) from Japan[21], which can accumulate Mn up to 7900 μg per g in leaf dry matter. Some plants that grow on naturally metal-contaminated soils may adapt and develop to survive and accumulate much greater concentrations of heavy metals in their shoots than other plant species[6]. Accumulation of manganese in leaf mesophyll of four tree species, *Gossia bidwillii*, *Virotia neurophylla*, *Macadamia integrifolia* and *Macadamia tetraphylla* has been reported[22].

The pH of the dump soil was 8.84 compared to 7.37 of the control soil and Mn content in the soil of the tailing dump was 1296.33 μg per g. This is approximately 44 times higher than the control soil (Table 1). The colour of the soil from the contaminated site and normal site varied distinctly (Figure 3). Comparison of Mn content (Table 2) in the organs of three tree species of local vegetation in Pune and samples from trees growing on Mn tailing dump, revealed the following.

(i) Manganese content in the samples of leaves and twigs collected from the tailing dump was higher than in the control. This indicates accumulation of Mn in all the plants growing on the tailing dump. Although all the three plants are growing under identical conditions, Mn content in their organs varied. This suggests that the mechanisms of Mn uptake and sequestration in these three plants may be different.

(ii) In the leaf tissues Mn content was significantly higher compared to the twigs irrespective of location of the plant, thereby maintaining a gradient between these two organs. This is possibly due to deposition of Mn in the leaves. The three trees under study have been growing in the high Mn-containing medium for several years, but Mn is not evenly distributed in the organs. There is a need to conduct detailed studies to understand the process of transfer of Mn from the twigs to the leaves to maintain the gradient.

(iii) Among the samples collected from normal vegetation, Mn content varied in the three species (Table 2). Mn content in this soil was 29.27 ± 1.55 μg per g. Cassia showed highest Mn content followed by Neem and Holoptelia in the leaves and twigs respectively. The values are several fold higher than the concentration of the metal in the soil. Further studies are needed to determine if these are the required concentrations in the organs for their normal functioning. The varying amounts of Mn in the organs of the three plants under normal and identical conditions indicate that the ability of a plant to uptake and accumulate Mn differs from species to species.

(iv) Among the three trees in the dump site, Mn content was highest in the tissues of Holoptelia (Table 2). This was

Table 1. Manganese content and pH in soil of dump site and normal vegetation

Soil	Mn (μg per g)[†]	pH
NCL (control)	29.27 ± 1.55	7.37
Mn tailing dump	1296.33 ± 102.89	8.84
NCL : Mn dump	Ratio is 1 : 44	
t-test	S1%	

[†]Mean of three repeats.

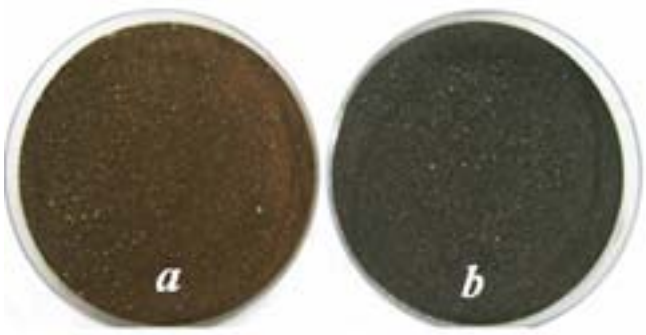

Figure 3. ***a***, Sample of normal soil; ***b***, Sample of soil from Mn mine dump.

Table 2. Distribution of manganese (μg per g) in leaf and stem of Holoptelia, Cassia and Neem

Tree		Leaf (A)†	Stem (B)†	*t*-test (A and B)	Dry fallen leaves†
Holoptelia	Control (a)	168.59 ± 57.20 (11)	98.78 ± 41.70 (6)	S-5%	143.12 ± 8.50 (6)
	Dump (b)	1744.06 ± 539.46 (11)	1248.15 ± 268.3 (9)	S-5%	2682.19 ± 94.11 (6)
	t-test (a and b)	S-1%	S-1%		S-1%
Cassia	Control (a)	437.56 ± 144.9 (9)	248.88 ± 13.34 (3)	S-1%	185.58 ± 32.1 (6)
	Dump (b)	1199.35 ± 296.9 (9)	642.96 ± 123.52 (3)	S-1%	2852.50 ± 131.7 (6)
	t-test (a and b)	S-1%	S-5%		S-1%
Neem	Control (a)	286.86 ± 122.6 (9)	174.78 ± 20.04 (3)	NS	139.36 ± 15.2 (6)
	Dump (b)	726.60 ± 177.1(9)	626.66 ± 113.40 (3)	NS	2513.30 ± 127.7 (6)
	t-test (a and b)	S-1%	S-5%		S-1%

†Figures in parenthesis indicate number of replicates.

followed by Cassia and Neem. In Holoptelia, the ratio of Mn in leaf samples of control and mine dump respectively, was approximately 1 : 8. In twigs, the ratio was 1 : 13. It appears that under Mn stress condition the uptake of Mn by Holoptelia is more. The optimum capacity of this plant to uptake and accumulate Mn needs to be determined by designing appropriate experiments.

(v) Uptake of Mn by Cassia in the dump site was more (1199.35 μg per g) as compared to the level of Mn in control (437.5 μg per g), but it was not as high as in Holoptelia. However, the ratio of Mn content between the control and dump site soil sample was 1 : 44 (Table 1), whereas the ratio between the control and dump site samples of both leaves and twigs of Cassia was only 1 : 3. Thus the amount of Mn accumulated in the two organs of Cassia may be the optimum for this plant. The mechanism of Mn tolerance in this plant is possibly different from that in Holoptelia.

(vi) In Neem leaf and twig samples from the control soil, Mn content was higher (Table 2) than in Holoptelia, whereas the amount in the organs of plants in the dump site was less than in Holoptelia. We presume that the amount of Mn detected in the organs of plants in normal soil is that required for their normal functioning and growth. In Mn-rich soil, uptake of the metal in each plant is according to its own specific ability.

(vii) Comparison of data generated from the three trees growing on the dump site (Table 2) suggests that Holoptelia has a special ability to accumulate higher amounts of Mn under stress condition. Till date there is no report on metal accumulation in this species. Further studies will reveal more information on its optimum ability to accumulate Mn and also other metals.

(viii) The metal contents in the mature fallen leaves of all three trees were estimated (Table 2). In the fallen leaves collected from trees growing in normal soil, Mn content was less than in their green counterparts. This observation cannot be explained with the present knowledge. It is assumed that the amount of Mn required by the plant organ for its normal function is taken up by the plants and is maintained. The cellular activities diminish gradually with aging prior to abscission. Presumably, demand for Mn as co-factor for cellular processes is reduced in the maturing tissues, causing mobilization and transfer of the metal to the more active organs, thereby resulting in reduction of Mn in the dry, fallen leaves. Foliar Mn sequestration in *Gossia bidwillii* (Myrtaceae), a species discovered relatively recently to be Mn-hyperaccumulating[23], has been shown to occur in the photosynthetic tissues[22]. A possible association of reduction in Mn in aging leaves with reduction in photosynthetic activity requires further investigations. The pattern of Mn content in dry leaves of the three species was similar to those in green leaves; Cassia showing the highest amount followed by Holoptelia and Neem respectively.

(ix) Unlike the dry leaves of plants from normal soil, in dry fallen leaves of the dump site Mn content was higher than in the green counterparts. In view of our assumption regarding reduced Mn in matured leaves of control plant, in the dump site there is unlimited supply of Mn from the soil. Thus, Mn from the aging leaves need not move to the more active parts, leaving the Mn content unaltered in the dry leaves. Due to optimum accumulation of Mn in leaves prior to abscission, Mn content is higher in the dry leaves than in the green leaves. Abscission of the leaves with high Mn may be the mechanism of these plants to eliminate excess Mn from the system. In Cassia, the ratio of Mn content between control and dump site fallen leaves was approximately 1 : 15 (Table 2). In Holoptelia, the ratio was approximately 1 : 19 times in fallen leaf samples (Table 2). In Neem, it was approximately 1 : 18. When all the three values are compared, they are almost similar.

All plants take up metals to varying degrees from the substrates in which they are rooted[24]. The flora of metal-contaminated sites is typically impoverished in comparison with that of the surrounding vegetation and populations of plants growing there are often genetically distinct from those of the same species in the adjacent location with soil of low heavy-metal content[25]. Moreover, the level of tolerance developed can often be related to the amount of metal in the soil[26].

There is evidence from natural establishment of trees on contaminated sites that some types of trees can survive under such adverse conditions, e.g. *Salix* (willow), *Betula*

(Birch), *Populus* (Poplar), *Alnus* (Alder) and *Acer* (Sycamore). The main characteristics of trees that make them suitable for phytoremediation is their large biomass, both above and below ground level. Physical phytostabilization can be readily achieved, and is often the main benefit of using trees on such sites. Vegetation of tree species helps in decreasing the risk of soil, water and wind erosion. Phytoremediation and especially the use of trees is an emerging and developing technology, and this has grown rapidly in recent years[27]. From the present study it may be concluded that accumulation of Mn in the three tree species and in their different parts varies. This could be due to differences in the mechanisms of uptake and sequestration of this metal in different plant systems[28]. These three trees are from different families. However, all three plants have the ability to thrive on Mn-rich dump. This common characteristic makes them suitable for restoration of Mn-contaminated sites, but all three may not be suitable for phytoremediation. The aim in a phytoremediation programme is to reduce toxic metal from the soil. Among the three trees tested, Holoptelia has the ability to take up more metal. Thus this plant is more suitable for removal of Mn from the soil. Further studies using higher concentrations of Mn need to be carried out to determine whether Holoptelia is a hyperaccumulator. The differential accumulation of Mn in the three tree species demands further studies to determine the mechanism of Mn tolerance in them.

1. Pittman, J. K., Manganese molecular mechanisms of manganese transport and homeostasis. *New Phytol*., 2005, **167**, 733–742.
2. Prasad, M. N. V., Phytoremediation of metals in the environment for sustainable development. *Proc. Indian Natl. Sci. Acad. Part B*, 2004, **70**, 71–98.
3. Raskin, I. and Ensley, B. D. (eds), *Phytoremediation of Toxic Metals: Using Plants to Clean up the Environment*, John Wiley, New York, 2000.
4. Glass, D. J., Economical potential of phytoremediation. In *Phytoremediation of Toxic Metals: Using Plants to Clean up the Environment* (eds Raskin, I. and Ensley, B. D.), John Wiley, New York, 2000, pp. 15–31.
5. Antonovics, J., Bradshaw, A. D. and Turner, R. G., Heavy metal tolerance in plants. In *Advances in Ecological Research* (ed. Cragg, J. B.), Academic Press, New York, 1971, vol. 7, pp. 1–85.
6. Long, X. X., Yang, X. E., Ye, Z. Q., Ni, W. Z. and Shi, W. Y., Difference of uptake and accumulation of zinc in four species of Sedum. *Acta Bot. Sin.*, 2002, **44**, 152–157.
7. Xue, S. G., Chen, Y. X., Reeves, R. D., Baker, A. J. M., Lin, Q. and Fernando, D., Manganese uptake and accumulation by the hyperaccumulator plant *Phytolacca acinosa* Roxb. (Phytolaccaceae). *Environ. Pollut*., 2004, **131**, 393–399.
8. Salt, D. E., Smith, R. D. and Raskin, I., Phytoremediation. *Annu. Rev. Plant Physiol. Plant Mol. Biol*., 1998, **49**, 643–668.
9. Pilon-Smits, E., Phytoremediation. *Annu. Rev. Plant Biol*., 2005, **56**, 15–39.
10. Pulford, I. D. and Watson, C., Phytoremediation of heavy metal-contaminated land by trees – A review. *Environ. Int*., 2003, **29**, 529–540.
11. Pulford, I. D. and Dickinson, N. M., Phytoremediation technologies using trees. In *Trace Elements in the Environment: Biogeochemistry, Biotechnology and Bioremediation* (eds Prasad, M. N. V., Sajwan, K. S. and Naidu, R.), CRC Press, Boca Raton, 2006, pp. 375–395.
12. Rosselli, W., Keller, C. and Boschi, K., Phytoextraction capacity of trees growing on a metal-contaminated soil. *Plant Soil*, 2003, **256**, 265–272.
13. Osteras, A. H. and Greger, M., Interactions between calcium and copper or cadmium in Norway spruce. *Biol. Plant.*, 2006, **50**, 647–652.
14. Lei, Y., Korpelainen, H. and Li, C., Physiological and biochemical response to high Mn concentrations in two contrasting *Populus cathayana* populations. *Chemosphere*, 2007, **68**, 686–694.
15. Shraddha, S., Susan, E. and Souza, S. F. D., Cadmium accumulation and its influence on lipid peroxidation and antioxidative system in a aquatic plant, *Bacopa monnieri* L. *Chemosphere*, 2006, **62**, 233–246.
16. Sparks, D. L. *et al*. (eds), *Methods of Soil Analysis. Part 3. Chemical Methods*, Soil Science Society of America, Inc., Madison, W.I., 1996, pp. 487–489.
17. *Lab Procedures*, Soil Testing and Plant Analysis Laboratory, Cooperative Extension Service, Athens, GA, 1970.
18. Reeves, R. D. and Baker, A. J. M., Metal accumulating plants. In *Phytoremediation of Toxic Metals: Using Plants to Clean up the Environment*, John Wiley, New York, 2000, pp. 193–229.
19. Proctor, J., Phillips, C., Duff, G. K. Heaney, A. and Robertson, F. M., Ecological studies in Gunung Silam, a small ultra basic mountain in Sabah, Malaysia II. Some forest processes. *J. Ecol*., 1989, **77**, 317–331.
20. Bidwell, S. D., Hyperaccumulator of metal in Australian native plants. Ph D, thesis, University of Melbourne, Australia, 2000.
21. Memon, A. R., Chino, M., Takeoka, Y., Hara, K. and Yatazawa, M., Distribution of manganese in leaf tissue of the manganese accumulator. *Acanthopanax sciadophylloides* as revealed by electronprobe X-ray microanalysis. *J. Plant Nutr*., 1980, **2**, 457–476.
22. Fernando, D. R., Bakkaus, E. J., Perrier, N., Baker, A. J. M., Woodrow, I. E., Batianoff, G. N. and Collins, R. N., Manganese accumulation in the leaf mesophyll of four tree species, a PIXE/EDAX localization study. *New Phytol*., 2006, **171**, 751–758.
23. Bidwell, S. D., Woodrow, I. E., Batianoff, G. N. and Sommer-Knusden, J., Hyperaccumulation of manganese in the rainforest tree *Austromyrtus bidwillii* (Myrtaceae) from Queensland, Australia. *Funct. Plant Biol*., 2002, **29**, 899–905.
24. Baker, A. J. M., McGrath, S. P., Reeves, R. D. and Smith, J. A. C., Metal hyperaccumulator plants: A review of the ecology and physiology of a biological resource for phytoremediation of metal-polluted soils. In *Phytoremediation of Contaminated Soil and Water* (eds) Terry, N. and Banuelos, G., Lewis Publishers, Florida, 2000, pp. 85–107.
25. Ye, Z. H., Baker, A. J. M., Wong, M. H. and Willis, A. J., Copper tolerance, uptake and accumulation by *Phragites australis*. *Chemosphere*, 2003, **50**, 795–800.
26. Foy, C. D., Chaney, R. L. and White, M. C., The physiology of metal toxicity in plants. *Annu. Rev. Plant Physiol*., 1978, **29**, 511–566.
27. Van der Lelie, D., Schwitzguébel, J. P., Glass, D. J., Vangronsveld, J. and Baker, A, Assessing phytoremediation's progress in the United States and Europe. *Environ. Sci. Technol*., 2001, **35**, 446A–452A.
28. Clemens, S., Molecular mechanisms of plant metal tolerance and homeostasis. *Planta*, 2001, **212**, 475–486.

ACKNOWLEDGEMENTS. We thank Dr A. A. Juwarkar, National Environment and Engineering Research Institute, Nagpur for help in procuring the plant material from Gumgaon mines. The project was supported by Council of Scientific and Industrial Research, New Delhi under the Network Programmes on Phytoremediation.

Received 19 September 2007; revised accepted 23 January 2008

Paper in Press (2008)

In

BIOREMEDIATION, BIODIVERSITY AND BIOAVAILABILITY

In Vitro Studies On Chromium And Copper Accumulation Potential Of *Pongamia Pinnata* (L.) Pierre Seedlings

Sunil Kumar, Urmil J. Mehta and Sulekha Hazra*

Original Research Paper

In Vitro Studies On Chromium And Copper Accumulation Potential Of *Pongamia Pinnata* (L.) Pierre Seedlings

Sunil Kumar, Urmil J. Mehta and Sulekha Hazra*

National Chemical Laboratory, Council of Scientific and Industrial Research, India

*Corresponding author: Dr. Sulekha Hazra

Plant Tissue Culture Division,

National Chemical Laboratory,

Pune- 411008, India.

Tel.: 091-020-25902217

Fax: 091-020-25902645

Email: **s.hazra@ncl.res.in**

Key words: AAS, In Vitro, absorption, Pongamia, phytoremediation,

Running title: Chromium and copper accumulation in *Pongamia*

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 *Pongamia pinnata seedlings* grown *in vitro* in medium containing Cr or Cu. Pongamia seeds were cultured in MS medium supplemented with various concnetration 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.

Abbreviations: AAS, atomic absorption spectrophotometer; **DW**, dry weight; **FW**, fresh weight, Cr, Chromium; Cu, Copper.

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 their concentration of metal in the soil, as long as the metal in question is present (Prasad and Freitas 2003). A *Holoptelia integrefolia* tree growing on manganese mine dump is identified for its ability to accumulate increased amount of manganese whereas two other species growing in the same location do not demonstrate 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 hyperaccmulate 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) (Rev; Pulford and Watson 2003; Pulford and Dickinson 2006) and on *Holoptelia integrifolia*-(Raju et al 2008) on different metals. However, these studies were carried out in field conditions. However, 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 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 oven at 90–100°C till constant weight was achieved. The data on the DW^{-g} FW was calculated to determine the change is 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. Cu or Cr content of each organ were 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**).

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. Decrease in germination frequency in the presence of Cr were reported for *Echinochloa colona* at 200 µM, *Phaseolus vulgaris* at 500 ppm (i.e. 9.61mM) (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 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 interesting 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}) 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}) 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 transfers 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. 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 investigation 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. *Pongamia 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) *Pongamia pinnata* seedling can tolerate 100-800 µM concentration of Cr in growth medium and 50-400 µM concentration 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.

ACKNOWLEDGMENT

We acknowledge C.S.I.R. India for a research fellowship awarded to Sunil Kumar and for a research grant provided in the network program (NWP0019) on phytoremediation.

REFERENCES

Barbosa RMT, Almeida AF, Mielke MS, Loguercio LL, Pedro AO, Mangabeira PAO, Gomes FP (2007) A physiological analysis of *Genipa mericana* L.: A potential phytoremediator tree for chromium polluted watersheds. *Environmental and Experimental Botany* **61**, 264-271

Bojarczuk k (2004) Effect of Toxic Metals on the Development of Poplar (*Populus tremul*(*Populus tremula* L. × *P. alba* L.) Cultured *in vitro*. *Polish Journal of Eviron. Studies* **13**,115-120

Boojar MMK, Goodarzi F (2007) The copper tolerance strategies and the role of antioxidative enzymes in three plant species grown on copper mine. *Chemosphere* **67**, 2138-2147

Castro RO, Trujillo MM, Bucio JL, Cervantes C, Dubrovsky J (2007) Effects of dichromate on growth and root system architecture of *Arabidopsis thaliana* seedlings. *Plant Science* **172**, 684-691

Chatterjee J, Chatterjee C (2000) Phytotoxicity of cobalt, chromium and copper in cauliflower. *Environmental Pollution* **109**, 69-74

Gardea-Torresdey JL, de la Rosa G, Peralta-Videa JR, Montes M, Cruz-Jimenez G, Cano-Aguilera I (2005) Differential uptake and transport of trivalent and hexavalent chromium by tumbleweed (*Salsola kali*). *Archives of Environmental Contamination and Toxicology* **48**, 225-232

Han FX, Sridhar BBM, Monts DL,Su Y (2004). Phytoavailability and toxicity of trivalent and hexavalent chromium to *Brassica juncea. New Phytologist* **162**, 489-499

Hoewyk DV, Garifullina GF, Ackley AR, Abdel-Ghany SE, Marcus MA, Fakra S, Ishiyama K, Inoue E, Pilon M, Takahashi H, Pilon-Smits EAH (2005) Overexpression of AtCpNifS Enhances Selenium Tolerance and Accumulation in Arabidopsis. *Plant Physiology* **139,** 1518-1528

Lin J, Jiang W, Liu D (2003) Accumulation of copper by roots, hypocotyls, cotyledons and leaves of sunflower (*Helianthus annuus* L.). *Bioresource Technology* **86**, 151-155

Mangabeira PA, Gavrilov KL, Furtado de Almeida A, Oliveira AH, Severo MI, Rosa TS, da Costa Silva D, Labejof L, Escaig F, Levi-Setti R, Mielke MS, Loustalot FG, Galle P (2006) Chromium localization in plant tissues of *Lycopersicum esculentum* Mill using ICP-MS and ion microscopy (SIMS). *Applied Surface Science* **252**, 3488-3501

Meera B, Kumar S, Kalidhar SB (2003) A review of the chemistry and biological activity of *Pongamia pinnata. Journal of Medicinal and Aromatic Plant Sciences* **25**, 441-465

Montes-Holguin MO, Peralta-Videa JR, Meitzner G, Martinez-Martinez A, de la Rosa G, Castillo-Michel H, Gardea-Torresdey JL (2006) Biochemical and spectroscopic studies

of the response of Convolvulus arvensis L. to Cr(III) and Cr(VI) stress. *Environmental Toxicology and Chemistry* **25**, 220-226

Nehnevajova E, Herzig R, Erismann KH, Schwitzguébel JP (2007) *In vitro* breeding of *Brassica juncea* L. to enhance metal accumulation and extraction properties. *Plant Cell Report* **26**, 429-437

Peng HY, Yang XE (2007) Characteristics of copper and lead uptake and accumulation by two species of *Elsholtzia*. *Bulletin of Environmental Contamination and Toxicology* **78**, 152-157

Peralta JR, Gardea Torresdey JL, Tiemann KJ, Gomez E, Arteaga S, Rascon E (2001) Uptake and effects of five heavy metals on seed germination and plant growth in alfalfa (*Medicago sativa* L.) *Bulletin of Environmental Contamination and Toxicology* **66**, 727-734

Prasad MNV, Freitas HM (2003) Metal hyperaccumulation in plants - Biodiversity prospecting for phytoremediation technology. *Electronic Journal of Biotechnology* **6**,285-305

Pulford ID, Watson C (2003) Phytoremediation of heavy metals-contaminated land by trees: a review. *Environment International* **29**, 529-540

Pulford ID, Dickinson NM (2006) "PhytoremediationTechnologies Using Trees" in: Prasad MNV, Sajwan KS, Ravi Naidu (eds) Trace elements in the environment: Biogeochemistry, Biotechnology and Bioremediation. CRC Press. Boca Raton. 726 pp. Taylor and Francis Group

Pulford ID, Watson C, McGregor SD (2001) Uptake of chromium by trees: prospects for phytoremediation. *Environmental Geochemistry and Health* **23**, 307-311

Raju D, Kumar S, Mehta UJ, Hazra S (2008). Differential accumulation of Manganese in three mature tree species (Holoptelia, Cassia, Neem) growing on a mine dump. *Current Science*: **94** , 639-643

Rosselli W, Keller C, Boschi K 2003. Phytoextraction capacity of trees growing on a metal contaminated soil. *Plant and Soil* **256**, 265-272.

Sanità di Toppi L, Fossati F, **Musetti R**, **Mikerezi I**, **Favali MA** (2002) Effect of hexavalent chromium on maize, tomato and cauliflower plants. *Journal of Plant Nutrition* **25**, 701-717

Shah K, Nongkynrih JM (2007) Metal hyperaccumulation and bioremediation. *Biologia Plantarum* **51**, 618-634

Shanker KA, Cervantes C, Loza-Tavera H, Avudainayagam S (2005a) Chromium toxicity in plants. *Environment International* **31**, 739-753

Shanker AK, Ravichandran V, Pathmanabhan G (2005b) Phytoaccumulation of chromium by some multi purpose tree seedlings. *Agroforestry Systems* **64**, 83-87

Singh S, Eapen S, D'Souza SF (2006) Cadmium accumulation and its influence on lipid peroxidation and antioxidative system in a aquatic plant, *Bacopa monnieri* L. *Chemosphere* **62**, 233-246

Street RA, Kulkarni MG, Stirk WA, Southway C, Vanstaden G (2007) Toxicity of metal elements on germination and seedling growth of widely used medicinal plants belonging to Hyacinthaceae. *Bulletin of Environmental Contamination and Toxicology* **79**, 371-376

Sujatha K, Hazra S (2006) *In vitro* regeneration of *Pongamia pinnata* Pierre. *Plant Biotechnology* **23**, 263-270

Sujatha K, Hazra S (2007) Micropropagation of mature *Pongamia pinnata* Pierre. *In Vitro Cellular and Developmental Biology – Plant* **43**, 608-613

Unterbrunner R, Puschenreiter M, Sommer P, Wieshammer G, Tlustoš P, Zupan M, Wenzel WW (2007) Heavy metal accumulation in trees growing on contaminated sites in Central Europe. *Environmental Pullution* **148**, 107-114

Vivek X, Gupta AK (2004) Biodiesel production from Karanja oil. *Journal of Scientific and Industrial Research* **63**, 39-47

Xia Y, Shen G (2007) Comparative studies of copper tolerance and uptake by three plant species of the genus Elsholtzia. *Bulletin of Environmental Contamination and Toxicology* **79**, 53-57

Yruela I (2005) Copper in plants. *Brazilian Journal of Plant Physiol* **17**, 145-156

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

Cr^{+6} µM	**Germination frequency Mean ± SD 10 d**	**Shoot Ht. Mean ± SD cm (6 weeks)**	**Root length Mean ± SD cm (6 weeks)**
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.

FIGURE LEGENDS

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.

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.

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

Fig. 1

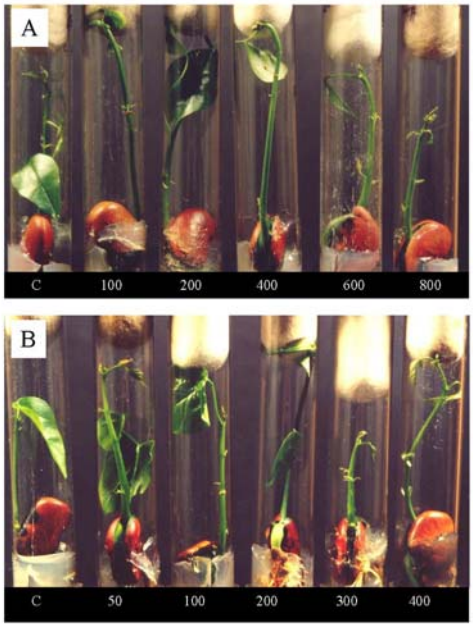

Fig. 2

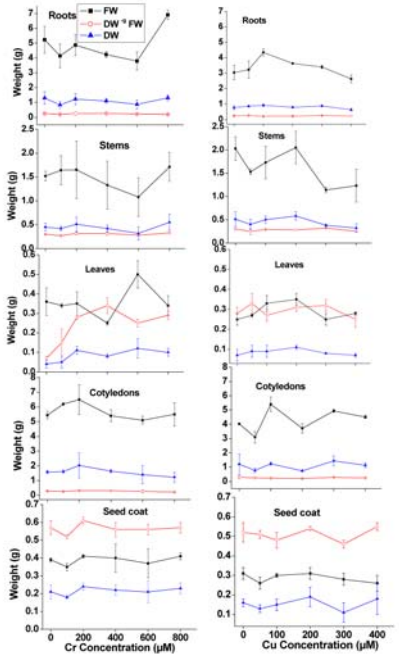

Fig. 3