TAPPING PHYTOREMEDIATION POTENTIAL FOR SOILS CO-CONTAMINATED WITH HEAVY METAL AND PESTICIDE

THESIS

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

LUCKNOW

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2020

Dedicated
To
My Family

DECLARATION

I **Arpna Ratnakar**, declare that the thesis entitled **"Tapping Phytoremediation Potential for soils co-contaminated with Heavy Metal and Pesticide"** which is being submitted to Department of Environmental Science, School for Environmental Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow in fulfillment of the degree of **Doctor of Philosophy** in Environmental Science has previously not formed the basis for award of any such degree by any university. This is my original research work carried out during 2014-2020 and also declared that the thesis is essentially free from all kinds of plagiarism.

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CERTIFICATE

This is to certify that the thesis entitled **"Tapping Phytoremediation Potential for soils co-contaminated with Heavy Metal and Pesticide"** submitted by **Ms. Arpna Ratnakar** is an original research work and has not been previously submitted in part or full, for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University, Lucknow, satisfies all the requirements as stipulated in the *Doctor of Philosophy (Ph.D.) Regulations – 1999 as amended in 2008/2010/2013* and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

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PREFACE

Contaminated land is a global problem that gives rise to noteworthy problems both to human as well as environmental health. Pollutants happen to be ubiquitous in the soil system with inorganic (heavy metals) and organic (xenobiotics) constituents. Generally, lands in either developed or developing countries are contaminated by more than one single pollutant. The mixed pollutants could be of same form e.g., land contaminated with different kinds of heavy metals or herbiicides or could be of different forms e.g., land contamination with heavy metals and herbicides. This has been the challenge for remediation as most of the contaminated soils do not contain one single pollutant but instead a number of different substances. In the above context, low energy and environmental friendly technologies are required and phytoremediation could solve this problem. Phytoremediation is a plant-based, cost-effective and on-site remediation process for treating contaminated soils. Research in this area is vast but very limited since there have been gaps in knowledge on the use of plants to remediate the co-contaminated land types. As phytoremediation has been mostly used on single contaminants or multiple contaminants of same type, this research work will try to address the problems posed by the mixture of organic (herbicide, Butachlor) and inorganic (heavy metals Cd & Hg) contaminants during phytoremediation of co-contaminated soils.

In the aforesaid context, the present research work has been designed to investigate the phytoremediation potential of C. roseus on co-contaminated soils. Although, the mechanism of hyperaccumulation is yet to be explored, the studies at cell and at tissue level will help in knowing how plants pick up and absorb extremely hazardous and omnipresent metals (Cd and Hg) which will be very helpful for designing effective methods to check the problem of food-chain contamination by such pollutants. The study encompasses the effects of chelating agents which may facilitate more uptakes of heavy metals as well as herbicide dissipation by C. roseus plant. The results of this study overall emphasizes that the plant species C. roseus could be applied to co-contaminated soils, and could act as a potential candidate for phytoremediation of co-contaminated soil.

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LIST OF ABBREVIATIONS AND SYMBOLS

Percent	**%**
Degree centigrade	**°C**
Micro	**µ**
Microgram per gram	**$\mu g g^{-1}$**
Microliter	**$\mu g l^{-1}$**
Microgram per milliliter	**$\mu g ml^{-1}$**
Micromoles	**µm**
Microsiemen per centimeter	**$\mu S\ cm^{-1}$**
Atomic Absorption Spectroscopy	**AAS**
Absorbance	**Ab**
Aluminum	**Al**
Analysis of variance	**ANOVA**
Ascorbate peroxidase	**APOX**
Analytical grade	**AR**
Arsenic	**As**
Adenosine triphosphate	**ATP**
Butachlor	**BC**
Bovine serum albumin	**BSA**
Carbon nitrogen ratio	**C/N**
Calcium	**Ca**
Carotenoids	**Car**
Catalase	**CAT**
Cadmium	**Cd**
Colony forming unit	**cfu**
Chlorophyll	**Chl**
CA	**Citric Acid**
Centimeter	**cm**
Carbon dioxide	**CO_2**
Chromium	**Cr**
Days After Showing	**DAS**
Double Distilled Water	**DDW**
Dry Weight	**DWt.**
Electrical Conductivity	**EC**
Alia (and others)	**et al**
EDTA	**Ethylenediaminetetraacetic acid**
Folin- Ciocalteu reagent	**FCR**
Iron	**Fe**
Fresh Weight	**FWt.**
Gram	**g**
Gas Chromatography	**GC**
Hour	**h**
Hydrogen Peroxide	**H_2O_2**
Hectare	**ha**
Hydrochloric acid	**HCl**
Perchloric acid	**$HClO_4$**
Helium	**He**
Mercury	**Hg**
High performance liquid chromatography	**HPLC**
Available Potassium	**K**
Kilogram	**Kg**
Litre	**L**
Lipid Peroxidation	**LPO**
Molar	**M**

Moisture Content	**MC**
Malondialdehyde	**MDA**
Milligram per gram	**mgg^{-1}**
Minute	**min**
Millimoles per litre	**mmol**
Manganese	**Mn**
Available Nitrogen	**N**
Nicotinamide adenine dinucleotide	**$NADH^{+}$**
Nitroblue terazolium chloride	**NBT**
Nickel	**Ni**
Nanometer	**nm**
Nitrate	**NO_3^{-}**
Chemical fertilizers	**NPK**
Superoxide radical	**O^{2-}**
Organic Carbon	**OC**
Degree Centigrade	**ºC**
Optical Density	**OD**
Hydroxyl radical	**OH**
Organic Matter	**OM**
Available Phosphorus	**P**
Poly aromatic hydrocarbons	**PAHs**
Lead	**Pb**
Phosphate buffer solution	**PBS**
Guaiacol peroxidase	**POD**
Parts per million	**ppm**
Reactive oxygen species	**ROS**
Revolution per minute	**rpm**
Standard deviation	**SD**
Standard error	**SE**
Superoxide dismutase	**SOD**
Software packaged used for statistical analysis	**SPSS**
Thiobarbituric acid	**TBA**
Trichloro acetic acid	**TCA**
Unit activity	**UA**
Ultra violet	**UV**
Volume per volume	**v/v**
Weight per volume	**w/v**
Weight	**Wt.**

Chapter 1

Introduction

1.1 Introduction

Nowadays pollutants are omnipresent in the soil systems that in particular are heavy metals and persistent organic pollutants (POPs) (**Tremolada et al, 2008**). An increase in industrial, agricultural and engineering practices, has led to severe degradation of soils **(Ghazaryan et al, 2019)**. Contaminated land is a term used to describe sites that includes wider land areas consisting of higher concentration of mixed pollutants due to anthropogenic and natural sources **(CLARINET, 2002)**. Global existence of contaminated land constitutes problem to both human and environmental health.

A real world situation reveal complex industrial processes and multiple land use which happens to create sites containing mixed pollutants. Lands in developing and developed countries are largely contaminated by more than one individual pollutant (**Yaqoob et al, 2019; Zhang et al, 2011**). Such multiple pollutants may be of the similar form like soil contaminated with either different kinds of metal or with different types of herbicides or xenobiotics, or that can also be of different forms such as, soil contaminated with inorganic pollutants (*viz*; metals) mixed with organic pollutants (*e.g.,* herbicides)

Over the past decades, the release of both inorganic and organic anthropogenic pollutants has been increased into the surroundings. Co-contaminants (organic and inorganic contaminants) such as solvents, heavy metals, petroleum-based hydrocarbon compounds and agricultural pesticides, can affect the quality of soil (**Khan et al, 2004**). Remediation of co-contaminated soils is a complex problem because remediation technologies and chemical processes are different for both group of pollutants (**Sandrin & Maier, 2003**). It was demonstrated that the kinetics and extent of metal phytoextraction mechanisms gets influenced by the presence of poly aromatic hydrocarbon compounds (PAHs) (**Almeida et al, 2008**). On the contrary **Singer et al** (**2007**) reported that the extraction of heavy metal (Ni) hyperaccumalator plant *Alyssum lesbiacum* is not affected by the presence of PAHs. More translocation was observed in soil co-contaminated by pyrene and Zn as compared to the soil treated with Zn alone (**Batty & Anslow, 2008**). Noteworthy changes were recorded in the bioavailability of heavy metals in the copresence of organic (2, 4- dichlorophenol) compound **(Chen et al, 2004)**.

The restoration and reclamation of contaminated soil is an old concept and different physical and chemical methods have been adopted *e.g.* soil washing, thermal

treatment, soil excavation, electro-reclamation, and other chemical techniques (**Muthusaravanan et al, 2018**). Although these treatment methods may shift pollution to a new phase (air pollution etc.) it will not eliminate the pollution problem **(Chen et al, 2004)**. So keeping this view in mind sustainable practices for land remediation which involves the application of microbes and plants for transformation and uptake of mixed pollutants was proposed.

Phytoremediation is a reliable, cost-effective, environmentally friendly, and greener technology as compared to other physical and chemical treatment methods **(Ashraf et al, 2019).** It offers the rational and alternative solution for aforesaid problems **(Bocuk & Ture, 2014)**. Some plants extraordinarily hold the ability of phytostabilization (to immobilize metals and store them below ground in roots) and hyperaccumulation (to remove metals from the soil and concentrate them in above ground tissues).

Co-contaminated land wherein both inorganic and organic substances coexist, very limited work has been done so far to explicit the phytoremediation for cleanup. It is very demanding to unveil the mechanism of interactions of both organic and inorganic substances with the surrounding medium. The metal concentration has been found to hinder the microorganism mediated biodegradation of organics **(Thavamani et al, 2013)** besides interacting with organic pollutants thereby affecting bioavailability of metals **(Gao et al, 2006)**. Co-planting in metal contaminated soils results into increased metal uptake (Cd and Zn) as well as increases growth stimulation also for particular plants (**Jiang et al, 2010**). Co-occurrence of multiple contaminants influences remediation processes because of their interactive effects on soil processes, plant growth, and rhizosphere biota (**Almeida et al**, **2008**).

Chelating agents increases the availability for plant uptake and root-to-shoot translocation by acting as soluble chemicals that are able to bind and mobilize other molecules (including both metals and several organic contaminants) into the soil solution (**Farid et al, 2017**). Both synthetic chelators (Ethylene diamine tetraacetic acid, Ethylene Diamine Disuccinic Acid) as well as natural (Citric Acid) chelating agents have been found to increase mobilization of plant metal as well as organic pollutant uptake since it forms stable chelates with most of heavy metals and PAHs compounds (**Almaroai et al, 2013**). This then leads to an increase in the metal uptake

of high biomass crop plants (e.g. *Helianthus annuus*, *Zea mays*, *Brassica juncea*, *Nicotiana tabacum*, etc.) (**Meers et al**, **2005**; **Di Gregorio et al**, **2006**).

Since research in the above mentioned area is small, besides gaps in knowledge exist on the exploitation of plants to remediate different land types, and most of the studies on phytoremediation of inorganic and organic contaminants in soil are considered as two separate topics either in the behavioral context of the contaminants and/or the remediation aspects the present study entitled "Tapping phytoremediation potential for soils co-contaminated with heavy metal and pesticide" is an effort to explore the difficulties posed by the admixture of inorganic (heavy metal) and organic (herbicide) contaminants at the time of phytoremediation of co-contaminated industrial soils.

Aims and objectives:

Since most of the phytoremediation studies are based on either single contaminants or multiple contaminants of the same type, the research overall aims to determine whether phytoremediation can be applicable for co-contaminated sites posed by mixture of organic (herbicide) and inorganic (heavy metal) contaminants. In the aforesaid context the study is undertaken with following objectives:

- A survey of agricultural lands in and around Lucknow city to ascertain the status of heavy metals & herbicides in contaminated soils.
- To determine whether single (heavy metal or herbicide) and mixed contaminants (heavy metal and herbicide) affect seed germination rate and early seedling growth of some test plant species.
- To establish a suitable plant species for phytoremediation having tolerance for co-contaminants and selection of suitable plant species.
- To establish the suitability of the selected plant species for phytoremediation by comparing the uptake, accumulation and translocation of heavy metals by the selected plant species in single and co-contaminated soils.
- To study whether metals uptake by the test species is affected in the presence of herbicides.

- To ascertain whether soil amendments (chelating agents) can facilitate phytoremediation in co-contaminant soils.

Chapter 2

Review of Literature

2.1 Co-Contamination

Recent years have witnessed an expanding area of soil contaminated with heavy metals, agrochemicals, hydrocarbons, organic solvents and miscellaneous pollutants. Owing to rapid increase in industrialization as well as population, and unreasonable directionless development, has ended into excessive utilization of mineral resources, erroneous application of pesticides and chemical fertilizers and sewage irrigation practices (**Marques et al, 2011**).

Soils contaminated with mixed pollutants like organic and inorganic compounds such as pesticides, Poly aromatic hydrocarbons (PAHs) *etc*., along with heavy metals are called co-contaminated soils. The problem of co-contamination has attracted global attention because more than one third of the sites has been ruled as co contaminated and the remediation of such co contaminated soil happens to be not only complicated but quiet difficult to achieve, due to the mixed nature of the pollutants (**Tang et al, 2010**; **Polti et al, 2014**).

Soils tend to get contaminated by undue accumulation of heavy metals and/or metalloids as well as organic pollutants through various anthropogenic activities, industrial emissions, mine tailings, spillage of petrochemicals, erroneous disposal of high metal wastes, paints, leaded gasoline, coal combustion residues, waste water irrigation, land application of fertilizers, pesticides, animal manures, sewage sludge, and various atmospheric depositions (**Subhashini & Swamy, 2013**), besides unavoidable natural causes like weathering of parent materials and occasional volcanic eruptions (**Sharma & Pandey, 2014**).

The cocktail of various organic and inorganic pollutants give rise to environmental pollution, precipitating serious health hazards in living beings (**Maszenan et al, 2011; Saxena & Bharagava, 2017**). Overall, the key component of multiple pollutants that contaminate the soil systems include heavy metals, organic pollutants, pesticides, petroleum hydrocarbons, crude oil, wood preservatives etc. The presence of toxic metals in soil may interfere in the biodegradation of organic contaminants and calls for protection and remediation at large (**Maslin et al, 2000**).

2.1.1 Heavy Metals

Presently heavy metal has become the most serious problem in terms of environmental pollution *viz*., air, water and soil. It poses higher threats to not only ecosystem but also soil quality as well as human health, through the various source of emission like industries, sewage sludge, pesticides, urbanisation, coal combustion residues, water irrigation and many more. Globally there are over 20 million hectare of land contaminated by the heavy metal(loid)s such as Lead (Pb), Chromium (Cr), Mercury (Hg), Arsenic (As), Cadmium (Cd), Cobalt (Co), Copper (Cu), Nickel (Ni), Zinc (Zn), and Selenium (Se) with significant soil concentration (**Liu et al, 2018**).

Cd is a silver-white, lustrous, ductile, very malleable metal. It is soluble in acids but not in alkalis. Cd occurs as the divalent Cd (II) ion. Cd is similar in many respects to zinc and its substitution by Cd may cause the malfunctioning of metabolic processes (**Safarzadeh et al**, **2007**). Cd is a highly bioactive and toxic element, its presence at elevated levels in soil and water is a threat to food safety and human health (**Sun et al, 2008**). Cd in environment is found dissolved in water or form insoluble complexes with organic and inorganic compounds **(Crea et al, 2013).** In environment cadmium occur as Cd (II) ions and is released in the environment through both natural as well as anthropogenic sources which includes production of pigments, batteries and alloys, application of sewage sludge and phosphate fertilizer to farm land and industrial emissions. Cd is thus mobilized in the environment and eventually accumulates in crops and vegetables, grown for human consumption (**Zhao et al, 2003; Tchounwou et al, 2012**). Being a severe pulmonary and gastrointestinal irritant, Cd can be fatal if inhaled or ingested.

Hg is a compound that can be found naturally in the environment. Hg can be found in organic mercury compounds or mercury salts as well as in metal form. Hg occurs naturally by evaporation from the oceans and the degassing of the earth's crust through volcanoes (**Boening, 2000**). Hg exists in three forms (Hg^0, Hg^+ and Hg^{+2}); Hg^{+2} are available as organic and inorganic forms in soil, and in liquid form at room temperature (**Tchounwou et al, 2012**). Hg is toxic for human beings since through biomagnification it enters the food chain as methylated mercury (**Copat et al, 2012**).

Bioaccumulation property of heavy metals poses a serious threat to human health, which necessitates adequate remediation strategy (**Wu et al, 2017**). Heavy metals are either acid or water soluble in contrast to organic pollutants which possess the properties of hydrophobicity (**Cang et al, 2013**). Therefore, it becomes easy for heavy metals to migrate through the soil and integrate with Fe-Mn and organic matter of soil, whereas, the organic pollutants integrate with soil organic matter. In addition, heavy metals are resistant to microbial remediation process (**Bolan et al, 2014**) and can be extracted through phytoremediation (**Peer et al, 2005**).

2.1.2 Pesticides

Although, agrochemicals and pesticides are important for improving quality and yield of crop production (**Ratnakar & Shikha, 2018**), they significantly contribute towards the global environmental pollutants which has reached great heights owing to industrial and intensive agricultural activities and practices (**Chen et al, 2014; Rajasankar et al, 2013**). Pesticides are required for enhancing crop production to meet the growing demands of the ever increasing population and reducing crop loss from pest injury (**Peshin & Zhang, 2014**). According to an estimate nearly one-third of the agricultural products contain pesticides (**Liu et al, 2002; Zhang et al, 2011**). Pesticides have been found to decline crop loss from pest injury by 35% to 42% (**Pimentel, 1997**). Percent contribution of different pest control chemicals globally and indigenously has been shown in **Figure 2.1** and **2.2.**

Out of the various pesticides used globally, herbicide classification tops the group, owing to their integral contribution in modern intensive cropping systems (**Sarma et al, 2015)**. Among the most commonly used herbicides, the chloroacetanilide group *viz*: butachlor, propachlor, alachlor, acetochlor and metachlor are the extensively used chemicals across the world in agriculture. Inhibition of very long chain fatty acid biosynthesis happens to be the principal mode of action of metazachlor which affects cell plasma membranes and results into shoot elongation (**Schmalfuβ et al, 2000**). According to **Alla et al (2007)** butachlor affects the lipid

synthesis of isolated leaf cells of *Phaseolus vulgaris L*. besides alleviating the glutathione and its associated enzymes in butachlor tolerant plants.

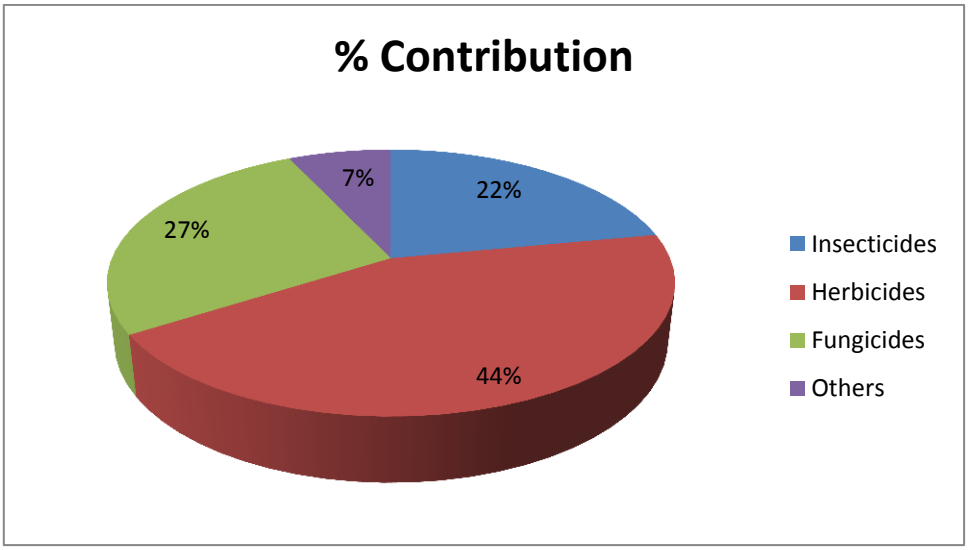


Figure 2.1 Global distribution of pesticides (Adopted from Devi et al, 2017).

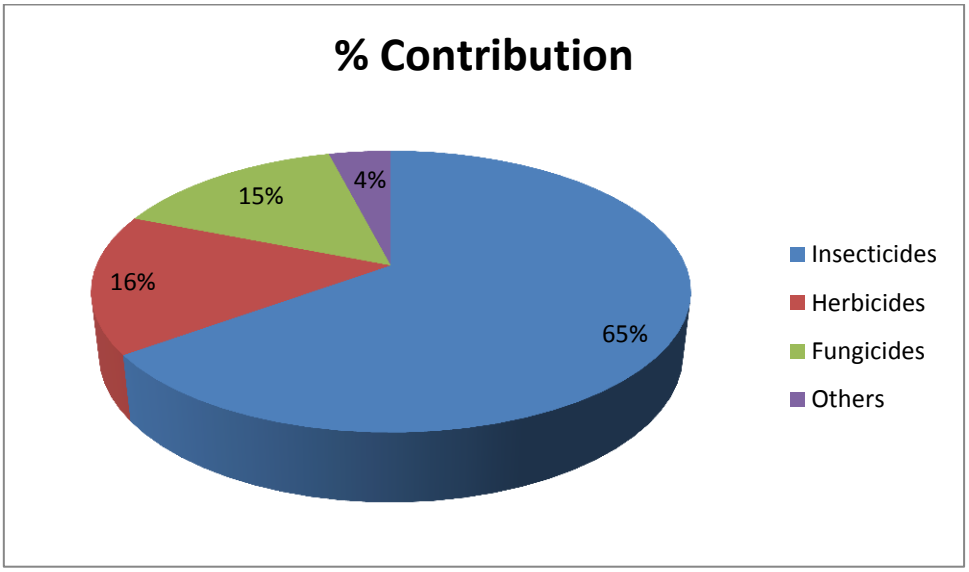


Figure 2.2 Distribution of pesticides in India (Adopted from Devi et al, 2017).

India stands 4th in global suppliers of agrochemicals after USA, Japan and China (**Devi et al, 2017**). Pesticide consumption was observed highest in Maharashtra, followed by Uttar Pradesh, Punjab and Haryana (**Table 2.1)**. Higher use of herbicides

during last couple of years for weed control has led to an increase in the cost of manual weed control due to increase in agricultural wages which results in pesticides increment **(FICCI, 2015)**. In Punjab use of fertilisers and pesticides is one of the factors that leads to deficiency of micronutrients and loss of fertility in the soil (**Khajuria, 2016**).

Table 2.1: State-wise pesticides consumption (Technical Grade)

States/UTs	Total consumption (tonnes)			Per ha (kg)
	2003-04	2008-09	2015-16	2016-17*
Punjab	6780	5760	5743	0.74
Haryana	4730	4288	NR	0.62
Maharashtra	3385	2400	11665	0.57
Kerala	326	273	1123	0.41
Uttar Pradesh	6710	8968	10457	0.39
Tamil Nadu	1434	2317	2096	0.33
West Bengal	3900	4100	3712	0.27
Chhattisgarh	332	270	1625	0.26
Andhra Pradesh	2034	1381	2713	0.24
Odisha	682	1156	723	0.15
Gujarat	4000	2650	1980	0.13
Bihar	860	915	831	0.11
Karnataka	1692	1675	1434	0.10
Rajasthan	2303	3333	2475	0.05
Madhya Pradesh	62	663	732	0.03
All India	**41020**	**43860**	**54121**	**0.29**

Source: **Ministry of chemicals and fertilizers, govt. of India.**

Note: **NR refers to not reported; *Gross Cropped Area based on 2014-15.**

2.1.3 Mixed or Co-contaminants

Co-contaminated sites are those sites that have mixed contamination of pollutants of different origin and nature. Examples include sites which are contaminated with inorganic contaminants as well as chemicals belonging to diverse nature with surfactants, pesticides, petroleum hydrocarbon, *etc*., (**Almeida et al, 2009**).

A number of conventional and modern technologies are being employed for removal of heavy metals and co contaminants from soil, but they are disadvantageous requiring higher capital cost (**Mahajan & Kaushal, 2018**). For example, amongst physical, chemical and biological methods for remediation, physical and chemical methods are not preferred because they are cost intensive and transfer contaminants from one place to another, besides not completely removing the contaminant. On the contrary, bioremediation is a technique which facilitates the remediation of toxic /harmful materials *via* natural processes (**US EPA, 2001**). It harnesses the adaptive property of plants and/or microorganisms as well as their metabolic diversity to bring degradation and transformation of various inorganic and organic contaminants (**Cunningham & Philip, 2000**). Apart from plants, commonly exploited organisms to meet this purpose include naturally occurring or genetically modified bacteria, fungi and/or protozoa (**Mathew, 2005**). Cadmium is the most important metal in the soil pollution problem, and it has been reported that human beings consume Cd through many crops along the food chain (**Asami, 1984; Khan et al, 2017**) whereas mercury contamination in soil is of paramount concern to the environment and public health (**Huang et al, 2019**). Although the heavy metal-organic pollutant co-contaminated soil may lower the phytoremediation potential of hyper accumulator plants due to the toxic effects of organic pollutants (**Tripathi et al, 2015**), hence, novel pursuits and potentially efficient plant species are required to reduce the mixed contaminated soil systems and make it amenable for remediation. The phytoremediation technology is aesthetically pleasing and eco-friendly; *in-situ* remediation technology (without transportation of pollutants to other place) which is useful in treating both inorganic as well as organic pollutant (**Laghlimi et al, 2015**). Residual plant biomass could be

converted into energy and land restoration could be attained for sustainable agricultural development or general habitation (**Mahar et al, 2016**).

In view of above, an attempt has been made here to summarize the problem and consequences of heavy metal toxicity as co-contamination with herbicide and other organic contaminants in soil with due exploration of the various studies that has been carried out so far and their cleaning technique.

2.2 Phytoremediation

The generic term 'phytoremediation' comprises of two Greek words including-phyto which means plant attached to the Latin root *remedium* which refers to clean or remove (**Cunningham et al, 1996**). The natural removal of toxic materials *viz.*, phytoremediation has been treated as potentially providing *in situ*, on-site solutions that requires a simple and cost-effective technique than other alternatives (**Sainger et al, 2011**). Phytoremediation encompasses the use of plants to reduce the volume, mobility, or toxicity of contaminants (metals, pesticides, oil spills etc.) in groundwater, soil, or other contaminated media (**USEPA, 2000**).

Different bioremediation strategies have evolved which may reduce the period needed to achieve degradation thereby reducing the incurring cost by augmenting the degradative ability of indigenous microorganisms (**Perfumo et al, 2007**). Phytoremediation is counted as one of the green technologies which is emerging as a potential tool for cleaning up the environment from various contaminants, besides being cost-effective and non-invasive alternative to the conventional techniques (**Ashraf et al, 2019**).

Different authors suggested different phytoremediation techniques to remove heavy metal from contaminated soil and water which includes- phytoextraction, phytosequestration, phytostimulaton etc. On the basis of uptake mechanism the phytoremediation is thus subdivided into following types:

2.2.1 Phytoextraction

This process establish an involvement of plant roots for uptake of contaminants which accumulates in the aerial parts of plants and then that part is harvested (**Mahar et al, 2016**), that can be further used in energy and/or biofuel production. For example: In a study done by **Smolinska and Szczodrowska (2017)**, phytoextraction was attempted employing *Lepidium sativum L*. for remediation of Hg contaminated soil using compost as soil substrate under pot experimentation. In order to reduce Hg contamination from soil, phytoextraction process was repeated six times under continuous assessment. The results revealed that compost as a soil substrate enhanced the removal efficiency of Hg contamination and also *L. sativum L*. was able to accumulate significant amount of Hg in the above ground parts.

2.2.2 Phytovolatilization

This process involves an uptake of contaminants by plants and its further transformation into less toxic gaseous forms by selective translocation to the aerial parts of plants (**Limmer & Burken, 2016**) ultimately to the atmosphere. Several plants have been used for phytovolatilization *viz.*, *Brassica juncea*, *Arabidopsis thaliana*, *Chara canescens* out of which *Brassica juncea* has been found to be very promising for Se removal from soil (**Mahar et al, 2016; Khalid et al, 2017**). Apart from the abovementioned species, certain trees were also reported useful for this process. Phytovolatilization is primarily used for Hg removal into less toxic gaseous elemental form (**Ghosh & Singh, 2005**).

2.2.3 Phytostablisation

It is the process of reduction of mobility of heavy metals in soil, thereby reducing the solubility or bioavailability of contaminant entering the food-chain (**Khalid et al, 2017**). Selecting optimal species of plant could alleviate the process of phytostablisation because different plants have different mechanisms to reduce the degree of toxicity. It is proven best for removal of Cd, Hg, Zn, Cu contaminated soil and the common plants species that have been used are *Agrostis* spp. and *Fescuta* spp. For example, deep rooted plants can be best suited for phytostabilization of Cr(VI)

(**Ali et al, 2013**). A number of plant species have been found suitable or tolerant towards different metals *viz*., *Lupinus luteus* (**Dary et al, 2010**), *Brassica juncea* (**Shiyab et al, 2009; Banuelos et al, 2005**), *Hordeum vulgare*, *Vicia villosa* (**Katoh et al, 2017**), *Typha domingensis* and *Phragmites australis* (**Bonanno, 2013**).

2.2.4 Phytodegradation

It is the process of enzymatic degradation to remove or decontaminate organic contaminants by plants containing enzymes like dehalogenase, oxygenase etc. while restricted to heavy metal decontamination as heavy metals are not degradable or non-biodegradable (**Yadav et al, 2018**). For example, *Cyperus alternifolius* has been found to degrade Ethalonamines in wastewater (**Dolphen & Thiravetyan, 2015**) and *Armoracia rusticana* can potentially degarade benzophenone (**Chen et al, 2016 a, b**).

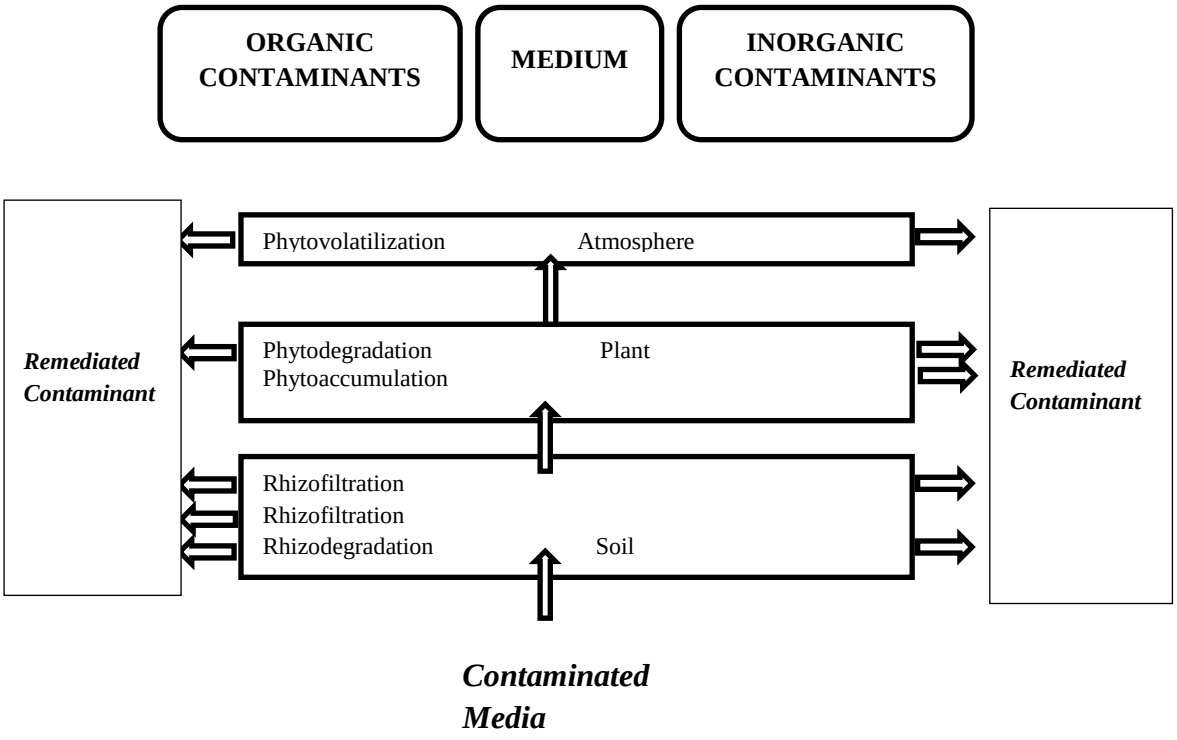


Figure 2.3: Uptake mechanisms of different phytoremediation processes (ITRC, 2009).

2.2.5 Rhizodegradation

It is the process of degradation of pollutants with the help of bacteria and fungi in the rhizospheric zone. Microbial remediation increases the efficiency of phytoremediation besides providing additional support to plant growth. For example, *Kocuria flava AB402* and *Bacillus vietnamensis AB403,* two isolated bacterial strains from mangrove rhizoshphere showed increased bioremediation potential towards As contamination (**Mallick et al, 2018**). Rhizodegradation varies differently according to the type and nature of pollutants or heavy metals. Hence, further investigation is needed in this field for its appropriate application (**Figure 2.3**).

2.3 Remediation using different plants

2.3.1 Remediation using tree species

Over the years, many studies have been done on phytoremediation and the technology is gradually being established as a promising and eco-friendly technique for environmental clean-up, particularly soil and water (**Raskin et al, 1997; Singh et al, 2003; Ali et al, 2013**). **Iqbal et al (2019)** suggested that removal of Cd from a contaminated site can be enhanced using hyper accumulator plants including tree species. Some examples of the plants which have been identified as hyperaccumulators are *Alyssum murale*, *Sebertia acumulnata*, *Phyllanthus balgooyi*, *Noccaea caerulescens*, *Thlaspi caerulescens*, *Arabidopsis halleri*, *Pimelea leptospermoides*, *Rorippa globulosa*, *Solanum nigrum*, *Sedum alfredii*, *Viola baoshanensis*, *etc.* (**Deng et al, 2008; Gao et al, 2010;Vollenwieder et al, 2011**). Preferably trees are best suited for remediation of heavy metal and other organic pollutant contaminated sites since trees are excellently found to accumulate numerous heavy metals. The suitability of various tree species *e.g.*, Willow (*Salix spp*.), *Delonex regia*, *Leucaena leucocephala*, *Thespesia populneoides*, *Populus tremula*, *Pinus sylvestris*, *Betula pendula*, etc. have been investigated for the phytoremediation of heavy metals (**Rosselli et al, 2003; Pulford & Watson 2003; Ismail et al, 2013**; **Dmuchowski et al, 2014**). **Jaiswal et al (2018)** studied removal of Cd contamination and microbial activity in artificially contamimated soil at different Cd concentration

using the saplings of Neem tree (*Azadirachta indica*). In this study the Neem was found to be a potential accumulator of Cd that revealed the efficiency of Neem in removal of Cd from contaminated site.

2.3.2 Remediation using native weed species

Kumar et al (2013) studied twelve native weed species viz. *Croton bonplandianum, Calotropis procera, Solanum nigrum, Datura stramonium, Cyperus rotundus, Phyllanthus amarus, Sida cordifolia, Solanum xanthocarpum, Spinacia oleracea, Euphorbia hirta, Parthenium hysterophorus* and *Tridax procumbens* growing naturally in the field having potential for heavy metals phytoremediation. They found that the concentration of heavy metals (Cr, Cu, Ni, Pb and Cd) in all the plants species having an enrichment factor > 1, which reflected their high metal accumulation potential. This study concluded that weed species may be used for phytoremediation of toxic heavy metals in the land. In another study **Tauqeer et al (2016)** evaluated the phytoremediation potential of *Alternanthera bettzickiana* (Regel) G. Nicholson plant at different level of Cd and Pb. They revealed that the *A. bettzickiana* successfully accumulated Cd and Pb at different levels in different parts of plants, hence possess significant tolerance towards both Cd and Pb.

2.3.3 Remediation using hydrophytes

Many invasive hydrophytes store a potential for phytoremediation and the best amongst them is *Eichhornia crassipes*, the most widely tested plant for phytoremediation (**Ting et al, 2018; Mishra & Maiti, 2017**). Some invasive species employed as hyperaccumulators are, *Typha latifolia*, *Phragmites australis*, *Chromolaena odorata*, *Ipomoea aquatic*, *Ipomoea carnia*, *Elodea canadensis*, *Egeria densa*, *Pistia stratiotes*, *Pluchea indica*, *Alternanthera philoxeroides etc*. for the removal of Ag, Cd, Cr, Cu, Hg, Ni, Pb, Zn and *Benzimidazole anthelmintics,* for the removal of crude oil (**Kaewtubtim et al, 2018; Hanks et al, 2015; Ohlbaum et al, 2018**).

2.3.4 Remediation using hyperaccumulators

Phytoremediation using invasive plant species has been found to be promising because invasive plants are capaable to (**Prabhakaran et al, 2019**) accumulate heavy metals and other pollutants and possess high properties of hyper-accumulation. For example: **Wei et al (2018)** in their research took three invasive plants namely *Chromolaena odorata, Bidens pilosa* and *Praxelis clematidea* for remediation of Cd contaminated soil using pot experiment and found them as potential accumulators of Cd. Among the three tested invasive plants, *C. odorata* was found to possess higher properties of hyperaccumulators, however, the other two were also good accumulators of Cd from the contaminated soil. Hence the invasive species of plants can be a good option for treatment of contaminated sites.

2.3.5 Remediation using ornamental plants

Many ornamentals plants have also been used for remediation of heavy metals and other pollutants, as many ornamental plants are not edible; hence the risk of contamination entering food chain is reduced. Ornamentals plants not only clean the environment but also add the aesthetic beauty to the environment; in addition, they provide employment opportunities. However the efficiency of phytoremediation can be alleviated by applying genetic or transgenic plants species, cheating agents *etc*., for example, Tobacco is the best transgenic plant for phytoremediation, because it possesses high biomass and high heavy metal accumulation properties apart from being fast growing, deep rooted and easily harvested **(Daghan, 2019)**. In order to enhance the efficiency of remediation of tobacco plant many researchers suggested for foreign gene transfer from different sources such as animals, plants as well as microbes. **Liu et al (2013)** studied the ecotoxicological responses and tolerance of seed germination parameters for different concentrations of Cd species by three ornamental plants *viz*; Italian ryegrass (*L. multiflorum Lam*.), Alfalfa (*M. sativa L*.), White clover (*T. repens L*.).

2.3.6 Remediation using herbs

Different plants have different mechanism for metal uptake and accumulation (**Nouri et al, 2009**) and this is the reason behind difficulty in selection of an efficient plant for phytoremediation. Tolerance capacity and biomass of selected plant play a major role because, if the biomass is higher, the ability of heavy metal removal from plants would be higher. Existing literature demonstrated that sunflower have high tolerance towards heavy metal accumulation from contaminated soil (**Chirakkara & Reddy 2015; Rizwan et al, 2016; Govarthanana et al, 2018**). **Alboudi et al (2018)** assessed remediation ability of *Helianthus annuus* for the Cd and Pb contaminated soil. The result showed that more Cd is accumulated in tissues of *H. annuus* compared to Pb, therefore, *H. annuus* could be considered favourable and potential candidate for heavy metal remediation measures. They suggested further investigation on the ability of *H.annus* in combination of heavy metals with plant growth promoters, to maximize the efficiency of removal of heavy metals. In an experiment done by **Li et al (2019)** phytoremediation potential of *Solanum nigrum L*. was checked using biochar/attpulgite as soil amendments. Results revealed that plant lengths and fresh weight under soil amendments was higher than in the non-amended control.

2.4 Phytoremediation of Organic pollutant

According to **Maslin & Maier (2000)** number of combination of technologies may be required for successful remediation of soil co-contaminated with metals and organics. They suggested that Rhamnolipid biosurfactant is biodegradable and could effectively stimulate the organic contamination in soil thus, would not be considered a long term problem if added to contaminated site. They identified that remediation can be enhanced for organic-metal co-contaminated site by applying biodegradable biosurfactant. Whereas, in another study **Madrid et al (2019)** demonstrated the effect of an admixture of rhamnolipid and Hydroxypropyl-β-cyclodextrin (HPBCD) for the remediation of an industrial soil co contaminated with PAH and potentially toxic elements (PTEs). The purpose to add rhamnolipid with mixed consortium of bacteria was to enhance the remediation efficiency and bioavailability of PAHs. Addition of

rhamnolipids did not show any effects on remediation efficiency, however, an increase in bioavailability of PAHs and mainly PTEs was observed in presence of rhamnolipids. While HPBCD at its highest dose (5%) enhanced PAH degradation with mixed consortium of bacteria.

Similarly in another study **Agnello et al (2016)** demonstrated that as compared to individual application of bioaugmentation, natural attenuation or phytoremediation, combined exploitation of plant together with microflora happens to be more efficient strategy for remediation of co-contaminated soil.

They evaluated following four distinct bioremediation strategies *i.e*., phytoremediation (using alfalfa), natural attenuation, bioaugmentation (using *Pseudomonas aeruginosa*) and phytoremediation assisted with bioaugmentation in order to treat co-contaminated soil with heavy metal (Zn, Cu and Pb) and petroleum hydrocarbons. They maximum removal of TPH (total petroleum hydrocarbon) was attained during phytoremediation assisted by bioaugmentation, as compared to other remediation strategies. Similarly, **Khudur et al (2019)** used combination of two technologies *i.e*., natural attenuation and biostimulation to evaluate the effect of Pb and total petroleum hydrocarbons (TPH) co-contaminated soil. The result showed decrease in TPH concentration under biostimulated treatment which was more than in that natural attenuation either as single or co-contamination scenario.

Organic compounds are diverse in nature owing to varied structural forms and chemical composition. For phytoremediation of organic compounds they should be mineralised into non-toxic components like NO^{3-}, NH^{+4}, Cl^{-} and CO_2, (**Meagher, 2000**), that can be readily available to plants or microorganisms. The diversity in structure and chemical compositions of organic compounds affects the mechanism of remediation since for phytoremediation to accomplish successfully the compounds should be in forms readily available to both plants and/ or microbes (**Parrish et al, 2005**).

Petroleum as well as it's by products are of major concern because they show slow biodegradability, high complexity and bio-magnification properties. There is an urgent requirement of technologies that can effectively remediate the persistent

pollutants from soil and that too in an eco-friendly manner. Phytoremediation is an eco-friendly approach for remediation of soils contaminated with polycyclic aromatic hydrocarbons (PAH), heavy metals and organic pollutants (**Kathi & Khan, 2011**). Numbers of studies have been done on soil contaminated with PAHs using various plants species (**Chen et al, 2016b; Mathur et al, 2010**). **Chen et al (2016b)** conducted a study to remediate soil having co-contamination of heavy metals (Cd and Zn) and PAHs and they used combination of technologies *viz,*. microbial remediation, phytoremediation and microbe-assisted phytoremediation for cleaning the soil. They obtained PAH removal, mineralisation and metal phytoextraction through interplanting *Seduce alfredii* with Ryegrass and *Microbacterium sp. KL5* & *Candida tropicalis C10* for re-inoculation in the co-contaminated soil. They concluded that interplanting with *S. alfredii* reduced metal concentration in Ryegrass tissue hence ryegrass showed stronger rhizosphere effect than *S. alfredii*. Diverse plant communities can be used to address the issue of co-contamination. Plant diversity affects microbial community in rhizospheric associations (**Kowalchuk et al, 2002**). Examples include association of *Medicago sativa* with *Lolium perenne* L. which besides increasing rhizospheric bacterial count also have capability of petroleum degradation (**Kirk et al, 2005**).

PAHs together with heavy metals co-contaminated soil and water makes the biodegradation more complex and challenging. **Mandal et al (2016)** introduced modified clay-modulated microbial degradation to provide a new and efficient revelation in those issues. They investigated the phenanthrene degradation pattern of *Mycobacterium gilvum VF1* and its growth in the presence of palmitic acid grafted organobentonite in co-contaminated water with Cd-phenanthrene. Their study revealed that organobentonite supported the growth and spreading of bacteria that eliminated the Cd toxicity by the process of adsorption and also provided a suitable microenvironment for the survival of bacteria. This study revealed new and innovative vision for bioremediation technologies involving clay modulated PAH in mixed water as well as soil co-contaminants. PAH degradation was investigated in an another study by **Lu and Zhang (2014)** in artificially contaminated soil, wherein the

remediation strategy for Polybrominated diphenyl ethers (PBDEs) and heavy metal (Cd, Pb and Zn) co-contaminated soil was developed. PBDEs under falls one of the major environmental concerns due to their persistent nature, bioaccumulation properties and other toxic effects. They are extensively used as retardants in electronic appliances and textile industry to check propagation of fire. The most widely used PBDEs are Deca BDE (BDE-209) (**Gandhi et al, 2011**). **Lu and Zhang (2014)**, used combination of plants for remediation of BDE-209 and heavy metal co-contaminated soil in which, a hyperaccumulator plant *Sedum alfredi* and Tall fescue *i.e*., *Festuca arundinaceae* were combined with a bacterial strain *Bacillus cereus* JP12. The result depicted that the bacterial strain was helpful in enhancing the degradation potential of BDE-209, in addition, the co-planting was suitable for remediation of PBDEs and heavy metal co-contaminated soil. In contrast, in an another study, **Li et al (2018)** demonstrated the remediation potential of *Solanum nigrum* for BDE-209 and Cd co-contaminated soil using pot experimentation combined with two arbuscular mycorrhizal fungi that is *Funneliformis mosseae* (FM) and *Rhizophagus intraradices* (RI) and surfactant β-cyclodextrin (β-CD). This study revealed that the uptake of BDE-209 and Cd by plants was alleviated due to the interaction in between them which led the conversion of BDE into less toxic form in plant and soil, in addition, *S. nigrum* added with β-CD treatments could achieve potential uptake of Cd and BDE-209. Hence, this could be a better alternative for remediation of PAHs and heavy metal co-contaminated sites and the finding of this study suggested that interaction between plants, microbes *etc.* should be considered while choosing a phytoremediation technology.

Li et al (2019) investigated the effect of combination of Pb and fluoranthene co contaminated soil on mushrooms and bacteria. Their toxicity can be reduced and co contaminated soil can be improved by addition of *Oudemansiella radicata* (*O. radicata*) combining with *Serratia marcescens* (*S. marcescens*). This study was carried out using pot experimentation and microbial count, enzymatic activity *etc*., were done by different methods. Results showed that the presence of *O. radicata* and *S. marcescens* could significantly promote the bioremediation of Pb-fluoranthene co-

Table 2.2: Phytoremediation potentialof different plant species for organic contaminants

Contaminant(s)	Soil Concentration (mg kg^{-1})	Plant Species	Experiment Duration	Results/ Remediation Efficiency	Source
TPH	6400	*Lolium perenne*	102 days	Loss of TPH	**Hou et al (2001)**
Cypermethrin	10 µg/g	*Plantago Major*	14 days	Results revealed that the plant able to remove cypermethrin from the soil by its roots and then its leaves	**Aioub et al (2019)**
Azoxystrobin	Unknown	*Plantago major L.*, *Helianthus annus L.* and *Glycine max L.*	14 days	*Glycine max L.* and *Plantago major L.* were the most suitable plant species as compared to *Helianthus* for phytoremediation of azoxystrobin contaminated soil.	**Romeh (2015)**
Polycyclic Aromatic hydrocarbons (PAHs)	100	*Medicago sativa*, *Brassica napus*, and *Lolium perenne*	90 days	Pyrene was successfully removed by plants used	**D'Orazio et al (2013)**
2,4,6 trinitrotoluene (TNT)	80	*Vetiveria zizanioides*	48 days	Removal of TNT helped by Urea.	**Das et al (2010)**
Phenanthrene and Pyrene	Unknown	*Panicum bisulcatum*, *Echinogalus crus-galli*, *Astragalus membranaceus*, and *Aeschynomene indica)*	80 days	*E. crus-galli* and *A. membranaceus* are suitable candidates for the phytoremediation of soils contaminated with recalcitrant pollutants because they are capable of robust growth and efficient extracellular enzyme production in soil	**Lee et al (2008)**

Pyrene	492	*Medicago sativa L.*	60 days	Enhancement of pyrene degradation was probably the result of an increased rhizosphere microbial community	**Fan et al (2008)**
Alkylated PAHs	9175	*Lolium arundinaceum, Lolium multiflorum* and *Cynodon dactylon.*	21 days	Greater degradation for anthracenes and phenanthracenes	**White et al (2006)**
Benzo[a]pyrene	Unknown	*Vetiveria zizanioides*	60 days	Results indicate that V. zizanioides may be useful for phytoremediation of B[a]P contaminated sites.	**Li et al (2006)**
Polycyclic Aromatic Hydrocarbons	Phenanthrene-200, Pyrene-199.3	*F. arundinacea, L. perenne, M. sativa* and *B. napus*	65 days	The presence of vegetation significantly increased the dissipation of phenanthrene and pyrene in the soil environment.	**Cheema et al (2010)**

contaminated soil; hence the combined effect of *O. radicata* and *S. marcescens* could potentially remediate the Pb-fluoranthene co-contaminateds soils. Whereas in another study **Li et al (2019)** used the combination of *Sauda salsa* plant together with an indigenous fungi *Trichoderma asperellum* for Pb and salinity (Na and Ca) co-contaminated soil. They suggested that the plant growth was found to be well tolerant towards Pb contamination especially under bioaugmentation with *T. asperellum*. They confirmed the biostimulation advantage over natural attenuation for remediation of TPH and TPH-Pb contaminated site. Phytoremediation potential of distinct plant varieties for organic pollutants is given in **Table 2.2**.

2.5. Phytoremediation of Inorganic Contaminants

Since, inorganic contaminants are primarily non-degradable; hence their degradation or mineralisation cannot be like organic pollutants. Heavy metals are naturally occurring throughout the earth's crust, but several heavy metals, such as Copper (Cu), Lead (Pb), Zinc (Zn), Cadmium (Cd), Nickel (Ni), Chromium (Cr), Mercury (Hg) and the metalloid Arsenic (As), are widely used by industries, agriculture and consequently released into the environment (**Tchounwou et al, 2012**). Although, the traditionally used physical and chemical methods have long been used to restore the various soil parameters but they have several limitations *viz*; they require excessive processing and cause changes in the soil properties, expensive, disturb the native vegetation *etc*., on the other hand, recent technologies with sustainable costs includes phytoremediation which is far better and more eco-friendly than the traditional ones (**Ali et al, 2013**). **Marmiroli et al (1999)**, have studied the phytoremediation ability of Walnut (*Juglans regia*) and Maple (*Acer saccharinum*) for Pb and Cr. Greater accumulation of Pb in roots was observed as compared to the stems. Out of the two plants, Walnut was having greater phytoremediation potential for both metals as comparison to the Maple.

Pseudometallophytes are plant groups having an ability to exist and reproduce under high metal contamination without metals hyperaccumulation; which

is obtained by tolerance developed due to rhizospheric metal precipitation. In *Agrostis tenuis* metal (Zn, Cu, Cd and Pb) concentration was greater in roots compared to leaves thereby suggesting metal immobilization in roots. During phytoremediation process, metal availability for uptake can be augmented by an application of soil amendments (**Dahmani-Muller et al, 2000**). For example, **Gupta et al (2008)** studied soil amendment with ethylenediaminetetraacectic acid (EDTA) which enhanced the uptake of Pb by *Vetiveria zizanioides.* Addition of compost was found to remove higher amount of chromium by *Pterocarpus indicus* and *Jatropha curcas* L in the soil (**Mangkoedihardjo et al, 2008**).

Phytoexcretion is a novel phytoremediation process (**Liang et al, 2016**) used by halophytes as metal detoxification strategy, wherein toxic metals are excreted through specialized salt glands from leaf tissue onto leaf surface (**Manousaki & Kalogerakis, 2011; Liang et al, 2016**). **de Souza et al (2014)** reported the halophyte *Atriplex nummularia* and its remediation potential for saline and sodic soils due to its high biomass and salt extraction capability.

According to **Demarco et al (2019)** native plant species *S. montevidensis* possess natural phytoextraction ability for Ca and K and also demonstrated rhizofiltration property for Mg, Cr, Zn, Mn, S, V, Cd, P, Fe, As, Al, Cu, Na, Pb, Ni, establishing its ability for bioaccumulation of these contaminants particularly in the roots.

Different plant species like *Elsholtzia splendens* (**Jiang et al, 2004**) has been reported to be used for phytoremediation of Cu; *Alnus sp*., *Salix sp*. and *Populus sp.* for zinc phytoextraction and nickel stabilization (**French et al, 2006**), and *Brassica carinata* and *Brassica juncea* for Cu, Cd, Zn and Pb phytoextraction (**Rio et al, 2000**). Plant species like *Pueraria lobata*, *Leucaena leucocephala*, *Bidens pilosa*, *Crotalaria micans*, and *Conyza canadensis* have been studied for their ability to remove Ni from a serpentine site for successful phytoremediation applications in Taiwan (**Ho et al, 2013**).

Premarathne et al (2019) reported that *Canna indica* is the best plant among the selected plant types for the removal of the different types of metal pollutants (Mn,

Table 2.3: Phytoremediation potentialof different plant species for inorganic contaminants

Contaminant(s)	Soil concentration ($mg\ kg^{-1}$)	Plant Species	Experiment Duration	Results/ Remediation Efficiency	Source
Cd, Pb, Ni, Zn, Cr	Cd- 27.45, Pb-77.05, Zn-47.82, Ni-47.75, Cr-36.73	*Catharanthu roseus*	60 days	Results shows that the plant species was good accumulator of lead, nickel, zinc, cadmium and chromium contaminated soils.	**Subhashini and Swamy (2013)**
Hg	3.8	Sweet sorghum KCS105	90 days	Sweet sorghum variety KCS105 could be considered a suitable candidate energy crop for phytoremediation of mercury contaminated soil.	**Oh et al (2015)**
Cd, Cr. Ni	Cd- 160, Cr-240, Ni-480	*Populus alba* and *Morus alba*	60 days	This study showed that leaves accumulate higher concentrations of Cd, Cr, and Ni than other organs.	**Rafati et al (2011)**
Hg	5	*B. juncea*	30 days	Induced plant- Hg accumulation for plant species grown in the modified GM mine tailings (Hg at 2.5 mg/kg) which was more pronounced in the presence of thiosulphate salts.	**Moreno et al (2005)**
Cd, Zn	Cd- 19, Zn-2920	*Thlaspi caerulescens*	391 days	Soil amendment with EDTA increases the phytoextraction capability.	**Lombi et al (2001)**
Hg	15 kg	*Lindernia crustacea (L.) F., Paspalum conjugatum L.* and *Cyperus kyllingia*	63 days	Addition of ammonium thiosulphate to the mercury contaminated soil increased mercury accumulation in plants viz; *P. conjugatum*, *C. kylingia* and *L.*	**Muddarisna et al (2013)**

		Endl		*crustacea*	
Cd, Zn	Zn- 600, Cd- 8	*Pennisetum americanum* and *Pennisetum atratum*	100 days	Removal of metal	**Zhang et al (2010)**
Cd, Zn, Pb	Cd- 20, Zn- 500, Pb- 1000	*Dianthus chinensis* and *Vetiveria zizanioides*	21 days	Removal of Metals greater with EDTA	**Lai and Chen (2004)**
Hg	10	*Jatropha curcas*	120 days	The results revealed that *J. curcas* is a good choice for the phytoremediation of mercury-contaminated soil because it is easy to plant and maintain and contributes to soil restoration and conservation	**Marrugo-Negrete et al (2015)**
Cr	90	*Pterocarpus indicus* and *Jatropha curcas L.*	60 days	Removal of chromium	**Mangkoedihardjo et al (2008)**
As, Co, Cu, Pb, Zn	As- 886 (av), Co- 100 (av), Cu-1735 (av), Pb-473 (av), Zn- 2404 (av)	*Populus alba*, *Populus Nigra*, *Populus tremula* and *Salix alba.*	2 years	Species successfully stabilized metals	**Vameralli et al (2009)**
Ni	Ni- 60, 100, 240	*Phalaris arundinacea*, *Salix viminalis* and *Zea mays*	2 year	These plants phytostabalize the Ni and checks soil erosion also.	**Korzeniowska and Stanislawska-Glubiak (2019)**

Cr, Cd, Mg and Cu) in textile wastewater *Ipomoea aquatic* was found to be the second highest effective plant which also signifies that, contaminated plant can cause health issues if consumed as a food. Phytoremediation potential of some established plant species for inorganic contaminants is given in **Table 2.3**.

2.6 Phytoremediation of Co-contaminants

Joint application of inorganic and organic contaminants may negatively affect both plant growth and survival (**Sun et al, 2011**).

In a study, removal of hydrocarbon was demonstrated by combination of plants that is *Pinus sylvestris* and *Populus deltoides* x wettsteinii in the presence of metals like Pb, Cu and Zn. However 80% of plants died due to toxicity (**Palmorath et al, 2006)**.

Occurrence of DDT (dichlorodiphenyltrichloroethane) and Cd or their metabolic residues in food or agricultural soils impose great threat to both animal and human health. A comparison of 23 different genotypes of *Ricinus communis* was carried out to study the mobilization and uptake of both DDT and Cd. Large variation in the plant genotypes were revealed both in terms of uptake as well as accumulation of both the contaminants Cd and DDTs. Overall it was deduced that *R. communis* has the capacity to remove both the above mentioned contaminants from soil which can be attributed to its robust growth, lush biomass, strong absorption and accumulation for both contaminants (**Huang et al, 2011)**.

Lin et al (2008) investigated pyrene and Cu co contamination on the growth of *Zea mays L.* and found that root and shoot biomass was affected by the Cu-pyrene co contamination. They observed the pyhtoextraction ability of Cu would be inhibited under high level of pyrene in highly Cu polluted soil. Whereas, **Zhang et al (2009)** demonstrated removal of Cd from pyrene co contaminated soil under the growth response, after 60 days of plantation of Maize CT38. Dissipation of Pyrene in soil, has been found to affect the uptake and accumulation of pyrene and Cd by maize. The presence of Cd stimulated the accumulation of pyrene but not the *vice versa* in the roots and shoots of maize. It was overall revealed that maize CT38 can grow normally

in soil co-contaminated with high level of Cd and pyrene and can effectively remediate the pyrene-Cd co-contaminated soil.

It has been shown that pyrene and Zn together significantly affected the growth of *B. juncea* wherein its ability for Zn accumulation was compromised whereas, *Festuca arundinacea* did not show any reduction in growth under pyrene and Zn co-contaminated soil, thereby suggesting that *F. arundinacea* can be a better candidate for remediation of mixed contaminated soil (**Batty & Anslow, 2008**).

Interaction of different plant species may lead to a change in the general response of a plant to a particular contaminant, *e.g*., *Salix caprea*, *Carex flava* and *Centaurea angustifolia* as a mixed culture improved the negative effect of Zn (**Koelbener et al, 2008**).

In one study by **Hechmi et al (2013),** remediation of Cd and Pentachlorophenol (PCP) co-contaminated soil was carried out under pot experimentation by *Zea mays L*. Being a pesticide, herbicide PCP is resistant for degradation in soil, although the use of PCP has decreased in recent years, but still it is present in the environment, hence, the remediation is necessary.

Ghosh et al (2015) reported the role of *Vetiver* for phytoremediation of fly ash and its amendments. The roots and leaves of *Vetiver* grown in different amendments were subjected to metal estimation to help understand the extent of remediation of fly ash under the influence of *Vetiver* grass. The study revealed marked decrease in concentration of heavy metals and a significant decrease in genotoxic potential, in the Fly Ash soil amendments over the period of 18 months (**Ghosh et al, 2015**). The study revealed that *Vetiver* grass is capable of remediating Fly Ash by stabilizing the metals in the root. Phytoremediation potential of different plant species for mixed contaminants is given in **Table 2.4**.

Existing remediation technologies (both sustainable and unsustainable) have shown promise for individual pollutants; which has not been the case for sites that are contaminated with more than one single pollutant. This has been the challenge for soil remediation, as many contaminated sites do not contain one single pollutant but instead a number of different substances of various origin. This literature survey

Table 2.4: Phytoremediation potential of different plant species for mixed contaminants

Contaminant(s)	Soil concentration (mg kg^{-1})	Plant Species	Experiment Duration	Results/ Remediation Efficiency	Source
Pyrene + Cu	Pyrene- 500, Cu- 400	*Zea mays L*	28 days	In *Zea mays* Cu toxicity was alleviated with an increase in pyrene concentration. Similarly an increase in residual pyrene was recorded with an increasing conc. of Cu.	**Lin et al (2008)**
Carbendazim +Cd		*Sedum alfredii*	180 days	The results revealed that, *S. alfredii* combined with carbendazim-degrading strains is a suitable plant–microbe interaction for the remediation of Cd and carbendazim co contaminated soil.	**Xiao et al (2013)**
Benzo[a]pyrene,+Cu, Cd, Pb	B[a] P – 5, Cd- 50, Cu- 500, Pb- 3000	*Tagetes patula*	92 days	Greater degradation of B[*a*]P in the presence of Cd	**Sun et al (2011)**
Penta Chloro Phenol (PCP) +Cu	PCP- 100 and Cu- 300	*Lolium perenne L.* and *Raphanus sativus*	84 days	Higher dissipation of PCP under 50 mg kg-1 with increasing Cu concentration.	**Lin et al (2006)**
Pyrene + Cd	Pyrene- 100, Cd- 4.5	*Zea mays*	60 days	Pyrene uptake stimulated by presence of Cd	**Zhang et al (2009)**

Pyrene, Phenanthrene +Cd	Pyrene- 250, Phe-250 Cd- 50	*Juncus subsecondus*	70 days	Dissipation of PAH influenced by Cd.	**Zhang et al (2011)**
Total Petroleum Hydrocarbons +Cd, Zn, Ni	TPH-50,000, Cd-2000, Zn-1000 and Ni-2000	*Chromolaena odorata (L)*	180 days	The growth of the plant was sustained and was able to cause the removal of both the contaminant oil by 82% and the present heavy metals by up to 65%.	**Atagana (2011)**
PCP + Cd	PCP-0-250, Cd-0-20	*Phragmites australis*	75 days	Results concluded that the emergent wetland *P. australis* was able accumulate both Cd and PCP from polluted soils.The, root depth, the resistance to different types of environmental stress, high biomass production, as well as stimulating effects for microbial activities shows the phytoremediation potential for organic and inorganic chemicals from polluted soils.	**Hechmi et al (2015)**

provides information about the application of phytoremediation processes in soils contaminated by inorganic and organic compounds using herbaceous and woody plants. This study encompasses the phytoremediation techniques available today for the recovery of soils contaminated by metals and organic substances. Chelating agents and surfactants are in general effective amendments for the enhanced removal of both heavy metals and organic contaminants.

Phytomining can be done to recover and reuse the heavy metals from plant tissues after phytoremediation. The product biomass may be employed again for cogeneration of energy and production of biofuels; their ashes could be used by brick-kiln industries, after removal of the extracted contaminants; the metals recovered after the incineration of the plants may become a raw material for various industrial processes.

In the above context for remediation of mixed contaminated soil cost-effective, less energy consuming and eco-friendly technologies are required to which phytoremediation could be a possible solution. Generally phytoremediation is usually employed to address threats posed by either single contaminant or a mixture of contaminants of similar types. To this end the present study will provide an insight on the problems posed by an admixture of inorganic and organic contaminants for phytoremediation of co-contaminated agricultural soils close to industries.

Chapter 3

Materials & Methods

3.1 Study area

The city of Lucknow, U.P., India having gps coordinates of 26° 51' 0.0000" N latitude and 80° 56' 59.9892" E longitude elaborates an elevation of approximately 123 metres (404 ft) above sea level.. The experiments in the present study were carried out in the greenhouse of research field station of the Department of Environmental Science, Babasaheb Bhimrao Ambedkar University, Lucknow, India. The minimum and maximum temperature ranged between (Jan) 7.9°C-22.1°C in winters and (May) 24.7°C-39.6°C in summers with relative humidity of 88% and rainfall 917.3 mm (**Source: IMD, http://www.imd.gov.in/pages/city_weather_show.php**).

3.2. Plant selection

Several ornamental plants (*viz*., Marigold, Celosia, Gaillardia *etc*.) were studied for their tolerance under heavy metal (Cd, Hg) and herbicide (Butachlor) stress. *Catharanthus roseus* (Sadabahar), formerly known as *Vinca rosea* was selected for the study.

Classification for kingdom plantae for *Catharanthus roseus*

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Asteridae
Order	Gentianales
Family	Apocynaceae
Genus	*Catharanthus* G. Don
Species	*Catharanthus roseus* (L.) G. Don

Source: United States Department of Agriculture, Natural Resources Conservation Service. (**https://plants.usda.gov/java/ClassificationServlet?source=display&classid=CARO14**)

3.2.1. *Catharanthus roseus* (Periwinkle, 2n=16)

Catharanthus roseus, commonly known as Periwinkle, belongs to genus, *Catharanthus*, and is a native of West Indies. *Catharanthus roseus* (Periwinkle) is a species of Apocynaceae family. Synonyms include *Ammocallis rosea*, *Vinca rosea*, and *Lochnera rosea*; other English names occasionally used include, Old-maid, Rose Periwinkle, Cape Periwinkle and Rosy Periwinkle. It is a perennial herb, flowering all the year round in tropical regions. A large number of cultivars are known for diversity in flower colour (peach, scarlet, mauve, reddish-orange and white), and tolerance to cooler growing conditions of temperate regions (**Gamble, 2008**). In the present study *C. roseus* has been selected due to its easy availability, evergreen nature, ornamental value and its endurance under dry and nutritionally deficient conditions.

Plate 3.1 *Catharanthus roseus* L.

3.3 Soil analysis

For the soil analysis samples were collected from top soils (0-15 cm), following Z-pattern of sampling. The samples were collected in a zip mouthed polyethylene bags, were properly labeled and stored in the laboratory of Department of Environmental Science, Babasaheb Bhimrao Ambedkar University, Lucknow, U.P., at 4˚C prior to analysis. Soil samples were air dried, pulverized and sieved (2 mm sieve) prior to analysis. Analytical grade reagents were used throughout the course of the study. For each soil sample pH, moisture content, electrical conductivity, organic carbon (OC),

total nitrogen (N), phosphorus (P), and potassium (K) were estimated using standard methods.

3.3.1 Soil pH

Potential of Hydrogenii (pH) of each soil sample was determined using Fischer's Digital pH meter in the ratio 1:2 of soil and distilled water (**Chopra & Kanwar, 1982**). Before measurement, the pH meter was calibrated using buffer solutions made by buffer tablets of known pH (pH 4, pH 7 and pH 9) in 100 ml distilled water. During the pH measurements, 10 g of soil sample was put into 25 ml of distilled water in a clean beaker. The mixture was stirred at least for 30 minutes using a magnetic stirrer and then allowed to settle down for few minutes. Then, the calibrated electrode of pH meter was dipped in the soil solution and pH reading was noted.

3.3.2 Electrical conductivity (EC)

Electrical conductivity of the soil samples was measured by digital electrical conductivity meter. Electrical conductivity is the ability of dissolved inorganic solutes to conduct current under the influence of electric field (**Chopra & Kanwar, 1982**).

10 g of soil was dissolved in 40 ml of freshly prepared distilled water. Soil solution was put in shaker for 1 hour so that soil particles get mixed properly and then left for undisturbed for 30 minutes for the soil to settles down. Calibrated the conductivity meter with standard KCl solution and the readings were recorded after dipping the conductivity rod in the soil solution.

3.3.3 Soil texture

Texture is related to certain physical properties of soil such as plasticity, permeability, ease of tillage, fertility, water holding capacity and overall soil productivity. For instance, for irrigation purposes, loamy and clay textures are classed as soils of high moisture holding capacity while loamy sands and sands have low moisture holding capacity **(Brady, 2008)**. Soil texture was determined through protocol given by **Shirazi and Boersma (1984)**.

3.3.4 Moisture content (MC)

Moisture content (MC) was estimated as % moisture content on oven dry weight basis method **(ASTM, 2010).** Soil samples of known weight were kept in hot air oven at a

temperature of 70°C for 48 h. Reweighted the sample to find out the loss in weight. Percentage moisture content of soil was calculated as per the formulae given below:

Weight of empty crucible = w_1

Weight of soil +crucible = w_2

Weight of soil + crucible after heating for 48 h = w_3

Percent moisture content (dry weight) = $\frac{\text{Loss of weight of dry matter}}{\text{Weight of dry soil}} \times 100$

3.3.5 Organic carbon (OC)

The soil organic carbon content in soil was determined using **Walkley and Black (1934)** method.

Reagents Required:

- Standard (1N) $K_2Cr_2O_7$ solution: Exactly 49.04 g is dissolved in water and diluted to 1lt.
- Standard (0.5 N) FAS solution: Dissolved 191.6g $Fe(NH_4)(SO_4)_2.6H_2O$ in 600ml water containing 20 ml H_2SO_4 and diluted to 1 lt. Standardized with 1N $K_2Cr_2O_7$.
- Orthophosphoric acid (85%) reagent grade
- Sulphuric acid (conc.)
- Sodium Fluoride (Na F) reagent grade
- Diphenylamine indicator: Dissolved approximately 0.5 g diphenylamine in 20 ml water and 100 ml H_2SO_4

Procedure:

Soil sample (1 g) was taken and passed through 5 mm sieve in a 500 ml conical flask. 10 ml of 1N $K_2Cr_2O_7$ solution was added through pipette. Swirled the flask and 2 ml of conc. H_2SO_4 was added and gently mixed for 1 minute. Flask was kept standing for 30 minutes for oxidation of organic carbon, and the contents were diluted with 200 ml water and 10 ml of 85% H_3PO_4, 0.2 g NaF powder and 30 drops of diphenylamine indicator was added. The unreacted potassium dichromate was titrated with FAS solution delivered from burette. The colour was dull green in the beginning, which shifts to turbid blue as the titration proceeds and finally becomes brilliant green at the end point. A blank titration was carried out in the same manner (with 10 ml of 1N

$K_2Cr_2O_7$) but, without soil. Then NaF was added Al, which forms AlF_3 while H_3PO_4 ensures a sharp end point. High Cl content, as in case of saline soil, interferes in the estimation which can be prevented by adding Ag_2SO_4 @ 1.25% to the conc. H_2SO_4.

Calculations:

- Milliequivalents of $K_2Cr_2O_7$ added = ml × N = 10 × 1 = 10
- Normality of FAS = (ml of $K_2Cr_2O_7$ × N) / ml of FAS used by blank = 10/ ml of FAS used by blank
- Milliequivalents of FAS used = FAS used in sample titration (ml) × N
- Organic C% in soil = [(Meq. of $K_2Cr_2O_7$- Meq of FAS) × 0.003×100×1.3] / Dry weight of soil (g)

  = [(Meq. of $K_2Cr_20_7$- Meq of FAS) × 0.39 / Dry weight of soil (g)]

  % Organic matter (OM) = Organic C% × 1.724 (assuming average 58% C in OM)

3.3.6 Available nitrogen (N)

The soil available nitrogen was determined using Alkaline permanganate ($KMnO_4$) procedure (**Subbiah & Asija, 1956)**. The method involves distilling the soil with alkaline potassium permanganate solution and determining the ammonia liberated.

Reagents Required:

- $KMnO_4$ solution (0.32%): Dissolved 3.2 g $KMnO_4$ in 250 ml distilled water in a 1 litre volumetric flask followed by volume make up and thorough mixing.
- NaOH solution (2.5%): Dissolved 25.0 g NaOH flakes in 250 ml distilled water, mixed well and the volume was made up to 1 litre.
- Liquid paraffin
- 0.02 N H_2SO_4
- Boric acid- indicator solution: Dissolved 20 g boric acid in 700 ml hot water. Transfered the cooled solution to 1 litre volumetric flask containing 200 ml ethanol and 20 ml of mixed indicator. Mixed the contents of flask and added a little 0.02 NaOH solution until the colour turns pink. Content was mixed properly and volume was made up in the volumetric flask.
- Mixed indicator: Dissolved 0.07 g of methyl red and 0.1 g bromocresol green in 100 ml of 95% ethanol.

Procedure:

Transferred 20.0 g of soil in a 500 ml Kjeldahl flask, and added 20 ml of distilled water and swirled the Kjeldahl flask gently. Added 1 ml of liquid paraffin and few glass beads and then finally added 100 ml each of $KMnO_4$ and NaOH solutions. Distilled the contents in a Kjeldahl assembly and collected the liberated ammonia in a 250 ml Erlenmeyer flask containing 20 ml of boric acid mixed indicator solution.100 ml distillate was collected in a volumetric flask after 30 minutes. The colour of the distillate was bluish green, which was further titrated with 0.02 N H_2SO_4 up to end point (pink colour) reached.

Calculations:

Available N (kg ha^{-1}) = (X ml H_2SO_4 sample – X ml H_2SO_4 blank) * 31.36

3.3.7 Available phosphorus (P)

The available Phosphorus content in soil sample was determined following **Olsen et al (1954)** method.

Reagents Required:

- Reagent A: Dissolved 12 g ammonium molybdate in 250 ml of distilled water. Also dissolved 0.2908 g potassium antimony tartarate in 100 ml distilled water. Added both the solutions to 1 litre of 5 *N* H_2SO_4, mixed thouroughly and diluted to 2 L. Stored it in Pyrex glass bottle in dark and cool compartment.
- Reagent B: Dissolved 1.056 g ascorbic acid in 200 ml of reagent A. It was freshly prepared, before experimentation.
- p-nitrophenol: Prepared 0.25% aqueous solution and filtered it.
- NH_4OH (4N) and HCl (4N) for adjusting the pH.

Procedure:

An aliquot were taken containing approximately 1- 40 µg P (5 ml in 25 ml volumetric flask), added 2 drops of p-nitrophenol indicator. pH was adjusted by adding dilute acid or alkali drop wise. In yellowish solution few drops of dilute acid was added to make the solution colourless. Then 4 ml of reagent B was added, after adequate shaking it was diluted upto the mark. Stoppered the flask and inverted 3-4 times to homogenize it well. The %Transmittance (T) or Absorbance (A) were measured at

882 nm after 15 minutes when the blue colour was fully developed. The colour was stable for about a day. Standard curve of concentration 0 to 0.4 ppm of P solution was prepared. For this 0, 0.5, 1.0, 2.0, 3.0, and 5.0 ml of 2 ppm P stock solution was taken to make 0, 0.04, 0.08, 0.16, 0.24, and 0.4 µg P/ml, respectively.

Calculations:

Plotted standard curve for absorbance (Ab) v/s concentration of P on simple graph paper, and calculated the corresponding concentration of P (ppm) in samples from standard curve (Ps). Since, 1 g soil was shaken with 20 ml extract, 5 ml of extract was taken for (blue) colour development in 25 ml solution, the dilution factor was (25 × 20)/(5 × 1) or 100. The conversion factor from ppm to kg/ha is 2.24 and from P to P_2O_5 was 2.29.

The content of available P in soil was calculated as follows:

Available P (kg P_2O_5/ha) = (Ps × 25 × 20 × 2.24 × 2.29) / 5 = Ps × 512.96

3.3.8 Available potassium (K)

The available Potassium in soil samples was determined using **Black (1965)** method.

Reagents Required:

- Ammonium acetate (1N pH 7): Dissolved 77g CH_3COONH_4 in 900 ml distilled water, mixed thoroughly and adjusted its pH to 7.0 by addition of diluted (3N) NH_4OH or CH_3COOH. Diluted it to 1L with distilled water (Extraction solution).
- Standard K solution (1000 ppm): Dissolved 1.908 g AR grade KCL (dried at 60 ºC for 1 h) in distilled water and diluted to 1L. Prepared 100 ppm K solution by diluting 100 mL of 1000 ppm solution to 1L by extracting solution.

Procedure:

1) **Extraction**: Placed 5 g soil in 150 ml conical flask and added 25 ml of extraction solution (1N ammonium acetate of pH 7). Shaken on a reciprocating shaker for 5 minutes at 180 oscillations per minute. Immediately filtered the suspension through Whatman no. 1 filter paper. First few mL of the filtrate was rejected. Alternatively, centrifugation at 2000 rpm for 10 minutes was also used to get

supernatant containing K. For better results, centrifuge washing and collection of supernatant was repeated twice and volume was made up to 100 ml in this case.

2) **Determination**: Pipetted 0, 10, 20, 30, 40 and 50 mL of 100 ppm K solution into 100 mL volumetric flasks and raise the volume to mark with extracting solution. These solution contained 0, 10, 20, 30, 40 and 50 ppm K, respectively. Adjusted the instrument using 0 ppm K extracting solution and maximum concentration (50 ppm K) placing K filter. Determined flame photo reading (FPR) for all concentrations of this set and constructed the standard curve by plotting FPR (Y axis) against K concentration (X axis). Also recorded the FPR for soil extracts and determined the concentration of K in final solution from standard curve.

Calculations:

Available K (kg/ha) ={R × Volume of extract (mL) × 2.24}/ Weight of soil taken (g)

= R × 11.2

Where, R = ppm K in the extract obtained from standard curve

and, 2.24 = Factor to convert ppm to kg/ha.

3.4 Heavy metal analysis in soil

For heavy metal determination 1g of oven dried samples were taken in 250 ml conical flasks and digested in HNO_3: $HClO_4$ (3:1) at 70 to 80 °C (**Odu et al, 1986)**. The digested samples were filtered and diluted with double distilled water up to 50 mL and analyzed for the metals using *Varian Spectra AA-250* plus Atomic Absorption Spectrophotometer (AAS) as described by **Odu et al (1986**) using acetylene gas as fuel and air as an oxidizer. Blanks were prepared to check for background contamination by reagents used. Average values of three replicates were taken for each determination.

Metal concentration (µg g^{-1}dwt) = $\frac{XV}{W}$

Where,

X = Reading in ppm on AAS

V = Final volume of digested samples (ml)

W = Dry weight of the sample (g)

3.5 Translocation factor (TF) and bioconcentration factor (BCF) of the metals

The Bioconcentration Factor (BCF) was used to determine the heavy metal accumulation by the plant from the soil (**Ali et al, 2002**) and was calculated using the formula:

$$BCF = \frac{Concentration\ of\ metal\ in\ roots/shoots}{Concentration\ of\ metal\ at\ contaminated\ site}$$

TF or mobilization ratio determines the ability of the plant to translocate metals from the roots to the aerial parts of the plant (**Marchiol et al, 2004**). It is represented by the ratio:

$$TF = \frac{\text{Concentration of metal in plant shoots}}{Concentration\ of\ metal\ in\ plant\ roots}$$

3.6 Biochemical parameters

3.6.1 Chlorophyll and carotenoid estimation

The amount of chlorophyll a and b were calculated by using the formulae described by **Maclachlan and Zalik (1963)** and carotenoids by **Duxbury and Yentsch (1956)**, respectively and their values were expressed on fresh weight basis. Total chlorophyll was calculated by adding the contents of chlorophyll a and b obtained from above mentioned formulae.

Method:

About 0.5 g of fresh leaf was crushed in 10 ml of 80% (v/v acetone/water) chilled acetone with the help of pestle mortar in dark. Then it was centrifuged at 5000 rpm at 10º C for 15 minutes. The supernatant was taken and optical density (O.D.) was measured at wavelength 663 nm, 645 nm, 510 nm, 480 nm, by spectrophotometer (*Model 2203, Double Beam Spectrophotometer, Systronics, India*). 80% acetone was used as blank.

Calculations:

$$\textbf{Chlorophyll a (mg g}^{-1}\textbf{ Fwt.)} = \frac{[12.3\ O\ D_{663} - 0.86\ \times O\ D_{645}] \times V}{d\ \times 1000\ \times W}$$

Chlorophyll b (mg g^{-1} Fwt.) = $$\frac{[19.3 \times O\,D_{645} - 3.6 \times O\,D_{663}] \times V}{d \times 1000 \times W}$$

Carotenoids (mg g^{-1} Fwt.) = $$\frac{[7.6 \times O\,D_{480} - 1.49 \times O\,D_{510}] \times V}{d \times 1000 \times W}$$

where, V= volume of the extract (ml)
d= length of the light path (cm)
W=dry weight of leaves (mg)

3.6.2 Carbohydrate content

Estimation of carbohydrate present in the samples were carried out using the Anthrone method (**Hedge, 1962**).

Reagents:

- 2.5 N HCl.
- **Anthrone Reagent**: Dissolved 200 mg anthrone in 100 ml of chilled ice cold 95% H_2SO_4. Freshly prepared before use.
- **Standard Glucose**: Stock – Dissolved 100 mg glucose in 100 ml of distilled water in a volumetric flask.
- **Working standard**: Diluted 10 ml of stock solution to 100 ml distilled water in a volumetric flask. Stored refrigerated after adding a few drops of toluene.

Procedure:

Taken 0.2 to 1ml of working standard solution in different test tubes and added distilled water to bring the volume to 1ml in each test tube. Then added 4 ml of anthrone reagent and after mixing the contents well the samples were boiled in a water bath for 10 minute. Cooled the test tube to the room temperature and measured the Ab. in spectrophotometer at 620 nm. A standard graph was plotted containing concentration on X-axis versus absorbance on the Y-axis. From the graph calculated the amount of carbohydrate present in the sample tube.

Calculations:

Carbohydrate present in 100 mg of the sample (mg/ml)

$$= \frac{\text{mg of glucose}}{\text{Volume of test sample}} * 100$$

3.6.3 Proline estimation

Proline content in fresh leaf samples were determined using the method described by **Bates et al (1973)**. Fresh leaf samples of control and treated plants (1 g) were homogenized in 10 ml of sulfosalicylic acid (3% w/v) and homogenate was centrifuged at 20000 × g for 5 min. After centrifugation, 2 ml of the supernatant was incubated at 100 ^{0}C for 60 min with 2 ml of ninhydrin reagent (prepared by dissolving 1.2 g of ninhydrin in 30 ml glacial acetic acid and 20 ml orthophosphoric acid) and then 2 ml of glacial acetic acid was added. After termination of the reaction in an ice bath, the reaction mixture was extracted with 4 ml of toluene and mixed vigorously using shaker for 15 - 20 min. and the absorbance of the chromophore-containing toluene was recorded at 520 nm. Standard curve was prepared with known concentrations of proline using the above methodology.

Proline content was calculated using the following formula:

Proline content (mg g^{-1} Fresh leaf) = $\frac{C*V}{115.5*W}$

Where, C is concentration of proline read from standard curve, V is volume of toluene (ml), W is weight of leaf sample (mg) and 115.5 is molecular weight of toluene.

3.6.4 Malondialdehyde (MDA) or lipid peroxidation (LPO) content estimation

LPO in both control and treated samples was determined by 2-thiobarbituric acid-malondialdehyde (TBA-MDA) adduct formation as described by **Heath and Packer (1968)**. For estimation of lipid peroxidation, fresh leaf tissues of both control and treated leaves were homogenized in 5% Tri chloro-acetic acid (TCA) agent. A volume of supernatant obtained was mixed with the same volume of thiobarbituric acid (TBARS) reagent and was then heated for 25 min at 95°C. The TBARS reagent consisted of 0.5% (w/v) thiobarbituric acid, prepared in 20% (w/v) trichloroacetic acid. Following a 15-min centrifugation at 3200 × *g*, an aliquot of the supernatant was taken and measured spectrophotometrically at 532 and 600 nm wavelengths (the latter for non-specific turbidity). The concentration of TBARS was calculated using a molar extinction coefficient of 156 mM^{-1} cm^{-1} and expressed as n moles/mg Protein equivalents.

3.6.5 Estimation of total phenol content

Total phenolic content was determined with Folin- Ciocalteu reagent using the method of **Bray and Thorpe (1954)**. For phenol determination, 100 mg fresh leaf samples were homogenized with 10 ml of 70% acetone and suspension was centrifuged at 6000 × g for 10 minutes. After centrifuging an aliquot of extracted solution, 1 ml of supernatant was added with 1 ml Folin- Ciocalteu reagent (1N), and allowed to stand for few minutes. Then 2.0 ml of saturated sodium carbonate (20% w/v) was added to the mixture and final volume was made up to 10 ml with distilled water. The mixture was boiled up to one minute and then cooled to room temperature. Absorbance of resulting solution was determined at 650 nm, using UV-VIS spectrophotometer (Model 2203, Double Beam Spectrophotometer, Systronics, India). Standard curve to estimate the concentration was prepared by using gallic acid.

Total phenolic content was calculated by following formula:

$$\textbf{Phenol content (GAE/Fresh wt.)} = \frac{C*V}{W*v*1000}$$

Where, C = = total phenolic content in mg/g, in GAE (Gallic acid equivalent), w = weight of leaf sample (mg), V = volume of extract (ml), v = volume of supernatant taken for analysis (ml).

3.6.6 Protein content and antioxidative enzyme activities

3.6.6.1 Preparation of plant extract

For the estimation of protein and antioxidative enzyme activities (*viz*., APOX, POD, SOD, GR, CAT), plant material (0.5 mg) was homogenized in mortar with pestle with 5.0 ml of 100 mM potassium phosphate buffer at pH 7.0 under ice cold conditions. The homogenate was centrifuged at 15,000 g for 20 minutes at 4ºC. Supernatant was used to measure the activities of various antioxidants.

3.6.6.2 Protein content estimation

Protein estimation was done by following the method of **Lowry et al (1951)**.

Reagents:

- Reagent A – 2.0 % sodium carbonate (Na_2CO_3) in 0.1 N sodium hydroxide (NaOH)
- Reagent B – 0.5% copper sulphate in 1.0 % potassium sodium tartarate

- Reagent C – 50 ml of reagent A and 1.0 ml of reagent B (prepared prior to use)
- Reagent D – 50 ml of 1N (normal) Folin-Ciocateau (FC) reagent in 50 ml of distilled water.
- Protein solution (stock standard) – 50 mg of bovine serum albumin (BSA) dissolved in distilled water and the final volume was made to 50 ml. Protein working standard solution was prepared by diluting the standard stock solution.

Procedure:

Fresh leaves (100 mg) of the control and treated plants were homogenized separately in 3 ml of 10% chilled trichloroacetic acid (TCA) in mortar with pestle and centrifuged at 5000 rpm for 15 min. After decanting the supernatants, the pellets were washed and heated for 7 min with 3 ml of 0.1N NaOH (sodium hydroxide), cooled and centrifuged again at 5,000 rpm for 15 min. Then, 0.5 ml of supernatant was taken in clean and dry test tube and 5 ml of reagent C was added and left for 10 min. After 10 min. 0.5 ml of reagent D was added rapidly, the solution turns blue in colour. The O.D. was taken at 700 nm by spectrophotometer.

Calculations:

A graph of absorbance vs. concentration for standard solutions of proteins was plotted and the amount of protein in the samples was calculated from the graph. The amount of proteins was expressed as mg/g FW (fresh weight).

3.6.6.3 Superoxide dismutase (SOD) (EC. 1.15.1.1)

The activity of superoxide dismutase (SOD, EC 1.15.1.1) was assayed according to the method of **Beauchamp and Fridovich (1971)** by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium chloride (NBT) at 560 nm.

Reagents:

- Polyvinylpolypyrrolidone (PVPP)
- Riboflavin
- L-methionine
- NaH_2PO_4 & Na_2HPO_2
- EDTA.Na_2
- Nitro blue tetrazolium chloride (NBT)

- Liquid nitrogen
- **Extraction Buffer** - 50 mM sodium phosphate buffer (pH 7.8) 1 mM EDTA•Na_2 2% (w/v) (PVPP).

Procedure:

The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 0.2 mM methionine, 1 mM NBT, 0.2 mM riboflavin and 0.1 ml enzyme extract. SOD activity was measured at absorbance 560 nm and expressed as Unit/g Fresh wt. and was defined as the amount of enzyme required to cause 50% inhibition in the rate of NBT photo-reduction.

Calculations:

% Inhibition of NBT reduction by SOD = $\frac{\text{Control OD} - \text{Treatment OD}}{\text{Control OD}} * 100$

= X% inhibition.

50% inhibition is equal to 1 unit of enzyme.

Then, X% is equal to 1/50 x X= Y unit.

3.6.6.4 Catalase (CAT) (EC. 1.11.1.6)

The catalase (CAT; EC 1.11.1.6) activity was determined according to the method of **Aebi (1984)**.

Reagents:

- Phosphate buffer – 100 mM, pH 7.0
- Hydrogen peroxide – 150 mM

Procedure:

The rate of decomposition of H_2O_2 was followed by decrease in absorbance at 240 nm in a reaction mixture containing 1.5 ml phosphate buffer, 1.2 ml of hydrogen peroxide and 300 µl of enzyme extract.

Calculations:

Unit Activity (Units/min/g FW) = $\frac{Change\ in\ abs\ /\ minute * Total\ Volume\ (ml)}{Ext.\ Coeffecient * Volume\ of\ sample\ taken\ (ml)}$

Where, Extinction coefficient = 6.93 * 10^{-3} $mM^{-1}cm^{-1}$

Specific Activity (mol UA/mg protein) = $\frac{Unit\ Activity\ (Units/min/\ g\ FW)}{Protein\ Content\ (mg\ /\ g\ FW)}$

3.6.6.5 Guaiacol peroxidase (POD) (EC. 1.11.1.7)

Guaiacol peroxidase (GPX, EC 1.11.1.7) activity was assayed according to the method given by **Putter (1974)**.

Reagents:

- Guaiacol solution – 20 mM
- H_2O_2 solution – 12.3 mM
- Phosphate buffer – 0.1 M, pH 7.0

Procedure:

The reaction mixture comprised of 3.0 ml phosphate buffer, 50 µl guaiacol solution, 100 µl enzyme sample and 30 µl H_2O_2 solution was taken. The rate of formation of guaiacol dehydrogenation product (GDHP) was monitored spectrophotometrically at 436 nm.

Calculations:

Unit Activity (Units/min/g FW) = $\frac{Change\ in\ abs\ /\ minute * Total\ Volume\ (ml)}{Ext.\ Coeffecient * Volume\ of\ sample\ taken\ (ml)}$

Where, Extinction coefficient = 25 $mM^{-1}cm^{-1}$

Specific Activity (mol UA/mg protein) = $\frac{Unit\ Activity\ (Units/min/\ g\ FW)}{Protein\ Content\ (mg\ /\ g\ FW)}$

3.6.6.6 Ascorbate peroxidase (APOX) (EC. 1.11.1.11)

Ascorbate peroxidase activity was estimated according to the method of **Nakano and Asada (1981)**.

Reagents:

- Ascorbate – 5.0 mM
- Hydrogen peroxide – 0.5 mM
- Phosphate buffer – 100 mM, pH 7.0

Procedure:

Three (3.0) ml of the reaction mixture containing 1.5 ml phosphate buffer, 300 µl ascorbate, 600 µl H_2O_2 and 600 µl enzyme extract was taken and the decrease in absorbance was recorded at 290 nm.

Calculations:

Unit Activity (Units/min/g FW) = $\frac{Change\ in\ abs\ per/\ minute * Total\ Volume\ (ml)}{Ext.\ Coeffecient * Volume\ of\ sample\ taken\ (ml)}$

Where, Extinction coefficient = 2.8 $mM^{-1}cm^{-1}$

Specific Activity (mol UA/mg protein) = $\frac{Unit\ Activity\ (Units/min/\ g\ FW)}{Protein\ Content\ (mg\ /\ g\ FW)}$

3.6.6.7 Glutathione reductase (GR) (EC. 1.6.4.2)

Glutathione reductase (GR, EC. 1.6.4.2) activity was determined according to the method given by **Carlberg and Mannervik (1975)**.

Reagents Required:

- Ethylene diamine tetraacetic acid disodium salt (Na-EDTA) - 3.0 mM
- Phosphate buffer – 50 mM, pH 7.6
- Nicotinamide adenine dinucleotide phosphate (NADPH) - 0.1mM
- GSSG (Oxidized glutathione) - 1.0 mM

Procedure:

GR activity was determined by measuring the oxidation of NADPH at 340 nm in a reaction mixture containing 1.8ml phosphate buffer, 300 µl each of EDTA, NADPH, oxidized glutathione (GSSG) and enzyme extract. The decrease in absorbance per minute was recorded at 340 nm.

Calculations:

Unit Activity (Units/min/g FW) = $\frac{Change\ in\ abs\ per/\ minute * Total\ Volume\ (ml)}{Ext.\ Coeffecient * Volume\ of\ sample\ taken\ (ml)}$

Where, Extinction coefficient = 6.22 $mM^{-1}cm^{-1}$

Specific Activity (mol UA/mg protein) = $\frac{Unit\ Activity\ (Units/min/\ g\ FW)}{Protein\ Content\ (mg\ /\ g\ FW)}$

3.7 Scanning electron microscopy coupled with energy-dispersive X-ray microanalysis (SEM-EDX)

Root and shoot specimens were prepared for SEM study using the protocol adapted from standard procedures (**O'Brien & McCully, 1981**). In order to determine the cellular differentiation of plant tissues treated with Hg, Cd and Butachlor (BC) alone and in combination, along with control, fresh root, shoot and leave samples (5 mm square from similar middle portion) were dissected and immediately fixed in a solution of 2.5 % gluteraldehyde at 4ºC temperature. The specimens were washed three times in 0.1 M sodium phosphate buffer (pH 6.8) and kept overnight at 4 °C and then dehydrated in absolute acetone using 30 minutes series with 30, 50,70, 95 and

100% acetone and then stored at 4°C until examination. The specimens were rinsed, post-fixed in 2% osmium tetraoxide, critical point dried and sputter coated with aluminum stubs with double-sided carbon tape. The specimens were viewed and photographed using Scanning electron microscope (*SEM, Model: JSM-6490LV, Make: JEOL, Japan*).

3.8 Statistical analysis

All data were analyzed by one-way as well as two-way ANOVA to test the individual and combined treatment of Hg, Cd and BC. Significantly different means were calculated using the 'Duncan and Tukey Post Hoc Test' to determine the significance of differences among treatments at P-values ≤ 0.05, ≤ 0.01, ≤ 0.001.The entire statistical tests were performed using SPSS software (SPSS Inc., version 25.00).

Chapter 4

Soil analysis of agricultural lands in and around Lucknow to ascertain the status of heavy metals & herbicides in contaminated soils

4.1 Introduction

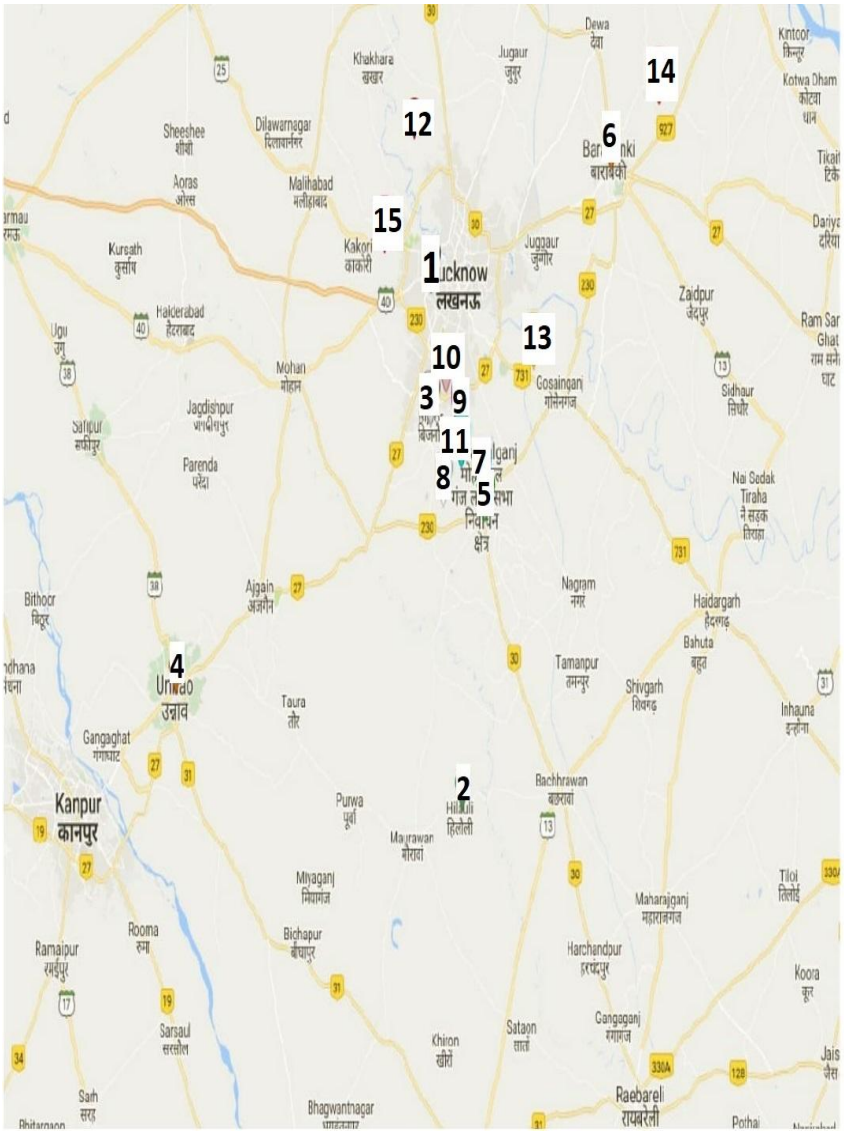
The agricultural land close to urban areas witness various processes like traffic emissions, energy and fuel production, power-transmission, mining, refining of metals, intensive agriculture and dumping of sludge which contributes to contamination of soil **(Nouri et al, 2009; Samarghandi et al, 2007**). Wastewater irrigation practices leads to the accumulation of heavy metals like Nickel (Ni), Copper (Cu), Zinc (Zn), Cadmium (Cd), Iron (Fe) and Lead (Pb) in the soil and chronic exposure to heavy metals can lead to their accumulation in plant parts affecting plant growth (**Sharma et al, 2006**; **Qu et al, 2012**; **Tsai & Lee, 2013**), and ground cover apart from having a negative impact on soil micro flora. Since heavy metals cannot be degraded chemically their physical removal is needed for transformation into nontoxic compounds.

Pesticides and agrochemicals happen to be important inputs for improved yield and better quality crop production. However, an unplanned, erroneous and indiscriminate use of these organic compounds leads to the destruction of bio-control agents, pesticide residues in agro-ecosystems, and environmental pollution, besides accumulating in the food chain (**Khan et al, 2010**).

Agricultural lands may be co-contaminated with both organic and inorganic substances, but limited work has been carried out for cleanup and monitoring of such co-contaminated lands. While studying such sites, it becomes important to consider the interactions of both organic and inorganic substances with respect to those that may affect both the form as well as availability of pollutants. Different metal concentrations have been shown to inhibit the microbial biodegradation of organics (**Sandrin & Maier, 2003**; **Thavamani et al, 2015**) and their further interaction with organic pollutants affects metal bioavailability (**Gao et al, 2006**).

In the present study, 15 representative agricultural soil samples from areas in and around Lucknow city, the capital of Uttar Pradesh, India was collected to assess the co-contamination of heavy metals and herbicide. Overall, the aim of the study was to assess the physico- chemical and microbial properties of soil which are co-contaminated with inorganic (heavy metals) and organic compounds (herbicides) and analysis of correlation matrix.

4.2 Materials and Methods



1	Aishbagh
2	Mohani
3	Bijnor
4	Unnao
5	Mohanlalganj
6	Barabanki
7	Mau
8	Diwanganj
9	Kalli West
10	Control
12	Kamlapur
13	Semarou
11	Kalli East
14	Suklai
15	Bhawaniganj

*Map drawn with the help of multiplottr (https://multiplottr.com/)

Figure 4.1: Study sites in and around Lucknow (U.P.)

4.2.1 Study area

Lucknow, a large city in northern India, is the capital of the state of Uttar Pradesh. It is situated between 23° 52' N and 31° 28' N latitudes and 77° 3' and 84° 39'E longitudes (**IMD, nidm.gov.in/pdf/dp/Uttar.pdf**). The study area includes agricultural lands situated in and around Lucknow city. **Figure 4.1** shows the location of area from where the soil samples were collected. Industries like brick Kiln, leather tanning and electroplating are situated close to the study area.

4.2.2 Sampling

For the present study, a total of 45 top soils (0-15 cm), samples were collected from fifteen (15) different agricultural lands (**Figure 1**) through Z- pattern of sampling. 300g portion of each soil sample was taken in a 50 m diameter area and a composite sample was formed. The samples were collected in a zip mouthed polyethylene bags with the help of an auger. The collected samples were properly labeled and immediately transferred to laboratory, where they were stored at 4˚C prior to analysis (**Sun et al, 2015**).

All the samples were treated and analyzed at the laboratory of Department of Environmental Science, Babasaheb Bhimrao Ambedkar University, Lucknow. To get the reference data soil samples were collected from the remotely placed agricultural soil, where no herbicide were used previously (Control Soil). These soil samples were also processed similarly to the test samples from the fields.

4.2.3 Soil analysis

The soil samples were air dried, pulverized and sieved (2 mm sieve) prior to analysis. Analytical grade reagents were used throughout the study.

4.2.4 Determination of physico-chemical parameters

The, pH, Electrical conductivity (EC), Moisture content (MC) and Organic Carbon (OC) content of the soil samples were determined following standard protocols. Detail methodology is given in Chapter 3 under Materials and Methods (Section 3.3.1 to 3.3.5).

Available nitrogen (N) was estimated by the methods proposed by **Subbiah and Asija** (**1956**). Potassium (K) was measured by using a Flame photometer (Systronics-130) after digesting the samples in a di-acid mixture ($HClO_4/HNO_3$ in a 4:1 ratio) Black (1965), while available phosphorus (P) was determined by sodium bicarbonate extraction method using spectrophotometer (*Thermo Scientific, Evolution 201*) (**Olsen et al, 1954**). Detail methodology is given in Chapter 3 Materials and Methods (Section 3.3.6 -3.3.8).

4.2.5 Heavy metal analysis

For heavy metal **(**Pb, Hg, Cu, Cr, Ni, As, Cd, Mn, Fe) determination 1g of oven dried soil samples were taken in 250 ml conical flasks and digested in HNO_3: $HClO_4$ (3:1)

at 70 to 80 °C (Odu *et al*. 1986). The digested samples were filtered and diluted with double distilled water up to 50 mL and analyzed for the metals Lead (Pb), Mercury (Hg), Copper (Cu), Chromium (Cr), Nickel (Ni), Arsenic (As), Cadmium (Cd), Manganese (Mn) and Iron (Fe) using Varian Spectra AA-250 plus Atomic Absorption Spectrophotometer (AAS) as described by **Odu et al,** (**1986**). Detail methodology is given in Chapter 3 under Materials and Methods (Section 3.4).

4.2.6 Soil bacteria

A spread-plate technique was used to estimate the soil bacterial population (**Martin, 1950**). Ten grams of each soil sample were added to 95 mL of deionized water. After homogenization for 30 min, this solution was serial diluted (10^{-1} to 10^{-7}) and aliquots of the resulting solutions plated on nutrient agar. Each dilution was spread onto five replicate. After incubation at 28 °C, for 24-72 h, the colony forming units (CFU) were counted. Colony forming units per g of soil (cfu/g) was calculated using the equation of **Johnson and Case** (**2007**).

4.2.7 Herbicide estimation in soil through HPLC

4.2.7.1 Soil extraction process

All the soil samples were air-dried at room temperature, pulverized, and passed through 100 µm sieves. The extraction procedures were as follows: 1.0 g of the soil sample was accurately weighed and extracted in a Soxhlet apparatus using 100 mL n-hexane for 8h, followed by rotatory-evaporation and concentration of the extract up to dryness (1-2 mL). The concentrated soil extract was cleaned up using 1 or 2 chromatographic columns (10 mm, diameter) packed with Na_2SO_4, Florisil and silica gel (from bottom to top) to remove the interferences. The target analyte was recovered in 100 mL of n-hexane. The final extract was concentrated, exchanged into hexane, and reduced to 1.0 mL. Before injection into HPLC – UV-Visible detector, butachlor was added as the internal standard.

4.2.7.2 HPLC and Chromatographic conditions

The HPLC system used for analyzing butachlor was model 2489 UV/Visible detector (Waters, USA). Analytical column used was C 18 column. The HPLC mobile phase was composed of methanol:water (72:28 v/v). Flow rate of the mobile phase was 1.0 mL/min. The wavelength of the detector was set at 248 nm. A 20 µL volume of

standard and sample was injected into the chromatographic column. The retention time was 9.9 min (**Yang et al, 2013**).

4.3 Results & Discussion

4.3.1 Physico-chemical properties of soil

Average values of physico-chemical properties of soil samples are given in **Tables 4.1** and **4.2**, respectively. The studied soil samples were found to vary between sandy loam to loam, which were in agreement to the texture observed in the regions of the studied area by **Upadhyay and Sharma (2016)**. The pH of the soil samples ranged from 6.76 to 9.03, more towards neutral to alkaline in nature. Soil electrical conductivity, an indicator of salinity of soil **(Solanki & Chavda, 2012)** was found to vary from 60 to 285.2µS/cm. The average moisture content of soil samples ranged from 9.16 to 33.52%. The highest moisture content (33.52%) was observed in Diwanganj soil and the lowest moisture content was observed in Kamlapur (9.16%).

Table 4.1 Physical characteristic of soil samples (Mean ± S.D.)

Soil Sampling site			Parameters			
	Sand (%)	Silt (%)	Clay (%)	pH	EC (µS/cm)	MC (%)
Control	35.2±1.31	21.3±1.41	43.5±2.01	7.56±0.36	80.21±3.11	20.32±0.17
Barabanki	41.9±2.14	22.8±1.42	35.3±2.15	8.7±0.163	119.63±2.73	22.46±0.58
Suklai	38.7±1.3	23.2±2.15	38.1±2.03	8.466±.047	167.93±3.41	32.23±2.45
Mohanlalganj	35.5±1.61	22.4±0.71	42.1±1.52	8.4±0.00	202.33±0.47	24.32±0.55
Bijnor	42.3±2.31	23.1±1.34	34.6±0.51	8.066±.047	123.23±5.06	17.56±0.37
Mohani	41.4±0.29	22.8±1.05	35.8±0.07	8.366±.047	118.86±4.81	14.08±0.15
Kamlapur	39.8±2.17	23.4±0.045	36.8±1.04	7.86±.047	109.86±2.14	9.16±0.12
Unnao	42.4±0.46	23.1±0.071	34.5±1.71	8.73±.094	132.06±2.62	19.20±0.68
Kalli East	36.4±1.63	24.9±2.14	38.7±3.16	6.76±.047	119.39±0.40	22.34±0.04
Kalli West	39.7±0.78	21.78±0.06	38.52±2.14	7.26±.094	60.00±1.29	17.75±0.02
Semarou	43.1±1.23	22.8±2.52	34.1±1.17	7.83±.047	163.94±1.85	22.04±0.04
Mau	36.5±1.04	24.2±0.061	39.3±0.62	8.26±.047	139.32±1.74	18.92±0.54
Diwanganj	37.3±0.05	23.2±1.26	39.5±0.39	9.03±.047	129.48±1.69	33.52±0.61
Bhawaniganj	38.6±0.48	21.2±2.11	40.2±1.28	8.76±.047	271.18±2.73	25.03±0.23
Aishbagh	35.3±0.73	22.7±0.042	42±2.10	7.33±.124	285.2±0.73	31.24±0.11
Soil Range	**35.2-43.1**	**21.2-24.9**	**34.1-43.5**	**6.76-9.03**	**60-285.2**	**9.16-33.52**
Soil Average	**38.94**	**22.85**	**38.20**	**8.09**	**142.84**	**17.41**

Table 4.2 Chemical characteristics of soil samples (Mean ± S.D.)

Soil Sampling Site	Parameters				
	OC %	OM (%)	N(Kg/ha)	P (kg/ha)	K(kg/ha)
Control	2.12±0.41	3.65±0.13	58.23±4.41	6.53±0.72	96.41±0.18
Barabanki	6.52±0.81	11.25±0.26	71.39±3.38	10.86±0.15	89.6±0.00
Suklai	5.95±1.32	10.25±0.05	81.53±5.43	16.06±0.22	306.13±0.57
Mohanlalganj	6.62±0.65	11.41±0.05	187.11±4.79	15.97±0.33	597.33±1.15
Bijnor	6.75±0.05	11.63±0.50	317.78±4.79	33.34±0.47	769.06±1.52
Mohani	6.73±0.89	11.61±0.59	127.53±3.62	11±.08	365.86±0.577
Kamlapur	7.19±0.43	12.39±0.26	251.92±1.81	10.84±0.07	548.8±1.00
Unnao	6.07±0.05	10.47±0.19	291.64±5.43	16.38±0.07	291.2±1.0
Kalli East	4.22±0.05	7.28±0.06	128.57±3.13	31.31±0.21	282.98±2.51
Kalli West	3.21±0.11	5.53±0.16	97.21±5.43	29.05±.039	477.86±0.57
Semarou	3.64±0.11	6.28±0.23	163.07±6.27	11.25±0.15	160.90±0.57
Mau	3.40±0.05	5.87±0.16	102.44±1.81	22.23±0.11	481.6±2.00
Diwanganj	3.22±0.1	5.56±0.19	91.98±1.81	18.95±0.10	162.0 ±2.30
Bhawaniganj	3.64±0.11	6.27±0.08	154.70±3.62	10.7±.15	117.973±2.08
Aishbagh	4.25±0.20	7.33±0.17	486.08±3.13	37.3±0.35	272.906±2.51
Soil Range	**2.12-7.19**	**3.65-12.39**	**58.23-486.08**	**6.53-37.3**	**89.6-769.06**
Soil Average	**4.90**	**8.45**	**174.07**	**18.78**	**431.90**

Organic Carbon (%) content of the soils varied from 2.12 to 7.19% (**Table 4.2**). The Kamlapur site soil was found to contain highest amount of organic carbon *i.e.*, 7.19 % and the lowest organic carbon was observed in Control soil (2.12 %). Soil Organic Matter (%) was found to vary from 3.65 to 12.39% which was directly related to the content of organic carbon in soil samples studied. The high organic carbon content results in plants taking up nutrients more easily and it leads to the change in the pH which improves soil condition for crop growth (**Aydinalp & Marinova, 2003**).

N, P and K are micronutrients essential for the plant growth. The available Nitrogen content varied from 58.23 kg/ha in Control soil to highest in Aishbagh Soil *i.e.*, 486.08 kg/ha (**Table 4.2**). The available Nitrogen content of most of the soil samples was high. The average amount of phosphorus in soil ranged from 6.53 kg/ha to 37.3 kg/ha in different agricultural sites. On the basis of the limits suggested by **Muhr et al (1965)**, most of the soil samples (94%) were low (< 20P_2O_5 Kg/ha) in available phosphorus status and rest were under medium (20-50 P_2O_5 kg/ha) category.

Potassium is an important nutrient for paddy crop which is mostly grown in the fields of Uttar Pradesh. The available potassium content in the soil samples were found varying from 89.6 kg/ha in Barabanki soil to 769 kg/ha in Bijnor kg/ha (**Table 4.2**) which was highest amongst all the sites. Most of the soil sample (96%) were in medium range for Potassium *i.e.,* 125-300 kg/ha (**Muhr et al, 1965**).

4.3.1.1 Correlation matrix among different physico-chemical properties

The relationships between different physico-chemical parameters were analyzed by Pearson's correlation coefficient (**Table 4.3**). The high correlation coefficient (near +1 or -1) means a good relation between two variables, and its concentration around 0 means no relationship between them at a significant level of 0.05% (level), it can be strongly correlated , if r >0.7, where r values lie between 0.5 to 0.7, shows moderate correlation.

Table 4.3 Correlation matrix between physico-chemical properties of soil

	pH	**EC (µS)**	**MC (%)**	**OC (%)**	**OM (%)**	**N (Kg/ha)**	**P (kg/ha)**	**K (kg/ha)**	**Sand (%)**	**Silt (%)**
EC (µS)	NS	1								
MC (%)	0.21	0.53*	1							
OC (%)	0.29	NS	-0.33	1**						
OM (%)	0.29	NS	-0.33	0.99**	1					
N(Kg/ha)	NS	0.51	0.00	0.29	0.29	1				
P (kg/ha)	-0.50	0.13	0.20	NS	NS	0.52*	1			
K(kg/ha)	0.31	-0.16	0.23	NS	NS	NS	NS	1		
Sand (%)	0.29	-0.27	-0.41	0.45	0.45	NS	NS	NS	1	
Silt (%)	NS	NS	NS	0.25	0.25	NS	0.37	NS	NS	1
Clay (%)	NS	0.316	0.40	-0.51	-0.51	NS	NS	NS	-0.94**	-0.32
**** Correlation is significant at the 0.01 level**										
***Correlation is significant at the 0.05 level.**										

Various notable significant correlations among different physico-chemical parameters have been summarized in **Table 4.3**. The pH established non-significant correlation with all parameters while showing negative correlation with P (r = -0.5). EC shows positive relationship with moisture content (r=0.53. p>0.05) while rest of the parameters did not reveal any significant relationship. MC have no significant relation with any other physico-chemical parameters.

Results (**Table 4.3**) revealed that OC showed positive correlation with organic matter (r = 0.99, p>0.01) while it showed negative correlation with clay particles (r=-0.51) and with rest of the parameters no significant correlation was observed.

Nitrogen showed positive correlation with phosphorus (r=0.52, p>0.05), similar results was reported by **Ray and Mukhopadhyay** (**2012**). Nitrogen established non-significant correlation with rest of the parameters. Relationships of P with all other physico-chemical properties were statistically not significant. Phosphorus showed moderate relationship with Nitrogen. Sand showed negative correlation with clay (r = -0.94, p<0.01), similar results for sand was observed by **Kumar et al** (**2011**) while no significant relationship was observed with rest of the parameters.

4.3.2 Soil bacteria count

The quantification of the number of soil bacteria reveals an indication of soil health *e.g.*, if there are 10^6 to 10^8 culturable bacteria present per gram of soil, this would be considered a healthy number. The soil samples from different agricultural sites were serially diluted and spread on nutrient agar plates. Results (**Figure 4.2**) revealed highest bacterial population in Control Soil (122 CFU/g $X10^5$) while lowest population was recorded in Diwanganj soil (13.7 CFU/g $X10^5$). A number less than

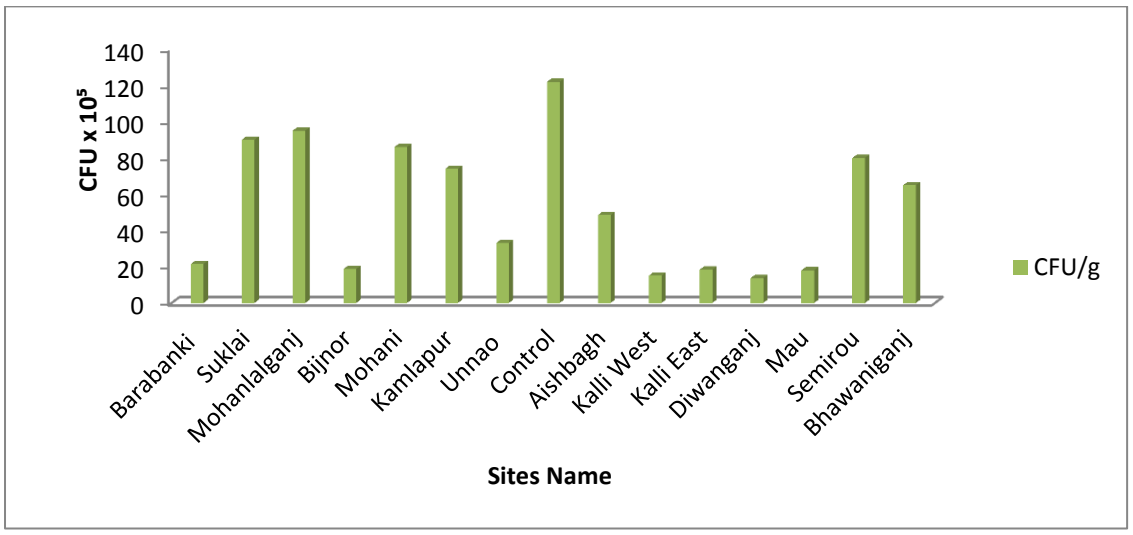


Figure 4.2 Soil bacteria count (CFU/g)

10^6 per gram indicates poorer soil health, which may be due to a lack of nutrients as found in low organic matter soils; abiotic stress imposed by extreme soil pH values

(pH < 5 or > 8); or toxicity imposed by organic or inorganic anthropogenic contaminants. Microbial analysis indicates that the high concentration of heavy metal and herbicide in the soil samples significantly affected the diversity of microbial community.

4.3.3 Distribution of heavy metals

Soils have a natural ability to hold on to metals. Acidification in the soil makes some metals to bind less tightly with soil particles, except mercury. Metals freed in this way become available to plants to which they may be or not be toxic. The concentration of heavy metals in soil at different sampling sites is presented in **Table 4.4**.

Table 4.4: Distribution of heavy metals and metal content at different sites

	mg/kg								
Sites Name	**Pb**	**Cr**	**Hg**	**Fe**	**Cu**	**Ni**	**Cd**	**As**	**Mn**
Control	6.28	5.17	0.02	1500	0.57	0.01	5.51	0.00	101.31
Barabanki	59	194.05	8.3	4600	0.58	0.86	45.92	1.6	307.82
Suklai	36.75	78.22	7.1	5745	1.39	1.51	38.1	2.45	405.35
Mohanlalganj	46.5	47.5	7.5	4345	2.17	1.42	61.76	2.6	343.82
Bijnor	49	50.5	20	5355	1.58	1.38	20.98	1.69	325.35
Mohani	28.5	72.7	ND	6450	0.59	0.61	55.73	2.79	213.22
Kamlapur	1.25	213.42	10	2050	1.46	2.20	39.09	3.61	348.9
Unnao	24	188.67	ND	3762.5	0.75	0.87	40.85	3.75	307.82
Kalli East	48	8.24	14.4	2359.5	1.63	26.34	22.85	ND	458.4
Kalli West	20	1.4	24	1643.46	0.78	25.15	58.15	ND	243.36
Semarou	28.4	3.72	6.8	1881.75	0.93	21.57	61.95	ND	403.6
Mau	21.2	2.48	12.0	2042.4	1.09	12.82	52.18	ND	289.6
Diwanganj	19.2	5.24	5.2	1519.05	0.90	17.05	10.18	ND	332
Bhawaniganj	18.8	ND	19.2	2129.4	0.84	15.67	37.28	ND	289.2
Aishbagh	57.2	13.72	10.0	1977.36	5.00	19.32	17.39	ND	413.2
Soil range	**1.25-59**	**0.0-213.42**	**0.0-24**	**1500-6450.0**	**0.57-5.00**	**0.01-26.34**	**5.51-61.76**	**0.0-3.75**	**101.31-458.4**
Soil Average	**30.93**	**59.0**	**9.63**	**3157.36**	**1.35**	**9.78**	**37.86**	**1.23**	**318.86**

Lead (Pb) levels in the collected soil samples were in the range of 1.25 mg/kg to 59 mg/kg. In all the collected soil samples concentration of Pb was recorded within the permissible limits set by **WHO** (**1992**).

Chromium (Cr) levels in the study sites ranged from 1.4 mg/kg to 213.42 mg/kg with an average of 59 mg/kg. Concentration of Cr in some agricultural sites

(Barabanki, Kamlapur, Unnao) were found higher than the permissible level which is 50 mg/kg for soil as recommended for agriculture by **MAFF** (**1992**) and **EC** (**1986**).

The Hg concentration in the studied soil samples was between 0 mg/kg (Control soil, Mohani and Unnao) to 24 mg/kg (Kalliwest). The Hg concentration in most of the soil samples (all soil samples except Control soil, Mohani and Unnao) was above the permissible limit (0.3-5 mg/kg) (**Table 4.5**).

Iron (Fe) had the highest mean concentration among all the metals studied in and around Lucknow areas and its level ranged from 1500 mg/kg to 6450 mg/kg and mean was 3157.36 mg/kg, while concentration of Cu in the soil samples (**Table 4.5**) studied was within the range *i.e.*, 0.57 mg/kg to 5.00 mg/kg as given by **WHO** (**2007**).

Table 4.5 Mean values of heavy metals (Pb, Cd, Cu, Cr, Mn, Ni, As, Hg and Fe) for paddy soils, worldwide normal surface soils, critical concentrations for contaminated soils, Indian standards, European Union standards, MEF compared with the values of present study.

Elements	Mean values for paddy soils [a] (mg/kg)	Mean values for worldwide normal surface soil [b] (mg/kg)	Critical soil concentration[c] (mg/kg)	Indian standard [d] (mg/kg)	European union standard [e] (EU 2002) (mg/kg)	Present study (mg/kg)
Pb	23.3	22–44	100–400	250–500	300	32.95
Cd	0.34	0.37–0.78	3–8	3–6	3	**38.89**
Cu	20.7	13–24	60–125	135–270	140	1.37
Cr	64	12–83	75–100	-	150	72.06
Mn	-	<1800	1500-3000	-	-	327.44
Ni	-	100*	150*	75-150	75	9.84
As	-	50*	100	-	20	2.68
Hg	-	0.005-0.5*	0.3-5*	-	-	**9.63**
Fe	-	-	-	75-150	-	3276.02

[a]Data from **Wong et al** (**2002**), **Wang et al** (**2003**); **Chandrajith et al** (**2005**).
[b] **Kabata-Pendias** (**1995**); **Essington** (2004).
[c] Data are from (**Alloway, 1990**).
[d]Indian standards (**Awashthi, 2000**) for agricultural soils.
[e]European standards (**EU, 2002**) for agricultural soils.
***MEF** (**2007**)

Concentration of Ni in soil sample ranged between 0.01 mg/kg to 26.34 mg/kg and was within permissible limits *i.e.*, 75-150 mg/kg (**WHO, 1992**; **Awasthi, 2000**) (**Table 4.5**). Cadmium (Cd) level in all the soil samples was found between 5.51

mg/kg to 61.76 mg/kg which was greater than the permissible limits (0.01-3.0 mg/kg) as observed by **MAFF** (**1992**) and **EC** (**1986**). Cd level in soil might be due to application of high dose of phosphate fertilizers and metal based pesticide in agricultural crops (**Singh & Kumar, 2006**).

Arsenic (As) is the common trace metals in super phosphate and rock phosphate. In most of the soil samples As was not detectable. However, in some of the soil samples where As was detectable, the level was within the permissible limits (**WHO, 1992**; **Awasthi, 2000**) (**Table 4.5**). The As level in the study sites was between 0.0 mg/kg to 3.75 mg/kg and mean was 1.23 mg/kg.

Sources of Manganese (Mn) are rocks and fertilizers. Mn levels around the study area ranged between 101.31 mg/kg to 458.4 mg/kg, with an average of 318.86 mg/kg which falls within the suggested range (170 mg/kg to 1200 mg/kg) as given by **Wang** (**1995**).

4.3.3.1 Relationship among different heavy metals

Pb showed positive correlation with Mn ($r=0.51$, $p>0.05$) and non-significant relationship with all heavy metals. Ni showed negative correlation with Cr ($r=-0.62$, $p<0.05$) and strong negative correlation with As ($r= -0.75$, $p<0.01$) and Fe ($r= -0.64$, $p<0.01$) while Fe showed significant correlation with Hg ($r = 0.50$) while with rest of

Table 4.6 Correlation among heavy metals

	Pb	**Hg**	**Cd**	**Cu**	**Ni**	**As**	**Cr**	**Fe**
Hg	0.149296	1						
Cd	0.023	0.087	1					
Cu	0.504	0.126	-0.24	1				
Ni	0.047	0.508	-0.009	0.225	1			
As	-0.027	-0.384	0.292	-0.113	-0.75**	1		
Cr	-0.0004	-0.314	0.169	-0.165	-0.62*	0.82**	1	
Fe	0.452	-0.215	0.269	-0.105	-0.64**	0.64**	0.388	1
Mn	0.51*	0.218	0.055	0.506	0.392	0.023	0.044	0.023
**** Correlation is significant at the 0.01 level**								
***Correlation is significant at the 0.05 level.**								

the heavy metals there was non-significant relationship. The similar results were also obtained for Ni by **Tripathi and Mishra** (**2012**). Hg showed negative correlation

with As (r = -0.38) and non- significant relationship with rest of the heavy metals (**Table 4.6**).

Cr shows strong positive correlation with As (r=0.82, p>0.01) and negative correlation with Ni (r=-0.6, p>0.05). Cd, and Cu displayed non-significant relationship with rest of the metals. As showed strong positive relationship with Cr (r=0.82, p>0.01) and positive relationship with Fe (r=0.64, p>0.05) while rest of the parameters showed no relationship with As.

4.3.3.2 Relationship among different physico-chemical parameters and heavy metals

Pb showed significant relationship with EC (r=0.42) and Silt (r=0.26), same results was observed by **Aysen** (**2013**). Hg showed positive correlation with phosphorus (r = 0.55, p >0.05) and negative correlation with pH (r = -0.32) and non-significant relationship with rest of the parameters (**Table 4.7**).

Cd was found to show non-significant statistical relationship with the soil parameters studied. Cu showed positive correlation with EC (r = 0.61, p>0.05) and N (r= 0.77, p>0.01), similar results were observed by **Mushtaq and Khan** (**2010**). Cu also showed positive correlation with P (r = 0.62, p>0.05) and negative correlation with Sand (r = -0.45). Ni shows strong negative correlation between MC (r = -0.52, p<0.05), OC and OM (r = -0.63, p<0.05).

Table 4.7 Correlation among Heavy metals and different physico-chemical parameters of soil.

	pH	EC	MC	OC	OM	N	P	K	Sand	Silt	Clay
Pb	-0.09	0.42	-0.01	0.33	0.34	0.32	0.50	-0.13	0.02	0.26	-0.10
Hg	-0.32	0.17	-0.29	-0.13	-0.13	0.09	0.55*	0.04	0.00	-0.03	0.00
Cd	0.14	0.11	-0.19	0.30	0.30	-0.19	-0.24	-0.27	0.40	-0.01	-0.37
Cu	-0.37	0.61*	-0.20	0.06	0.06	0.77**	0.62*	-0.00	-0.45	0.12	0.38
Ni	-0.49	0.21	-0.52*	-0.63*	-0.63*	0.01	0.49	0.08	-0.21	0.15	0.15
As	0.37	-0.05	0.24	0.87**	0.87**	0.19	-0.30	-0.01	0.41	0.13	-0.43
Cr	0.32	-0.13	0.11	0.74**	0.75**	0.14	-0.34	-0.13	0.49	0.16	-0.51*
Fe	0.36	0.05	0.39	0.77**	0.77**	0.00	-0.09	-0.09	0.41	0.11	-0.42
Mn	-0.15	0.56*	-0.08	0.24	0.24	0.37	0.44	0.09	0.02	0.61*	-0.22
** Correlation is significant at the 0.01 level.											
*Correlation is significant at the 0.05 level.											

As shows strong positive correlation with OC and OM (r=0.87, p>0.01). Cr shows strong positive correlation with OC (r = 0.74, p>0.01) and OM (r = 0.75, p>0.01) and negative correlation with Clay (r = -0.51, p<0.05). Similar correlation results was observed by **Mushtaq and Khan** (**2010**). Fe have positive correlation with OC and OM (r = 0.77, p>0.01) and non-significant relation with rest of the physico-chemical parameters. It indicates that soils with high organic carbon content in soil are rich in Fe content. **Mali et al** (**2002**) reported similar correlation between OC and Fe content. Mn has positive correlation with EC (r=0.56, p>0.05) and Silt (r=0.61, p>0.05) while it shows non- significant parameters with all parameters (Table 4.7).

4.3.4 Cluster analysis of different heavy metal

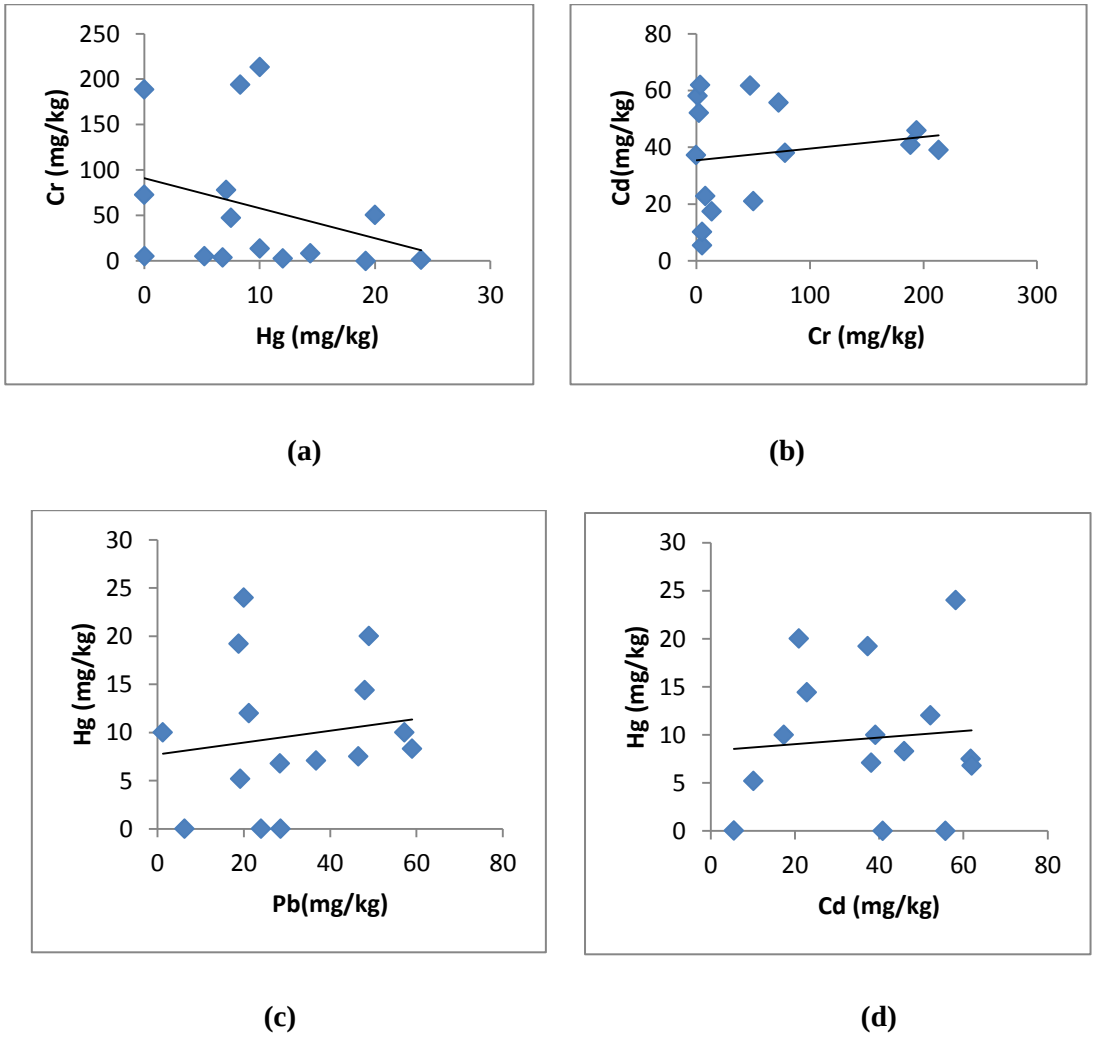


Figure 4.3 (a) Correlation between Hg and Cr (b) Correlation between Cr and Cd. (c) Correlation between Hg and Pb (d) Correlation between Hg and Cd.

Scatter analysis of different metals have been shown in **Figure 4.3**. Hg shows strong negative relation with Cr while Cd shows positive correlation with Cr. Hg shows positive correlation with Pb and Cd. The results revealed that any soil having high concentration of Cr might have low amount of Hg.

4.3.5 Herbicide

In India approximately several thousand tons of herbicides are used for weed control, mainly in irrigated crops and on plantations (**Kumar et al, 2008**). Chemical, biological and physical forces play an important role deciding the fate of herbicides in soil. Herbicide residues persist in the soil from days to years depending upon the type of herbicide, organic matter, pH, soil moisture and temperature.

While collection of soil samples a survey was carried out to find out the herbicides used by the farmers in the agricultural lands at the selected sites. The survey revealed that the majority of farmers were using the herbicide Butachlor. Butachlor (N-butoxymethyl-2-chloro-2, 6-diethylacetanilide), is a herbicide of acetanilide class. It is used as a selective pre-emergent herbicide (**Janaki et al, 2009**; **Chinnusamy et al, 2012**). Integration of inorganic (metals and heavy metals) and organics source of nutrients for crop production is most significant factor influencing the herbicide behaviour in soils (**Akay, 2013)**. Butachlor has also been found at significant levels in agricultural areas around Delhi (**Arora & Gopal 2004**, **Kumar et al, 2008**, **2011**).

In the light of the aforesaid context, HPLC chromatogram of Standard Butachlor was obtained and was compared with the HPLC chromatogram of extracts of untreated soil samples from different agricultural sites. Out of the fifteen soil samples studied, eight soil samples revealed Butachlor residues. In the HPLC chromatogram several small peaks were present apart from the standard peak corresponding to Butachlor that revealed the presence of other pestides, herbicide or insectides residues corresponding to Atrazine (Pesticide), Benomyl (Pesticide), Cypermethrin (Pesticide), Endosulfan (Insecticide), Methyl Parathion (Insecticide), Isoproturon (Herbicide), , Chloropyriphos (Insecticide) and Dichlorvos (Insecticide).

Since, the maximum residues of butachlor were found in soil samples, a preliminary comparison undertaken with the corresponding residue limits used in other countries, considering the shortage of local standard for identifying soil pollution of the herbicides.

Table 4.8 Herbicide concentration in soil samples from different sites

Sampling sites	Concentration (µg/kg) mean S.D.
Control	1.21 (0.4)
Suklai	4.78 (0.6)
Bijnor	4.91 (0.8)
Kalli East	**46.56 (2.1)**
Kamlapur	**43.93 (1.0)**
Kalli West	18.97 (0.9)
Mau	**111.88 (2.7)**
Mohanlalganj	5.60 (0.6)
Unnao	12.06 (0.7)

S.D.= Standard Deviation; Each datum is the mean of triplicate analyses (n=3).

The results on butachlor residues determined in soil samples are summarized in **Table 4.8**. The concentration of butachlor in the soil samples ranged from 4.78 µg/kg to 111.8 µg/kg, the measured concentration at Kalli East (46.56), Kamlapur (43.93) and Mau (111.88) soils were found above maximum allowable concentration. The national regulations relevant for pesticide residues in agricultural soils (**The Official Gazette of the RS, 1994**) define the maximum residue level (MRL) upto, 40 µg/kg for Butachlor.

4.4 Conclusion

The present study was carried out on analyzing the heavy metal concentration of soils in different land uses practicing extensive agricultural activities to assess its soil quality in terms of its physico-chemical properties. The data collected in the present study gives an insight into the level of co-contamination of agricultural area with herbicide residues and heavy metals. Among the heavy metals tested, content of Hg and Cd were found higher than the maximum permissible limits. Residues of several herbicides, herbicides and insecticides were detected in some of the soil samples. Based upon the Laboratory analysis of the soil sample through HPLC, a peak of Butachlor was detected in most of the soil samples and in some of the soil samples it was beyond the permissible limit. Hence, on the basis of survey and laboratory

analysis Butachlor herbicide was chosen along with Hg and Cd for co-contamination study.

However, the presence of Butachlor exceeding the relevant Minimum Residual, indicated potential risk to the crop ecosystem apart from having a negative impact on human health. The observed heavy metals and herbicides in agricultural soils studied may affect food chain accumulation and human health and hence is a matter of concern for future. A regular investigation of herbicide and heavy metals is therefore, required to maintain soil health and contamination levels.

Chapter 5

Seed germination rate and early seedling growth of C. roseus in presence of single (heavy metal or herbicide) and mixed contaminants (heavy metal and herbicide)

5.1 Introduction

Among the d-orbital elements of the modern periodic table Mercury (Hg), Cadmium (Cd), lead (Pb), and Arsenic (As) have attained due importance because of their patho-physiological impact owing to their bioaccumulation in living systems (**Tchounwou et al, 2012**). An elevated concentration of heavy metals in soil may be attributed to both natural and/or anthropogenic activities (**Chibuike & Obiora, 2014**). On the other hand, erroneous use of pesticides has caused severe environmental pollution leading to health hazards (**Ouyang et al, 2016**). The living beings are frequently exposed to mixtures of heavy metals and herbicides compared to individual exposure of either of the two. Any phytoremediation approach addressing the individual exposure could not deal with the consequence observed under joint toxicities which is likely under real life situations. Hence, a study on the combined interactions between xenobiotics and xenobiotics and plant systems are equally important for effective mitigation of toxicity. Our earlier studies **(Ratnakar & Shikha, 2018)** demonstrated widespread distribution of xenobiotics in agricultural land with co contamination of Hg, Cd and BC. In the present study, in order to test the phytoremediation potential of co contaminated soil as above, a preliminary study on the effect of Hg and Cd alone and in combination with BC on seed germination and early seedling growth of *C. roseus* was carried out under simulated soil contamination.

Catharanthus roseus is a herbaceous, perennial herb and flowers throughout the year in tropical regions. The ability of the plant to take up Pb is well documented **(Imam, 2017)**. However no preview is available so far on the effect of Hg, Cd either alone or along with any other herbicide on the phytoremediation potential of the herb. In the present study *C. roseus* has been chosen because it is ornamental, easily available and is well known for its endurance in dry and deficient conditions.

5.2 Materials and Methods

Catharanthus roseus seeds were purchased from a certified shop Neelkanth Agroforestry, Kaiserbagh, Lucknow, U.P. Healthy seeds were selected and surface sterilized with 10% (v/v) hydrogen peroxide for 20 min to prevent fungal growth, washed with distilled water several times before transferring into petri dish having a

double layer of filter paper. Initially a dark condition was provided for germination followed by a photoperiod of 16/08 h, light/dark period. All glass wares were autoclaved at 121°C for 15 min, prior to use.

5.2.1 Experimental procedure

For the treatments, salts of Hg (Hg_2Cl_2), Cd ($CdNO_3$) and herbicide BC (Butachlor, $C_{17}H_{26}ClNO_2$) were used. Concentrations of each metal representing 5, 20, 50, 80 ppm of Hg and 5, 20, 50, 80, 100 ppm of Cd were used while herbicide BC concentration was 1, 2, 4, 6, 10 ppm. The seeds grown in distilled water were referred to as controls. Each treatment was added with 3 ml of the respective solution after every 48 h. Germination percentage was recorded for 08 days at different intervals. Three different treatments were opted throughout the course of the present study:-

(i) Individual treatment of Cd (5 to 100 ppm)

(ii) Individual treatment of Hg (5 to 80 ppm)

(iii) Joint treatment Cd (5-100 ppm) / Hg (5-80 ppm) and BC (4 ppm). BC (4 ppm) was selected based on EC 50 values.

5.2.2 Determination of germination

Seed germination was observed at regular intervals for 08 days. A seed was considered as germinated if the radicle was emerged. The germination percentage was calculated from the total number of seeds and germinated seeds in a Petri plate.

- The germination percentage (G %) was calculated, by the formula given by **Tanveer et al, (2010)**:

  Germination Percentage (G %) = $\frac{Germinated\ seeds}{Total\ Seeds} * 100$

- Seedling Vigour index (VI) was calculated by the following formula (**Vashisth and Nagarajan, 2010**).

  VI = $Seedling\ length * Germination\ Percentage\ \%$

- Germination index (GI) was calculated according to the formula given by (**USDA, 2001**; **Tiquia et al, 1996**).

  Germination Index (GI) = $E * G/100$

  Relative root elongation (E) = (Mean root length with treatment)/Mean root length with control)* 100

Seed germination (G) = (seeds germinated with treatment)/ Seeds germinated with control) * 100

- The Tolerance Index (T.I.) was calculated using the formula given by **Iqbal and Rahmati (1992)**.

  Tolerance Index= T. I = $\frac{Mean\ root\ length\ in\ treatment}{Mean\ root\ length\ in\ control} * 100$

- The percent phytotoxicity (P.P.) was calculated according to **Chou and Lin (1976)** and **Ray and Banerjee (1981)**.

  Phytotoxicity (%) = $\frac{Radicle\ length\ of\ Control - Radicle\ length\ of\ treatment}{Radicle\ length\ of\ Control} * 100$

5.2.3 Scanning electron microscopic (SEM) study

Detail methodology is given in Chapter 3 (Material & Methods) under Section 3.7.

5.2.4 Statistical analysis

Chapter 3 (Materials & Methods) Section 3.8 is referred for details.

5.3 Results

5.3.1 Effect of Cd and BC alone and in combination on seed germination and early seedling growth of *C. roseus*

5.3.1.1 Effect on seed germination

Seeds of *C. roseus* were treated with diverse concentrations of heavy metal ($CdNO_3$) and herbicide (BC) individually, and in combination, to study the effect on germination. The germination was scored after 8^{th} day of imbibitions.

a) Effect of Cadmium on seed germination:

The final germination percentage reduced from 100 to 23.12% relative to control treatments as the concentration of Cd in solution increased from 5 to 120 ppm. The final germination percentage of seeds of *C. roseus* at different concentrations of Cd showed 5.1% inhibition for the lowest concentration (5 ppm) and 91.67% **(Table 5.1)** for the highest concentration (120 ppm) studied. Germination was also inhibited by 29.63% at 50 ppm of Cd in solution. However, there was no total inhibition of seed germination in all concentrations of Cd **(Figure 5.1)**. One-way ANOVA showed that

the inhibition of germination was significant ($p<0.05$) as the concentration of Cd in solution increased from 5-120 ppm.

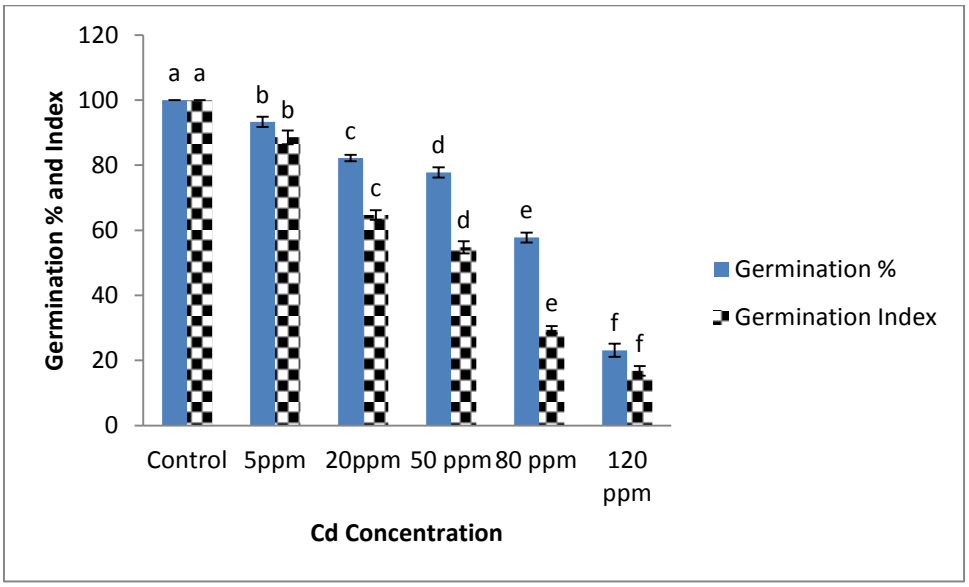


Figure 5.1: Effect of different concentration of Cd on germination percentage and index of *C. roseus* (±S. D., n=3)
***Means with different letters are significantly different from each other ^{a,b,c,d,e} ($p < 0.05$) .**

Over an 8-day germination period GI significantly decreased from 88.5 to 16.81 with an increase in Cd concentration from 5 to 120 ppm in solution. Significant difference in GI was recorded as the concentration of Cd in the solution increased from 5-120 ppm (**Figure 5.1**).

***b) Effect of BC on seed germination*:** The final germination percentage reduced from 93.33 to 24.21% relative to control as the concentration of BC increased from 1 to 10 ppm (**Figure 5.2**). The final germination percentage of seeds of *C. roseus* at different concentrations of BC showed 5.07% inhibition for the lowest concentration (1 ppm) and 90.32% inhibition **(Table 5.2)** for the highest concentration (10 ppm) studied. However, there was no total inhibition of seed germination in all concentrations of BC studied. One-way ANOVA showed that the inhibition of germination was significant ($p<0.05$) as the concentration of BC increased from 1-10 ppm.

Over an 8-day germination period GI significantly decreased from 88.5 to 3.45 with an increase in BC concentration from 1 to 10 ppm **(Figure 5.2)**.

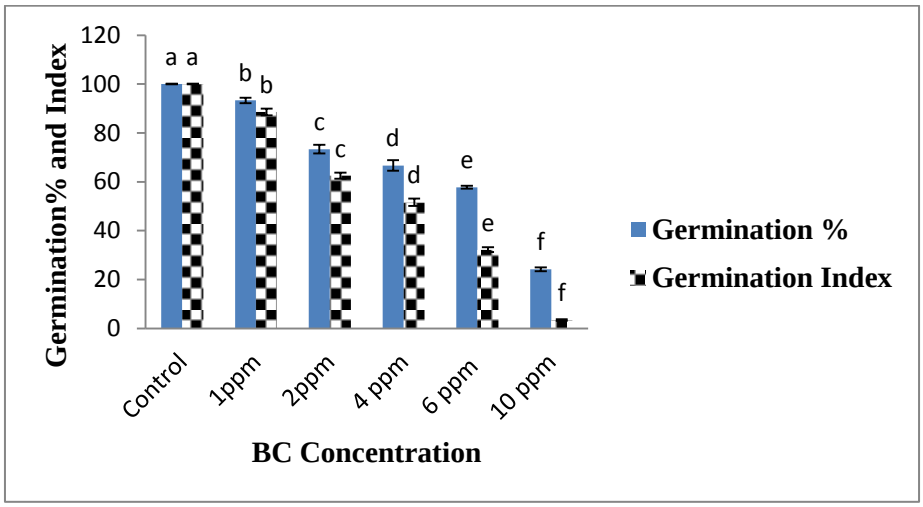


Figure 5.2: Effect of different BC concentration on germination percentage and index (±S.D., n=3)

***Means with different letters are significantly different from each other ^{a,b,c,d,e,f} ($p < 0.05$).**

***c) Joint toxicity of Cd and BC on seed germination*:** Combined effect of Cd (5-120 ppm) and BC (4 ppm) on seed germination showed a significant difference in both germination percentage and index as compared to the individual effects of heavy metal and herbicide. The final germination percentage under combined treatment reduced from 100 to 21.18% relative to control as the concentration of Cd in solution increased from 5-120 ppm **(Figure 5.3)**. The final germination percentage of seeds of *C. roseus* under combined treatment showed 94.94% inhibition for the highest concentration (120 ppm) (**Table 5.3**). However, there was no total inhibition of seed germination in all concentrations of combinations studied.

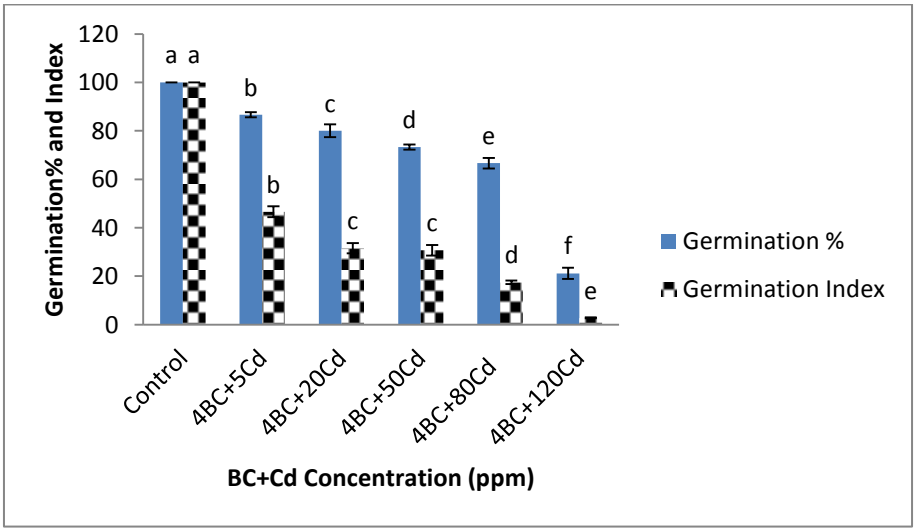


Figure 5.3: Effect of different BC and Cd concentration on germination percentage and index (±S. D., n=3)

***Means with different letters are significantly different from each other ^{a,b,c,d,e,f} ($p < 0.05$).**

Results showed a significant change in germination percentage under combined effect of Cd and BC as compared to individual treatments, respectively. An 80% germination was observed under combined treatment of 20 ppm of Cd and 4 ppm of BC which was higher than the individual treatment of BC (66.66 %) at 4ppm and marginally less than the isolated treatment of Cd, being 82.21% at 20 ppm **(Figure 5.1, 5.2 and 5.3)**

One-way ANOVA showed that the inhibition of germination was significant ($p<0.05$) as the concentration of Cd increased from 5-120 ppm in the presence of 4 ppm BC.

Over an 8-day germination period, the GI significantly decreased from 46.63 to 2.82% **(Figure 5.3)** with an increase in Cd concentration from 5 to 120 ppm in the presence of BC (4 ppm). Significant difference in GI was recorded as the concentration of Cd in the presence of BC in the solution increased from 1-10 ppm.

5.3.1.2 Effect on seedling growth

The effect of different concentrations of heavy metal ($CdNO_3$) and herbicide (BC) alone, and in combination, was studied on the root and shoot length of *C. roseus.*

***a) Effect of Cd on seedling growth*:** The effect of various Cd concentrations (5-120 ppm) on the root and shoot length of *C. roseus* is shown in **Figure 5.4**. The shoot length and root length as measured on the 8^{th} day of incubation revealed a gradual decline in root length from 2.16 cm in control to 0.08 cm at 120 ppm of Cd. However, the length of the plumule was found to be greater than the root length at all concentrations studied. The shoot length was 2.62 cm in the control and showed a concentration (5-120 ppm) dependent decline reaching to 0.11 cm at 120 ppm. It was further revealed that relative to control treatments, the shoot length of *C. roseus* was adversely affected by Cd and results showed a 13.98% and 81.73% significant ($p<0.05$) inhibition for 5 and 120 ppm of the metal in solution. Similar results were observed for roots, but the root length inhibition was lesser than the shoot length inhibition. The present result showed a marginal decrease (5.1% inhibition) in root length at 5 ppm of cadmium, however, as the concentration of Cd in solution increased from 20-120 ppm the inhibition to the root length of *C. roseus* increased significantly ($p<0.05$) from 21.3% to 91.67% relative to control treatments.

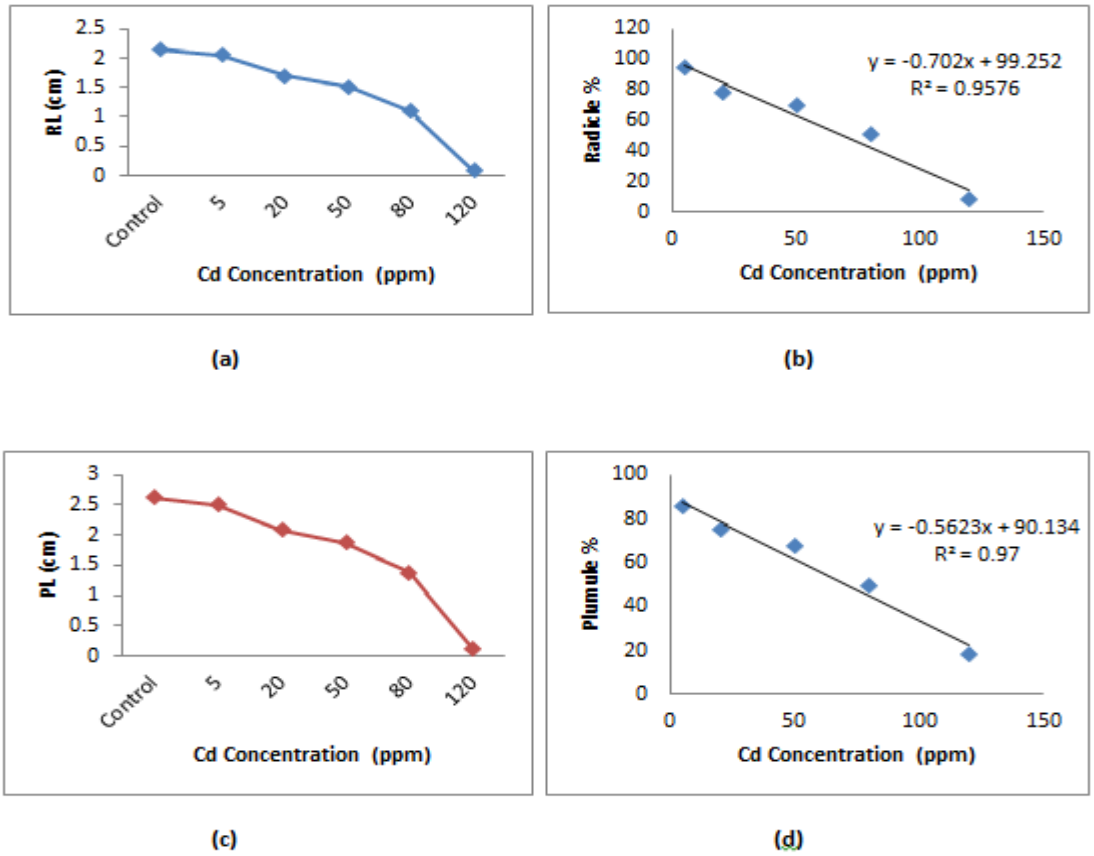


Figure 5.4: Effect of Cd on (a) (b) radicle and (c) and (d) plumule length of *C. roseus* seedlings

Early seedling growth of *C. roseus* showed a differential trend for R: S, it showed decrease as the concentration of Cadmium increased from 5 to 120 ppm relative to control. A 12.19% decrease in R: S ratio recorded at the highest concentration (120 ppm) of Cd may be attributed to limitations of nutrient and water under metal stress.

***b) Effect of BC on seedling growth*:** The effect of BC concentrations (1-10 ppm) on the root and shoot length of *C. roseus* is shown in **Figure 5.5**. The shoot length and root length as measured on the 8th day of incubation revealed a gradual decline in root length from 2.17 cm in control to 0.21 cm at 10 ppm of BC. However, the length of the plumule was found to be greater than the root length at all concentrations studied. The shoot length was 3.39 cm in the control and showed a marginal decrease up to 2 ppm of BC, followed by a major decline in length with increase in concentration. A 79.64% decrease in plumule length was recorded at the highest concentration of BC (10 ppm) relative to control. It was further revealed that relative to control treatments,

the root length and shoot length of *C. roseus* was adversely affected by BC. The present result showed a marginal decrease (5% inhibition) in root length at 1 ppm of BC, however, as the concentration of BC increased from 2-10 ppm the inhibition to the root length of *C. roseus* increased significantly ($p<0.05$) from 14.75% to 90.32% relative to control treatments **(Table 5.2)**.

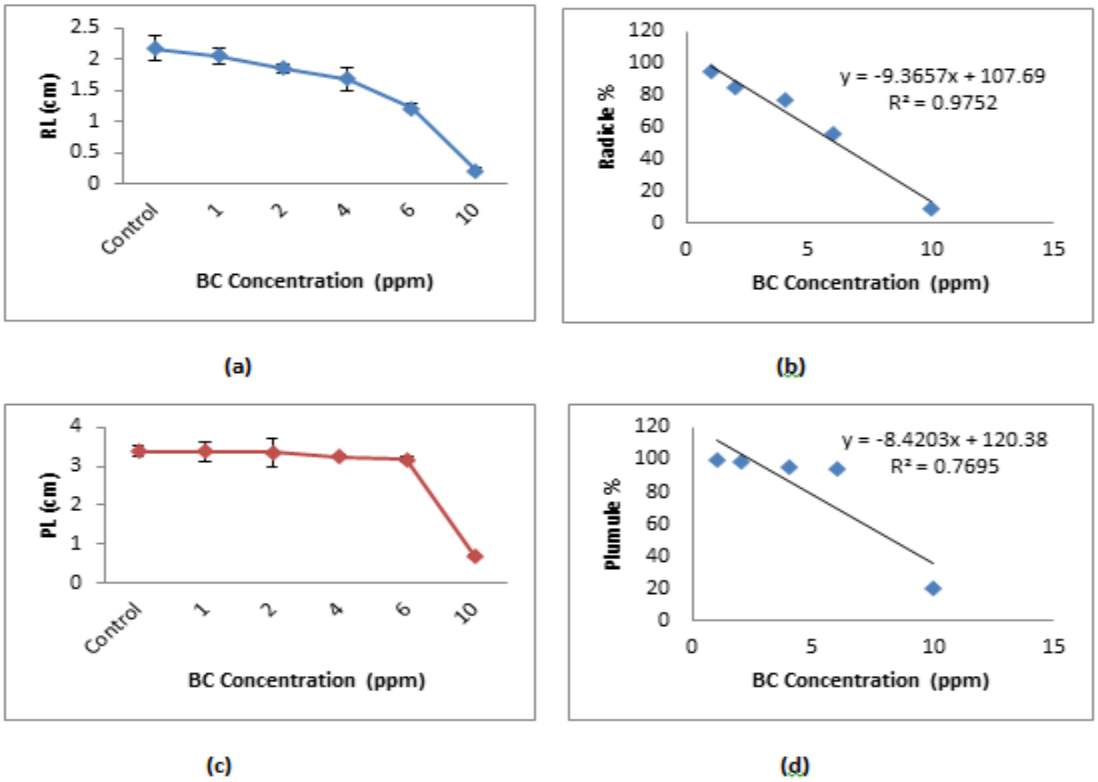


Figure 5.5: Effect of BC concentration on (a) (b) radicle and (c) and (d) plumule length of *C. roseus* seedlings (±S. D., n=3)

Early seedling growth of *C. roseus* showed a fairly consistent trend for R:S to decrease as the concentration of BC increased from 1 to 10 ppm relative to control. A 53.12% decrease in R: S ratio recorded at the highest concentration (10 ppm) of BC may be attributed to limitations of nutrient and water under metal stress.

***c) Joint toxicity of Cd and BC on seedling growth*:** Combined effect of Cd (5-120 ppm) and BC (4 ppm) on the root and shoot length of *C. roseus* is shown in **Figure 5.6**. The shoot length and root length as measured on the 8th day of incubation revealed a gradual decline in root length from 2.17 cm in control to 0.11 cm at 120 ppm of Cd in the presence of BC. However, the length of the plumule was found to be greater than the root length at all concentrations studied. The shoot length was 3.9 cm in control and showed a concentration (20-120 ppm) dependent decline reaching to

0.39 cm at 120 ppm. It was further revealed that relative to control treatments, the shoot length of *C. roseus* was adversely affected by Cd and results showed a 15.65% and 90% significant ($p<0.05$) inhibition for 20 and 120 ppm of the metal in solution. Similar results were observed for roots, but the root length inhibition was greater than the shoot length inhibition. The present result showed a significant decrease (47.9% inhibition) in root length at 5 ppm of Cd, however, as the concentration of Cd in solution increased from 20-120 ppm the inhibition to the root length of *C. roseus* increased significantly ($p<0.05$) from 50.24% to 94.94% relative to control treatments.

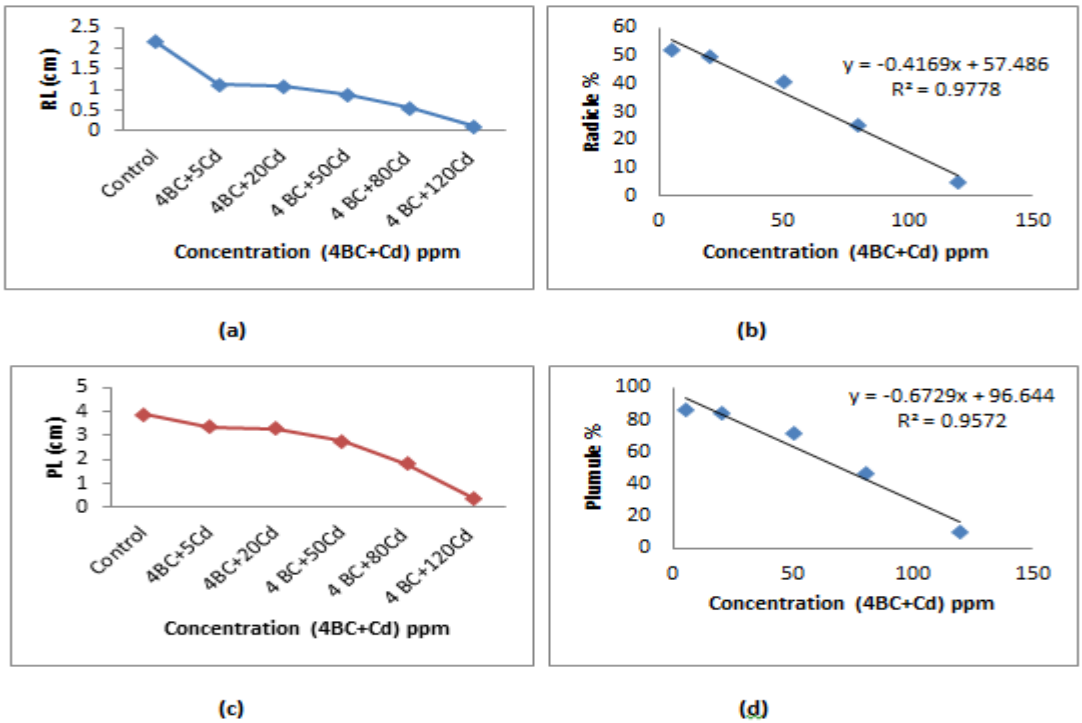


Figure 5.6: Effect of Cd +BC (4 ppm) on (a) (b) radicle and (c) and (d) plumule length of *C. roseus* seedlings (±S. D., n=3)

Early seedling growth of *C. roseus* showed R:S to decrease as the concentration of Cd increased from 20 to 120 ppm relative to control. An approximate 1.13 fold decrease in R: S ratio recorded at the highest concentration (120 ppm) of Cd and (4 ppm) BC, may be attributed to limitations of nutrient and water under metal stress.

5.3.1.3 Effect on seed vigour

Seed vigour of *C. roseus* declined with increase in Cd concentration (5-120 ppm), BC (1-10 ppm), both alone and in combination (Cd-5-120 ppm and BC-4 ppm) as

measured after 48 h of germination. The seedling vigour index was reduced from 96 to 2.19 as the concentration of Cd increased from 5-120 ppm (**Table 5.1**). However, the vigour index in the presence of BC was found to decrease from 72.79 to 5.81 with the increase in concentration from 1-10 ppm **(Table 5.2)**. The vigour index in combination on the contrary decreased from 70.19 to 3.38 when the concentration of Cd increased from 5-120 ppm in the presence of BC **(Table 5.3**). Results thus revealed that treatment of Cd alone bears greater impact on seed vigour followed by combination and BC alone. Under stress conditions there may be a decrease in uptake of water both during imbibitions and seedling establishment (**Prisco & Vieira, 1976**), which bears physiological and biochemical changes in the metabolism of both seed and seedling (**Gomes & Sodek, 1988**).

5.3.1.4 Effect on tolerance indices

The seedlings of *C. roseus* were tested for tolerance to heavy metal and herbicide (BC), using different concentrations of Cd (5-120 ppm) and BC (1-10 ppm), both alone and in combination. *C. roseus* showed high percentage of tolerance at 5 ppm of Cd. An increase in Cd concentration (20-120 ppm) gradually decreased the tolerance of *C. roseus*. The treatment of Cd at 120 ppm showed the lowest percentage of tolerance in *C. roseus* as measured on the eighth day of exposure as compared to control. The tolerance index was found to decrease from 91.3 to 5.21 as the concentration of Cd increased from 5-120 ppm (**Table 5.1**). However, as compared to Cd better tolerance index was reported in the presence of BC. The tolerance index decreased from 92.59 to 14.28 as the concentration of BC increased from 1-10 ppm (**Table 5.2**). Under combination treatment, the tolerance index was lesser than individual treatments of Cd and BC decreasing from 60.66 to 4.58 as the concentration of Cd in the presence of BC (4 ppm) increased from 5-120 ppm (**Table 5.3**). Hence, according to tolerance test it may be inferred *C. roseus* was more tolerant to BC compared to Cd and combination treatments.

5.3.1.5 Effect on percent phytotoxicity

The phytotoxicity of Cd (5-120 ppm) and butachlor (1-10 ppm), both alone and in combination on root length of *C. roseus* is given in **Tables 5.1, 5.2 and 5.3**. The increase of Cd levels significantly increased the percent phytotoxicity on root length under all treatments ($p<0.05$). The lowest percent phytotoxicity (5.09%) was observed

at the lowest concentration of Cd (5 ppm). However, in the presence of BC, it was 5.06% at the lowest concentration (1 ppm). The joint toxicity of Cd and BC revealed 46.19% phytoxicity at the lowest concentration (5 ppm Cd in the presence of 4 ppm BC). Results clearly revealed that the joint treatment of Cd and BC was more toxic to root growth followed by the individual treatment of Cd and BC on the radical length of *C. roseus.*

Table 5.1: Effect of different Cd concentration on seedling growth parameters (±S. D., n=3)

	Inhibitory %	Vigour Index	Tolerance Index	Phytotoxicity %	R/S Ratio
Control	0 ± 0.00^{f}	96 ± 4.58^{a}	100 ± 0.00^{a}	0 ± 0.0^{e}	$0.82\pm.15^{a}$
5ppm	$5.1\pm.26^{e}$	76.5306 ± 6.13^{b}	91.3 ± 2.60^{b}	$5.09\pm.08^{d}$	$0.81\pm.81^{a}$
20ppm	$21.3\pm.99^{d}$	50.1481 ± 3.56^{c}	78.69 ± 3.09^{c}	24.07 ± 1.04^{c}	$0.81\pm.14^{a}$
50 ppm	$29.63\pm.55^{c}$	42.7735 ± 2.02^{d}	66.08 ± 1.78^{d}	37.96 ± 1.05^{b}	$0.80\pm.02^{a}$
80 ppm	49.08 ± 1.04^{b}	32.9289 ± 2.22^{e}	63.91 ± 1.56^{d}	39.35 ± 1.53^{b}	$0.79\pm.06^{a}$
120 ppm	91.67 ± 1.68^{a}	$2.1964\pm.253^{f}$	$5.21\pm.23^{e}$	94.9 ± 1.03^{a}	$0.72\pm.18^{a}$

****Means with different letters are significantly different from each other ^{a,b,c,d,e,f} ($p < 0.05$).**

Table 5.2: Effect of different BC concentration on seedling growth parameters (±S. D., n=3)

	Inhibitory %	Vigour Index	Tolerance Index	Phytotoxicity %	R/S Ratio
Control	0.00 ± 0.00^{f}	111 ± 8.88^{a}	100 ± 0.00^{a}	0.031 ± 0.002^{f}	0.64 ± 0.07^{a}
1ppm	5.07 ± 0.56^{e}	72.79 ± 3.27^{b}	92.59 ± 1.13^{b}	5.06 ± 0.24^{e}	$0.61\pm0.01^{a,b}$
2ppm	14.75 ± 0.37^{d}	52.79 ± 3.47^{c}	77.77 ± 1.52^{c}	14.74 ± 0.63^{d}	$0.55\pm0.07^{a,b}$
4 ppm	22.59 ± 0.36^{c}	43.32 ± 3.86^{d}	70.37 ± 1.12^{d}	22.58 ± 0.94^{c}	0.51 ± 0.05^{b}
6 ppm	44.24 ± 0.80^{b}	35.23 ± 3.68^{d}	68.23 ± 0.97^{e}	44.23 ± 1.03^{b}	0.38 ± 0.01^{c}
10 ppm	90.323 ± 1.70^{a}	5.81 ± 0.42^{e}	14.28 ± 0.94^{f}	85.71 ± 1.57^{a}	0.30 ± 0.05^{c}

****Means with different letters are significantly different from each other ^{a,b,c,d,e,f} ($p < 0.05$).**

Table 5.3: Effect of different Cd and BC concentration on seedling growth parameters of *C. roseus* (±S. D., n=3)

	Inhibitory %	Vigour Index	Tolerance Index	Phytotoxicity %	R/S Ratio
Control	0 ± 0.0^{e}	111 ± 4.35^{a}	100 ± 0.00^{a}	0 ± 0.00^{e}	0.55 ± 0.05^{a}
4BC+5Cd	47.93 ± 2.26^{d}	70.19 ± 2.90^{b}	60.66 ± 2.09^{b}	46.19 ± 0.97^{d}	0.33 ± 0.03^{b}
4BC +20Cd	50.24 ± 2.62^{d}	53.60 ± 5.17^{c}	46.66 ± 2.01^{c}	58.09 ± 1.03^{c}	0.32 ± 0.04^{b}
4BC +50Cd	59.45 ± 1.52^{c}	47.66 ± 4.65^{c}	36.25 ± 2.00^{d}	60.47 ± 3.07^{c}	0.31±0.01b
4BC +80Cd	74.66 ± 1.22^{b}	40.66 ± 3.68^{d}	23.75 ± 2.21^{e}	73.8 ± 1.35^{b}	0.30 ± 0.01^{b}
4BC+120Cd	94.94 ± 1.60^{a}	3.38 ± 0.46^{e}	4.58 ± 0.46^{f}	94.93 ± 2.59^{a}	0.28 ± 0.08^{b}

****Means with different letters are significantly different from each other ^{a,b,c,d,e,f} ($p < 0.05$).**

5.3.1.6 Scanning electron microscopy study (SEM) analysis

The result of Scanning Electron microscope studies indicated the structural deformation in the tissues of root and shoot in comparison to control **(Figures 5.7 and 5.8).** The shrinkage in the parenchymatous cells of plant tissues is clearly visible which may be attributed to limitation of nutrient supply and toxic effect of combined treatment.

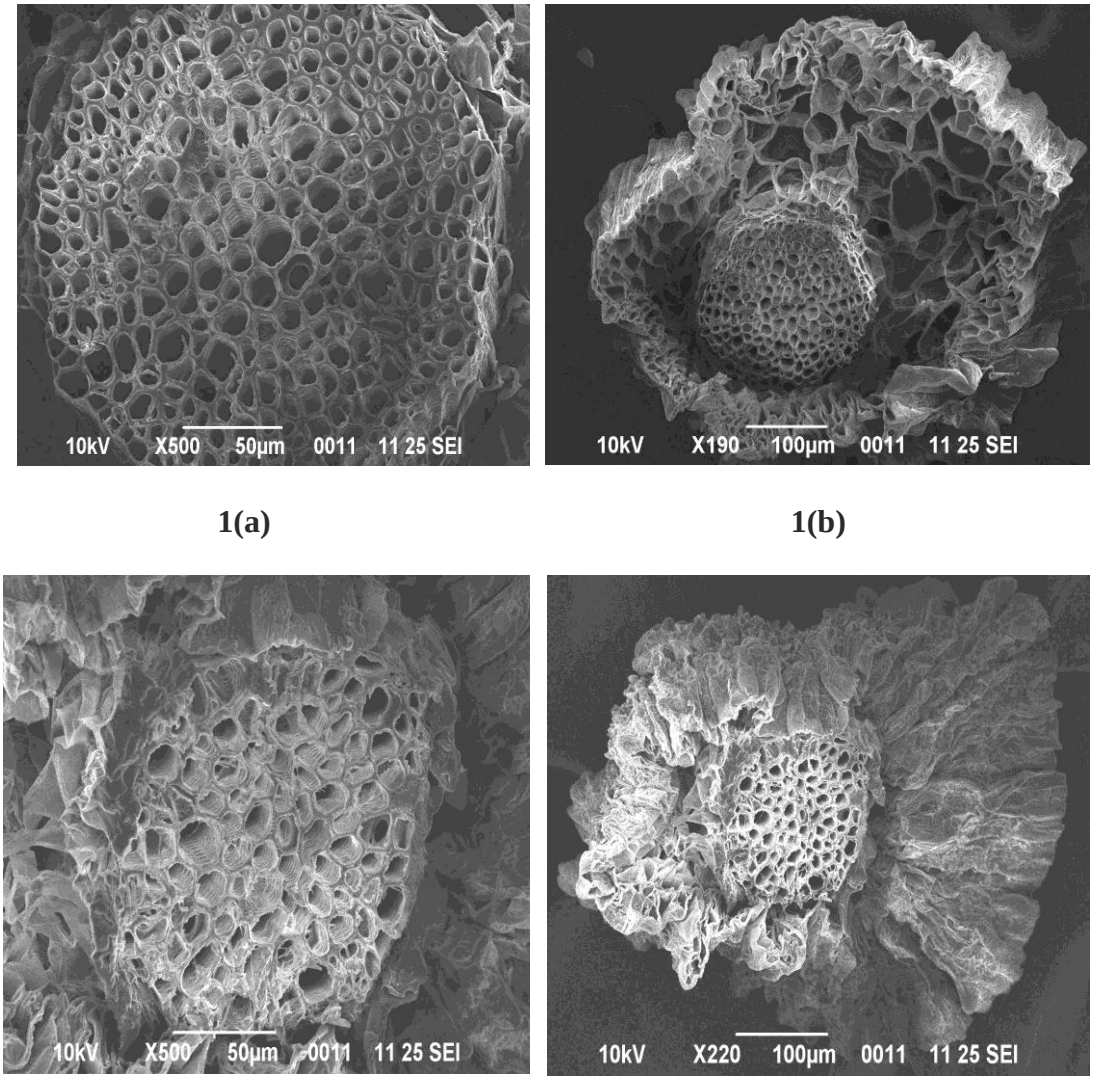
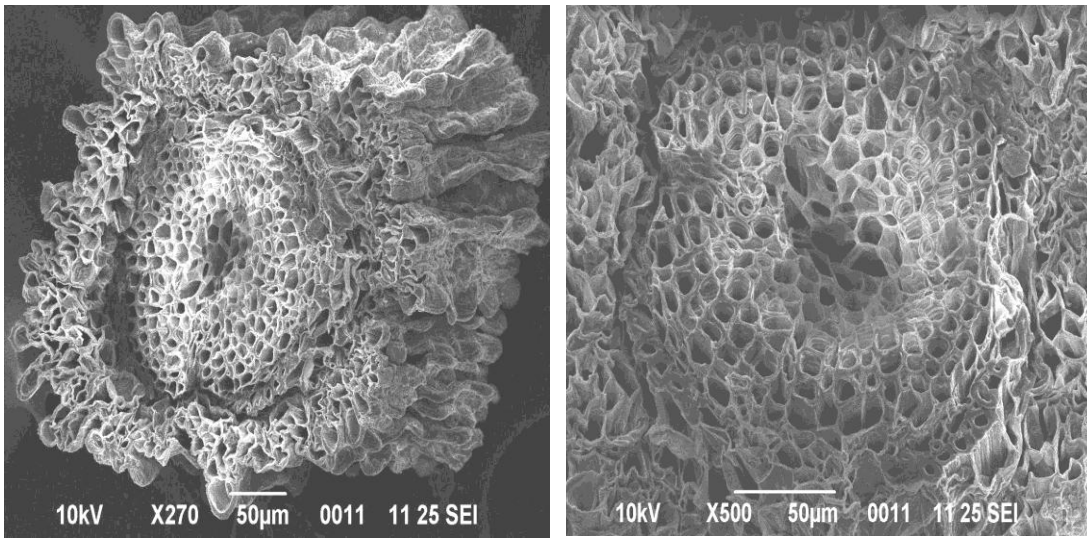
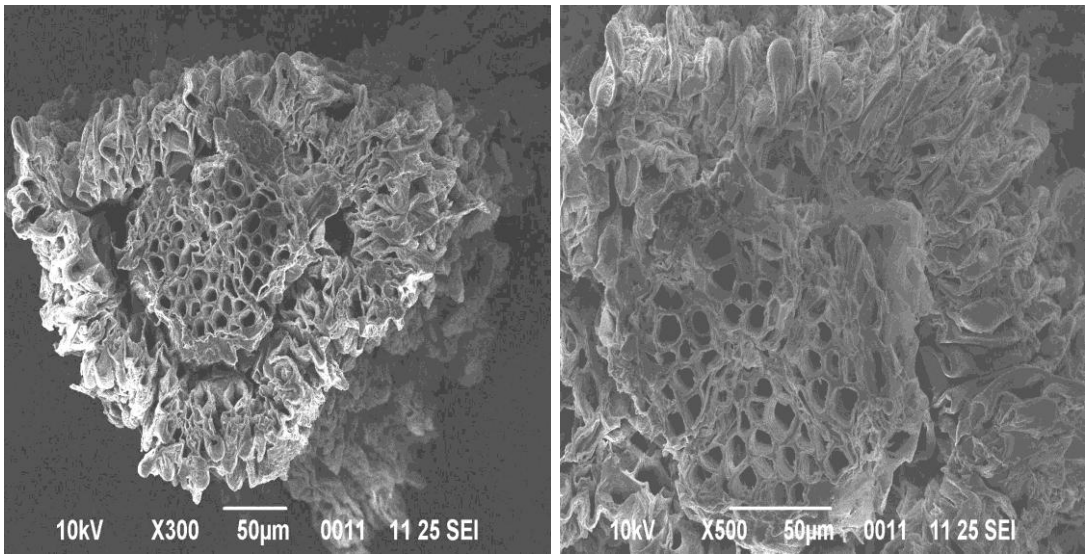


Figure 5.7: Scanning electron micrograph (SEM) of untreated and treated radicle of *C. roseus* under early seedling growth: 1(a,b) Control; 2(a,b) Cd+BC treated root



1(a) **1(b)**



2(a) **2(b)**

Figure 5.8: Scanning electron micrograph (SEM) of untreated and treated plumule of *C. roseus* under early seedling growth: 1(a,b) Control; 2(a,b) Cd+BC treated Shoot

5.3.1.7 Toxicity thresholds

The Cd, BC and combination toxicity thresholds were determined for radical and plumule of C. *roseus*. EC 50 is the contaminant concentration that produced 50% inhibition in seedling length (root and shoot) with respect to the control treatment. The EC50 values for Cd, BC alone and in combination is given in **Table 5.4**. The 50% inhibitory concentration of Cd for radicle and plumule of *C. roseus* was found to be 70.15 and 71.37 ppm,, respectively, which is contrary to the the observation of **de**

Souza Guilherme et al (**2015**) who reported that Cd at 0.12 mM inhibited the growth of *T. aestivum* and thus inhibited the germination of 50% of seeds**.**

A 50% reduction in radicle and plumule length of *C. roseus* in the presence of herbicide BC was recorded at 6.15 and 8.35 ppm, respectively. **Ateeq et al** (**2002**) reported Butachlor induced dose-dependent root growth inhibition in *Allium* root tip with EC 50 value registered at 5.13 ppm which is contrary to our results. However, the EC 50 value for BC + Cd was found to be 17.95 ppm for radicle and 69.32 ppm for plumule. Overall results clearly revealed that joint treatment of Cd and BC was more toxic compared to individual toxicities of Cd and BC for both root and shoot.

Table 5.4: Effective concentration 50 (EC 50) values for Cd, BC alone and in combination

Treatment	EC-50	
	Radicle	Plumule
Cd (ppm)	70.15	71.37
BC (ppm)	6.15	8.35
BC+Cd (ppm)	17.95	69.32

5.3.2 Effect of Hg and BC alone and in combination on seed germination and early seedling growth of *C. roseus*

5.3.2.1 Effect on seed germination

Seeds of *C. roseus* were treated with diverse concentrations of heavy metal (Hg_2 Cl_2) and herbicide (BC) alone, and in combination, to study the consequence on germination. The germination was scored after 8^{th} day of imbibitions.

a) Effect of Hg on Seed Germination: The final germination percentage reduced from 100 to 13.3% relative to control treatments as the concentration of Hg in solution increased from 5 to 80 ppm (**Figure 5.9**). The final germination percentage of seeds of *C. roseus* at different concentrations of Hg showed 0.99% inhibition for the lowest concentration (5 ppm) and 64.22% for the highest concentration (80 ppm). Germination was also inhibited by 42.22% at 50 ppm of Hg in solution. However, there was no total inhibition of seed germination in all concentrations of Hg.

One-way ANOVA showed that the inhibition of germination was significant ($p<0.05$) as the concentration of Hg in solution increased from 5-80 ppm.

Over an 8-day germination period, the germination index (GI) significantly decreased from 85.8 to 4.7 with an increase in Hg concentration from 5 to 80 ppm in

solution. Significant difference in germination index was recorded as the concentration of Hg in the solution increased from 5-80 ppm (**Figure 5.9**).

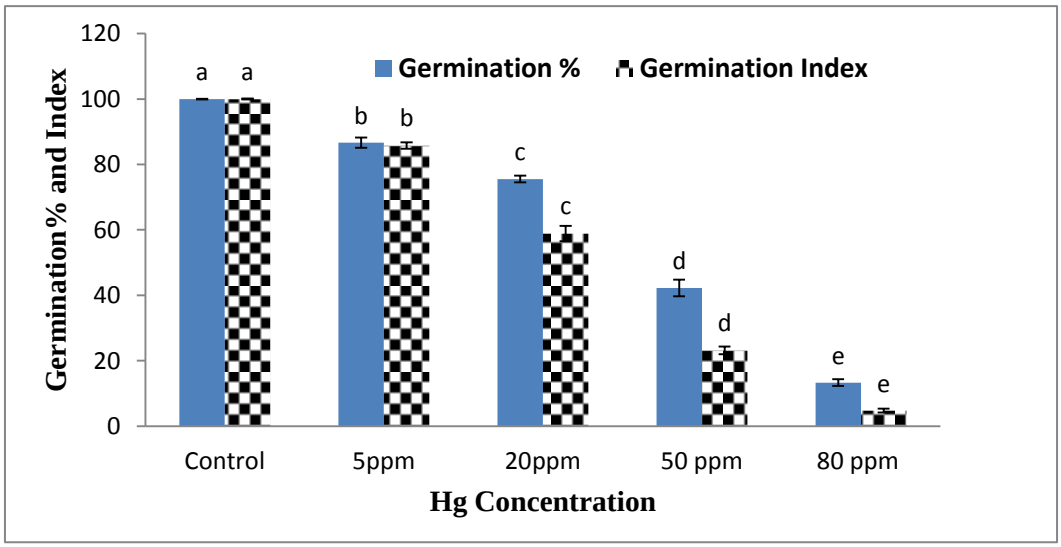


Figure 5.9: Effect of different concentration of Hg on germination percentage and index of *C. roseus* (±S. D., n=3)
***Means with different letters are significantly different from each other ^{a,b,c,d,e} ($p < 0.05$) .**

***b) Joint toxicity of Hg and BC on seed germination*:** Combined effect of Hg (5-80 ppm) and BC (4ppm) on seed germination showed a significant difference in both germination percentage and index as compared to the individual effects of heavy metal and herbicide.

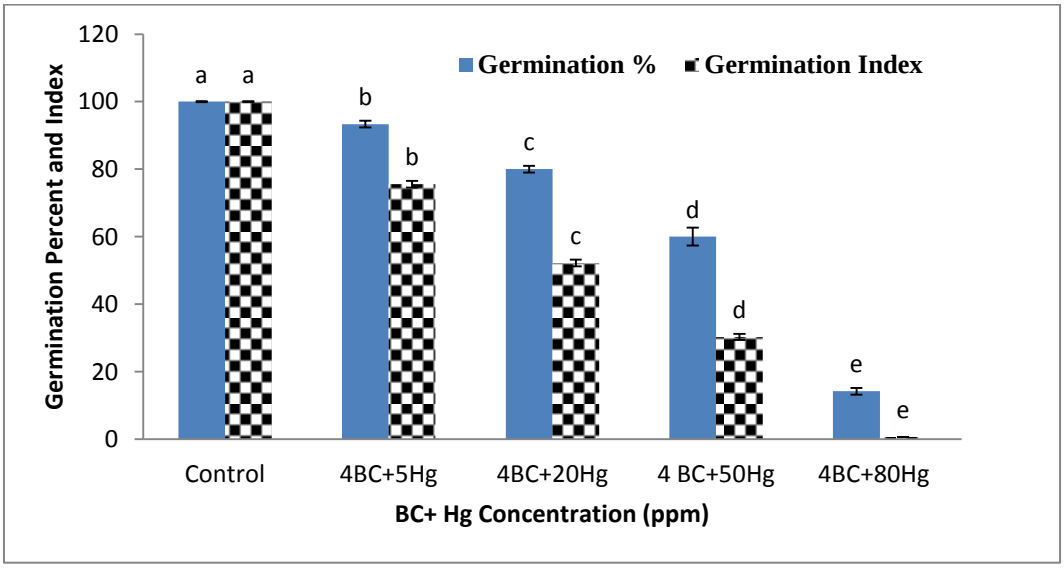


Figure 5.10: Effect of Different BC and Hg concentration on germination percentage and index (±S. D., n=3)
***Means with different letters are significantly different from each other ^{a,b,c,d,e,f} ($p < 0.05$).**

The final germination percentage under combined treatment reduced from 100 to 14.22% relative to control as the concentration of Hg in solution increased from 5-80

ppm (**Figure 5.10**). The final germination percentage of seeds of *C. roseus* under combined treatment showed 95.4% inhibition for the highest concentration (80 ppm). However, there was no total inhibition of seed germination in all concentrations of combination studied.

Results showed an improvement in germination percentage under combined effect of Hg and BC as compared to Hg alone. 80 % germination was observed in the combination treatment of 20 ppm of Hg and 4 ppm of BC which was higher than the individual treatments, being 75.55% in the presence of 20 ppm Hg and 66.66% in the presence of BC (4ppm) (**Figure 5.9 and 5.2**)

One-way ANOVA showed that the inhibition of germination was significant ($p<0.05$) as the concentration of Hg increased from 5-80 ppm in the presence of 4 ppm BC.

Over an 8-day germination period, the germination index (GI) significantly decreased from 75.55 to 0.67 with an increase in Hg concentration from 5 to 80 ppm in the presence of BC (4 ppm). Significant difference in germination index was recorded as the concentration of Hg in the presence of BC in the solution increased from 5-80 ppm.

5.3.2.2 Effect on seedling growth

The effect of diverse concentrations of heavy metal (Hg_2Cl_2) and herbicide (BC) alone, and in combination, was studied on the root and shoot length of *C. roseus.*

***a) Effect of Hg on seedling growth*:** The effect of various Hg concentrations (5-80 ppm) on the root and shoot length of *C. roseus* is shown in **Figure 5.11 (a, c)**. The shoot length and root length as measured on the 8^{th} day of incubation revealed a gradual decline in root length from 2.04 cm in control to 0.73 cm at 80 ppm of Hg. However, the length of the plumule was found to be greater than the root length at all concentrations studied. The shoot length was 2.85 cm in the control and showed a concentration (5-80 ppm) dependent decline reaching to 0.90 cm at 80 ppm. It was further revealed that relative to control treatments, the shoot length of *C. roseus* was adversely affected by Hg and results showed a 0.99% and 64.22% significant ($p<0.05$) inhibition for 5 and 80 ppm of the metal in solution. Similar results were observed for roots, but the root length inhibition was lesser than the shoot length inhibition. The present result showed a marginal decrease (0.98% inhibition) in root

length at 5 ppm of Hg, however, as the concentration of Hg in solution increased from 20-80 ppm the inhibition to the root length of *C. roseus* increased significantly ($p<0.05$) from 22% to 64.2% relative to control treatments.

Early seedling growth of *C. roseus* showed a fairly consistent trend for R: S to increase as the concentration of Hg increased from 5 to 80 ppm relative to control. Albeit small a 14% increase in R:S ratio recorded at the highest concentration (80 ppm) of Hg may be attributed to limitations of nutrient and water under metal stress.

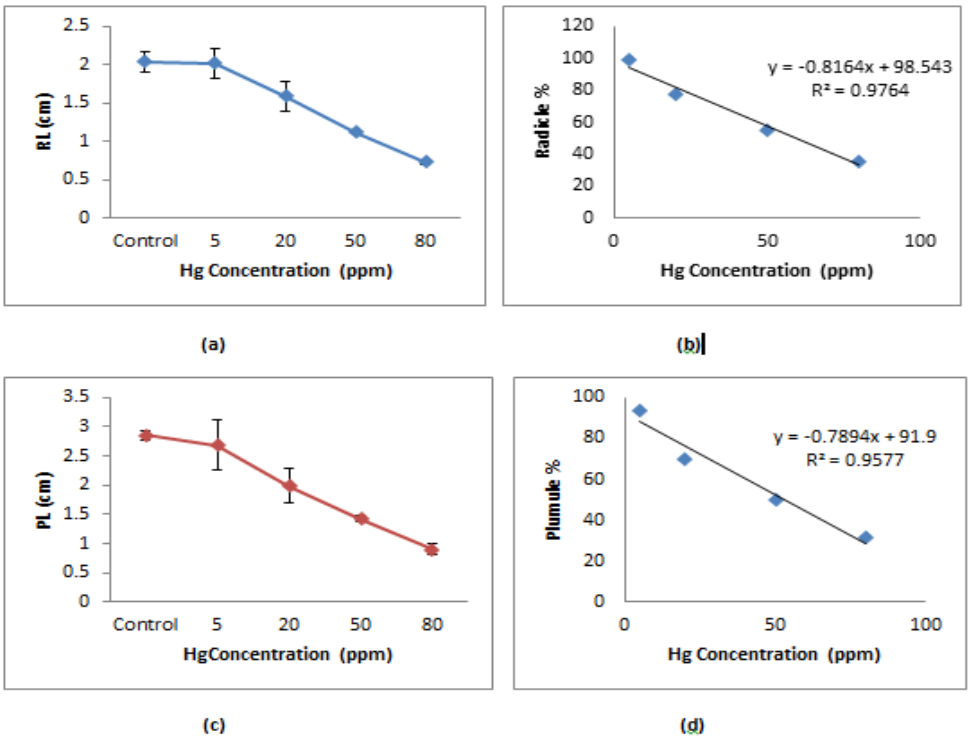


Figure 5.11: Effect of Hg on (a) (b) radicle and (c) and (d) plumule length of *C. roseus* seedlings

***b) Joint toxicity of Hg and BC on seedling growth*:** Combined effect of Hg (5-80 ppm) and BC (4 ppm) on the root and shoot length of *C. roseus* is shown in **Figure 5.12 (a, c)**. The shoot length and root length as measured on the 8th day of incubation revealed a gradual decline in root length from 2.17 cm in control to 0.10 cm at 80 ppm of Hg in the presence of BC. However, the length of the plumule was found to be greater than the root length at all concentrations studied. The shoot length was 3.8 cm in the control and showed a marginal (0.52%) decrease at 5 ppm concentration of Hg with respect to control, in the presence of BC (4 ppm), beyond which a concentration (20-80 ppm) dependent sharp decline was recorded reaching to 0.20 cm at 80 ppm. It was further revealed that relative to control treatments, the shoot length

of *C. roseus* was adversely affected by Hg and results showed a 30.18% and 68.43% significant ($p<0.05$) inhibition for 20 and 80 ppm of the metal in solution. Similar results were observed for roots, but the root length inhibition was lesser than the shoot length inhibition. The present result showed a significant decrease (0.99% inhibition) in root length at 5 ppm of Hg, however, as the concentration of Hg in solution increased from 20-80 ppm the inhibition to the root length of *C. roseus* increased significantly ($p<0.05$) from 22.06% to 64.22% relative to control treatments.

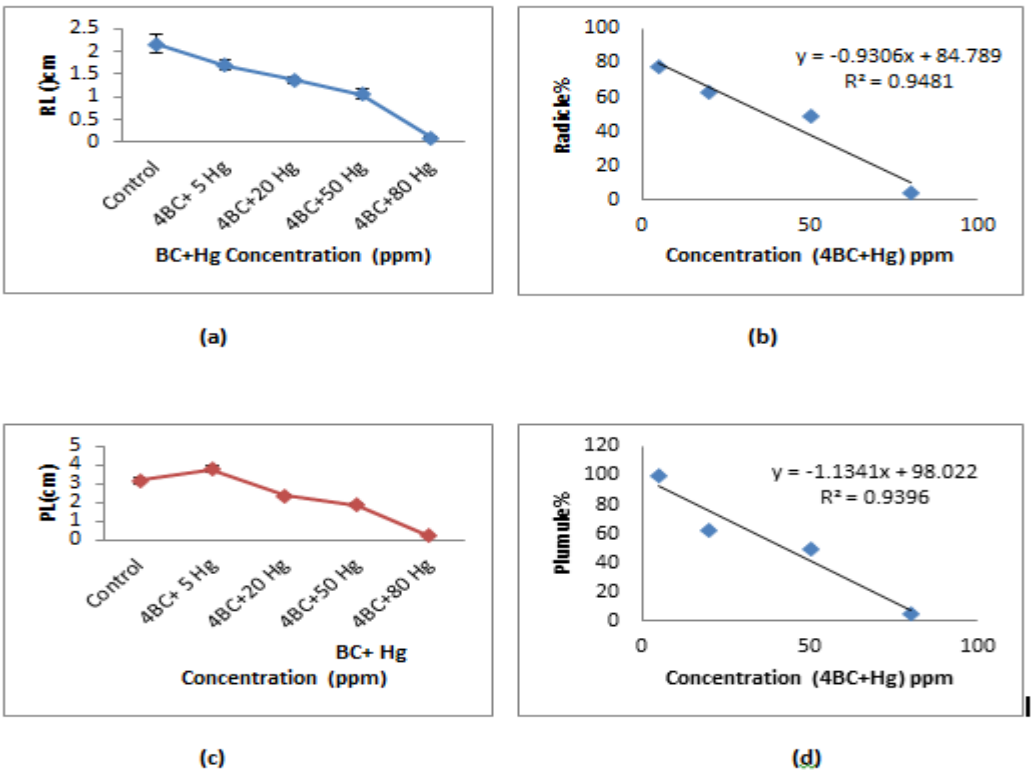


Figure 5.12: Effect of Hg +BC (4 ppm) on (a) (b) radicle and (c) and (d) plumule length of *C. roseus* seedlings (±S. D., n=3)

Early seedling growth of *C. roseus* showed R:S to increase as the concentration of Hg increased from 5 to 80 ppm relative to control. An approximate 0.87 fold increase in R:S ratio recorded at the highest concentration (80 ppm) of Hg may be attributed to limitations of nutrient and water under metal stress (**Table 5.5**).

5.3.2.3 Effect on seed vigour

Seed vigour of *C. roseus* declined with increase in Hg (5-80 ppm) and BC (1-10 ppm), concentration in both alone and in combination (Hg-5-80 ppm and BC-4 ppm) as measured after 48 h of germination. The seedling vigour index was reduced from

78.8 to 1.7 as the concentration of Hg increased from 5-20 ppm. However, the vigour index in the presence of BC was found to decrease from 72.2 to 5.81 with the increase in concentration from 1-10 ppm. The vigor index in combination on the contrary decreased from 74.66 to 3.1 when the concentration of Hg increased from 5-80 ppm in the presence of BC. Results thus revealed that treatment of Hg alone bears greater impact on seed vigour followed by combination and BC alone. It has been reported **(Prisco & Vieira, 1976)** that under stress conditions there may be a decrease in uptake of water both during imbibitions and seedling establishment, which may bring physiological and biochemical changes in the metabolism of both seed and seedling **(Gomes & Sodek, 1988**).

Table 5.5: Effect of different Hg concentration on seedling growth parameters (±S. D., n=3)

	Inhibitory %	Vigour Index	Tolerance Index	Phytotoxicity %	R/S Ratio
Control	0 ± 0.0^{a}	102 ± 5.29^{a}	100 ± 0.0^{a}	0 ± 0.0^{a}	0.71 ± 0.06^{a}
5ppm	0.99 ± 0.03^{a}	78.86 ± 3.84^{b}	87.5 ± 1.77^{b}	0.98 ± 0.05^{a}	0.75 ± 0.06^{a}
20ppm	22.06 ± 1.61^{b}	53.64 ± 3.21^{c}	65.6 ± 2.48^{c}	22.05 ± 2.0^{b}	0.79 ± 0.10^{a}
50 ppm	45.1 ± 1.50^{c}	31.24 ± 1.68^{d}	40.6 ± 1.97^{d}	46.07 ± 2.80^{c}	0.78 ± 0.03^{a}
80 ppm	64.22 ± 1.92^{d}	1.73 ± 0.27^{e}	21.42 ± 2.90^{e}	77.18 ± 1.76^{d}	0.81 ± 0.05^{a}

***Means with different letters are significantly different from each other ^{a,b,c,d,e} ($p < 0.05$).**

Table 5.6: Effect of different Hg and BC concentration on seedling growth parameters of *C. roseus* (±S. D., n=3)

	Inhibitory %	Vigour Index	Tolerance Index	Phytotoxicity %	R/S Ratio
Control	0.0 ± 0.0^{e}	111 ± 3.60^{a}	100 ± 0.0^{a}	0 ± 0.00^{e}	0.57 ± 0.06^{a}
5Hg+4BC	21.66 ± 0.93^{d}	74.66 ± 2.3^{b}	84 ± 2.64^{b}	19.04 ± 0.82^{d}	0.44 ± 0.05^{a}
20Hg+4BC	36.87 ± 1.18^{c}	54.4 ± 2.70^{c}	76 ± 1.00^{c}	34.76 ± 0.97^{c}	0.57 ± 0.04^{a}
50Hg+4 BC	51.16 ± 0.97^{b}	39.6 ± 2.11^{d}	44 ± 1.00^{d}	49.52 ± 1.04^{b}	0.56 ± 0.08^{a}
80Hg+4BC	95.4 ± 0.87^{a}	3.12 ± 0.13^{e}	23.47 ± 1.50^{e}	90.01 ± 1.00^{a}	0.5 ± 0.21^{a}

***Means with different letters are significantly different from each other ^{a,b,c,d,e,f} ($p < 0.05$).**

5.3.2.4 Effect on tolerance indices

The seedlings of *C. roseus* were tested for tolerance to heavy metal and herbicide (BC), using different concentrations of Hg (5-80 ppm) and BC (1-10 ppm), both alone and in combination.

C. roseus showed high percentage of tolerance at 5 ppm of Hg. An increase in Hg concentration (20-80 ppm) gradually decreased the tolerance of *C. roseus*. The treatment of Hg at 80 ppm showed the lowest percentage of tolerance in *C. roseus* as

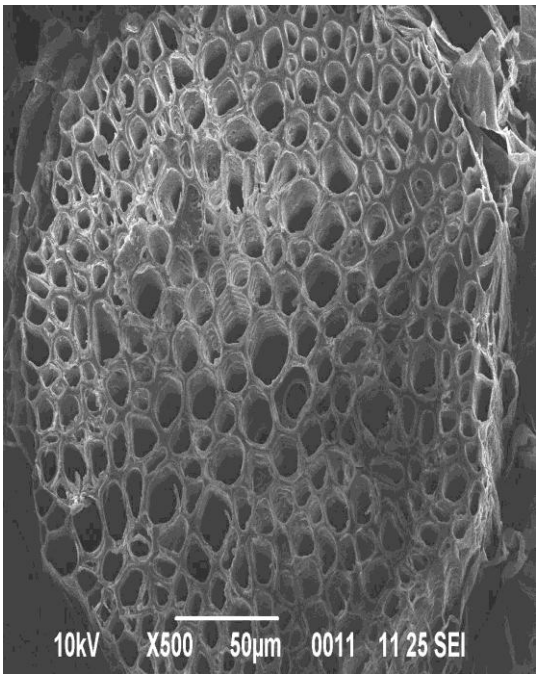
measured on the eight day of exposure as compared to control. The tolerance index was found to decrease from 87.5 to 21.42 as the concentration of Hg increased from 5-80 ppm. However, as compared to joint treatment BC and Hg to Hg alone, better tolerance index was reported in the presence of BC. The tolerance index decreased from 92.59 to 14.28 as the concentration of BC increased from 1-10 ppm. Under combination treatment, the tolerance index was found at par with Hg alone treatment decreasing from 84 to 23.47 as the concentration of Hg in the presence of BC (4 ppm) increased from 5-80 ppm. Hence, according to tolerance test it may be inferred *C. roseus* was more tolerant to BC compared to Hg and combination treatments.

5.3.2.5 Effect on percent phytotoxicity

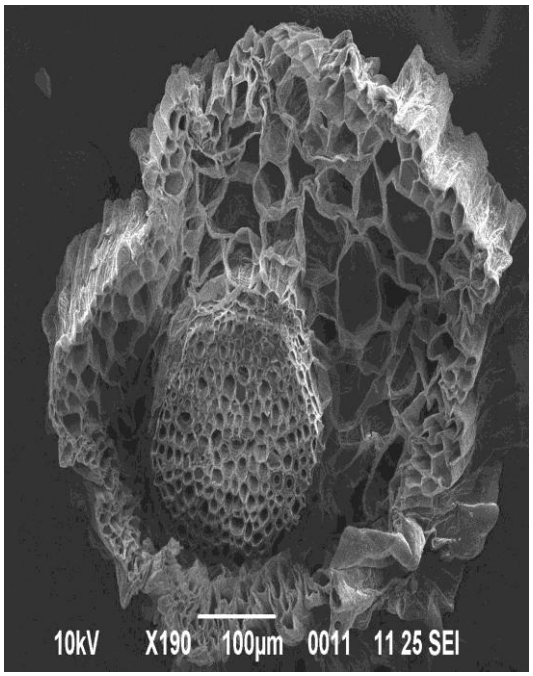
The phytotoxicity of Hg (5-80 ppm) and BC (1-10 ppm), both alone and in combination on root length of *C. roseus* is given in **Table (5.2, 5.5 and 5.6)**. The increase of Hg levels increased significantly the percent phytotoxicity on root length under all treatments ($p<0.05$). The lowest percent phytotoxicity (0.98%) was observed at the lowest concentration of Hg (5 ppm). However, in the presence of BC, it was 5.06% at the lowest concentration (1 ppm). The joint toxicity of Hg and BC revealed 19.04% phytotoxicity at the lowest concentration (5ppm Hg in the presence of 4ppm BC). Results clearly revealed that the joint treatment of Hg and BC was more toxic to root growth followed by the individual treatment of BC and Hg on the radicle length of *C. roseus.*

5.3.2.6 Scanning electron microscopy study (SEM) analysis

The result of Scanning Electron microscope studies indicated the structural deformation in the tissues of root and shoot in comparison to control (**Figure 5.13, 5.14**). The shrinkage in the parenchymatous cells of plant tissues is clearly visible which may be attributed to limitation of nutrient supply and toxic effect of combined treatment.



1(a)



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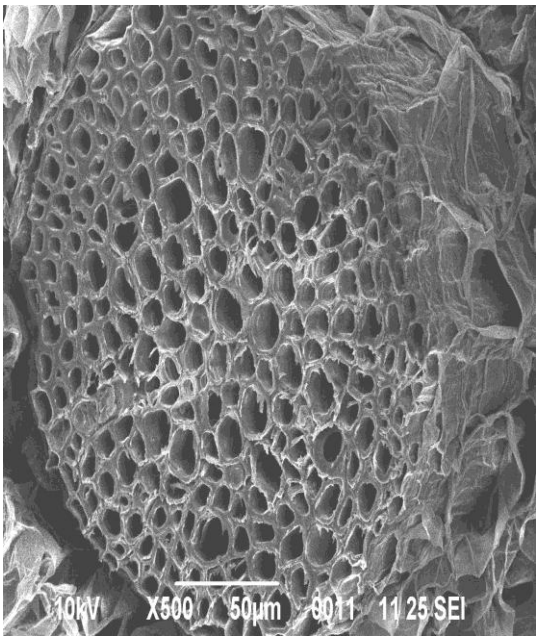
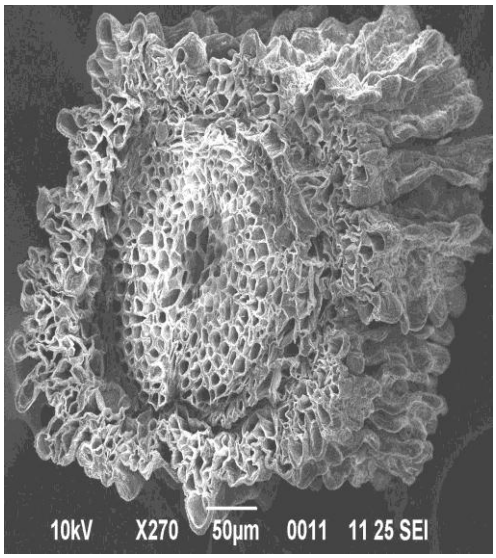


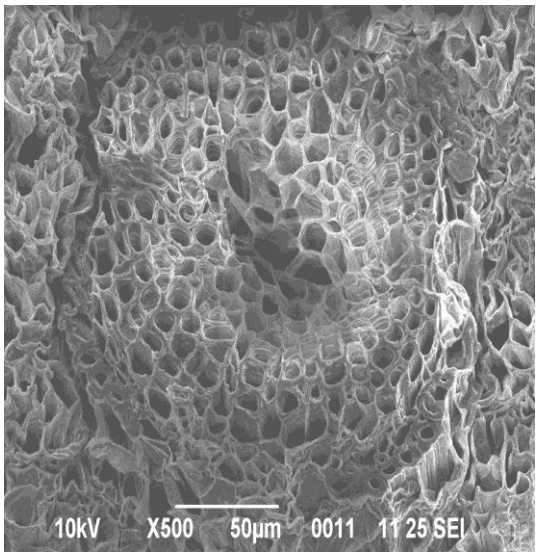
2(a)

2(b)

Figure 5.13: Scanning electron micrograph (SEM) of untreated and treated radicle of *C. roseus* under early seedling growth: 1(a,b) Control; 2(a,b) Hg+BC treated root



1(a)



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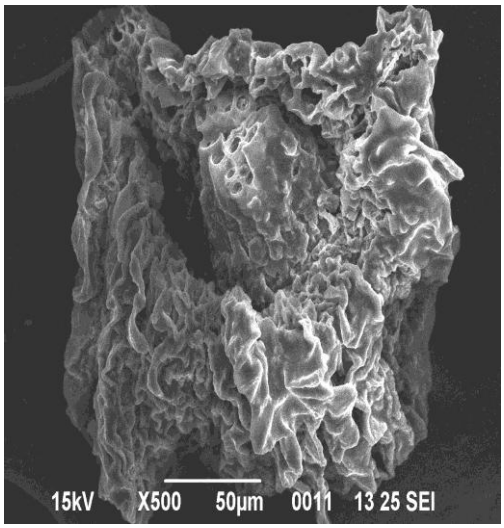

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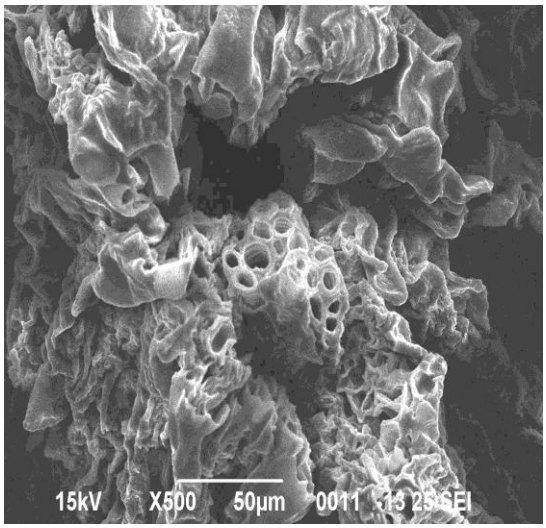


2(b)

Figure 5.14: Scanning electron micrograph (SEM) of untreated and treated plumule of *C. roseus* under early seedling growth: 1(a,b) Control; 2(a,b) Hg+BC treated shoot

5.3.2.7 Toxicity thresholds

The Hg, BC and combination toxicity thresholds were determined for radical and plumule of *C. roseus*. EC 50 is the contaminant concentration that produced 50% inhibition in seedling length (root and shoot) with respect to the control treatment. The EC 50 values for Hg, BC alone and in combination is given in **Table 5.7**. The 50% inhibitory concentration of mercury for radicle and plumule of *C. roseus* was found to be 59.45 and 53.38 ppm, respectively.

A 50% reduction in radicle and plumule length of *C. roseus* in the presence of herbicide BC was recorded at 6.15 and 8.35 ppm respectively. However, The EC 50 for BC + Hg was found to be 37.38 ppm for radicle and 42.34 ppm for plumule.

Results clearly revealed that joint treatment of Hg and BC was more toxic compared to individual toxicities of Hg and BC for both root and shoot. EC 50 of root for Hg was observed at 59.45 ppm which is contrary to the observation of **Ivanov et al (2003)** who reported that Hg at 10^{-6} M inhibited the growth of *Zea mays L.* roots by 50 % reduction in root.

Table 5.7: Effective concentration 50 (EC 50) values for Hg, BC alone and in combination

Treatment	EC-50	
	Radicle	Plumule
Hg (ppm)	59.45	53.38
BC (ppm)	6.15	8.35
BC+Hg (ppm)	37.38	42.34

5.4 Discussion

Filter paper soaked with heavy metal and or herbicide for seed incubation may reduce the effects of any other metals or xenobiotics that might be present in soil under natural conditions, due to their synergistic and/or antagonistic effect (**Munzuroglu & Geckil, 2002**). Heavy metals and herbicides are known to affect the development of plants and can be studied by determining the characteristics of seed germination.

In the present study, the effect of increasing concentrations of Cd (5-120 ppm) and Hg (5-80 ppm) and BC (1-10 ppm) alone and in combination [Cd (5-120 ppm), Hg (5-80 ppm) and BC (4 ppm)] on germination and early seedling growth of *C. roseus* was studied. Results revealed that the individual and combined effect of the heavy metal and herbicide affected both, the percentage of germination and early seedling growth of *C. roseus,* differently.

There was no complete inhibition of germination even for the highest Cd and/or BC concentration used either singly or jointly **(Figure 5.1, 5.2, 5.3).** The joint effect of Cd and BC was found to be more toxic as compared with the individual toxicities of Cd and BC. Seed germination under Cd stress may decrease due to accelerated breakdown of reserved food material in seed embryo **(Ahmad et al, 2012)**. The negative impact of heavy metals on seed germination has earlier been

reported by several researchers (**Sethy & Ghosh, 2013**). It is well documented that the effect of metals on seeds germination may lower the water uptake and transport, causing embryonic damage and/or death. Selection of a specific plant species and metal element or herbicide, either alone or in combination may vary the degree to which the above mentioned toxicities affect negative germination.

Similar results were obtained for root elongation as well. Lower concentration of Cd (5 ppm) and BC (1 ppm) did not seem to affect the root length of *C. roseus* and revealed a marginal decrease of 5.09 and 5.06%, at the respective concentrations compared to control. However, a significant decrease in root length (47.92%) was observed at the lowest concentration under joint toxicity of Cd and BC. The overall results indicated that the joint effect of Cd and BC was more toxic followed by individual treatments of Cd and BC as far as root growth is concerned.

On the contrary the results obtained for shoot length revealed that lower concentration of Cd (5 ppm) and BC (1 ppm) affected a marginal decrease of 4.19% and 0.58% in shoot length of *C. roseus* respectively, as compared to control.

However, under joint treatment of Cd and BC although a 14% decrease was observed in the length of shoot of *C. roseus* at lowest concentration (5 ppm Cd + 4 ppm BC) studied which was similar to Cd treatment alone, rest of the concentration dependent decline was improved. Overall results indicated that combined effect of Cd and BC was more pronounced followed by the application of Cd and BC alone. Similar results were observed by **Cailin et al (2009),** their results confirm the inhibitory effects of Cd and Tri Chloro Benzene on the growth of wheat seedlings.

Roots are the initial part of the plant which comes in touch with any soil and any contamination therein; therefore, they tend to be more sensitive to toxicity compared to shoots (**Magna et al, 2013**). In the present investigation, length of the shoot was found greater than the root length at all concentrations studied, and a greater sensitivity was revealed on the root length (**Khatamipour et al, 2011 ; Subin & Francis, 2013**). An increase in root/shoot ratio observed in the presence of BC and Cd, and under joint toxicities as well, might be attributed to structural and morphological changes in root (less root hairs, thickening of roots) induced by metal and/or herbicide.

The tolerance index of *C. roseus* was significantly reduced under joint application of Cd and BC, compared to individual application of BC and Cd. Results

of vigour index revealed that effect of Cd alone is more pronounced followed by combination treatment and BC alone. Although with the increase in concentration phytotoxicity was found to be increased continuously in Cd and BC either alone or under combined treatment. These findings are in line with **Raziuddin et al (2011)** who reported that Cd stress decreased germination index, vigour index and seed germination, of *Brassica*.

Similarly, the effect of increasing concentrations of Hg (5-80 ppm) and BC (1-10 ppm) alone and in combination Hg (20-80 ppm) and BC (4 ppm) on germination and early seedling growth of *C. roseus* was also investigated. The results obtained showed that the individual and combined effect of the heavy metal and herbicide affected the percentage of germination and early seedling growth of *C. roseus* differently. Similar results were observed by **Karaye et al (2014)** wherein Alachlor and Propachlor herbicides were found to reduce seed germination and other seedling parameters of barley by interfering with metabolic processes related to it.

There was no complete inhibition of germination even for the highest Hg and/or BC concentration used either singly or jointly (**Figure 5.2, 5.9, 5.10**). The joint effect of Hg and BC was found to be more toxic as compared with the individual toxicities of Hg and BC. The negative impact of heavy metals on seed germination has been reported by several authors. The heavy metals affects seeds germination by lowering the water uptake and transport, causing embryonic damage or death **(Gao et al, 2010; Malar et al, 2015)**. Selection of a specific plant species and metal element or herbicide, alone or in combination can vary the degree to which the above toxicities affect negative germination.

Similar results were obtained for root elongation as well. Lower concentration of Hg (5 ppm) and BC (1 ppm) did not seem to affect the root length of *C. roseus* and revealed a marginal decrease of 0.98% and 5%, at the respective concentration compared to control.

However, a significant decrease in root length (21.6%) was observed at the lowest concentration under joint toxicity of Hg and BC. The overall results indicated that the joint effect of Hg and BC was more toxic followed by individual treatments of BC and Hg as far as root growth is concerned.

On the contrary the results obtained for shoot length revealed that lower concentration of Hg (5 ppm) and BC (1 ppm) affected a marginal decrease of 5.96 %

and 0.59 % in shoot length of *C. roseus* respectively, as compared to control. Under joint treatment of Hg and BC a marginal decrease of 0.52 % was observed in the length of shoot of *C. roseus* at lowest concentration (5 ppm Hg+ 4 ppm BC) studied followed by a concentration dependent decline. Overall results indicated that combined effect of Hg+BC was more pronounced followed by the application of BC and Hg alone**.** Similar results for radicle and plumule length was observed by **Cailin et al** (**2009**) for wheat seedlings in presence of Hg and Trichlorobenzene.

A root happens to be the initial part of the plant that comes in touch with any contamination; hence they are likely to be more sensitive to toxicity than shoots. Since the length of the plumule was found to be greater than the root length at all concentrations studied, and a greater sensitivity was revealed on the root length, a decrease in root/shoot ratio observed in the presence of BC and under joint toxicities might be attributed to structural and morphological changes in root (less root hairs, thickening of roots) induced by metal and/or herbicide, which was further affirmed by the SEM micrograph obtained under different treatments. Hg is one of the most toxic heavy metals to living organisms and its conspicuous effect is the inhibition of root growth (**Wang et al, 2013).**

The tolerance index of *C. roseus* was significantly reduced in the presence of BC, but *C. roseus* seemed to be more tolerant to joint application of BC and Hg compared to Hg alone. Highest vigour index was reported in BC followed by combination of BC and Hg and Hg alone. However, a marginal increase in phytotoxicity was recorded under lower concentration of Hg and BC either singly or under combined treatment which was found to increase at higher concentrations. Kumar and Jagannath (2015) also reported similar results for BC phytotoxicity and root-shoot length in different cultivars for maize.

The SEM images under combination treatment (Cd/Hg+BC) showed a significant anatomical difference between root and shoot. This study showed the structural deformation more in shoots as comparison to the root structure, which is contrary to observations of **Godbold and Huttermann (1986)** who highlighted that the structural deformation in root is very prominent than shoot which may have serious consequences for nutrient and water supply to aboveground plant parts.

5.5 Conclusion

This study revealed that Cd, Hg and BC either alone or in combination have differential effects on seed germination and early seedling growth of *C. roseus*. In spite of the fact that, morphologically root was more sensitive to shoot both under single and/or joint toxicities, the SEM micrographs revealed more structural deformation in shoot as compared to roots. Based on EC 50 values it was inferred that the joint effect of Cd and BC was more toxic followed by individual treatments of BC and Cd as far as root and shoot growth is concerned. In case of Hg treatment, based on EC 50 values it was inferred that the joint effect of Hg and BC was more toxic followed by individual treatments of BC and Hg as far as root and shoot growth is concerned.

A differential toxicity of heavy metal and herbicide on seed germination and root and shoot elongation of *C. roseus* under different germination parameters revealed the ability of the plant to germinate and grow in the admixture of both Hg, Cd and BC and its prospective phytoremediation potential.

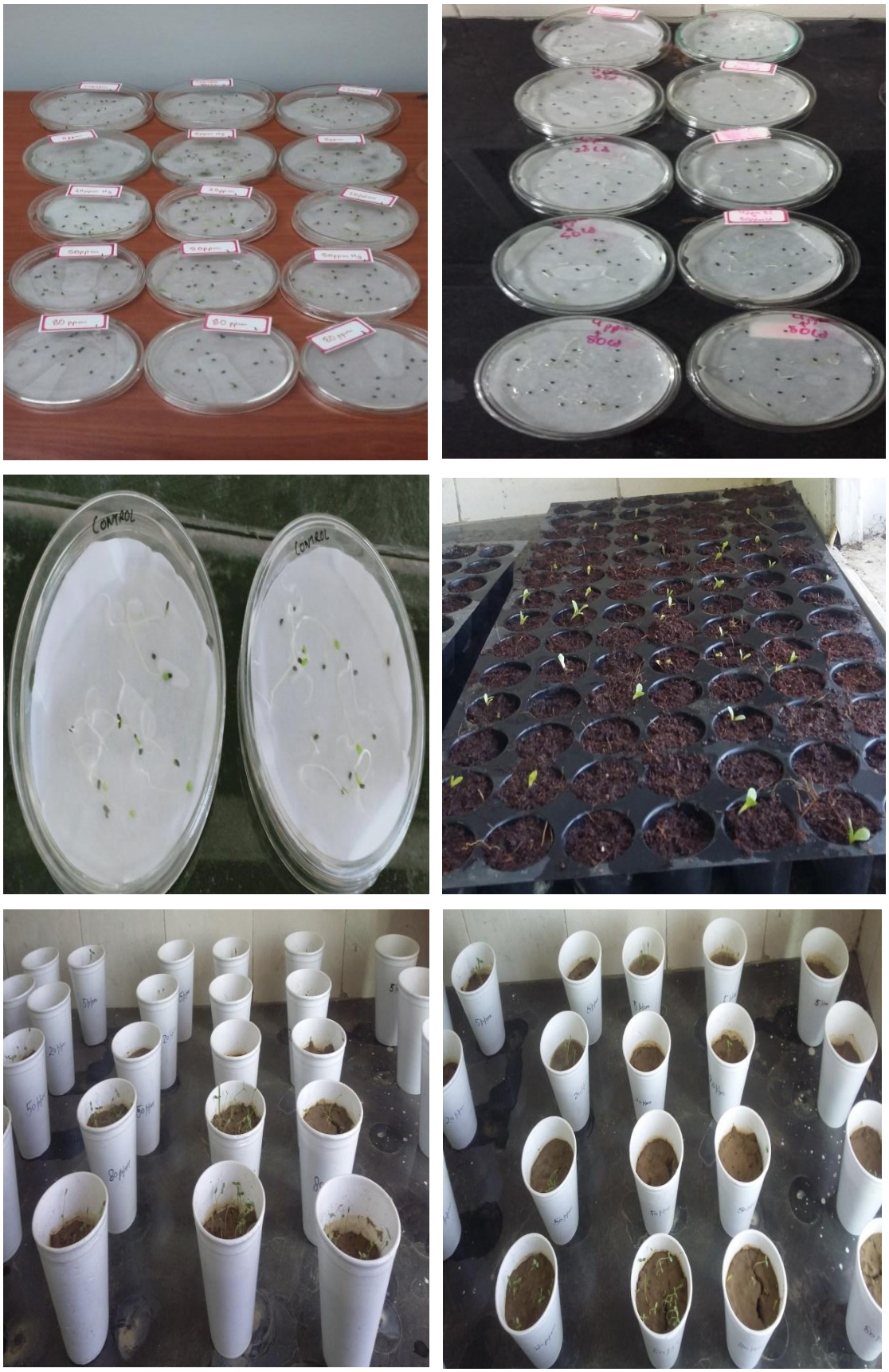


Plate 5.1: Seed germination and early seedling growth of *C. roseus*

Chapter 6

Screening of phytoremediation potential of C. roseus under Cd/Hg and Butachlor co-contamination using biochemical parameters and SEM-EDX analysis

6.1 Introduction

Trace elements, cadmium (Cd) and Mercury (Hg) are two major soil contaminants that mostly results from industrial activities, municipal wastes, irrigation with sewage effluent, paints, dyes and fertilizers (**Zhang et al, 2010**). Both mercury and Cd lack any biological function (**Wang & Shi, 2001**), are nonessential metals, and at high concentrations, are extremely reactive and toxic (**Akinola & Ekiyoyo, 2006**). Contamination by hazardous metals needs immediate reclamation. Large scale remediation practices by physical means are often costly and affect both soil quality and fertility (**Marques et al, 2009**).

Apart from heavy metals, pesticides are also being recognized as global pollutant. Butachlor (BC) (N-(butoxymethyl)-2-chloro-2',6'-diethyl acetanilide) has emerged as a systemic selective pre-emergent herbicide widely applied on beans, tea, wheat, rice and other crops (**Dwivedi et al, 2012**). It is used to manage a wide range of broad leaf weeds and annual grass (**Wang et al, 2013**). Since, plants facilitate the contaminant degradation by creating a favorable environment, besides being economically cheap and environmentally friendly; phytoremediation of potentially toxic metals and pesticides from soil has fascinated significant recent attention (**Cho-Ruk et al, 2006**).

Selection of appropriate plants for phytoremediation purposes needs careful consideration (**Gratao et al, 2005**). The plants should be able to mitigate oxidative stress associated with elevated heavy metals and herbicide levels. Oxidative stress occurs when the balance between the generation and the scavenging of reactive oxygen species (ROS) is disturbed due to external factors like drought, salinity, heavy metals, pesticides, pollution etc. (**Miller et al, 2010**).

In plants, the effect of oxidative stress can be appraised by malondialdehyde (MDA), an outcome of membrane lipid peroxidation that is generated by Cd and Hg (**Cuypers et al, 2011**) as well as the herbicide (BC). In plants, the primary ROS scavenging system includes antioxidative enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), guaiacol peroxidase (GPx, EC 1.11.1.7) and ascorbate peroxidase (APX, EC 1.11.1.11), and several non-enzymatic antioxidants (**He et al, 2011**). Further, the soil Cd/Hg or Butachlor enters the plant primarily via root; therefore, it is worthwhile to study the root anatomy. Due to explicit features of root cell tonoplast, plants promptly and proficiently translocate

metals to the shoot, via the xylem (**Seregin & Kozhevnikova, 2008**). Hence, plant leaf might serve as one of the final destinations for these metals.

Use of ornamental plants in remediation of metal and or herbicide infested soil is now very widely accepted (**Nakbanpote et al, 2016**). Such plants have practical value due to very little food chain contamination, apart from generating revenues; they also beautify the area (**Nakbanpote et al, 2016**). *C roseus* is naturalized and widely cultivated in tropical and subtropical areas of the world. It is an evergreen sub-shrub or herbaceous plant. The genus *Catharanthus* has wide assemblage of over 100 alkaloids including ajmalicine, serpentine, ajmaline, vincristin, and vinblastin which are extremely important (**Pandey et al, 2007**). It is having long flowering period, throughout the year in tropical conditions (**Subhashini & Swamy, 2017**). Some reports are available on Cd remediation potentials of *C. roseus* as evaluated by **Marchiol et al (2004)**.

However no reports are available on remediation potential of the selected plant species under co-contamination. In the aforesaid context the present objective aims to study whether heavy metal and herbicide induced stress affects the antioxidant defense system, under both single (heavy metals) and/or joint toxicities (heavy metals and herbicide).

However, the remediation property of this plant or the biochemical and physiological responses of this plant to elevated levels of either Cd or Hg in the presence of broad spectrum herbicide BC are largely unknown. Hence, this study is an attempt to analyze the effects of Cd and Hg alone and in combination with selected herbicide (BC) on physiological and biochemical responses of treated plants. Scanning electron microscopic (SEM) analysis in the presence of heavy metal and herbicide on *C. roseus* root, shoot and leaf will provide any anatomical changes during stress. The contrast between two highly toxic metals Cd and Hg in the presence of BC will help to understand the mechanism underlying their accumulation, as well.

6.2 Materials and Methods

6.2.1 Preparation of heavy metals stock solutions

A high purity analytical grade of Cd and Hg standard solutions were used as the source of Cd and Hg stock solutions. The required solutions were prepared using

analytical reagents and double-distilled water. A single stock standard solution from each studied metal was used to contaminate the soil with Cd and Hg at different concentrations of 25, 50, 100, 150, 200, and 20, 40, 80, 100, 120 $mgkg^{-1}$ respectively.

6.2.2 Preparation of herbicide stock solutions

BC (Butachlor, $C_{17}H_{26}ClNO_2$) 50EC was purchased from a local certified dealer Neelkanth Agroforestry, Kaiserbagh, Lucknow. A standard stock solution was prepared from the original herbicide and was used to prepare solution of different $mgkg^{-1}$concentrations. Then, soil was spiked with BC by dissolving stock of BC in 25 mL of acetone and 0.5,1, 2, 4, and 8 $mgkg^{-1}$ treatment of BC was prepared. The solution of acetone and BC was transferred into 250 g of soil as a portion and then mixed with remaining soil untill the acetone had volatilized completely in the fume hood. 25 mL of acetone was also added to control and other soil treatments. 25-200 $mgkg^{-1}$ and 20-120 $mgkg^{-1}$ of Cd and Hg respectively were added in BC (4 $mgkg^{-1}$) spiked soils. Through EC 50 values concentration of BC 4 $mgkg^{-1}$ was observed suitable for the co-contamination study. The spiked soil was thoroughly mixed by sieving and stored in a dark room for equilibration before planting.

6.2.3 Estimation of chlorophyll, carotenoid and carbohydrate contents

Detailed methodology is given in Chapter 3 (Materials & Methods). Please see Section 3.6.1 & 3.6.2.

6.2.4 Protein and total phenol estimation

Chapter 3 (Materials and Methods) Section 3.6.5 and 3.6.6.2 is referred for more details.

6.2.5 Determination of lipid peroxidation (LPO) and proline

Proline was determined according to the procedure described by **Bates et al (1973)**. The level of lipid peroxidation products in leaf samples was expressed as malondialdehyde (MDA) content and was determined by **Heath and Packer (1968)**. Please see Section 3.6.3 and 3.6.4 of Chapter 3 (Materials & Methods) for details.

6.2.6 Estimation of antioxidant enzymes

The activity of SOD was assayed according to the method of **Beauchamp and Fridovich (1971)**. The CAT activity was determined according to the method given

by **Aebi (1984)**. POD activity was determined according to the method given by **Putter (1974)**. APOX activity was assayed according to the protocol given by **Nakano and Asada (1981)**. GR activity was estimated by according to the procedure given by **Carlberg and Mannervik (1975)**. The detailed methodologies for estimation of these parameters are given in Chapter 3 (Materials and Methods) Section 3.6.6.3 to 3.6.6.7.

6.2.7 Scanning electron microscopy coupled with energy-dispersive X-ray microanalysis (SEM-EDX)

Root and shoot specimens were prepared for SEM study using the protocol adapted from standard procedures **(O'Brien and McCully 1981)**, for details please refer Chapter 3 (Materials & Methods), Section 3.7.

6.2.8 Stastical Analysis

The statistical design was completely random. The graphs were drawn by using EXCEL, and the statistical tests were performed using SPSS software (SPSS Inc., version 25.00). The Duncan method was applied in order to compare the means ($p<0.05$) of the obtained data and one-way analysis of variance.

6.3 Results

6.3.1 Effects of Cd, Hg and BC alone and in combination on the photosynthetic pigment of *C. roseus*

Cd and Hg induced a substantial decrease in chlorophyll a (*Chl a*), Chlorophyll b (*Chl b*), total chlorophyll and carotenoid contents in *C. roseus* (**Figure 6.1**).

The total chlorophyll contents of control plant was 0.664 mg g^{-1} fresh weight (n=3), for the plants treated with different concentrations of Cd (25-200 mg kg^{-1}), an initial increase in total chlorophyll values were observed upto 50 mg kg^{-1} Cd and then substantial drop in total chlorophyll contents was found with increasing concentration (100-200 $mgkg^{-1}$). A 72.44% increase was recorded at 25 $mgkg^{-1}$ of Cd while maximum decrease (62.05%) was observed at 200 $mgkg^{-1}$ of the metal as compared to control. Carotenoid content for sole Cd treatments showed an increase upto 100 $mgkg^{-1}$ (*i.e.*, 18.43%) while maximum decrease (7.83%) was recorded at 200 $mgkg^{-1}$, with respect to control (**Figure 6.1**).

At 20, 40, 80, 100 and 120 mg kg^{-1}, Hg treatments no significant effect ($p<0.05$) was observed on total chlorophyll content. Results showed an increase in total chlorophyll content for Hg treatments from 20 to 100 $mgkg^{-1}$ being 6.17% at 100 $mgkg^{-1}$ and marginal decrease (8.88%) at 120 $mgkg^{-1}$, with respect to control. Overall Hg treatment did not show a significant difference in total chlorophyll contents, as compared to that of control, predominantly due to *Chl b*, which resisted Hg stress at this dose. Though both *Chl a* and *b* are involved in photosynthesis, increased levels of *Chl b* have been shown to suspend senescence, paving the way to a longer period of growth in plants (**Scheumann et al, 1999**). On the other hand, carotenoids content for individual Hg treatment did not show any significant effect with the increasing concentration as compared to control (**Figure 6.2**).

Increased concentration of BC inhibited the photosynthetic pigment as well as the carotenoid contents. Chlorophyll and carotenoid contents showed a decrement of 28.01% & 43.31%, respectively at lowest concentration of BC (0.5 $mgkg^{-1}$) while a decrement of 87.95% and 88.02%, respectively were recorded at highest concentration of BC (8.0 $mgkg^{-1}$) as compared to control. *Chl a* and *Chl b* content also declined (89.18 and 84.97%), respectively, with the increase in concentration (25-200 $mgkg^{-1}$) respectively as compared to control (**Figure 6.3**).

Effect of the joint treatment of Cd (25-200 $mgkg^{-1}$) and BC (4 $mgkg^{-1}$) on chlorophyll and carotenoid content of *C. roseus* is given in **Figure 6.1.** Results showed a decrease of 81.17% in total chlorophyll and 81.56% in carotenoid content at highest concentration (200Cd+4BC) of metal, herbicide co-contamination, which might be attributed to the possibility of increased stress due to co-contamination. Cd in presence of BC showed a decrease in both *Chl a* and *Chl b* content with increase in concentration.

The joint toxicities of Hg (20-120 $mgkg^{-1}$) and BC (4 $mgkg^{-1}$) showed an increase in total chlorophyll content upto 40 Hg+4BC (*i.e*., 4.36%) and then there was a sharp decline observed upto 120 Hg+4BC (*i.e*., 60.24%). Similar results were also observed for *Chl a* and *Chl b*. Carotenoid content showed a decrease with the increasing concentration of Hg (20-120 $mgkg^{-1}$) under co-contamination with BC (4 $mgkg^{-1}$) being 25.34% to 58.98%, respectively for both the doses (**Figure 6.2**).

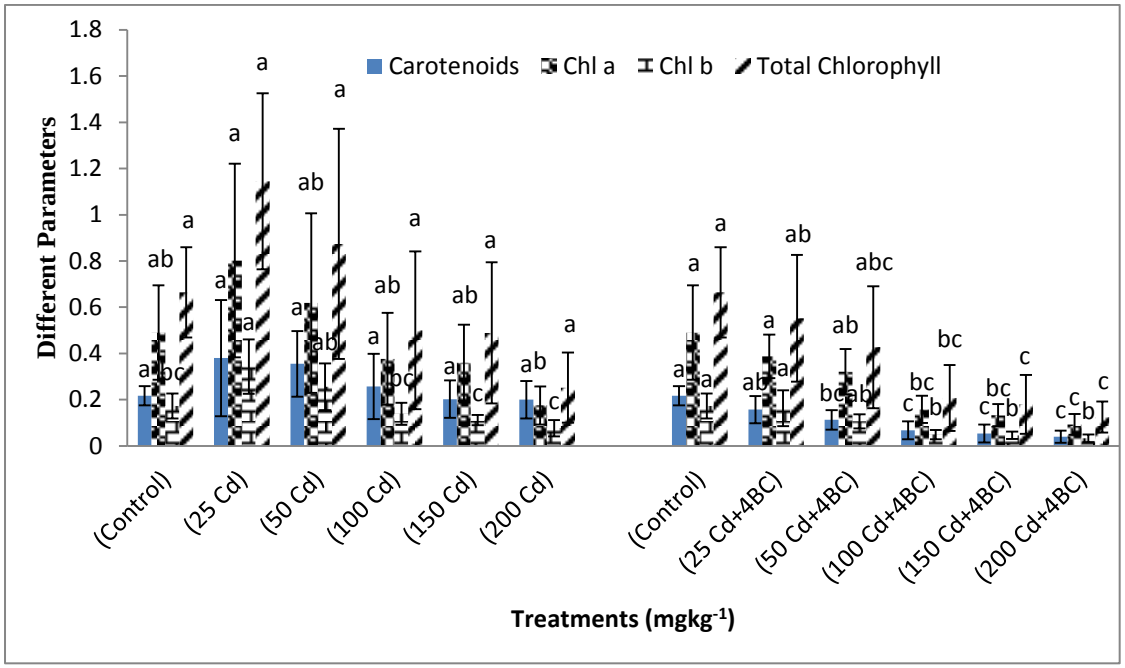


Figure 6.1: Effect of Cd alone and Cd+4BC on photosynthetic pigments of leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

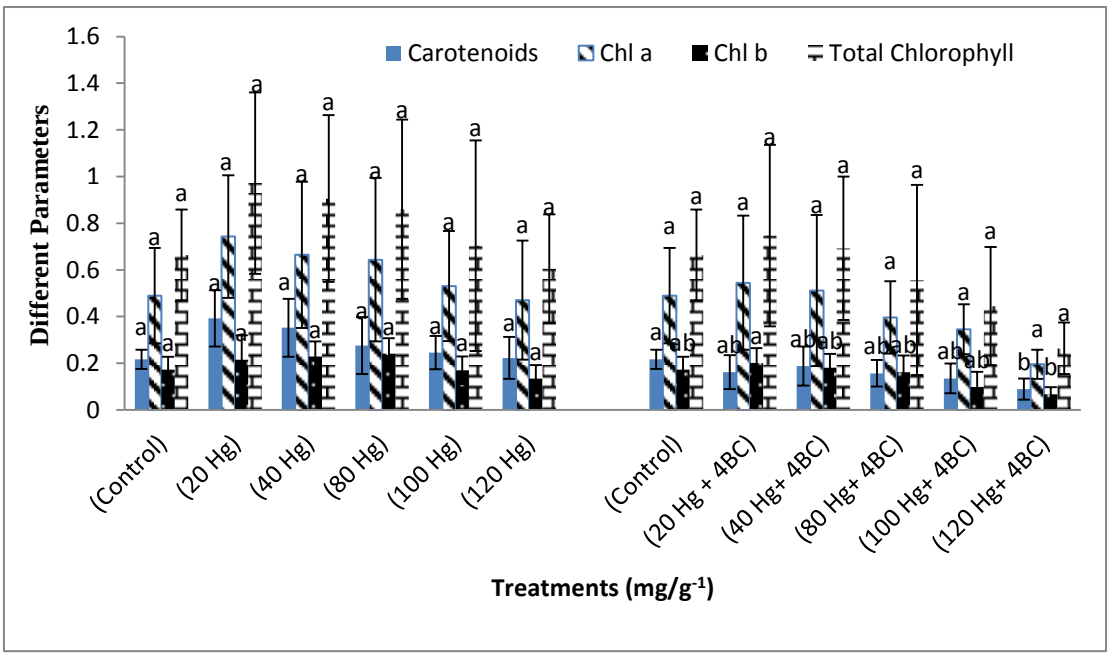


Figure 6.2: Effect of Hg alone and Hg+4BC on photosynthetic pigments of leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

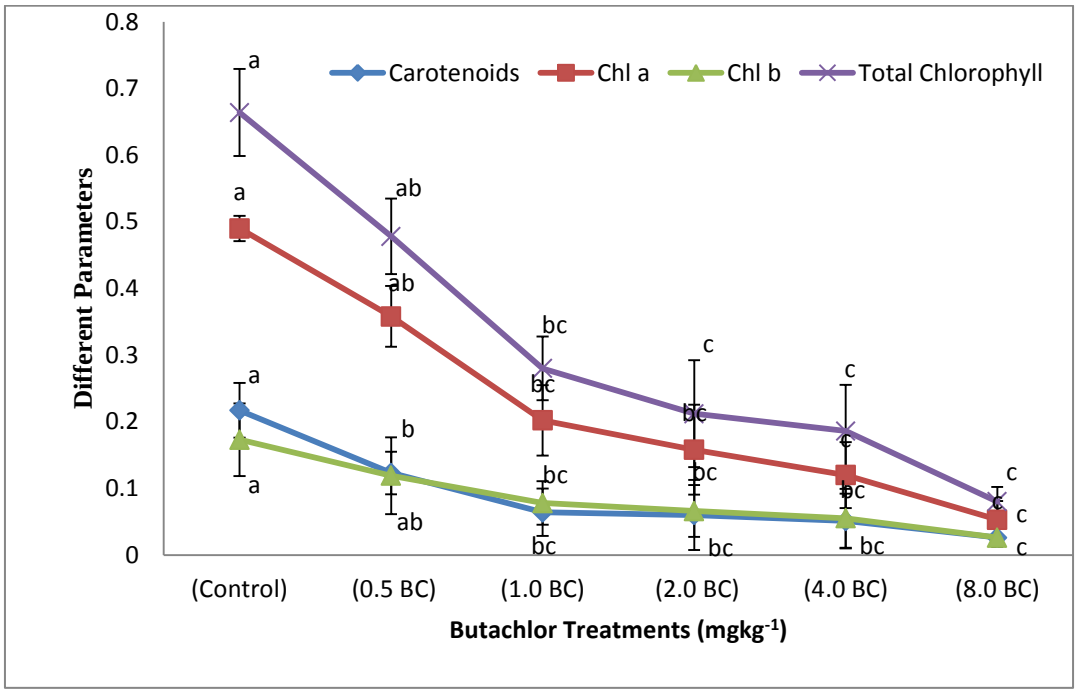


Figure 6.3: Effect of BC on photosynthetic pigments of leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

6.3.2 Effects of Cd, Hg and BC alone and in combination on the carbohydrate, protein, total phenol and proline contents of *C. roseus*

The effect of heavy metal and/or herbicide on the carbohydrate, protein, total phenol and proline contents were studied in the leaves *C. roseus* grown in different concentration (mgkg^{-1}) of $CdNO_3$, Hg_2Cl_2 and BC. The control was maintained with distilled water.

Carbohydrate Content: The carbohydrate content showed a decline (23.81% to 77.30%) with increasing concentration of Cd (50-200 mgkg^{-1}, respectively) with respect to control, except at 25 mgkg^{-1} of Cd (**Figure 6.4**). The present results for Hg alone have showed a marginal increase in carbohydrate content *i.e.* 29.99% upto 40 mgkg^{-1} which then reduced to 33.13% at 120 mg kg^{-1} (**Figure 6.5**) as compared to control. Results revealed that in the presence of BC alone there was an initial increase in carbohydrate content up to 1 mgkg^{-1} (1.51%) followed by a decrease in content as recorded at 8 mgkg^{-1} (18.74%,), in comparison to control (**Figure 6.6)**.

While under co-contamination of Cd (25-200 mgkg^{-1}) and BC (4 mgkg^{-1}) with the increase in concentration of Cd from 25-150 mgkg^{-1} there was decrease (48.17% to 9.06%, respectively) in carbohydrate content except at Cd (200 mgkg^{-1}), with respect to control. Results revealed that joint treatment (Cd and BC) as compared to

alone treatment (Cd) did not show any significant effect on overall carbohydrate content (**Figure 6.4**). Under joint toxicity of Hg (20-120 $mgkg^{-1}$) and BC (4 $mgkg^{-1}$) the carbohydrate content was found to decline upto 80 $mgkg^{-1}$ and then showed a marginal increase up to highest concentration (120 $mgkg^{-1}$), respectively to control (**Figure 6.5**).

Protein: The protein content of Cd, Hg and BC both alone and in combination on *C. roseus* is given in **Figures** (**6.4 - 6.6**). In the presence of Cd alone with the increase in concentration (25-200 $mgkg^{-1}$) a significant decline in protein values (*i.e.*, 28.43 to 57% respectively) was observed compared to control as shown in (**Figure 6.4)**. Results revealed that Hg alone treatment induced a decrease of 1.09 and 1.74 fold at 20 and 120 $mgkg^{-1}$, respectively, as compared to control (**Figure 6.5**). However, in the presence of BC alone there was a significant decline in protein content from 1 $mgkg^{-1}$ (4.25 %) to 8 $mgkg^{-1}$ (53.82 %) of BC was observed with respect to control (**Figure 6.6**).

While under combined treatment of Cd (25-200 $mgkg^{-1}$) and BC (4 $mgkg^{-1}$) the values of protein increased up to 28.86% at 50Cd+4BC which then showed a decline with respect to control (**Figure 6.4**).Co-contamination of Hg (20-120 $mgkg^{-1}$) and BC (4 $mgkg^{-1}$) showed a significant ($p<0.05$) decline in protein content from 2.74 to 42.64 % respectively at 20-120 $mgkg^{-1}$ Hg in the presence of BC (**Figure 6.5**). Overall results revealed higher degradation of protein content in the leaves of *C. roseus* in response to Cd, Hg and/or BC toxicity.

Proline: Proline content in the leaves of the selected plant species was found to be increase significantly ($p<0.05$) with increase in Cd, Hg and/or BC alone or combined contamination (**Figures 6.4 - 6.6**).

In the presence of single treatment of Cd the proline content showed a 2.11 to 4.91 fold increase at 25 and 200 $mgkg^{-1}$, respectively, to control. While alone Hg treatment showed a significant ($p<0.05$) increase in proline concentration from 20 $mgkg^{-1}$ Hg (69.35%) to 120 $mgkg^{-1}$ Hg (372.98%) as compared to control. Alone BC have showed an increase in the values of proline from 0.98 to 8.96 fold at 0.5 and 8.0 $mgkg^{-1}$ concentration of BC.

While co-contamination of Cd/Hg along with BC showed a significant ($p<0.05$) increase in proline content. At Cd 25 and 200 $mgkg^{-1}$ along with BC 4 $mgkg^{-1}$ there was an increase of 2.76 and 8.28 fold respectively, in proline content as

compared to control (**Figure 6.4**). Likewise, under joint toxicity of Hg (20 and 120 $mgkg^{-1}$) in presence of BC (4 $mgkg^{-1}$) there was an increase of 1.84 and7.88 fold, respectively, to control (**Figure 6.5**).

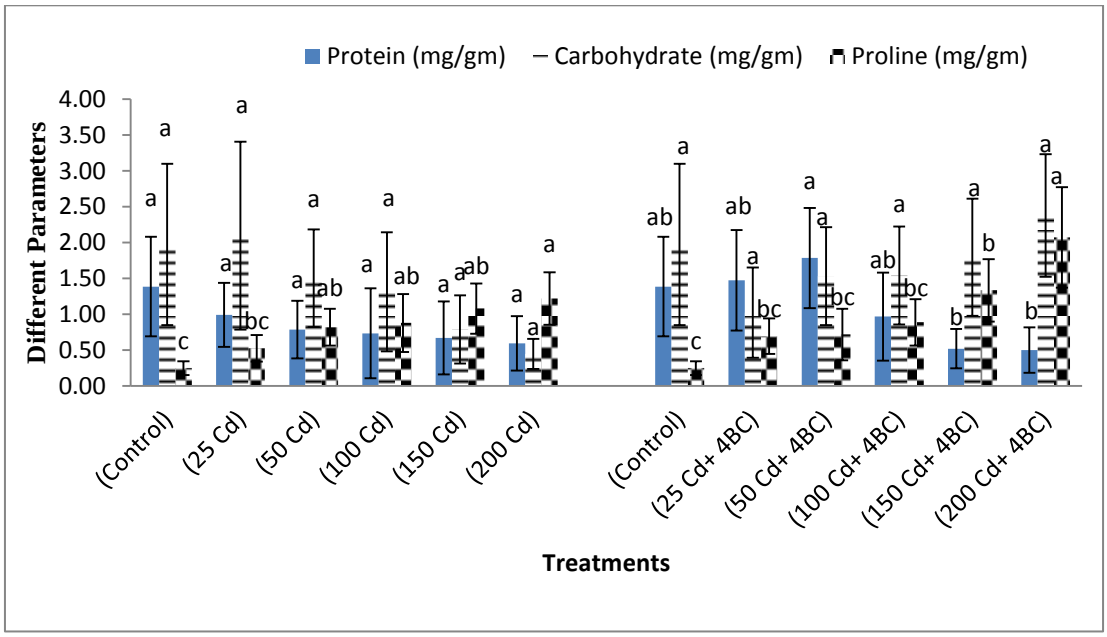


Figure 6.4: Effect of Cd alone and Cd+4BC in combination on protein, carbohydrate and proline content on leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

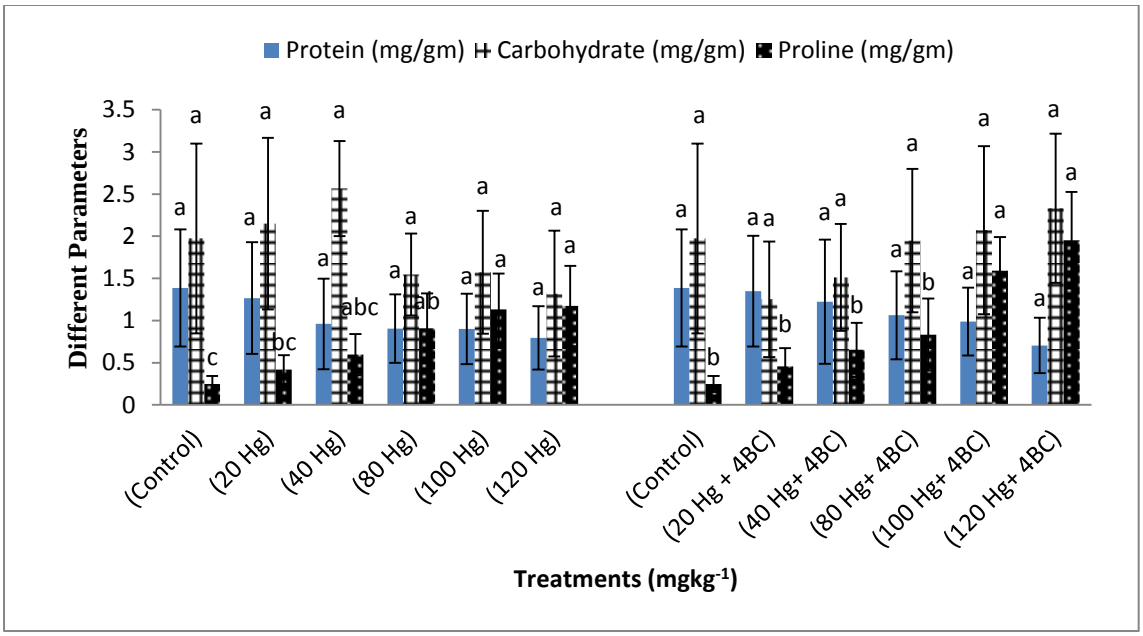


Figure 6.5: Effect of Hg alone and Hg+4BC in combination on protein, carbohydrate and proline content on leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

Overall studies revealed that co-contaminated treatments have higher proline level as compared to the individual treatments. Moreover, an increase in proline

accumulation was revealed with the increase in protein degradation at respective concentrations (**Chen et al, 2001**).

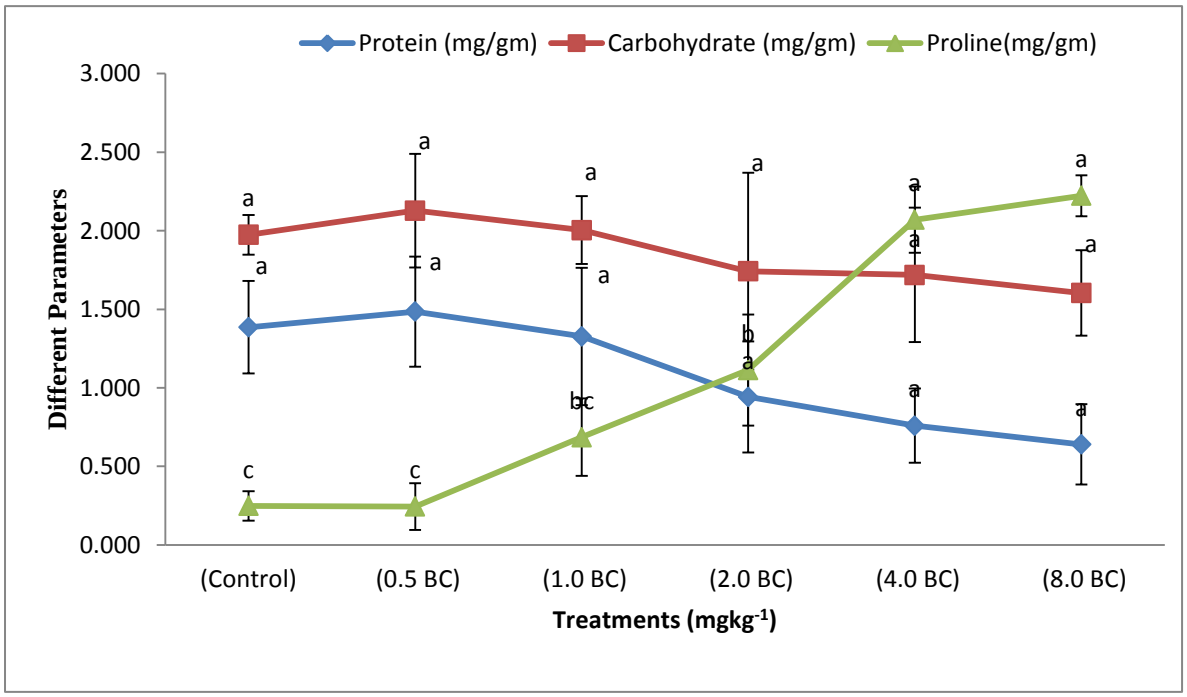


Figure 6.6: Effect of BC on protein, carbohydrate and proline content on leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

Total Phenol: *C. roseus* total phenolic content were tested for tolerance to heavy metal and herbicide (BC), using different concentrations of Hg (20-120 $mgkg^{-1}$), Cd (25-200 $mgkg^{-1}$) and BC (0.5-8.0 $mgkg^{-1}$), both alone and in combination.

The treatment of Cd alone revealed that there was no significant difference in total phenolics content with the increase in concentration. There was an increase in total phenolics content with the increasing concentrations upto 150 $mgkg^{-1}$ Cd (29.54%) which further showed a decrement of 56.30% at 200 $mgkg^{-1}$ Cd, as compared to control (**Figure 6.7**).

Similar trend as of Cd was also observed in Hg alone treatments. Results revealed an increase in total phenolics content with the increasing concentration from 20-100 $mgkg^{-1}$ Hg (42.35 to 6.89%, respectively) while at 120 $mgkg^{-1}$ a 5.25% decrease in phenolic content was observed, in comparison to control (**Figure 6.8**).

In the presence of BC alone there was an increase in phenolics content upto 2.0 $mgkg^{-1}$ (25.50%), and then a marginal decrease in its content was observed at higher concentration *i.e.*, 8.0 $mgkg^{-1}$ (35.24%) with respect to control (**Figure 6.9**).

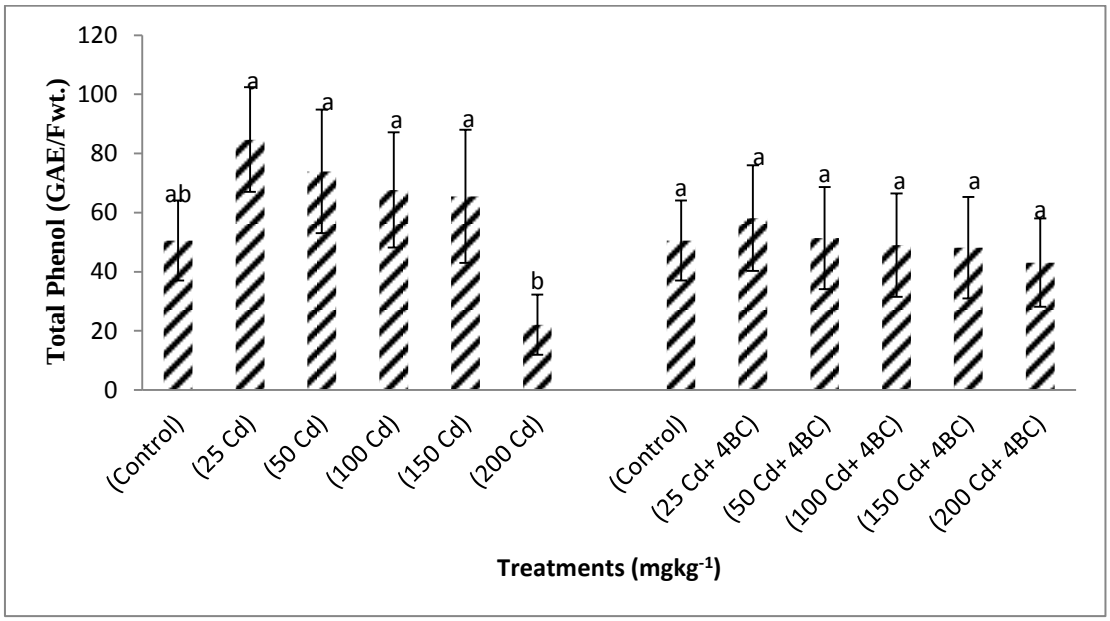


Figure 6.7: Effect of Cd alone and Cd+4BC in combination on total phenol content on leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

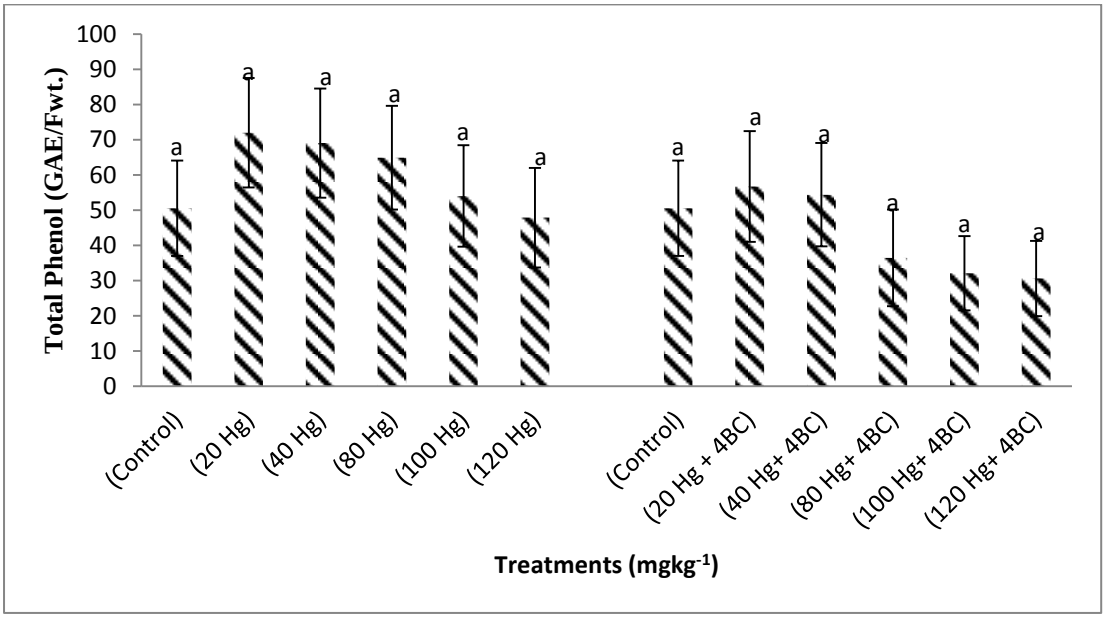


Figure 6.8: Effect of Hg alone and Hg+4BC in combination on total phenol content on leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

However, Cd in the presence of BC showed an increase in phenolics upto 50 mgkg^{-1} Cd (1.01 fold) and then showed a decrement at 200 mgkg^{-1} (1.17 fold), in comparison to control (**Figure 6.7**).

Similar results were observed for Hg in the joint presence of BC. The increase in phenolics contents was observed at 40 mgkg^{-1} Hg+ 4 mgkg^{-1} BC (7.61%) while at 120 mgkg^{-1} there was a decrease (39.39%) in total phenolics (**Figure 6.8**). Results

revealed that co-contamination poses more stress to the plants which may be the cause of lower phenolic content under joint treatment as compared to the individual treatment.

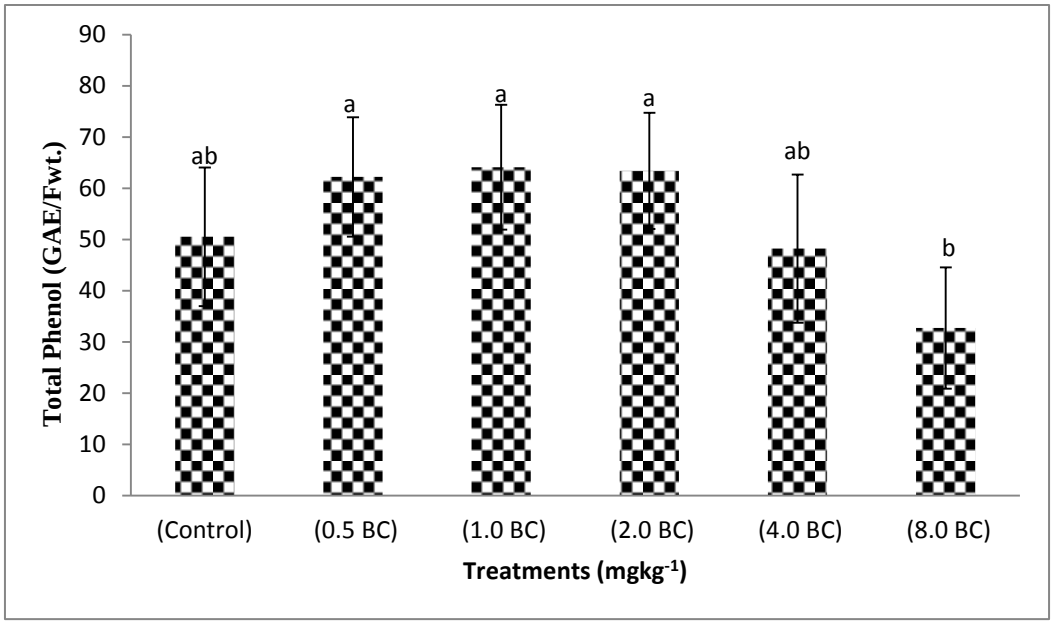


Figure 6.9: Effect of BC on total phenol content on leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

6.3.3 Effects of Cd, Hg and BC alone and in combination on Lipid Peroxidative Damage (LPO)

An estimate of MDA contents served as an excellent indicator of Cd/Hg and/or BC-induced oxidative stress in *C. roseus* (**Figure 6.10-6.12**).

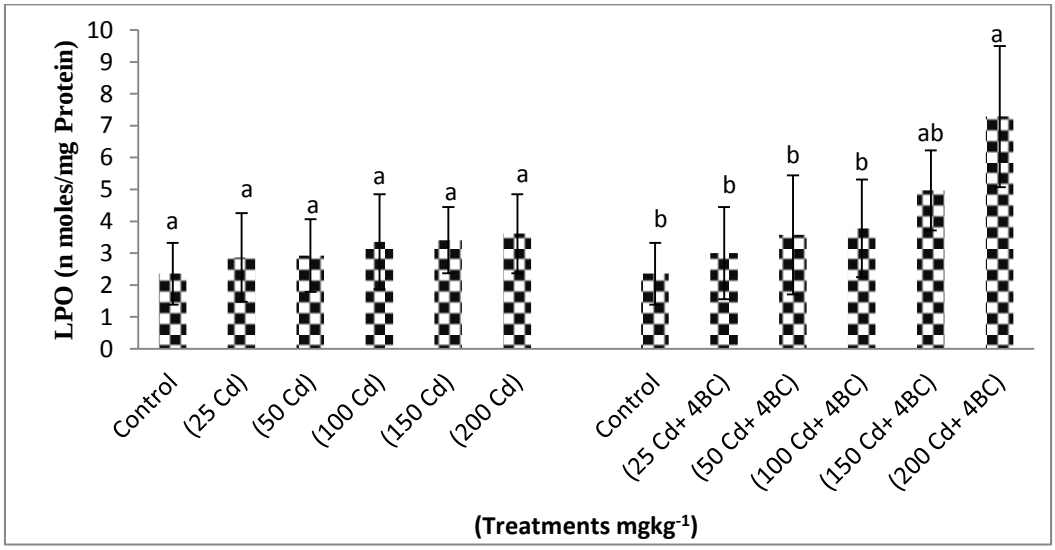


Figure 6.10: Effect of Cd alone and Cd+4BC on lipid peroxidase content of leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

In 25, 50, 100, 150 and 200 mg kg^{-1} Cd, the leaf MDA contents increased by 21.55, 24.10, 42.34, 44.50 and 53.29 %, respectively as compared to control. Similarly Cd (25-200 $mgkg^{-1}$) in the presence of BC (4 $mgkg^{-1}$) augmented MDA contents by 27.24, 51.46, 60.20, 110.94 and 209.16 %, with respect to control (**Figure 6.10**).

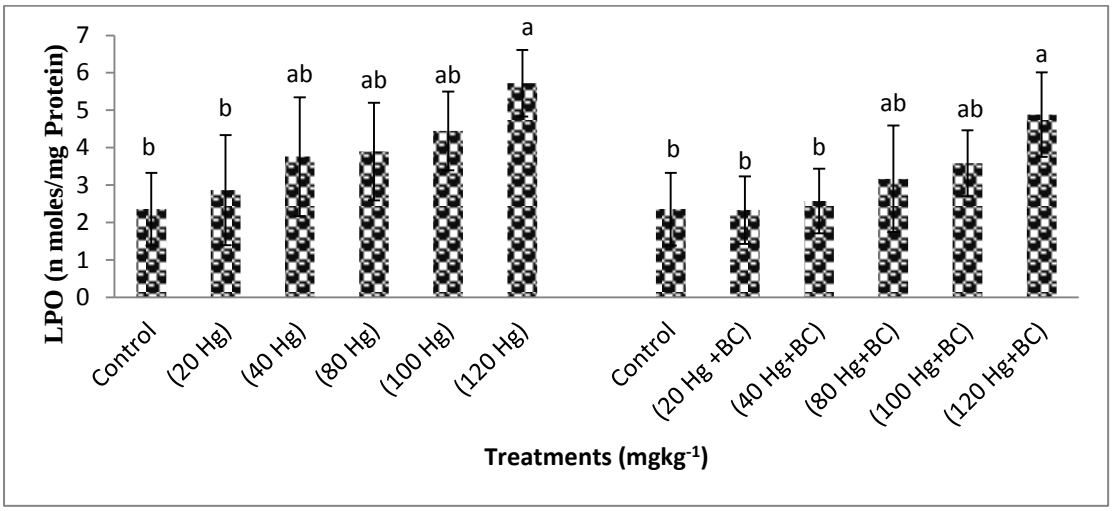


Figure 6.11: Effect of Hg alone and Hg+4BC on lipid peroxidase content of leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

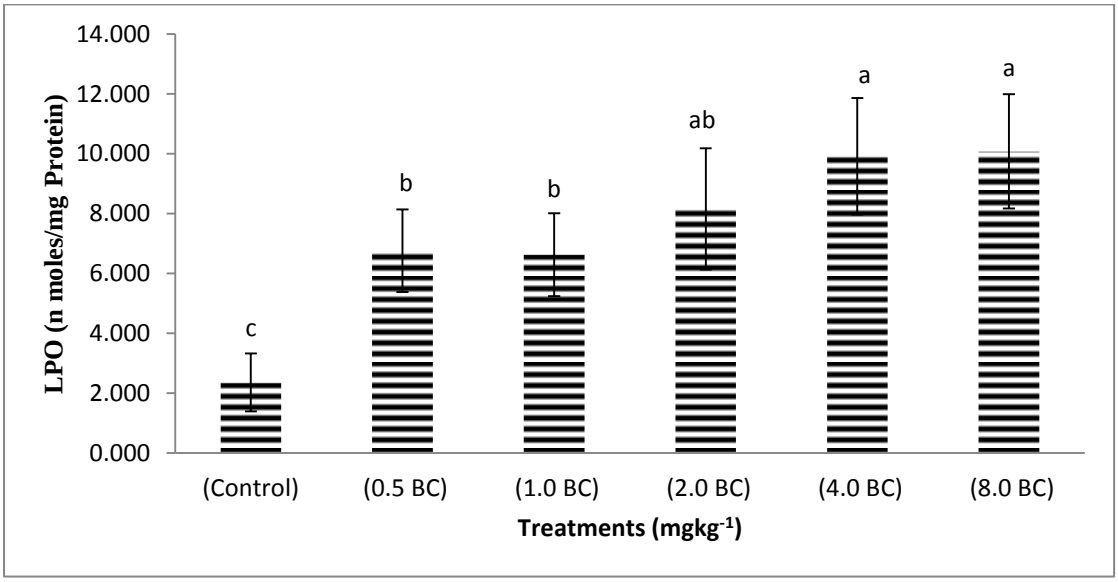


Figure 6.12: Effect of BC on lipid peroxidase content of leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

However, in the presence of 20, 40, 80, 100 and 120 $mgkg^{-1}$ Hg, the leaf MDA augmented by 21.55, 59.48, 65.34, 88.67 and 142.68 % in comparison to control. Likewise, the combined treatment of Hg (40-120 $mgkg^{-1}$) along with BC (4 $mgkg^{-1}$)

showed an increased in LPO content by 9.12, 34.32, 52.18 and 107.30 % respectively to control (**Figure 6.11**).

While BC alone treatments have significant ($p<0.05$) effect on LPO contents. Results revealed an increase in LPO contents ranging from 186.50-327.36 %, with respect to control (**Figure 6.12**), for lowest (0.5 $mgkg^{-1}$) and highest (8.0 $mgkg^{-1}$) concentration of BC respectively.

6.3.4 Effects of Cd, Hg and BC alone and in combination on Antioxidant enzyme activities

The activities of enzymes involved in the mitigation of oxidative stress in *C. roseus* are given in **Figure 6.13-6.21**.

CAT Activity: CAT activity has been shown in **Figure 6.13-6.15** with all the doses of Cd and Hg alone and in the presence of BC.

At 25, 50, 100, 150 and 200 mg kg^{-1} Cd, CAT activity was found to decrease by 2.60, 2.27, 2.11, 1.51, and 1.24 folds, respectively as compared to control (**Figure 6.13**). While Hg (20-120 mg kg^{-1}) alone treatment decreased CAT activity by 1.318 and 2.52 folds respectively at lowest and highest concentration studied, over control (**Figure 6.14**).

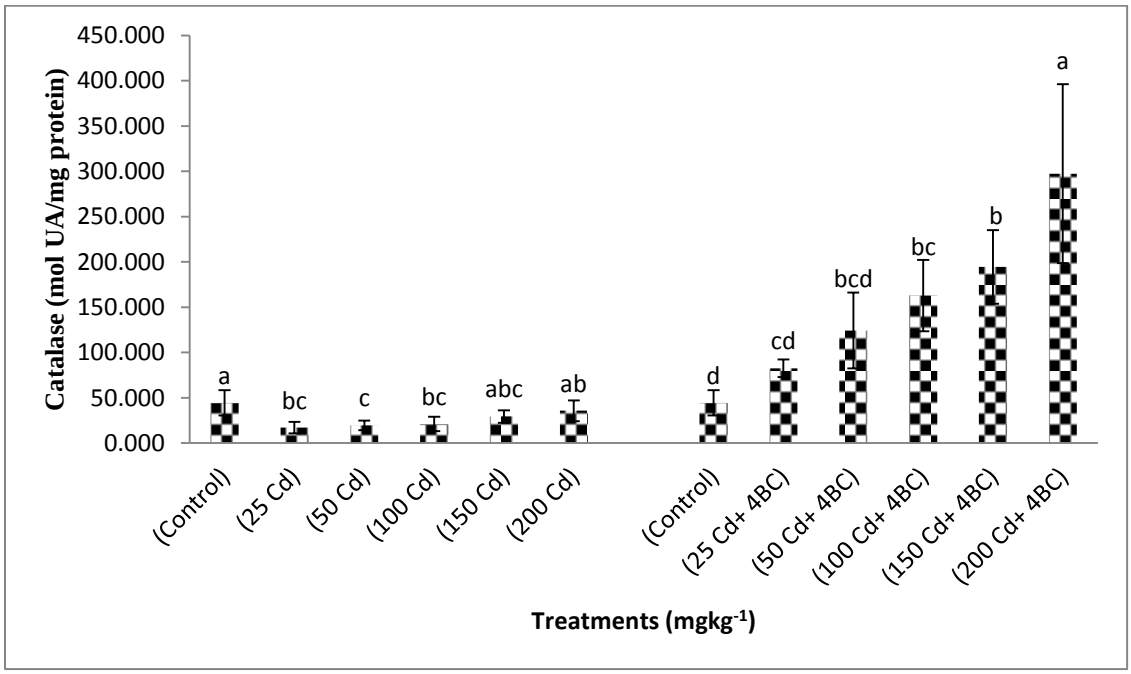


Figure 6.13: Effect of Cd alone and Cd+4BC in combination on catalase activity of leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

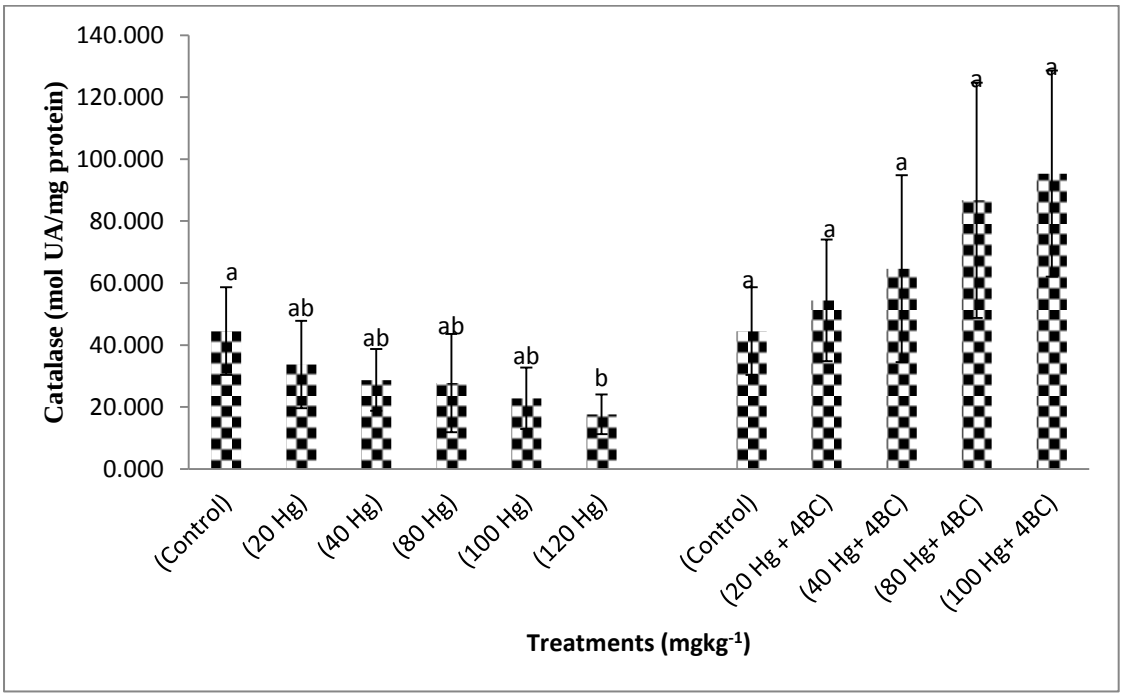


Figure 6.14: Effect of Hg alone and Hg+4BC in combination on catalase activity of leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

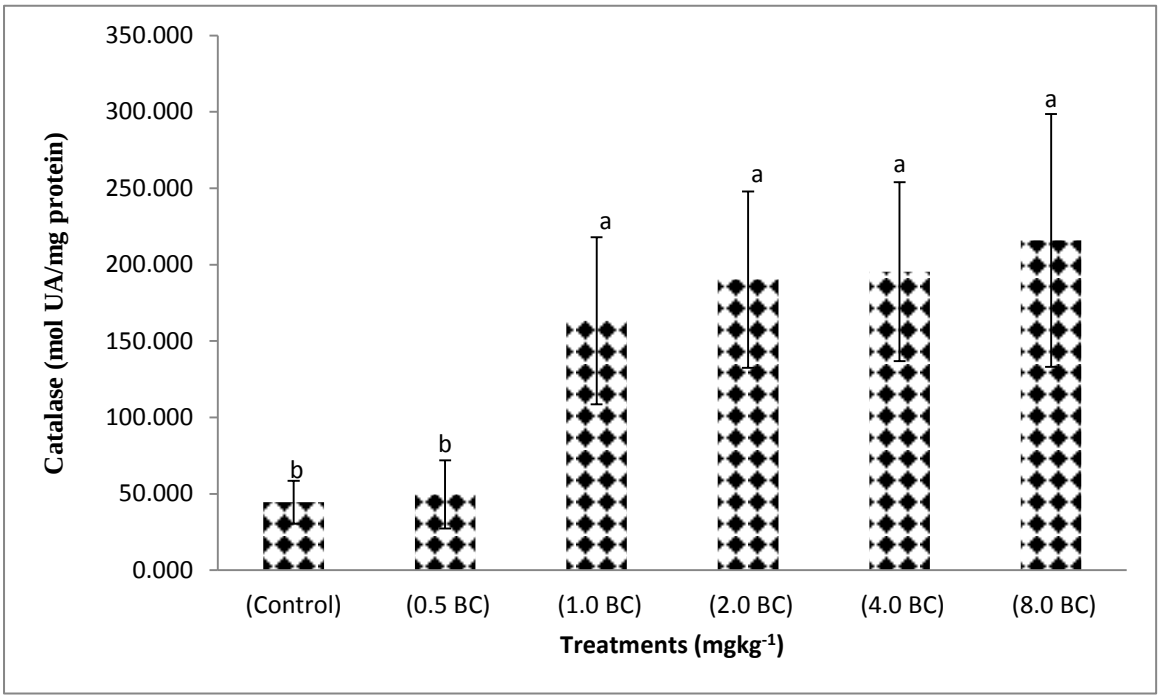


Figure 6.15: Effect of BC on catalase activity of leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

In the presence of different treatments of BC *i.e.*, 0.5 to 8.0 mg kg^{-1}, CAT activity in leaves improved about 1.11- 4.85 folds respectively over the control (**Figure 6.15**).

Co-contamination of Cd (25-200 $mgkg^{-1}$) along with BC (4 $mgkg^{-1}$) showed an increase in CAT activity by 1.85-6.68 folds for lowest (25 $mgkg^{-1}$ Cd and 4 $mgkg^{-1}$ BC) and highest (200 $mgkg^{-1}$ Cd and 4 $mgkg^{-1}$ BC) treatments respectively, as compared to control (**Figure 6.13**).

Under joint toxicities of Hg & BC an increase of 1.22 fold in CAT activity was observed for 20 $mgkg^{-1}$ and 2.25 fold for 120 $mgkg^{-1}$ Hg, with respect to control (**Figure 6.14**).

SOD Activity: At all Cd/Hg alone and combined treatments with BC, tissue SOD activity gradually increased with doses. In the presence of different concentrations of Cd (25, 50, 100, 150 and 200 mg kg^{-1}), SOD activity of about 0.053, 0.109, 0.145, 0.195 and 0.276 units/mg fwt respectively, was observed over the control. Further Cd (25-200 mg kg^{-1}), in the presence of BC (4 mg kg^{-1}) induced SOD activity by 2.54, 2.64, 3.53, 3.60 and 4.16 folds respectively, over the control (**Figure 6.16**).

In the presence of different Hg (40-120 $mgkg^{-1}$) concentrations, SOD activity amplified about 2.15-3.59 folds, while under co-contamination of Hg (20-120 $mgkg^{-1}$) +BC (4 $mgkg^{-1}$) the activity was approximately 2.75-6.63 folds respectively, to lowest and highest treatment over the control (**Figure 6.17**).

In the presence of alone BC treatment, SOD activity at lowest (0.5 $mgkg^{-1}$) concentration was 0.306 units/mg FWt. and at highest (8.0 $mgkg^{-1}$) concentration was 0.193 units/mg FWt. (**Figure 6.18**). Overall, results revealed that co-contamination poses more stress as compared to individual treatments (**Figure 6.16-6.18**).

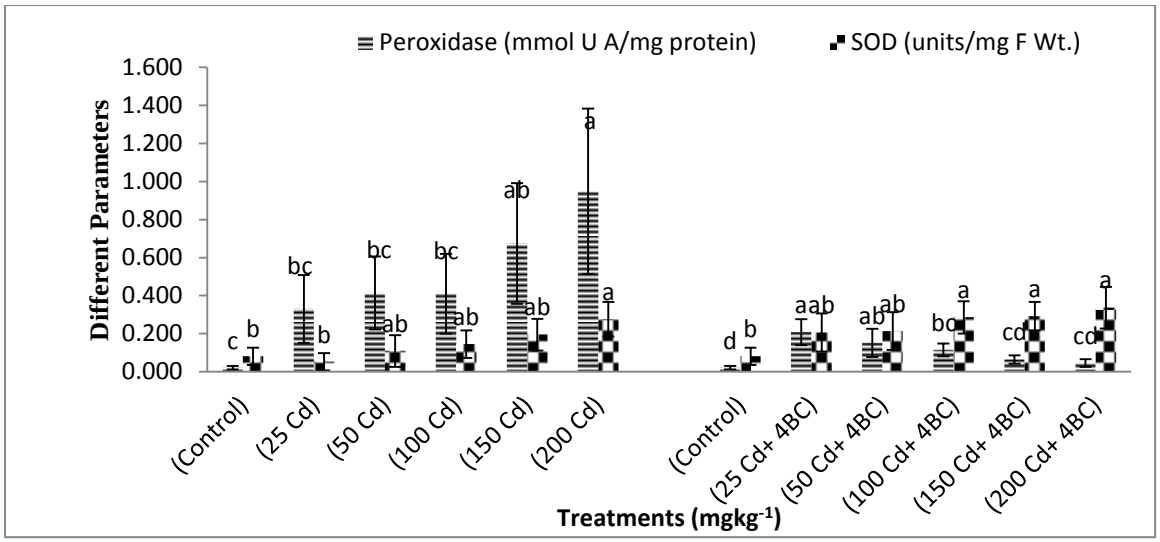


Figure 6.16: Effect of Cd alone and Cd+4BC in combination on POD and SOD activity on leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

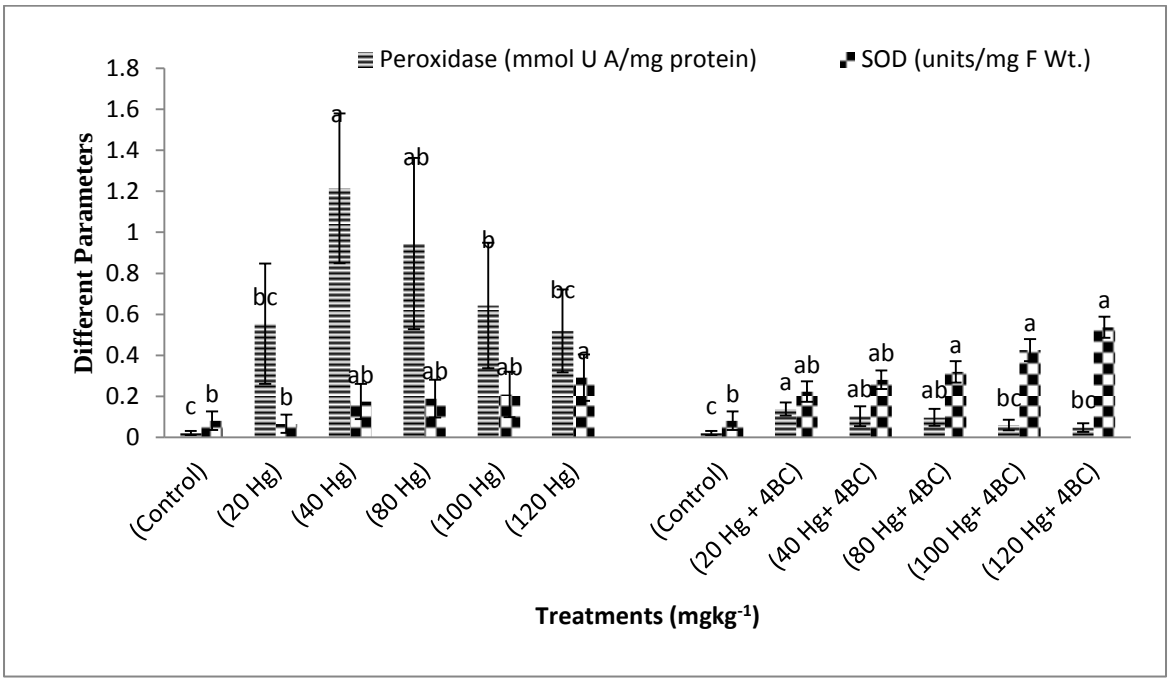


Figure 6.17: Effect of Hg alone and Hg+4BC in combination on POD and SOD activity on leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

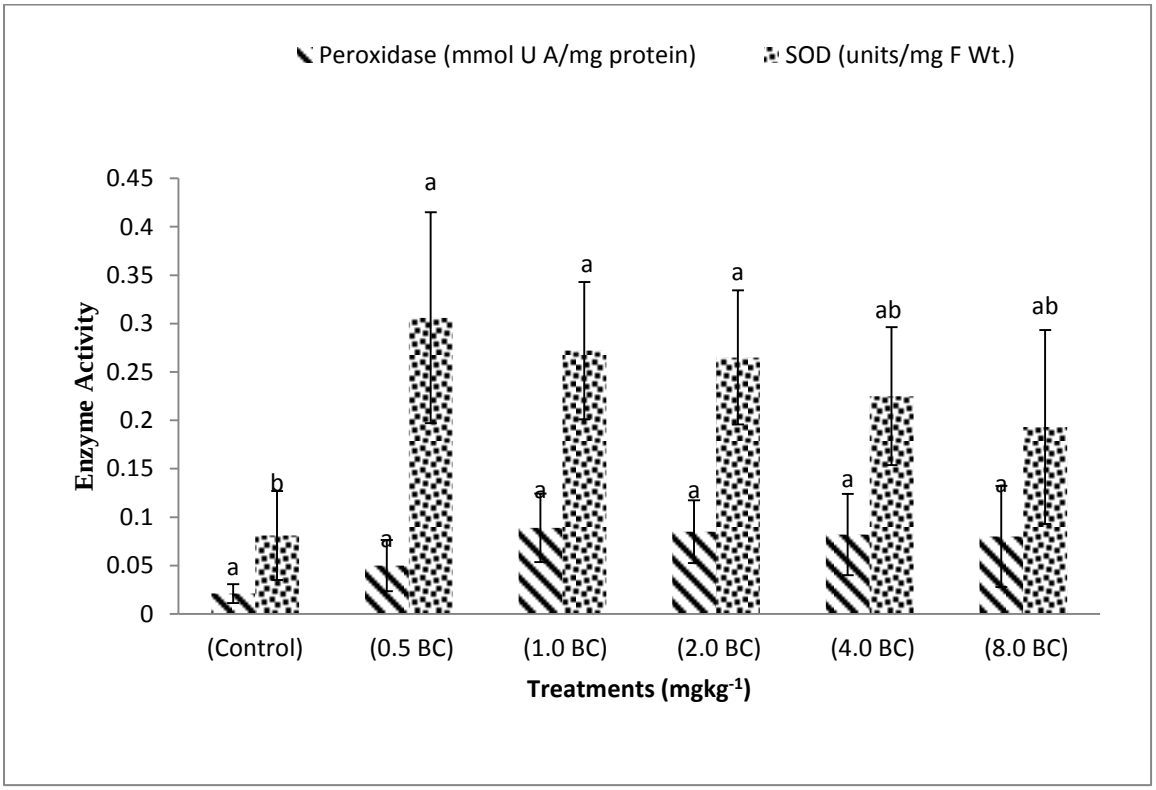


Figure 6.18: Effect of BC on POD and SOD activity of leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

POD activity: **Figures 6.16-6.18** shows peroxidase (POD) activity with all the doses of Cd/Hg and/or BC treatment.

Under individual treatment of Cd (25-200 mg kg^{-1}), POD activity increases significantly, with the increase in concentration. The POD values showed an increment of approximately 15.71 fold for 25 $mgkg^{-1}$ Cd and 45.14 fold for 200 $mgkg^{-1}$, in comparison to control (**Figure 6.16**).

In the presence of different concentration of Hg (20 - 120 mg kg^{-1}), POD activity was found to increase upto 40 $mgkg^{-1}$ (1.21 mmol UA/mg protein) and then declined with the increase in concentration (**Figure 6.17**).

POD activity under BC treatment increased upto 1 $mgkg^{-1}$ (0.089 mmol U A/mg protein) and then showed a marginal decline from 2.0-8.0 $mgkg^{-1}$ (0.085-0.080 mmol U A/mg protein), respectively (**Figure 6.18**).

Under the joint treatment of Cd (25-200 $mgkg^{-1}$) and BC a decrease in POD activity (0.208-0.046 mmol UA/mg protein) was observed with the increasing concentration of Cd respectively at lowest and highest doses studied.

Under joint toxicities of Hg (20-120 $mgkg^{-1}$) and BC, the POD values decreased with the increasing concentration (0.138-0.048 mmol UA/mg protein, respectively) of Hg.

APOX activity: APOX activity also progressively and significantly increased (**Figure 6.19-6.21**) with all the doses of Cd/Hg alone and under combined treatment with BC.

In the presence of different concentration of Cd (25-200 mg kg^{-1}), APOX activities augmented almost 7.27-12.24 fold, while under co-contamination with BC augmentation was 3.22 and 13.69 folds respectively, at lowest and highest doses over the control (**Figure 6.19**).

Results obtained for different treatments (20, 40, 80, 100 and 120 mg kg^{-1}), of Hg revealed that the APOX activities increased to nearly 6.96, 8.59, 8.65, 8.90 and 10.28 folds, while Hg (20-120 $mgkg^{-1}$) in the presence of BC induced about 3.12, 3.36, 3.42, 4.82 and 4.86 folds activity respectively, as compared to control (**Figure 6.20**).

BC (0.5-8.0 $mgkg^{-1}$) lone treatment showed significant (**$p<0.05$**) difference in APOX activities with the increase in concentration. The APOX activity for 0.5 $mgkg^{-1}$ BC was 2.77 fold while that of 8.0 $mgkg^{-1}$ was 7.04 fold as compared to control (**Figure 6.21**).

Overall results revealed that Cd in presence of BC was having high APOX activity as compared to alone treatments. On the contrary of above results, Hg alone revealed higher values for APOX activity as compared to joint treatment of Hg with BC. *C. roseus* leaves showed higher antioxidative enzymes activities at all the concentrations, in general.

GR activity: GR activity increased significantly ($p<0.05$) in the presence of different concentrations of Cd (at 25 $mgkg^{-1}$ (2.92 fold) and 200 $mgkg^{-1}$(4.403 fold)), while under the joint treatment of Cd and BC, GR activity at the lowest concentration (25 $mgkg^{-1}$) was 1.855 fold and at the highest concentration (200 $mgkg^{-1}$) was 15.94 fold, as compared to control (**Figure 6.19**).

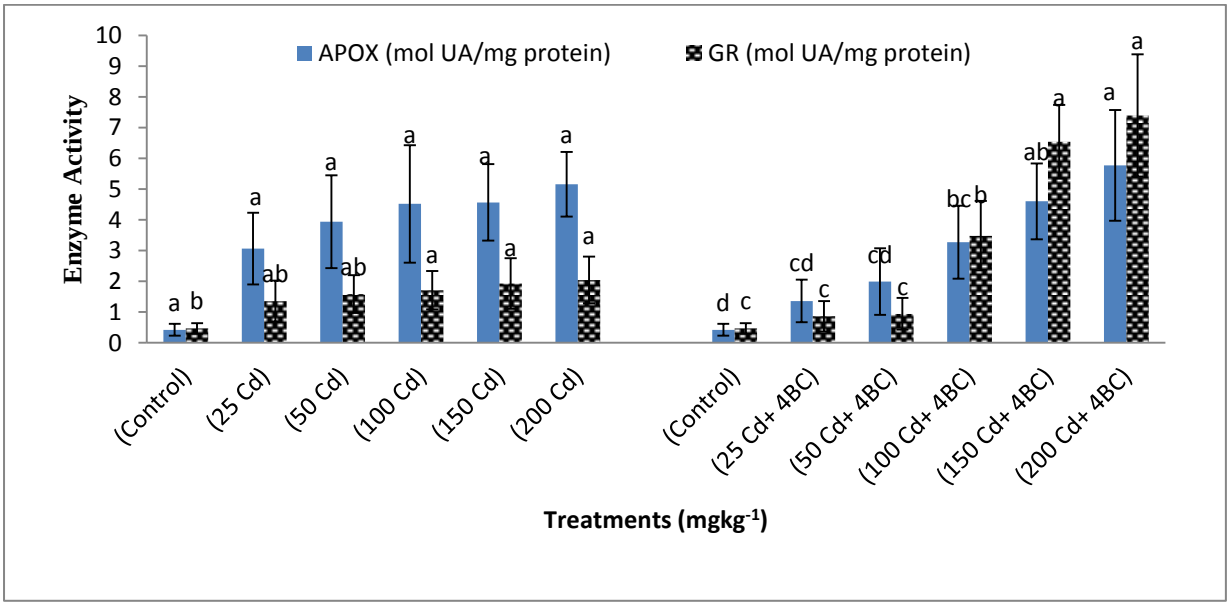


Figure 6.19: Effect of Cd alone and Cd+4BC in combination on APOX and GR activity on leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

Under different concentrations of Hg (20-120 $mgkg^{-1}$) GR activity was increased from 1.73 - 4.11 folds, respectively with respect to control. Likewise, co-contamination of Hg (20-120 $mgkg^{-1}$) with BC showed an increase in APOX activity from 2.89-5.35 folds respectively, as compared to control (**Figure 6.20**).

However, GR activity under BC treatment showed a significant (**$p<0.05$**) increase with the increasing concentration of BC from 0.5 $mgkg^{-1}$ (2.23 fold) to 8.0 $mgkg^{-1}$ (4.87 fold), with respect, to control (**Figure 6.21**).

Elevated levels of GR activity could increase the ratio of NADP+/NADPH thereby ensuring the availability NADP+ to accept electrons from photosynthetic

electron transport chain, and minimizing the reduction of oxygen and formation of superoxide radicals (**Srivastava et al, 2011**).

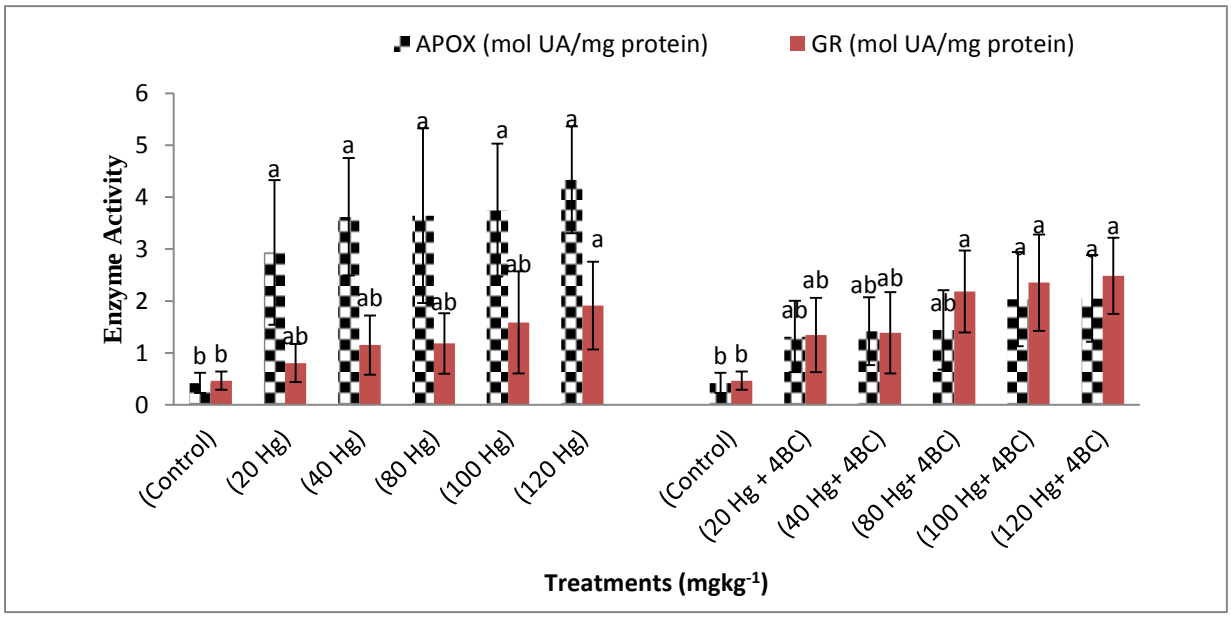


Figure 6.20: Effect of Hg alone and Hg+4BC in combination on APOX and GR activity on leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

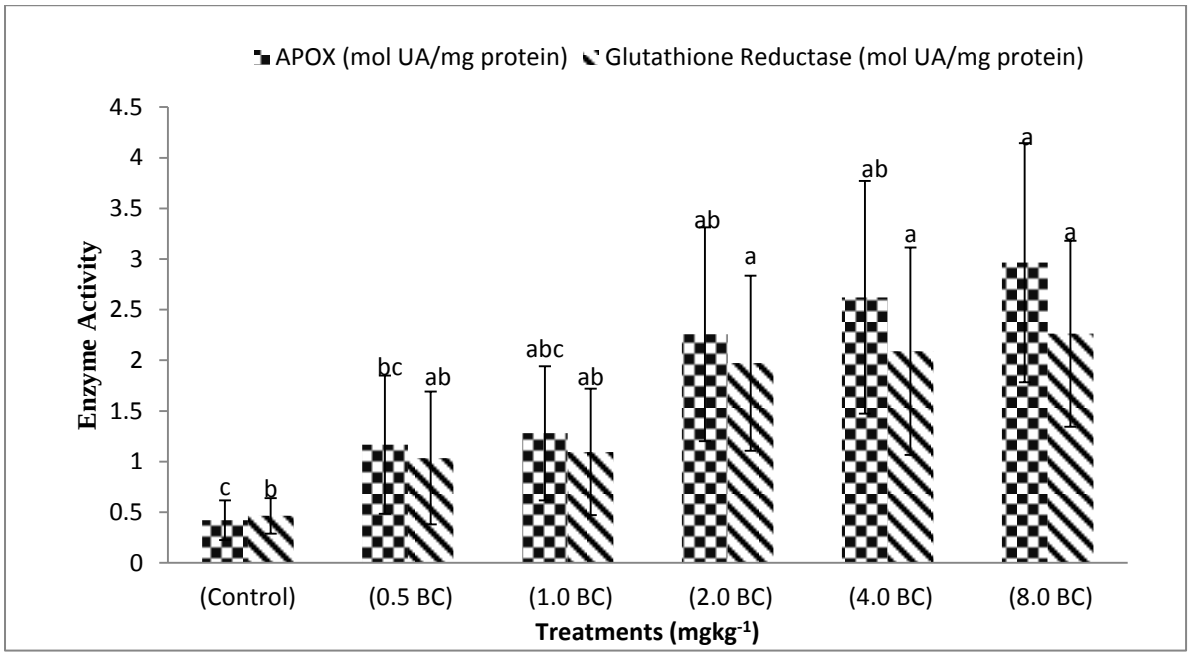


Figure 6.21: Effect of BC on APOX and GR activity on leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

6.3.5 SEM- root, shoot, leaf and EDX of root

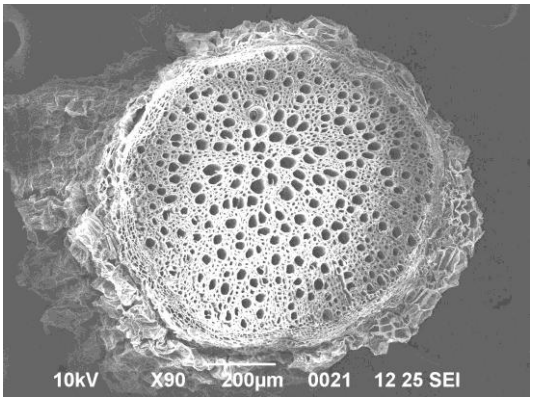
At 25 mg kg^{-1} Cd, xylem showed higher Mg-K, Ca-K peaks (weight% 2.77, 3.86 respectively), as compared to control (weight% 2.28 and 2.57), respectively. No data was obtained for 200 $mgkg^{-1}$ Cd (data not shown). This showed that with the increase in dose, Cd accumulation increased and Ca levels decreased in xylem. Besides, anatomical damage to root tissues was evident at higher Cd exposure (**Figure 6.22**). While under co-contamination, (25Cd+4BC and 200Cd+4BC mg kg^{-1}), xylem showed peak of Al-K, Si-K in lower value (weight% 0.29, 0.21 and 0.0, 0.0, respectively) with respect to control (weight% 0.77 and 23.92). Results revealed that in the presence of BC with the increase in concentration of Cd, Al and Si level decreases in the xylem. Under Hg treatment at 20 and 120 mg kg^{-1}, xylem showed higher Na-K, Mg-K peak (weight% 0.0, 0.52 and 0.84, 0.91, respectively) compared to that of control (weight% 8.90 and 2.28, respectively). However, in combined treatment 20Hg+4BC and 120Hg+4BC mg kg^{-1}, xylem showed peak of Na-K, Si-K (weight% 0.64, 0.37 and 0.61, 0.33, respectively) with respect to control (weight% 8.90 and 23.92, respectively).

While in BC alone treatment the lower peak values were recorded for Na-K, Cl-K at lower dose *i.e.*, 0.5 mgkg-1 (weight% 0.92 and 0.35, respectively) as well as higher dose 8.0 mg kg^{-1} (weight% 0.00 and 0.24, respectively), compared to control (weight% 8.90 and 0.44). It is known that calcium is important for plant growth and metal detoxification. Growing evidence showed that Ca^{+2} signal plays an important role in ion uptake in plants (**Lauer et al, 2008**). Therefore, it may be predicted that Ca plays a role in plant Cd/Hg detoxification to some extent at respective exposure doses (**Suzuki, 2005**).

The analysis of scanning electron microscopy showed a significant anatomical difference between root, shoot and leaves. SEM images from transverse sections of root and shoot samples under control conditions showed normal stellar structure. SEM images of control root and shoot clearly revealed the proper arrangement of xylem and phloem (**Figures 6.22 (a-k) and 6.23 (a-k)**). From the SEM images it is clearly visible that distinct feature of xylem and phloem are gradually distorted in the presence of Cd, Hg and/or BC. However, more structural deformation was observed under co-contamination as compared to individual treatments. The structural disintegration might be due to breakdown of spongy parenchyma cells resulting in a

decrease in intercellular spaces, as compared to control (**Mondal et al, 2015**). Above, mentioned ultrastructural deformation clearly revealed that in comparison to shoot more structural formation was observed in roots. This is perhaps due to poor translocation of heavy metals from root to shoot and most of the metals are sequestered in the root vacuoles (**Shanker et al**, **2004**). **Mondal et al (2015)** reported similar observation for *V. radiata* L. wherein most prominent effect was recorded on ultrastructure of root followed by shoot, leaf and nodules in the presence of mercury.

The SEM of the dorsal side of *C. roseus* leaves, for both Cd, Hg and/or BC, showed stomatal structural changes, as against the stomata of control leaves (**Figures 6.24 a-k**). The effects of co-contamination were more severe compared to alone treatments. The turgor pressure of the guard's cell controls the opening and closing of stomata. An increase in the turgor pressure of the guard cells regulates the widening of the stomatal aperture (**Mitra et al, 1998**). Similar results for SEM study was found by **Mondal et al (2013)** who reported disruption in normal orientation of leaves as well as associated tissues and vascular tissues while the stomatal complexes with guard cells were highly affected.



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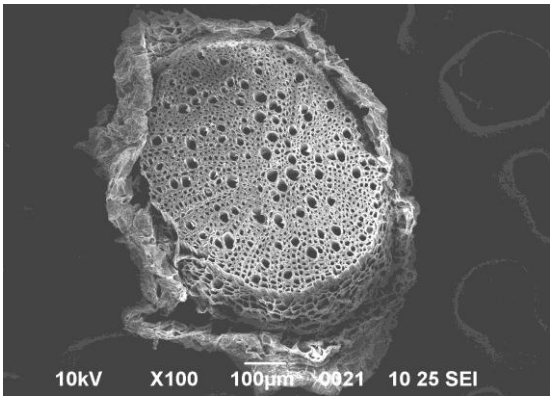

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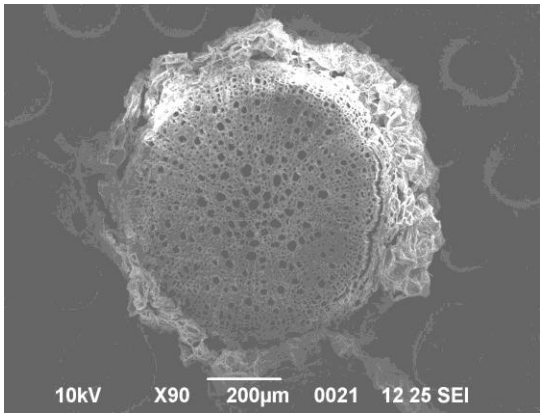

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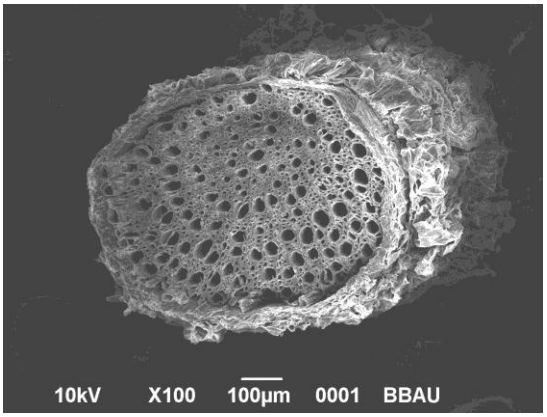

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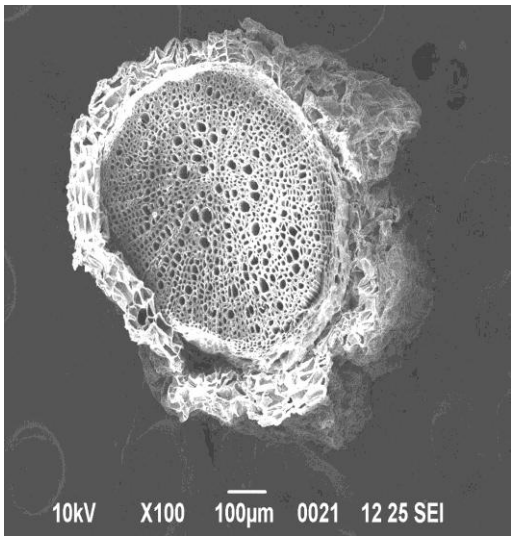

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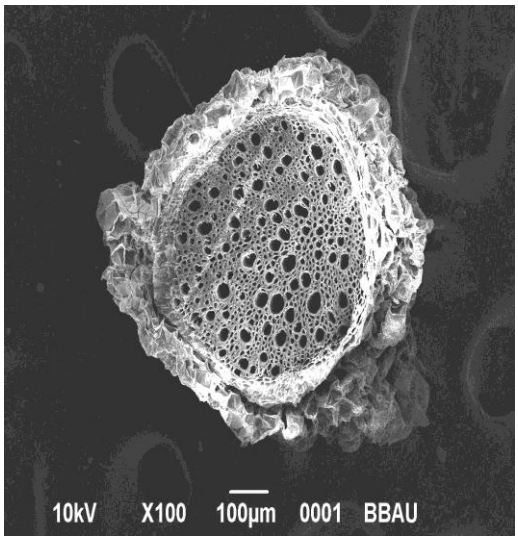

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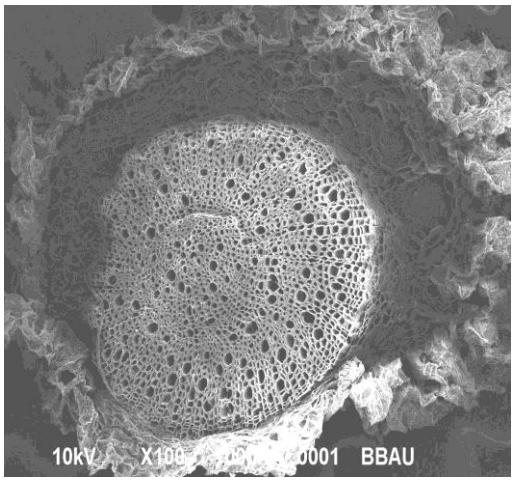

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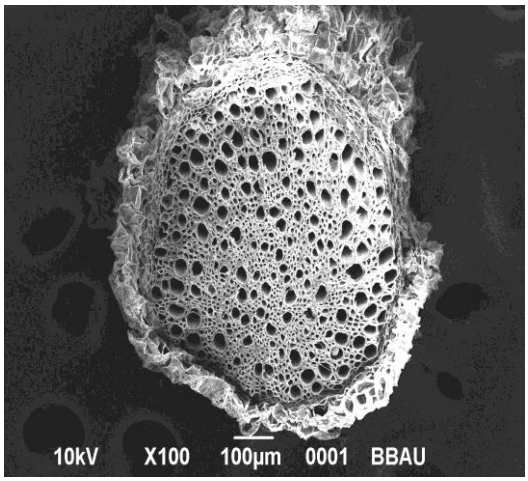

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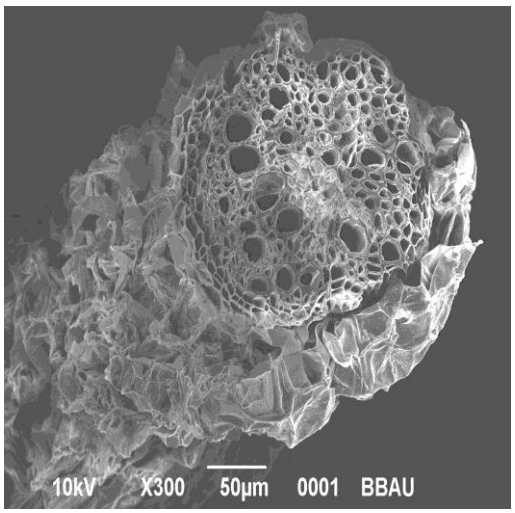

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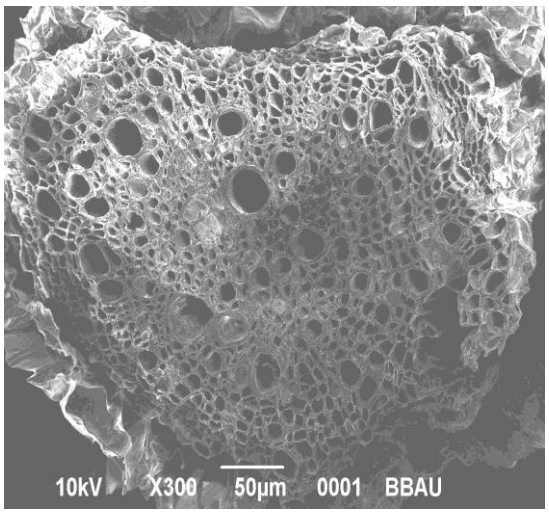

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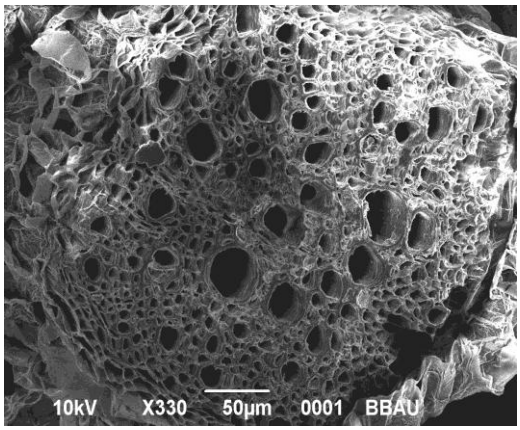
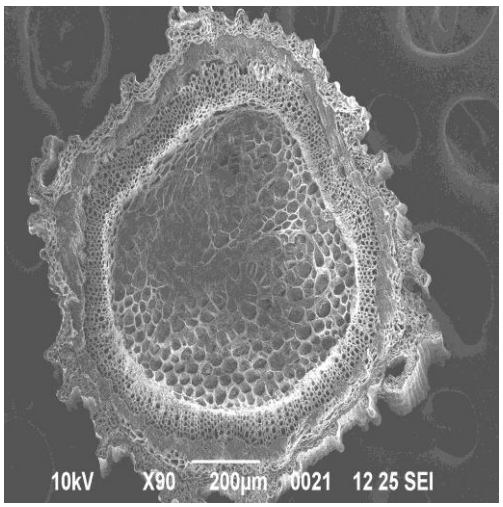


1(k)

Figure 6.22: Scanning electron micrograph (SEM) of untreated and treated root of *C. roseus* under different treatments of Cd, Hg, BC alone and in combination: 1(a) Control; (b) 25Cd;(c) 200Cd; (d) 20Hg; (e) 120Hg; (f) 0.5BC; (g) 8.0 BC; (h) 25Cd+4BC; (i) 200Cd+4BC; (j) 20Hg+4BC; (k) 120Hg+4BC



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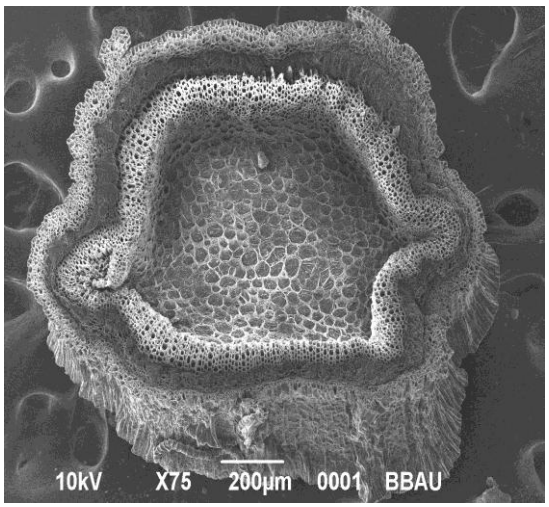

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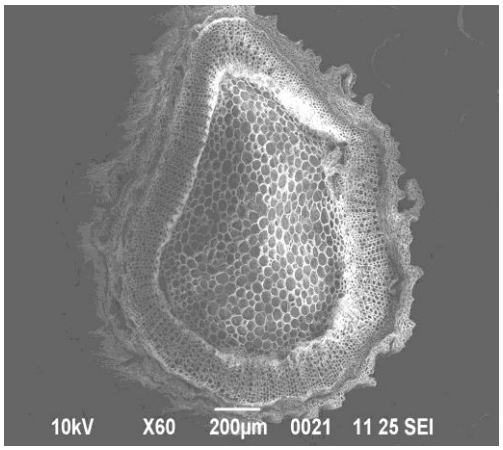

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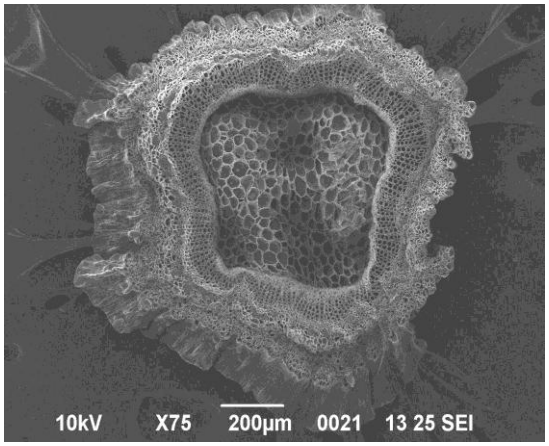

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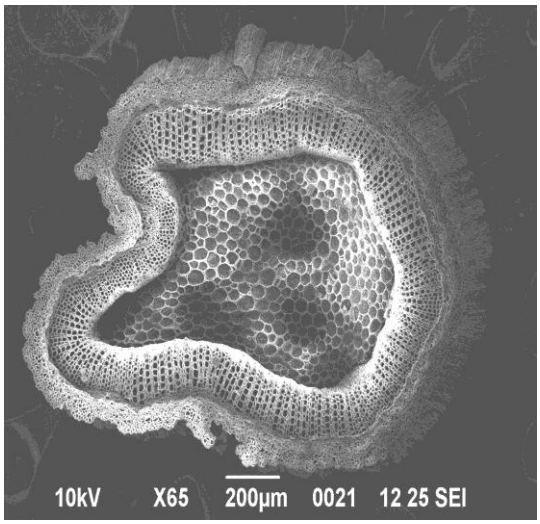

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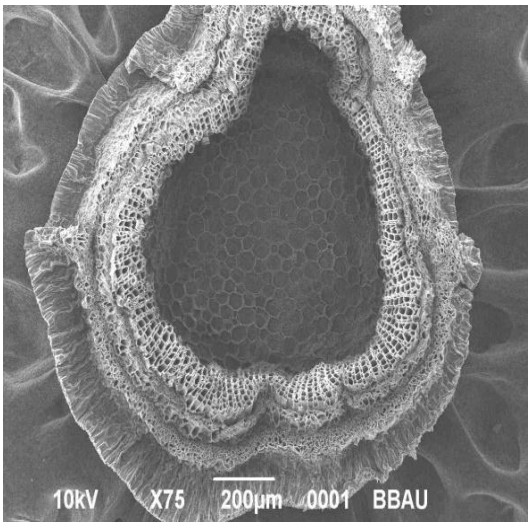

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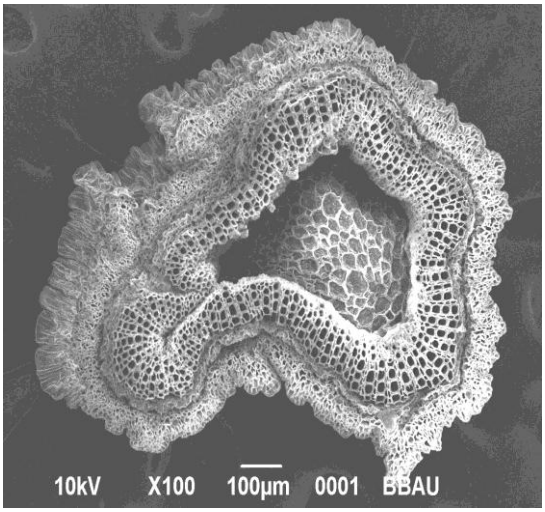

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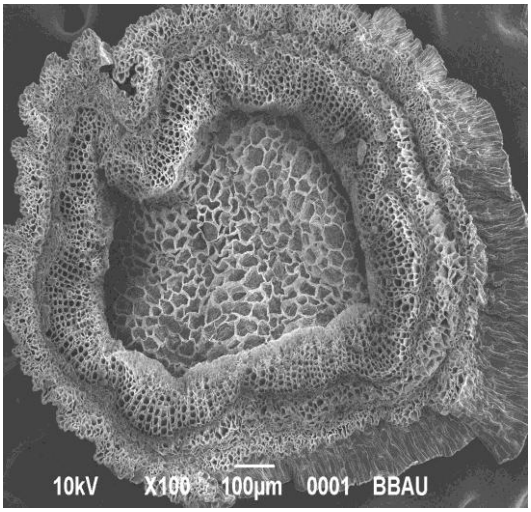

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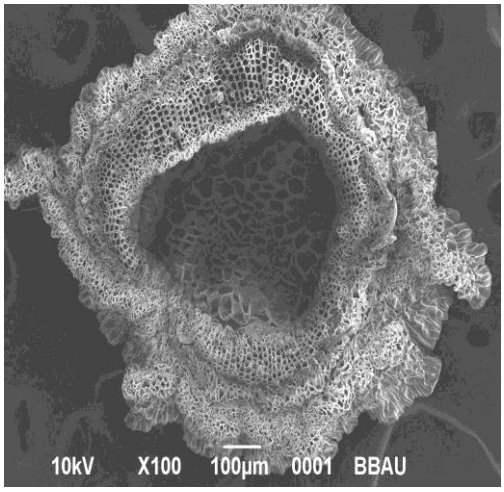

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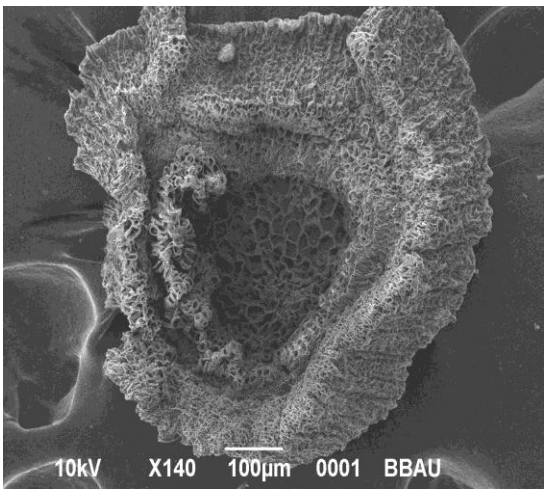

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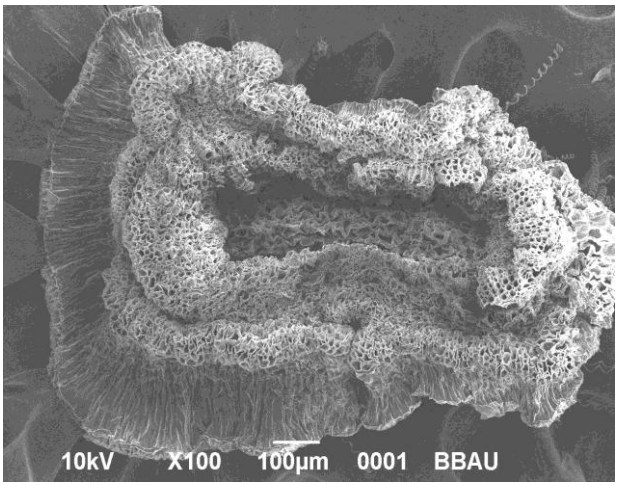
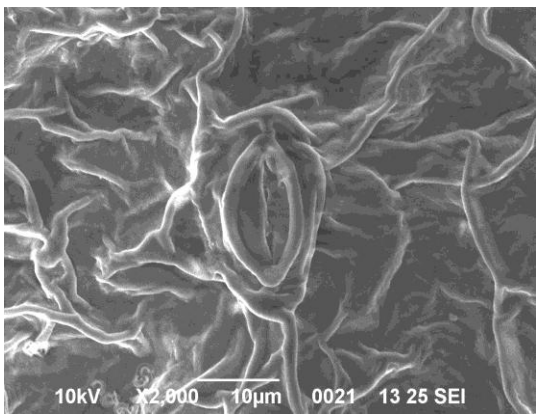


2(k)

Figure 6.23: Scanning electron micrograph (SEM) of untreated and treated shoot of *C. roseus* under different treatments of Cd, Hg, BC alone and in combination: 2(a) Control; (b) 25Cd; (c) 200Cd; (d) 20Hg; (e) 120Hg; (f) 0.5BC; (g) 8.0 BC; (h) 25Cd+4BC; (i) 200Cd+4BC; (j) 20Hg+4BC; (k) 120Hg+4BC



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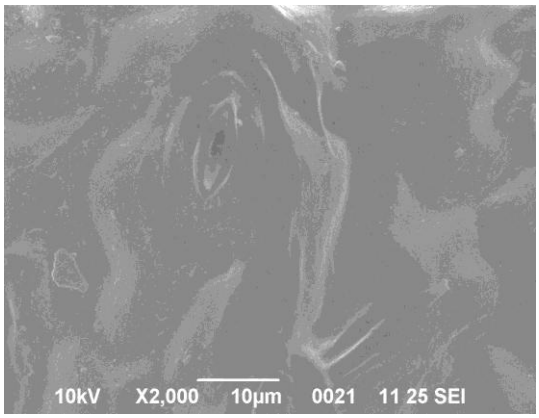

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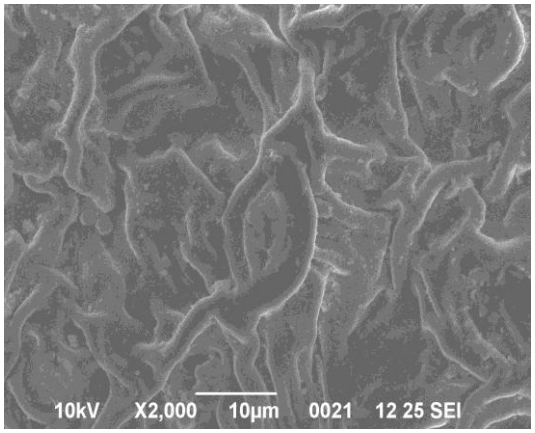

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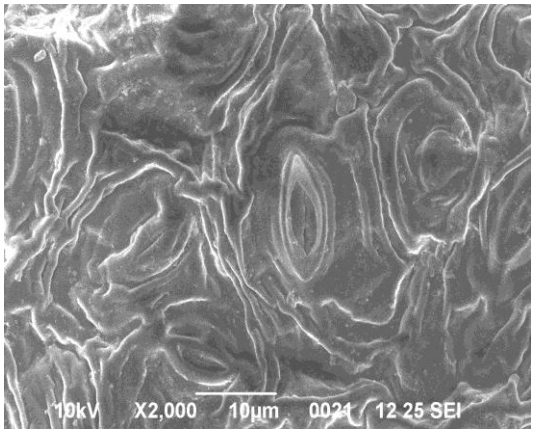

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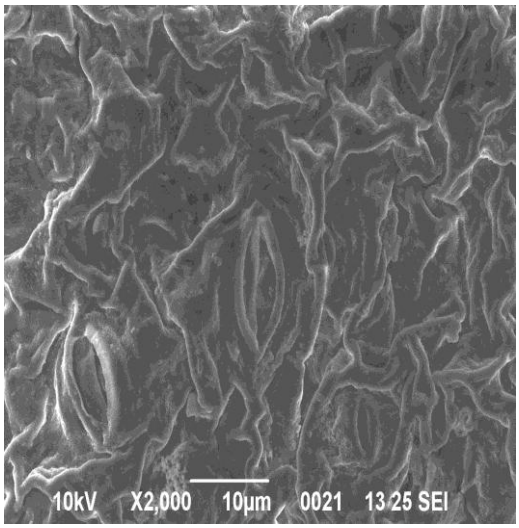

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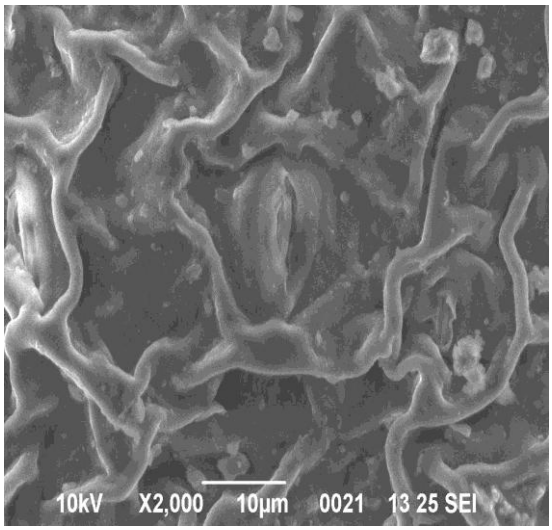

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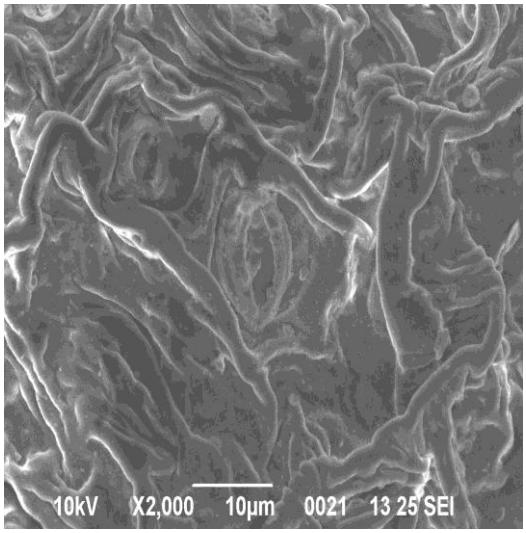

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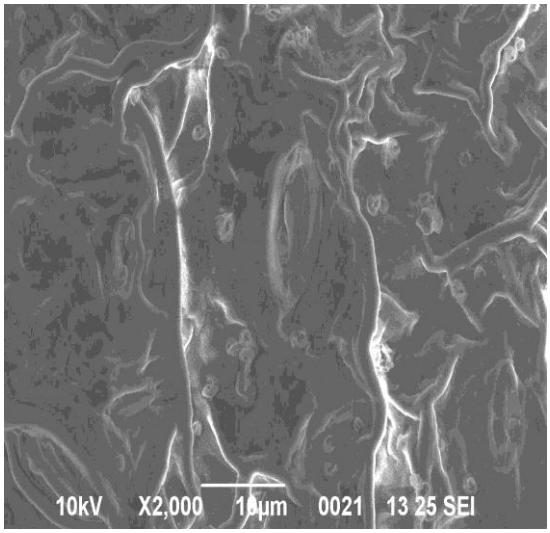

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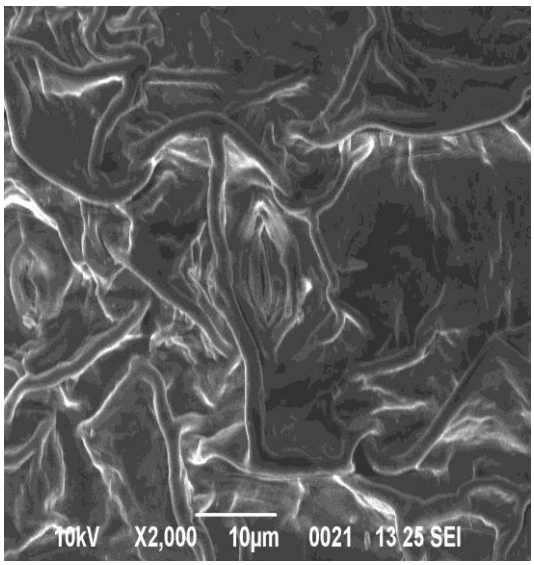

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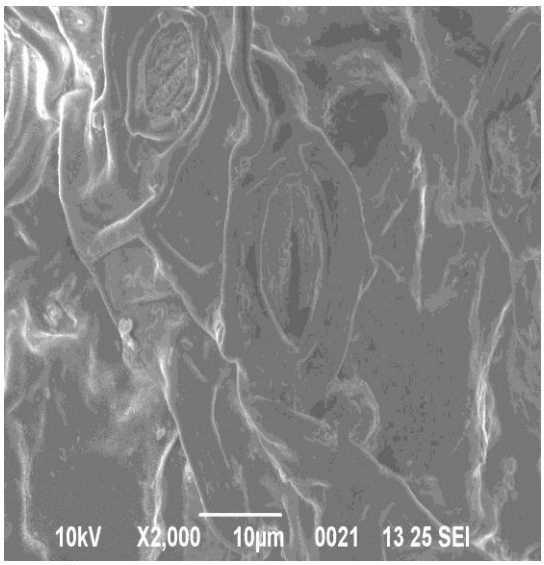

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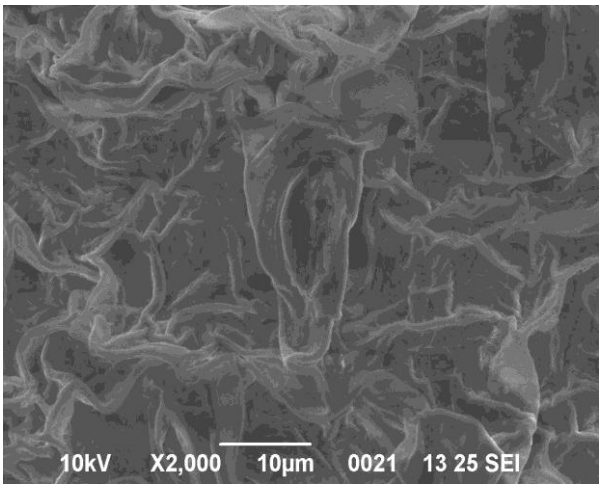


3(k)

Figure 6.24: Scanning electron micrograph (SEM) of untreated and treated leaves of *C. roseus* under different treatments of Cd, Hg, BC alone and in combination: 3(a) Control; (b) 25Cd; (c) 200Cd; (d) 20Hg; (e) 120Hg; (f) 0.5BC; (g) 8.0 BC; (h) 25Cd+4BC; (i) 200Cd+4BC; (j) 20Hg+4BC; (k) 120Hg+4BC

6.4 ANOVA

One-way analysis of ANOVA showed that the total phenol, CAT, APOX, POD, *Chl b* and proline content were having significant ($p<0.05$) effect as the concentration of Cd increased from 25-200 $mgkg^{-1}$. While under co-contamination of Cd (25-200 $mgkg^{-1}$) with BC (4 $mgkg^{-1}$) except for protein, carbohydrate and total phenol rest of the parameters were having significant effect. Results also revealed that Hg (20-120 $mgkg^{-1}$) alone treatments have significant ($p<0.05$) effect on APOX, POD, proline and LPO, activities, while Hg in the presence of BC showed significant difference on the activity of POD, Proline and GR only. BC alone was having significant ($p<0.05$) effect on LPO, proline, catalase, APOX and photosynthetic pigments.

6.5 Discussion

In the present study the effect of heavy metal and/or herbicide on total photosynthetic pigment, carotenoids, LPO, protein, proline, total phenolics and antioxidants were carried out. Results of our study on *C. roseus* revealed a decrease in photosynthetic pigments values as well as carotenoid content, as toxicity increases (**Figures 6.1-6.3**). Cd is non-essential, impairs development and chlorophyll synthesis, leading to death in plants (**Hassan & Mansoor, 2014**). Cd is also found to replace Mg in chlorophylls

and leads to chlorophyll degradation (**Kupper et al, 1996**). In a plant, Hg is a vital nutrient that forms the structure of several proteins and enzymes involved in the photosynthetic and respiratory processes (**Emamverdian et al, 2015**). Yet, a surplus of Hg may weaken nutritional and water balance, hampering photosynthesis and growth (**Zengin & Munzuroglu, 2005**). The ROS generated by Hg excess has been found to damage chloroplasts and plant pigments, and damage the photosystem II, thus affecting the photosynthetic rate. Both Cd and Hg are known to inhibit carotenoid levels in plants (**Sharma et al, 2012**). Thus, degeneration of carotenoid might reflect Cd/Hg-induced oxidative stress, as this component is known to be a major antioxidant (**Shaw et al, 2004**).

Under heavy metal (Cadmium) stress a metabolite proline is produced by plants which plays an important role in osmotolerance and osmoregulation (**Weihong et al, 2009**; **Muneer et al, 2011**; **Zhao, 2011**). Similar findings for proline content were obtained in our study on *C. roseus* when grown in the presence of soil contaminated with heavy metal and/or herbicide (**Figures 6.4-6.6**).

The protein content in the leaves of plant species was found to decrease significantly ($p<0.05$) with increasing concentration of Cd, Hg, BC alone and in combination in the soil (**Figures 6.4-6.6**). **Chen et al** (**2001**) reported that decrease in protein values might lead to proline accumulation in heavy metal stressed plants.

In the present study on *C. roseus* an estimation of MDA contents served as an excellent indicator of heavy metal and/or herbicide induced oxidative stress in the plant (**Figures 6.10-6.12**). Although Cd is non-redox active, it can stimulate ROS production and subsequent stress and lipid peroxidation (**Shah et al, 2001**). Hg, on the other hand, is redox-active, and induces ROS via Fenton and Haber–Weiss reactions and subsequently causes lipid peroxidation (**Opdenakker et al, 2012**). Malondialdehyde is the ultimate end produce of peroxidative damage of membrane lipids and stimulates ion seepage through the plasma membrane, whereby, it promotes damage to cell turgidity leading to changes and alterations of proteins and DNA (**Ayala et al, 2014**).

Some antioxidant proteins and proteins involved in glutathione synthesis have been reported to be upregulated more strongly in an Hg-tolerant rice variety than a non-tolerant variety in response to Hg stress (**Tanou et al, 2009**). Similarly, an enhanced abundance of molecular chaperones was observed in a strongly cadmium-

accumulating cultivar, which may represent an additional layer of protection against metal stress (**Patra et al, 2011**). However, both Cd and Hg excess often generate ROS, which may target sulfur-containing amino acids, such as cysteine and methionine, and thiol groups of proteins, making them inactivate (**Mukwevho et al, 2014**). However, *C. roseus* showed tolerance to 100 mg kg^{-1} Hg, hinting towards favorable hyper accumulation attributes.

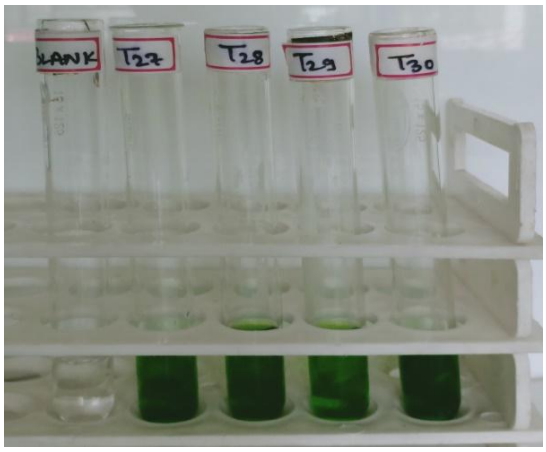
The activities of enzymes involved in the mitigation of oxidative stress in *C. roseus* were shown in **Figures 6.13-6.21**. Antioxidant enzymes form a part of the battery of stress mitigation repertoire of plants against metal excess (**Sharma et al, 2012**). SOD swiftly helps in the dismutation of $O^{-2\bullet}$ to H_2O_2 and O_2, while, the major amount of H_2O_2 is controlled by both peroxisomal CAT, and the vacuolar, cell wall, and cytosolic peroxidases (**Mittler, 2002**). Peroxidases are hemoproteins that use H_2O_2 as an electron acceptor and a variety of substrates as electron donors. In the current study, GR and APOX activities increased with the increasing concentration, both in the presence of heavy metal and/or herbicide (**Figures 6.19-6.21**). Two classes of peroxidases, viz., APOX and POD, occur in higher plants (**Takeda et al, 2000**). Data obtained in this study established the fact that the activities of all antioxidant enzymes inclined to escalate with the Cd/Hg in soil. Therefore, a noteworthy surplus of such enzymes can be regarded as a contingent signal for a greater ROS moderation as well as creation. Greater production of key antioxidant enzymes in hyperaccumulator, therefore act as a tactic to strengthen the cell antioxidant system and manage the threat of reactive oxygen species upsurge due to metal excess. This also indicated that the increased MDA in plant tissues were not due to the reductions in antioxidant enzymes in *C. roseus*.

The scanning electron microscopy analysis showed a structural deformation between roots, shoots and leaves (**Figures 6.22, 6.23 and 6.24**). However, at higher doses, biochemical and structural damages are evident, which can aid in the biomonitoring of environmental contaminants. However, various root tissue barriers, such as endodermis and Casparian strips, may aid in reducing the damage, by limiting uptake (**Goyal et al, 2003**). However, higher Cd/Hg can potentially damage the root. Such alterations in the root organization may be implicated in a compromised plant development (**Horst et al, 1983**). It is known that the cell membrane of the root cortical tissue takes up Cd^{2+}, which is further carried via the xylem and deposited in

the vacuoles (**Clemens et al, 2002**). Cd^{2+} causes peroxidative damage of root cell membranes and consequently damages root structures (**Ge et al, 2012**). Cd is a poisonous metal that can alter leaf morphology (**Benavides et al, 2005**). Photosynthesis is sensitive to disturbances in gas exchange through the stomata and both Cd and Hg stress-stimulated stomatal closure. Besides, the rapid response of stomata closure due to heavy metals would be a result of changes in water balance in leaves that also influence the rate of transpiration (**Kumar et al, 2000**). Stimuli, both exogenous and endogenous, are known to boost intracellular Ca^{+2} levels. The increase in the guard cell cytosolic Ca^{+2} appear to be the major regulator of stomatal functioning and therefore of plant water status (**Hamilton et al, 2000**). Such rapid stomatal closure, as seen the present study, might have been a physiological response to reduce water loss by transpiration and help mitigate stress. However, the mode of augmentation of Ca^{+2} as well its relation to other endogenous signals needs to be studied further.

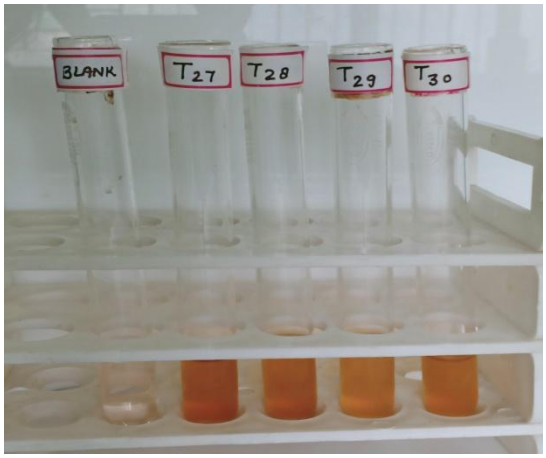
6.5 Conclusion

C. roseus demonstrated high Cd/Hg tolerance under butachlor co contamination, by triggering a number of stress mitigation strategies. The fact that this plant tolerated Cd and Hg above phytotoxic levels, accumulated high metal content in its above ground tissues, we suggest that this plant alleviated stress via augmenting the antioxidative defense pathways, involving a battery of enzymes, which helped with ROS dissipation. However, both Cd and Hg stress induced changes in root, shoot and leaf structural architecture, as evidenced by SEM. EDX showed specific Ca^{+2} signals in root xylem and which could be part of signaling pathway leading to increased root metal uptake and stomatal closure. These novel outcomes comprise, to our understanding, the first suggestion to show *C. roseus*, a hyperaccmulator elaborating the biochemical and physiological responses to elevated levels of either Cd or Hg, under co contamination with BC which were largely unknown. Although, the intricacy of hyperaccumulation is yet to be comprehended, the studies at cell and at tissue level will help in knowing how plants pick up and absorb extremely hazardous and ubiquitous metals, such as Cd and Hg, which will be very helpful for devising effective methods to check the problem of food-chain contamination by such pollutants.

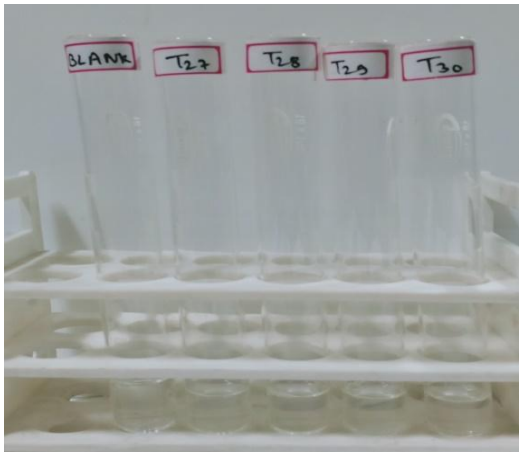


Chlorophyll

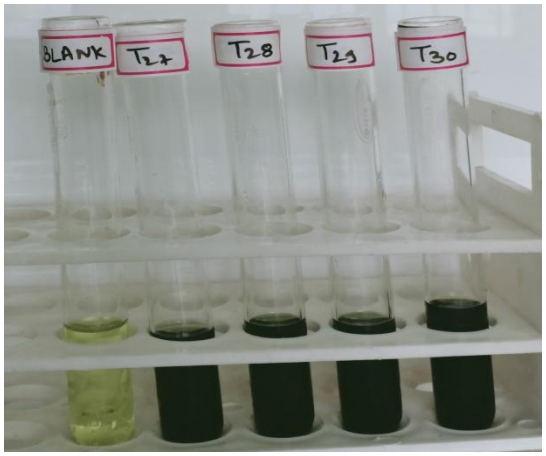
Protein



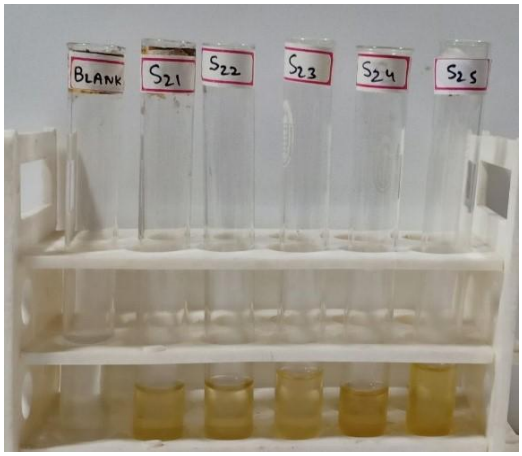
LPO



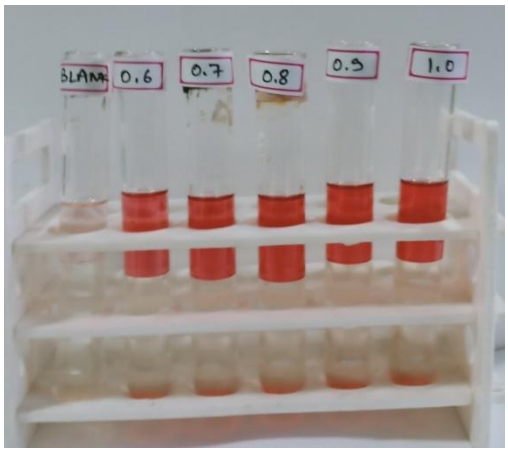
APOX



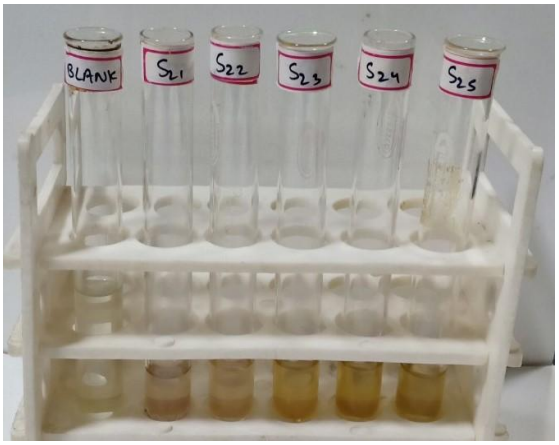
Carbohydrate



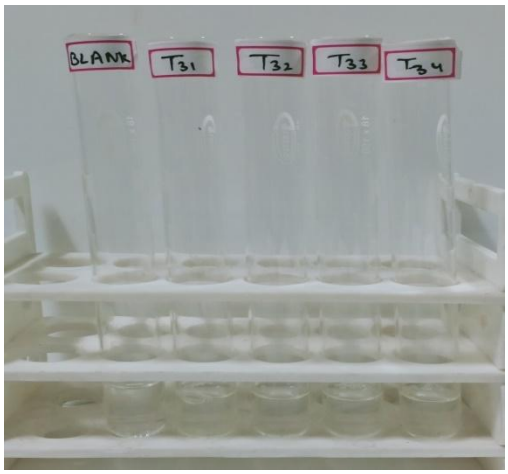
CAT



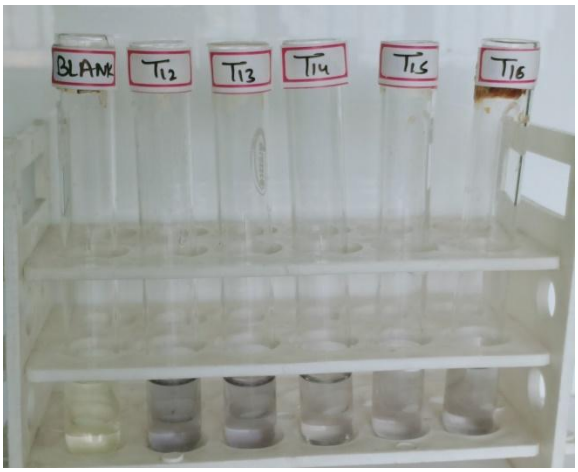
Proline



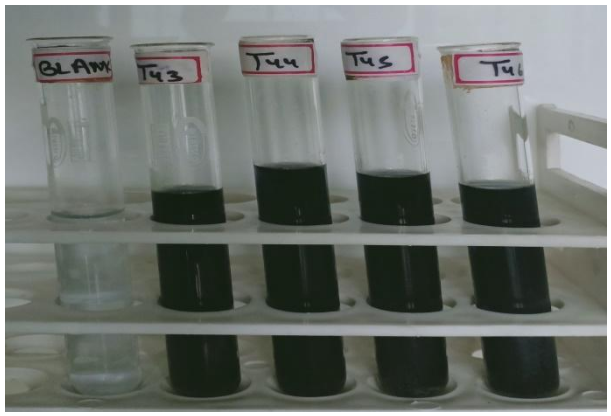
POD



GR



SOD



Total Phenol

Plate 6.1 Different biochemical parameters

Chapter 7

Effect of chelate addition on biochemical parameters of C. roseus under single and joint toxicities of Cd/Hg along with Butachlor and SEM-EDX analysis

7.1 Introduction

Extraction of heavy metal by plants is generally limited by their availability in soils. Chelating agents like EDTA (Ethylenediamine-tetraacetic acid), CA (Citric acid), *etc*. are mostly employed to increase the bioavailability of heavy metals and their enhanced uptake by the plants. It has been reported that chemical amendments may improve metal accumulation in different plant parts without significantly affecting the plant growth. This often gives rise to a question about the amount and mechanisms of such chemical amendments that may be required for potential plant growth and metal phytoremediation.

Several chelating agents including organic acids such as oxalic, citric, malic, succinic, tartaric, glutamic acids and EDTA, have been extensively investigated for their ability to mobilize metals thereby increasing metal accumulation by different plant species (**Madrid et al, 2003; Wu et al, 2003**). Among the abovementioned chelating agents, EDTA has been recognized as a strong chelator for different metals and hence suggested widely, being most effective in mobility, solubility, and bioavailability in the soil solution phase, root uptake, and soil-bound metal accumulation (**Evangelou et al**, **2007**; **Leleyter et al, 2012**).

Therefore, objective of the present study was to investigate the effect of both Cd and Hg in the presence and/or absence of chelators (EDTA and Citric acid) along with BC on biochemical alterations in *C. roseus*, so as to elucidate their roles in plant adaptation to stress both under single and joint toxicity.

7.2 Materials and Methods

7.2.1 Soil spiking

Detailed methodology is given in Chapter 6 (Materials & Methods) Section 6.2.1 and 6.2.2

7.2.2 Experimental set up

A completely randomized design of 21 treatments with three replicates of each was opted for present study. Pots with no plants were included to monitor on-plant facilitated dissipation of BC. 150 mg/kg^{-1} Cd/100 $mgkg^{-1}$ Hg and/or 4 $mgkg^{-1}$ BC in presence of individual and joint application of EDTA and CA.

7.2.3 Planting

Plastic pot planters of 30 cm in height were used for experimentation. Spiked soil approximately, 2 kg were placed in each pots. 3 weeks old 2-3 seedling of *C. roseus* having uniform size were transferred to each pot. EDTA, Citric acid and a combination of EDTA and Citric acid were used in the present study. The chelates application was done after 15 days of transplanting the *C. roseus* so as to ease the acclimatization with the surrounding environment. As a result of the preliminary experiments and literature findings, CA amendments were added at 1.0 g/kg and EDTA amendments were added at 0.1 g/kg. The chelates were equally divided into three parts with single application per week for 3 weeks. Treatments without addition of chelate served as control. Leachate collector plates were placed underneath the pots so as to collect potential leachates during the experimentation. Post 60 days of growth, plants were harvested through cutting shoots immediately above the soil surface and washed with double distilled water (ddw). The pots were then emptied after that the roots were washed with running tap water to separate the soil. The roots were further rinsed with ddw, thrice to remove any remaining soil particles. All samples were subsequently dried in an oven at 70^{o}C to constant wt. The dried samples were weighed to calculate biomass used for plant analysis.

7.2.4 Determination of chlorophyll, carotenoid and carbohydrate contents

The amount of chlorophyll was calculated by using the formulae described by **Maclachlan and Zalik (1963)** and carotenoids by **Duxbury and Yentsch (1956)**, respectively. Carbohydrate content was determined by the method **Hedge (1962)** and for details refers to Chapter 3 (Materials & Methods) Section 3.6.1 and 3.6.2.

7.2.5 Estimation of Proline and Malondialdehyde (MDA)

Proline was carried out according to the procedure given by **Bates et al (1973)**. The level of lipid peroxidation content was estimated through protocol given by **Heath and Packer (1968)**. The details of procedures have been given in Chapter 3, (Materials and Methods) Section 3.6.3 and 3.6.4.

7.2.6 Determination of protein and total phenol estimation

Protein and total phenolics content in the leaves of *C. roseus were* estimated by method of **Lowry et al. (1951)**, **Bray and Thorpe (1954)** respectively as described in Chapter 3,

(Materials and Methods) Section 3.6.5 and 3.6.6.2.

7.2.7 Antioxidant estimation

The activity of superoxide dismutase (SOD, EC 1.15.1.1) was assayed by the method of **Beauchamp and Fridovich (1971)**. The activity of ascorbate peroxidase (APOX, EC 1.11.1.11) was measured by estimating the rate of ascorbate oxidation (extinction coefficient 2.8 mM^{-1} cm^{-1}) at 290 nm (**Nakano & Asada 1981)**. Guaiacol peroxidase (GPX, EC 1.11.1.7) activity was assayed according to the protocol given by **Putter (1974)**. For catalase (CAT; EC 1.11.1.6) activity measurement; the method of **Aebi (1984)** has been used. Detail methodology is given in Chapter 3 (Materials & Methods), Section 3.6.6.3 - 3.6.6.7.

7.2.8 Statistical analysis

Data (*n*=3) were analyzed statistically by one way analysis of variance (SPSS Inc., version 25.00) using Duncan's Multiple Range Tests (DMRT) to determine the significance of differences among treatments at probability (*p*) 0.05.

7.3 Results

7.3.1 Photosynthetic pigment content

Chlorophyll a (*Chl a*) chlorophyll b (*Chl b*), total chlorophyll and carotenoids value are presented in **Figure 7.1**. The *C. roseus* plants experienced a significant reduction in *Chl a*, *Chl b*, total chlorophyll and carotenoids concentration when exposed to 150 $mgkg^{-1}$ of Cd. However, individual application of EDTA and CA, in the presence of Cd (150 $mgkg^{-1}$) increased the photosynthetic pigments of Cd stressed plants as compared to 150 $mgkg^{-1}$ Cd alone. On the other hand, joint application of EDTA and CA displayed a significant increase in photosynthetic pigments of Cd (150 $mgkg^{-1}$) stressed plants. The increase in *Chl a*, *Chl b*, total chlorophyll and carotenoids was about 6%, 55%, 28% and 23%, as compared to respective Cd only treatments.

Contrary to the results as above, both single and joint application of EDTA and CA displayed a marginal decrease in photosynthetic pigments of Hg (100 $mgkg^{-1}$) stressed plants. The decrease in *Chl a*, *Chl b*, total chlorophyll and carotenoids content in 100 Hg+EDTA+CA was about 7.1%, 6.7%, 6.8% and 8.70%, as compared to respective Hg only treatments, respectively.

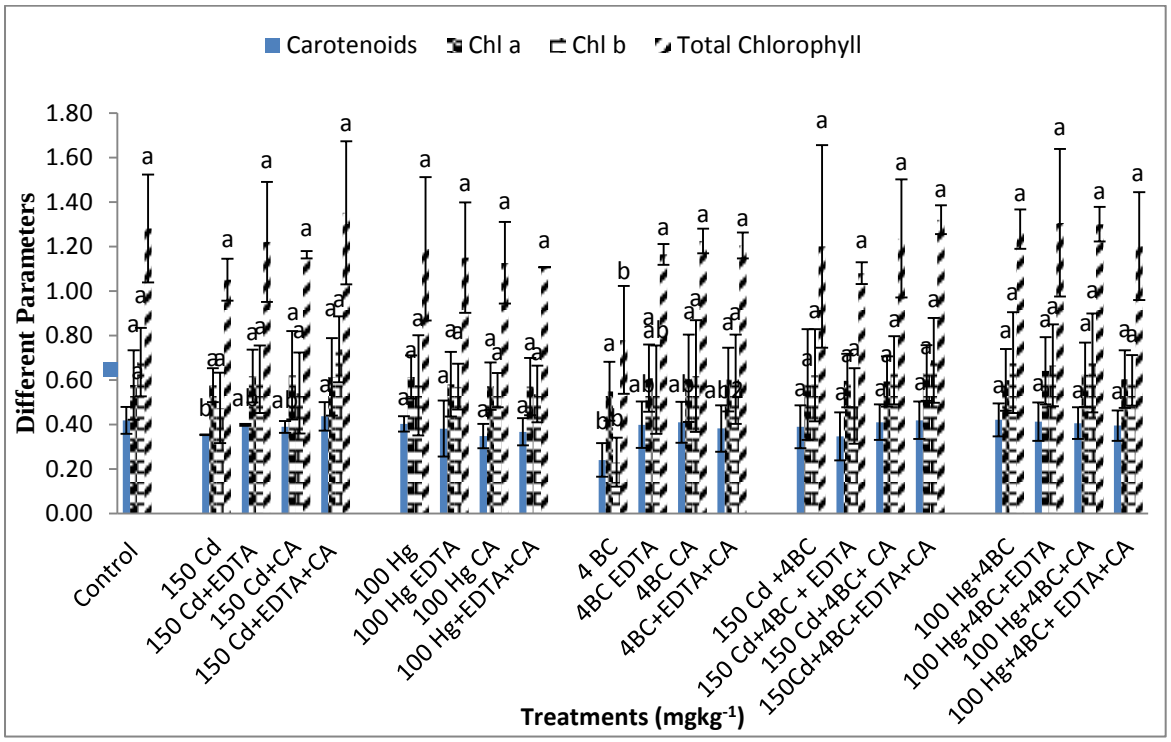


Figure 7.1: Effect of Hg, Cd, BC, Cd+4BC, Hg+4BC with chelating agents on photosynthetic pigments of leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

Both joint and individual application of EDTA and CA in the presence of BC (4 $mgkg^{-1}$) increased the photosynthetic pigments of plants as compared to BC alone. The increase in *Chl a*, *Chl b*, total chlorophyll and carotenoids in BC+EDTA+CA was about 11.2%, 62%, 54% and 59%, as compared to BC only treatments, respectively.

Effect of chelating agents under joint toxicities of heavy metal (Cd) and herbicide revealed that in the presence of EDTA and CA the joint application of Cd (150 $mgkg^{-1}$) and Butachlor (4 $mgkg^{-1}$) resulted in an increase in photosynthetic pigments as compared to 150 Cd+4BC ($mgkg^{-1}$). The increase in *Chl a*, *Chl b*, total chlorophyll and carotenoids in Cd+ BC+EDTA+CA was about 11.2%, 62%, 54% and 59%, as compared to respective Butachlor only treatments, respectively.

Effect of chelating agents under joint toxicities of heavy metal (Hg) and herbicide revealed that in the presence of EDTA and CA the joint application of Hg (100 $mgkg^{-1}$) and Butachlor (4 $mgkg^{-1}$) resulted in a marginal increase in photosynthetic pigments as far as *Chl a*, *Chl b* and total chlorophyll is concerned compared to Hg+BC and a marginal decrease in carotenoid content. However, in Hg+ BC+EDTA+CA a marginal decrease in *Chl a*, *Chl b*, total chlorophyll and carotenoids was found as compared to respective BC only treatments respectively.

The overall results revealed the positive effect of EDTA and CA under all treatments except for Hg+ BC+EDTA+CA wherein a marginal decrease was recorded. Chelate induced decrease in photosynthetic pigments as above may be attributed to ultrastructural alteration of chloroplast under metal toxicity (Hg), (**Gill et al, 2015**). An increase in photosynthetic pigments in the rest of the treatments may be due to EDTA and/or CA induced chelation of metals, decreasing free metal ions in plants, as suggested by **Najeeb et al (2011)**.

7.3.2 Lipid peroxidation (LPO) activity

Results of lipid peroxidation (LPO) showed that both the metals (Cd and Hg) and BC induced an increase in MDA levels as compared to control. The results are presented in **Figure 7.2**. Results of LPO increase is as expected; since both investigated metals have a redox potential contributing to the production of ROS and subsequently to oxidative damage to membrane lipids.

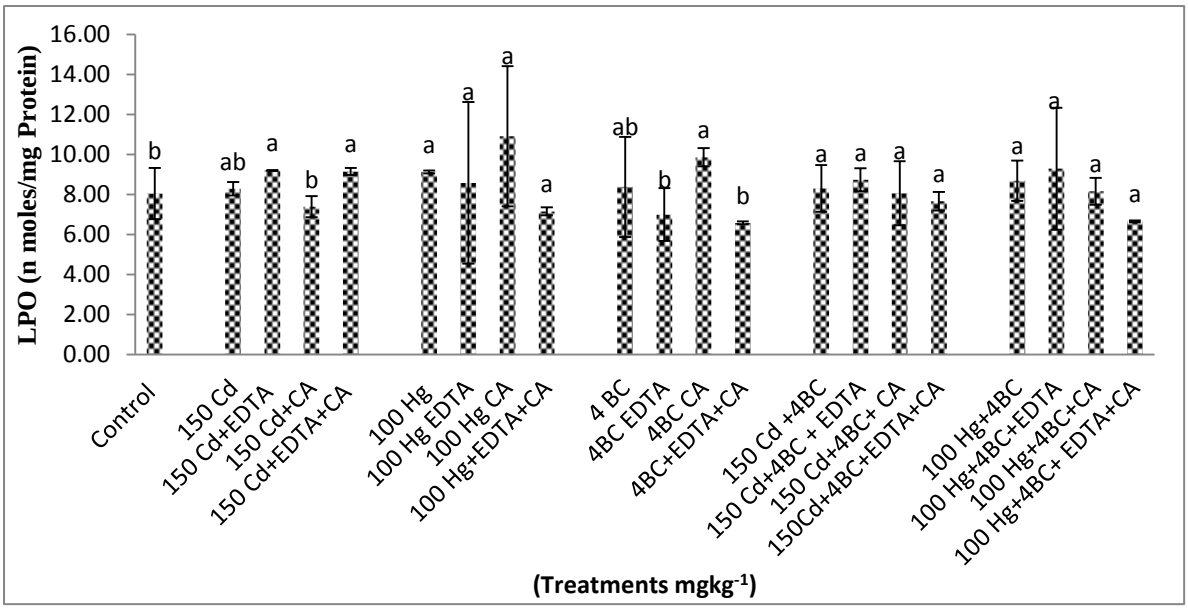


Figure 7.2: Effect of Hg, Cd, BC, Cd+4BC, Hg+4BC with chelating agents on lipid peroxidase content on leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

However, in the presence of chelating agents a differential response to LPO was revealed. In 150 $mgkg^{-1}$ Cd +EDTA, an 11.01% increase in LPO was observed compared to Cd alone, while a 10.80% decrease in LPO activity was recorded in presence of CA which might be due to antioxidant activity of CA in addition to its action as a metal chelator.

Likewise LPO in the presence of chelating agents revealed a differential response in Hg stressed cells. In 100 $mgkg^{-1}$ Hg+EDTA there was a 6.04% decrease in LPO as compared to Hg alone while a 19.51% increase in LPO activity was observed in the presence of CA which was *vice versa* to the results obtained above with Cd. Moreover, the joint application of both the chelating agents on Hg toxicity revealed a significant decrease in LPO (21.58%), as compared to Hg alone.

Effect of chelating agents on BC toxicity revealed results similar to Hg toxicity, discussed as above, in which addition of EDTA lowered the values of LPO while the same was increased in the presence of CA. The joint application of chelating agents was found to drop the LPO activity.

LPO activity was also monitored after chelate application under co contamination. Results obtained under co contamination Cd+BC and Hg+BC showed an increase in LPO activity in the presence of EDTA (5.23% and 6.90%, respectively), while LPO activity decreased in the presence of CA (2.85% and 6.15%) and EDTA+CA (7.62% and 23.38%), both with respect to Cd+BC and Hg+BC treatments. Altogether, it could be summarized that addition of chelating agents have a differential response to heavy metals, as far as toxicity is concerned and joint application of both EDTA and CA attributes improved tolerance to the heavy metal and herbicide induced oxidative stress in *C. roreus* under co contamination.

7.3.3 Carbohydrate, protein and proline values

Carbohydrate: In **Figure 7.3**, it was shown that the carbohydrate content were significantly decreased by 36.64% and 36.49% in plants treated with Cd and Hg, respectively, as compared to control, which might be due to the inhibition of chlorophyll biosynthesis leading to decrease in carbohydrate content. An addition of EDTA and CA to 150 $mgkg^{-1}$ of Cd could only marginally improve the carbohydrate values in individually and under joint application of chelates. On the contrary, chelate addition in the presence of 100 $mgkg^{-1}$ of Hg, resulted in a significant ($p<0.05$) increase (1.74 fold) in carbohydrate values, especially in the presence of CA, rest of the values being marginal with respect to 100 $mgkg^{-1}$ Hg. However, in BC (4 $mgkg^{-1}$) alone treatment in the presence of chelates EDTA and CA, there was an increase of 18.75% in the presence of EDTA, 11.97% in presence of CA and 18.04% in joint application respectively with BC (4 $mgkg^{-1}$). Effect of chelating agents under joint

toxicities of heavy metal (Hg) (100 mgkg^{-1}) and herbicide (BC) (4 mgkg^{-1}) revealed that there is a marginal increase in carbohydrate content under single and joint application of EDTA and CA as compared to alone Hg+BC treatments. While contrary of that was observed in combined treatment (Cd+BC) in the presence of chelates. Except in the presence of EDTA, all treatments showed decrease in carbohydrate content as compared to alone (Cd+BC) treatment (**Figure 7.3**).

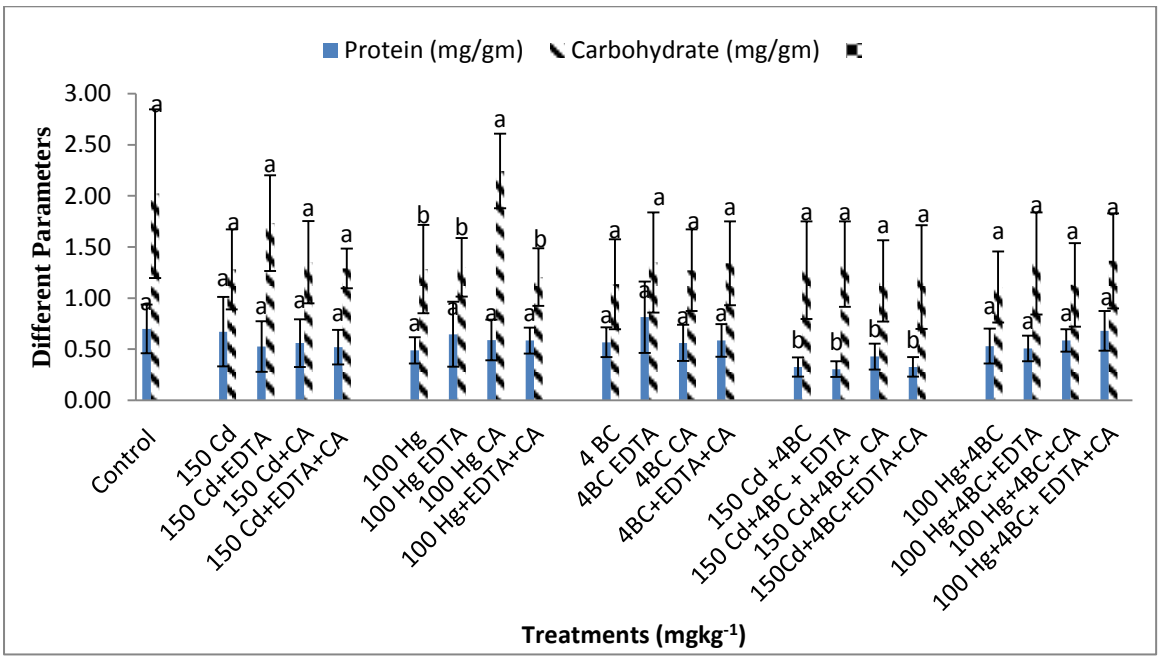


Figure 7.3: Effect of Hg, Cd, BC, Cd+4BC, Hg+4BC with chelating agents on protein, carbohydrate content on leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

Protein: The protein content of Cd, Hg and BC both alone and in combination in the presence of chelate EDTA and CA individually or jointly on *C. roseus* is given in **Figure** (**7.3**). Results revealed a decrease in protein content in the presence of Cd (150 mgkg^{-1}) and chelating agents (EDTA, CA alone and jointly). In the presence of EDTA, protein content showed a 21.46% decrease, while in the presence of CA, it was 16.69% and under joint toxicity, it was 22.65% as compared to Cd alone.

In the presence of Hg (100 mgkg^{-1}) a significant increase in protein content was observed in the presence of chelating agents EDTA and CA. In the presence of BC (4 mgkg^{-1}) a significant decrease in protein content was revealed in the presence of CA (1.01 fold) while an increase was recorded in the presence of EDTA and EDTA+CA (1.432 and 1.03 fold), respectively, to BC.

Protein content under the joint treatment of Cd (150 $mgkg^{-1}$) and BC (4 $mgkg^{-1}$) in the presence of chelating agents, was found to decrease in the presence of EDTA (1.07 fold), while marginal increase was observed under joint application of EDTA and CA and highest value of protein was observed in presence of CA as compared to Cd+BC, which may be due to less toxicity or stress produced by *C. roseus* in the presence of CA as compared to other chelates. However, Hg (100 $mgkg^{-1}$) and BC (4 $mgkg^{-1}$) in the presence of EDTA showed a marginal decrease (1.04 fold) in protein content while it increased (1.10 fold) in the presence of CA and was found to be highest (1.28 fold) under joint application in comparison to Hg+BC.

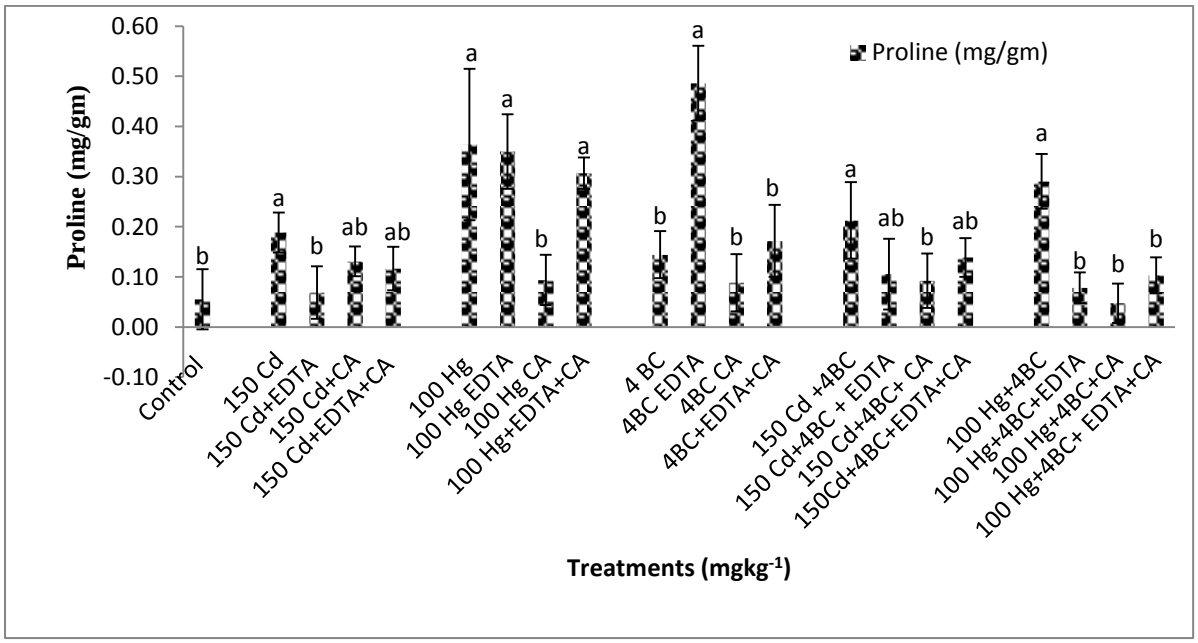


Figure 7.4: Effect of Hg, Cd, BC, Cd+4BC, Hg+4BC with chelating agents on proline content on leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

Proline: C. roseus proline content was tested for tolerance to heavy metal (Cd, Hg) and herbicide (BC), individually and under joint application of chelates (**Figure 7.4**). Results revealed that, chelate addition in the presence of 150 $mgkg^{-1}$ of Cd induced significant ($p<0.05$) decrease (63.30%) in proline content, especially in the presence of EDTA, while rest of the treatments (CA and EDTA+CA) revealed approximately 30.32% and 37.76% reduction in proline content with respect to 150 $mgkg^{-1}$ Cd.

Likewise proline values in the presence of chelating agents revealed a differential response in the presence of Hg (100 $mgkg^{-1}$). Proline content in the presence of CA showed decrement (approximately 3.87 fold) as compared to EDTA

(1.04 fold) and joint application of chelates (1.18 fold), as compared to control (Hg 100 $mgkg^{-1}$).

In contrast to the results stated above, joint and individual application of EDTA and CA in the presence of BC (4 $mgkg^{-1}$) have shown an increase in proline content under joint application of EDTA and EDTA+CA (3.375 and 1.19 fold) in comparison to BC (4 $mgkg^{-1}$). Effect of chelating agents under joint toxicities of heavy metal (Cd/Hg) and herbicide (BC) showed a significant ($p<0.05$) decrease in the proline content. In the presence of joint application of EDTA and CA alongwith co-contamination of Cd (150 $mgkg^{-1}$)/Hg (100 $mgkg^{-1}$) and BC (4 $mgkg^{-1}$) resulted in a significant decrease (1.53 and 2.81 folds, respectively) in proline content with respect to Cd+BC and Hg+BC, respectively.

7.3.4 Total phenolics content

The total phenolics content of Cd, Hg and BC both alone and in combination along with chelating agents on *C. roseus* is given in **Figure** (**7.5**). Chelating agent application tends to decrease the phenolics content in alone Cd (150 $mgkg^{-1}$) treatment as compared to untreated control. However, in the presence of EDTA, CA and EDTA+CA, a 13.76, 4.76, & 25.28% increase in phenolic content was observed under Cd treatment.

Similarly the total phenol content in the presence of different chelating agents under Hg treatment also revealed an increment of 38.05, 8.60, & 26.76% in the presence of EDTA, CA and EDTA+CA, respectively. In the presence of BC, addition of chelating agents *viz*, EDTA, CA and EDTA+ CA revealed a 1.062, 1.036 and 1.21 fold increase in values of total phenol content with respect to BC alone.

Combined treatment of Cd (150 $mgkg^{-1}$) and BC (4 $mgkg^{-1}$) under joint and single application of EDTA and CA, revealed a decrease of 32.12% in presence of EDTA followed by 2.22% in presence of CA and 17.91% in the presence of EDTA+CA in phenolics content with respect to Cd and BC treatments without any chelates. Similar decreasing trend in total phenolic content in the presence of chelating agents was also observed in Hg (100 $mgkg^{-1}$) along with BC (4 $mgkg^{-1}$).

Overall results revealed that total phenolic content was higher under BC treatment and was only marginally reduced under the joint application with heavy metals (Cd/Hg) under chelate.

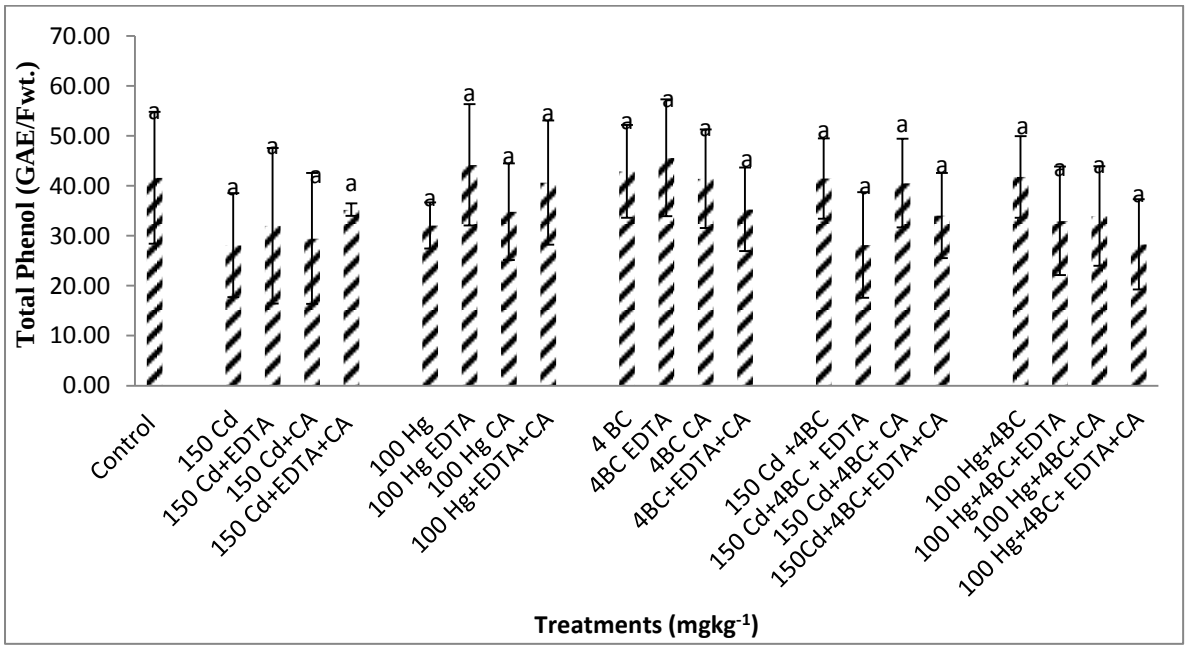


Figure 7.5: Effect of Hg, Cd, BC, Cd+4BC, Hg+4BC with chelating agents on total phenol content of leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

7.3.5 Assay of antioxidants

The effect of antioxidants involved in the mitigating stress induced generation of free radicals by Cd, Hg and BC alone and in combination on *C. roseus* in the presence of chelating agents EDTA, CA individually and jointly is given in **Figures 7.6-7.8**.

CAT Activity: Effect of chelating agents (EDTA, CA, EDTA+CA) on CAT activity under Cd (150 $mgkg^{-1}$) treatment, revealed a marginal increase in CAT activity (5.27%, 4.99% and 9.04%, respectively) with respect to Cd (150 $mgkg^{-1}$) alone. Similar trends was observed under Hg treatment too, wherein except for joint application (EDTA+CA) of chelates all other treatments revealed marginal decrease in CAT activity. However, in the presence of chelating agents a differential response to CAT activity was revealed under BC (4 $mgkg^{-1}$) treatment. In treatment BC +EDTA a 20.61% decrease in CAT activity was observed compared to BC alone, while a 4.29 % marginal increase in CAT activity was recorded in presence of CA, which might be due to CA application which regulates the anti-oxidant activities under stress environment (**Shakoor et al, 2014**).

Effect of chelating agents on catalase activity under joint presence of metal and herbicide revealed a differential response under Cd and Hg induced stress. A significant increase (5.28%) in CAT activity was recorded when EDTA was added to Cd+BC treatment, alone, as well as along with CA (5%).However, in the presence of

CA without EDTA a significant decrease in CAT activity was reported in Cd+BC treated samples which might be attributed to CA induced quenching of free radicals.

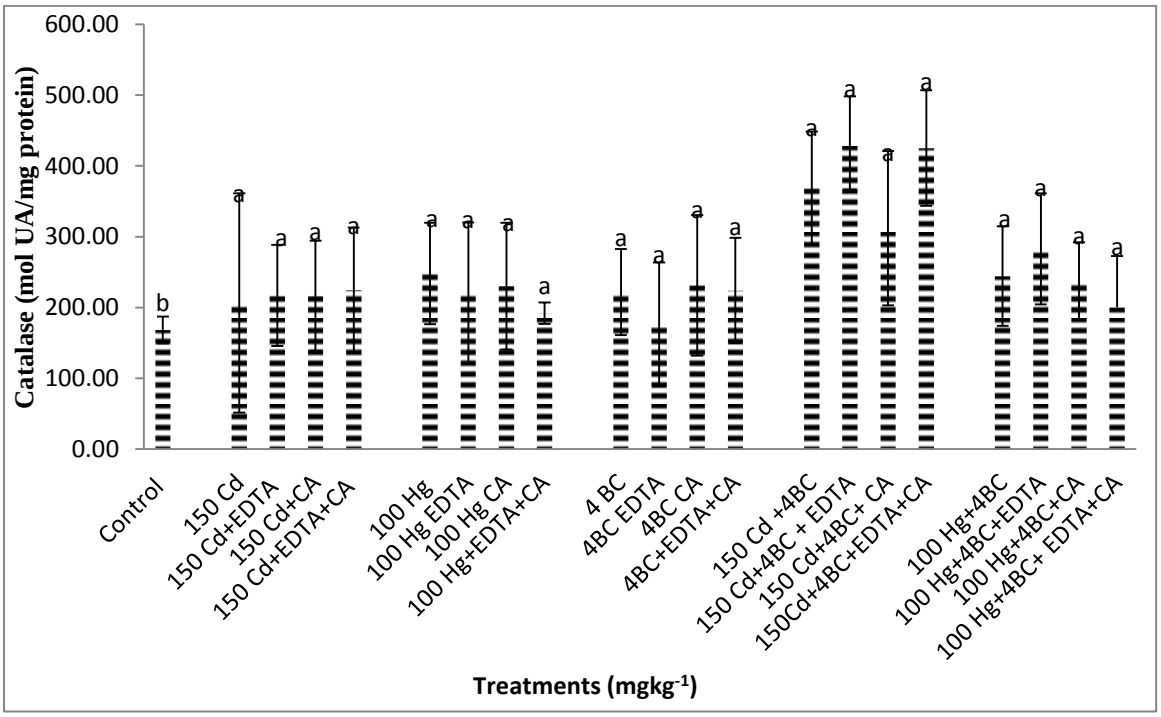


Figure 7.6: Effect of Hg, Cd, BC, Cd+4BC, Hg+4BC with chelating agents on catalase activity on leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

Since an increase in CAT activity is observed when Cd+BC are treated with both the chelates jointly, it can be inferred that EDTA affects the antioxidant activity of CA as far as Cd toxicity is concerned.

However, addition of EDTA to Hg+BC treated samples only revealed a marginal increase in CAT activity (1.16 fold), as compared to without chelates. Addition of CA, on the contrary resulted in a marginal decrease in CAT activity (1.03 fold), which further decreased marginally (1.22 fold) under joint application of chelates.

Overall, it could be summarized that both the heavy metals *i.e.*, Cd and Hg as well as BC induce oxidative stress which is revealed by an elevated values of CAT under various treatments. A substantial increase in CAT activity under joint application of Cd and BC shows that the combination is more toxic as compared to Hg+BC treatment with respect to control.

SOD Activity: An effect of chelating agents (EDTA, CA and EDTA+CA) was monitored on SOD activity Cd (150 $mgkg^{-1}$)/Hg (100 $mgkg^{-1}$) and BC stressed plants (**Figure 7.7**)

A significant decrease in SOD activity was revealed both under individual and joint application of chelate in Cd stressed plants. Presence of EDTA, CA and EDTA+ CA resulted in 33.79, 26.48 and 15.07% decrease in SOD activity under Cd treatment as compared to chelate free Cd (150 $mgkg^{-1}$).

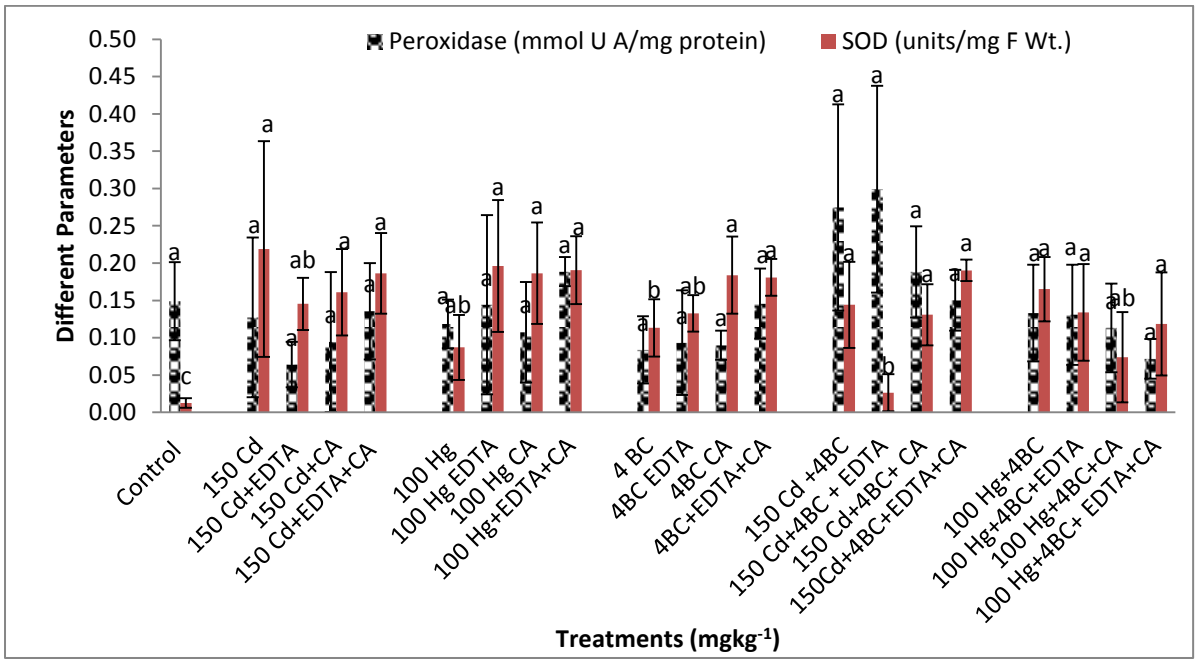


Figure 7.7: Effect of Hg, Cd, BC, Cd+4BC, Hg+4BC with chelating agents on POD and SOD activity on leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

On the contrary under Hg treatment, SOD activity was found to increase by 2.25, 2.14 and 2.19 fold, respectively, in the presence of EDTA, CA and EDTA+CA compared to Hg treatment without any chelate.

Similarly in the presence of BC too, the SOD activity increased by 1.17, 1.63 and 1.6 fold, respectively in the presence of EDTA, CA and joint application of chelates, respectively, as compared to BC treatment without any chelate.

Addition of chelates under co-contamination *i.e.*, Cd (150 $mgkg^{-1}$) and BC (4 $mgkg^{-1}$) revealed decrease in SOD activities, whereas, maximum reduction was observed in the presence of EDTA (81.94%). In the presence of CA a marginal decrease (9.03%) was observed in activity while, under joint application of chelates a 24.21% increase in the activity was recorded as compared to Cd+BC without any chelate.

SOD activity under Hg+BC treatment revealed 18.78, 55.15 & 28.48% decrease in the presence of EDTA, CA and EDTA+CA, respectively as compared to joint application of metal and herbicide without any chelate (**Figure 7.7**).

POD Activity: **Figure 7.7** shows the peroxidase activity (POD) activity monitored under Cd/Hg and/or BC treatment both in the presence and absence of chelates. Under individual Cd treatment, addition of EDTA and CA induced a reduction in POD activity, while joint application of EDTA and CA results in an increase in the activity, with respect to Cd (150 $mgkg^{-1}$) in the absence of chelates.

On the contrary under individual Hg treatment addition of EDTA and EDTA+ CA induced 1.21 and 1.59 fold increase in POD activity and approximately 0.9 fold decrease in the activity, in the presence of CA alone as compared to Hg (100 $mgkg^{-1}$) in the absence of chelates.

Effect of chelation on POD values under BC (4 $mgkg^{-1}$) revealed a marginal decrease of 1.07 and 7.14% in the presence of EDTA and CA, respectively while the joint application of chelates brought a significant (73.81%) decrease in the POD activity, as compared to BC alone treatment, without chelates.

Results obtained under co-contamination (Cd-150 $mgkg^{-1}$ and BC- 4 $mgkg^{-1}$) revealed a marginal increase in POD activity in the presence of EDTA (0.92 fold), while a significant decrease in activity was obtained in the presence of CA (1.46 fold). Under joint application of chelates approximately 1.83 fold decrease in activity was observed as compared to Cd + BC treatment without chelation

Co-contamination with Hg (100 $mgkg^{-1}$) and BC (4 $mgkg^{-1}$) showed a significant (<0.05) decrease in POD activity in the presence of chelating agents as compared to Hg+BC treatment without chelation.

APOX Activity: Effect of chelating agents (EDTA, CA and EDTA+CA) on APOX activity under Cd/Hg and/or BC treatment is given in **Figure 7.8**.

Application of chelating agents, either alone or in combination induced a significant increase in APOX activity under Cd (150 $mgkg^{-1}$) treatment. However, on the contrary the same application induced a decrease in the activity under Hg (100 $mgkg^{-1}$) treatment when compared to individual treatments without chelation.

On the other hand, application of chelating agents under BC (4 $mgkg^{-1}$) treatment induced approx. 1.01 and 1.19 fold increase in APOX activity in the

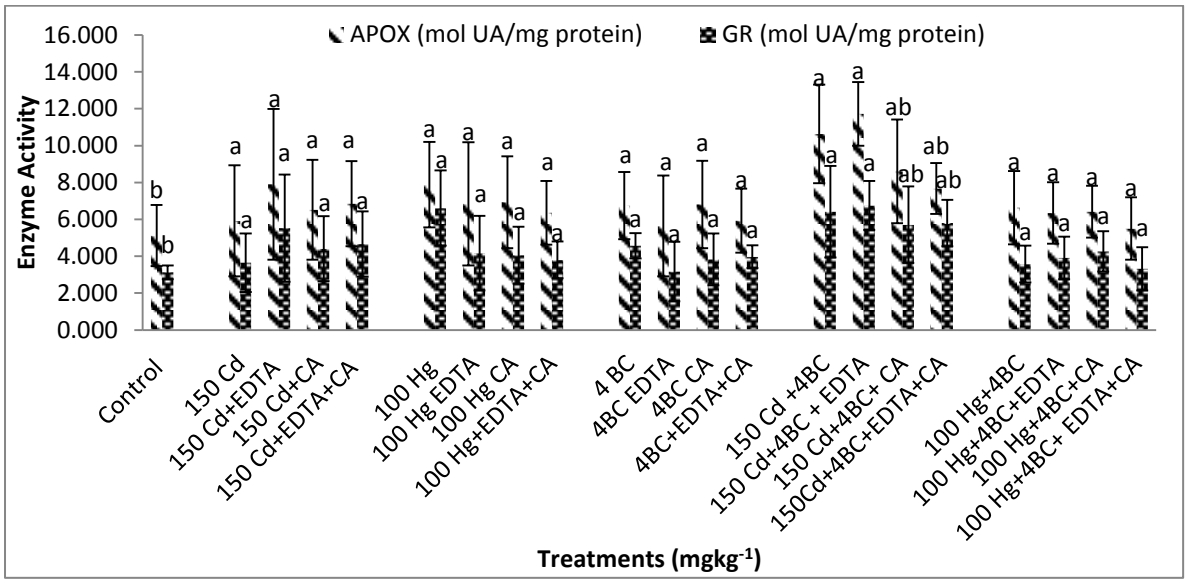


Figure 7.8: Effect of Hg, Cd, BC, Cd+4BC, Hg+4BC with chelating agents on APOX and GR Activity on leaves of *C. roseus* (±S.D., n=3, Duncan test, P≤0.05)

presence of CA and EDTA, respectively, while an approximately 1.14 fold decrease in the activity was observed under joint application of chelates as compared to individual BC treatment without any chelation.

Evaluation of chelation on APOX activity under co-contamination (Cd 150 $mgkg^{-1}$) + BC 4 $mgkg^{-1}$) revealed an approximately 10% increase in activity in the presence of EDTA while approximately 19 and 27% decrease in activity was recorded in the presence of CA and joint application of EDTA+CA, respectively. However, the presence of chelates had no significant effect on the activity under Hg (100 $mgkg^{-1}$) and BC (4 $mgkg^{-1}$) co-contamination as compared to respective treatment without chelation (**Figure 7.8**).

GR Activity: Effect of chelating agents (EDTA, CA, EDTA+CA) on GR activity under Cd (150 $mgkg^{-1}$) treatment revealed a significant increase in values. An approximate 1.15, 1.21 and 1.27 fold increase in GR activity was recorded in the presence of EDTA, CA and EDTA+CA, respectively as compared to Cd (150 $mgkg^{-1}$) treatment without any chelation.

On the contrary, under Hg (100 $mgkg^{-1}$) treatment addition of chelating agents induced an overall decrease in the GR activity with respect to Hg (100 $mgkg^{-1}$) treatment without chelation.

Similarly, chelate application under BC (4 $mgkg^{-1}$) treatment showed 30.69, 16.31 and 13.03% decrease in GR activity in the presence of EDTA, CA and EDTA +

CA when compared to BC treatment without chelation.

However, GR activity under co-contamination (Cd+BC) revealed an approximate 1.05 fold increase in activity in the presence of EDTA while 1.12 and 1.10 fold decreases in activity was observed for CA and EDTA + CA applications, respectively.

Contrary to above the GR activity under Hg (100 $mgkg^{-1}$) + BC (4 $mgkg^{-1}$) co-contamination revealed an increase in activity in the presence of individual chelates. However, joint application of chelates induced a decrease in activity with respect to chelate deficit co-contamination.

Effects of chelating agents either EDTA, CA alone or in combination on various biochemical parameters under individual and co-contamination have revealed differential responses for different parameters. An increase in various antioxidant and lipid peroxidation enzymes under various treatments could be correlated to a protective role of CA/EDTA in increasing antioxidant responses in *C. roseus*.

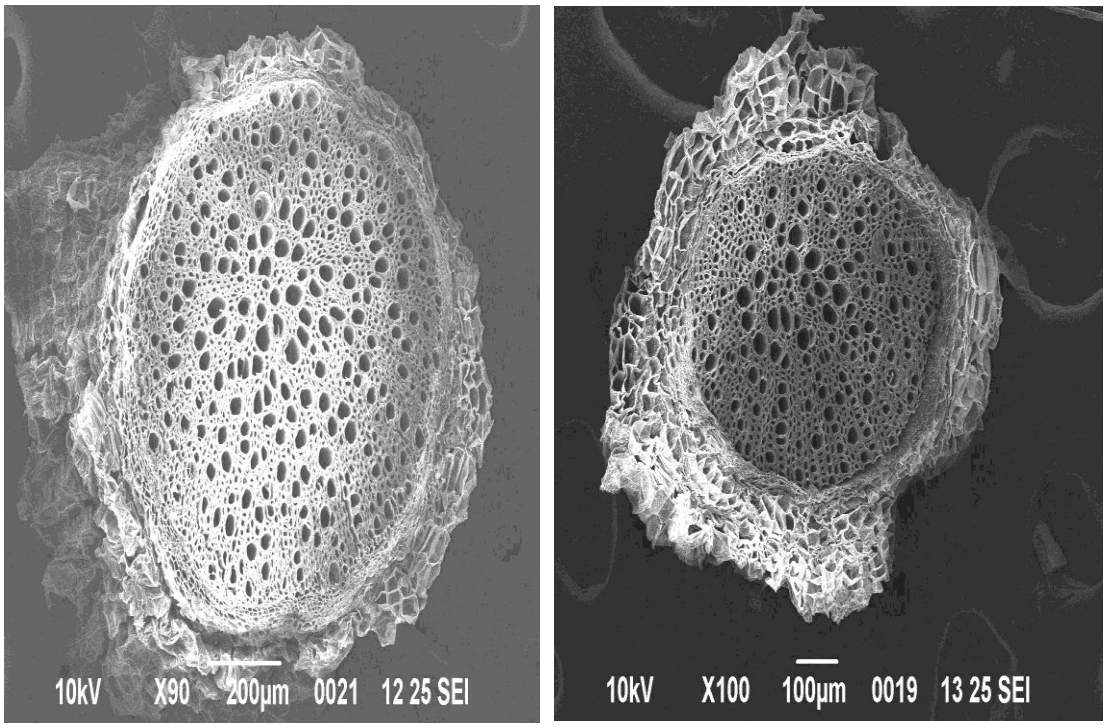
7.3.6 SEM- root, shoot, leaf and EDX of root

SEM-EDX analysis of root, shoot and leaf of *C. roseus* plants were carried out under co-contamination in the presence of chelating agents to ascertain their protective role under stress. Two different combinations, one each from Hg and Cd co-contamination with BC were selected based upon good plant growth as compared to other treatments over control.

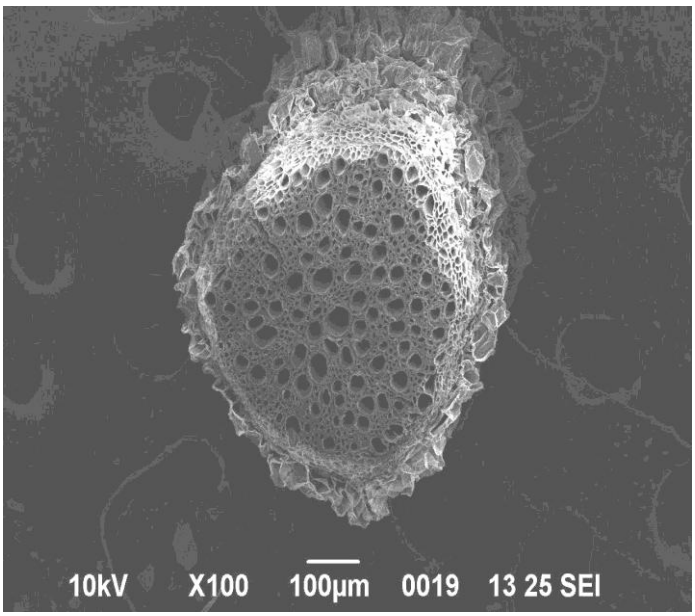
The first combination that was subjected to SEM-EDX analysis was Hg (100 $mgkg^{-1}$) + BC (4 $mgkg^{-1}$) +EDTA + CA and another was Cd (150 $mgkg^{-1}$) + BC (4 $mgkg^{-1}$) + CA.

EDX analysis of plant treated with 100 $mgkg^{-1}$ Hg and 4 $mgkg^{-1}$ BC in the presence of EDTA and CA both, showed Al-K, Ca-K peak values (weight% 0.15 and 0.00 respectively, Data not shown) compared to that of control (weight% 0.77 and 2.57). Effect of CA on joint toxicities of 150 $mgkg^{-1}$ Cd and 4 $mgkg^{-1}$ BC revealed Al-K, Si-K to be values 0.23 and 0.25 (weight%), respectively, as compared to control (0.77 and 23.92 weight%), respectively.

Metal stress plants induce ultrastructure modification in shoots as well as root cells due to deposition or binding of the metal element, when they enter into the cell of plants (**Hamim et al, 2018**).

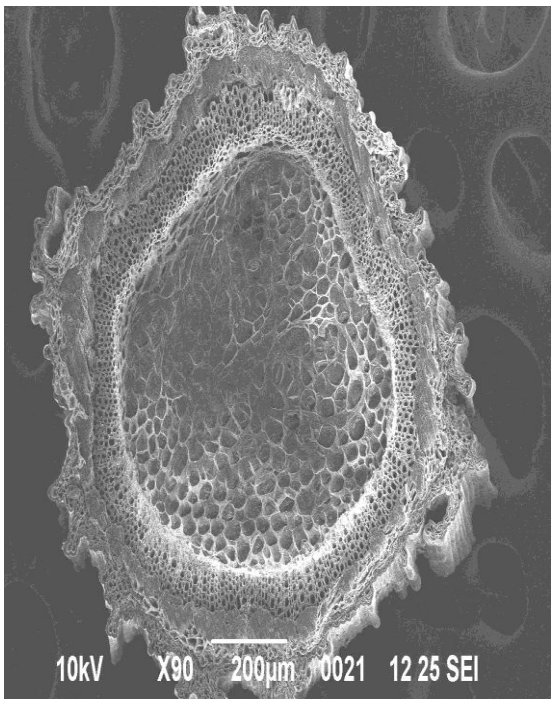


1(a) **1(b)**

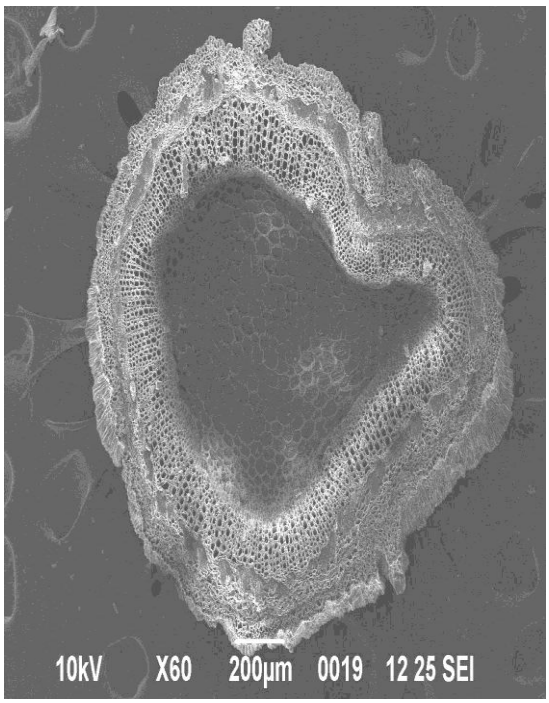


1(c)

Figure 7.9: Scanning electron micrograph (SEM) of untreated and treated root of *C. roseus* under different treatments of Cd, Hg, BC alone and in combination with chelating agents: 1(a) Control; (b) 100Hg+4BC+EDTA+CA; (c) 150 Cd+4BC+CA



2(a)



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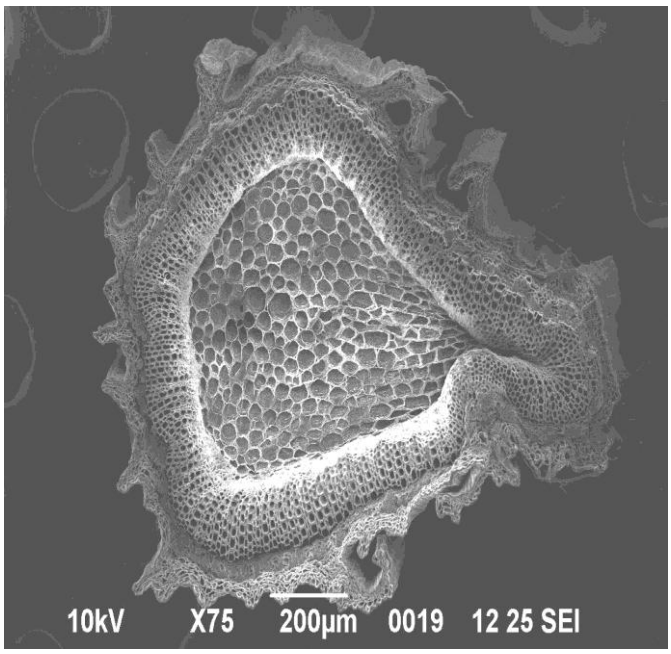
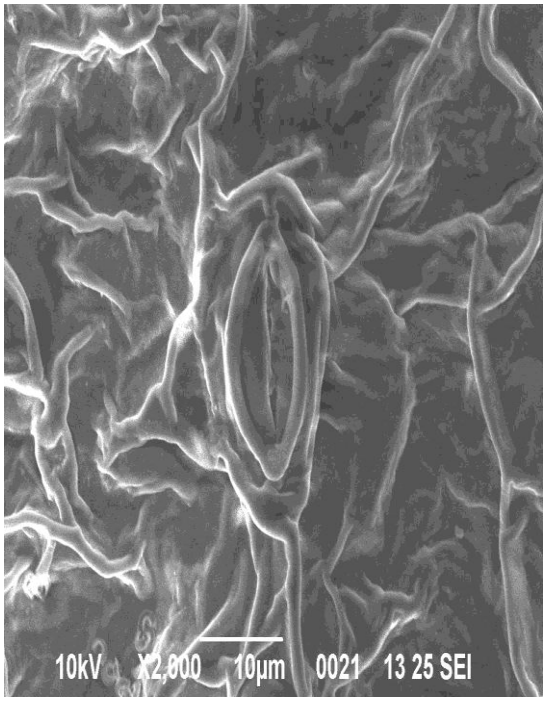
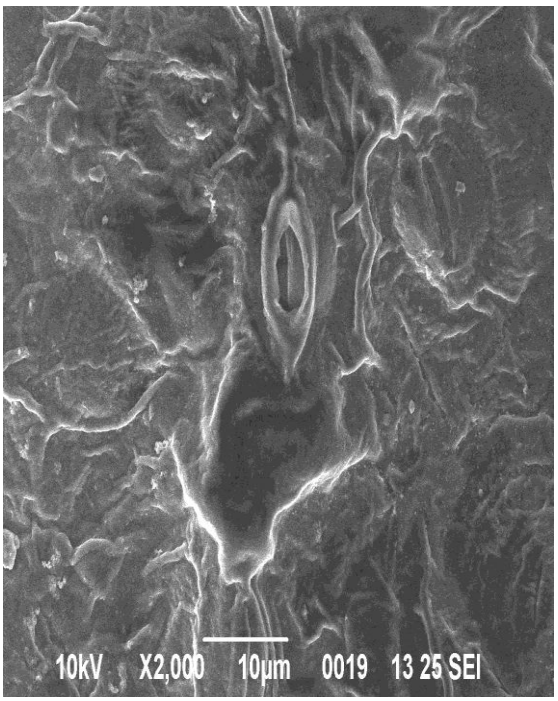


2(c)

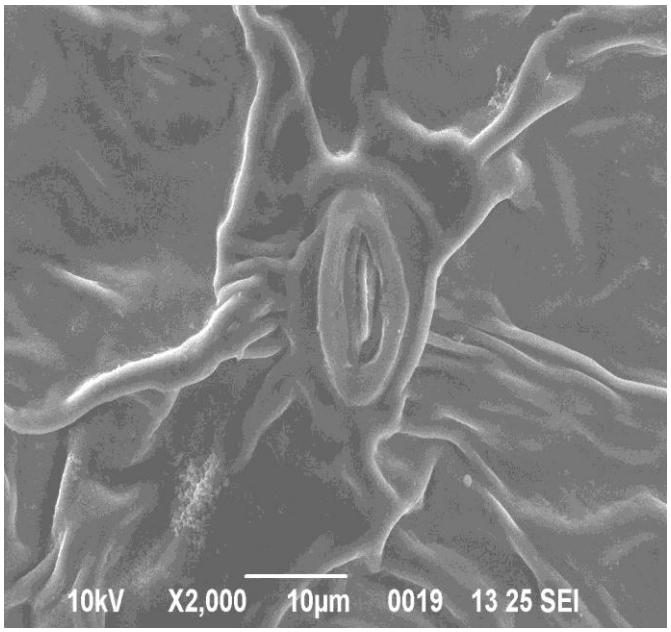
Figure 7.10: Scanning electron micrograph (SEM) of untreated and treated shoot of *C. roseus* under different treatments of Cd, Hg, BC alone and in combination with chelating agents: 2(a) Control; (b) 100Hg+4BC+EDTA+CA; (c) 150 Cd+4BC+CA



3(a)



3(b)



3(c)

Figure 7.11: Scanning electron micrograph of untreated and treated leaves of *C. roseus* under different treatments of Cd, Hg, BC alone and in combination with chelating agents: 3(a) Control; (b) 100 Hg+4BC+EDTA+CA; (c) 150 Cd+4BC+CA

SEM ultrastructure analysis of transverse sections of root and shoot samples of *C. roseus* revealed normal arrangement of xylem and phloem in control (**Figures 7.9 (a-c) and 7.10 (a-c)**). However, the SEM image in treated plants under both combinations shows a thickening of both xylem and phloem as compared to untreated control root. Shoots, on the other hand, revealed more structural deformation in comparison to roots as shown in **Figure 7.10**.

The SEM analysis of treated leaves of *C. roseus* under co-contamination in the presence of chelating agents results in considerable damages in stomata revealed as compared to control. The size of stomata in leaves of control was 15.34* 7.42 µm while that under co-contamination (Hg+BC+EDTA+CA) showed decrease in stomatal size *i.e.*, 13.20*6.50 µm. On the contrary stomatal size in the combination Cd+BC+CA revealed a marginal increase (16.91*8.61 µm) was recorded with respect to control.

7.4 ANOVA

One-way analysis of ANOVA showed that the LPO and proline were having significant ($p<0.05$) effect on Cd (150 $mgkg^{-1}$) in the presence of EDTA/CA, alone and jointly. While under co-contamination of Cd (150 $mgkg^{-1}$) with BC (4 $mgkg^{-1}$), results revealed APOX, SOD, CAT and protein values being significantly effected ($p<0.05$) in the presence of chelators. Results further revealed that Hg (100 $mgkg^{-1}$) alone treatments have significant ($p<0.05$) effect on Carbohydrate, Proline and SOD while Hg (100 $mgkg^{-1}$) in the presence of BC (4 $mgkg^{-1}$) along with application of chelating agents showed significant difference in the activity of Proline and SOD only. BC (4 $mgkg^{-1}$) alone with EDTA and CA revealed significant ($p<0.05$) effect on SOD, Proline and total chlorophyll.

7.5 Discussion

In this chapter, we elucidated the physiological changes in *C. roseus* plants when subjected to Cd, Hg, BC alone and in combination and modulation of these traits when a natural (CA) and a synthetic (EDTA) chelating agent was added in the soil to induce heavy metal and herbicide stress.

In the present study, when chelating agents were applied to plants under heavy metal and herbicide stress, an increase in photosynthetic pigments content values were

observed (**Figure 7.1**). Similar findings were observed by **Wang et al** (**2004**), who reported an increase in chlorophyll content and elevating light harvesting potential of the treated plants in the presence of chelating agents.

Phenolic compounds acting as antioxidants may function as terminators of free radical chains and as chelators of redox-active metal ions that are capable of catalyzing lipid peroxidation. Addition of chelating agents have a differential response to heavy metals and herbicide as far as toxicity (LPO activity) is concerned (**Figure 7.2**). Our results revealed that in most of the treatments addition of chelating agents enhanced antioxidant enzymes, which decreased the production of MDA contents with respect to various treatment (Cd, Hg, BC alone and jointly). Similarly, **Markovska et al** (**2013**) stated that application of EDTA lowered MDA content and oxidative damage was not strongly expressed in the presence of EDTA.

Results of our study on *C. roseus* revealed an increase in protein contents in the presence of chelating agents (**Figure 7.3**), our study are in concurrence with the findings of **Aderholt et al** (**2017**) who stated that increased soluble proteins content might be due to the efficient working of photosystem and gas exchange of plants which was regulated by the addition of CA under Cr stress. Similarly, **Saeed Akram et al** (**2009**) and **Park et al** (**2012**) also reported that the scavenging of ROS and decrease in electrolyte leakage may be the key factors for increased soluble proteins content in both roots and leaves of sunflower.

During stress condition plants stimulates an amino acid compound proline (**Sun et al, 2007**; **Ahmad et al, 2015**). In the current study, proline content was found to decrease in the presence of chelating agents, which might be due to the reason that chelators form a complex structure which reduces the toxicity of heavy metals and herbicide in the soil. An increase in the activities of antioxidative enzymes under the combined treatment of heavy metal and herbicide in the presence of chelating agents suggest that it might improve defense mechanism in the current study on *C. roseus*. For mitigation of ROS-induced damages plant developes protective mechanism in the form of enhancement of SOD, APX, CAT, GR and POD activity (**Sharma et al, 2012**). In the current study, GR and APOX activity revealed that there is decrease in activity in the presence of chelators in most of the treatments (**Figure 7.8**). SOD activity of *C. roseus* in individual treatment (Cd, Hg and BC) showed an increase in the values while joint toxicities of Cd+BC and Hg+BC showed a decrease in activity

in the presence of chelating agents repectively, with alone treatment (Cd, Hg, BC, Cd + BC and Hg + BC) which might be due to quenching of free radicals produced under metal and herbicide stress by CA, a natural chelating agent which is also an antioxidant. However, POD results did not give a significant difference. Similar results were also observed in recent past by **Rizwan et al** (**2017**) that increasing and then decreasing trend was observed by sunflower plants in antioxidant enzymes activities in the presence of heavy metal contaminated soils. Exposure to BC poses significant cell damage in the plants which might be closely related to the hydrogen peroxide- induced oxidative stress than the superoxide-induced oxidative stress (**Wang et al, 2013**).

Antioxidants like proline, carbon monoxide and glutathione (GSH) are non-enzymatic systems which plays a crucial role in plants to respond under stress conditions (**Sharma & Dietz**, **2009**). According to **Iannelli et al** (**2002**) mechanism of heavy metal stress (Cd) induced oxidative stress results in ROS species production which stimulates antioxidant enzymes. Antioxidative mechanism for cleansing of ROS depends upon the plant types, nature of the organic acid applied and stress severity; the exogenous application of chelating agents may alters the antioxidant activities under non-stress and stress environments (**Shakoor et al**, **2014**; **Ashraf et al**, **2015**; **Farid et al**, **2017**).

Heavy metal induced ROS promptly react with poly-unsaturated fatty acids leading to structural, functional and biological changes in membranes (**Bhattacharjee, 2005**). The SEM structure of treated root, shoot and leave showed a deformation in the epidermal layer as well as thickening of xylem and phloem with respect to control (**Figures 7.9-7.11**). The stomatal changes may be due to toxicity of rapid and preferential absorption of heavy metal and herbicide by subsidiary cells which results in changes in membrane permeability which is likely to create decrease in cell turgor pressure.

7.6 Conclusion

In the present study, it was found that for *C. roseus* addition of chelating agents like CA and EDTA can be used for phytoremediation of heavy metals and herbicide in co-contaminated soil. Co-contaminants significantly reduced transpiration rate, stomatal conductance and photosynthesis, whereas it enhanced activities of lipid peroxidation

and antioxidative enzymes. Application of chelating agents in heavy metal and herbicide contaminated soil significantly increased the uptake of co-contaminants and the potential of antioxidative defense system against oxidative injury, persuaded by heavy metal and herbicide toxicity in *C. roseus* plants. Co-contaminant induced oxidative stress in *C. roseus*, by reducing photosynthetic pigments, increasing ROS and MDA content, and disrupting the antioxidant defense systems. However, application of EDTA/CA in the soil with heavy metal (Cd/Hg) and herbicide (BC) noticeably alleviated the detrimental effect through upregulating the antioxidant defense mechanism. Cd/Hg and BC concentrations caused ultra-structural alteration in root, shoot and leave cells. CA/EDTA application significantly enhanced accumulation in *C. roseus* by recovering plant roots from co-contaminants induced ultra-structural change. Biochemical parameters have observed different responses for different parameters in the presence of chelating agents. Overall, it can be inferred that CA/EDTA would be beneficial in accelerating the environmentally safe phytoextraction of heavy metals and herbicide in co-contaminated soil through enhanced accumulation and antioxidant activity in *C. roseus* plant. Soil amendments facilitate more accumulation of heavy metals and their antioxidant potential makes their use suitable for phytoremediation of Cd and Hg in *C. roseus* plant in the presence of BC.

Plate 7.1: View of pot experiment to study the impact of co-contaminants on *C. roseus*

Chapter 8

Phytoremediation of Cd/Hg and Butachlor contaminated soils by C. roseus under individual treatment and joint contamination

8.1 Introduction

Contamination with polycyclic aromatic hydrocarbons (PAHs) and heavy metals (HMs) poses severe threats to soil, water resources and human health besides their capability to destroy natural ecosystems (**Khan et al**, **2008**; **Chen et al**, **2015**). **Zhang et al** (**2011**) investigated the combined toxicity of PAHs and metals and suggested that toxicity may be independent, additive, antagonistic (less than additive toxicity), or synergistic (greater than additive toxicity).

The World Health Organization (WHO) has alloted a hazard ranking of III (slightly hazardous) for metolachlor and acetochlor and a ranking of U (unlikely to be hazardous) for butachlor (**WHO**, **2004**), whereas heavy metal Cd/Hg is a big three heavy metal (Pb, Hg and Cd) and regarded as poisons and yet not reported for any essential biological function either for plants or animals. Occurrence of heavy metals may together with PAHs in the environment (**Thavamani et al**, **2012**; **Viana et al**, **2012**), makes the remediation processes difficult because different types of pollutants may either interact among themselves and/or with their rhizosphere microbial communities as well as with plants.

It has been demonstrated that the interactions between organic and inorganic contaminants may increase the complexity of their remediation. Other obstructions are related to the inherent toxicity of heavy metals that can restrain the biodegradation of organic contaminants by the microbes in soil (**Said & Lewis**, **1991**; **Sandrin & Maier**, **2003**). The transport and removal of heavy metals in soils gets affected positively or negatively due to presence of organic contaminants.

Sun et al (**2008**) reported that phytoremediation is an environment friendly technology that employs plants to sequester organic contaminants as well as metal through processes including degradation, assimilation, detoxification, metabolization or extraction. Earlier researchers have addressed the plant induced dissipation of PAH in the contaminated soil (**Khan et al, 2013**) or uptake and accumulation of heavy metal by plants in soils contaminated with heavy metal (**Babu et al, 2013**).

Present study has been undertaken to evaluate the phytoremediation potential of *C. roseus* in Cd/Hg- BC co-contaminated soils. Therefore, the aim of the present study was to study the impact of co-contamination of Cd/Hg and BC on *C. roseus* growth and also to investigate the fate of contaminants both in plants as well as soil. BC and Cd/Hg were selected for this study since BC is an herbicide which represents

a class of organic compounds that could be naturally present in most polluted sites and Cd/Hg are one of the commonly present heavy metals in most of the sites. Hence, the objectives of the present study were to- (i) to study the growth response of *C. roseus* to individual Cd/Hg or BC and co-contaminated Cd/Hg and BC soils. (ii) to examine the potential of *C. roseus* for uptake, accumulation and translocation of Cd/Hg (iii) to explore the role of *C. roseus* in dissipation of BC.

8.2 Materials and Methods

8.2.1 Preparation of heavy metals stock solutions

Detail methodology is given in Chapter 6 (Materials and Methods) Section 6.2.1.

8.2.2 Preparation of herbicide stock solutions

For detail methodology please refers to Chapter 6 (Materials & Methods) Section 6.2.2.

8.2.3 Soil preparation and pot experimentation

To start the experiments, air-dried loamy sand soil was collected from the Green house of Babasaheb Bhimrao Ambedkar University, Lucknow, (U.P.), India, campus. Cd and Hg concentrations were below the detection limit of the instrument used. The soil sample were mixed with equal volume of perlite (1:1, v/v) to ensure aeration of plant roots with aeration 2 kg of the soil mixture were placed in plastic planter pots in which *C. roseus* seeds were sown. The pots were irrigated for two weeks with distilled water. The two weeks old seedlings were treated with different concentrations of Cd/Hg while one set without treatment served as control. The moisture content was adequately maintained at 70% of water holding capacity. Number of seedlings per pot were thinned to two plants per plot, after 3 weeks and further grown for 8 weeks.

All the treatments were replicated three times. Pots were kept in the green house. Soil was fertilized with micronutrients fertilizer mixture. The pots were kept in naturally illuminated green house of research field. All the measurements were performed at 20 and 60 days after sowing (DAS). Physicochemical properties of experimental soil were analyzed and given in the **Table 8.1**.

Table 8.1 Physicochemical characteristics of experimental soil

Parameters	Values
Sand (%)	35.2
Silt (%)	21.3
Clay (%)	43.5
MC (%)	20.32
pH	7.56
EC(µS/cm)	80.21
OC (%)	2.12
OM (%)	3.65
N (kg/ha)	58.23
K(kg/ha)	96.41
P (Kg/ha)	6.53
Pb (mg/kg)	6.28
Cr (mg/kg)	5.17
Hg (mg/kg)	0.02
Fe (mg/kg)	1500
Cu (mg/kg)	0.57
Ni (mg/kg)	0.01
As (mg/kg)	0.00
Mn (mg/kg)	101.31
Cd (mg/kg)	5.51

8.2.4 Plant growth analysis

The plants were removed from pots at 20 and 60 DAS and dipped in a water filled bucket. The plants were moved smoothly to remove the adhering soil particles. Fresh weight of roots and shoots was taken through digital weighing balance. The root and shoot were then placed in an oven at 70 °C till the weight became constant. The dried plant parts were weighed to record the dry mass of roots and shoots.

Biomass (mg) day^{-1} $plant^{-1}$ of 20 days old plant (B_1) = Biomass of 20 days old plant/20

Biomass (mg) day^{-1} $plant^{-1}$ of 60 days old plant (B_2) = Biomass of 60 days old plant/60

Rate of biomass production (mg) day^{-1} $plant^{-1}$ = $\frac{B_1+B_2}{2}$

8.2.5 Plant harvest, preparation and analyses

After eight weeks of cultivation, *C. roseus* plants were removed from the pots. After separation of shoot and roots from plants, measurement of lengths and weights of each were measured and dried (70 ºC) to measure dry wt.

8.2.6 Estimation of Cadmium and Mercury content in Plant (*C. roseus*)

Plant materials were ground and 0.5 g portions of sieved plant matter was digested with a mixture of perchloric acid–nitric acid mixture (1:3, v/v). The concentrations of Cd and Hg in the samples were determined using Varian Spectra AA-250 plus Atomic Absorption Spectrophotometer (AAS). Per plant (by roots and shoots) the metal accumulation was calculated as follows:

Total metal extraction by roots/shoot (µg/plant) =

Dry biomass of roots/shoot (g) * metal accumulated (µg/g dry wt) by roots/shoot

8.2.7 Soil analysis for heavy metal Hg and Cd

Cd and Hg contents in the soil samples were measured following the method given by **Odu et al, 1986**. Samples were subsequently measured for metal content using atomic absorption spectroscopy (AAS). Detailed methodology is given in Chapter 3 (Materials & Methods) Section 3.4.

8.2.8 Bioconcentration and translocation of heavy metals

Please refer to Chapter no. 3, Section 3.5 for details.

8.2.9 Herbicide estimation in soil through GC-MS

8.2.9.1 Soil extraction process

All the soil samples were air-dried at room temperature, pulverized, and passed through 100 µm sieves. The extraction procedures were as follows: 10 g of the soil sample was accurately weighed and extracted in a Soxhlet apparatus using 100 mL n-hexane for 8 h, followed by rotatory-evaporation and concentration of the extract up to dryness (1-2 mL). The concentrated soil extract was cleaned up using 1 or 2

chromatographic columns (10 mm, diameter) packed with Na_2SO_4, Florisil and silica gel (from bottom to top) to remove the interferences. The target analyte was recovered in 100 mL of n-hexane. The final extract was concentrated, exchanged into hexane, and reduced to 1.0 mL. Before injection into GC-MS detector, butachlor was added as the internal standard.

8.2.9.2 GC/MS Conditions

Helium gas was used as carrier. A constant column flow rate (1 mL min^{-1}) was maintained. The temperature program was as follows: the initial temperature of 120°C was increased to 200°C at 20°C min^{-1}, to 230°C at 5°C min^{-1}, to 260°C at 30°C min^{-1}, finally held for 2 min. The total run time was 13.00 min. The temperature of the injection port was 250°C and 1.0 μL of sample was injected into the GC in pulsed splitless mode. Electron impact ionization source with ionization energy of 70 eV and selected ion monitoring (SIM) mode were used. The SIM program was 8.8–11.3 min for butachlor (m/z 176, 160,188), The ion source and MS Quad temperatures were 230°C and 150°C, respectively. The solvent delay was 6 min (**Qiu et al, 2010**).

8.3 Statistical analysis

All results were statistically analyzed. Means of three replicates for all analyses were subjected to 1-way as well as 2-way ANOVA (SPSS Inc., version 25.00). Tukey's honestly significant difference based on F values was applied for testing the significant differences among means ($P< 0.05$, <0.01,<0.001).

8.4 Results

8.4.1 Plant growth and biomass

An exposure of heavy metals to plants, at higher concentrations, causes severe damage to different metabolic activities ultimately leading to the death of plants. An excessive metals exposure to plants has been reported to inhibit enzymes that are physiologically active (**Gadd, 2007**), may inactivate photosystem I and II (**Sandmann and Boger, 1980**), and bring about mineral metabolism destruction (**Janas et al, 2010**). Hence, it is obvious that for effective bioremediation the biomass of the plant selected for the study plays a significant role as it ensures maximum removal of heavy metals. *C. roseus* an ornamental plant was chosen in the present

study based on its adequate biomass, fast growth rate and its suitability for removal of heavy metals and herbicides from contaminated soils (**Forte & Mutiti, 2017**). The experimental plants appeared healthy both in the control as well as soil amended with low concentration of metals, whereas plants grown on higher Cd and Hg contaminated soils revealed yellowing and/or browning of the leaves. Growth parameters *viz*. fresh and dry biomass of root and shoots of *C. roseus* were analyzed on 20 and 60 days after sowing (DAS). In the present study, significant differences were noted in shoot, root weight and length of plant grown on soil amended with various concentrations (25,50,100,150,200 and 20,40,80,100,120 $mgkg^{-1}$) of different metals (Cd and Hg) and herbicide BC (0.5,1.0,2.0,4.0,8.0 $mgkg^{-1}$) alone and in joint treatment (**Tables 8.2-8.16**).

Results revealed that Cd induced more decrease in plant biomass of the growing plants as compared to Hg. The plant weights and length was found to be reduced under high metal contaminated soils with respect to control and those growing under low to moderate metal contamination. The fresh and dry weights of plants significantly decreased in shoot by 63.79 and 70.64% and root by 69.56 and 65%, respectively, to control, when the plants were grown on soil amended with 200 mg kg^{-1} of Cd (**Table 8.2**) in soil and a decline of 65.89 % and 64.84 % in root and 36.47% and 60.68% in shoot fresh and dry wt. at 120 mg kg^{-1} of Hg (**Table 8.3**), respectively in comparison to control as measured on 60 DAS.

Analysis of variance test was conducted for biomass and morphological parameters of *C. roseus* grown in soil amended with different concentration of Cd. Results revealed a significant variation with respect to days and concentration individually but in combination of both there was non-significant variation observed in root length, root and shoot dry wt. (**Table 8.8**). Multivariate analysis showed a significant variation in morphological parameters of *C. roseus* in alone Hg treatment with concentration and days. Interaction between days and concentration in different parameters showed a non-significant variation in all parameters except shoot length (**Table 8.10**). Shoot biomass (fresh and dry wt.) was significantly reduced ($p<0.05$, 0.01) in comparison to control by 41.75 and 60.48% in presence of 8.0 mg kg^{-1} BC, respectively. Root fresh and dry wt. was also reduced by 49.80% and 48.08% respectively upon exposure to the same concentration of BC (**Table 8.4**). Multivariate analysis of *C. roseus* grown in soil amended with BC revealed that individual factors

Table 8.2: Effect of Cd on fresh and dry biomass accumulation in *C. roseus* (±S. D., n=3)

	20 DAS				60 DAS			
Soil Cd Concentration (mg kg^{-1} soil)	**Fresh Wt. of Roots (g plant^{-1})**	**Dry Wt. of Roots (g plant^{-1})**	**Fresh Wt. of Shoots (g plant^{-1})**	**Dry Wt. of Shoots (g plant^{-1})**	**Fresh Wt. of Roots (gplant^{-1})**	**Dry Wt. of Roots (g plant^{-1})**	**Fresh Wt. of Shoots (g plant^{-1})**	**Dry Wt. of Shoots (g plant^{1})**
Control	1.305±0.55^{a}	0.452±0.07^{a}	1.203±0.35^{a}	0.598±0.09^{a}	3.197±0.37^{a}	0.763±0.16^{a}	7.943±0.73^{a}	1.863±0.58^{a}
25 Cd	0.937±0.21^{a}	0.353±0.11^{ab}	1.030±0.44^{a}	0.470±0.06^{ab}	2.817±0.30^{ab}	0.636±0.06^{ab}	5.147±0.61^{b}	1.465±0.64^{a}
50 Cd	0.883±0.16^{a}	0.341±0.01^{ab}	0.760±0.16^{a}	0.436±0.13^{abc}	2.136±0.34^{abc}	0.597±0.05^{ab}	4.927±0.77^{b}	1.395±0.08^{a}
100 Cd	0.731±0.20^{a}	0.278±0.06^{ab}	0.750±0.26^{a}	0.338±0.07^{abc}	1.643±0.49^{bc}	0.490±0.10^{abc}	4.435±0.56^{b}	1.388±0.08^{a}
150 Cd	0.503±0.07^{a}	0.227±0.07^{ab}	0.697±0.25^{a}	0.280±0.05^{bc}	1.100±0.36^{c}	0.378±0.02^{bc}	3.295±0.66^{b}	0.824±0.09^{a}
200 Cd	0.463±0.04^{a}	0.185±0.04^{b}	0.594±0.30^{a}	0.156±0.05^{c}	0.973±0.24^{c}	0.267±0.04^{c}	2.876±0.52^{b}	0.547±0.08^{a}

Table 8.3: Effect of Hg on fresh and dry biomass accumulation in *C. roseus* (±S. D., n=3)

	20 DAS				60 DAS			
Soil Hg Concentration (mg kg^{-1} soil)	**Fresh Wt. of Roots (gplant^{-1})**	**Dry Wt. of Roots (g plant^{-1})**	**Fresh Wt. of Shoots (g plant^{-1})**	**Dry Wt. of Shoots (g plant^{-1})**	**Fresh Wt. of Roots (gplant^{-1})**	**Dry Wt. of Roots (g plant^{-1})**	**Fresh Wt. of Shoots (g plant^{-1})**	**Dry Wt. of Shoots (g plant^{1})**
Control	1.028±0.20^{a}	0.409±0.06^{a}	3.467±0.59^{a}	0.723±0.092^{a}	2.747±0.79^{a}	0.839±0.29^{a}	9.267±2.28^{a}	2.037±0.58^{a}
20 Hg	0.929±0.21^{ab}	0.383±0.06^{a}	2.787±0.46^{ab}	0.522±0.09^{ab}	2.330±0.57^{a}	0.657±0.29^{a}	7.747±2.79^{a}	1.574±0.53^{a}
40 Hg	0.657±0.12^{ab}	0.326±0.05^{ab}	2.073±0.24^{bc}	0.397±0.06^{bc}	2.197±0.68^{a}	0.585±0.13^{a}	6.950±2.62^{a}	1.547±0.76^{a}
80 Hg	0.567±0.10^{ab}	0.272±0.06^{ab}	1.360±0.37^{c}	0.270±0.04^{c}	1.953±1.02^{a}	0.558±0.11^{a}	6.893±1.27^{a}	1.228±0.31^{a}
100 Hg	0.485±0.17^{ab}	0.248±0.05^{ab}	1.227±0.23^{c}	0.212±0.05^{c}	1.810±0.53^{a}	0.411±0.03^{a}	6.377±0.97^{a}	1.119±0.30^{a}
120 Hg	0.377±0.06^{b}	0.133±0.03^{b}	0.893±0.21^{c}	0.160±0.04^{c}	0.937±0.73^{a}	0.295±0.08^{a}	5.887±1.04^{a}	0.801±0.05^{a}

Table 8.4: Effect of BC on fresh and dry biomass accumulation in *C. roseus* (±S. D., n=3)

	20 DAS				60 DAS			
Soil BC Concentration (mg kg^{-1} soil)	**Fresh Wt. of Roots (g plant^{-1})**	**Dry Wt. of Roots (g plant^{-1})**	**Fresh Wt. of Shoots (g plant^{-1})**	**Dry Wt. of Shoots (g plant^{-1})**	**Fresh Wt. of Roots (gplant^{-1})**	**Dry Wt. of Roots (g plant^{-1})**	**Fresh Wt. of Shoots (g plant^{-1})**	**Dry Wt. of Shoots (g plant^{1})**
Control	1.9640±0.39^{a}	0.555±0.08^{a}	3.250±0.41^{a}	0.967±0.22^{a}	3.347±0.41^{a}	0.990±0.04^{a}	9.035±1.14^{a}	1.941±0.12^{a}
0.5 BC	1.719±0.39^{ab}	0.478±0.12^{ab}	3.168±0.20^{a}	0.873±0.17^{a}	3.127±1.40^{a}	0.892±0.39^{a}	8.500±4.41^{a}	1.760±1.02^{a}
1.0 BC	1.277±0.48^{ab}	0.384±0.06^{ab}	2.367±0.05^{ab}	0.688±0.14^{a}	3.091±0.31^{a}	0.839±0.29^{a}	7.541±1.45^{a}	1.533±0.27^{a}
2.0 BC	0.947±0.19^{ab}	0.373±0.09^{ab}	2.061±0.30^{b}	0.585±0..10^{a}	2.767±0.57^{a}	0.780±0.15^{a}	6.700±2.81^{a}	1.373±0.40^{a}
4.0 BC	0.761±0.21^{ab}	0.286±0.03^{ab}	1.923±0.26^{b}	0.463±0.08^{a}	2.047±0.33^{a}	0.684±0.10^{a}	6.557±4.57^{a}	1.163±0.18^{a}
8.0 BC	0.623±0.18^{b}	0.189±0.08^{b}	1.469±0.23^{b}	0.423±0.10^{a}	1.680±0.65^{a}	0.514±0.11^{a}	5.263±2.04^{a}	0.767±0.27^{a}

Table 8.5: Effect of Cd+BC on fresh and dry biomass accumulation in *C. roseus* (±S. D., n=3)

	20 DAS				60 DAS			
Soil Cd+BC Concentration (mg kg^{-1} soil)	**Fresh Wt. of Roots (g plant^{-1})**	**Dry Wt. of Roots (g plant^{-1})**	**Fresh Wt. of Shoots (g plant^{-1})**	**Dry Wt. of Shoots (g plant^{-1})**	**Fresh Wt. of Roots (gplant^{-1})**	**Dry Wt. of Roots (g plant^{-1})**	**Fresh Wt. of Shoots (g plant^{-1})**	**Dry Wt. of Shoots (g plant^{1})**
Control	1.897±0.22^{a}	0.423±0.09^{a}	4.003±0.81^{a}	0.870±0.24^{a}	2.857±0.22^{a}	0.860±0.20^{a}	9.903±1.55^{a}	2.617±0.42^{a}
25 Cd+4BC	1.340±0.28^{ab}	0.413±0.11^{a}	3.617±0.76^{ab}	0.682±0.25a	2.358±0.41^{ab}	0.847±0.26^{a}	7.686±0.86^{ab}	1.285±0.40^{b}
50 Cd+4BC	1.164±0.35^{abc}	0.388±0.08^{a}	3.483±0.77^{ab}	0.646±0.27^{a}	2.122±0.31^{abc}	0.740±0.30^{a}	5.623±0.59^{bc}	1.034±0.33^{b}
100 Cd+4BC	0.924±0.12^{bc}	0.315±0.09^{a}	2.907±0.61^{ab}	0.577±0.18^{a}	1.720±0.51^{abc}	0.522±0.15^{a}	4.890±0.65^{bcd}	1.020±0.19^{b}
150 Cd+4BC	0.541±0.06^{bc}	0.270±0.05^{a}	1.700±0.35^{ab}	0.440±0.16^{a}	1.510±0.14^{bc}	0.480±0.15^{a}	3.237±0.32^{cd}	0.527±0.29^{b}
200 Cd+4BC	0.387±0.09^{c}	0.163±0.01^{a}	1.253±0.41^{b}	0.285±0.06^{a}	0.950±0.28^{c}	0.240±0.06^{a}	2.343±0.55^{d}	0.439±0.27^{b}

Table 8.6: Effect of Hg+BC on fresh and dry biomass accumulation in *C. roseus* (±S. D., n=3)

	20 DAS				60 DAS			
Soil Hg+BC Concentration (mg kg^{-1} soil)	**Fresh Wt. of Roots (gplant^{-1})**	**Dry Wt. of Roots (gplant^{-1})**	**Fresh Wt. of Shoots (gplant^{-1})**	**Dry Wt. of Shoots (gplant^{-1})**	**Fresh Wt. of Roots (gplant^{-1})**	**Dry Wt. of Roots (gplant^{-1})**	**Fresh Wt. of Shoots (gplant^{-1})**	**Dry Wt. of Shoots (gplant^{1})**
Control	1.763±0.24^{a}	0.403±0.04^{a}	2.911±0.29^{a}	0.820±0.17^{a}	2.351±0.33^{a}	0.897±0.21^{a}	6.113±1.34^{a}	1.717±0.44^{a}
20 Hg+4BC	1.243±0.16^{ab}	0.418±0.11^{a}	2.610±0.19^{ab}	0.742±0.15^{a}	2.317±0.51^{a}	0.860±0.20^{a}	5.547±1.37^{a}	1.627±0.31^{a}
40 Hg+4BC	1.057±0.23^{ab}	0.316±0.03^{a}	2.470±0.16^{abc}	0.578±0.29^{a}	1.923±0.70^{a}	0.720±0.27^{a}	5.297±0.48^{a}	1.483±0.14^{a}
80 Hg+4BC	0.987±0.23^{ab}	0.302±0.07^{a}	1.823±0.11^{bcd}	0.570±0.17^{a}	1.883±0.47^{a}	0.703±0.19^{a}	4.595±0.69^{a}	1.370±0.58^{a}
100 Hg+4BC	0.898±0.22^{b}	0.236±0.06^{a}	1.530±0.31^{cd}	0.511±0.22^{a}	1.826±0.42^{a}	0.683±0.10^{a}	4.400±0.30^{a}	0.983±0.24^{a}
120 Hg+4BC	0.823±0.20^{b}	0.214±0.08^{a}	1.170±0.40^{d}	0.278±0.10^{a}	1.081±0.32^{a}	0.380±0.05^{a}	3.34±.71^{a}	0.521±0.09^{a}

Table 8.7: Root and shoot length of *C. roseus* grown in soil amended with different concentration of Cd (mg kg^{-1} soil, ±S. D., n=3)

	20 DAS		60 DAS	
Soil Cd Concentration (mg kg^{-1} soil)	**Root Length (cm)**	**Shoot Length (cm)**	**Root Length (cm)**	**Shoot Length (cm)**
Control	9.703 ± 1.202^{a}	11.233 ± 2.034^{a}	16.267 ± 0.959^{a}	19.500 ± 0.661^{a}
25 Cd	6.433 ± 1.305^{ab}	9.700 ± 1.166^{ab}	14.80 ± 1.934^{ab}	19.233 ± 2.285^{a}
50 Cd	6.303 ± 0.931^{ab}	8.800 ± 0.779^{ab}	14.207 ± 1.336^{ab}	18.305 ± 1.443^{ab}
100 Cd	6.067 ± 0.987^{ab}	7.667 ± 0.755^{ab}	13.667 ± 0.854^{ab}	17.207 ± 1.102^{ab}
150 Cd	5.767 ± 0.769^{b}	7.000 ± 0.427^{ab}	13.500 ± 0.978^{ab}	14.404 ± 0.628^{bc}
200 Cd	5.000 ± 0.608^{b}	6.367 ± 0.747^{b}	11.0 ± 1.709^{b}	$10.433\pm.0.655^{c}$

Table 8.8: F-ratio and level of significance of two- way ANOVA test for biomass and morphological parameters of *C. roseus* grown in soil amended with different concentration of Cd

Factors	RL	SL	RFWt.	RDWt.	SFWt.	SDWt.
Days	344.262^{***}	407.405^{***}	123.133^{***}	68.633^{***}	539.879^{***}	99.757^{***}
Concentration	11.181^{***}	27.843^{***}	21.815^{***}	18.737^{***}	23.535^{***}	8.698^{***}
Days*Concentration	0.845^{ns}	4.792^{**}	5.548^{**}	1.826^{ns}	14.593^{***}	2.418^{ns}

RL: root length; SL: shoot length; RFWt.: root fresh weight; RDWt.: root dry weight; SFWt.: shoot fresh weight; SDWt.: shoot dry weight. ns: not significant $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

Table 8.9: Root and shoot length of *C. roseus* grown in soil amended with different concentration of Hg (mgkg^{-1} soil, ±S. D., n=3)

	20 DAS		60 DAS	
Soil Hg Concentration (mg kg-1 soil)	**Root Length (cm)**	**Shoot Length (cm)**	**Root Length (cm)**	**Shoot Length (cm)**
Control	10.333 ± 2.673^{a}	12.847 ± 1.312^{a}	17.75 ± 0.908^{a}	21.907 ± 1.826^{a}
20 Hg	9.467 ± 1.922^{a}	11.283 ± 1.005^{a}	15.947 ± 1.197^{ab}	18.314 ± 2.025^{ab}
40 Hg	8.300 ± 2.516^{a}	10.033 ± 1.387^{ab}	14.133 ± 1.701^{abc}	15.333 ± 2.111^{bc}
80 Hg	7.167 ± 1.456^{a}	9.170 ± 1.059^{ab}	11.837 ± 1.055^{bcd}	13.837 ± 1.342^{bc}
100 Hg	6.533 ± 1.137^{a}	8.567 ± 1.266^{ab}	10.827 ± 1.294^{cd}	12.273 ± 1.403^{bc}
120 Hg	5.200 ± 0.963^{a}	6.710 ± 0.799^{b}	9.467 ± 0.833^{d}	10.537 ± 0.766^{c}

Table 8.10: F-ratio and level of significance of two- way ANOVA test for biomass and morphological parameters of *C. roseus* grown in soil amended with different concentration of Hg

Factors	RL	SL	RFWt.	RDWt.	SFWt.	SDWt.
Days	108.143^{***}	139.491^{***}	55.35^{***}	33.52^{***}	120.558^{***}	76.261^{***}
Concentration	15.398^{***}	29.326^{***}	3.807^{*}	6.706^{***}	3.481^{*}	5.14^{**}
Days*Concentration	1.005^{ns}	3.221^{*}	0.841^{ns}	0.783^{ns}	0.1^{ns}	0.665^{ns}

RL: root length; SL: shoot length; RFWt.: root fresh weight; RDWt.: root dry weight; SFWt.: shoot fresh weight; SDWt.: shoot dry weight. ns: not significant; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

Table 8.11: Root and shoot length of *C. roseus* grown in soil amended with different concentration of BC (mg kg^{-1} soil, ±S. D., n=3)

	20 DAS		60 DAS	
Soil BC Concentration (mg kg-1 soil)	**Root Length (cm)**	**Shoot Length (cm)**	**Root Length (cm)**	**Shoot Length (cm)**
Control	10.147±2.352^{a}	12.847±1.050^{a}	18.287±0.653^{a}	21.907±1.880^{a}
0.5 BC	9.867±1.464^{a}	12.500±1.052^{a}	16.600±0.648^{a}	19.900±1.192^{ab}
1.0 BC	9.600±1.398^{a}	11.900±1.065^{a}	16.100±0.728^{a}	17.223±0.961^{bc}
2.0 BC	8.400±0.511^{a}	10.600±0.912^{ab}	12.733±1.118^{b}	15.600±1.205^{bc}
4.0 BC	7.733±0.947^{a}	8.000±0.846^{b}	11.267±1.258^{b}	13.327±0.861^{cd}
8.0BC	5.100±0.813^{a}	7.350±0.752^{b}	10.077±1.056^{b}	9.743±0.533^{d}

Table 8.12: F-ratio and level of significance of two- way ANOVA test for biomass and morphological parameters of *C. roseus* grown in soil amended with different concentration of BC

Factors	**RL**	**SL**	**RFWt.**	**RDWt.**	**SFWt.**	**SDWt.**
Days	209.440***	258.538***	61.328***	54.049***	45.990***	40.724***
Concentration	27.647***	58.519***	6.590**	4.837**	1.393 ^{ns}	4.798**
Days*Concentration	3.166*	6.742***	0.437 ^{ns}	0.110 ^{ns}	0.154 ^{ns}	0.585 ^{ns}

RL: root length; SL: shoot length; RFWt.: root fresh weight; RDWt.: root dry weight; SFWt.: shoot fresh weight; SDWt.: shoot dry weight. ns: not significant; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

Table 8.13: Root and shoot length of *C. roseus* grown in soil amended with different concentration of Cd+BC (mg kg^{-1} soil, ±S. D., n=3)

	20 DAS		60 DAS	
Soil Cd +BC Concentration (mg kg-1 soil)	**Root Length (cm)**	**Shoot Length (cm)**	**Root Length (cm)**	**Shoot Length (cm)**
Control	9.667±0.767^{a}	12.563±1.056^{a}	18.253±1.584^{a}	23.267±1.002^{a}
25 Cd+4BC	7.600±1.323^{ab}	11.730±1.036^{ab}	15.933±0.777^{a}	21.533±2.240^{ab}
50 Cd+4BC	7.067±0.922^{ab}	10.300±0.860^{abc}	13.563±1.566^{ab}	17.343±1.149^{abc}
100 Cd+4BC	6.320±0.871^{ab}	9.133±0.950^{abc}	10.033±1.429^{bc}	16.567±2.159^{bc}
150 Cd+4BC	4.533±0.949^{b}	8.300±0.954^{bc}	9.867±1.531^{bc}	14.833±1.583^{c}
200 Cd+4BC	4.217±0.916^{b}	8.003±0.856^{c}	7.967±0.306^{c}	11.900±1.493^{c}

Table 8.14: F-ratio and level of significance of two- way ANOVA test for biomass and morphological parameters of *C. roseus* grown in soil amended with different concentration of Cd+4BC

Factors	RL	SL	RFWt.	RDWt.	SFWt.	SDWt.
Days	250.09***	278.544***	86.17***	30.794***	122.292***	38.712***
Concentration	40.559***	29.595***	27.723***	7.389***	39.513***	18.861***
Days*Concentration	5.289**	4.809**	0.548 ns	1.314 ns	8.774***	7.309***

RL: root length; SL: shoot length; RFWt.: root fresh weight; RDWt.: root dry weight; SFWt.: shoot fresh weight; SDWt.: shoot dry weight. ns: not significant; *p<0.05; **p<0.01; ***p<0.001.

Table 8.15: Root and shoot length of *C. roseus* grown in soil amended with different concentration of Hg+BC (mg kg^{-1} soil, ±S. D., n=3)

	20 DAS		60 DAS	
Soil Hg +BC Concentration (mg kg-1 soil)	**Root Length (cm)**	**Shoot Length (cm)**	**Root Length (cm)**	**Shoot Length (cm)**
Control	9.330±1.068a	11.633±1.607a	16.098±0.947a	26.153±0.857a
20 Hg +4BC	8.960±1.210a	10.503±0.784a	13.867±1.301ab	21.233±1.930ab
40 Hg+4BC	7.767±2.021a	10.003±1.327a	12.467±1.629abc	18.967±0.528bc
80 Hg+4BC	6.800±0.500a	9.033±0.484a	10.867±1.102bc	14.800±3.297cd
100 Hg+4BC	5.033±1.484a	8.467±0.635a	9.233±1.834bc	13.080±1.490cd
120 Hg+4BC	4.733±2.250a	7.767±0.960a	7.800±0.458c	10.733±0.586d

Table 8.16: F-ratio and level of significance of two- way ANOVA test for biomass and morphological parameters of *C. roseus* grown in soil amended with different concentration of Hg+4BC

Factors	RL	SL	RFWt.	RDWt.	SFWt.	SDWt.
Days	95.036***	274.513***	38.681***	68.267***	155.868***	55.745***
Concentration	18.316***	37.228***	6.213**	4.968**	9.102***	7.659***
Days*Concentration	1.129 ns	13.452 ***	0.956 ns	0.996 ns	0.388 ns	1.464 ns

RL: root length; SL: shoot length; RFWt.: root fresh weight; RDWt.: root dry weight; SFWt.: shoot fresh weight; SDWt.: shoot dry weight. ns: not significant; *p<0.05; **p<0.01; ***p<0.001.

of days and concentration significantly affected all parameters except shoot fresh wt. whereas interaction between individual factors significantly affect root and shoot length only (**Table 8.12**). There were a 4.22 and 5.96 fold decrease of shoot fresh and dry matter compared to control in the presence of Cd (200 $mgkg^{-1}$) and BC (4 $mgkg^{-1}$) mixed in approx. 2 kg of soil and a further 3.01 and 3.58 fold decrease in root fresh and dry biomass, respectively, with control (**Table 8.5**). Results of two-way ANOVA in co-contamination of Cd along with BC revealed that root length, shoot length, and shoot fresh and dry weight varied with individual factors of concentration and days as well as with their interactions. While root fresh and dry wt. varied with individual factors only (**Table 8.14**).

Similar results as above were observed for Hg co-contaminated with BC (**Table 8.6**). Two-way ANOVA showed that individual factors of days and concentration significantly affected all the morphological parameters whereas their interaction showed no significant variation except shoot length in soil co-contaminated with Hg and BC (**Table 8.16**).

Co-contaminated treatments have shown more decline in biomass of *C. roseus* as compared to the alone heavy metal and herbicide treatment. *C. roseus* growth and metabolism was found to be variably affected by the heavy metal stress. Elevated concentrations of Cd and Hg resulted in reduction in growth & biomass and displayed distinctly visible effects akin to those described earlier in various plant species (**Zhou & Qiu, 2005; Gajewska & Sklodowska, 2006**). Such observations were further substantiated by a significant increase in the metal concentration in the plant tissue as discussed further in the chapter.

The biomass reduction was found to be significantly high in Cd followed by Hg, compared to control treatment. This may be an outcome of the synergistic effect of damaged or altered/or inhibited metabolism under stress. The decrease manifested in production of dry matter production may be a result of metal stress which is in concurrence with earlier findings in plants other than *C. roseus* (**Ryser & Sauder, 2006**). Similarly, Cd in high concentration induced a significant decrease in both shoot and root length followed by Hg with respect to control. The maximum shoot and root length was recorded in plants grown on control soil having average values of 19.50 cm and 16.27 cm, respectively, while the shortest plant shoot (10.433 cm) and root (11.0 cm) was recorded at 200 mg kg^{-1} Cd concentration recorded at 60 DAS (**Table 8.7**). Similarly, Hg was found to significantly decrease, both plant shoot and root length by 46.66% and 51.90%, respectively when the soil was amended with 120 mg kg^{-1} of Hg (**Table 8.9**).

Under BC treatment a significant decline in root (44.89%) and shoot (55.52%) length was observed with the increasing concentration (**Table 8.11**). While under joint toxicity of Cd (150 $mgkg^{-1}$) in presence of BC (4 $mgkg^{-1}$) there was a 18.50 and 15.68 fold decrease in root and shoot length respectively, compared to control (**Table 8.13**). The root and shoot length observed a 2.06 and 2.43 folds decline at highest concentration of Hg (120$mgkg^{-1}$) along with herbicide BC (4 $mgkg^{-1}$) (**Table 8.15**).

8.4.2 Metal accumulation in plant tissues

The concentration of heavy metals (Cd and Hg) in plant tissues was affected by joint treatments of heavy metal (Cd, Hg) and herbicide (BC) and significant interactions were detected. The concentrations of Cd and Hg in different plant tissues was found to increase with an increase in soil Cd and Hg which increased further with BC additions both in roots and shoots (**Tables 8.17 - 8.20**). A contamination level dependent increase in Cd and Hg in roots and shoots of *C. roseus* was found on each determination from samples uprooted after 20-60 DAS (**Tables 8.17- 8.20**). The Cd content in shoot and root systems of *C. roseus* increased as soil metal concentration increased from 25 to 200 mg kg^{-1} in soil (**Table 8.17**). The highest Cd (612.07 and 1343.24 $\mu g g^{-1}$) contents in plant shoot and root, respectively, were recorded in plants grown on soil amended with 200 $mgkg^{-1}$ of the test metal. Cd contents in shoot ranged from 126.33 to 612.07 $\mu g g^{-1}$ while, that in root ranged from 512.69 to 1343.24 $\mu g g^{-1}$ respectively, under the lowest (25 mg kg^{-1}soil) and highest (200 mg kg^{-1}soil) metals concentration. Cd contents in roots was found to be higher than the Cd contents in shoot, respectively, in plants grown on soil amended with different concentrations of heavy metals.

The Hg contents in shoot and root systems of *C. roseus* increased as metal concentration in soil increased from 20 to 120 mg kg^{-1} soil (**Table 8.18**). When 80 and 120 mg kg^{-1} Hg was added to soil, the shoot and root Hg concentrations were increased by 119.032 and 316.780 $\mu g g^{-1}$ in shoots while, 267.416 and 611.746 $\mu g g^{-1}$ in roots, respectively. Roots of *C. roseus* revealed high Hg contents as compared to the shoot in plant grown on soil amended with different concentrations of heavy metals. The addition of 4 mg kg^{-1} of BC to 25-200 mg kg^{-1} of Cd and 20-120 $mgkg^{-1}$ of Hg significantly ($p<0.05$) increased the concentration of Cd and Hg in root as well as shoot of *C. roseus* (**Tables 8.19 and 8.20**). Plants grown under co-contamination revealed higher concentration of heavy metals in their plant tissues as compared to those plants grown under individual treatment of Cd and Hg. Results revealed that co-contamination through herbicide may facilitate translocation of metals which results in increase in concentration of metals in *C. roseus* root and shoot.

Table 8.17: Cd accumulation in roots and shoots of *C. roseus* (±S. D., n=3)

Soil Cd concentration (mg kg-1 soil)	Cd content in Roots(μgg^{-1})		Cd content in Shoots (μgg^{-1})	
	20 DAS	60 DAS	20 DAS	60 DAS
Control	2.899±0.092^{d}	7.807±0.042^{d}	0.393±0.058^{c}	1.236±0.418^{e}
25 Cd	93.124±10.933^{cd}	512.699±19.277^{c}	31.057±3.183^{bc}	126.339±6.470^{d}
50 Cd	178.800±25.466^{c}	916.157±51.138^{b}	39.850±5.707^{bc}	278.291±21.659^{c}
100 Cd	201.810±26.748^{bc}	1297.775±34.553^{a}	77.656±8.076^{ab}	331.786±65.636^{bc}
150 Cd	307.440±37.447^{ab}	1325.586±71.665^{a}	94.511±7.611^{a}	437.165±32.926^{b}
200 Cd	350.611±57.007^{a}	1343.240±49.537^{a}	100.731±4.869^{a}	612.076±26.415^{a}

Table 8.18: Hg accumulation in roots and shoots of *C. roseus* (±S. D., n=3)

Soil Hg concentration (mg kg-1 soil)	Hg content in Roots(μgg^{-1})		Hg content in Shoots (μgg^{-1})	
	20 DAS	60 DAS	20 DAS	60 DAS
Control	0.111±0.019^{e}	1.293±0.136^{f}	0.052±0.013^{e}	0.695±0.135^{e}
20 Hg	35.083±5.421^{de}	78.252±8.019^{e}	18.819±3.411^{d}	44.980±5.706^{de}
40 Hg	75.107±5.345^{cd}	157.440±14.563^{d}	38.283±2.976^{c}	93.548±4.449^{cd}
80 Hg	110.012±20.552^{bc}	267.416±19.370^{c}	52.848±6.252^{bc}	119.032±16.433^{bc}
100 Hg	144.795±14.673^{b}	372.723±18.476^{b}	66.196±6.402^{ab}	165.318±18.999^{b}
120 Hg	194.441±17.499^{a}	611.746±31.844^{a}	83.732±8.223^{a}	316.780±35.606^{a}

Analysis of variance for Cd, Hg alone and in joint treatment with BC with respect to their accumulation in roots and shoots of *C. roseus*, showed a significant variation with respect to days, concentration and under combination. The results showed that days (20, 60) and different concentration of heavy metal and herbicide significantly affected the accumulation efficiency of the studied plant (**Tables 8.21-8.22**).

Table 8.19: Cd+BC accumulation in roots and shoots of *C. roseus* (±S. D., n=3)

Soil Cd+BC concentration (mg kg-1 soil)	Cd+BC content in Roots(μgg^{-1})		Cd+BC content in Shoots (μgg^{-1})	
	20 DAS	60 DAS	20 DAS	60 DAS
Control	0.154±0.011^{d}	5.346±0.289^{d}	0.121±0.008^{d}	2.529±0.1860^{e}
25 Cd+4BC	110.958±9.662^{c}	526.181±34.201^{c}	39.444±4.438^{c}	160.815±16.462^{d}
50 Cd+4BC	151.201±12.284^{c}	988.662±10.281^{b}	49.292±8.166^{c}	210.873±17.359^{d}
100 Cd+4BC	289.701±36.495^{b}	1374.715±44.083^{a}	74.557±7.539^{bc}	311.114±33.374^{c}
150 Cd+4BC	317.890±22.890^{b}	1414.518±20.323^{a}	95.669±5.876^{b}	410.784±17.049^{b}
200 Cd+4BC	416.034±16.172^{a}	1476.208±48.100^{a}	156.105±5.503^{a}	608.831±18.397^{a}

Table 8.20: Hg+BC accumulation in roots and shoots of *C. roseus* (±S. D., n=3)

Soil Hg+BC concentration (mg kg-1 soil)	Hg+BC content in Roots(μgg^{-1})		Hg+BC content in Shoots (μgg^{-1})	
	20 DAS	60 DAS	20 DAS	60 DAS
Control	0.164±0.027^{d}	4.145±0.192^{d}	0.107±0.020^{e}	1.555±0.305
20 Hg +4BC	46.065±6.591^{cd}	99.162±10.909^{d}	26.909±7.922^{d}	56.000±7.394
40 Hg+4BC	82.728±10.609^{bc}	245.096±32.323^{c}	36.251±7.125^{cd}	112.807±17.583^{bc}
80 Hg+4BC	106.361±15.044^{b}	310.124±20.388^{c}	60.946±8.586^{bc}	147.016±15.873^{bc}
100 Hg+4BC	110.992±15.252^{b}	547.087±37.137^{b}	71.351±2.524^{b}	210.050±31.816^{b}
120 Hg+4BC	225.332±27.430^{a}	672.291±44.514^{a}	98.357±10.868^{a}	343.581±54.375^{a}

Table 8.21: F-ratio and level of significance of two- way ANOVA test for Cd, Hg accumulation in roots and shoots of *C. roseus*

	Cd		Hg	
Factors	Root	Shoot	Root	Shoot
Days	3052.724***	819.002***	861.099***	326.721***
Concentration	447.656***	154.633***	511.855***	164.779***
Days*Concentration	182.158***	76.734***	138.283***	59.493***

Level of Significance: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

Table 8.22: F-ratio and level of significance of two- way ANOVA test for Cd+4BC and Hg+4BC accumulation in roots and shoots of *C. roseus*

	Cd+4BC		Hg+4BC	
Factors	Root	Shoot	Root	Shoot
Days	7312.135***	1692.229***	811.573***	204.043***
Concentration	1180.004***	426.037***	308.655***	88.713***
Days*Concentration	434.696***	151.148***	100.613***	27.927***

Level of Significance: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

8.4.3 Bioconcentration (BCF) and translocation factors

Bioconcentration and translocation factors have been recognized as important tools to identify the potential of any plant for uptake of metal ions. The shoot and root concentration factor (SCF/RCF), are the compartmentalized concentration ratio of heavy metals in plant to that of soil, and is used to evaluate the plants accumulation capacity. Data given in **Figures 8.1 - 8.4** presents the values of bioaccumulation factors of Cd and Hg. *C. roseus* generally accumulates the greater amount of Cd and Hg in its root as compared to shoot. But, accumulation of Cd in shoot of *C. roseus* was found greater as compared to Hg. Cd transfer from soil into plant root reflected values of BCF > 1 at lowest concentrations of Cd in soil (25 mg kg^{-1}) with an average value of 1.81.

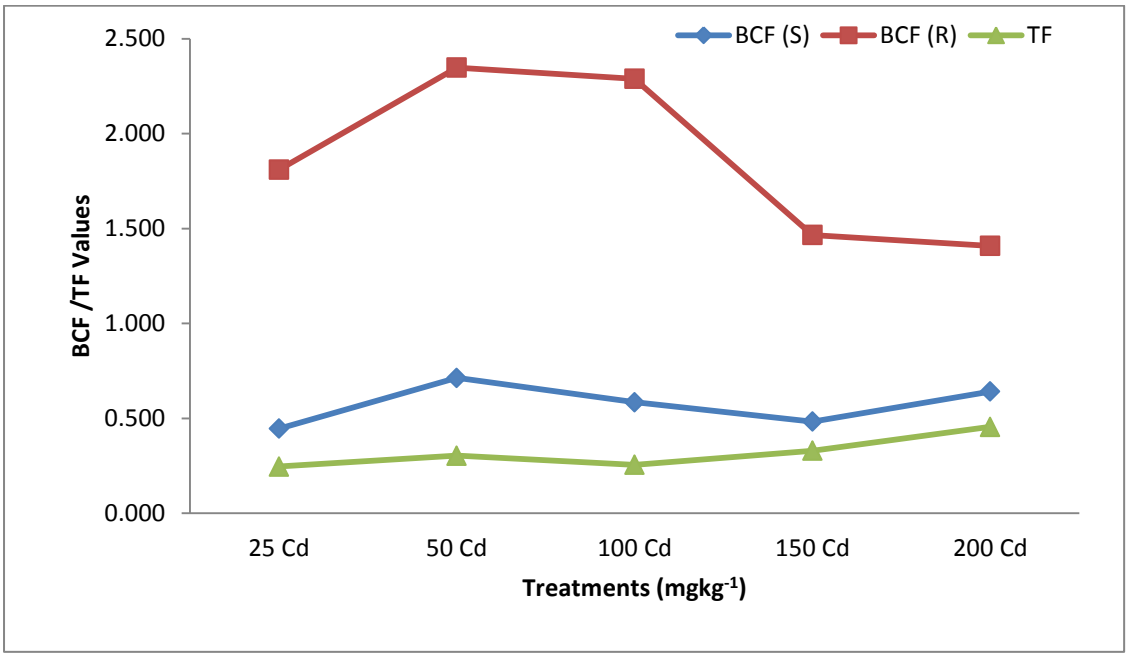


Figure 8.1: Bioconcentration factor (BCF) and translocation factor (TF) of Cd in *C. roseus*

However, an increase in the Cd concentration in soil beyond 200 mg kg^{-1} resulted in a decrease in the value of BCF as compared to lowest concentration of Cd (25 $mgkg^{-1}$). A BCF value > 1 indicated the potential of the growing plants for metal accumulation ability (**Yanqun et al, 2005**). Therefore, the obtained results showed that *C. roseus* can accumulate much amounts of Cd in its tissues compared to Hg. Hence it is deduced that, the studied plant can be used more efficiently for the remediation of Cd contaminated soils. **Figure 8.1 - 8.4** shows the translocation factor (TF) of Cd and Hg individually and in combination with BC. Clearly, *C. roseus* is more suitable for uptake of Cd compared to Hg; as a result, the amount of Cd transferred into plant tissues was found to be much higher than Hg. The obtained results are in agreement with the previous studies of **Oh et al** (**2015**) and **Subhashini and Swamy** (**2013**) who reported that Sweet sorghum KCS105 and *C. roseus* plants are favorable for accumulating Cd/Hg in their tissues compared to other heavy metals.

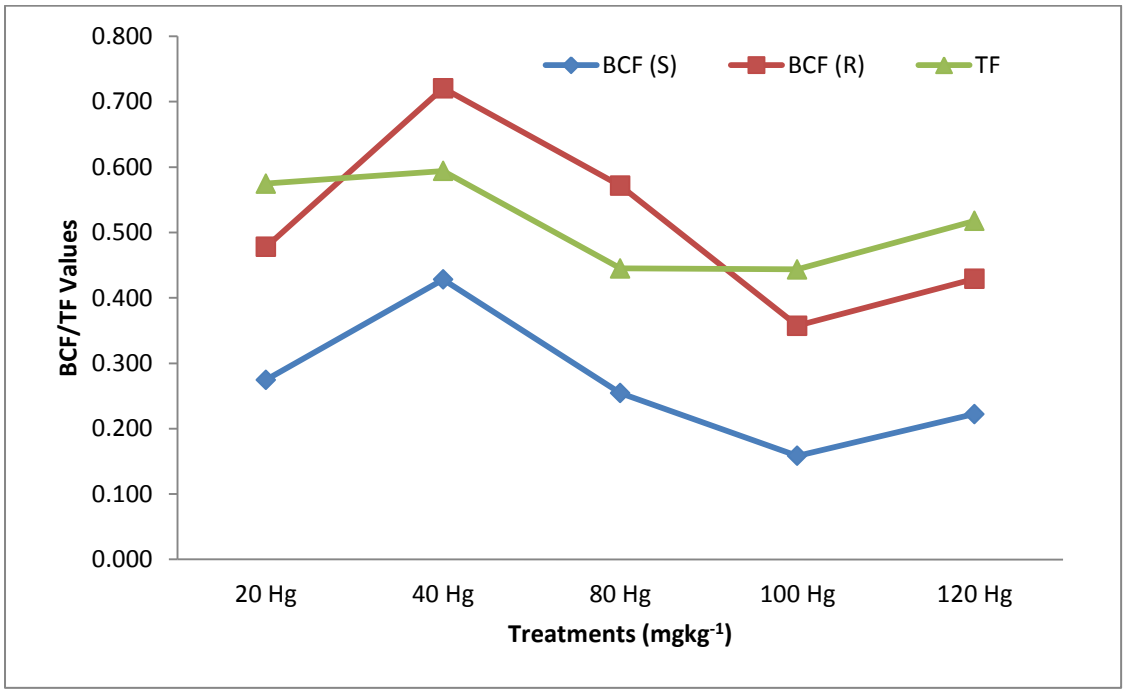


Figure 8.2: Bioconcentration factor (BCF) and translocation factor (TF) of Hg in *C. roseus*

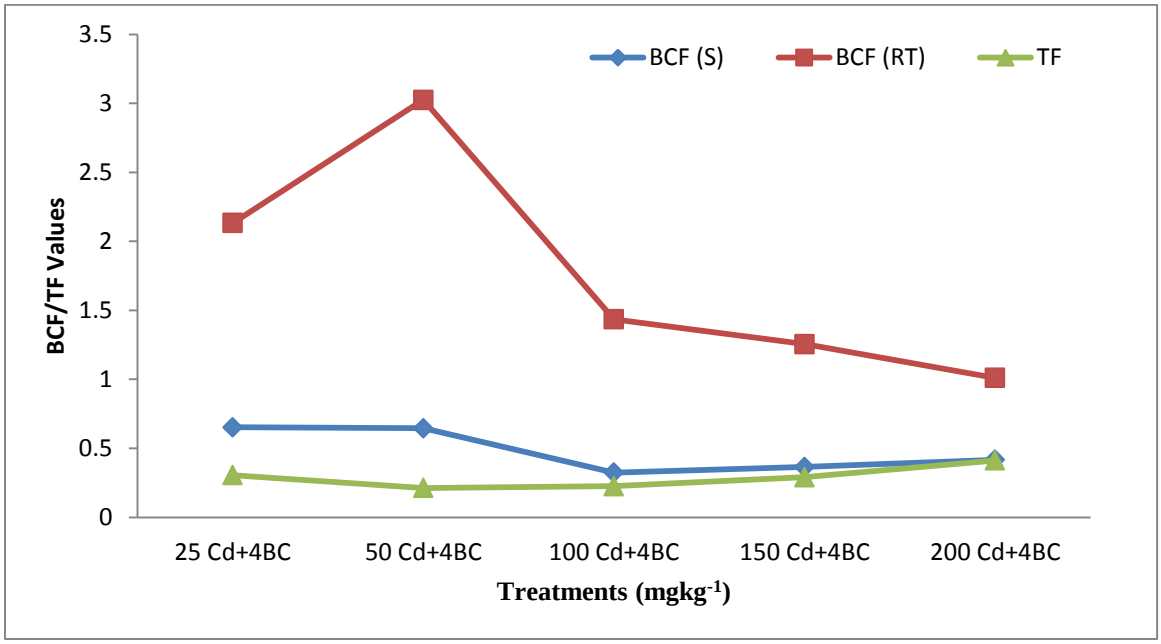


Figure 8.3: Bioconcentration factor (BCF) and translocation factor (TF) of Cd+4BC in *C. roseus*

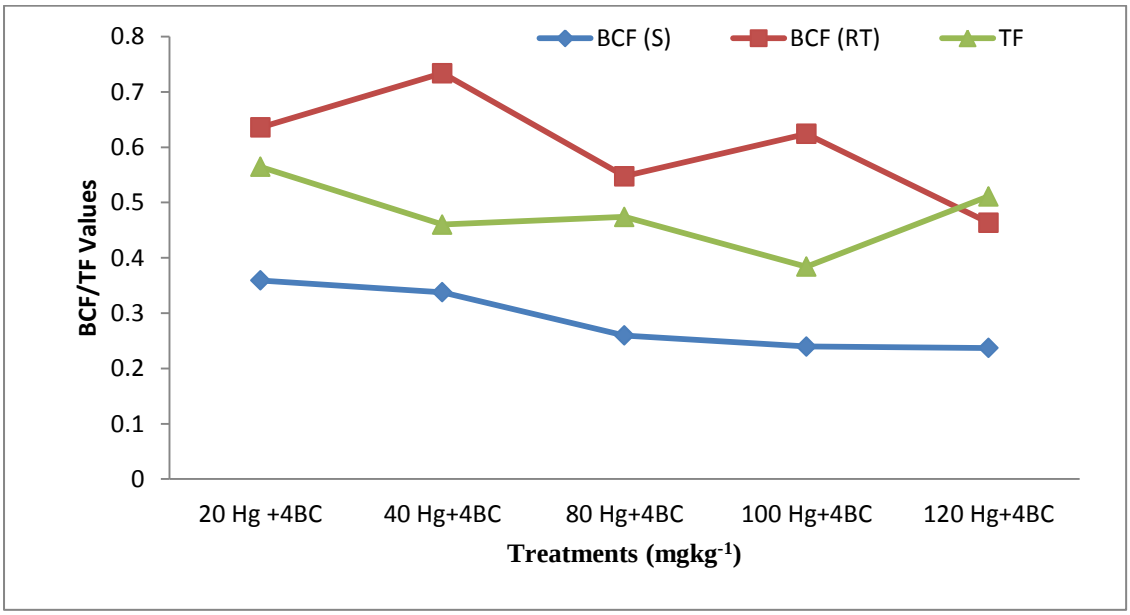


Figure 8.4: Bioconcentration factor (BCF) and translocation factor (TF) of Hg+4BC in *C. roseus*

In co-contaminated treatment of Cd/Hg in combination with BC, the BCF values for roots and shoots showed higher values in comparison to individual treatments of Cd and Hg. An increase of 46.17% and 17.90%, respectively, in shoot and root BCF values were recorded for joint treatment of Cd and BC (4mgkg^{-1}) in comparison to Cd (25 mgkg^{-1}) alone. While at higher concentration of Cd (200 mgkg^{-1}) along with BC (4 mgkg^{-1}) a marked decrease in the values of BCF for shoot and root (35.04% and 28.24%, respectively) was recorded (**Figure 8.3**). Although similar results were obtained for BCF of root and shoot under lower concentration of Hg, a *vice versa* was figured for at higher concentration of Hg (120 mgkg^{-1}) with joint treatment of BC (4 mgkg^{-1}, **Figure 8.4**).

8.4.4 Butachlor dissipation

After 60 days of growth, the extractable BC in individual as well as in combination with heavy metal (Hg and Cd) treatment decreased significantly ($p<0.05$) in soil planted with *C. roseus* compared to unplanted soil (**Figure 8.5-8.7**). This resulted in approximately 32 to 62.25% removal of BC in planted soil for soil treated with BC alone. BC removal percentage was however, found to be influenced by, an interaction of Cd and Hg, with BC by *C. roseus* plants accounting for 46% (at 25Cd+BC) to 56.25% (at 200 Cd+BC) and 47.75% (at 20 Hg+BC) to 65.75% (at 120 Hg+BC) for

planted soils, respectively. The percent removal data was showing that co-contamination facilitates more BC dissipation as compared to the BC alone. The residual BC in soil planted with *C. roseus* was found to be significantly ($p<0.05$) lower compared to unplanted soil when soil was contaminated with BC alone and in co-contamination of BC along with Cd and Hg (**Figures 8.5-8.7**).

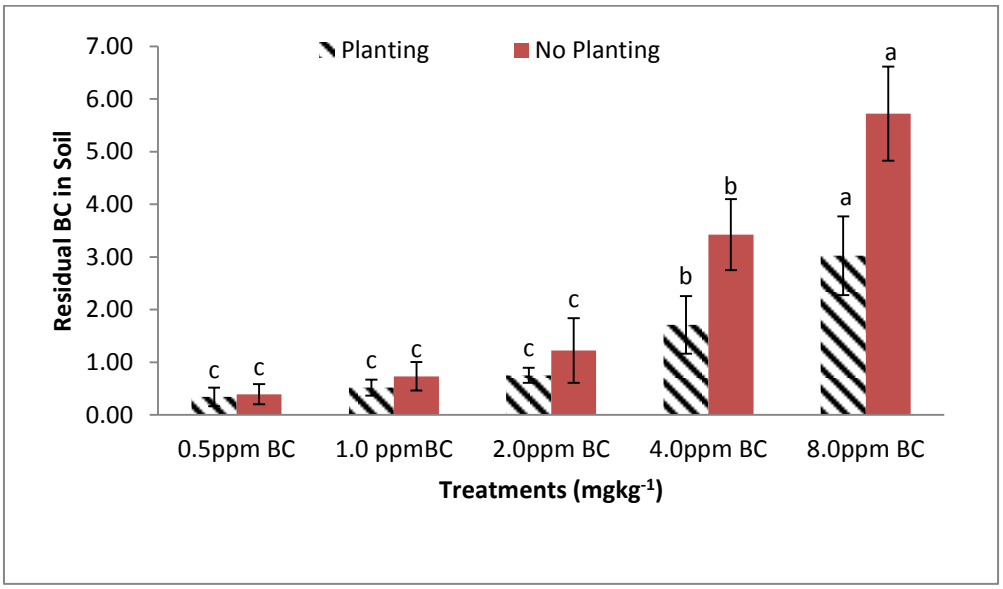


Figure 8.5: Residual butachlor in planted and non-planted soil after 60 days of planting. Bars (means ± SE, n = 3) with different letters indicate a significant difference (Duncan test, P≤0.05)

The residual concentration of BC in soil planted with *C. roseus* after 60 DAS, for single BC contaminated soil was found to range between 0.34 to 3.02 $mgkg^{-1}$. **Figure 8.5** shows that the residual BC concentration decreased for 0.5, 1.0, 2.0, 4.0 and 8 $mgkg^{-1}$ treatments in lone BC contaminated soil when compared to non-planted soils. After 60 days of planting in 0.5 $mgkg^{-1}$ BC contaminated soil, the residual BC remained at 0.34 $mgkg^{-1}$. When planted and non-planted soils were compared, the residual BC concentration in soils contaminated with Butachlor alone was significantly affected by planting at higher concentrations (2, 4 and 8 $mgkg^{-1}$ of BC) contaminated soil. As shown in **Figure 8.5**, the presence of *C. roseus* plants was found to significantly decrease the residual BC concentration from 5.72 to 3.02 $mgkg^{-1}$ in BC (8 $mgkg^{-1}$) contaminated soil as compared to soil, without planting.

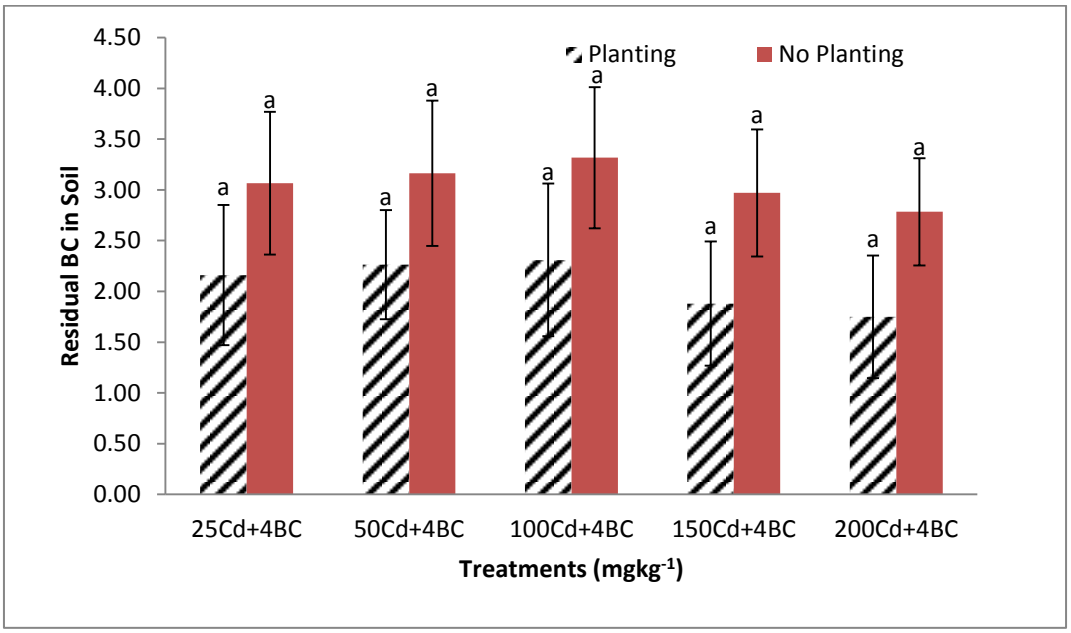


Figure 8.6: Residual butachlor in joint treatment with cadmium in planted and non-planted soil after 60 days of planting. Bars (means ± SE, n = 3) with different letters indicate a significant difference (Duncan test, P≤0.05)

Under co-contamination, the effect of planting on BC dissipation varied. A significant effect of planting on BC dissipation was observed in soil co-contaminated with 150, 200 mgkg^{-1} Cd and 4mgkg^{-1} BC (**Figure 8.6**). It is obvious from the **Figure 8.6** that at BC concentration of 4mgkg^{-1}, co-contamination with 150 and 200 mg kg^{-1} Cd was found to decrease the residual BC concentration significantly from 2.97 to 1.88 mg kg^{-1} and 2.78 to 1.75mgkg^{-1}, respectively in planted soil. However, in the presence of 50 mg kg^{-1} Cd + 4 mg kg^{-1} BC under co-contamination, the residual BC in planted soil were found to significantly decrease by 28.48% with respect to non-planted soil.

Under joint toxicity of Hg (20-120 mgkg^{-1}) and BC (4 mgkg^{-1}), no significant dissipiation of BC was observed upto 40 mgkg^{-1} while with the increase in concentration of Hg (120 mgkg^{-1}) a residual decrease in BC 49.25% concentration was observed in comparison to non-planted soil (**Figure 8.7**).

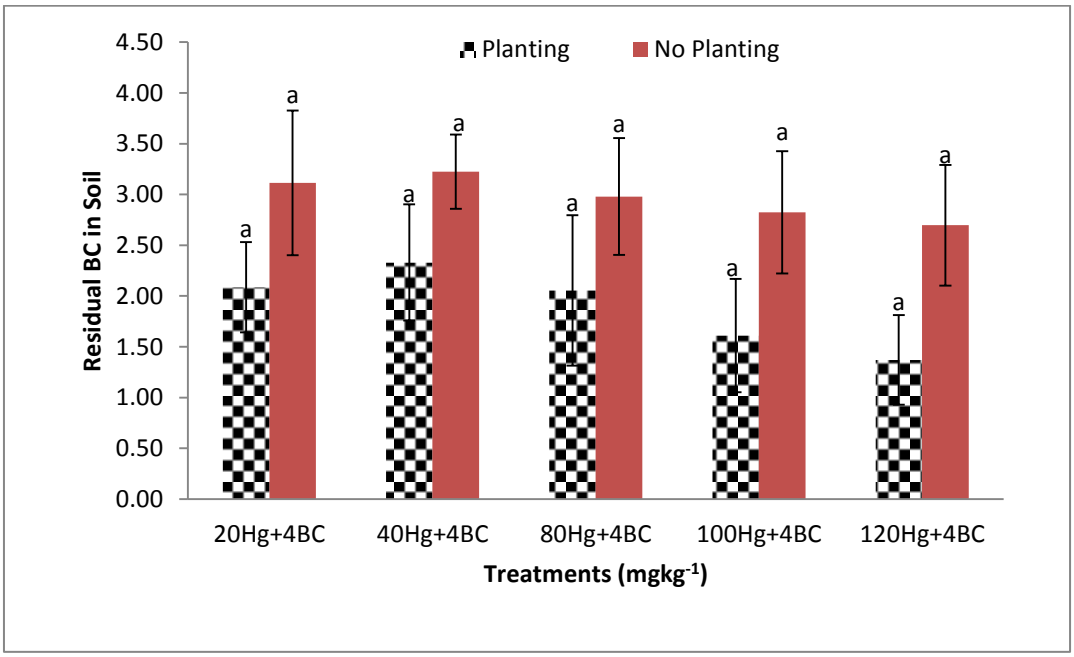


Figure 8.7: Residual butachlor in joint treatment with mercury in planted and non-planted soil after 60 days of planting. Bars (means ± SE, n = 3) with different letters indicate a significant difference (Duncan test, P≤0.05)

8.5 Discussion

Cd and Hg contamination in the soil resulted in general reduction in the growth of *C. roseus* plants in terms of root and shoot length, fresh and dry biomass accumulation. A reduction in root and shoot biomass and length was observed as a function of metal concentration and exposure time, in *C. roseus*. According to **Pedron et al** (**2009**), if plant species are able to reduce soil metal content through uptake processes than phytoremediation could be considered as successful treatment method. Results revealed that Cd could effectively decrease plant biomass of the growing plants with respect to Hg (**Tables 8.1 and 8.2**). **Ahmed et al** (**2010**) reported that Cd induce growth inhibition which results in decrease in water use efficiency and net photosynthesis in the plant species. Results given in **Table 8.3** revealed that there was a significant decline in root (44.89%) and shoot (55.52%) length as the toxicity of herbicide (BC) increases. **Alla et al** (**2008**) reported similar results as reported in the present study on *C. roseus*, wherein it was noticed that butachlor, when applied at recommended field dose resulted in differentially less shoot fresh and dry weight after about 16 days of exposure in the selected plants. In another study, **Gao and Zhu** (**2004**) reported a lower biomass in 12 species of studied plant (*Brassica chinensis* L)

grown under PAH treatment as compared to un-spiked control soils. An interactive effect of BC and Cd, Hg on growth of *C. roseus* was observed. Plants showed better growth under BC treatment alone in comparison to an admixture of Cd, Hg and BC. The combined treatment of heavy metal and herbicide have been reported to exert an antagonistic effect on both plant growth as well as performance, however, reported results were not found significant (**Chigbo & Batty**, **2014**).

Results revealed that co-contamination through xenobiotics may facilitate the greater accumulation of metals which results in an increase in concentration of metals in *C. roseus* root and shoot as compared to individual Cd and Hg treatment (**Tables 8.16-8.19**). The general trend in **Tables 8.16-8.19** showed that the concentration of Cd and Hg in root tissues was greater than in shoot of *C. roseus*. Similar trend was also observed in Cd/Hg in combination with BC. **Cardwell et al** (**2002**) and **Fitzgerald et al** (**2003**) also supported similar findings in freshwater macrophytes grown in polluted areas; they observed that the heavy metals concentration in the root tissues was found to contain higher concentrations of most metals in general as compared to the shoot. The movement of metals from roots to shoots was likely to occur via the xylem and to be driven by transpiration from the leaves.

Our results showed that in soil contaminated with mixed contaminants of Cd and Hg along with BC, the BCF values for shoot and root showed higher values than alone treatments of Cd/Hg as shown in **Figures 8.1-8.4**. There are various factors which influence the translocation factor *viz*., soil, temperature, pH, plant species and type of metals etc. (**Deng et al**, **2004**; **Seregin & Kozhevnikova, 2006**). In phytoremediation, roots are the first organs which come in to contact with the soil containing toxic metal and a higher amount of the metal is uptaken in comparison to shoots (**Simonova et al**, **2007**; **Bauddh & Singh**, **2011**). BC dissipation in planted soil was found to be greater than in non-planted soil in the presence of heavy metal and herbicide alone and in combination (**Figures 8.5-8.7**). The results revealed the benefit of vegetation in BC contaminated soils. BC uptake by *C. roseus* was not studied since in earlier studies uptake of PAHs by plants have been reported to be either low or negligible, besides the uptake of BC might have taken place from elsewhere instead of soil (**Gao & Zhu**, **2004**). A high removal rate of BC observed in the presence of *C. roseus* in sole BC contaminated soil might be correlated to the

microorganisms inhabiting rhizospheric zone which is known to plays a vital role in degradation of organics. Researchers have reported that plant growth enhances in-soil degradation of BC in the rhizosphere of soil (**Yu et al, 2003**; **Wang et al, 2013**). **Sun et al** (**2010**) reported that the dissipation of PAH compound pyrene was found higher in soil amended with root exudates relative to the soil harboring growing root of *L. perenne*. Previous researches revealed that the plants contribution for dissipation of PAH *via* processes like immobilization or degradation mainly depends on the activities which occur in the rhizosphere of soil (**Pilon-Smits, 2005**). The results of present study for BC dissipation revealed that higher degradation of BC was observed at higher concentration of heavy metal Cd and Hg, *i.e.*, 150, 200 and 80, 100, 120 $mgkg^{-1}$, respectively (**Figures 8.6 and 8.7**). Previous findings reported phytoremediation as a cost-effective method for degrading butachlor from the contaminated sites (**Wang et al, 2013**). Plant species *Acorus calamus* was found to exhibit greater degradation efficiency compared to *Phragmites australis* and *Zizania aquatic* and hence reported greater phytodegradation potential for butachlor remediation of agricultural nonpoint sources (**Abigail et al**, **2015**). It has been reported that co-contamination can affect a wide range of microbial processes in soil which includes nitrogen conversion, methane metabolism, and various other reductive processes, which results in an indirect or direct degradation of organic contaminants (**Knight et al**, **1997**).

8.6 Conclusion

This study was carried out to find out the ability of *C. roseus* to remove Cd and Hg from contaminated or co-contaminated soils. Results revealed that *C. roseus* has the ability to accumulate Cd and Hg in the tissues (roots and shoots). Cd accumulation in plant shoot was found to be more favorable than Hg. The BCF values for roots were recorded more than 1 for the treatments of Cd alone or in combination with BC indicating its potential for uptake of metal ion. On the contrary, the BCF of Hg as recorded for all the treatments did not exceed 1. The values of translocation factor obtained in the present study showed the ability of *C. roseus* to accumulate greater amounts of Cd as compared to Hg. Hence, in order to improvise the phytoremediation performance of *C. roseus* for heavy metals alone and under co contamination

experiments in combination with synthetic and natural chelating agents were conducted (Chapter 9) so as to maximize the removal efficiency of heavy metals under co contamination.

Plate 8.1: Cd treated plants (T0:Control, T1-T5:25-200 mgkg^{-1})

Plate 8.2: Hg treated plants (T0:Control, T6-T10:20-120 mgkg^{-1})

Plate 8.3: Cd+BC treated plants (T0:Control, T12-T16:25-200 mgkg^{-1}Cd + 4 mgkg^{-1} BC)

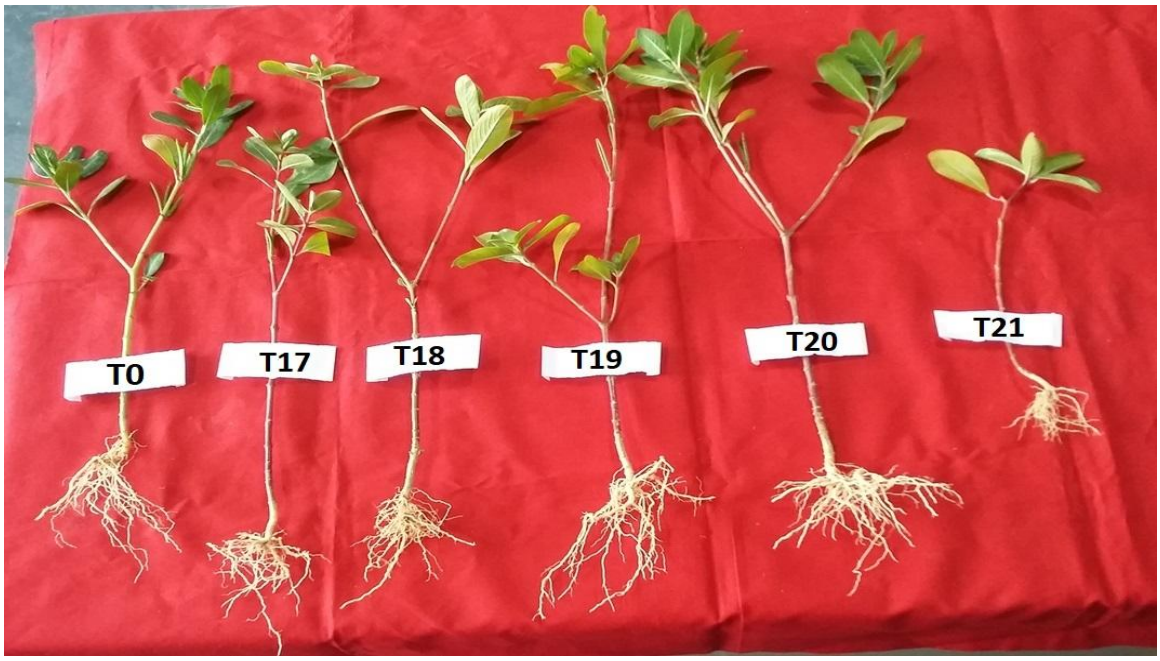


Plate 8.4: Hg+BC treated plants (T0:Control, T17-T21:20-120 mgkg^{-1} Hg + 4 mgkg^{-1} BC)

Plate 8.5: BC treated plants (T0: Control, T22-T26:0.5-8.0 mgkg^{-1} BC)

Chapter 9

Chelate-assisted phytoremediation of Cd/Hg and Butachlor co-contaminated soils using C. roseus

9.1 Introduction

Industrial revolution leads to the release of major anthropogenic pollutants as well as heavy metals (Cd, Hg, etc.) into the environment (**Zhou & Huang, 2001**). **Bell et al (1991)** reported the variation in toxicity of heavy metals which affected the vegetation growth. The chloroacetanilide herbicides are suspected as endocrine disruptors and designated as B-two carcinogen classified by USEPA (**PAN, 2001**). Chloroacetanilide include butachlor well known to cause problem to aquatic invertebrates (**Vallotton et al**, **2009**), microbial communities (**Widenfalk et al**, **2008**) besides being a possible carcinogen in animals and humans (**Geng et al**, **2005**). **Alloway** (**1995**) and **Diels et al** (**2002**) reported that few heavy metals are classified as carcinogenic and mutagenic as well; it poses a critical concern to human health together with the environment due to low solubility in biota and high occurrence as a contaminant. Therefore, a robust and economical and eco-friendly technology for treatment of these pollutants is required. Phytoremediation could solve this purpose because of the potential of plants to either sequester or remediate environmental contaminants, finding application in the treatment of polycyclic aromatic hydrocarbon (PAH) and heavy metal contaminated soil (**Turgut et al**, **2004**). **Lee and Sung** (**2014**) studied that low availability of heavy metals in soil may reduce the phytoremediation efficiency. Application of chelating agent's leads to the dissolution of heavy metals in activated soils, is one of the methods to increase bioavailability of heavy metals in soils, to improve efficiency of phytoremediation, and to promote the absorption of heavy metals by plants (**Ali et al**, **2013**; **Usman et al**, **2013**; **Li et al**, **2018)**.

The addition of chelating agent increases the uptake and improves the bioavailability of heavy metals by plants. Several researches have reported that chelating agents such as citric acid (CA), N-(2-hydroxyethyl)-ethylene diamine triacetic acid (HEDTA) and ethylene diamine triacetic acid (EDTA), increases the metal mobility, thereby enhancing phytoextraction (**Elless & Blaylock**, **2000**; **Chen & Cutright**, **2001**; **Chen et al**, **2003**). **Piechalak et al** (**2003**) reported that soil having 200 mg/kg Pb content when amended with chelating agent EDTA, increases the Pb accumulation by 67%, in comparison to control.

Previous studies have also shown the impact of chelating agents on heavy metal extraction from contaminated soil; however, very scanty information is available on the possible role of chelates during phytoremediation of co-contaminated

(heavy metal and herbicide) soil. The present objective was carried out to investigate the role of chelating agents particularly EDTA (a synthetic chelate) and citric acid (naturally occurring), alone and in combination on BC degradation and the concurrent extraction of Cd/Hg by *C. roseus*. Selection of *C. roseus* for present study was due to ability of this plant to tolerate higher concentrations of heavy metals.

9.2 Materials and Methods

For soil spiking and planting please refer section 7.2.1, 7.2.2 and 7.2.3 of Chapter 7.

9.2.1 Plant growth analysis

Detail methodology is given in Chapter 8 (Materials & Methods) Section 8.2.4.

9.2.2 Estimation of Cadmium and Mercury content in Plant

Plant materials were ground and 0.5 g portions of sieved plant matter was digested with a mixture of perchloric acid–nitric acid mixture (1:3, v/v). The concentrations of Cd and Hg in the samples were determined using Varian Spectra AA-250 plus Atomic Absorption Spectrophotometer (AAS). Details methology is given in Chapter 8 under section 8.2.6.

9.2.3 Soil analysis for heavy metal Hg and Cd

Cadmium and mercury content were estimated after digesting the sample in perchloric acid–nitric acid mixture (1:3 v/v) by atomic absorption spectrophotometer (AA 240 FS, Varian). Per plant (by roots and shoots) Cd/Hg accumulation was calculated (**Odu et al, 1986**). For more details please refer to Chapter 3 (Materials & Methods) Section 3.4.

9.2.4 Heavy metals bioconcentration and translocation factor

Bioconcentration factor (BCF) and translocation factor (TF) have been widely used to assess the translocations of heavy metals into the growing plant tissues. The same were calculated following the methods given by **Ali et al (2002)** and **Marchiol et al (2004)**, respectively given in Chapter 3 (Materials & Methods) Section 3.5.

9.2.5 Analysis of plants and soil samples

Butachlor concentration in soil samples were analyzed using the GC-MS as described by **Qiu et al (2010)** in sections of 8.2.9 of Chapter 8.

9.3 Statistical analysis

The data (n=3) were analyzed statistically by one way as well as two way anova analysis of variance (SPSS Inc., version 25.00). Tukey's honestly significant difference based on F values was applied for testing significance of differences among treatments at probability (p) 0.05, 0.01 and 0.001.

9.4 Results

9.4.1 Effects of EDTA and CA under individual and co-contamination on plant growth and biomass

At harvest, all plants were in growth stage with flowering. Some chlorosis in leaves were found when EDTA alone and EDTA + citric acid jointly were applied to 150 mg kg^{-1} Cd/100 mg kg^{-1} Hg and 4 mg kg^{-1} BC contaminated soils as recorded on final day after planting (60 DAS). The effect of chelating agents on *C. roseus* biomass as well as length were found to be reduced in all treatments compared with alone treatments (Cd/Hg and BC).

The fresh and dry weights of shoots significantly decreased in the presence of chelating agents. In the presence of EDTA shoot fresh and dry wt. were 14.23 and 32.06% while in presence of CA, it were 19.69 and 13.50%, respectively, and upon joint application of CA and EDTA both the shoot fresh and dry wt. were 36.16 and 25.22%, respectively compared to Cd (150 mg kg^{-1}) alone treatment with no chelates. Cd (150 mg kg^{-1}) in the presence of EDTA, CA and both (EDTA+CA) showed a decline of 17.06 and 32.06%, 18.58 and 35.25% and 28.71 and 46.45% in root fwt. and dwt., respectively, in comparison to Cd 150 mg kg^{-1} as measured on 60 DAS (**Table 9.1**).

Similar results were observed for Hg (100 mg kg^{-1}) in the presence of chelating agents wherein there was a decrease in root and shoot fresh and dry wt. as compared to Hg treatment without chelating agents (**Table 9.2**).

Both under individual and joint toxicities addition of EDTA and CA were found to affect the plant biomass. The highest decline in root fresh (1.64 fold) and dry wt. (1.77 fold) was observed in the presence of EDTA followed by joint application of EDTA and CA (1.46 and 1.22 fold, respectively) and finally by CA (1.022 and 1.10

Table 9.1: Effect of Cd with soil chelating agents on fresh and dry biomass accumulation in *C. roseus* (±S. D., n=3)

	20 DAS				60 DAS			
Soil Concentration (mg kg-1 soil)	**Fresh Wt. of Roots (g plant^{-1})**	**Dry Wt. of Roots (g plant^{-1})**	**Fresh Wt. of Shoots (g plant^{-1})**	**Dry Wt. of Shoots (g plant^{-1})**	**Fresh Wt. of Roots (gplant^{-1})**	**Dry Wt. of Roots (g plant^{-1})**	**Fresh Wt. of Shoots (g plant^{-1})**	**Dry Wt. of Shoots (g plant^{1})**
Control	1.898±0.38^{a}	0.754±0.11^{a}	3.240±0.18^{a}	0.780±0.21^{a}	3.773±0.25^{a}	1.691±0.24^{a}	7.557±3.24^{a}	1.508±0.72^{a}
150 Cd	1.663±0.46^{a}	0.548±0.16^{ab}	2.478±0.49^{ab}	0.747±0.11^{a}	2.497±0.44^{a}	0.973±0.32^{ab}	7.007±1.40^{a}	1.237±0.25^{a}
150 Cd+EDTA	1.340±0.20^{a}	0.445±0.08^{ab}	2.610±0.84^{ab}	0.664±0.15^{a}	2.071±0.43^{a}	0.661±0.10^{b}	6.010±3.74^{a}	1.163±0.69^{a}
150 Cd+CA	1.141±0.14^{a}	0.400±0.01^{ab}	1.767±0.35^{ab}	0.592±0.02^{a}	2.033±0.70^{a}	0.630±0.31^{b}	5.627±0.73^{a}	1.070±0.09^{a}
150Cd+EDTA+CA	0.644±0.41^{a}	0.201±0.12^{b}	1.177±0.28^{b}	0.433±0.12^{a}	1.780±0.73^{a}	0.521±0.09^{b}	4.473±1.86^{a}	0.925±0.35^{a}

Table 9.2: Effect of Hg with soil chelating agents on fresh and dry biomass accumulation in *C. roseus* (±S. D., n=3)

	20 DAS				60 DAS			
Soil Concentration (mg kg-1 soil)	**Fresh Wt. of Roots (g plant^{-1})**	**Dry Wt. of Roots (g plant^{-1})**	**Fresh Wt. of Shoots (g plant^{-1})**	**Dry Wt. of Shoots (g plant^{-1})**	**Fresh Wt. of Roots (gplant^{-1})**	**Dry Wt. of Roots (g plant^{-1})**	**Fresh Wt. of Shoots (g plant^{-1})**	**Dry Wt. of Shoots (g plant^{1})**
Control	1.738±0.45^{a}	0.642±0.10^{a}	4.016±0.54^{a}	1.293±0.30^{a}	3.852±0.27^{a}	1.798±0.15^{a}	9.360±1.09^{a}	2.774±0.55^{a}
100 Hg	1.597±0.22^{a}	0.502±0.07^{a}	3.943±0.44^{a}	0.906±0.06^{ab}	3.238±0.32^{ab}	1.104±0.13^{b}	8.633±0.74^{a}	2.280±0.34^{a}
100 Hg EDTA	1.463±0.42^{a}	0.434±0.12^{a}	3.790±1.20^{a}	0.887±0.26^{ab}	2.943±0.58^{ab}	0.927±0.20^{b}	8.010±0.60^{a}	2.050±0.15^{a}
100 Hg CA	0.761±0.12^{a}	0.283±0.05^{a}	1.447±0.24^{a}	0.303±0.03^{b}	1.853±0.41^{b}	0.741±0.09^{b}	6.577±0.73^{a}	1.508±0.51^{a}
100Hg+EDTA+CA	1.400±0.25^{a}	0.431±0.10^{a}	2.120±0.13^{a}	0.585±0.06^{ab}	2.520±0.20^{ab}	0.762±0.05^{b}	7.331±0.79^{a}	1.698±0.33^{a}

Table 9.3: Effect of BC with soil chelating agents on fresh and dry biomass accumulation in *C. roseus* (±S. D., n=3)

	20 DAS				60 DAS			
Soil Concentration (mg kg-1 soil)	**Fresh Wt. of Roots (g plant^{-1})**	**Dry Wt. of Roots (g plant^{-1})**	**Fresh Wt. of Shoots (g plant^{-1})**	**Dry Wt. of Shoots (g plant^{-1})**	**Fresh Wt. of Roots (gplant^{-1})**	**Dry Wt. of Roots (g plant^{-1})**	**Fresh Wt. of Shoots (g plant^{-1})**	**Dry Wt. of Shoots (g plant^{1})**
Control	1.803±0.31^{a}	0.651±0.10^{a}	3.023±0.22^{a}	1.191±0.17^{a}	3.915±0.38^{a}	1.852±0.38^{a}	10.360±0.98^{a}	3.376±0.26^{a}
4 BC	0.665±0.13^{b}	0.240±0.03^{b}	2.147±0.44^{a}	0.391±0.04^{b}	3.087±0.58^{ab}	1.228±0.37^{a}	9.454±0.67^{a}	2.713±0.30^{ab}
4BC EDTA	0.352±0.06^{b}	0.164±0.02^{b}	1.973±0.24^{a}	0.496±0.07^{b}	1.876±0.25^{b}	0.693±0.12^{a}	5.921±1.02^{b}	1.508±0.34^{bc}
4BC CA	0.460±0.11^{b}	0.194±0.04^{b}	2.135±0.40^{a}	0.450±0.05^{b}	3.018±0.37^{ab}	1.118±0.31^{a}	8.720±0.82^{ab}	2.355±0.31^{abc}
4BC+EDTA+CA	0.417±0.06^{b}	0.157±0.03^{b}	2.110±0.36^{a}	0.528±0.07^{b}	2.110±0.29^{b}	1.003±0.26^{a}	7.167±0.54^{ab}	1.892±0.25^{c}

Table 9.4: Effect of Cd+BC with soil chelating agents on fresh and dry biomass accumulation in *C. roseus* (±S. D., n=3)

	20 DAS				60 DAS			
Soil Concentration (mg kg^{-1} soil)	**Fresh Wt. of Roots (g plant^{-1})**	**Dry Wt. of Roots (g plant^{-1})**	**Fresh Wt. of Shoots (g plant^{-1})**	**Dry Wt. of Shoots (g plant^{-1})**	**Fresh Wt. of Roots (gplant^{-1})**	**Dry Wt. of Roots (g plant^{-1})**	**Fresh Wt. of Shoots (g plant^{-1})**	**Dry Wt. of Shoots (g plant^{1})**
Control	1.934±0.20^{a}	0.912±0.11^{a}	7.150±0.97^{a}	1.589±0.30^{a}	4.812±0.82^{a}	1.798±0.26^{a}	11.135±0.90^{a}	3.238±0.25^{a}
150 Cd+BC	1.867±0.28^{a}	0.747±0.13^{ab}	5.343±2.02^{a}	0.929±0.37^{ab}	4.067±1.72^{a}	1.326±0.69^{a}	8.780±0.83^{a}	1.980±0.25^{ab}
150 Cd+BC + EDTA	1.297±0.54^{a}	0.469±0.19^{ab}	3.627±1.34^{a}	0.601±0.18^{b}	2.913±0.24^{a}	1.049±0.08^{a}	7.553±1.20^{a}	1.620±0.22^{ab}
150 Cd+BC+ CA	1.793±0.27^{a}	0.552±0.12^{ab}	4.520±0.58^{a}	0.724±0.05^{ab}	3.057±0.66^{a}	1.246±0.34^{a}	7.843±0.98^{a}	1.68±0.90^{ab}
150Cd+BC+EDTA+CA	1.13±0.12^{a}	0.344±0.04^{b}	3.497±0.30^{a}	0.515±0.12^{b}	2.737±0.45^{a}	0.906±0.26^{a}	7.483±0.78^{a}	1.324±0.30^{b}

Table 9.5: Effect of Hg+BC with soil chelating agents on fresh and dry biomass accumulation in *C. roseus* (±S. D., n=3)

	20 DAS				60 DAS			
Soil Concentration (mg kg-1 soil)	**Fresh Wt. of Roots (g plant^{-1})**	**Dry Wt. of Roots (g plant^{-1})**	**Fresh Wt. of Shoots (g plant^{-1})**	**Dry Wt. of Shoots (g plant^{-1})**	**Fresh Wt. of Roots (gplant^{-1})**	**Dry Wt. of Roots (g plant^{-1})**	**Fresh Wt. of Shoots (g plant^{-1})**	**Dry Wt. of Shoots (g plant^{1})**
Control	2.833±0.37^{a}	0.912±0.10^{a}	7.490±0.83^{a}	1.395±0.24^{a}	4.342±0.36^{a}	1.565±0.38^{a}	12.425±0.49^{a}	3.238±0.44^{a}
100 Hg+BC	1.914±0.42^{ab}	0.760±0.14^{ab}	5.053±0.21^{b}	0.863±0.11^{b}	3.567±0.23^{a}	1.237±0.07^{a}	10.205±1.62^{a}	2.017±.27^{ab}
100 Hg+BC+EDTA	1.367±0.20^{b}	0.422±0.05^{bc}	3.120±0.19^{b}	0.524±0.04^{b}	3.013±1.81^{a}	0.996±0.65^{a}	7.370±2.19^{a}	1.341±.36^{b}
100 Hg+BC+CA	1.480±0.21^{b}	0.443±0.08^{bc}	4.680±0.65^{b}	0.810±0.12^{b}	3.299±1.23^{a}	1.011±0.40^{a}	9.820±1.11^{a}	1.447±.45^{b}
100Hg+BC+EDTA+CA	1.093±0.21^{b}	0.336±0.06^{c}	4.241±0.82^{b}	0.716±0.05^{b}	3.197±0.32^{a}	1.045±0.08^{a}	9.193±1.41^{a}	1.18±.32^{b}

fold, respectively) with respect to alone BC treatment as far as Hg toxicity is concerned under co-contamination. Shoot biomass of *C. roseus* in the presence of chelating agent was lower as compared to the alone treatment. Marginal decline was observed in shoot fwt. & dwt. in the presence of CA along with joint application of EDTA and CA and lowest shoots fresh and dry wt. was observed in the presence of chelating EDTA (**Table 9.3**).

Both joint and individual application of EDTA and CA in the presence of Cd (150 $mgkg^{-1}$) +BC (4 $mgkg^{-1}$) showed a decrease in shoot fresh and dry biomass. Joint application of EDTA+CA showed a decline in shoot fresh and dry wt. by 14.77 and 33.13%, along with EDTA the decline was 13.97 and 18.18% while with CA it was 10.67 and 15.15%, respectively, as compared to Cd+BC treatments without chelates. Similar trend was observed for root biomass too (**Table 9.4**).

Likewise Hg (100 $mgkg^{-1}$) +BC (4 $mgkg^{-1}$) in the presence of chelating agents showed a significant (<0.001) decrease in root fresh and dry biomass. The highest decline in root fresh and dry wt. was observed in EDTA (1.18 and 1.24 folds, respectively) followed by joint application of EDTA+CA (1.11 and 1.18 folds) and lastly by CA (1.08 and 1.22 folds), respectively, in comparison to Hg+BC without chelates. Similar trend was observed for root biomass in the soil co-contaminated with Hg and BC along with the chelating agents (**Table 9.5**). The reduction in biomass was found to be significantly high in the presence of chelating agents compared to treatment (Cd, Hg and BC alone and jointly) without chelating agents. This may be an outcome of higher uptake of heavy metals and herbicide which results into reduction in plant biomass under individual and combined stress.

Table 9.6: Root and shoot length of *C. roseus* grown in soil amended with chelating agent and different concentration of Cd (mg kg^{-1} soil, ±S. D., n=3)

	20 DAS		60 DAS	
Soil Concentration (mg kg^{-1} soil)	**Root Length (cm)**	**Shoot Length (cm)**	**Root Length (cm)**	**Shoot Length (cm)**
Control	9.300 ± 0.819^{a}	14.373 ± 0.755^{a}	15.707 ± 1.064^{a}	21.437 ± 2.962^{a}
150 Cd	6.560 ± 0.692^{ab}	12.053 ± 3.616^{a}	14.933 ± 1.756^{a}	18.670 ± 2.455^{a}
150 Cd+EDTA	5.993 ± 1.414^{ab}	11.620 ± 1.119^{a}	13.077 ± 1.416^{ab}	17.923 ± 1.332^{a}
150 Cd+CA	5.473 ± 0.775^{b}	10.010 ± 2.006^{a}	12.160 ± 1.316^{ab}	16.187 ± 2.000^{a}
150 Cd+EDTA+CA	4.323 ± 0.921^{b}	9.403 ± 1.962^{a}	9.630 ± 0.708^{b}	15.157 ± 1.154^{a}

Table 9.7: F-ratio and level of significance of two- way ANOVA test for biomass and morphological parameters of *C. roseus* grown in soil amended with chelating agent and different concentration of Cd.

Factors	RL	SL	RFWt.	RDWt.	SFWt.	SDWt.
Days	263.505***	68.392***	43.732***	41.170***	35.665***	16.743**
Concentration	19.743***	6.425**	11.088***	20.269***	1.841^{ns}	1.405^{ns}
Days*Concentration	1.422^{ns}	0.081^{ns}	1.558^{ns}	4.027*	0.140^{ns}	0.132^{ns}

RL: root length; SL: shoot length; RFWt.: root fresh weight; RDWt.: root dry weight; SFWt.: shoot fresh weight; SDWt.: shoot dry weight. ns: not significant; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

The effect of chelating agents on root and shoot length of the studied plant *C. roseus* revealed that the maximum plant root and shoot length was achieved at 150 mg kg^{-1} Cd, alone treatment having average values of 14.93 cm and 18.67 cm, respectively, while the shortest plant root (9.63 cm) and shoot (15.15 cm) length were recorded under the joint application of EDTA+CA as recorded at 60 DAS (**Table 9.6**). Results of two-way ANOVA for Cd along with chelating agents revealed that there was a significant variation in all parameters for individual factors *i.e.*, 'days' whereas concentration does not show any significant impact on shoot fresh and dry wt. Interaction of both factors (days and concentration) showed non-significant difference on all parameters except root dry weight (**Table 9.7**).

Table 9.8: Root and shoot length of *C. roseus* grown in soil amended with chelating agent and different concentration of Hg (mg kg^{-1} soil, ±S. D., n=3)

	20 DAS		60 DAS	
Soil Concentration (mg kg^{-1} soil)	**Root Length (cm)**	**Shoot Length (cm)**	**Root Length (cm)**	**Shoot Length (cm)**
Control	8.397±0. 0.650^{a}	11.217±0.920^{a}	16.317±0.766^{a}	23.137±1.127^{a}
100 Hg	7.740±0.945^{a}	10.917±2.404^{a}	11.420±0.828^{b}	18.997±1.151^{ab}
100 Hg EDTA	7.663±1.294^{a}	9.477±0.693^{a}	11.300±0.458^{b}	17.037±1.202^{b}
100 Hg CA	6.383±0.908^{a}	9.857±0.575^{a}	10.007±2.366^{b}	16.570±0.993^{b}
100Hg+EDTA+CA	5.630±1.255^{a}	8.297±3.104^{a}	9.460±1.111^{b}	14.810±1.982^{b}

Root and shoot length in soil treated with Hg under amendments with chelating agents was found to significantly decrease both plant shoot and root by 22.04 and 17.16%, respectively, when the soil was amended with EDTA+CA, followed by CA 12.67 and 12.37 %, respectively and finally by EDTA 10.21 and 1.05% respectively, in comparison to Hg with no chelation (**Table 9.8**). Multivariate analysis for Hg showed significant variability between 'days' and 'concentration' as individual factors on root and shoots length, fresh wt, dry wt. While the interaction of

these two factors showed no significant variability except root length and dry wt (**Table 9.9**).

Table 9.9: F-ratio and level of significance of two- way ANOVA test for biomass and morphological parameters of *C. roseus* grown in soil amended with chelating agent and different concentration of Hg.

Factors	RL	SL	RFWt.	RDWt.	SFWt.	SDWt.
Days	112.704***	191.747***	135.799***	205.471***	343.685***	122.198***
Concentration	14.962***	10.465***	15.130***	34.249***	14.221***	11.414***
Days*Concentration	3.92*	2.782 ns	2.163 ns	11.487***	0.605 ns	0.364 ns

RL: root length; SL: shoot length; RFWt.: root fresh weight; RDWt.: root dry weight; SFWt.: shoot fresh weight; SDWt.: shoot dry weight. ns: not significant; *p<0.05; **p<0.01; ***p<0.001.

Under BC treatment a significant decline was induced in the joint application of chelating agents (EDTA+CA) both in roots (31.45%) as well as shoots (16.83%) with respect to BC without chelating agents (**Table 9.10**). Analyses of variance for BC in the presence of chelators showed a significant variability among root and shoot for individual factors 'concentration' and 'days'. The interactions of these two factors showed no significant effect on root dry wt, root and shoot length (**Table 9.11**).While under joint toxicity of Cd (150 $mgkg^{-1}$) and BC (4 $mgkg^{-1}$) in presence of EDTA + CA there was a 1.47 and 1.27 fold decrease in root and shoot length respectively, with control (**Table 9.12**).

Table 9.10: Root and shoot length of *C. roseus* grown in soil amended with chelating agent and different concentration of BC (mg kg^{-1} soil, ±S. D., n=3)

	20 DAS		60 DAS	
Soil Concentration (mg kg^{-1} soil)	**Root Length (cm)**	**Shoot Length (cm)**	**Root Length (cm)**	**Shoot Length (cm)**
Control	8.300±0.819^{a}	15.247±1.060^{a}	13.703±0.856^{a}	23.437±0.775^{a}
4 BC	6.160±1.729^{ab}	13.417±2.075^{ab}	11.257±0.903^{ab}	20.167±2.466^{ab}
4BC EDTA	5.430±0.854^{ab}	11.350±1.083^{ab}	10.530±0.586^{ab}	18.347±0.842^{ab}
4BC CA	4.370±1.045^{ab}	9.283±1.325^{b}	8.470±0.905^{b}	19.480±1.781^{ab}
4BC+EDTA+CA	3.583±0.749^{b}	8.273±0.897^{b}	7.717±2.035^{b}	16.773±1.714^{b}

Table 9.11: F-ratio and level of significance of two- way ANOVA test for biomass and morphological parameters of *C. roseus* grown in soil amended with chelating agent and different concentration of BC.

Factors	RL	SL	RFWt.	RDWt.	SFWt.	SDWt.
Days	132.135^{***}	218.556^{***}	351.834^{***}	126.938^{***}	681.587^{***}	484.51^{***}
Concentration	20.377^{***}	17.826^{***}	31.206^{***}	12.328^{***}	16.667^{***}	30.053^{***}
Days*Concentration	0.428^{ns}	1.258^{ns}	3.324^{*}	1.889^{ns}	8.324^{***}	9.676^{***}

RL: root length; SL: shoot length; RFWt.: root fresh weight; RDWt.: root dry weight; SFWt.: shoot fresh weight; SDWt.: shoot dry weight. ns: not significant; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

Table 9.12: Root and shoot length of *C. roseus* grown in soil amended with chelating agent and different concentration of Cd+BC (mg kg^{-1} soil, ±S. D., n=3)

	20 DAS		60 DAS	
Soil Concentration (mg kg_{-1} soil)	**Root Length (cm)**	**Shoot Length (cm)**	**Root Length (cm)**	**Shoot Length (cm)**
Control	10.31 ± 1.314^{a}	12.127 ± 0.650^{a}	19.417 ± 1.198^{a}	25.217 ± 1.936^{a}
150 Cd+4BC	8.203 ± 0.949^{a}	9.397 ± 0.833^{a}	12.553 ± 0.671^{ab}	19.783 ± 2.046^{ab}
150 Cd+4BC + EDTA	7.787 ± 1.620^{a}	8.250 ± 2.717^{a}	11.513 ± 3.004^{ab}	17.180 ± 0.976^{b}
150 Cd+4BC+ CA	7.327 ± 2.588^{a}	7.220 ± 0.896^{a}	10.410 ± 3.618^{b}	16.097 ± 1.212^{b}
150Cd+4BC+EDTA+CA	5.963 ± 1.199^{a}	7.937 ± 1.193^{a}	8.523 ± 0.666^{b}	15.587 ± 0.716^{b}

Results of two-way ANOVA for Cd+BC in the presence of chelating agents revealed that the interaction of both the parameters 'days' and 'concentration' showed no variability among different parameters except root length. A significant variation was observed among the individual factors for all morphological parameters of *C. roseus* (**Table 9.13**). Under joint toxicity of Hg (100 mg kg^{-1}) along with herbicide BC (4 $mgkg^{-1}$) showed a highest decline in the presence of chelating agent CA *i.e.*, 1.24 and 1.18 folds, respectively in root and shoot length as compared to Hg+BC (**Table 9.14**). Multivariate analysis for Hg along with BC in the presence of chelating agents revealed that individual factors were significantly affecting the root and shoots length, fresh, dry weight of *C. roseus*. Interaction between 'days' and 'concentration' showed a non-significant effect on all parameters except shoot dry weight (**Table 9.15**).

Table 9.13: F-ratio and level of significance of two- way ANOVA test for biomass and morphological parameters of *C. roseus* grown in soil amended with chelating agent and different concentration of Cd+4BC.

Factors	RL	SL	RFWt.	RDWt.	SFWt.	SDWt.
Days	127.610***	123.994***	41.285***	33.287***	139.659***	88.887***
Concentration	15.730***	10.935***	3.827*	4.335*	13.710***	24.430***
Days*Concentration	2.735 ns	2.895*	1.25 ns	0.339 ns	0.023 ns	1.484 ns

RL: root length; SL: shoot length; RFWt.: root fresh weight; RDWt.: root dry weight; SFWt.: shoot fresh weight; SDWt.: shoot dry weight. ns: not significant; *p<0.05; **p<0.01; ***p<0.001.

Table 9.14: Root and shoot length of *C. roseus* grown in soil amended with chelating agent and different concentration of Hg and 4BC (mg kg^{-1} soil, ±S. D., n=3)

	20 DAS		60 DAS	
Soil Concentration (mg kg^{-1} soil)	**Root Length (cm)**	**Shoot Length (cm)**	**Root Length (cm)**	**Shoot Length (cm)**
Control	11.210±0.976^{a}	16.360±2.359^{a}	16.750±0.895^{a}	24.033±1.229^{a}
100 Hg+4BC	9.207±1.238^{ab}	14.210±2.880^{a}	14.370±1.101^{ab}	20.800±2.185^{a}
100 Hg+BC+4EDTA	7.657±0.785^{ab}	10.617±1.295^{a}	12.950±1.262^{ab}	19.907±2.206^{a}
100 Hg+4BC+CA	6.450±0.687^{b}	11.577±1.764^{a}	11.553±0.869^{b}	17.573±1.603^{a}
100Hg+4BC+EDTA+CA	8.287±1.703^{ab}	9.853±1.293^{a}	11.780±1.840^{b}	18.597±1.200^{a}

Table 9.15: F-ratio and level of significance of two- way ANOVA test for biomass and morphological parameters of *C. roseus* grown in soil amended with chelating agent and different concentration of Hg+4BC.

Factors	RL	SL	RFWt.	RDWt.	SFWt.	SDWt.
Days	127.61***	123.994***	41.285***	33.387***	139.659***	88.887***
Concentration	15.730***	10.935***	3.827*	4.335*	13.710***	24.430***
Days*Concentration	0.699ns	0.446 ns	0.141 ns	0.148 ns	0.160 ns	5.456**

RL: root length; SL: shoot length; RFWt.: root fresh weight; RDWt.: root dry weight; SFWt.: shoot fresh weight; SDWt.: shoot dry weight. ns: not significant; *p<0.05; **p<0.01; ***p<0.001.

9.4.2 Effect of EDTA/CA alone and in combination on metal accumulation in plant tissues

Effect of application of EDTA/CA alone and in combination on the accumulation of heavy metals (Cd and Hg) with or without herbicide (BC) showed significant interactions. Soil application of 150 mg kg^{-1} Cd and 100 mg kg^{-1} Hg with 4 mg kg^{-1} BC, under co-contamination was found to increase the concentration of Cd/Hg in roots and shoots significantly, in this study, elaborating a synergistic effect of Cd/Hg and BC co-contamination.

An absorption of Cd and Hg by above ground parts or roots of *C. roseus* increased significantly with the application of chelating agents EDTA and CA. The trend of enhanced Cd absorption through application of chelating agents was as

follows EDTA+CA>CA>EDTA. Cd accumulation increased by 1.36 and 2.05 folds under joint application, followed by CA where it was increased by 1.20 and 1.85 folds and finally by EDTA (1.09 and 1.28 folds) in roots and shoots respectively as compared with Cd treatment having no chelates (**Table 9.16**).

Table 9.16: Cd accumulation in roots and shoots in the presence of soil chelating agents in *C. roseus* (±S. D., n=3)

Soil Cd+Soil Amendments concentration (mg kg^{-1} soil)	Cd content in Roots($\mu g g^{-1}$)		Cd content in Shoots ($\mu g g^{-1}$)	
	20 DAS	60 DAS	20 DAS	60 DAS
Control	2.217 ± 0.661^{b}	6.993 ± 0.837^{b}	0.443 ± 0.137^{b}	3.337 ± 0.453^{c}
150 Cd	333.847 ± 44.622^{a}	489.237 ± 13.467^{a}	87.267 ± 9.522^{a}	121.460 ± 26.225^{b}
150 Cd+EDTA	317.497 ± 34.364^{a}	533.743 ± 34.470^{a}	76.553 ± 10.519^{a}	156.007 ± 29.058^{ab}
150 Cd+CA	385.197 ± 70.294^{a}	590.887 ± 50.406^{a}	70.597 ± 13.229^{a}	225.713 ± 27.814^{ab}
150 Cd+EDTA+CA	290.300 ± 50.466^{a}	668.130 ± 79.976^{a}	56.580 ± 15.056^{a}	249.273 ± 36.052^{a}

Table 9.17: F-ratio and level of significance of two- way ANOVA test for Cd and Hg accumulation in roots and shoots in the presence of soil chelating agents in *C. roseus*

	Cd		Hg	
Factors	**Root**	**Shoot**	**Root**	**Shoot**
Days	131.842^{***}	153.696^{***}	396.488^{***}	208.206^{***}
Concentration	117.222^{***}	53.169^{***}	119.639^{***}	57.562^{***}
Days*Concentration	12.823^{***}	22.772^{***}	38.509^{***}	27.271^{***}

Level of Significance: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

The Hg contents in shoot and root systems of *C. roseus* increased due to application of chelating agents. The highest Hg (1.89 and 2.57 fold) contents were recorded in plant roots and shoots respectively, on soil amended with CA (**Table 9.18**). The addition of 4 mg kg^{-1} of BC to 150 mg kg^{-1} of Cd in the presence of chelating agents significantly ($p<0.05$) increased the accumulation of Cd in roots as well as shoots of *C. roseus* (**Tables 9.19**). The highest concentration of Cd was recorded under co-contamination with BC in root and shoot of *C. roseus* in the presence of CA (1.45 and 1.15 fold) followed by joint application of EDTA+CA (1.35 and 0.87 fold) and in the last by EDTA (1.10 and 1.13 fold).

Table 9.18: Hg accumulation in roots and shoots in the presence of soil chelating agents in *C. roseus* (±S. D., n=3)

Soil Hg+Soil Amendments concentration (mg kg-1 soil)	Hg content in Roots(µgg-1)		Hg content in Shoots (µgg-1)	
	20 DAS	60 DAS	20 DAS	60 DAS
Control	0.118±0.004[b]	1.048±0.069[d]	0.079±0.016[b]	0.572±0.105[d]
100 Hg	158.653±12.885[a]	338.483±52.799[c]	62.593±10.223[a]	124.607±20.799[c]
100 Hg EDTA	163.843±31.073[a]	430.850±41.471[bc]	74.900±6.951[a]	189.737±37.104[bc]
100 Hg CA	175.067±22.379[a]	641.733±51.640[a]	66.147±11.057[a]	320.007±32.107[a]
100Hg+EDTA+CA	165.403±16.280[a]	527.180±57.024[a,b]	58.913±10.408[a]	255.243±49.503[ab]

Table 9.19: Cd+BC accumulation in roots and shoots in the presence of soil chelating agents in *C. roseus* (±S. D., n=3)

Soil Cd+Soil Amendments concentration (mg kg-1 soil)	Cd+BC content in Roots (µgg-1)		Cd+BC content in Shoots (µgg-1)	
	20 DAS	60 DAS	20 DAS	60 DAS
Control	0.187±0.030[b]	5.737±0.55[d]	0.133±0.01[b]	3.650±0.46[b]
150 Cd+4BC	335.090±20.84[a]	612.637±34.89[c]	49.657±12.48[a]	156.493±17.67[a]
150 Cd+4BC + EDTA	377.093±31.40[a]	675.330±55.20[bc]	53.203±15.49[a]	177.327±17.27[a]
150 Cd+4BC+ CA	363.740±32.67[a]	890.827±48.76[a]	48.720±15.21[a]	179.813±54.33[a]
150Cd+4BC+EDTA+CA	382.690±50.13[a]	825.270±48.37[ab]	59.657±11.71[a]	136.750±38.29[a]

Effects of chelating agents were also studied under joint treatment of Hg+BC and its effect on accumulation of Hg by *C. roseus*. CA induced increase in the heavy metal uptake was approximately 2.37 and 1.85 folds in root and shoot of *C. roseus* followed by EDTA+CA (1.84 and 1.49 folds, respectively) and finally by EDTA (1.35 and 1.28 folds, respectively) with respect to Hg alone treatment with no chelating agents (**Table 9.21**).

Table 9.20: F-ratio and level of significance of two- way ANOVA test for Cd+BC and Hg+BC accumulation in roots and shoots in the presence of soil chelating agents in *C. roseus*

	Cd+BC		Hg+BC	
Factors	Root	Shoot	Root	Shoot
Days	516.034***	101.463***	810.346***	157.545***
Concentration	280.274***	23.738***	197.149***	42.755***
Days*Concentration	42.523***	6.972**	88.619***	15.22***

Level of Significance: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

Table 9.21: Hg+BC accumulation in roots and shoots in the presence of soil chelating agents in *C. roseus* (±S. D., n=3)

Soil Hg+Soil Amendments concentration (mg kg^{-1} soil)	Hg+BC content in Roots(µgg^{-1})		Hg+BCcontent in Shoots (µgg^{-1})	
	20 DAS	60 DAS	20 DAS	60 DAS
Control	0.297±0.112^{b}	4.617±0.677^{d}	0.144±0.032^{b}	1.883±0.12^{b}
100 Hg+4BC	127.097±24.08^{a}	355.867±42.77^{c}	66.917±10.23^{a}	154.670±35.05^{a}
100 Hg+BC+4EDTA	147.927±23.12^{a}	481.070±62.47^{c}	63.503±10.42^{a}	199.027±39.73^{a}
100 Hg+4BC+CA	161.437±12.21^{a}	843.253±50.20^{a}	59.983±11.59^{a}	285.947±46.89^{a}
100Hg+4BC+EDTA+CA	172.027±17.61^{a}	656.253±35.80^{b}	92.790±10.22^{a}	230.483±33.78^{a}

Multivariate analysis revealed that individual factors like 'days', 'concentration' and the interaction of both significantly affected the root and shoot bioaccumulation factor both in Cd/Hg and BC alone an in combination in the presence of chelating agents EDTA and CA singly or combined (**Tables 9.17 and 9.20**).

9.4.3 Effect of EDTA and CA on bioconcentration (BCF) and translocation factor (TF)

Cd/Hg translocation from the root to the shoot of *C. roseus* was affected by chelates. In sole Cd-contaminated soil, significantly ($p>0.05$) higher translocation ratio of Cd was observed post application of CA, as recorded after 60 days of sowing. The present results showed that the translocation of Cd from root to the shoot of *C. roseus* in the presence of CA had increased by 1.54 fold when compared to lone Cd treatments (metal without chelates). Cd translocation in the presence of EDTA+CA was less efficient as compared to CA but however, was found to increase significantly from 0.24–0.37 (**Figure 9.1**) at Cd (alone) to Cd (EDTA+CA), respectively.

Translocation factor of Hg was found highest in the presence of chelating agent CA (1.35 fold) followed by EDTA+CA (1.30 fold) and the by EDTA (1.19 fold, **Figure 9.2**). In co-contaminated soils (Cd+BC and Hg+BC), the chelating agents did not show any significant effect on translocation factor for Cd and Hg (**Figures 9.3 and 9.4**).

BCF or Bioconcentration Factor is defined as the ratio of metal concentration present in the plant to the metal concentration present in the soil. In addition, BCFs were calculated, as indicators of the ability of the plant to accumulate metals in plant tissues from soils. **Figures 9.1-9.4** shows the values of BCF/TF factors of Cd/Hg

alone and in combination with BC in the presence of chelating agents. A preferential accumulation of metals in plant roots was also reflected by the BCF values, which were higher for roots than for shoots in the presence of chelating agents and experimental conditions.

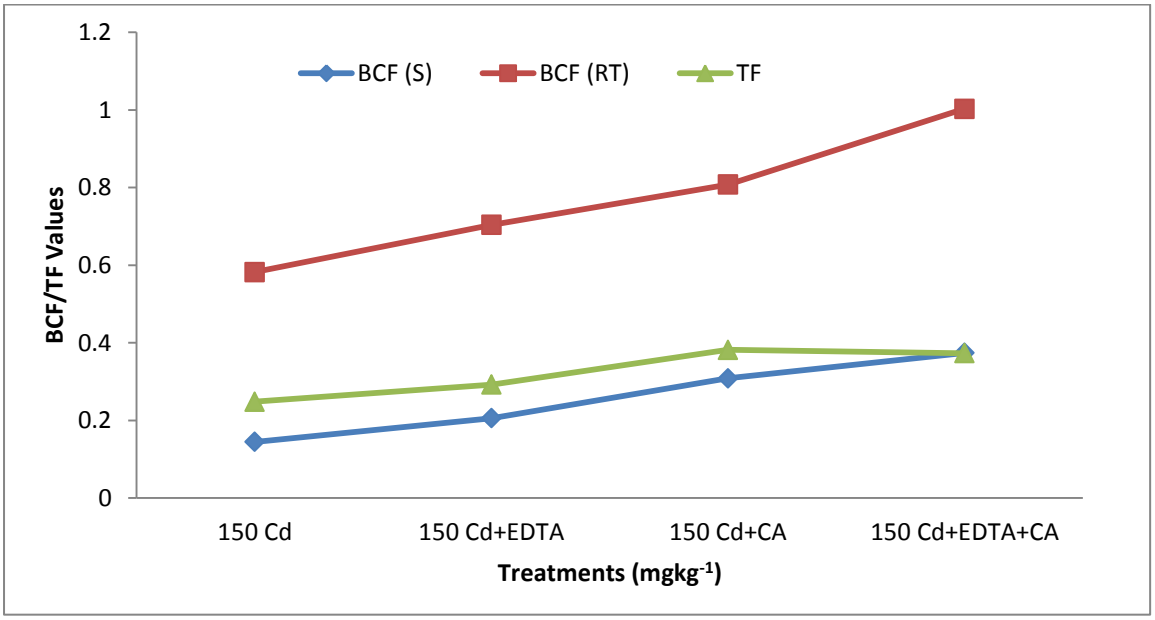


Figure 9.1: Bioconcentration factor (BCF) and translocation factor (TF) of Cd in soil amended with chelating agents in *C. roseus*

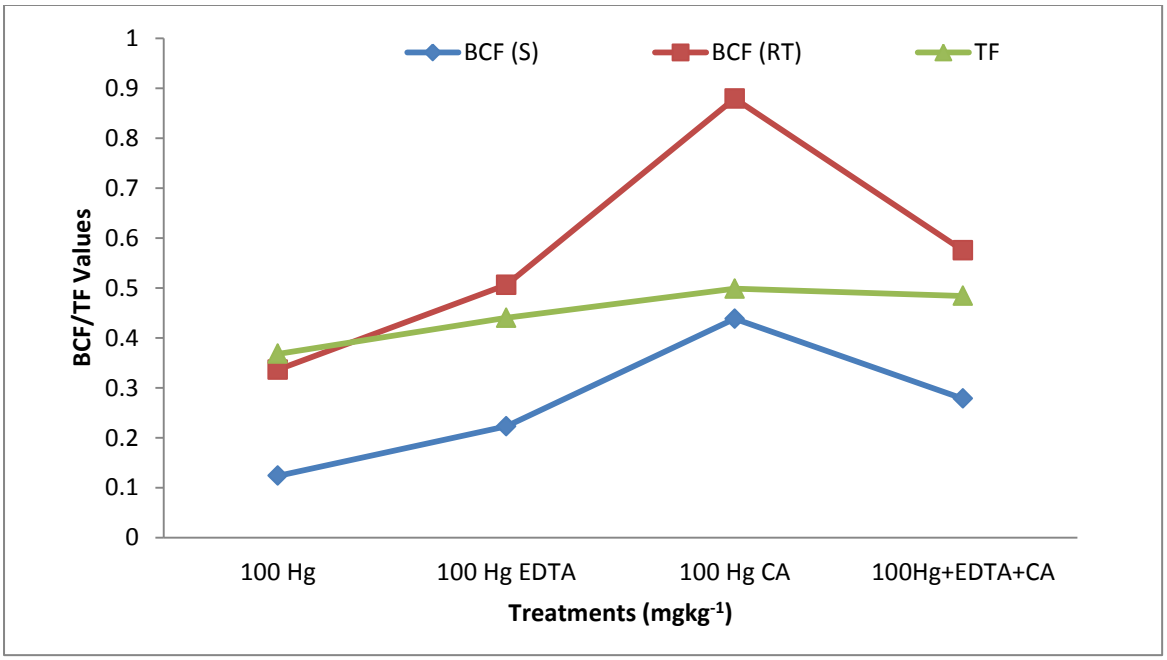


Figure 9.2: Bioconcentration factor (BCF) and translocation factor (TF) of Hg in soil amended with chelating agents in *C. roseus*

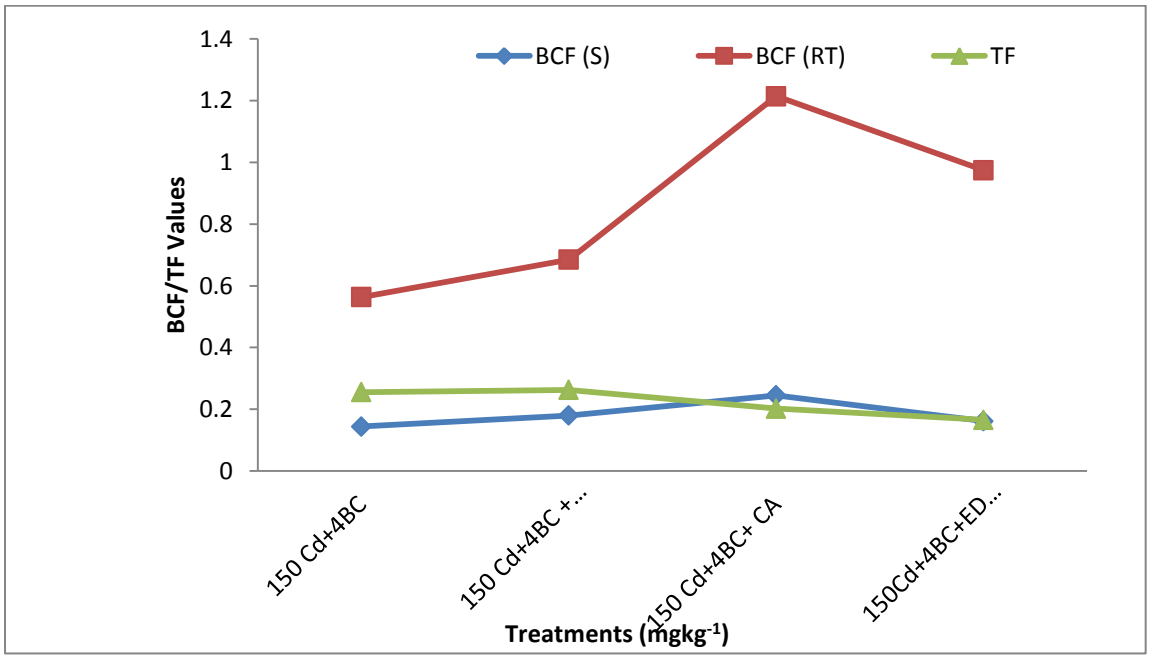


Figure 9.3: Bioconcentration factor (BCF) and translocation factor (TF) of Cd+4BC in soil amended with chelating agents in *C. roseus*

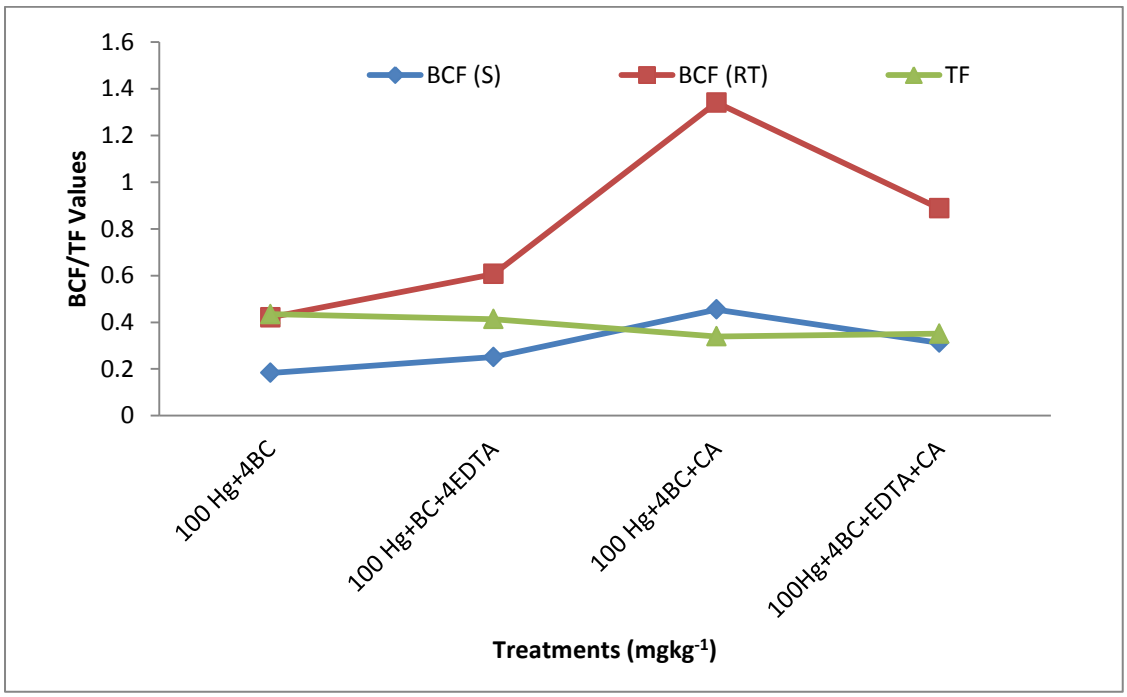


Figure 9.4: Bioconcentration factor (BCF) and translocation factor (TF) of Hg+4BC in soil amended with chelating agents in *C. roseus*

Application of chelating agents significantly increased the BCF factor of root and shoot in the presence of Cd (150 $mgkg^{-1}$) as compared to the Cd alone (no chelates). The roots showed an approximately 1.42 fold increase in the BCF values

under Cd treatment in presence of chelating agent EDTA+CA as compared to EDTA and CA alone. Similar trend was observed for shoots too, but roots displayed higher BCF values as compared to shoot (**Figure 9.1**).

BCF values for Hg (100 $mgkg^{-1}$) on the contrary showed an increase in root and shoot values in the presence of CA (2.61 and 3.53 folds) followed by EDTA+CA (1.71 and 2.24 folds) and finally by EDTA (1.50 and 1.80 folds) respectively, compared to Hg alone (**Figure 9.2**).

On the other hand, application of chelating agents under co-contamination of Cd (150 $mgkg^{-1}$) along with BC (4 $mgkg^{-1}$) was found to increases the Cd accumulation both in root as well as shoot as compared to individual treatments of Cd and BC. There was 2.15 and 1.70 fold increase in the BCF values for root and shoot in presence of CA while approximately 1.73 and 1.12 fold as well as 1.21 and 1.25 fold increase in BCF values were recorded in the presence of joint application of EDTA+CA and CA, respectively (**Figure 9.3**). Similar results were observed for joint toxicity of Hg along with BC in the presence of chelating agents (**Figure 9.4**).

The overall results revealed that the total metal uptake by *C. roseus* was significantly ($p<0.001$) increased in the presence of chelating agents with respect to metals with no chelates. This outcome reflects the success of amendments to enhance metal concentration in plant tissues.

9.4.4 Effect of chelating agents (EDTA and CA) on soluble butachlor dissipation

BC concentration in soil was found to decrease for all treatments along with soils with no planting and no chelate application (**Figure 9.5**). The initial concentration of BC in soil amended with heavy metals was approximately 4 mg kg^{-1}.

The result of this study showed that the dissipation of polyaromatic hydrocarbon compounds such as BC along with heavy metals (Cd/Hg) in soils might be increased under chelates application. EDTA and CA are well known and efficient chelating agents of heavy metals such as Cd and Hg. The percentage removal of BC reached 51.89% for sole BC contaminated soil, respectively with soils compared to BC with no chelates (**Figure 9.5)**.

Under joint toxicities the BC dissipation was 45.17% for Cd+BC and 49.28% for Hg+BC co-contaminated soils compared to BC in combination with Cd/Hg with no chelates (**Figure 9.5**).

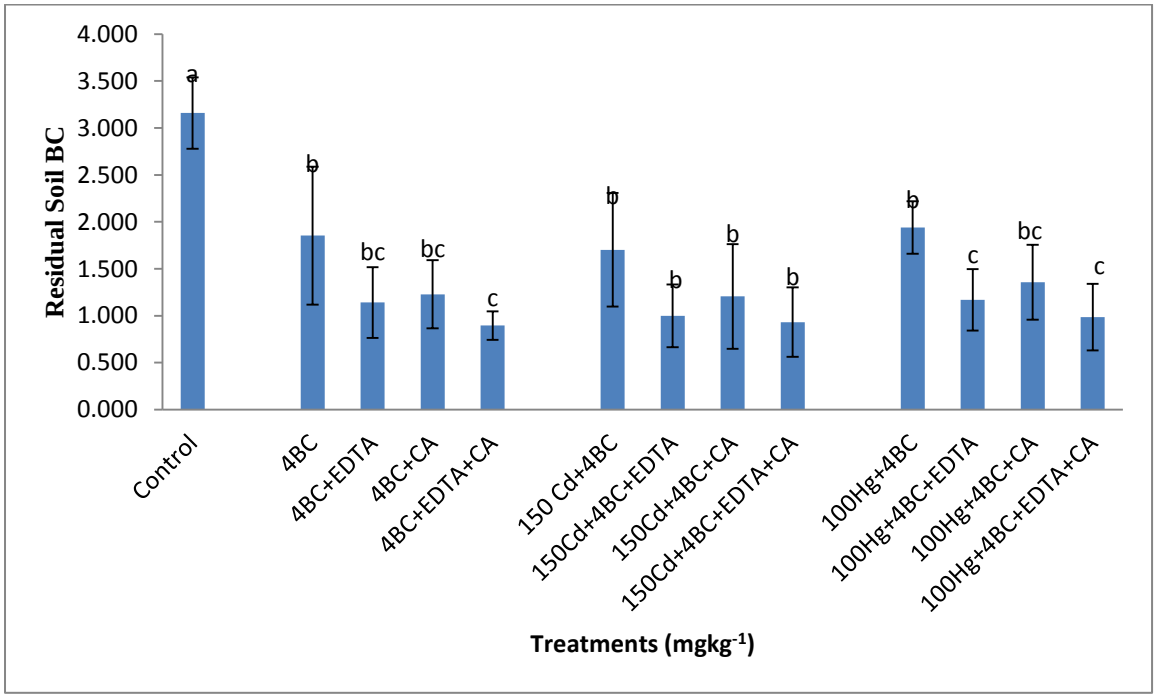


Figure 9.5: Residual butachlor alone and in joint treatment with Cd and Hg in planted and non-planted soil after 60 days of planting. Bars (means ± SE, n = 3) with different letters indicate a significant difference (Duncan test ,P≤0.052).

Application of EDTA or CA alone and in combination (EDTA + CA) to sole BC contaminated soil, significantly ($p \leq 0.05$) lowered the residual BC concentration in soil from 1.85 to 1.14, 1.227 and 0.895 $mgkg^{-1}$ respectively as compared to *C. roseus* planted soil with no chelating agents (**Figure 9.5**).

Under joint toxicity of Cd+BC in co-contaminated soils, an application of EDTA + CA was found to decrease the BC concentration significantly in final (60 DAS) soil from 1.70 to 0.932 in joint application of EDTA+CA and 0.998 $mgkg^{-1}$ under sole application of EDTA, respectively (**Figure 9.5**). Similarly, Hg+BC have observed highest BC dissipation (49.48%) in presence of EDTA+CA with respect to Hg+BC without chelates (**Figure 9.5**). **Yang et al (2001)** reported that heavy metals in the presence of chelating agent's makes organic matter less constrained which results in an increase in the diffusion capability of PAH compounds. The present

study revealed that EDTA + CA displayed greater potential for BC desorbtion from co-contaminated soil.

9.5 Discussion

The presence of Cd/Hg and BC alone and in combined treatment in the soil along with the chelating agents affects the growth of *C. roseus*, as indicated by a significant decrease in growth of *C. roseus* monitored after 60 days of planting. The biomass (roots and shoots) of the plant *C. roseus* was affected by the presence of chelating agents EDTA and CA as represented in **Tables 9.1-9.5**. The joint application of EDTA and CA reduced the biomass of plants in all the treatments compared with treatments with no chelate. **Gunawardan et al** (**2010**) reported similar findings for reduction in shoot biomass of *L. perenne* grown in soil amended with combined application of rhamnolipid + sulfate and rhamnolipid + EDDS with the different metal concentration. Soil spiking with EDTA significantly affects the plant root and shoot dry biomass and plant growth. With the increasing concentration of chelating agent in the soil there was decrease in all the morphological parameters of plant (**Wang et al**, **2006**). The root and shoot length of all the treatments Cd/Hg and BC alone and in combination in the presence of chelating agents showed a decrease in plant height of *C. roseus*. Chelates present in the soil tends to form complexes to the metal present in soil solution and cations compete, which leads to an imbalance of the chemical components in the soil solution and results into toxicities, because unbound amendments and free metal ions are well known for their plant toxicity (**Vassil et al**, **1998**). Researchers reported that heavy metals decreased the elasticity and viscosity of cell walls of plant and results into inhibition of root growth (**Ma et al**, **2004**; **Suthar et al**, **2014**).

The present study showed the effectiveness of CA in enhancing the root and shoot accumulation of Hg alone as well as under co-contamination (Cd/Hg in combination with BC) which was significantly higher than that of EDTA and EDTA+CA. Sole Cd treatment revealed high accumulation in the presence of EDTA irrespective of other chelating agents (**Tables 9.16-9.21**). In the present study Cd accumulation in root and shoot of *C. rsoeus* increased in the presence of EDTA+CA by 1.36 and 2.05 fold while increase by 1.20 and 1.85 folds, and 1.09 and 1.28 folds

was observed in the presence of CA and EDTA respectively compared to Cd lone treatments having no chelates (**Table 9.16**). **Wu et al** (**2004**) had similar findings related to the present study, wherein Cu uptake was reported to enhance significantly in the presence of EDTA (3 mmol kg^{-1}).

The accumulation of Cd under joint treatment with BC in root and shoot of *C. roseus* was found highest in the presence of CA (1.45 and 1.15 folds) followed by combination of EDTA+CA (1.35 and 0.87 folds) and EDTA (1.10 and 1.13 folds), respectively. Similar, findings for co-contamination with Hg+BC in presence of chelating agents was observed (**Tables 9.19, 9.21**). **Sun et al** (**2013)**, found an increase in Cd accumulation in the plant *Tagetes patula* (shoots, roots and leaves) grown in soil co-contaminated with benzo (a) pyrene and Cd in the presence of chelating agent Tween® 80. **Jean et al** (**2008**) reported that uptake of Cr by plant *D. innoxia* was enhanced in the presence of chelating agents EDTA.

Previous researches have reported that the chelating agent EDTA increased the bioavailability of any metals through soil, but the ability to improve translocation from root to shoot varied according to different metals (**Lombi et al**, **2001**; **Madrid et al, 2003**).

In the presence of Hg (100 $mgkg^{-1}$), TF values was found highest for CA *i.e*., 1.35 folds compared to control, followed by EDTA+CA and EDTA the values being 1.30 and 1.19 folds respectively, compared to treatment without chelates (**Figure 9.2**). While in joint toxicity of Cd/Hg along with BC in the presence of chelators did not show significant effect on translocation factor for Cd and Hg (**Figures 9.3 and 9.4**). On the contrary of above results **Qu et al** (**2011**), reported low translocation for Cr by *M. sativa* in the presence of chelating agents CA and sodium hydrogen phosphate.

The overall results for BCF values for roots and shoots revealed that the total metal uptake by *C. roseus* was significantly ($p<0.001$) increased in the presence of EDTA and CA alone as well as in combined application with respect to metal treatments without chelates (**Figures 9.1-9.4**). Similar results were observed by **Agnello et al** (**2016**) that accumulation of metals in plant roots, also denoted by the BCF values, were higher for roots irrespective of shoots for all the treatments. However, BCFs were the indicators of the ability of the plant to accumulate metals in plant biomass from soils.

Present study revealed that the dissipation of PAH compound such as BC along with heavy metal in soils increased in the presence of chelating agents. Soil contaminated with mixed pollutants enhanced the benzo-alpha-pyrene dissipation in the presence of chelators EDTA or EDTA+ CA also suggested that the presence of organic acids may improve the microbial activity in soil (**Chigbo & Batty**, **2013**). Results revealed that BC alone and along with heavy metals under contamination along with chelating agents significantly increased the dissipation rate of BC both in individual and co-contaminated soil (**Figure 9.5**). However, joint application of EDTA+CA had a more significant effect as compared to individual EDTA or CA applications. BC dissipation rate was found to enhance by 51.89% for lone BC treatment at the end of the 60 days experiment (**Figure 9.5**) in presence of chelators. Chelating agents have the ability to desorb the PAH compounds and make them bioavailable for degradation of microbes. In the present study, due to the presence of EDTA and CA, the rate of degradation of BC increases due to availability of compounds which can be degraded by microbes (**Harms & Bosma**, **1997**). Similar results were reported by **Bach et al** (**2005**) wherein joint application of chelating agents enhanced the PAH dissipation more than single application.

9.6 Conclusion

Result of the present study showed that EDTA and Citric acid in aqueous solutions may be applied in single Cd/Hg and/or BC co-contaminated soils in the presence of *C. roseus*, to efficiently remove Cd/Hg or BC in individual or co-contaminated soils.

Natural chelating agents such as citric acid can be recommended as potential chelating agent for increasing metal translocation from root to shoots in plants as compared to other chelate treatments carried out in the present study.

Since the binding capacities of chelates are limited, a restricted amount of ions can be carried by their molecule which depends on the number of binding sites. For example, lone CA treatment enhanced more Cd/Hg solubility under individual and/or co-contamination, revealed in terms of an increase in the amount of Cd/Hg absorbed by roots and translocated to shoots in the present study. Since CA is biodegradable, increased leaching during plant trial is likely to cause lower environmental risk as compared to EDTA amended soils.

Overall the study showed that alone application of CA could be considered as an effective chelate candidate for the phytoextraction of Cd/Hg and dissipation of butachlor by *C. roseus*. An increase in TF as well as the metal extraction ratio observed after the chelate application (CA) reveals, the ability of *C. roseus* to simultaneously accumulate Cd/Hg and remove BC in the presence of EDTA or EDTA + citric acid.

Plate 9.1: Cd (150 mgkg^{-1}) treated plants (T0:Control, T27:Cd, T28:Cd+EDTA, T29:Cd+CA, T30:Cd+EDTA+CA)

Plate 9.2: Hg (100 mgkg^{-1}) treated plants (T0:Control, T31:Hg, T32:Hg+EDTA, T33:Hg+CA, T34:Hg+EDTA+CA)

Plate 9.3: BC (4 mgkg $^{-1}$) treated plants (T0:Control, T35:BC, T36:BC+EDTA, T37:BC+CA, T38:BC+EDTA+CA)

Plate 9.4: Cd (150 mgkg^{-1}) + BC (4 mgkg^{-1}) treated plants (T0:Control, T39:Cd+BC, T40:Cd+BC+EDTA, T41:Cd+BC+CA, T42: Cd+BC+EDTA+CA)

Plate 9.5: Hg (100 mgkg^{-1}) + BC (4 mgkg^{-1}) treated plants (T0:Control, T43:Hg+BC, T44:Hg+BC+EDTA, T45:Hg+BC+CA, T46: Hg+BC+EDTA+CA)

Chapter 10

General Discussion

Contamination of agricultural soils is emerging as a grievous environmental problem since it imposes threat to human health *via* sneaking into food chains and eventually to environment through groundwater leaching (**Romic & Romic, 2003**). The coexistence of organic contaminants and heavy metals at global scale is gradually increasing, leading to soil pollution to which phytoremediation has been recommended as a remediation technology. Studies on the interaction of organic (*e.g.*, PAH, xenobiotics, organometallic compounds) and inorganic (*e.g.*, heavy metals) compounds has been found to impose synergistic or antagonistic effect on plant growth (**Lin et al, 2006**). **Almeida et al (2008)** proposed that an interaction of organic contaminant with heavy metals extraction potential of the plants affects, uptake kinetics and mechanism. The present study investigates the phytoremediation of soils co-contaminated by metals (Cd/Hg) and Butachlor (BC) by *C. roseus* which has not been fully investigated as yet. Since most of the work on phytoremediation has been carried out either on single contaminants or multiple contaminants of similar type, the present study was an effort to address the problems posed by an admixture of both organic and inorganic contaminants.

Prior to phytoremediation studies, a survey of agro-industrial soil was carried out so as to ascertain the predominance of pollutants, which can be selected for experimentation. To this end soil samples were collected from agricultural and rural areas existing close to industrial sites, situated in and around Lucknow (U.P.) city. The analysis of different physico-chemical properties of soil samples revealed that the texture of the collected samples varied between sandy loam to loam, which were in agreement to the findings by **Upadhyay and Sharma (2016)**. Soil was further analysed for different parameters *viz*., pH, EC, OC(%), OM(%), MC(%), N, P, K. Besides physicochemical analysis, an analysis of the heavy metal (Pb, Hg, Cu, Cr, Ni, As, Cd, Mn, Fe) content present in the soil samples was also carried out. Results revealed Hg and Cd to be more prevalent compared to rest of the heavy metals detected in the preliminary analysis and the same were above the tolerable limits (**MAFF**, **1992**; **EC, 1986**) especially in the soil sample collected from Kalli West followed by Bijnor and Bhawaniganj and finally by Mau (for Hg); in case of Cadmium, however, Semarou was found to have highest concentration followed by Mohanlalganj and Kalli West. The soil samples were also analysed for presence of herbicides. Results revealed the predominance of BC in most of the soil samples

compared to other herbicides which were in traces. The concentration of BC in the soil samples ranged from 4.78 μgkg^{-1} to 111.8 μgkg^{-1}. To ensure further a HPLC chromatogram of Standard Butachlor was obtained and was compared with the HPLC chromatogram of extracts of untreated soil samples from different agricultural sites. The national regulations relevant for pesticide residues in agricultural soils (**The Official Gazette of the RS, 1994**) define the maximum residue level (MRL) range upto, 40 µg/kg for Butachlor. Depending upon the above mentioned findings, on the basis of survey and laboratory analysis, BC herbicide was chosen along with Hg and Cd for co-contamination study.

To begin with, the phytoremediation of soil co-contaminated with Cd, Hg alone and jointly with BC, was carried out, to examine the effect on some test plant species particularly on seed germination and early seedling growth. Seeds of different plant species (*Catharanthus, Rauwolfia, Celosia* and *Gaillardia*) were checked for their germination potential in soil co-contaminated with heavy metals (Cd and Hg) and herbicides (BC). Results revealed that treatment of the individual Cd, Hg and/or BC affected both, the germination percentage as well as the early seedling growth of the plants differently. The preliminary studies revealed the greater tolerance of *C. roseus* under co-contamination; hence it was selected for further studies. Overall studies on germination and early seedling growth of *C. roseus* revealed that the joint effect of Cd/Hg and BC was found to be more toxic as compared to the individual toxicities of Cd/Hg and BC. Previous researches on co-contamination have shown that Cd and Tri Chloro Benzene impose inhibitory effects on wheat seedlings (**Cailin et al, 2009)**. The tolerance index of *C. roseus* was found to be significantly reduced under joint application of Cd and BC, as compared to individual application of BC and Cd. However, vigour index revealed that Cd alone was more toxic followed by combination and finally by BC sole treatment. On the contrary the tolerance index of *C. roseus* was significantly declined in the presence of BC, but seemed to be more tolerant under joint toxicity of BC and Hg with repect to Hg alone. Seed vigour index was found to be highest in BC followed by combination of BC and Hg and Hg alone.

The present study on *C. roseus* revealed that the length of the shoot was greater in comparison to root length at all treatments, while root was found to be more sensitive which was in concurrence to earlier studies (**Khatamipour et al, 2011 ; Subin & Francis, 2013**). There was a significant structural deformation in root and

shoots of *C. roseus*. The SEM images revealed the anatomical differences more in shoots as comparison to the root structure, which is contrary to findings of **Godbold and Huttermann (1986)**. This study revealed that the co-contaminants especially heavy metals and herbicides affect the early seedling growth in different ways.

The effect of heavy metal (Hg/Cd) and/or herbicide (BC) on different biochemical parameters of *C. roseus*, revealed a decrease in carotenoid content and photosynthetic pigments values with the increasing concentration of contaminants in all treatments. **Hassan & Mansoor (2014)** reported that heavy metal (Cd) was non-essential element but impedes chlorophyll synthesis and development which finally leads to death in plants. Decline of carotenoid content might reflect Cd/Hg-induced oxidative stress, since carotenoids are well known to be major antioxidant (**Shaw et al, 2004**). There was decrease in protein content significantly ($p<0.05$) with increasing concentration in Cd/Hg and/or BC in all treatments. Heavy metal stress results decline in protein contents which may leads to accumulation of proline content in the plant species (**Chen et al, 2001**). Likewise, estimation of LPO activity served as an excellent indicator of heavy metal and/or herbicide induced oxidative stress in the plant species *C. roseus*. Cd is non-redox active, it can stimulate ROS production and subsequent stress and lipid peroxidation (**Shah et al, 2001**). Heavy metal (Cd/Hg) interaction leads to generation of ROS, which may target sulfur-containing amino acids, like methionine and cysteine, and thiol groups of proteins and make them inactivate (**Mukwevho et al, 2014**).

The present study elucidated changes in biochemical and physiological parameters in *C. roseus* plants when subjected to Cd, Hg, BC alone and in combination with the addition of natural (CA) and a synthetic (EDTA) chelating agent in the soil to induce heavy metal and herbicide uptake. There was increase in total photosynthetic pigments values of *C. roseus* in presence of chelating (EDTA and/or CA) agents. **Wang et al** (**2004**) also revealed similar findings, where in the presence of chelating agents an increase in chlorophyll content was observed for the treated plants. Results further revealed that the application of chelating agents decreased LPO activity which results in enhancement of antioxidant enzymes with respect to different treatments (Cd, Hg, BC alone and jointly). In support of our findings, earlier observation could be quoted wherein application of chelating agent EDTA, lowered MDA content and oxidative damage was not strongly expressed in the presence of

EDTA (**Markovska et al, 2013**). The value of protein content of *C. roseus* was found to be enhanced in the presence of chelating agents. Likewise, **Saeed Akram et al** (**2009**) and **Park et al** (**2012**) also investigated that the scavenging of ROS and decrease in electrolyte leakage may be the key factors for increased soluble protein content in both roots and leaves of the plant (sunflower). The antioxidant activities of enzymes were involved in the mitigation of oxidative stress in *C. roseus*. **Sharma et al (2012)** reported that plants develop antidefence mechanism in the form of enhancement of SOD, APX, CAT, GR and POD activity for mitigation of ROS-induced damages. However, the results in the present revealed that both the heavy metals *i.e*., Cd and Hg as well as BC induced oxidative stress which resulted in elevated values of CAT under various treatments. SOD activity of *C. roseus* showed an increase under individual treatment while contrary of above was observed under joint toxicities of Cd+BC and Hg+BC, wherein, a decrease in activity in the presence of chelating agents was observed as compared to lone treatments (Cd, Hg, BC, Cd + BC and Hg + BC without chelates) which may be due to quenching of free radicals produced under metal and herbicide stress by CA, a natural chelating agent which is also an antioxidant. Similar findings on sunflower plants by **Rizwan et al** (**2017**) showed an increasing followed by decreasing trend in antioxidant enzymes activities in the presence of heavy metal contaminated soils. The SEM images revealed a structural deformation between shoots, roots and leaves. However, at higher doses, biochemical and structural damages are evident, which can aid in the biomonitoring of environmental contaminants. Studies by **Ge et al (2012)** supported our findings, stated that Cd^{2+} causes peroxidative damage of root cell membranes and which leads to damages in root structures. The stomatal changes revealed in SEM micrographs under co-contamination may be due to toxicity induced by rapid and preferential absorption of heavy metal and herbicide by subsidiary cells which results in changes in membrane permeability, likely to create decrease in cell turgor pressure. SEM micrograph further revealed the deformation in the epidermal layer as well as thickening of xylem and phloem of treated root and shoot. **Bhattacharjee** (**2005**) reported that heavy metal stress induced ROS generation by the plant and it reacts with poly-unsaturated fatty acids leading to structural and functional in biological changes membranes.

In the present study reduction in root and shoot biomass and length was observed in *C. roseus* with the increasing concentration of co-contaminants monitored on 60 days after sowing. Results revealed that Cd was found to be more toxic to plant growth in comparison to Hg. Cd are known to inhibit plant growth which leads to decrease in water use efficiency and net photosynthesis in the plant species. Plants showed better growth under BC treatment alone in comparison to an co-contamination of Cd/Hg and BC as measured after 60 DAS. **Chigbo and Batty (2014)** also reported similar results, that co-contamination stimulates an antagonistic effect on plant growth and performance. The presence of xenobiotics (BC) may facilitate the greater uptake of metals which leads to increase in concentration of metals in *C. roseus* root and shoot as compared to individual Cd and Hg treatment. The accumulation of heavy metals (Cd/Hg) was found to be more in root as compared to shoots of *C. roseus*. Similar results were observed for co-contamination of Cd/Hg along with BC. Effect of an admixture of Cd and Hg along with BC showed that the BCF values for shoot and root were higher than alone treatments of Cd/Hg. Previous researches on phytoremediation revealed that roots are the first organs which come in contact with the soil containing heavy metal, so this might be the reason that a higher amount of the metal is uptaken by roots as compared to shoots (**Simonova et al**, **2007**; **Bauddh & Singh**, **2011**). In BC contaminated soils, the dissipation of herbicide was greater in planted soil in comparison to non-planted soil for the different treatments of BC alone or in combination with heavy metal. BC dissipation revealed that higher degradation of BC was observed at higher concentration of heavy metal Cd (150, 200) and Hg, (80, 100, 120). **Wang et al (2013)** reported that phytoremediation was found to be cost-effective method for degrading butachlor from the polluted sites.

Effect of chelating agents on the growth of *C. roseus* was studied in the presence of different treatments Cd/Hg and BC alone and under joint application in the soil. The combined application of EDTA and CA reduced the biomass (roots and shoots) of plant *C. roseus* in all the treatments compared with treatments with no chelate. Similar to the present study, the effect of chelating agents on reduction in shoot biomass of *L. perenne* for soil co-contaminated with rhamnolipid + sulfate and rhamnolipid and EDDS with a different metal was reported (**Gunawardan et al, 2010**). It was overall deduced that the citric acid would be more effective in enhancing the root and shoot accumulation of Hg alone as well as under co-

contamination (Cd/Hg in combination with BC) with respect to EDTA and EDTA+CA. While individual Cd treatment revealed high accumulation in root and shoot in the presence of EDTA, irrespective of other chelating agents alone or in combination. However, under co-contamination Cd/Hg and/or BC in the presence of chelating agents did not show significant effect on translocation factor for Cd and Hg. The overall results for BCF (roots and shoots) values revealed that metal accumulation by *C. roseus* was increased in the presence of chelating agent EDTA and CA alone as well as in joint application, respectively, to metal treatments without any chelates. Similar findings were observed by **Agnello et al** (**2016**), wherein the BCF values denoting accumulation of metals in plant roots, were found to be higher for roots irrespective of shoots for all the treatments. Presence of chelating agents significantly increased the dissipation rate of BC both in individual (BC) and co-contaminated (Cd/Hg and/or BC) soil. While joint application of EDTA+CA was found to be more effective in BC dissipation in comparison to individual EDTA or CA applications. The rates of BC dissipation were enhanced by 51.89% for lone BC treatment at the end of the 60 days experiment in the presence of chelating agents. **Bach et al** (**2005**) also reported similar results for enhancement of PAH dissipation in the presence of joint application of chelating agents.

Moreover, the conclusion drawn from the present study infers in a nutshell that, that *C. roseus* could be considered as a potential candidate for phytoremediation of co-contaminated soil.

Chapter 11

General Conclusion

The overall conclusion of the present study has been summarized in this chapter. The physico-chemical as well as heavy metal and herbicide analysis of lands near industrial areas in and around Lucknow city, practicing extensive agricultural activities was carried out to know the status of the soil. Results revealed the prevalence of heavy metals Cd and Hg among rest of the heavy metals studied. Since, nowadays most of the soils are contaminated with more than one single pollutant; hence the soil samples were also studied for residual presence of pesticides, herbicides or insecticides. Herbicides analysis showed the presence of Butachlor (BC) exceeding the relevant minimum residual recommended above other herbicides. Hence, depending upon the findings, BC was chosen along with Hg and Cd for co-contamination studies. In order to address the problem of co-contamination phytoremediation potential of few ornamentals plants were tested and finally *C. roseus* was selected for further study.

The co-contaminants Cd/Hg and/or BC revealed differential effects on germination parameters, like seed germination and root and shoot elongation of *C. roseus* which ensured the ability of the plant to germinate and their prospective phytoremediation capability. SEM results revealed more structural deformation in shoot as compared to roots under both individual (Cd/Hg or BC) and/or joint toxicities (Cd/Hg and BC). The inhibitory concentration EC 50 values inferred that the joint effect of Cd and BC was more toxic followed by individual treatments of BC and Cd as far as root and shoot growth is concerned. In case of Hg/Cd treatment, based on EC 50 values it was deduced that joint toxicity of Hg/Cd and BC was more toxic followed by alone treatments of BC and Cd/Hg.

C. roseus demonstrated high Cd/Hg tolerance in the presence of herbicide BC under co contamination, by stimulating a number of stress antidefense mechanism. *C. roseus* tolerated heavy metals (Cd/Hg) above phytotoxic levels and accumulated high metal concentration in their tissues which revealed the plants stress mitigation strategies which helped with ROS dissipation. EDX showed specific Ca^{+2} signals in root xylem, which could be part of signaling pathway leading to increased root metal uptake and stomatal closure. Stress stimulation in the plants under various treatments was revealed in terms of structural deformation as shown in SEM micrograph images. The present study was an effort to understand the potential of *C. roseus*, as an accumulator based on the biochemical and physiological responses of this plant to

elevated levels of heavy metals (Cd/Hg) co-contaminated with herbicide (BC) in the presence of chelating agents which are not well studied as yet.

The application of chelating agents on biochemical parameters of the studied plant *C. roseus* was investigated in the contaminated soil. Chelating agents results into increase in heavy metal uptake by *C. roseus* and noticeably alleviated the antioxidative defense system against oxidative stress under co-contamination. Co-contaminant also induced oxidative stress in *C. roseus* by reducing photosynthetic pigments, increasing ROS and MDA content. Individual and joint treatments of chelating agents in presence of Hg/Cd and/or BC were found to cause ultra-structural alteration in root, shoot and leave tissues. The overall findings of this study was that CA/EDTA would be beneficial in accelerating the environmentally safe phytoextraction of heavy metals and herbicide in co-contaminated soil and leads to antioxidant response in the studied plant *C. roseus*.

The phytoextraction potential of *C. roseus* was evaluated for the removal of heavy metal (Cd/Hg) from contaminated and co-contaminated soils as well. Results revealed that the accumulation of Cd in *C. roseus* plant shoot was more favorable than Hg. The BCF values for roots was >1 for individual Cd treatment as well as under joint toxicity of BC, while contrary of the aforesaid findings was observed under Hg treatments, wherein the BCF values <1was observed for all treatments. The TF values of *C. roseus* were found to be more for Cd with respect to Hg. The overall study inferred that *C. roseus* could have more phytoextraction potential for Cd as compared to Hg.

This study revealed that EDTA and CA facilitate increase in metal uptake within the plant *C. roseus* under individual Cd/Hg and/or BC co-contaminated soils. Results showed that natural chelate CA was having more potential for enhancing the translocation of metals from plant root to shoots as compared to other chelate treatments. The dissipation of BC in individual as well as co-contaminated soil showed that individual application of CA could be more efficient in herbicide dissipation. The increase in TF values as well as the metal extraction ratio following the application of CA provides a basis for further detailed study in the ability of *C. roseus* to simultaneously accumulate Cd/Hg and remove BC in the presence of EDTA or EDTA + citric acid.

Although, the intricacies of hyperaccumulation are yet in need to be comprehended, the studies at cell and tissue level gives an understanding about the uptake, absorbance and survival of plants in the presence of extremely hazardous and omnipresent metals (Cd and Hg) which is likely to be helpful for proposing effective methods to check the problem of food-chain contamination by such pollutants. Likewise, the presence of chelating agents has been found to facilitate more uptakes of heavy metals as well as herbicide dissipation by *C. roseus* plant. The final conclusion drawn from the study states that that plant species *C. roseus* could be applied to co-contaminated soils, and could act as a potential candidate for phytoremediation of such soils.

Chapter 12

Summary

The overall aim of this thesis entitled, "Tapping phytoremediation potential for soils co-contaminated with heavy metal and pesticide" was to determine whether application of phytoremediation could be carried out for co-contaminated soils. Cd/Hg and BC were used as mixed contaminants. The overall aim was initiated by selecting suitable heavy metals and herbicide through survey of agro-industrial lands and laboratory analysis of soil samples, followed by studying the effects of co-contaminants on germination of seeds, biochemical parameters of plants, the role of soil amendments (chelating agents) on biochemical parameters, the phytoextraction potential of *C. roseus* plants under individual (Cd/Hg/BC) and joint contamination (Cd/Hg and BC) and the effect of chelate assisted phytoremediation of heavy metal and herbicide co-contaminated soils in six distinct but complementary chapters, as mentioned below:

- Chapter 4 Soil analysis of agricultural lands in and around Lucknow to ascertain the status of heavy metals & herbicides in contaminated soils.
- Chapter 5 Seed germination rate and early seedling growth of *C. roseus* in presence of single (heavy metal or herbicide) and mixed contaminants (heavy metal and herbicide).
- Chapter 6 Screening of phytoremediation potential of *C. roseus* under Cd/Hg and Butachlor co-contamination using biochemical parameters and SEM-EDX analysis.
- Chapter 7 Effect of chelate addition on biochemical parameters of *C. roseus* under single and joint toxicities of heavy metals along with Butachlor and SEM-EDX analysis.
- Chapter 8 Phytoremediation of Cd/Hg and Butachlor contaminated soils by *C. roseus* under individual treatment and joint contamination.
- Chapter 9 Chelate-assisted phytoremediation of Cd/Hg and Butachlor co-contaminated soils using *C. roseus*

This study was carried out on agricultural industrial soil samples from rural areas situated in and around Lucknow city. The physico-chemical and microbial properties of soil co-contaminated with inorganic and organic compounds at different sites was carried out. The results of correlation matrix among different physico chemical parameters, revealed positive correlation with organic matter while negative

correlation with clay particles and no correlation with rest of the parameters. Whereas, in terms of soil bacterial count the highest was revealed in control soil while the lowest was observed in Diwanganj soil. Hence it can be said that high concentration of heavy metal and herbicide in soil sample significantly affected the diversity of microbes. An analysis of the heavy metal (Pb, Hg, Cu, Cr, Ni, As, Cd, Mn, Fe) content in the soil samples revealed Hg and Cd to be more prevalent and above the tolerable limits in soil sample collected from Kalli West followed by Bijnor and Bhawaniganj and finally from Mau (For Hg), while in case of Cd, Semarou was found to have highest concentration followed by Mohanlalganj and Kalli West. Depending upon the findings, the two heavy metals Hg and Cd were selected for the proposed research work. In addition, the result of cluster analysis showed that any soil having high concentration of Cr might have low amount of Hg. Based upon the laboratory analysis of the soil sample through HPLC, a peak of BC was detected in most of the soil samples. Hence, on the basis of survey and laboratory analysis Butachlor herbicide was chosen along with Hg and Cd for co-contamination study.

Different plant species were checked for their phytoremediation potential, out of which *Catharanthus roseus* revealed high phytoremediation potential for co-contamination study based upon preliminary studies on seed germination and growth. The results for seed germination in the presence of heavy metal (Cd and Hg) and herbicide (BC) alone and in combination affected both, the percentage of germination and early seedling growth of *C. roseus*, differently. In specific, the joint effect of Cd and BC was found to be more toxic as compared with the individual toxicities. Based on EC 50 values for the effect of Hg and BC, the results indicated that the joint effect of Hg and BC was more toxic followed by individual treatments of BC and Hg. Although, morphologically root was more sensitive to shoot both under single and/or joint toxicities, however, the SEM micrographs revealed more structural deformation in shoot as compared to roots.

The effect of Cd, Hg and BC on *C. roseus* on different biochemical parameters was observed differently. For example the effect of metals and herbicide on photosynthetic pigment of plant revealed that Cd in presence of BC induced a decrease in *Chl a* and *Chl b*, carotenoid and total chlorophyll content with increase in concentration. While that of Hg (20-120 $mgkg^{-1}$) and BC (4 $mgkg^{-1}$) revealed an increase in chlorophyll content upto 40Hg+4BC (12.5% to 4.63%) and then there was

a sharp decline observed upto highest concentration 120 Hg+4BC (60.24%). Similarly, co-contamination was found to pose more stress to the plants which may be the cause of lower phenolic content in joint treatments as compared to the alone treatment. Data obtained in this study established the fact that the activities of all antioxidant enzymes inclined to escalate with the Cd/Hg in soil. The SEM images clearly defined that the distinct feature of xylem and phloem was gradually distorted with increasing concentration from 25-200 $mgkg^{-1}$Cd; 20-120 $mgkg^{-1}$ Hg and 0.5-8.0 $mgkg^{-1}$ BC. But more deformation was observed under joint toxicity of heavy metals and BC as compared to alone treatments.

In addition, the effect of heavy metals with and without BC was also observed after adding chelating agent (CA and EDTA) to the soil, to induce heavy metal and herbicide uptake. The results revealed that addition of chelating agents increased the photosynthetic pigment value, protein content and also enhanced antioxidant enzymes, which decreased the production of MDA under co contamination. Results for proline content showed a decline in the presence of chelating agents, which might be due to the reason that a chelator forms complex compounds which tends to reduce the toxicity in co-contaminated soil. For mitigation of ROS-induced in the present study *C. roseus* plant stimulates protective mechanism in the form of enhancement of SOD, APX, CAT, GR and POD activity for all the treatments. The SEM micrograph of treated root, shoot and leave showed a structural deformation in the epidermal layer, with respect to control.

The effect of heavy metal and/or herbicide on plant growth and their anatomical structure was studied. Individual and joint toxicity not only affects heavy metal accumulation but BC dissipation as well. Our study on the plant *C. roseus* revealed that Cd was more effective for decreasing plant biomass of the growing plants as compared to Hg. Results revealed that there was a significant decline in root (44.89%) and shoot (55.52%) length as the toxicity of herbicide (BC) increases. *C. roseus* showed better growth under BC treatment alone in comparison to co-contamination of Cd, Hg and/or BC. In presence of herbicide a higher uptake of metals was observed which results in an increase in concentration of metals in *C. roseus* root and shoot as compared to individual Cd and Hg treatment. The BCF values for shoot and root of *C. roseus* showed higher values under co-contaminated treatments (Cd/Hg and BC) than alone treatments of Cd/Hg. The results of present

study for BC dissipation revealed higher degradation of BC at higher concentration of heavy metal both Cd and Hg.

Chelate-assisted phytoremediation of Cd/Hg and BC co-contaminated soils using plant *C. roseus* was carried out. The biomass (roots and shoots) of the plant *C. roseus* was found to decline in the presence of chelating agents EDTA and CA, in comparison to control. The overall results for BCF values for roots and shoots further revealed that the total metal uptake by *C. roseus* was significantly ($p<0.001$) increased due to application of chelating agents EDTA and CA alone as well as in combined application with respect to metal treatments without chelates. Results also revealed that chelating agents enhanced the dissipation of PAH compound such as BC along with heavy metal in co-contaminated soils. Chelating agents have the ability to desorb the PAH compounds and make them bioavailable for degradation by microbes.

This investigation on the phytoremediation potential of *C. roseus* on co-contaminated soils with different concentrations of heavy metal and herbicide along with the presence of organic and inorganic chelates in this study represented an exhaustive assessment of this area of research. Moreover, *C. roseus* emerged as a good and a potential candidate for phytoremediation more significantly when it comes under co-contamination.

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Appendix

The authoress was born on April 27, 1991 at Ramnagar, District Nainital in Uttarakhand. She passed her High School with 71.4% in 2006 and Intermediate with 74.6% in 2008 (CBSE Board) from Sri D. D. Chhimwal Memorial Public School Dhikuli, Ramnagar, Uttarakhand. She completed her Graduation in Environmental Science with 73.24% in 2011 from Kurukshetra University, Haryana. She completed her Post Graduation in Environmental Science with 8.001 CGPA in 2013 from G. B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand. She has cleared UGC-NET Environmental Science in the year Dec-2013. She was awarded RGNF-National Fellowship for the year 2014, by University Grant Commission.

She has joined Ph.D degree programme in Department of Environmental Science at Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow, in August, 2014.

List of Publications

Research Papers:

- **Ratnakar, A.** and **Shikha.** (2018). Assessment of co-contamination in soil samples from agricultural areas in and around Lucknow city (U.P.), India. *Current Science*. 115(12):2267-74.
- **Ratnakar, A.** and **Shikha.** (2019). Effect of co-contamination of cadmium and butachlor on seed germination and early seedling growth of *C. roseus* under simulated soil contamination. *Plant Archives* 19(2):769-779.
- **Ratnakar, A., Shankar, S.** and **Shikha.** (2016**).** An overview of biodegradation of organic pollutants. *International Journal of Scientific and Innovative Research*. 4(1):73-91.
- **Shankar, S., Shikha., Ratnakar, A.** and **S. Singh.** (2018). Removal of synthetic dyes from textile wastewater using microbes as bioadsorbents: A Review. *Indian Journal of Environmental Protection*. 38(2):116-133.
- **Agnihotri, V., Ratnakar, A., Kanwal, M. S., Vishwakarma, S.C.R.** and **Joshi, R.** (2014). Soil Nutrient Status of Crop Fields in Two Villages of Kosi Watershed, Uttarakhand. *ENVIS Bulletin Himalayan Ecology*. 22:43-45.

Book Chapters:

1. **Ratnakar, A.** and **Shikha.** (2019). Role of Microbial Genomics in Plant Health Protection and Soil Health Maintenance. (Tripathi V. et al, Eds) Springer Singapore. Microbial Genomics in Sustainable Agroecosystems, Volume II (pp. 163-179).Springer, Singapore. ISBN 978-981-329-860-6.
2. **Ratnakar, A., Sankhwar, A. K., Sangeetha, V.** and **Melkania, U.** (2018). Possibilities of Wastelands Phyto-remediation using Native Species of India. (Singh, V. et al, Eds). Dimensions of Agriculture: Farming for Environment, Health, Clothing, Beauty and Happiness (pp. 111-120). Avon Publications. ISBN 978-93-8184-504-2.
3. **Shankar, S., Singh, S., Rawat, S., Ratnakar, A.** and **Shikha.** (2019). Environmental contamination, toxicity profile and bioremediation approaches for detoxification of pulp and paper mill wastewater. (Bhargava R.N and Saxena G Eds) Springer Verlag. Bioremediation of Industrial Waste for Environmental Safety, Volume I: Industrial waste and its management. ISBN 978-981-13-3426-9.

Papers/Posters Presented in Conferences:

1. **Arpna Ratnakar** and **Shikha** (2018). An oral presentation entitled "Assessment of some co-contaminated soil samples in and around Lucknow (U.P.), City" in the *International Conference on Science and Technology for Sustainable Future (1st North Indian Science Congress)* organized by the Babasaheb Bhimrao Ambedkar University, Lucknow Jan 10-11, 2018.
2. **Arpna Ratnakar** and **Shikha** (2015). Presented a poster entitled "Degradation of organic pollutants by microorganisms" in the *3^{srd} Lucknow Science Congress and National Conference on Science for Society: An Interdisciplinary Approach* organized by the Babasaheb Bhimrao Ambedkar University, Lucknow Oct 31- Nov 2, 2015.
3. **Arpna Ratnakar** and **Shikha** (2017). Presented a poster entitled "Need of IPR: Plant Patent" and won *II prize* in the *National Symposium on IPRs in Agricultural Research* organized by the Babasaheb Bhimrao Ambedkar University, Lucknow and U. P. Council of Agricultural Research, Lucknow, Aug 30-31, 2017.
4. **Arpna Ratnakar** and **Shikha** (2017). Presented a poster entitled ""Phyllo-remediation of Volatile Organic Carbons (VOCs): Tapping the potential of Plant leaves and Associated Microbes" and in the *International Symposium on Microbes for Sustainable Development:Scope & Applications (MSDSA-2017), 58th Association of Microbiologists of India* by the Babasaheb Bhimrao Ambedkar University, Lucknow Nov 16-19, 2017.
5. **Arpna Ratnakar, Sarita Joshi** and **A. S. Nain** (2013). Presented a paper (ORAL) in the *Workshop on Geospatial Technology for Natural Resource Management* by the Department of Agrometerology, College of Agriculture G. B. Pant Univ. of Agri. & Tech. May 21-22, 2013.

Seminars, Conferences, Workshops, Symposia/Training program etc. (Participated/ Attended):

1. Participated in one week workshop on Statistical Analysis for Engineers and Researchers (SAFER - 2017) at Indian Institute of Technology Kanpur.
2. Participated in Two Days Workshop (Winter Training School) on *Instrumentation of trace the signature of materials* at USIC, Babasaheb Bhimrao Ambedkar University, Lucknow on Jan 30-31, 2019.
3. Participated in 103rd Indian Science Congress held at University of Mysore, organized by the The Indian Science Congress Association 14,Dr. Biresh Guha Street, Kolkata, on Jan 3-7, 2016.
4. Participated in seminar on "*Gomti Yatra and National Seminar on Rejuvenation of River Gomti: Past, Present and Future"* (GY&NSRRG-2015) organized by the Department of Environmental Science, Babasaheb Bhimrao Ambedkar University, Lucknow on May 9-11, 2015
5. Participated in International Workshop on "*Bridging Development Divide for Inclusive Growth through Science, Technology and Innovation*" organized by the DST- Centre for Policy Research, Babasaheb Bhimrao Ambedkar University, Lucknow on Jan 16-17, 2015
6. Participated in 102nd Indian Science Congress held at University of Mumbai, organized by the The Indian Science Congress Association 14, . Biresh Guha Street, Kolkata, on Jan 3-7, 2015.
7. Participated in International Symposium on *Agricultural Communication and Sustainable Rural Development From Information to Knowledge Wisdom- Envisioning a Food Sovereign World in the Third Millenium* organized by the Directorate of Communication, G. B. Pant University of Agri & Tech., Pantnagar, Uttarakhand, on Nov 22-24, 2012

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Assessment of co-contamination in soil samples from agricultural areas in and around Lucknow city, Uttar Pradesh, India

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An environmental evaluation of agricultural lands situated close to industrial areas in and around Lucknow city was carried out to determine the effect of co-contamination in the study area. Analysis of soil samples revealed the presence of mercury and cadmium at higher levels than their normal distribution in soil. Apart from heavy metals, the herbicide Butachlor was also detected in most of the soil samples studied. Co-contaminated soils pose a major threat to agricultural ecosystems since the presence of different concentrations of heavy metals may inhibit biodegradation of organic pollutants, which further affects metal bioavailabilty and phytoremediation.

Keywords: Agricultural lands, co-contamination, heavy metals, industrial area, soil samples.

THE agricultural lands close to urban areas are subjected to various processes like traffic emissions, energy and fuel production, power transmission, mining, metal refining, intensive agriculture and dumping of sludge that contribute to contamination of soil[1]. Wastewater irrigation practices lead to the accumulation of heavy metals like nickel (Ni), copper (Cu), zinc (Zn), cadmium (Cd), iron (Fe) and lead (Pb) in the soil and chronic exposure to heavy metals can lead to their accumulation in plant parts affecting plant growth[2], ground cover apart from having a negative impact on soil micro flora. Pesticides and agrochemicals are important inputs for improved yield and better quality crop production. However, an unplanned, erroneous and indiscriminate use of these organic compounds leads to the destruction of bio-control agents, pesticide residues in agro-ecosystems and environmental pollution, besides accumulating in the food chain[3].

Agricultural lands may be co-contaminated with both organic and inorganic substances, but limited work has been carried out for clean-up and monitoring of such co-contaminated lands. While studying such sites, it becomes important to consider the interactions of both organic and inorganic substances with respect to soil physico-chemical and biological properties that may affect both the form as well as availability of pollutants. Different metal concentrations have been shown to inhibit the microbial biodegradation of organics[4] and their further interaction with organic pollutants affects metal bioavailability[5].

In the present study, 15 representative agricultural soil samples from areas in and around Lucknow city, Uttar Pradesh (UP), India, were collected to assess the co-contamination of heavy metals and herbicides. Overall, the aim of the study was to assess the physico-chemical and microbial properties of soils which are co-contaminated with inorganic (heavy metals) and organic compounds (herbicides), and analysis of correlation matrix.

Materials and methods

Study area

Lucknow, a large city in northern India, is the capital of UP. It is situated between 23°52′–31°28′N and 77°3′–84°39′E (ref. 6). The study area includes agricultural lands in and around Lucknow city (Figure 1). Industries such as brick kiln, leather tanning and electroplating are situated close to the study area.

Sampling and analysis

For the present study, a total of 45 topsoil (0–15 cm) samples were collected from 15 different agricultural lands through Z-pattern of sampling. Next, 300 g of each soil sample was taken in a 50 m diameter area and a composite sample was formed. The samples were collected in polyethylene bags, properly labelled and stored in the laboratory at 4°C prior to analysis[7]. To obtain reference data, soil samples were collected from remotely situated agricultural lands, where no herbicides were used previously (control soil). These soil samples were processed similar to test samples from the fields.

The soil samples were air-dried, pulverized and sieved (2 mm sieve) prior to analysis. Analytical-grade reagents were used throughout the study.

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EFFECT OF CO-CONTAMINATION OF CADMIUM AND BUTACHLOR ON SEED GERMINATION AND EARLY SEEDLING GROWTH OF *C. ROSEUS* UNDER SIMULATED SOIL CONTAMINATION

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Abstract

Cadmium (Cd) and Butachlor (BC) alone or in combination revealed diverse effects on seed germination and early seedling growth of *C. roseus*. Root elongation was more sensitive compared to shoot both under single and/or joint toxicities. The effect of Cd alone was found to be more toxic followed by combined treatment of Cd and BC and BC alone as far as root growth is concerned. On the contrary, a 14.1% decrease in shoot length was observed at the lowest concentration under joint treatment, however, the individual effect of Cd toxicity was more pronounced followed by the combination treatment and application of BC alone. The study reveals the ability of the plant to germinate and grow in the admixture of Cd and BC and its prospective phytoremediation potential.

Keywords : Co-contaminants, Butachlor, Cadmium, Phytoremediation, Seedlings

Introduction

Cd, Arsenic (As) and (Pb) lead are the d-orbital elements of the contemporary periodic table, which have attained due importance owing to their patho-physiological impact attributed to their bioaccumulation in living organisms (Sharma *et al*., 2014). At the same time, large scale application of pesticides further leads to environmental pollution causing health hazards (Igbedioh, 1991). The living systems are more often exposed to admixtures of heavy metals and pesticides as compared to isolated exposure of either of them (Chen *et al*., 2004). A phytoremediation approach accounting individual exposure may not encounter the consequence witnessed under joint toxicity which is universally reflected under real life conditions. Therefore, a study encompassing the combined interactions between pesticides and pesticide and plant systems has parallel importance for effective mitigation of toxicity (Chen *et al*., 2004). An earlier study carried out by us (Ratnakar and Shikha, 2018) reflected extensive distribution of pesticides in agricultural land in and around Lucknow city, (U.P.) India, having co contamination of Cd, Cd and BC. The present study, examines the phytoremediation potential of co contaminated soil as above wherein the effect of Cd alone and jointly with BC on seed germination and early seedling growth of *C. roseus* was carried out under simulated soil contamination.

Catharanthus roseus, commonly known as Periwinkle, belongs to genus, *Catharanthus* and is a native of West Indies. It is perennial herb flowering all the year round in tropical regions. Although the lead uptake ability of this plant is widely documented (Imam, 2017), but no preview is available as yet on the effect of co contamination with Cd along with any xenobiotics or BC on phytoremediation potential of the herb. In this study *C. roseus* has been opted because it is an evergreen ornamental herb, available easily and is well known widely for its endurance under arid and deficient conditions.

Materials and Methods

The seeds of *C. roseus* were obtained from a certified dealer "Neelkanth Agroforestry, Kaiserbagh", in Lucknow city, U.P., India.

Seed Sterilization: The seeds of *C. roseus* were sterilized by rinsing with 10% (v/v) hydrogen peroxide for 20 min to prevent fungal growth for 5 min and then washed with distilled water for two to three times. All glass wares were autoclaved at 121°C for 15 min, prior to use.

Experimental procedure: A total of 15 seeds were placed maintaining appropriate distance in each petriplate lined with double layered filter paper, and initially soaked with 10 ml of the respective solution. Initially a dark condition was provided for germination followed by a photoperiod of 16/08 h, light/dark period. Each treatment was added with 3 ml of the respective solution after every 48 h. Germination percentage was recorded for 08 days at different intervals.

Determination of germination: Seed germination was observed at regular intervals for 08 days. A seed was considered as germinated if the radicle was emerged. The germination percentage was calculated from the total number of seeds and germinated seeds in a Petri plate.

- The germination percentage (G %) was calculated, as given by Tanveer *et al*. (2010):

$$\text{Germination Percentage (G \%)} = \frac{\text{Germinated seeds}}{\text{Total Seeds}} \times 100$$

- Seedling Vigour index (VI) was calculated by the following formula (Vashisth and Nagarajan, 2010).

  VI = Seedling length * Germination Percentage %

- Germination index (GI) was calculated according to the formula given by (USDA, 2001) (Tiquia *et al*.,1996)

$$\text{Germination Index (GI)} = E \times \frac{G}{100}$$

Relative root elongation (E) = (Mean root length with treatment)/Mean root length with control)* 100