Phytoremediation of Arsenate, Zinc and Cadmium with the help of *Ceratophyllum demersum L.*

A

THESIS

Submitted in partial fulfillment of the requirement for thre award of Degree of

DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

By

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CERTIFICATE OF ORIGINAL WORK

This is to certify that the study conducted by **Ms Aparna Gautam, I.D. No. 10PHBT220**, during **March 2011** to **March 2014** reported in the present thesis was under my guidance and supervision. The results reported by her are genuine and the candidate herself has written the script of thesis. Her thesis entitled "**Phytoremediation of Arsenate, Zinc and Cadmium with the help of *Ceratophyllum demersum* L.**" is therefore, being forwarded for acceptance in partial fulfillment of the requirements for the award of the Degree of **DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY** of Sam Higginbottom Institute of Agriculture, Technology & Sciences (Deemed-to-be-University)Allahabad - 211007, India

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SELF-ATTESTATION

I, **Aparna Gautam (10PHBT220)** student of Ph.D Biotechnology, 6th semester in the Jacob School of Biotechnology & Bioengineering, Sam Higginbottom Institute of Agriculture, Technology & Sciences (Deemed-to-be-University), Allahabad have work on the thesis title "Phytoremediation of Arsenate, Zinc and Cadmium with the help of *Ceratophyllum demersum L.*" from Sam Higginbottom Institute of Agriculture, Technology & Sciences (Deemed-to-be-University), Allahabad under guidance of **Dr.(Mrs.)Poonam Singh** during the session January 2011-June 2014submitted for the degree of (**Doctor of Philosophy**) in biotechnology is my original work. Data obtained from other agencies have been duly acknowledged. None of the findings pertaining to this work has been concealed. The results embodied in this thesis have not been submitted to any other university or institute or the award of any other degree or diploma. I hereby affirm that the work has been done in all its respects and reported in this study are genuine and authentic.

Date:

Place: Aparna Gautam

ACKNOWLEDGEMENT

Success depends on motivation, inspiration, dedication, hard work and above all blessing of god. I bow in the reverence of the Almighty for blessing me with strong will power, patience and confidence that helped me in completing the present work.

First of all I would like to thank ***Prof. (Dr.) Ram Take and Prof. (Dr.) B. P. Mashi*** *for allowing me to complete my thesis work from Department of Molecular and Cellular Engineering, SHIATS, Allahabad.*

With deep sense, veneration and obligation from the core of my heart, I beseech the entire array of words of thanks and reverence to mark gratitude towards ***Prof. (Dr.) George Thomas****, Head, Department of Molecular and Cellular Engineering, SHIATS, Allahabad, Uttar Pradesh.*

I express my sincere gratitude and indebtedness to my advisor ***Dr.(Mrs.)Poonam Singh****, Assistant Professor, Department of Molecular and Cellular Engineering, SHIATS under whose guidance I had the privilege to do my thesis work. I am grateful to her for providing me the necessary facilities, meticulous guidance, constructive criticisms, encouragement and valuable suggestions during my thesis work.*

I would also like to give my esteem to ***Er. Sujeet Kr. Singh,*** *Director, CytoGene Research & Development, Lucknow for giving permission to do my part of work in this institute and thereby bestow platform for students to gain familiarity and working opportunity.*

I would like to extend my indebtedness to ***Dr. O.P.Verma, Preetam Verma, Dr. Eapen P. Koshy, Dr. Rohit lal, Dr. Amit A. Charan*** *and* ***Dr. Jishnu Naskar*** *for their kind lend a hand and insinuation endowed with me during the course of this work.*

I would also like to thanks the ***non-teaching staff*** *of the Department of Molecular and Cellular Engineering for their help during the course of my work.*

My efforts will remain incomplete if I do not express my gratitude to ***Ashwani S Krishnay*** *for his encouragement, moral support and suggestions without which my thesis work was a tough task.*

I am extremely thankful to my friends especially ***Shruti*** *for their useful advice, moral support and timely help during the course of work.*

Words often fail to express my humble gratitude and profound regards to my family members especially my sister ***Jayati Gautam*** *for their affectionate encouragement and blessing*

which always being a source of inspiration for me without which it could not have been possible to achieve my targets.

At last I dedicate my work to my most lovable and adorable ***Parents*** *and* ***In-laws*** *who took so much pain to make my career, and never let anything come in between the path of my higher education. Whenever I will be achieving in my life will always be because of them.*

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Place: *Aparna Gautam*

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LIST OF ABBREVIATIONS

C. demersum	*Ceratophyllum demersum*
TCA	Trichloroacetic acid
PVP	Polyvinylpyrrolidone
cDNB	1-chloro 2,4-dinitro benzene
PMSF	phenylmethanesulfonylfluoride
MOPS	4-Morpholinepropanesulfonic acid
MES	4-Morpholineethanesulfonic acid sodium salt
GSH	reduced glutathione
GSSH	oxidized glutathione
EDTA	Ethylene Diamine Tetra Acetic Acid
gm	Gram
g/l	Grams per liter
h	Hour
U/ml	Unit/mililiter
M	Molar
mg	Milligram
µg/ml	Micrograms per liter
min	Minute
ml	Milliliter
µl	Microliter
mM	Millimolar
OD	Optical Density
rpm	Revolutions Per Minute
UV	Ultra Violet
v/v	Volume per volume

w/v	Weight per volume
wt.	Weight
%	Percentage
+ve	Positive
−ve	Negative
<	Less than
>	Greater than

ABSTRACT

In the present study plants of *Certophyllum demersum* was collected and grown for six months in large hydrophobic tubes. The effect of zinc ion concentration was studied on plant; the concentrations used for the study were 10mg/ml, 50mg/ml, 100mg/ml, 150mg/ml, 200mg/ml and 300mg/ml for 7 days in 10% Hoagland media. After day 1 no significant effect was observed on plant for all concentration of zinc. After day 2, 3 and 4 change in colour from green to yellow was observed with different color intensity. It was observed that after day 5, 6 and 7 the leaves of *Certophyllum demersum* become black in colour the intensity of blacking in colour was increased as concentration of zinc ion increased.

Accumulation of Cd by *C. demersum* plants was found to be correlated to both concentration and duration of the treatment. The plant showed maximum accumulation of cadmium after 7 day at 20µM concentration.

The effect of Zinc-Cadmium interaction was studied on *Ceratophyllum demersum* after interval of 2, 4, 6, 8, 10 and 12 day. It was observed from the results that with increasing concentration of Zinc along with the Cadmium, there was reduction in growth of the plant.

The level of thiol compounds increased significantly at all the cadmium exposure concentrations. The maximum level of thiol compound was observed at 10µM after 3 days.The levels of cysteine synthetase compounds increased significantly at all the cadmium exposure concentrations. The maximum level of cysteine synthetase was observed at 10µM after 3 days.

The level of glutathione S-transferase compounds increased significantly at all the cadmium exposure concentrations. The maximum level of Glutathione-S-transferase was observed at 10µM after 4 days.

The level of glutathione reductase compounds increased significantly at all the cadmium exposure concentrations. The maximum level of glutathione reductase was observed at 10µM after 4 days.

Accumulation of arsenic was found to depend on both concentration and duration of exposure and showed a linear increase. The plant showed maximum accumulation of arsenic after 7 day at 20µM concentration.

The level of cysteine compounds increased significantly at all the arsenate exposure concentrations. The maximum level of cysteine concentration was observed at 15µM after 4 days.

The level of reduced and oxidized glutathione compounds increased significantly at all the arsenate exposure concentrations. The maximum reduced glutathione concentration was observed at 10µM and 20µM respectively. The level of enzyme extracted from plant treated with arsenate increased significantly at all the concentrations. The maximum level of arsenate reductase was observed at 15µM on 3rdday.

The enzymes extracted from *Ceratophyllum demersum* treated with cadmium and arsenic were cysteine synthetase, glutathione S-transferase, glutathione reductase and arsenate reductase were identified by SDS-PAGE. The molecular weight determined was found to be 42kD, 75kD, 86kD and 45kD respectively.

Keywords: *C. demersum*, Arsenate, Arsenate reductase, Thiol metabolism, Cysteine synthase, Glutathione S-transferase, Glutathione reductase, Zn-Cd interaction, etc.

1.0 Introduction

Heavy metals, industrial pollutants, in variance with organic materials cannot be degraded and therefore collect in water, soil, floor sediments and alive organisms. Water contamination with heavy metals is a very important problem in the present world. Use of such aquatic food stuff enriched with toxic metals may cause serious health hazards through food-chain enhacment**(Khan *et al.*, 2000).**

It is a well-known fact that aquatic plants accumulate metals that they take from the environment and concentrate on the trophic chains with accumulative result **(Outridgeet al., 1991)** Accumulation of metal in plant is usually larger than in the water and this fact attract investigators in the toxicity of these metals of plant **(Ernst *et al.*, 1992; van Steveninck *et al.*, 1992).**

Previously metal uptake kinetics was inspected, but in laboratory scale only **(Lee and Hardy, 1987; Thornton and Macklon, 1989;Brune *et al.*, 1994).** Accumulation and distribution of metal have important consequences in the capacity and removal of metal removable rate**(Ellis *et al.*, 1994).**

Toxicity of Cd may result fromits binding to sulfydryl groups of proteins leading to inhibition of activity or structural disruption, cellular redox control disturbance **(Schutzendubel *et al.*, 2002),** and/or generate the productionof reactive oxygen species **(Romero-Puertas *et al.*, 2004).** From various detoxification pathways activated in plants under the stress of heavy metal, raise the synthesis of sulfur containing defense compounds namely, cysteine, glutathione (GSH) and phytochelatins(PCs), is considered to be of prime importance for the tolerance and survival of plants **(Rausch and Wachter, 2005; Grill *et al.*, 2006).** An increased synthesis of PCs and cysteine and GSH has been reported in a various plants, algae and fungi **(Grill *et al.*, 2006; Mishra *et al.*, 2006a,b)** upon exposure of metals.

Enzymes involved in GSH synthesis are lacking in Arabidopsis mutants or lacking in PCS activity**((Howden *et al.*, 1995a; Howden *et al.*, 1995b, Cobbett *et al.*, 1998)** are against to Cd Chelation of metalsby PCs and compartmentalization of PC-metal complexes in vacuoles**(Tong *et al.*, 2004; Clemens, 2006; Grill *et al.*, 2006)** are ‘‘first line” of defense mechanisms.

The tolerance toheavy metal toxicity has been found to be correlated to the levelof thiols **(Cobbett, 2000; Perez-Rama *et al.*, 2002),** however this induces requirement for the reduced

sulfur. This expansion in demand is reflected by both increased activities of sulfate assimilation enzymes and an elevated expression of genes encoding these proteins. They are found in plants which are exposed to Cd stress. Cysteine is synthesized by the enzyme cysteine synthase (CS) at the final step of sulfate assimilation pathway and high cysteine biosynthesis rate has been demonstrated to increase the synthesis of downstream peptidesGSH and PCs under stress conditions **(Dominguez-Solis *et al.*, 2001).**

It has been shown that massive PC production is accompanied by a coordinated transcriptional induction of enzymes involved in sulfate uptake and assimilation**(Xiang and Oliver, 1998;Nocito*et al.*, 2002, Harada *et al.*, 2002; Saito, 2004; Wawrzyn´ ski *et al.*, 2006).** The necessity to conserve a balance between GSH biosynthesis and PCs production is suggested by mixed findings. Transgenic plants of *Brassica Juncea* over expressing GSH synthetase or γ-glutamyl cysteine synthetase were found to be more tolerant to Cd stress **(Zhu *et al.*, 1999a, b; Wawrzyn´ ski *et al.*, 2006),** whereas transgenic Arabidopsis lines of over expressing PCS were hypersensitive to Cd since these were probably depleted in GSH pools and thus more susceptible to Cd-induced oxidative stress **(Lee *et al.*, 2003; Li *et al.*, 2004).** In last few years, concurrent over expression of GSH synthetase and PCS in Arabidopsis has been found to increase the tolerance and accumulation of Cd **(Guo *et al.*, 2008),** which moreover supports to the need of maintaining proper balance of thiol metabolism under stress conditions.

GSH also protects against a range of toxicants including heavy metals by conjugating metabolites arising from oxidative damage provoked by them through glutathione-S-transferase (GST) catalyzed reaction **(Moons, 2003; Sobkoviak and Deckert, 2006).**A number of redox reactions utilized it to combat oxidative stress such as in ascorbate-glutathione cycle and by glutathione peroxidases (GPX)**.** In detoxification mechanisms the oxidation of GSH to form oxidized glutathione (GSSG) takes place. Therefore the role of glutathione reductase (GR), which reduces GSSG back to GSH, becomes essential for the maintenance of the redox state of the cells under metal stressed conditions **(Mishra *et al.*, 2006a).**

Arsenic (As) is a ubiquitously distributed and toxic metalloid. It has affected the health of many people in more than 23 countries. Arsenic toxicity can be found both in water and food chain contamination **(Mondal *et al.*, 2006; Tripathi*et al.*, 2007).** Phosphate and arsenate both belong to the same group of periodic table and, therefore arsenate show high affinity towards phosphate transporter**.** In biomolecules phosphate be replaced and can exert its toxicity to plants.

When there is an increase in the rate of PC biosynthesis under As stress, it might induce sulphate uptake and reduction pathways **(Rauschand Wachter, 2005)** to maintain the increased demand for GSH and cysteine.

Cysteine is produced in the final step of the sulphate reduction pathway by the enzyme cysteine synthase(CS). In a given organ the amount of GSH is the result of the combined action of consumption, biosynthesis and degradation.GSH is produced in two ATP-dependent steps which are catalyzed byγ-glutamyl cysteine synthetase (ECS) which is a rate-limiting enzyme,and glutathione synthetase (GS) **(Noctor and Foyer, 1998).** GS consumed in a number of redox reaction to combat with oxidative stress which result into the oxidation of GSH into oxidized glutathione(GSSG), again which is recycled into its reduced form by glutathione reductase (GR) **(Mishra *et al.,* 2006).**GSH also protects the plants against a range of toxicants by conjugating the more their metabolites through glutathione-*S*-transferases (GSTs)**(Moons, 2003).** The enzyme called glutamyl transpeptidases (GTs) is supposed to initiate the degradation of GSH, it is an important step in its metabolism. GTs is restricted outsidethe cell membrane **(Martin and Slovin, 2000)** and in vacuoles **(Nakano and Sekiya, 2005).** This enzyme catalyzes the hydrolysis of uniquely linked N-terminal Glu from GSH, GSSG, GS-conjugates and probably PCs **(Połec-Pawlak *et al.,* 2005).**

Plant of *C. demersum* L. (family Ceratophyllaceae) is selected for the experiment which is a rootless aquatic plant. It is recorded to be a potential accumulator ofAs **(Robinson *et al.,* 2006),** but the overall mechanism of As detoxification is not known **(Zheng *et al.,* 2003).** Present topic deals with the study of Phytoremidiation of *Ceratophyllum demersum* on Arsenate, Zinc and Cadmium exposure in order to trap the Phytoremediation.

Objectives: Objectives of the present study are as followes

- To study the visual symptom of different concentration of Zinc in *Ceratophyllum demersum*.
- To measure the efficiency of *Ceratophyllum demersum* towards Phytoremediation of Zn, Cd andAs.
- To study the effect of heavy metal concentration on stress marker enzymes.
- To determine the molecular weight of extracted enzyme i.e Cysteine synthetase, Glutathione S- transferase, Glutathione reductase and Arsenate reductase from *Ceratophyllum demersum* treated with Zinc, Cadmium and Arsenic**.**

2.0 Review of Literature

Arsenic (As) and cadmium (Cd) are of highly bioactive and toxic elements, their presence at elevated levels in soils and drinking water is threatening food safety and human health **(Kang and Jin, 2004; Geng *et al.*, 2005).** They adversely affect biological activities as a teratogen, carcinogen or mutagen as well as having detrimental effects on the digestive system, respiratory system and immune system**(Zhou and Huang, 2000; Zhou and Song, 2004; Liao*et al.*, 2005).**

Large areas of cultivated land in many countries have been contaminated by As and Cd due to agricultural land industrial practices such as application of pesticides and chemical fertilizers, waste water irrigation, precipitation from heavy coal combustion, and smelter wastes and residues from metalliferous mining **(Boisson*et al.*, 1999; Zhou and Huang, 2000; McGrath *et al.*, 2001; Xie *et al.*, 2006; Verma *et al.*, 2007).**

Recognition of the ecological and human health hazards of some toxic pollutants has led to development of reliable and cost-effective technologies such as bioremediation capable of reducing As and Cd in soils and wastes to environmentally acceptable levels **(Zhou and Song, 2004; Kertulis-Tartar *et al.*, 2006; Yoshida *et al.*, 2006).**

Phytoremediation can be defined as the use of plants including trees and grasses, to remove, destroy, or sequester hazardous contaminants from media, such as soil,water, and air **(Chaney *et al.*, 1997; Salt *et al.*, 1998; Prasad, 2003),** is gaining a lot of importance in recent years since itis a cost effective, promising technology, as well as a desire‘green’, sustainable process **(Wei and Zhouand Song, 2004).** Plants with metal resistance mechanisms based on exclusion can be efficient for phytostabilization technologies **(Wei *et al.*, 2005b).**

Hyperaccumulating plants, in contrast, may become useful for extracting toxic elements from the soil and thus decontaminate and restore fertility in polluted areas **(Barceloet al. (2003).** Hyperaccumulators are plants that have an innate capacity to absorb metal at levels 100 times greater than average plants **(Baker, Yang *et al.*, 2004; Zhou and Song, 2004).**

They are often found inmetal-rich regions where those traits probably give them a competitive advantage **(Ma*etal.*2001; Sun *et al.*, 2005; Gonzaga *et al.*, 2006).**Hyperaccumulators are defined based on the following characteristics: (1) shoots metal concentrations (thresholdvalues) are >10,000 mg/kg dry weight of shoots for Znand Mn, 1000 mg/kg for Co, Cu, Ni, As and Se, and100 mg/kg for Cd **(Baker et al.(1989), Ma *et al.*, 2001;**

Zhou and Song, 2004); (2) bio concentration factor(ratio of metal concentration in plant to soil) is greater than 1.0, sometimes reaching 50–100 **(Brooks, 1998; Cluis,2004);** (3) translocation factor (ratio of metal concentrationin shoots to roots) is greater than 1.0 **(Wei and Zhou,2004a,b).**

So far, more than 400 species of natural metal hyperaccumulators belonging to 45 families have been documented in the world, but hyperaccumulation of Cd and As are a rare phenomenon in the plant kingdom **(Zhou and Song,2004).**wetland plants are being used successfully for the phytoremediation of trace elements in natural and constructed wetlands **(Abdallah MA 2012,).** Compared with crops, weed plants display their characteristics of strong endurance to adverse environmental conditions, and high capacity to absorb water and fertilizers **(Gardea-Torresdey *et al.*, 2004; Wei *et al.*, 2005a; Wei and Zhou, 2006).** In this sense, weed is an important type of ideal natural resources for the remediation of contaminated soils.

Black Night shade herb is a very common species in a field in north China. It is tolerant to adverse environment, fast growing and with high biomass, under feasible environmental conditions, its biomass could increase rapidly**.** So it could fill a gapof known hyperaccumulating plants and it has the potential for phytoremediation of metal contaminated soils **(Zhou and Song, 2004, Wei *et al.*, 2005a).**

The Anzali lagoon is located in the province of Gilan in Northern Iran. It is situated at the southwestern coast of the Caspian Sea, near to the city of Bandar-e-Anzali, and made of a pretty diverse wetland flora and fauna. It's located between 37°28′ N and 49°25′E. It is 26 km long and 2.0 - 3.5 km wide, surrounded by reed-beds extended to its eastern limits by amargin up to 7 km wide. Over 11 rivers are entered the Anzali lagoon in their way towards the CaspianSea, the water system consists of large, eutrophic fresh water basins, shallow impoundments, marshes land seasonally flooded grasslands.

The wetland is bordered to the north by sand dunes and to the south by cultivated land (mainly rice fields) and patches of woodland **(Ramezanpoor, 2004).** The Anzali, like other lagoons, is affected by human activities resulting in contamination of the water. The pollution with petroleum, heavy metals, xenobiotics, organic compounds and other contaminants is an emergent environmental factor that harms both terrestrial and aquatic ecosystems. Heavy metal pollution is mainly due to the result of human activities such asagriculture, mining, construction and industrial processes **(Hoseinizadeh *et al.*, 2011).**

The commercial use of phytoremediation is acceptable way to refine contaminated regions, so therole of macrophytes in the heavy metal cycling, in aquatic ecosystems must first be noticed. Macrophytes are important in the biological monitoring of aquatic ecosystems, as changes in the composition of the aquatic vegetation are considered a great biological indicator of the quality of water **(Schneider and Melzer, 2003).** Most macrophytes which are mainly submerged and floating, have the capacity to tolerate high levels of heavy metal by forming chelates and by subcellular compartmentation. In phytoremediating aquatic plants, phytochelatines and metalothionines are the main cytoplasmic chelators of heavy metals. Macrophytes are involved in bioremediation due to their high tolerance to metals and the effect on ion solubility through the release of O_2 from their roots **(Stoltz, 2004).**

The ability to effectively absorbing metal ions out of solution and to concentrate high levels of these contaminants in plant tissue are dependent on plant species and metal ions, Some factors involved in the determining such differences is the rate of chelation,ionic exchange, chemical precipitation, translocation of metal ions and precipitation made by root exudatesor by microorganisms **(Suñe *et al.,* 2007).**The accumulation of heavy metal ions by the biomass of aquatic macrophytes was studied by **(Bunluesin *et al.,* 2007).** They found that *Hydrilla verticillata* is liable biosorbent for treatment of waste water with heavy metal contaminants. Body observed that aquatic plants are suitable for waste water treatment because they have tremendous capacity of absorbing nutrient and metals from the water so bring the pollution load down. Since plant species can differ inrates of metal uptake, allocation and excretion, metal dynamics in wetlands may be influenced by the composition of plant communities, use of plants in waste water treatment or phytoremediation could not be exceeded without environmental damage **(Pajević *et al.,* 2008).**

Arsenic (As) is a naturally occurring element in the earth's crust. It ranks 20th in natural abundance, 14^{th} in sea water, and 12th in the human body **(Mandal and Suzuki, 2002).** Natural As elevation of drinking water supplies has been reported from more than 70 countries, posing a serious health hazard to an estimated 150 million people world-wide **(Brammer and Ravenscroft, 2009).** As-contaminated groundwater used for irrigation may pose an equally serious health hazard to people eating food from irrigated crops **(Williams *et al.,* 2006),** and As accumulating in irrigated soils poses a serious threat to sustainable agriculture in affected areas **(Heikens, 2006).** Phytoremediation, a plant-based green technology, is a promising technology for environmental pollution caused by unavoidable limitations of traditional technologies

(Rahman *et al.*, 2008a). The use of some submerged aquatic macrophytes and floating plants in the process of phytoremediation is more commonly known as phytofiltration. As accumulation by aquatic plants, such as *Eichhornia crassipes*, *Lemna minor* **(Alvarado *et al.*, 2008),** *Salvinianatans* **(Rahman *et al.*, 2008a),** *Spirodela polyrhiza* **(Rahman *et al.*, 2008b, c),***Hydrilla verticillata***(Srivastava *et al.*, 2007),** and *Wolffia globosa***(Zhang *et al.*, 2009)**. The promising results of phytoremediation technology for As removal from contaminated water has gained the attention of researchers. There are three mechanisms proposed for the uptake of As species in aquatic macrophytes: (i) Active uptake through phosphate uptake transporters, (ii) Passive uptake through aquaglyceroporins, and (iii) Physicochemical adsorption on plant surfaces **(Rahman and Hasegawa, 2011).**

Physicochemical adsorption, an alternative mechanism for As accumulation by aquatic plants, was hypothesized by **Robinson et al. (2006).** In this mechanism, oxides/hydroxides of iron (Fe plaque) suspended on the aquatic plant surfaces adsorb and accumulate As. In most As-affected areas of South and South East Asia, groundwater is rich in Fe **(Brammer and Ravenscroft, 2009).** Water soluble $Fe^{+2}$are oxidized when water is exposed to the air and is then precipitated as Fe hydroxides that adsorb As**(Tripathi et al. 2014).**

If aquatic macrophytes are used for phytoremediation of As, the elevated Fe concentrations in water could be presumed to be a factor affecting the accumulation characteristics. Coontail (*Ceratophyllum demersum L.),* a completely submerged aquatic macrophyte is reportedly a scavenger of As(V) in contaminated water **(Mishra *et al.*, 2008).**This technique utilizes the plants and its associated microbes to decontaminate the low and moderately contaminated sites efficiently. Many plant species are successfully used for remediation of contaminated soil and water systems **(Sharma *et al.*, 2015).**

The existence of heavy metals in the environment, dueto industrial activities from smelters, tanneries, the textile industry, the chemical industry and urban sewage, is of serious concern owing to the toxicity of these metals to human and other forms of life **(Vieira and Volesky, 2000)**. Contamination of the aquatic environment by heavy metals has become a serious concern in the developing world. Considering its effectson human beings and other aquatic organisms, appropriate treatment of heavy metals in wastewater is of utmost importance. Their treatment usually requires removal through some technology. The technologies used for their treatment are reverse osmosis, ion exchange, electrodialysis, adsorption, etc. Most of these technologies are quite costly,energy intensive and metal specific.

None of them could claim to treat all the heavy metals in an economically feasible manner **(Singh *et al.*, 1996)**. The economies of developing countries,such as Egypt, have other investment priorities; therefore,such countries cannot afford the high price involved in the removal of heavy metals from wastewater.

Aquatic macrophytes have great potential to take upmetals from water, producing an internal concentration several times greater than the surroundings. They can accumulate heavy metals up to concentrations 100,000times greater than in the associated water **(Tripathi &Mishra, 2008)**.

In the past few years, among the various plant groups tested for phytoremediation, emphasis has been placed on the use ofaqua-vascular plants such as water hyacinth (*Eichhornia*sp.), duckweeds (*Lemna* sp., *Spirodella* sp.), a small waterfern (*Azolla* sp.) and water lettuce (*Pistia* sp.) for the removal of heavy metals from waste water **(Liu *et al.*, 2007; Denga *et al.*, 2004; Weis & weis, 2004)**. Thus the use of plants for removal of heavy metal pollutants has sofar proved a most promising technology **(Singh *et al.*, 1997; Hiretzy *et al.*, 2004)**.

The aquatic macrophytes are thought to remove metals by hyperaccumulation where by larger than normal amounts of metals are concentrated in the plant parts. The hyperaccumulative capacities of these aquatic macrophytes are beneficial for the removal of heavy metals. Most of the studies regarding the phytoremediation of heavy metals are confined to a few plants and metals. Still, there are a few studies available regarding the removal efficiencies of different plant groups and the removal of several heavy metalsat a time. Heavy metals, industrial pollutants, in contrast with organic materials cannot be degraded and there for accumulate in water, soil, bottom sediments and living organisms. Water contamination with heavy metals is a very important problem in the current world. Occurrenceof toxic metals in pond, ditch and river water affect the lives of local people that depend upon these water sources for their daily requirements**.** Consumption of such aquatic food stuff enriched with toxic metals may cause serious health hazards through food-chain magnification **(Khan *et al.*, 2000, Rai *et al.*, 2002).**

A number of methods are available to remove toxicmetals from water: ion exchange, reverse osmosis, electrolysis, precipitation and adsorption. The latter is byfar the most versatile and widely used. However, the methods present different efficiencies for different metals and they can be very expensive especially if large volumes, low metal concentration and high standards of cleaning are required. The main processes by which heavy metals are removed from aquatic environment are physical, biological and biochemical and they take placein water, biota

and suspended solids. The predominance of one of them will depend on the composition of the system, the pH, the redox condition, and the pollutant nature.

It is a well-known fact that aquatic plants accumulate metals that they take from the environment and concentrateon the trophic chains with accumulative effect**(Outridge and Noller, 1991; Tremp and Kohler, 1995).**The final metal concentration in plants is usually significantly larger than in the water column and this fact hasled investigators to be interested in the toxicity of these metals for the plants, in the plant tolerance **(Ernst *et al.*, 1992; van Steveninck *et al.*, 1992);** in the roll of the aquatic plants in the biochemical cycles **(Jackson *et al.*, 1994;St-Cyr *et al.*, 1994);** in their use as biological filters for polluted waters **(Brix and Schierup, 1989; Dunbabinand Bowmer, 1992; Ellis *et al.*, 1994)** and in their use as biomonitors of environment metal levels **(Whitton,1985; Phillips, 1994; Market, 1995; Whitton and Kelly,1995; Mal *et al.*, 2002).**

Some investigators have studied the metal uptake kinetics, but always in laboratory scale**(Lee and Hardy, 1987; Thornton and Macklon, 1989;Brune*et al.*, 1994).** The extent of metal adsorption and its distribution in plants seems to have important consequences in the capacity and rate of metal remove,in the metal residence time and in the eventual metal releaseto the environment **(Ellis *et al.*, 1994).**

Phytotechnologies are effective and valid alternative for the remediation of contaminated water bodies, not only under experimental conditions but also undernatural conditions. Aquatic macrophytes have been used during the last two decades for water metal removal competing with other secondary treatments, being the principal mechanism for metal uptake adsorption trough roots **(Denny and Wilkins, 1987).** For aquatic macrophytes that possess roots but do not have a close physical association with sediments, the water is undoubtedly the principal source of elements. The uptakeof trace metals through the root systems and subsequent release of metals during decomposition of plant material and transmission of these metals to organisms of higher trophic levels represent a pathway of cyclingof trace metals in aquatic ecosystems.The aquatic floating macrophytes *Pistia stratiotes L.*(water lettuce), *Spirodela intermedia* W. Koch and *Lemna minor L.* (duckweed) present a highgrowth rate and have been used for the removal of Cd, Cr and Pb from water column **(Nasu and Kugimoto,1981; Sridahar, 1986; Bassi *et al.*, 1990; Jain *et al.*, 1990;Taraldzen and Norberg-King, 1990; Wang, 1990; Maine*et al.*, 2001; Cardwell *et al.*, 2002).***S. intermedia* and L.minor present the additional advantage of growing under varied climatic conditions. Also they diminish algal production as they compete for

nutrients and extend all over the water surface, restricting the light penetration and consequently photosynthesis **(Hammouda*et al.,* 1990).** The above studies refer, in general, to theremoval of only one or two metals, few of them referto the simultaneous removal of various metals, and the synergy and antagonism between the metal removing is scarcely evaluated.

Cadmium is important widespread trace pollutant with high toxicity to plants, animals and humans. It is non-essential heavy metal having long biological persistence **(Wagner *et al.,* 1993)**. Cadmiumis released into the environment by power stations, heating systems,metal working industries, nickel-cadmium batteries and phosphate fertilizers **(Toppi and Gabbrielli, 1999; Wagner *et al.,* 1993)**, as well as from geo-chemical weathering of rocks. It has large solubility in water thus poses greater risk to aquatic ecosystem. Its entry into human via food chain is of major concern due to its known neurotoxic, mutagenic and carcinogenic effects **(Stohs and bagchi, 1995; Toppi and Gabbrielli, 1999)**.

Cadmium causes various phytotoxic symptoms including chlorosis, growth inhibition, water imbalance, phosphorus and nitrogen deficiency, reduced manganese transport and accelerated senescence **(Benavides *et al.,* 2005)**. Cadmium causes oxidative stress either by inducing oxygen free radical production or by decreasing enzymatic and non-enzymatic antioxidants due to its high affinity towards sulfur containing peptides and protein **(Benavides *et al.,* 2005;Romero-Puertas *et al.,* 2004 and Sandalio *et al.,* 2001).**

Defense system of the plant to reactive oxygen species constitutes enzymes like SOD, CAT, APX, GPX, GR and other antioxidant compounds such as GSH, carotenoids etc **(Larson, 1998)**. SOD constitutes the first line of defense converting superoxide radicalto hydrogen peroxide (H2O2), which is then reduced towater and oxygen either by APX in ascorbate–glutathione cycleor by GPX and CAT in cytoplasm and other cellular compartments.GR is another important enzymic constituent of this orchestrated antioxidative defense system that helps in maintaining a high GSH/GSSH ratio, crucial for protection against oxidative damage **(Asada *et al.,* 1994)**. GSH, present in plant cell is a major non-enzymatic scavenger of ROS being itself less susceptible to attack by oxidants. Metal entered within the cell may be scavenged by amino acids, organic acids, and tripeptide GSH or by specific metal binding ligands the phytochelatins (PCs). PCs are small metal binding peptides with the structure (γ-Glu-Cys)n-Gly, where value of n varies from 2 to 11, and are induced by various metals particularly by Cd **(Grill *et al.,* 1995).**

PCs are synthesized from GSH by the action of enzyme γ-glutamyl cysteine dipeptidyl transpeptidase, trivially named as phytochelatin synthase (PCS). The enzyme is constitutively expressed but may be regulated at transcriptional and translational levels by metals and metalloids **(Heiss *et al.*, 2003).** After chelation ofCd with PCs, the Cd-PC complexes are transported inside vacuoles by the function of an ATP binding cassette (ABC) type transporter **(Rai *et al.*, 1995).** PCs may transport metal from root to shoot **(Gong *et al.*, 2003)** and thus quantification of metal in different plant partmay correlate PC production with metal accumulation and its transport.

Aquatic plants are well known to accumulate heavy metals **(Rai *et al.*, 1995).** They respond to metal stress by increased production of antioxidants **(Arvind *et al.*, 2003; Edreli *et al.*, 2004)** and also PCs **(Gupta *et al.*, 1995; Gupta *et al.*, 1998; Rai *et al.*, 1995; Tripathi *et al.*, 1996)**. *Bacopa monnieriL.*is an emergent, wetland macrophyte. It grows very fast with its creeping stem in wetlands as a weed. It is well knownfrom ancient times for its medicinal properties having active ingredients like bacosides and bacopasides. The accumulation potential of *B. monnieri* for various heavy metals **(Ali *et al.*, 2001; Sinha *et al.*, 1999)** warrants its evaluation for metal tolerance and detoxification mechanism for its suitability in phytoremediation.

Free radical mediated damage in plants is one of several mechanisms involved in the cytotoxic effect of many xenobiotics. Xenobiotics sometimes termed ‘‘protertogens,’’which are relatively nontoxic, can be enzymatically bioactivatedin the embryo to highly toxic, electrophilic, or freeradical reactive intermediates **(Juchau *et al.*, 1992).** If not detoxified, electrophilic reactive intermediates can bind covalentlyto embryonic cellular macromolecules (proteins,DNA), while free radical reactive intermediates can react directly or indirectly with molecular oxygen to form reactive oxygen species (ROS), such as superoxide anion(O_2^{-2}), hydrogen peroxide (H_2O_2), and hydroxyl radicals(OH^-) **(Wells *et al.*, 2005).**

Free radicals and ROS are produced under normal physiological condition and can be detected in all plant tissues.Under environmental stress, changes in free radical processes are expected to occur and these are in turn to affect the radical scavenging ability of a plant **(Pirker *et al.*, 2002).**Namely, when ROS generation exceeds the capacityof the cellular antioxidants, it will cause oxidative stress and significant oxidative damage to a plant. These cytotoxic ROS can strongly disrupt normal metabolism through oxidative damage of chlorophyll, lipids, protein, and nucleicacids **(Wise and Naylor, 1987; Herbinger *et al.*, 2002).**

Since internal O_2 concentrations are high during photosynthesis, chloroplasts are especially prone to generate ROS **(Asada and Takahashi, 1987).** Electrons leaked from electron transport chains can react with O_2 during normal aerobic metabolism to produce ROS. Once produced, O_2^{-2}will rapidly dismutate, either enzymatically or no enzymatically, to yield H_2O_2 and O_2. In addition, H_2O_2 and O_2^{-2}may interact in the presence of Fe21 to yield the highly reactiveOH^-**(Imlay and Linn, 1988).** ROS production and subsequent oxidative damage may be an important mechanism of toxicity in organisms exposed to xenobiotics **(Livingstone,2001).** To protect them against oxidative stress,plant cells produce both antioxidant enzymes, such assuperoxide dismutase (SOD), catalase (CAT), peroxidase(POD), glutathione-S-transferase (GST), and non-enzymatic antioxidants such as glutathione and ascorbate. Some ofthese constitute good molecular bioindicators for contaminant-mediated oxidative stress **(Shao *et al.*, 2002).** Also,in-vivo free radical formation has been cited as a contributing factor to the deleterious effects of many chemical pollutants**(Pedrajas *et al.*, 1995).**

Submerged macrophytes (*Ceratophyllum demersum*) can cover large areas and dominate primary productivity in aquatic environments. Their ability to accumulate toxic metals from water has been well documented **(Mishra *et al.*, 2006),** but in general less is known about the effects of organic contaminants. The plants growing in polluted waterbodies can absorb toxic xenobiotics which thus enter intothe food chain, posing a serious threat to human health **(Gupta and Chandra, 1998).** Few studies was undertaken onorganic xenobiotics (such as PAH) accumulation capacityof *C. demersum* and its physiological responses to stress particularly concerning oxidative stress protection.

Cadmium (Cd) is a widespread pollutant. Anthropogenic emissions of Cd are estimated at about 30,000 t annually **(diToppi *et al.*, 1999)**, resulting in increased levels of Cd in agricultural soils and consequently in food crops, so that Cd contamination of food poses a danger for human health. Cd has no known biological function, and is highly toxic for human body and plants. Visible symptoms oftoxicity such as chlorosis and growth inhibition are induced when plants are exposed to high levels of Cd. Even before any visible symptoms of toxicity become apparent, lower levels of Cd have been found to induce oxidative stressin cells, commonly accompanied by an increase of reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), superoxide ($O^{\cdot -2}$) and hydroxylradicals ($OH^{\cdot}$) **(Somashekaraiah *et al.*1992; Milone*et al.*2003).**

Since a longtime, ROS are being considered mainly as dangerous molecules, whose levels should be kept as low as possible in order to avoid damage **(Schutzendubel and Polle,**

2002). Control of oxidant levels is achieved by a complex antioxidative system which includes enzymatic and non-enzymatic compounds. Antioxidant enzymes include catalase (CAT, E.C. 1.11.1.6.), guaiacol peroxidase(GPX, E.C.1.11.1.7), ascorbate peroxidase (APX, E.C.1.11.1.11), and superoxidedismutase (SOD, E.C. 1.15.1.1), and the non-enzymatic mechanisms consistof radical-scavenging compounds such as glutathione (GSH), ascorbate,etc. **(Schutzendubel *et al.*, 2001).** There is ample evidence that Cd exposureof result in changes of antioxidant levels and activities of antioxidant enzymes **(Hegedus *et al.*, 2001; Schutzendubel *et al.*, 2001; Olmos *et al.*, 2003).**

A large number of studies have indicated interactions between the essential trace element Zn and toxic Cd. In most of the cases, Zn has an antagonistic effect on Cd uptake by plants **(Choudhary *et al.*, 1994; Grant and Bailey, 1997, 1998; Clarke *et al.*, 1997; Cakmak *et al.*, 2000; Zhu *et al.*, 2003).** It is well known thatZn, as an essential trace element, has many biological functions. For example,Zn plays an important role in stabilizing plasma membrane and protecting membrane integrity, and is a cofactor in many essential antioxidant enzymes **(Vallee and Auld, 1990).**

In coastal belts of Bangladesh, India and China **(Rosen and Liu, 2009)** arsenic in drinking water exceeds the safe limit of10 lg L^{-1} described by WHO**(WHO, 2001)** and even 50 lg L^{-1} specifiedin India and Bangladesh **(Tuli *et al.*, 2010).** Widespread use ofarsenic contaminated groundwater for irrigation in rice field elevatesits concentration in surface soil and eventually into rice plantsand grains which is one of the major staple foods around the world.

Field trials conducted for screening of rice germplasm with respectto grain arsenic accumulation showed major differences among accessions which suggest that arsenic accumulation in rice grain may also be dependent on genetic differences of different cultivars **(Norton *et al.*, 2009; Tuli *et al.*, 2010).** Four rice cultivars that are commonly cultivated in West Bengal in India were selected for this study which showed Specific Arsenic Uptake (SAU) in the order Triguna (134) > IR-36 (71.5) > PNR-519 (53) > IET-4786 (29), however,the grain As order was IR-36 (1.5) > Triguna (1) > PNR-519(0.5) > IET-4786 (0.3) in a simulated pot experiment **(Dwivedi *et al.*, 2010a).**

Although there have been several studies related to arsenic in rice, mechanisms involved in arsenic uptake, efflux from roots,loading into xylem, transport, partitioning, arsenate reduction, arsenic sequestration in vacuoles, volatilization from leaves, accumulationin grains etc. are still poorly understood **.**The protective effect of reduced glutathione(GSH) and cysteine

(Cys) to As(III)/As(V) stressed seedlings showed that both GSH and Cys imparted enhanced tolerance to seedlings against arsenic stress. Germination percentage and seedling growth improved while the level of malondialdehyde(MDA) declined significantly when GSH and Cys were supplemented to As(III)/As(V) treatments suggesting GSH and Cys mediated protection against oxidative stress **(Shri *et al.*, 2009).**

The effect of arsenic (As) exposure on genome-wide expression examined in rice **(Chakrabarty *et al.*, 2009, Tuli *et al.*, 2010)** suggested that a group of defence and stress-responsive transporters, heat-shock proteins,metallothioneins, sulfate-metabolizing proteins, and regulatory genes are differentially expressed in rice seedlings challenged with arsenate (AsV) and arsenite (AsIII). Interestingly, one of the membersof the sulfate transporter gene family (Os03g09970) was observedto be up-regulated more than 10-fold in both As(V) andAs(III) stresses suggesting its role in arsenic accumulation in rice.

The members of this gene family are known to differ in their intracellular locations, expression patterns, and kinetic properties andhelp in transport of sulfate **(Nocito *et al.*, 2006)** leading to enhanced production of S-rich metal-binding peptides (such asGSH, Cys and phytochelatins), thus providing metal tolerance and resulting in metal accumulation **(Mishra *et al.*, 2009).** Apart fromone Nramp1, two glutathione-related transporters, two MATE-effluxfamily proteins, and one zinc/iron-transport family protein were also shown to be up-regulated specifically in AsV stress in rice**(Chakrabarty *et al.*, 2009).**

Arsenite and arsenate (AsV) are the predominant As species in nature, and under anaerobic flooded condition, however, rice is efficient in accumulating AsIII than AsV in comparison to other cereal crops **(Zhao et al. 2010).** Being a strong accumulator of silicon **(Yamaji et al. 2008),** rice isable to transport both silicic acid and arsenous acid via thenodulin 26-like intrinsic aquaporin channels due to physiochemical similarities between them **(Ma et al. 2008).** Arsenite induces toxicity in plants through reacting with sulfhydryl groups of enzymes and tissue proteins leading to inhibition of cellular function **(Tripathi et al., 2007).** It also stimulates the formation of free radicals and reactive oxygen species (ROS) causing oxidative damage to lipids and proteins**(Meharg and Hartley-Whitaker, 2002).**

To combat As stress, plants modulate a number of pathways that operate to keep the cellular concentration of free metalloid ion to a minimum level via thiol-mediated complexation **(Bleeker et al., 2006).** S-rich metal-binding peptides such as glutathione (GSH) and phytochelatins (PCs)are particularly synthesized in response to As stress and providing tolerance

to plant via effective complexation ofAs **(Tripathi et al., 2007; Mishra et al., 2008).** Recently, **Rother et al. (2008)** reported that rapid denovo synthesis of PCs under metalloid stress requires an increase in GSH biosynthesis, which in turn depends on an enhancement of sulphur assimilation into cysteine.

Cysteine is synthesized in the final step of the sulphate reduction pathway by the enzyme cysteine synthase (CS); however, for GSH synthesis γ-glutamyl cysteine synthetase (γ-ECS) is a rate-limiting enzyme **(Xiang and Oliver, 1998).** GSH is consumed in a number of redox reactions to combat oxidative stress resulting into its oxidation to oxidized glutathione (GSSG) **(Mishra et al., 2008).** In addition, GSH protects the plants against a range of toxicants by conjugating them or their metabolites through glutathione-S-transferase (GST) **(Moons, 2003).**

Degradation of GSH, an important step in its metabolism, is supposed to be initiated by the enzyme called γ-glutamylranspeptidase (γ-GT), which is localized outside the cell membrane and in vacuoles **(Nakano and Sekiya, 2005).** It thus appears that exposure of plants to metalloid might lead to sulphur deficiency and could mimic the responses induced by sulphur starvation of plants. Therefore, a detailed investigation of As tolerance with respect to whole thiol metabolism might improve our understanding about the involvement of PCs in As detoxification.

Amino acids , the building blocks of proteins, can be peroxidized by free radicals turning them into second toxic messengers in cells and tissues consequently, even resulting in the oxidation and depletion of vital antioxidants in vivo **(Gebicki and Gebicki, 1993).** Arsenic also affects concentration of macro and micronutrients in plant tissues either through direct competition with their uptake or due to interruption in nutrient metabolism consequently leading toAAs loss **(Tu and Ma, 2005; Dwivedi et al., 2010a).** On the other hand, As may play an important role in plant stress resistance through osmotic adjustment and the accumulation of compatible osmolytes, detoxification of active oxygen species and pH regulation **(Singh., 1999).**

Arsenic toxicity, plant responses and As accumulation profile vary among plant species with the given As species treatment. Some authors reported that AsV interferes with various metabolic processes that cause physiological and morphological disorders leading to inhibited plant growth including rice **(Srivastava et al., 2009; Dwivedi et al., 2010a; Tripathi et al., 2012).**Several As like histidine (His), proline (Pro), cysteine (Cys) and glycine (Gly) are known to be induced significantly in rice grain upon AsV exposure during pot experiment **(Dwivedi et al. 2010a);** however, contrast response of essential and nonessential As was observed in high and low As-accumulating rice cultivars during field conditions **(Dwivedi et al., 2012).** The

contrasting behaviour of thiolic ligands and prooxidant enzymes was observed in tolerant and sensitive rice cultivars during AsV stress **(Tripathi et al., 2012).**

Some studies demonstrated that AsIII is more toxic than AsV, and the transcripttomic responses of various rice cultivars were also differential during AsV and AsIII exposure **(Rai et al., 2011).** Besides, rice is particularly efficient in accumulating AsIII due to anaerobic flooded condition and sharing of silicon transport pathway for AsIII transport **(Zhao et al. 2010).** No comparative study has been performed till date to elucidate the utility of thiol metabolic pathway and stress responsive As to determine its coordinated role for AsIII tolerance in root and shoot part of the rice plant, therefore the present study has been conducted to explore the possible role of amino acids and thiolic ligand in AsIII tolerance.

In mammal cells, arsenic is known to interrupt ATP production in several ways and affects voltage-gated potassium channels by disrupting cellular electrolytic function, resulting in neurological disturbances, cardio vascular episodes, high blood pressure, anemia and death **(Kitchin and Conolly, 2010; Ventura-Lima *et al.,* 2011).** Thus, arsenic is one of the leading causes of mortality in the world. In relation to plants , arsenic studies focus on numerous areas of research, including metabolism, transport, accumulation, toxicity and detoxification strategies such as phytochelatin and hyper-accumulator plant species **(Raab *et al.,* 2005; Rathinasabapathi *et al.,* 2007**). However, the biochemical and molecular mechanisms involved in plant toxicity are less advanced than in animal cells. Under arsenic stress conditions, plants are subject to different types of changes, which include element uptake and transport, metabolism, and gene expression **(Catarecha *et al.,* 2007; Verbruggen *et al.,* 2009; Guo *et al.,* 2012).**

The metabolism of reactive oxygen species (ROS) is well known to be affected under many types of environmental conditions **(Valderrama *et al.,* 2006).** This is corroborated by several reports in the case of arsenic stress. Furthermore, the involvement of enzymatic components in regulating the production of antioxidant molecules such as GSH and NADPH proves that the redox state of the cell is a cornerstone of there gulation mechanism. Nitric oxide (NO), both a gas and a free radical, plays a fundamental role in controlling physiological functions during plant growth and development that include seed germination, primary and lateral root growth, flowering, pollen tube growth regulation, fruit ripening and senescence (**Lamattina*et al.,* 2003; Besson-Bard *et al.,* 2008**). In addition, NO also belongs to a family of related molecules named reactive nitrogen species (RNS) such as S-nitrosoglutathione (GSNO), peroxynitrite(ONOO_), dinitrogen trioxide (N_2O_3) and nitrogen dioxide (NO_2),which also play

an important role in numerous physiological processes, although information on plant cells as compared to that for animal cells remains limited. Nevertheless, under stress conditions, plants can undergo deregulated synthesis and overproduction of NO and NO-derived products, which can have toxic physiological consequences **(Chaki *et al.*, 2009; Corpas *et al.*, 2011)** such as increased nitration. This latter effect could deactivate and damage specific proteins, lipids and nucleic acids **(Corpas *et al.*, 2011),** a phenomenon known as nitrosative stress **(Valderrama *et al.*, 2006)**.

The molecular and physiological basis of crop plant interactions with the environment as attracted considerable interest in recent years. Being sessile organisms, plants are constantly exposed during their life cycle to adverse environmental conditions that negatively affect growth, development, or productivity. The presence of toxic compounds, such as heavy metals (HMs), is one important factor that can cause damage to plants by altering major plant physiological and metabolic processes **(Dalcorse *et al.*, 2008).** In a strict sense, the term HM includes only elements with specific gravity above five but frequently biologist's use this term for a vast range of metals and metalloids which are toxic to plants such as copper (Cu), iron (Fe), manganese (Mn), zinc (Zn), nickel (Ni), cobalt(Co), cadmium (Cd), and arsenic (As) etc. Importantly, few HMs and transition metals such as sodium (Na), potassium(K), calcium (Ca), magnesium (Mg), Fe, Cu, Zn, Co, or Ni are, at certain concentrations, essential micronutrients that are critically involved in the functional activities of large numbers of proteins involved in sustaining growth and development of living organisms. However, at excess concentrations, these metal ions can become detrimental to living organisms, including plants. Although HMs are natural constituents of soils and occur naturally in the environment, nowadays, contamination of soils by toxicmetals and metalloids is of major concern worldwide **(Rascio and Navari-Izzo, 2011).**

The problem of HM pollution is continuously worsening due to a series of human activities, leading to an intensification of research dealing with the Phototoxicity of these contaminants and with the mechanisms used by plants to counter their detrimental effects **(Rascio and Navari-Izzo, 2011).** Transfer of toxic elements to the human food chain is a concrete danger that has to be faced in the near future. Therefore, a complete understanding of the molecular mechanisms and genetic basis of phytoremediationis an important aspect of developing plants as agents for phytoremediating contaminated sites.

Depending on their oxidation states, HMs can be highly reactive, resulting in toxicity of plant cells in many ways. At the cellular and molecular level, HM toxicity results inalterations of

different plant physiological processes, including inactivation and denaturation of enzymes, proteins,blocking of functional groups of metabolically important molecules, displacement/substitution of essential metal ions from biomolecules and functional cellular units, conformational modifications and disruption of membrane integrity **(Villiers *et al.*, 2011),** which is finally attributed to altered plant metabolism, inhibition of photosynthesis, respiration, and alerted activities of several key enzymes **(Hossain *et al.*, 2009).** In addition, HM sare known to disturb redox homeostasis by stimulating the formation of free radicals and reactive oxygen species (ROS)such as singlet oxygen ($1O^2$), superoxide radicals (O_2-),hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\cdot OH$) **(Hosain *et al.*, 2010).**

Recently, methylglyoxal (MG), a cytotoxic compound, was also found to increase in response various stresses including HMs. An increase in MG level in plant cells further intensifies the production of ROS by interfering with different plant physiological and metabolic processes such as inactivation of the antioxidant defense system **(Hoque *et al.*, 2010)** and interfering with vital plant physiological processes such as photosynthesis. This increase in ROS and MG exposes cells to oxidative stress leading to lipid peroxidation, biological macromolecule deterioration, membrane dismantling, ion leakage, and DNA-strand cleavage and finally death of plants **(Hossain *et al.*, 2010; Rascio and Navari-Izzo, 2011).**

Plant tolerance mechanisms require the coordination of complex physiological and biochemical processes, including changes in global gene expression **(Dalcorso *et al.*, 2010).** Plants employ various strategies to cope with the toxic effects of metals or metalloids. Resistance to HM stress can be achieved by "avoidance" when plants are able to restrict metal uptake, or by "tolerance" when plants survive in the presence of high internal metal concentration. Avoidance involves reducing the concentration of metal entering the cell by extracellular precipitation, biosorption to cell walls, reduced uptake, or increased efflux. In a second type of situation, HMs are intracellularly chelated through the synthesis of amino acids, organic acids, GSH, or HM-binding ligands such as metallothioneins(MTs), phytochelatins (PCs), compartmentation within vacuoles, and upregulation of the antioxidant defense and glyoxalase systems to counter the deleterious effects caused by ROS and MG **(Hossain *et al.*, 2009; Hossain *et al.*, 2010).** A large number of recent studies in plants involving sensitive, tolerant, mutant, transgenic, and hyperaccumulator-adopting strategies in the fields of physiology, genomics, proteomics, and metabolomics suggest that GSH by itself and its related metabolizing enzymes, proteins, and

peptides play a pivotal role in HM tolerance by controlling different plant physiological processes, including ROS and MG detoxification, HM uptake, translocation, chelation, and detoxification.

Uptake of excess metal ions is toxic to most plants. The biochemical impact of metal ions on the cells is as diverseas their chemical nature. From the approximately 90 elements present in the earth's crust, about 80% are metals and 60% are heavy metals with specific weights higher than5 g cm^{-3}. In context of metal toxicity, other elements with only partial metal properties such as As and with a specific weight lower than 5 g cm^{-3} such as aluminum also need to be considered due to their toxicity to plants. In the literature, the term 'heavy metal' is occasionally used with a very broad and misleading meaning. Based on metal coordination chemistry, **Nieboer and Richardson (1980)** provided a biologically relevant classification of metals into three categories namely, class A with affinity for O-containing ligands, class B with affinity for N- or S-containing ligands, and borderline, i.e. intermediate between the two with affinity for all three groups of ligands with definite preferences. This categorization reflects a general manner in which different metal ions interact with biological systems. Al^{+3}, AsO^{-2}, AsO^{-3}, Au^{+2}, Cd^{2+},Cu^{+2}, Cu^{+2}, Co^{+2}, $Cr^{+}3$,CrO , Fe^{+2}, Fe^{+3}, Hg^{+2}, Mn^{+2}, Ni^{+2}, Pb^{+2}, SeO_2^{-3} , SeO_2^{-} , Sn^{+2} and Zn^{+2} are examples of ions with known toxicity to plants. At present, most studies focus on a few metal ions such as Al, Cd, Hg, Ni, and Zn. Typically, root inhibition is observed at micromolar concentrations, i.e. at concentrations more than three to four orders of magnitude lower than is usually encountered in salinity stress.

Phytotoxicity of heavy metals in most parts can be attributed to symplastic accumulation of heavy metals, particularly in the plasmatic compartments of the cells, such as the cytosol and chloroplast stroma **(Brune *et al.*, 1995).** Metal-induced changes in development are the result of either a direct and immediate impairment of metabolism **(Woolhouse, 1983; Van Assche and Clijsters, 1990)** or signalling processes that initiate adaptive or toxicity responses that need to be considered as active processes of the organism **(Jonak *et al.*, 2004).** Transport processes have been recognized as a central mechanism of metal detoxification and tolerance **(Hall, 2002; Hall and Williams, 2003).** Some metals, for example, Zn and Cu, are essential for normal plant growth and development as they serve asstructural and functional components of specific proteins.

Other metals, for example, Cd and Pb, have no known function in plants although a Cd requirement for carbonicanhydrase from marine diatoms has been reported **(Lane and Morel,**

2000). The generalized dose–response curves for the two kinds of metals differ with regard to their effects on plant growth. Whereas for non-essential metals these curves comprisea no-effect and a toxicity zone, for essential metals there sponse curves show an additional deficiency zone preceding the no-effect zone of adequate supply. It is implied in both cases that the plants are endowed with an inherent capability of tolerating toxic metals to some extent. Metalions turn toxic as soon as their concentration exceeds a metal-specific threshold which varies strongly among plant species and ecotypes, and also with metal properties. As an exception to the rule, certain low concentrations of nonessential elements have occasionally been associated with some promotion of plant growth, as for example seen for Alyssum species and *Thlaspi goesingense* **(Kupper *et al.,* 2001).**

Populations of certain plant species that chronically experience exposure to elevated metal concentrations, for example, those inhabiting metal-enriched locations, have repeatedly evolved tolerance to the metal(s) in question **(Ernst *et al.,* 1990; Schat *et al.,* 1996).** Such metal-tolerante cotypes and genotypes are the examples of accelerated microevolution when the selection pressure is acute. Examples of tolerant species are *Arabidopsis halleri,* a Zn-hyperaccumulator, *Thlaspi species,* that are Cd-Zn or Ni-hyperaccumulators, *Silene vulgaris* with Zn-, Cu-,and Cd-resistant ecotypes, and Alyssum bertolonii, a Ni-hyper accumulator **(Ernst and Nelissen, 2000; Kupper *et al.,* 2001).**

Correspondingly, the dose–response curves of metal-tolerant variants exhibit a wider no-effect or even limited beneficial zone when compared with their non-tolerant counterparts. Apparently, while evolving tolerance, they acquired some specific molecular means of efficiently detoxifying the surplus toxic metal ions through a combination of complexation and safe deposition. Such acquisition of special tolerance traits is not available to non-tolerant genotypes. Metal hyperaccumulation is defined based on certain metal specific thresholds of metal levels detected in the shoots. Thus, plants with an ability to accumulate Zn, Ni and Cd in excess of 1, 0.1, and 0.01% of dry weight, respectively are considered as hyperaccumulators for these metals **(Chaney *et al.,* 1997; Clemens, 2001).** The metal hyperaccumulators hold a high potential for being used in clean-up of toxic metal-contaminated soils (phytoremediation). It appears possible to engineer efficient plants for remediation purposes by combining the hyperaccumulation trait with the high biomass production ability of crop species. A clearer understanding of metal tolerance/hyperaccumulation mechanisms will greatly facilitate the realization of this goal. Several transporters for micronutrients have been characterized which

exhibit varying degrees of specificity, implyingthat non-essential metals share some of them for entering the cell **(Demidchik *et al.*, 2002; Hall and Williams, 2003).** Furthermore, the vacuolar compartmentation of surplus metal concentrations seem to be a strongcomponent of the cellular metal detoxification strategy **(Dietz *et al.*, 2001).**

Pollution of soil and water by heavy metals represents a major environmental problem to human health **(Sachaure *et al.*, 2006)**. From the approximately 90 elements present in the earth's crust, about 60% are heavy metal with specific weights higher than $5gcm^{-3}$. Different plants have been documented with remarkable ability to take up and accumulate heavy metals from their external environment. However, high concentrations of these metals, including those essential for growth and metabolism, exert toxic effects on the metabolic pathways of plants. Most studies related to adverse effects of heavy metals on plants have been focused only on few metal ions such as Al, As, Cd, Cu, Co, Cr, Hg, Ni, Pb, Se, Zn.

Toxic effects of these metals have been correlated with blocking of functional groups of important molecules, e.g. enzymes, polynucleotides, transport systems for essential nutrients and ions, displacement and/or substitution of essential ions from cellular sites, denaturation and inactivation of enzymes, and disruption of cell and organellar membrane integrity **(Fiehn *et al.*, 2000).** In addition, heavy metals also exert toxic effects through generation of reactive oxygen species (ROS) including superoxide radical ($O2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) which has to be kept under tight control **(Brautigam *et al.*, 2009).** Presence of heavy metals lead to excessive production of ROS causing cell death due to oxidative stress such as membrane lipid peroxidation, protein oxidation, enzyme inhibition and damage to nucleic acid. Though numerous studies have been carried out to study response of heavy metals to plants with respect to antioxidant system, metal accumulation and gene regulation, not much has been done to study modulation of metabolome.

It has been documented that heavy metal toxicity can elicit a variety of adaptive responses in plants. To repair the heavy metal-induced inhibitory effects of ROS, plants employ ROS-detoxifying antioxidant defense machinery which includes non-enzymatic (glutathione, GSH; ascorbic acid, AsA; α-tocopherol and carotenoids) and enzymatic (superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; glutathione reductase, GR; monodehydro ascorbate reductase, MDHAR; dehydroascorbate reductase, DHAR; glutathione peroxidase, GPX; guaicol peroxidase, GOPX and glutathione-S-transferase, GST). Apart from indigenous

production of these molecules, protective effects of reduced glutathione (GSH) and cysteine (Cys) to heavy metal stressed plants have been documented. A ubiquitous mechanism for heavy metal detoxification is the chelation of the metal ion by a ligand. Such ligands include organic acids, amino acids, peptides and polypeptides. Peptide ligands include the phytochelatins (PC), and small gene-encoded cysteine-rich polypeptides (metallothionins) are of signifcant importance for heavy metal stress tolerance **(Kaplan *et al.*, 2004, Sawada *et al.*, 2009).** Sulphur metabolism tightly regulates the biosynthesis of PCs in plants. As a detoxification mechanism, such complexes are compartmentalized in vacuoles.

It has been noticed that upon exposure to heavy metals, plants often synthesize a set of diverse metabolites that accumulate to concentrations in the millimolar range, particularly specific amino acids, such as proline and histidine, peptides such as glutathione and phytochelatins, and the amines spermine, spermidine, putrescine, nicotianamine, and mugineic acids. Thus, it can be speculated that nitrogen metabolism plays central role to the response of plants to heavy metals. The case of proline exemplifies the functional diversification of a compound initially addressed as a compatible solute in the context of osmotic and salinity stress. Proline is also suggested to quench ROS and reactive nitrogen species (RNS) and to relieve the oxidative burden from the glutathione system. This may facilitate phytochelatin synthesis and enhance metal tolerance **(Saito *et al.*, 2008).**

Using NMR-based metabolic fingerprinting and metabolite profiling few studies have been done to study metabolic consequences of heavy metal stress. Metabolic fingerprinting using NMR spectroscopy combined with multivariate statistics analysis was used to discriminate between control and Cd-treated *Silene cucubalus* cell cultures. An increase in the content of malic acid and acetate was observed in cadmium-treated cells. Metabolite profiling of Arabidopsis cells exposed to cesium stress using NMR showed that metabolite changes due to Cs stress, included products mainly from sugar metabolism and glycolytic fluxes, and depended on potassium levels in the cell[145]. Transcriptome and metabolome of Cr stressed rice roots where content of several metabolites including lactate, fructose, uracil and alanine increased following exposure to Cr stress **(D'Auria *et al.*, 2005).** Proline accumulated 3-fold in comparison to control. In plants, proline is synthesized not only from glutamate but also from arginine/ornithine. In limited metabolites profiling, ornithine content also increased suggesting ornithine may be used as substrate for enhanced biosynthesis of proline. Higher accumulation of various fatty acids was also observed in Cr exposed rice roots. Results showed that the content of

linoleic acid (C18:2) was highest in rice root under Cr (VI) stress. Levels of most common phytosterols, stigma sterol and sitosterol, decreased in the roots. Simultaneous analysis of microarray and metabolite content suggested that sucrose degradation pathway was modulated in Cr stress response.

The metalloid arsenic and the heavy metal cadmium are often considered to be biologically nonessential. However, under Zn limitation many diatoms use Cd instead of Zn in carbonic an hydrase **(Morel, 2008).** As (III) can be used as the sole electron donor for anoxygenic photosynthesis inbacteria from hot spring biofilms **(Kulp *et al.*, 2008).** Arsenic may also be beneficial in methionine metabolism and gene silencing in animals **(Huntis *et al.*, 2008).**

Essential or beneficial functions for Cd or As have not been reported in higher plants, except for some Cd-hyperaccumulating populations of *Thlaspic aerulescens*, which require Cd for optimum growth **(Roosens *et al.*, 2003).**However, the possibility cannot be excluded that the growth promoting effects of Cd in these populations may be an indirect effect of interference with the plant-internal availability of 'real' nutritional elements. The essential character of elements is thought to be a consequence of their chemical characteristics and availability **(Liu et al. 2008).** Arsenic and cadmium are found naturally at low concentrations in the earth's crust and may not have been recruited during evolution because of their lower abundance compared to phosphorus and zinc, respectively, which are the neighboring elements in the respective columns of the periodic table. The similarity between these pairs makes Cd and As potentially toxic for the cell because they tend to substitute for Zn and P, respectively, in cellular metabolism. However, unlike P, which is ``always present as phosphate in cells, cellular As can be present as arsenate, As(V), which is a phosphate chemical analog, but also as arsenite, As(III), which behaves as a sulphur-seeking heavy metal ion, rather like Cd2+.Since cellular As(V) is usually rapidly reduced to As(III) in cells, either enzymatically or nonenzymatically, there is a degree of similarity between the toxicologies of, and the sequestration machineries for Cd and As.

Arsenic and cadmium are both potential threats for human health and the environment, through their accumulation in the soil, in the food-chain and locally in drinking water **(Clemens *et al.*, 2006)**. Human activities (metallic industries, contaminated fertilizers, herbicides or insecticides, irrigation with As contaminated ground water, and use of contaminated sewagesludge) are largely responsible for the accumulation of above-background levels of As and Cd in soils. Despite the toxicity of As and Cd, high tolerance levels to these elements have evolved in a number of plant species, mainly through mechanisms of exclusion. Some

plantspecies, belonging to the class of hyperaccumulators, can accumulate exceptional concentrations of As (above 0.1%) and Cd (above 0.01%) in their shoot dry weight without toxicity symptoms. Arsenic hyperaccumulation seems to be confined to the Pteridaceae family of ferns. Cd hyperaccumulation is present only in some populations of *T. caerulescens*, *T. praecox*, and *Arabidopsis halleri*, all belonging to the Brassicaceae family, and *Sedum alfredii* (Crassulaceae).

Interest in As and Cd tolerance and accumulation capacities of plants is driven by potential applications in phytoremediation and food security. The close relatedness of *A. halleri* and *T. caerulescens* to *Arabidopsis thaliana* has allowed the use of high through puttechnologies, in particular *Arabidopsi* DNA chips. Large arrays of genes are constitutively highly expressed in Cd hyperaccumulators compared to a non hyperaccumulating related species. Gene duplication and modification of cis-regulatory elements are demonstrated mechanisms of enhanced expression **(Morel, 2008).** The importance of trans-regulatory elements or modified epigenetic regulation has not or been little studied. The molecular study of these Cd hyperaccumulators has further unraveled the role ofgenes involved in metal homeostasis and detoxification previously identified in *A. thaliana*. The major metal (loid) detoxification mechanisms in plants are transport to the major storage organs or tissues, chelation, subcellular compartmentalization, or efflux from the plant body.

Industrialization in developing countries has contaminated the environments by heavy metals, where advance treatment technologies are neither available nor affordable. Environmental exposure to toxic heavy metals is one of the main critical issues on environmental and public health. Heavy metals are common pollutants in aquatic ecosystems. These ecosystems are particularly susceptible and often final receptor of heavy metals. These ecosystems are sensitiveto pollutants due to the presence of relatively small biomassin a variety of trophic levels, which may lead to accumulation of heavy metals. Hence, hydrophytes are often the first link inrelation to metal contents of aquatic environments.

Environmental exposure to toxic trace elements is one of the main critical issues on environmental and public health. Ni is an essential trace element for plants an ecosystems survival in low concentration. Ni exceeding its critical level might bring about serious lung and kidney problems except from gastrointestinal distress, pulmonary fibrosis and skin dermatitis. And it is known that Ni is human carcinogen **(Ahn *et al.,* 2009)**.

Several health hazards caused by heavy metals and many technologies have been used for their removal from aquatic medium, including membrane filtration like: reverse osmosis and electro dialysis, ion exchange, ion adsorptive membrane, bioreactor system, adsorption , like: carbon nano tubes and activated carbon, chemical precipitation, addition of chemical substance, catalytic reduction **(Alyuz *et al.*, 2009) .**

Phytotechnology is an emerging technology that has a potential to treat a wide range of contaminants for a lower cost than traditional technologies. This technology uses various types of plants to degrade, extract, contain, or immobilize contaminants in soil and water. Phytotechnology has been used for remediation of chlorinated solvents, metals, explosives and propellants, pesticides, polycyclic aromatic hydrocarbons, radionuclides, and petroleum hydrocarbon compounds. Hydrophytes (aquatic macrophyte) are known to heavy metals accumulators and being for phytoremediation **(Andrade *et al.*, 2004).** Aquatic phytoremediation of metals from aqueous environments is very promising area, and several highly efficient examples have shown the applicability of this process to clean industrial waste streams, to preserve drinking water and aquatic biodiversity. *Ceratophyllum demersum L.* (hornweed or coontail) grows fast in shallow, muddy, quiescent water bodies at low light intensities **(Aravind *et al.*, 2005).** It is a submerged, rootless, free floating, perennial and is cosmopolitan in distribution. This submerged macrophyte has a high capacity for vegetative propagation and biomass production even under the modest nutritional conditions. It is useful as an oxygenator for use in the Closed Equilibrated Biological Aquatic System (CEBAS).C. demersum can be biofilter for heavy metals, such as Cd **(Borba *et al.*, 2006)** and Ni **(Chen *et al.*, 2011).**

Contamination of the aquatic environment by heavy metals has become a serious concern in the developing world. Considering its effects on human beings and other aquatic organisms, appropriate treatment of heavy metals in wastewater is of utmost importance.Their treatment usually requires removal through some technology. The technologies used for their treatment are reverse osmosis, ion exchange, electrodialysis, adsorption etc. Most of these technologies are quite costly, energy intensive and metal specific. None of them could claim to treat all the heavy metals in an economically feasible manner **(Alyuz *et al.*, 2009)**. The economies of developing countries, such as Egypt, have other investment priorities; therefore,such countries cannot afford the high price involved in theremoval of heavy metals from wastewater.

Aquatic macrophytes have great potential to take upmetals from water, producing an internal concentration severaltimes greater than the surroundings. These plants can accumulate

heavy metals to concentrations up to 100,000 times greater than in the associated water **(Andrade *et al.*, 2004).** In the past few years, among the various plant groups tested for phytoremediation, emphasis has been placed on the use of aqua-vascular plants such as water hyacinth (*Eichhornia sp*.), duckweeds (*Lemna sp., Spirodella sp*.), a small water fern (*Azolla sp.)* and water lettuce (*Pistia sp*.) for the removal of heavy metals from waste water **(Aravind*et al.*, 2005).** Thus theuse of plants for removal of heavy metal pollutants has sofar proved a most promising technology **(Vieira *et al.*, 2000; Singh *et al.*, 1996; Mishra and Tripathi, 2008; Liu *et al.*, 2007; Denga *et al.*, 2004).**

The aquatic macrophytes are thought to remove metals by hyperaccumulation where by larger than normal amounts of metals are concentrated in the plant parts. The hyperaccumulative capacities of the aquatic macrophytes are beneficial for the removal of heavy metals. Most of the studies regarding the phytoremediation of heavy metals are confined to a few plants and metals. Still, there are a few studies available regarding the removal efficiencies of different plant groups and the removal of several heavy metals at a time.

3.0 Materials and Methods

The materials used and different methods adopted in the present investigation are being presented as follows**:**

3.1 Biological Materials

Plant of Ceratophyllum demersum was collected from the near by ponds of yammuna bank and were grown in hydroponic tubes for the period of six months so that bulk amount of 1 inches tip can used for the experiment .

3.2 Chemicals and Glasswares/Plasticwares

The chemicals were purchased from different manufactures; major chemicals used in this investigation are given in Table 31.2. All plastic ware purchased from Axygen (India) Pvt. Ltd., and Tarsons Products Pvt. Ltd. The glassware was purchased from Borosil Glass Pvt. Ltd., India (Table 3.3). The details of equipment used to carryout this investigation and their manufactures are given in Table 3.4.

3.3: List of chemicals used

3.3.1Determination of Cadmium

- Cadmium chloride
- $HCLO_4$:HNO_3

3.3.2 Hoagland media composition

- 2M KNO_3
- 2.5M Ca $(NO_3)_2$•$4H_2O$
- 2.5M Iron (Sprint 138 iron chelate)
- 1.5 M $MgSO_4$•$7H_2O$
- 1M NH_4NO_3
- H_3BO_3
- $MnCl_2$•$4H_2O$
- $ZnSO_4$•$7H_2O$
- $CuSO_4$•$5H_2O$
- H_3MoO_4•H_2O
- Na_2MoO_4•$2H_2O$
- 1M KH_2PO_4 (pH to 6.0)

3.3.3 Quantification of Cadmium

- 16mM Nitric acid
- Hydrogen peroxide

3.3.4 Estimation of thiol compound

- Ninhydrin
- Acetic acid
- 0.6M phosphoric acid
- Conc. HCl
- Aq. Cysteine HCL l
- Ethanol

3.3.5 Assay of Cysteine Synthetase

- 50mM Phosphate buffer
- 1mM EDTA
- 2mM dithiothreitol
- Triton X-100
- PMSF
- O-acetyl L-serine
- 7.5% trichloroacetic acid

3.3.6 Assay of Glutathione S- transferase

- 100mM Phosphate buffer
- 0.1mM EDTA
- 1% polyvinyl pyrrolidone
- 0.1mM 1-chloro 2,4-dinitro benzene

3.3.7 Assay of Glutathione reductase

- 100mM Potassium Phosphate buffer
- 0.5mM EDTA
- 3mM 5,5 dithiobis (2-nitrobenzoic acid)
- H_2O_2
- 2mM NADPH

3.3.8 Quantification of Arsenic

- Na_2HAsO_4
- HNO_3
- 10%HCl
- 10%KI

- 5% ascorbic acid

3.3.9 Assay of arsenate reductase

- 50mM MOPS
- 50mM MES
- 375µM NADPH
- Yeast
- 1mM GSH
- 0.5µM glutardixin-2
- 10mM MAsV

3.3.10 Bradford reagents

- Crude sample
- Distilled water
- Water bath
- Spectrophotometer
- Coomassie blue
- 95% Ethyl alcohol
- 85% Hydrogen phosphate

3.3.11 SDS-PAGE

- 1.5M Tris HCl pH 8.8
- 0.5M Tris HCl pH 6.8
- 10% SDS Buffer
- Acrylamide Solution

  Acrylamide:Bisacrylamide
- APS(Ammonium Persulphate)
- **Tank Buffer(1 liter)**
- Glycine
- SDS
- Tris-HCL
- Water
- **Resolving Gel (For 10 ml)**
- Distilled water

- ❖ Acrylamide solution
- ❖ 1.5M Tris HCl
- ❖ 10% SDS
- ❖ 10% APS
- ❖ TEMED

➢ **Stacking Gel (For 3 ml)**

- ❖ Distilled Water
- ❖ Acrylamide solution
- ❖ 0.5M Tris-HCl
- ❖ 10% SDS
- ❖ 10% APS
- ❖ TEMED

➢ **Loading Dye**

- ❖ 0.5M Tris HCl
- ❖ Distilled Water
- ❖ Glycerol
- ❖ 10%SDS
- ❖ β-mercaptoethanol
- ❖ Bromophenol Blue

➢ **Staining Solution (100 ml)**

- ❖ Methanol
- ❖ Acetic Acid
- ❖ Water
- ❖ Comassie Brilliant Blue

➢ **Destaining Solution**

- ❖ Methanol
- ❖ Acetic Acid

Table 3.1: List of Glassware/Plasticware used

S. No.	Glassware/Plasticware used
1	Beakers
2	Centrifuge tubes (10 ml, 50ml)
3	Conical Flasks
4	Cover slip (for microscopy)
5	Dessicator
6	Eppendroff Tubes
7	Glass Rods
8	Liquid Nitrogen can
9	Ice Box
10	Measuring cylinders
11	Cuvet
12	Microtips (blue and yellow tips)
13	Mortar and Pestle
14	Pipette Stand
15	Reagent Bottles
16	Sieve (120-mesh size)
17	Test Tubes
18	Test Tube Stand
19	Volumetric Flasks
20	Wash Bottles
21	Watch glasses
22	Weigh pans

Table 3.2: Details of Equipments used in experiment

S. No.	Equipments
1	SDA-PAGE Electrophoresis UNIT
2	Analytical balance
3	Autoclave
4	Deep freezer (-80ºC)
5	Deep freezer (-20ºC)
6	Double distillation unit
7	Electronic Balance
8	Magnetic stirrer
9	Micropipette
10	Microwave Oven
11	pH meter
12	Refrigerated high speed centrifuge
13	Spinner
14	UV-spectrophotometer
15	Water bath

3.4Source of collection

Plant of *ceratophyllum demersum* was collected from near by pond of yammuna bank and grown for six months in large hydroponic tub.

3.5Methodology

3.5.1Methods for determination of cadmium accumulation in *C. demersum*

3.5.1aPlant materials and treatment conditions

Explants of *C. demersum*were collectedfrom near by pond of yammuna bank and were grown hydroponically.Than one inch. Tip were collected andwere acclimatized in 10% Hoagland's solution **(Hoagland and Arnon, 1950)**for one week in laboratory conditions using a light intensity of 115μmol m^{-2} s^{-1}, a 14 h photoperiod at 25±2ºC. The plants were maintained in 10%

Hoagland's solution in 250 ml conical flasks under above mentioned laboratory conditions and were exposed to different concentrations of Cd (0, 1, 5, 10, 15. 20µM; prepared by using the salt $CdCl_2$) for a period of 1, 2, 3, 4, 5, 6 and7 days. Flasks without Cd kept with each set of experiment served as control. After harvesting, plants were washed with demineralized water, blotted and were used to study the degree of Cadmium accumulation.

3.6 Quantification of cadmium

Harvested sample were washed thoroughly with demineralized water, blotted and oven dried at 80^{o}C for 2 days. The samples were digested in $HClO_4$:HNO_3 (1:3 v/v) at 100^{o}C and then diluting them with demineralized water. Cadmium concentration was determined on a UV-Visible double beam Spectrophotometer at 229 nm.

3.7 Method for Zinc Cadmium interaction

3.7.1Determination of Zinc cadmium content in *Ceratophyllum*

Plants of *C. demersum*were collected and were grown in large hydroponic tubs. Before treatment, plants tip portion approx. 2 inch were acclimatized in 10% Hoagland's solution **(Hoagland and Arnon, 1950)**for one week in laboratory conditions using a light intensity of 115µmol m^{-2} s^{-1}, a 14 h photoperiod at 25±2ºC. The plants Sample were exposed to different concentrations of Cd + Zn (Cd-10, Cd+Zn-10, Cd+Zn-50, Cd+Zn-100, Cd+Zn-150, Cd+Zn-200, Cd+Zn-300) **(P.Aravid et al; 2005)**for a period interval of 2, 4, 6, 8, 10 and12 days. Flasks without Zn kept with each set of experiment served as control. After harvesting, plant sample were washed with demineralized water, blotted and used to study the degree of Cadmium accumulation.Plant material was oven-dried at 80°C for 24h. Subsequently, plant tissue was digested in microwave oven in two steps. The first step was digestion in 10 ml of 16 mM HNO_3at 70°C for 5 min, then at 100°C for another 5 min. The second step included digestion in 1 ml of H_2O_2 at 85°C for 5 min and then at 120°C for 4 min. After cooling, the samples were diluted with 1% (v/v) HNO_3 to achieve the total volume of 50 ml. Cd-Zn concentrations were determined spectrophotometrically at 350nm.

3.8Estimation of thiol compound

Estimation of thiol compound was performed by using reaction with acid ninhydrin reagent (Appendix 1.2). The level of thiol compound was determined spectrophotometrically at 350nm.

3.8.1Procedure

The reaction mixture containing 0.5ml of aq. cysteine hydrochloride solution (0.05-0.5µmole), 0.5ml of acetic acid and 0.5ml of acid ninhydrin reagent (Appendix 1.2) was mixed thoroughly. The reaction may be performed with a sample volume of 1.0ml. The tubes were covered with aluminum caps or glass marbles and heated in a boiling-water bath for 10min. They were then rapidly cooled in tap water; the contents of the tube were diluted to 5with 95% ethanol and mixed. A reagent blank without cysteine was prepared under the same conditions. The spectral measurements of the reaction products were made against the reagent blank at 350nm using double beam spectrophotometer.

3.9 Assay of cysteine synthetase on cadmium exposure

For the assay of cysteine synthetase activity, 1 gram plant sample was extracted in 50 mM phosphate buffer (pH 7.5) containing 1 mM EDTA, 5 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 0.1% Triton X-100 and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was subjected to centrifugation at 16,000g for 15 min at 4^oC and the supernatant was used for the enzymatic assays. The reaction mixture containing 50 mM phosphate buffer (pH 8.0), 4 mM Na_2S, 12.5 mM O-acetyl L-serine (OAS) and 0.5ml of enzyme extract was incubated at 30^oC for 20 min. The reaction was terminated by the addition of 0.1 ml of 7.5% trichloroacetic acid (TCA) and the amount of cysteine synthesized was determined by the Bradford assay using BSA as standard.

3.9.1 Bradford reagent preparation for assay of cysteine synthetase

- Taken 2.5mg CBB dye and dissolved in 5 ml of 95% C_2H_5OH.
- Added 5ml of 85% H_3PO_4 and make up the final volume 50 ml with distilled water.
- Taken 11 test tubes. In a test tube, take aliquots of standard BSA (0, 2, 1.5, 1, 0.75, 0.50, 0.25 and 0.125ml).
- Added distilled water in decreasing order in each test tube (0.125, 0.25, 0.50, 0.75, 1, 1.5, 2ml).
- 2.5ml of Bradford reagent was added in each tube.
- The test tubes were incubated in dark at room temperature for 10 minutes.
- Absorbance was recorded at 595 nm.

3.10 Assay of Glutathione-S-transferase

For the assay of Glutathione-S-transferase, the plants (1 g) wereextracted in 2 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% polyvinyl pyrrolidone (PVP).Homogenate was centrifuged at 10,000g for 10 min and supernatant was used for the enzymatic assay.The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 6.5), 0.1 mM 1-chloro 2, 4-dinitrobenzene (CDNB) and a suitable aliquot of enzyme extract. The absorbance was calculated at 340 nm. The protein content in the supernatants was measured accordingto Bradford assay with reference**JH Best, S Pflugmacher, C Wiegand, FB Eddy 2002**

3.10.1 Bradford reagent preparation for assay of Glutathione-S-transferase

- Taken 2.5mg CBB dye and dissolved in 5 ml of 95% C_2H_5OH.
- Added 5ml of 85% H_3PO_4 and make up the final volume 50 ml with distilled water.
- Taken 11 test tubes. In a test tube, take aliquots of standard BSA (0, 2, 1.5, 1, 0.75, 0.50, 0.25 and 0.125ml).
- Added distilled water in decreasing order in each test tube (0.125, 0.25, 0.50, 0.75, 1, 1.5, 2ml).
- 2.5ml of Bradford reagent was added in each tube.
- The test tubes were incubated in dark at room temperature for 10 minutes.
- Absorbance was recorded at 340 nm.

3.11 Assay of glutathione reductase

For estimation of the glutathione reductase, plant sample wasextracted in 100 mM potassium phosphate buffer (pH 7.5) containing0.5 mMEDTA. The reaction was initiated by adding in the following order; 1.0 ml of 0.2 M potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 0.5 ml 3 mM 5,5-dithiobis (2-nitrobenzoicacid) (DTNB) in 0.01 M phosphate buffer (pH 7.5), 0.2 ml H_2O,0.1 ml 2 mM NADPH, 0.1 ml enzyme extract. The absorbance was recorded at 412 nm. The amount of glutathione reductase synthesized was determined by the Bradford assay using BSA as standard in reference **Best, Pflugmacher, Wiegand, Eddy (2002).**

3.11.1 Bradford reagent preparation for assay of glutathione reductase

- Taken 2.5mg CBB dye and dissolved in 5 ml of 95% C_2H_5OH.

- Added 5ml of 85% H_3PO_4 and make up the final volume 50 ml with distilled water.
- Taken 11 test tubes. In a test tube, take aliquots of standard BSA (0, 2, 1.5, 1, 0.75, 0.50, 0.25 and 0.125ml).
- Added distilled water in decreasing order in each test tube (0.125, 0.25, 0.50, 0.75, 1, 1.5, 2ml).
- 2.5ml of Bradford reagent was added in each tube.
- The test tubes were incubated in dark at room temperature for 10 minutes.
- Absorbance was recorded at 412 nm.

3.12 Effect of arsenic accumulation in plant

Plants of *C. demersum* were collected and were grown in large hydroponic tubes. Before treatment, plants (about 5 cm tip portion) were acclimatized in 10% Hoagland's solution **(Hoagland and Arnon, 1950)** for 1 week in laboratory conditions 25±2◦C). The plants were exposed to different concentrations of Arsenic (0, 1, 5, 10, 15. 20µM; prepared using Na_2HAsO_4) maintained in 10% Hoagland's solution in 250 ml conical flasks under above mentioned laboratory conditions for a period of 7 days. To investigate the effect arsenic uptake, plants were exposed different concentrations prepared in 10% Hoagland nutrient solution. After harvesting, plants were washed with demineralized water, blotted to remove water, and used to determine various parameters.

3.13Quantification of arsenic

The plant sample was oven-dried and powdered (100 mg) then digested in 1ml of concentrated HNO_3 on a heating block at 100◦C for 1 h and subsequently at 180◦C to evaporate the samples to dryness. The residue was taken up in 10 ml of 10% (w/v) HCl containing 10% (w/v) KI and 5% (w/v) ascorbic acid. Arsenic concentrations were determined on (UV –Visible) double beam spectrophotometer at 230nm**Abedin et al. (2002).**

3.14Estimation of cysteineinarsenic treated plant

Estimation of cysteine was performed by using reaction with acid ninhydrin reagent (Appendix1.2). The level of cysteine was determined spectrophotometrically at 350nm.

3.14.1 Procedure

The reaction mixture containing 0.5ml of aq cysteine hydrochloride solution (0.05-0.5µmole), 0.5ml of acetic acid and 0.5ml of acid ninhydrin reagent 1 or 2**Gaitonde (1967),**was mixed thoroughly. The reaction may be performed with a sample volume of 1.0ml. The tubes were

covered with aluminum caps or glass marbles and heated in a boiling-water bath for 10min. They were then rapidly cooled in tap water; the contents of the tube were diluted to 5 or 10ml. with 95% ethanol and mixed. A reagent blank without cysteine was prepared under the same conditions. The spectral measurements of the reaction products were made against the reagent blank at 350nm using double beam spectrophotometer.

3.15Estimation of thiol compounds in arsenate treated plant

Effect of various concentration of arsenate on non-protein thiol compound was studied such as reduced glutathione and oxidized glutathione. The level of reduced glutathione was studied spectrophotometrically at 340nm using Bradford assay using o-phthaldialdehyde.

3.15.1 Bradford reagent preparation for estimation of reduced glutathioneand oxidized glutathione

- Taken 2.5mg CBB dye and dissolved in 5 ml of 95% C_2H_5OH.
- Added 5ml of 85% H_3PO_4 and maked the final volume 50 ml with distilled water.
- Taken 11 test tubes. In a test tube, taken aliquots of standard BSA (0, 2, 1.5, 1, 0.75, 0.50, 0.25 and 0.125ml).
- Added distilled water in decreasing order in each test tube (0.125, 0.25, 0.50, 0.75, 1, 1.5, 2ml).
- 2.5ml of Bradford reagent was added in each tube.
- The test tubes were incubated in dark at room temperature for 10 minutes.
- Absorbance was recorded at 340 nm.

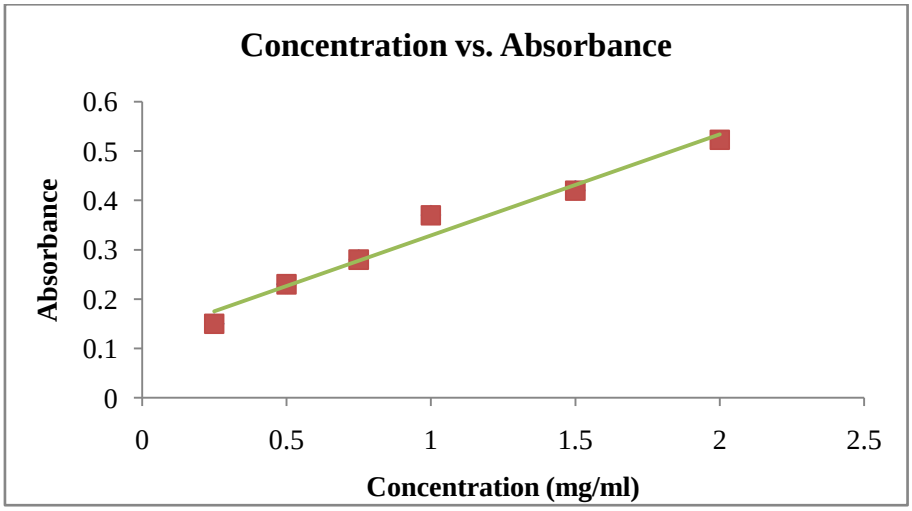


Fig 3.2 Standard curve of BSA

3.16 Assay of arsenate reductase

For the assay of arsenate reductase (AR; EC 1.20.4.1), plants sample were frozen in liquid nitrogen and then homogenized in buffercontaining 50mM MOPS and 50mM MES (pH 6.5). Supernatantobtained after centrifugation (at 10,000×*g* for 30 min) was filtrated to remove interferingmolecules.The reaction mixture contained 50mMMOPS and 50mM MES (pH 6.5) buffer, 375μM NADPH, 0.2 unit yeast (*Saccharomycescerevisiae*), 1mM GSH, 0.5 μM *Escherichiacoli* glutaredoxin-2 and 10mMAsV. GR-specific NADPH oxidation was monitored at 340 nm **Shi et al. (1999).**

3.17 Electrophoresis of enzymes extracted from *Ceratophyllum demersum* by SDS-PAGE

3.17.1 Preparation of Gel

1. Cleaned the surface thoroughly on which work has to be done.
2. Cleaned the glass plates with soap and water, then with ethanol. Assemble the glass plates and spacers.
3. Assembled the glass plates by putting the shorter glass plate in front of the longer one.
4. Fixed the glass plates and filled it with distilled water to check any leakage from the bottom. If leakage was occurs, seal the end of the plate with molten agar.
5. First the resolving gel is prepared which is usually more basic and has higher polyacrylamide content than the loading gel.
6. Taken a small beaker; add distilled water, Tris- HCl (pH-8.8), acrylamide, SDS with the help of pipette.
7. Ammonium per sulphate and TEMED are added when the gel is ready to be polymerized.
8. Mixed it properly by moving the beaker in circular motion on the surface.
9. Poured the gel between the plates before it polymerizes, then add water at the surface to remove un-polymerized gel.Similarly prepare stacking Gel.

3.17.2Preparation of samples

1. Mixed the sample with an equal volume of 2X sample buffer.
2. Boiled the sample in boiling water for 5 minutes, cool to room temperature before loading.
3. If particulate is present, centrifuge samples for 2 minutes.

3.17.3Loading the sample

1. Clamped the gel plates properly and fill it resolving gel. When resolving gel polymerizes, remove the water by using tissue paper, pour the stacking gel and placed the comb to create the wells.

2. After the stacking gel polymerizes, removed the comb and the gel is re buffer chamber with running buffer.

3. Loaded 50 µg prepared sample into the wells. The tip was wiped with distilled water every time the next sample is to be loaded.

4. Marker was loaded in the first lane to determine the size of desired protein.

5. Then samples were loaded in the adjacent lanes. Bubbles were avoided in the tip

3.17.4Running the gel

1. Placed the lid on top of the buffer chamber.

2. connected the electrical leads to the power pack with the proper polarity (black to black and red to red) and run the gel at 60V.

3. Runed the gel till the dye reaches the end of the gel.

4. As soon as the dye reaches at the end, switched off the power.

3.17.5 Staining the Gel

1**.** Taken out the glass plates from the electrophoretic tank and tear apart the two plates using spatula.

2. Carefully placed the gel into staining solution(Methanol, Acetic Acid, Water, Comassie Brilliant Blue).

3. Incubated the gel into this staining solution overnight.

Note: The staining of gel is always done in a covered container.

3.17.6 De-staining the Gel

1. When gel was stained, the gel was then transferred from a staining solution to a de-staining solution.

2. De-staining was done until all the extra dye was removed from the gel except the dye bound to protein in the form of bands.

3. After de-staining clear bands of protein were seen and compared to corresponding bands of ladder to determine the molecular weight of the protein.

4.0 Results and Discussion

4.1 Culture condition of *Certophyllum demersum*

After plants were grown for six months, before treatment plant tips were acclimatized in 10% Hoagland's solution for one week in laboratory conditions at 25± 2^{o}C.

4.2 Treatment of plant with different concentration of zinc

4.2.1 Effect of different concentration of zinc on plant species

The effect of zinc ion concentration (10mg/ml, 50mg/ml, 100mg/ml, 150mg/ml, and 200mg/ml) for 7 days in 10% Hoagland media (suspension culture)was studied on plant.

After day 1 no significant effect was observed on plant for all concentration of zinc(Fig 4.1). After day 2, 3 and 4 change in colour from green to yellow was observed with different colour intensity (Fig 4.2, Fig 4.3, and Fig 4.4).

It was observed that after regular interval, i.e day 5, 6 and 7 the leaves of *Certophyllum demersum* become black in colour the intensity of black colour was increased as concentration of zinc ion increased.

Fig 4.1: Effect of zinc ion concentration on plant after day 1

Fig 4.2: Effect of zinc ion concentration on plantafter day 2

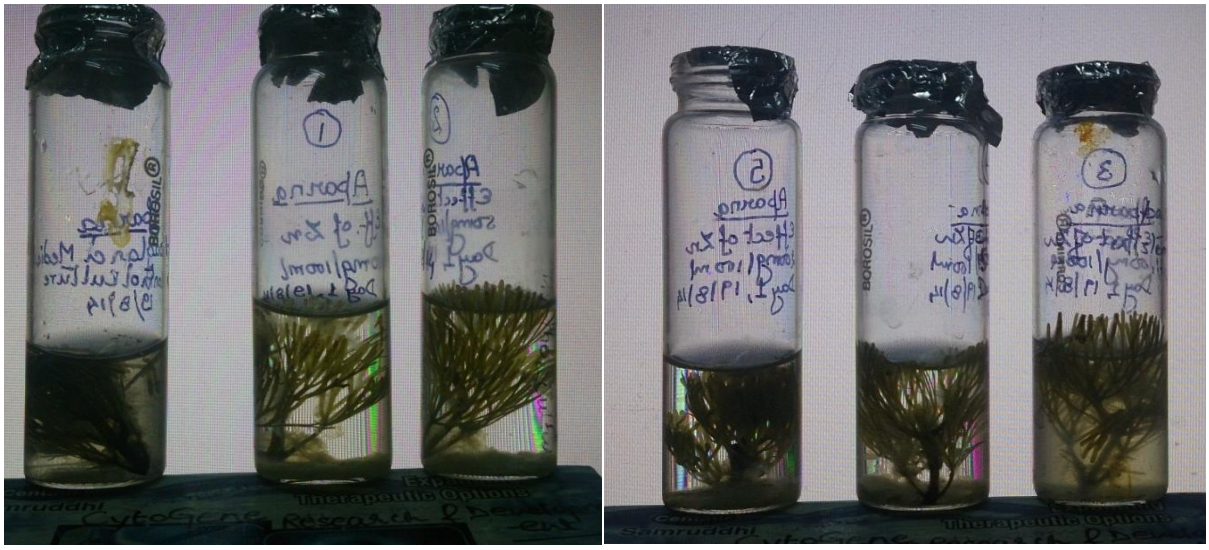


Fig 4.3: Effect of zinc ion concentration after day 3

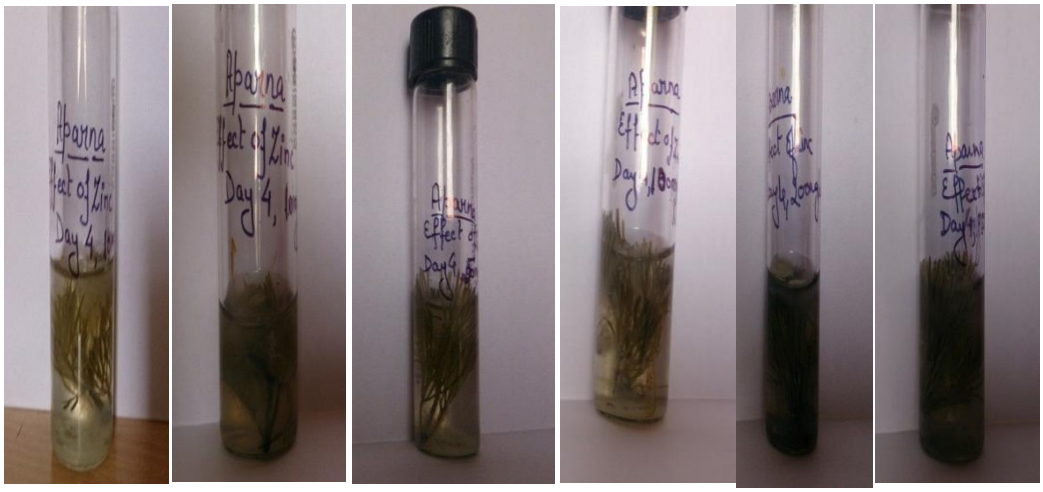


Fig4.4: Effect of zinc ion concentration after day 4

Fig4.5: Effect of zinc ion concentration after day 5

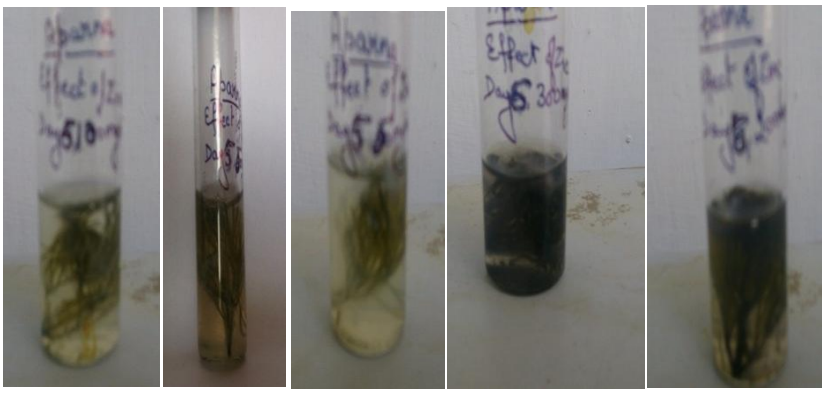


Fig4.6: Effect of zinc ion concentration after day 6

Figure 4.7: Effect of zinc ion concentration after day 7

4.4Effect of Cadmium on *Certophyllum demersum*

The plant sample wereprepared by using the salt $CdCl_2$ maintained in 10% Hoagland's solution in 250 ml conical flasks and exposed to different concentrations of Cd (0, 1, 5, 10, 15. 20µM); underlaboratory conditions for a period of 1, 2, 3, 4, 5, 6 and 7 days. Flasks without Cd kept with each set of experiment served as control. After harvesting, plants were washed with demineralized water, blotted and used to study the degree of Cadmium accumulation.Accumulation of Cd by *C. demersum* plants was found to be correlated to both concentration and duration of the treatment (Table 4.1). The plant showed maximum

accumulation of cadmium after 7 day at 20µM concentration (Fig 4.8) chart and figure indicates that pland had an ability to accumulate cadmium, and it is proposnal to days and concentration.

Table 4.1Accumulation of Cadmium in *Certophyllum demersum* after 1,2,3,4,5,6 and 7 days

S.No.	Conc. of Cadmium	Effect of Cadmium						
		1days	2 days	3 days	4 days	5 days	6 days	7 days
1	0.00 µM	0.00µg	0.00 µg	0.00 µg	0.00 µg	0.00 µg	0.00 µg	0.00 µg
2	1µM	131 µg	162 µg	168 µg	174 µg	189 µg	209 µg	234 µg
3	5 µM	136 µg	164 µg	173 µg	182 µg	194 µg	224 µg	252 µg
4	10 µM	157 µg	188 µg	194 µg	202 µg	199 µg	243 µg	297 µg
5	15 µM	173 µg	196 µg	217 µg	247 µg	214 µg	288 µg	320 µg
6	20 µM	262 µg	281 µg	290 µg	310 µg	369 µg	376 µg	417 µg

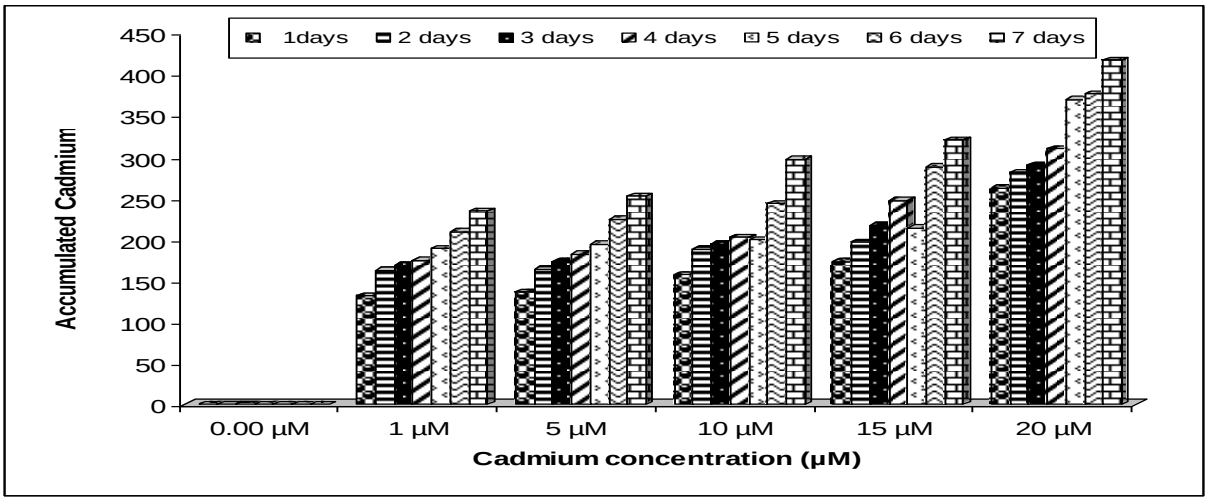


Fig 4.8 Accumulation of cadmium by *Ceratophyllum demersum* exposed to different concentrations after day 1,2,3,4,5,6 and 7 days

4.5 Study of Zinc-Cadmium interactionon *Ceratophyllumdemersum*

The effect of Zinc-Cadmium interaction was studied on *Ceratophyllumdemersum* on day 2,4,6,8,10, 12 days. It was observed from the results that with increasing concentration of Zinc along with the Cadmium, Zinc suppress the toxicity of the cadmium in plant hence less accumulation of cadmium in plant as the concentration of zinc increases(Fig 4.9, Fig4.10,Fig 4.11, Fig 4.12, Fig 4.13, Fig 4.14) .

Table 4.2: Effect of Zinc and Cadmium interaction on *Ceratophyllum demersum* (Day 2)

S. No.	Treatments	Cadmium effect	Zinc effect
1.	Cd-10	75µg	-
2.	Cd + Zn-10	147 µg	35 µg
3.	Cd + Zn-50	148 µg	142 µg
4.	Cd + Zn-100	142 µg	125 µg
5.	Cd + Zn-150	100 µg	227 µg
6.	Cd + Zn-200	113 µg	257 µg
7.	Cd + Zn-300	132 µg	286 µg

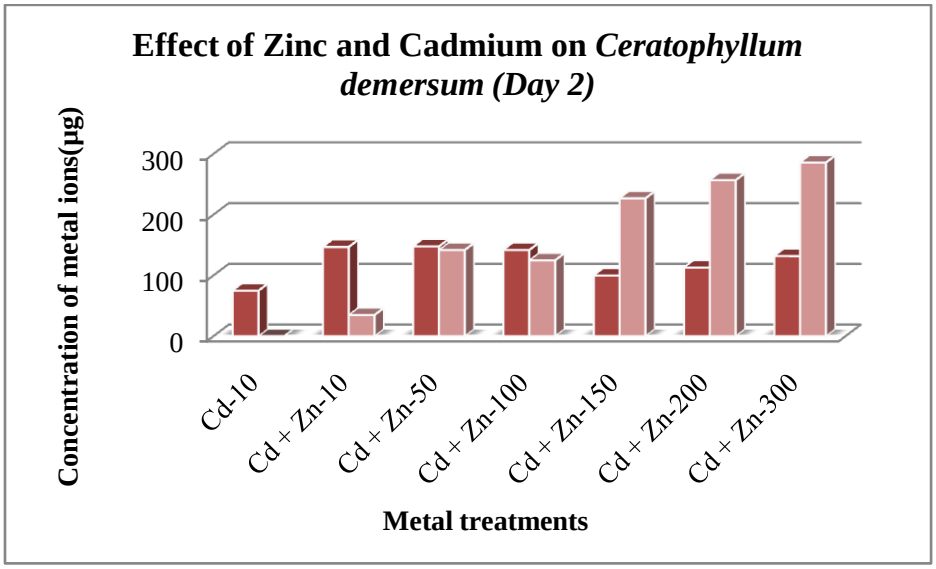


Fig 4.9: Metal accumulation in *Ceratophyllum demersum* treated with Cd-10 & Zn (10, 50, 100, 150, 200 & 300µg) after day 2

Table 4.3: Effect of Zinc and Cadmium interaction on *Ceratophyllum demersum* (Day 4)

S. No.	Treatments	Cadmium effect	Zinc effect
1.	Cd-10	77µg	0
2.	Cd + Zn-10	151 µg	42 µg
3.	Cd + Zn-50	151 µg	144 µg
4.	Cd + Zn-100	139 µg	132 µg
5.	Cd + Zn-150	115 µg	235 µg
6.	Cd + Zn-200	113 µg	254 µg
7.	Cd + Zn-300	147 µg	293 µg

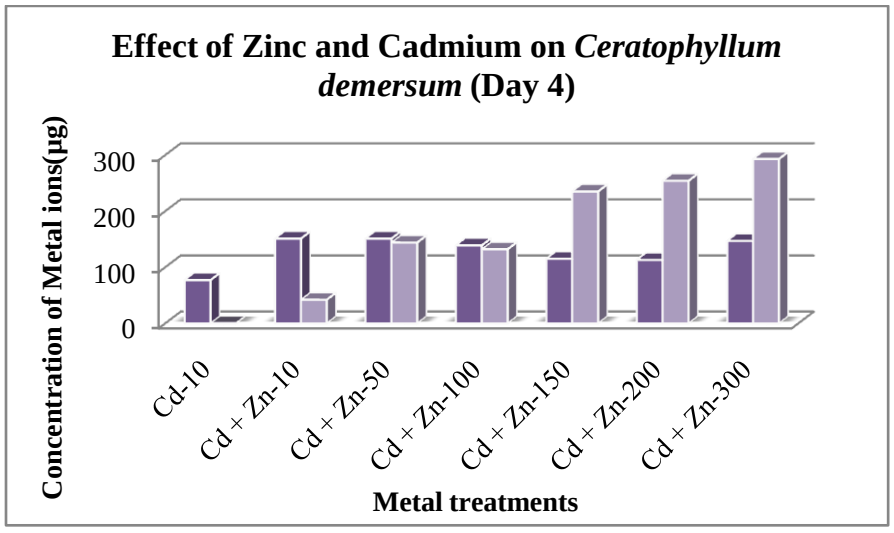


Fig4.10: Metal accumulation in *Ceratophyllum demersum* treated with Cd-10 & Zn (10, 50, 100, 150, 200 & 300µg) after day 4

Table 4.4: Effect of Zinc and Cadmium interaction on *Ceratophyllum demersum* (Day 6)

S. No.	Treatments	Cadmium effect	Zinc effect
1.	Cd-10	73 µg	0
2.	Cd + Zn-10	159 µg	29 µg
3.	Cd + Zn-50	165 µg	159 µg
4.	Cd + Zn-100	132 µg	125 µg
5.	Cd + Zn-150	97 µg	222 µg
6.	Cd + Zn-200	111 µg	236 µg
7.	Cd + Zn-300	149 µg	264 µg

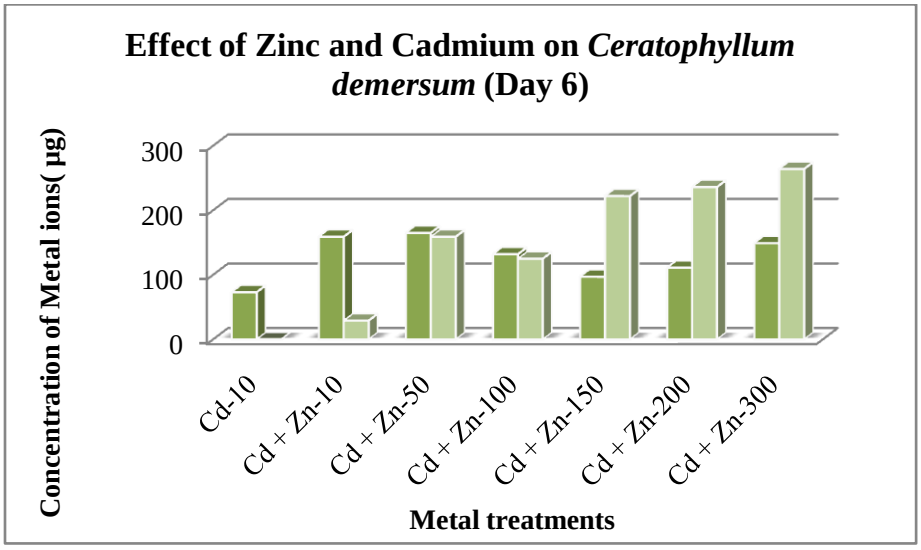


Fig4.11: Metal accumulation in *Ceratophyllum demersum* treated with Cd-10 & Zn (10, 50, 100, 150, 200 & 300µg) after day 6

Table 4.5: Effect of Zinc and Cadmium interaction on *Ceratophyllum demersum* (Day 8)

S. No.	Treatments	Cadmium effect	Zinc effect
1.	Cd-10	75µg	-
2.	Cd + Zn-10	162 µg	33 µg
3.	Cd + Zn-50	167 µg	163 µg
4.	Cd + Zn-100	135 µg	128 µg
5.	Cd + Zn-150	101 µg	224 µg
6.	Cd + Zn-200	114 µg	239 µg
7.	Cd + Zn-300	152 µg	267 µg

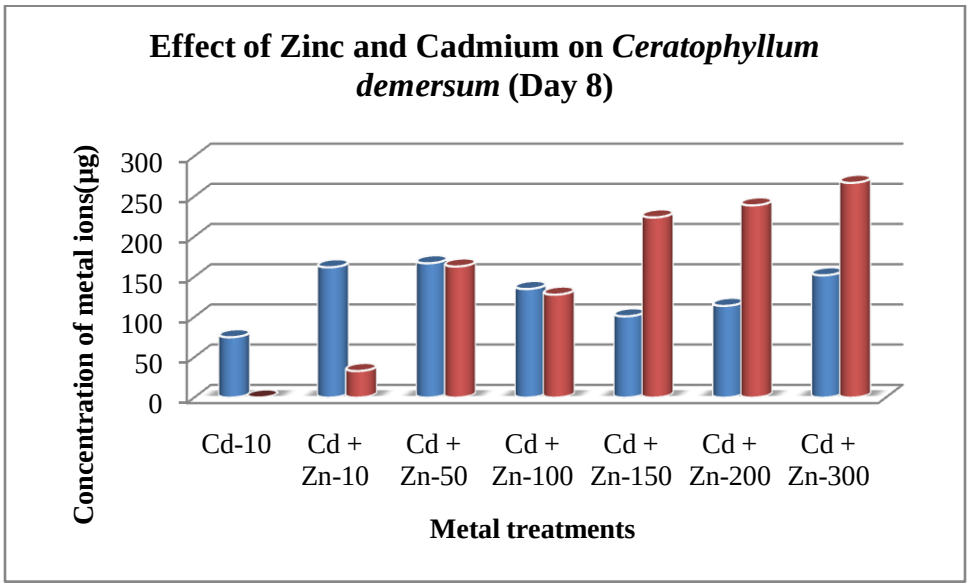


Fig 4.12: Metal accumulation in *Ceratophyllum demersum* treated with Cd-10 & Zn (10, 50, 100, 150, 200 & 300µg) after day 8

Table 4.6: Effect of Zinc and Cadmium interaction on *Ceratophyllum demersum* (Day 10)

S. No.	Treatments	Cadmium effect	Zinc effect
1.	Cd-10	78µg	0
2.	Cd + Zn-10	165 µg	37 µg
3.	Cd + Zn-50	169 µg	167 µg
4.	Cd + Zn-100	138 µg	132 µg
5.	Cd + Zn-150	105 µg	228 µg
6.	Cd + Zn-200	119 µg	243 µg
7.	Cd + Zn-300	157 µg	271 µg

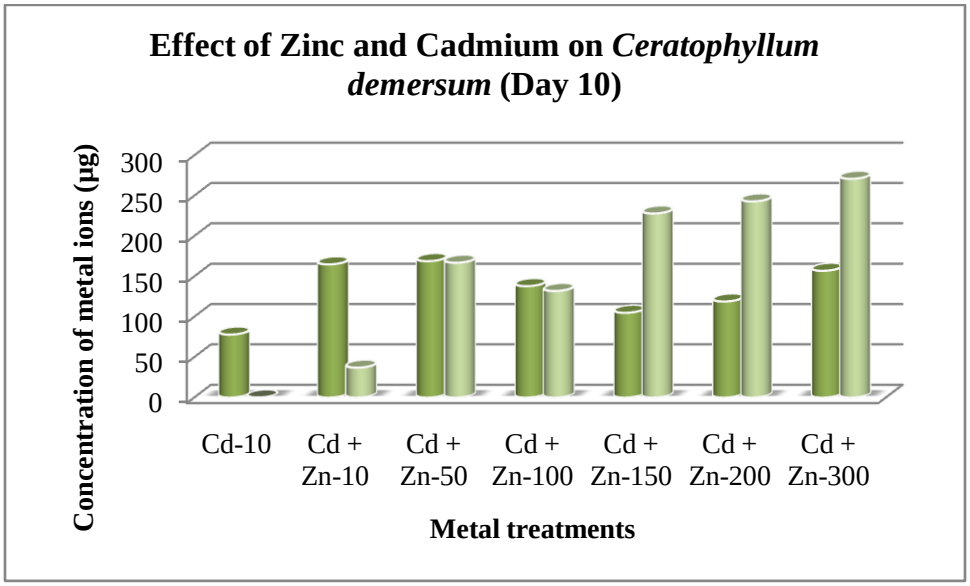


Fig 4.13: Metal accumulation in *Ceratophyllum demersum* treated with Cd-10 & Zn (10, 50, 100, 150, 200 & 300µg) after day 10

Table 4.7: Effect of Zinc and Cadmium interaction on *Ceratophyllum demersum* (Day 12)

S. No.	Treatments	Cadmium effect	Zinc effect
1.	Cd-10	81 µg	0
2.	Cd + Zn-10	170 µg	40 µg
3.	Cd + Zn-50	173 µg	171 µg
4.	Cd + Zn-100	142 µg	138 µg
5.	Cd + Zn-150	110µg	232 µg
6.	Cd + Zn-200	123 µg	249 µg
7.	Cd + Zn-300	160 µg	276 µg

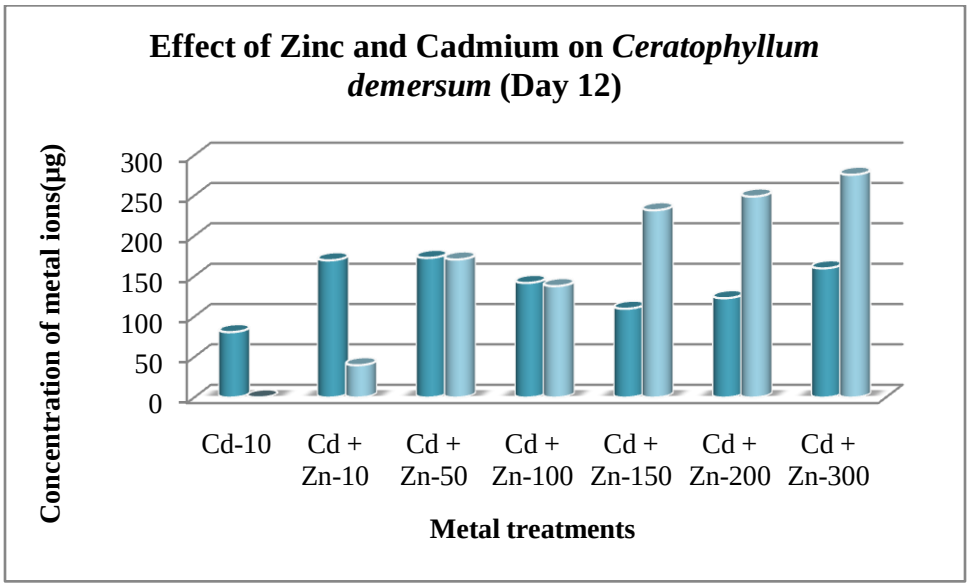


Fig 4.14: Metal accumulation in *Ceratophyllum demersum* treated with Cd-10 & Zn (10, 50, 100, 150, 200 & 300µg) after day 12

4.6 Estimation of Cystein compound from plant treated with cadmium

The thiol concentration was estimated using acid ninhydrin reagent at different concentrations of cadmium ($CdCl_2$) exposure (0, 1, 5, 10, 15 and 20µM) for a period interval of 1, 2, 3, 4, 5, 6 and 7 days. The level of thiol compounds increased significantly at all the cadmium exposure concentrations. The maximum level of thiol compound was observed at 10µM after 3 days (Fig 4.15), it show that the activity of this stress marker increace its activity upto day 3 at 10µM but after that it is unable to tolerate the toxicity and its activity decreases.

Table 4.8: Estimation of Cystein on *Ceratophyllum demersum*after day 1, 2, 3, 4, 5, 6, 7 days

S.No.	Conc. of Cadmium	cysteine estimation in (nmol g-1 fw)						
		1days	2 days	3 days	4 days	5 days	6 days	7 days
1	0.00 µM	1.2	1.3	1.4	1.5	1.2	1.2	1.25
2	1µM	1.6	2.3	2.9	3.2	2.4	1.9	1.8
3	5 µM	2.91	2.52	3.3	3.01	2.39	2.34	2.33
4	10 µM	3.0	3.1	3.4	2.9	2.6	2.57	2.43
5	15 µM	2.7	2.6	2.65	2.46	2.41	2.33	2.29
6	20 µM	2.2	1.9	1.9	1.6	1.2	1.25	1.27

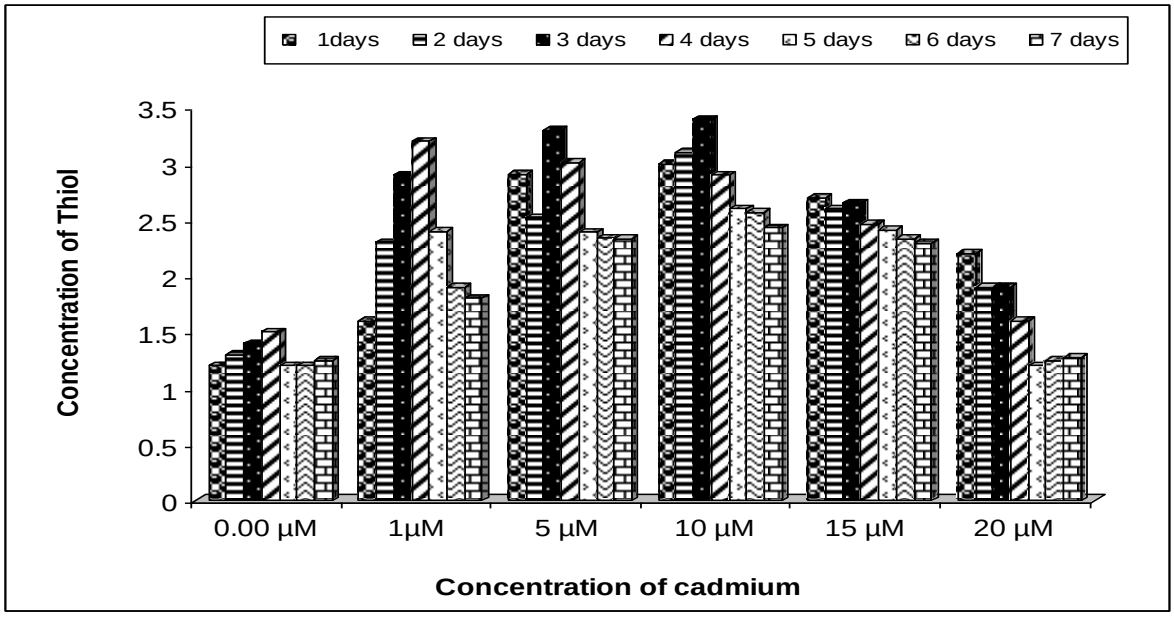


Fig 4.15: Effect of various concentration of cadmium on thiol content of *Ceratophyllum demersum* exposed for different durations after day 1, 2, 3, 4, 5, 6, 7 Days

4.7 Assay of cysteine synthetase from plant treated with cadmium

The cysteine synthetase was estimated using Coomassie Brilliant Blue (Bradford) reagent at different concentrations of cadmium ($CdCl_2$) exposure (0, 1, 5, 10, 15 and 20µM) for a period interval of 1, 2, 3, 4, 5, 6 and 7 days. The level of cysteine synthetase compounds increased significantly at all the cadmium exposure concentrations. The maximum level of cysteine synthetase was observed at 10µM after 3 days (Fig 4.16). In every concentration upto day 3 cysteine synthetase activity increases but after day 3 its tolerance quality decreases in every concentration, hence it can handle any concenteration upto day 3 after that it is unable to maintain its work.

Table 4.9: Estimation of cysteine synthetase on *Ceratophyllum demersum* after day 1, 2, 3, 4, 5, 6, 7 days

S.No.	Conc. of Cadmium	Concentration of CS (nmols cysteine min-1 mg-1 protein)						
		1days	2 days	3 days	4 days	5 days	6 days	7 days
1	0.00 µM	0.85	0.97	1.40	0.36	0.36	0.17	0.17
2	1µM	1.03	1.34	1.58	0.85	0.91	0.66	0.54
3	5 µM	1.03	1.58	1.76	1.21	1.09	0.97	0.42
4	10 µM	0.60	0.54	2.01	0.97	0.72	0.48	0.36
5	15 µM	0.36	0.66	1.21	0.17	0.17	0.66	0.17
6	20 µM	0.30	0.36	0.97	0.05	0.30	0.66	0.05

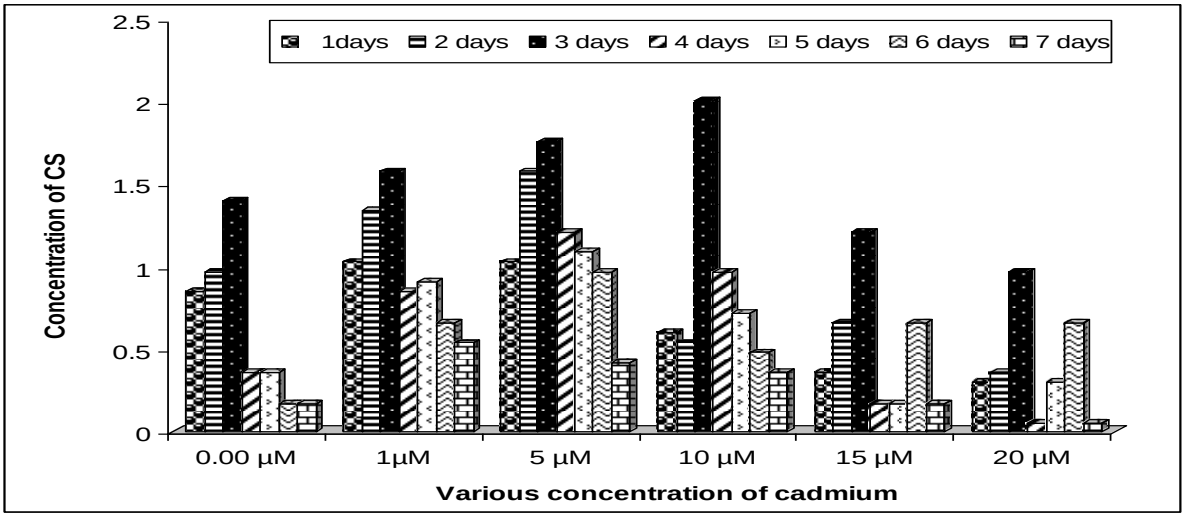


Fig 4.16: **Effect of various concentration of cadmium on level of cysteine synthetase in *Ceratophyllum demersum* exposed for different durations after day 1, 2, 3, 4, 5, 6, 7 Days**

4.8 Assay of Glutathione-S-transferasefrom plant treated withcadmium

The Glutathione-S-transferase was estimated using Coomassie Brilliant Blue (Bradford) reagent at different concentrations of cadmium exposure (0, 1, 5, 10, 15 and 20µM; prepared by using the salt $CdCl_2$) for a period interval of 1, 2, 3, 4, 5, 6 and 7 days. The level of glutathione reductase compounds increased significantly at all the cadmium exposure concentrations upto day 3. The maximum level of Glutathione-S-transferase was observed at 10µM after 4 days (Fig 4.17).

Table 4.10: Estimation of Glutathione-S-transferase on *Ceratophyllum demersum*after days 1, 2, 3, 4, 5, 6, 7

S. No.	Conc. of Cadmium	Concentration of GST units mg -1 protein						
		1days	2 days	3 days	4 days	5 days	6 days	7 days
1	0.00 µM	0.82	1.10	0.95	1.20	0.69	0.50	0.22
2	1µM	1.13	1.26	1.20	1.26	0.79	0.60	0.10
3	5 µM	0.85	1.48	1.07	1.20	1.07	0.63	0.41
4	10 µM	1.20	1.01	0.95	1.73	0.85	0.66	0.73
5	15 µM	0.47	0.88	1.07	0.95	0.47	0.41	0.35
6	20 µM	0.63	0.60	0.79	0.69	0.47	0.35	0.32

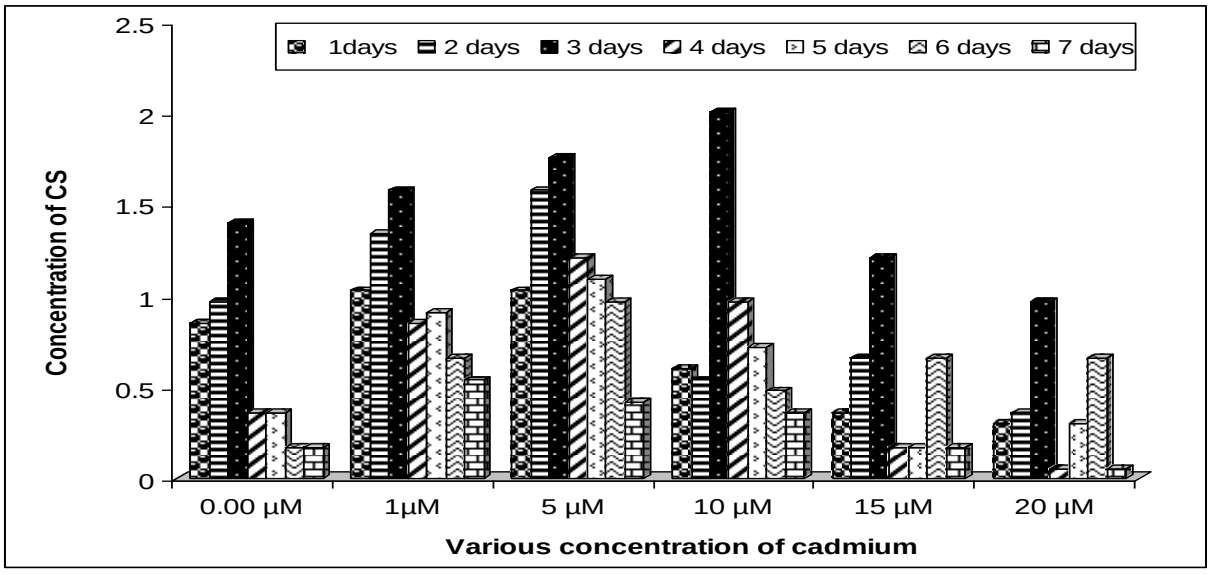


Fig 4.17: Effect of various concentration of cadmium on activity of Glutathione-S-transferase of *Ceratophyllum demersum* exposed for different durations (after days 1, 2, 3, 4, 5, 6, *7)*

4.9 Assay of glutathione reductase from cadmium treated plant

The glutathione reductase was estimated using Coomassie Brilliant Blue (Bradford) reagent at different concentrations of cadmium exposure (0, 1, 5, 10, 15 and 20µM; prepared by using the salt $CdCl_2$) for a period interval of 1, 2, 3, 4, 5, 6 and 7 days. The level of glutathione reductase compounds increased significantly at all the cadmium exposure concentrations upto day 4 and after that its activity gradually decrese. The maximum level of glutathione reductase was observed at 10µM after 4 days (Fig 4.18).

Table 4.11: Estimation of glutathione reductase on *Ceratophyllum demersum*after days 1, 2, 3, 4, 5, 6, 7

S. No.	Conc. of Cadmium	ConcenConcentration of GR in units mg -1 protein						
		1days	2 days	3 days	4 days	5 days	6 days	7 days
1	0.00 µM	0.66	1.04	1.48	1.61	0.54	0.32	0.35
2	1µM	1.13	1.23	1.57	1.89	0.60	0.35	0.32
3	5 µM	0.91	1.39	1.67	2.17	0.88	0.57	0.60
4	10 µM	0.79	0.95	1.80	2.30	1.10	0.63	0.50
5	15 µM	0.69	0.82	1.39	1.92	0.95	0.54	0.44
6	20 µM	0.60	0.54	1.26	1.51	0.88	0.47	0.38

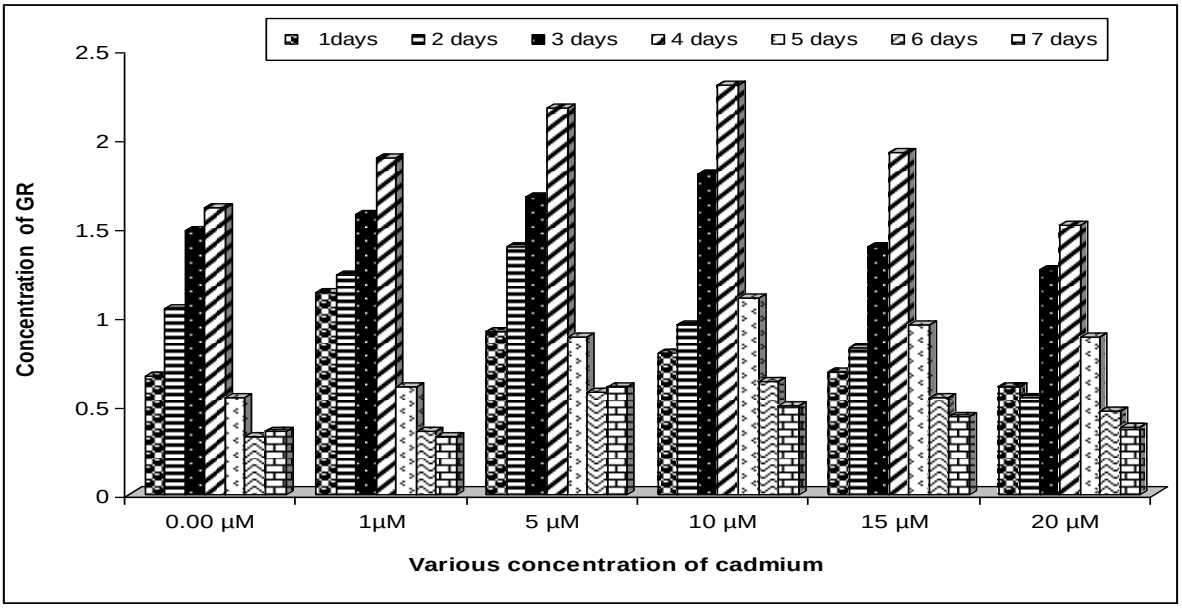


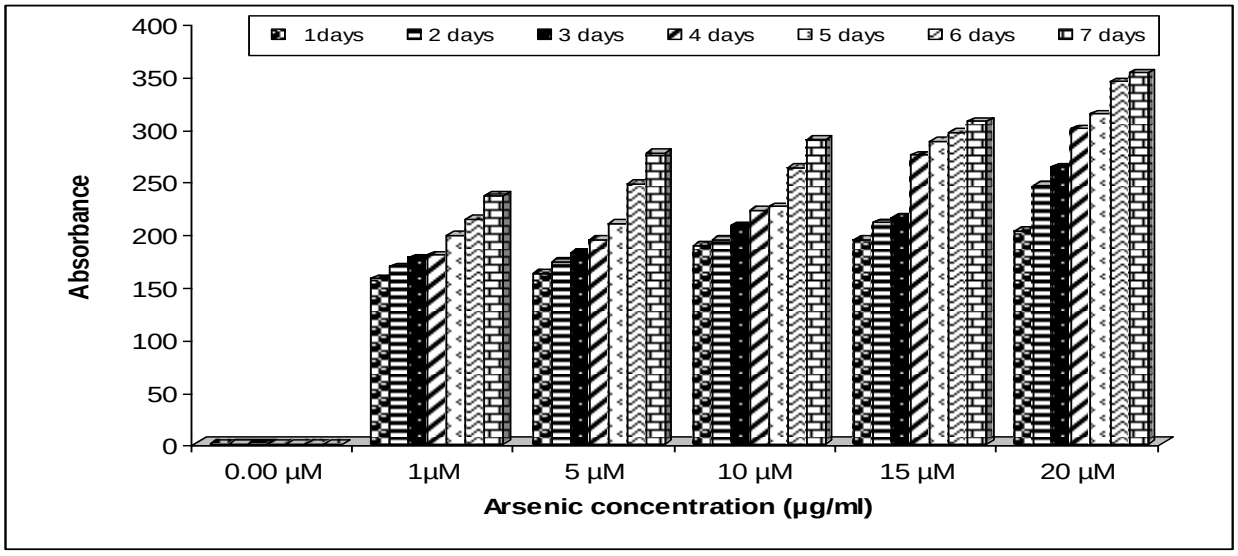
Fig 4.18: Effect of various concentration of cadmium on activity of glutathione reductase of *Ceratophyllum demersum* exposed for different durations (after day 1, 2, 3, 4, 5, 6, 7).

4.10 Accumulation of arsenic and its effect on *Certophyllum demersum*

Accumulation of arsenic was found to depend on both concentration and duration of exposure and showed alinear increase. The plant was exposed to different concentrations of arsenic (0, 1, 5, 10, 15. 20µM)maintained in 10% Hoagland's solution in 250 ml conical flasks underlaboratory conditions for a period of 1, 2, 3, 4, 5, 6 and 7 days. Flasks without arsenic kept with each set of experiment served as control. After harvesting, plants were washed with demineralized water, blotted and used to study the degree of arsenic accumulation. The plant showed maximum accumulation of arsenic after 7 day at 20µM concentration (Fig 4.19).

Table 4.12 Estimation of arsenate ion concentration in *Certophyllum demersum* after days 1,2 3, 4, 5, 6, 7.

Si. No.	Conc. of Arsenic	Estimation of arsenicaccumulation						
		1days	2 days	3 days	4 days	5 days	6 days	7 days
1	0.00 µM	0.00µg	0.00µg	0.00µg	0.00µg	0.00µg	0.00µg	0.00µg
2	1µM	157 µg	168 µg	176 µg	179 µg	198 µg	213 µg	236 µg
3	5 µM	162 µg	173 µg	181 µg	194 µg	209 µg	247 µg	276 µg
4	10 µM	188 µg	194 µg	207 µg	222 µg	225 µg	262 µg	289 µg
5	15 µM	194 µg	210 µg	215 µg	274 µg	287 µg	296 µg	306 µg
6	20 µM	202 µg	245 µg	263 µg	299 µg	313 µg	344 µg	352 µg



Graph 4.19: Accumulation of arsenic in *Ceratophyllum demersum* exposed to different concentrations after day 1, 2, 3, 4, 5, 6, 7.

4.11 Assay of cysteine from plant treated with arsenic

The cysteine concentration was estimated using acid ninhydrin reagent at different concentrations of arsenate exposure (0, 1, 5, 10, 15 and 20µM; prepared by using the salt Na_2HAsO_4) for a period interval of 1, 2, 3, 4, 5, 6 and 7 days. The level of cysteine increased significantly at all the arsenate exposure concentrationspto day 5 after that the concentration or can say the activity of cysteine decreases hence it is unable to handle the toxicity of arsenic after day 5 (fig 4.20).

Table 4.13 Estimation of cysteine concentration in *Certophyllum demersum* after days1 , 2, 3, 4, 5, 6, 7.

Si. No.	Conc. of Arsenic	Estimation of Cysteine concentration in µ mol g^{-1} fw						
		1days	2 days	3 days	4 days	5 days	6 days	7 days
1	0.00 µM	2.65	3.13	3.315	4.55	5.06	4.85	3.52
2	1µM	4.28	5.84	6.05	6.79	5.80	6.18	3.90
3	5 µM	5.54	5.93	6.18	7.96	7.13	6.94	2.63
4	10 µM	8.84	9.25	9.41	9.54	8.84	7.74	2.99
5	15 µM	12.12	12.16	12.31	12.67	10.36	9.22	3.20
6	20 µM	6.02	6.12	6.18	8.08	4.47	3.90	2.76

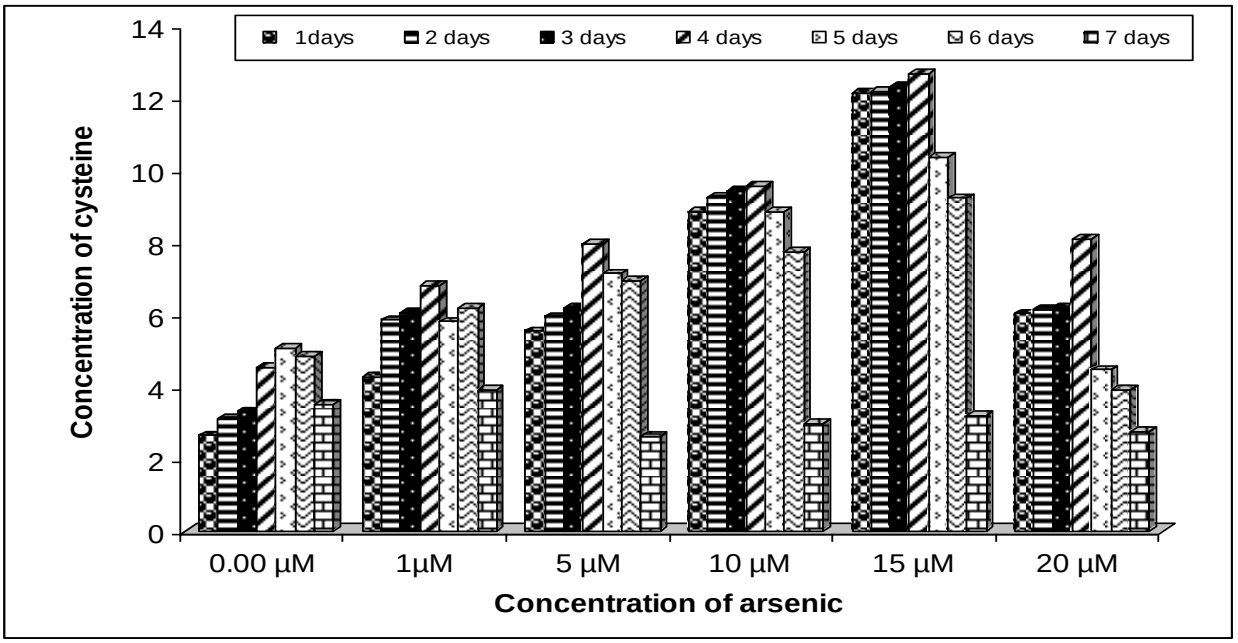


Fig 4.20: Effect of various concentration of arsenic on cysteine estimation of *Ceratophyllum demersum* exposed after days 1, 2, 3, 4, 5, 6, 7

4.13 Assay of oxidized glutathione and reduced glutathione plant treated with arsenate

4.13.1 Assay of oxidized glutathione plant treated with arsenate

The oxidized glutathione was estimated using Coomassie Brilliant Blue (Bradford) reagent at different concentrations of arsenate exposure (1, 5, 10, 15 and 20µM) for a period of 7 days. The level of oxidized glutathione compounds increased significantly at all the arsenate exposure concentrations. The maximum level of concentration was observed on 4th day at 20µMafter 4 day af each concentration its activity very slowly decreses. (Fig 4.21).

Table 4.14 Estimation of oxidized glutathione in *Certophyllum demersum* after day 1, 2, 3, 4, 5, 6, 7.

Si. No.	Conc. of Arsenic	Concentration of GSSH in µ mol g^{-1} fw						
		1days	2 days	3 days	4 days	5 days	6 days	7 days
1	1µM	0.20	0.26	0.27	0.29	0.28	0.26	0.23
2	5 µM	0.23	0.30	0.31	0.32	0.29	0.27	0.25
3	10 µM	0.31	0.34	0.34	0.42	0.39	0.31	0.27
4	15 µM	0.32	0.36	0.39	0.52	0.48	0.32	0.33
5	20 µM	0.35	0.40	0.45	0.55	0.54	0.5	0.36

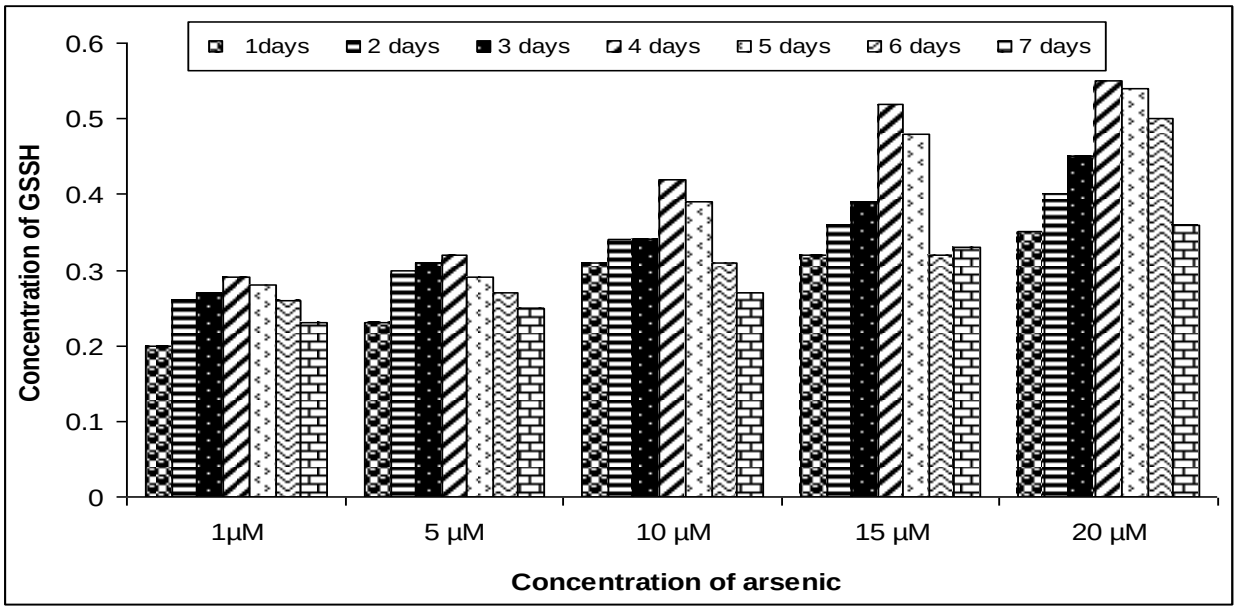


Fig 4.21: Effect of various concentration of arsenic on activity of oxidized glutathione of *Ceratophyllum demersum* exposed for different durations (after days 1,2 3, 4, 5, 6, 7).

4.13.2 Assay of Reduced glutathione plant treated with arsenate

The reduced glutathione was estimated using Coomassie Brilliant Blue (Bradford) reagent at different concentrations of arsenate exposure (0, 1, 5, 10, 15 and 20µM) for a period of 7 days. The level of reduced glutathione compounds increased significantly at all the arsenate exposure concentrations (Fig 4.22).

Table 4.15 Estimation of Reduced glutathione in *Certophyllum demersum* after day 1, 2, 3, 4, 5, 6, 7.

Si. No.	Conc. of Arsenic	Concentration of GSH in µ mol g^{-1} fw						
		1days	2 days	3 days	4 days	5 days	6 days	7 days
1	1µM	0.20	0.20	0.22	0.23	0.22	0.21	0.17
2	5 µM	0.26	0.27	0.29	0.30	0.26	0.25	0.23
3	10 µM	0.38	0.43	0.44	0.79	0.88	0.83	0.75
4	15 µM	0.33	0.40	0.40	0.73	0.81	0.69	0.38
5	20 µM	0.31	0.36	0.35	0.63	0.49	0.58	0.16

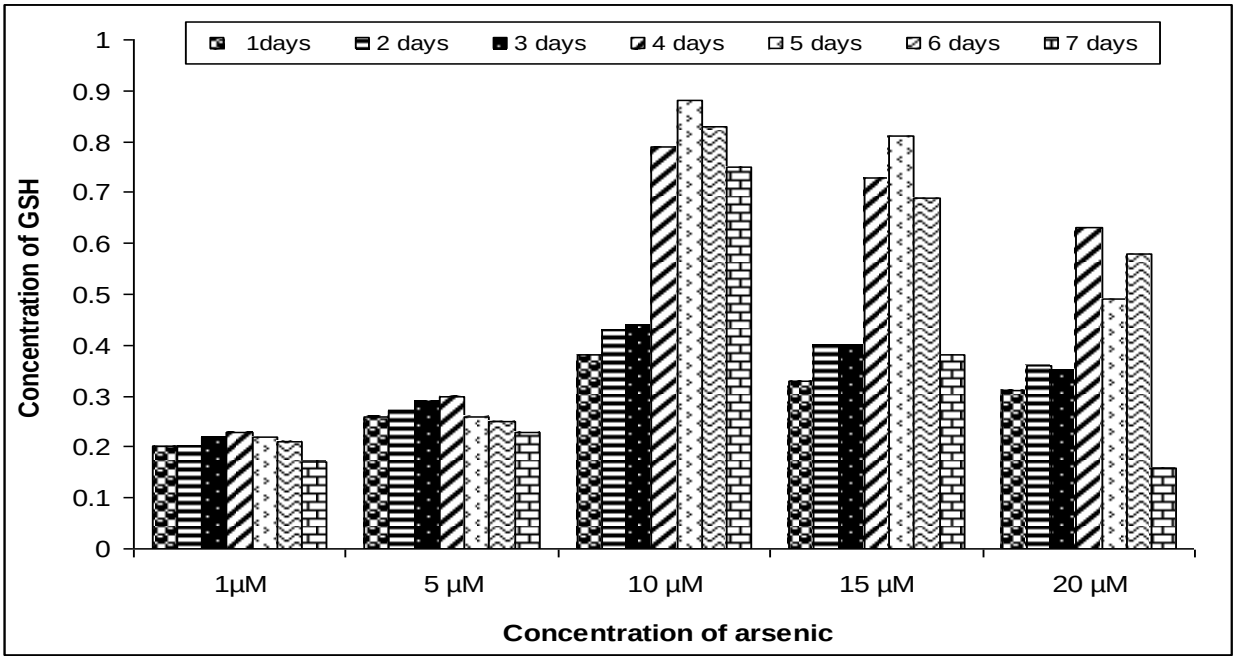


Fig4.22: Effect of various concentration of arsenic on activity of Reduced glutathione of *Ceratophyllum demersum* exposed for different durations (after days 1,2 3, 4, 5, 6, 7).

4.15Molecular weight determination of extracted enzyme by SDS-PAGE

The enzymes extracted from *Ceratophyllum demersum* treated with cadmium and arsenic were cysteine synthetase, glutathione S-transferase, glutathione reductase and arsenate reductase were identified by SDS-PAGE. The molecular weight determined was found to be 42kD, 75kD, 86kD and 45kD respectively Fig 4.23, Fig 4.24).

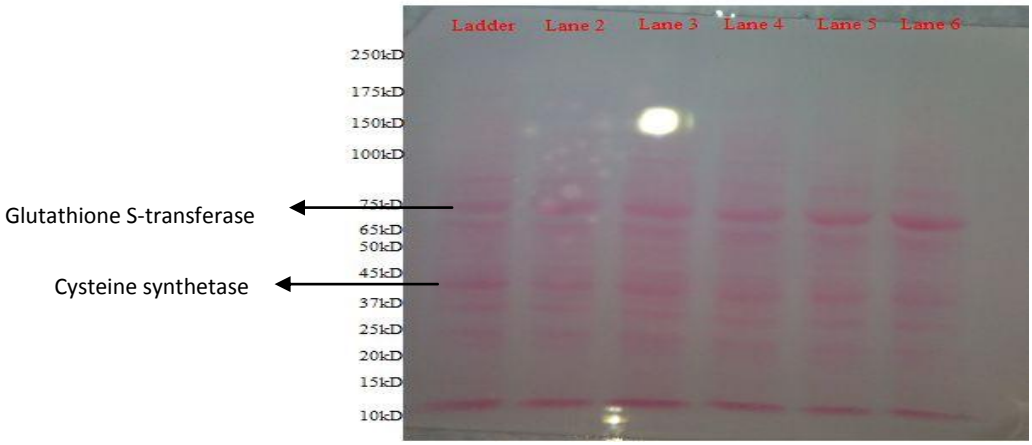


Fig.4.23. Molecular weight determinations from SDS-PAGE (a) Lane 1 shows protein ladder from 10kD to 250kD (b) Lane 2-4 represents protein bands of cysteine synthetase (c) Lane 5-6 represents protein bands of glutathione S-transferase

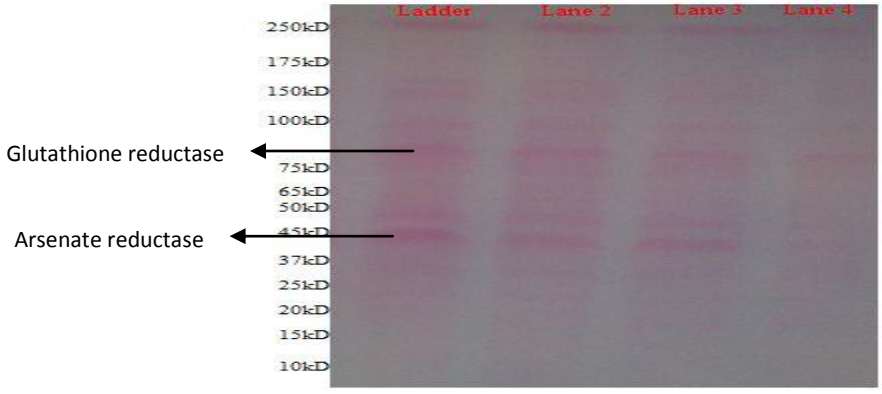


Fig 4.24 Molecular weight determinations from SDS-PAGE (a) Lane 1 shows protein ladder from 10kD to 250kD (b) Lane 2-3 represents protein bands of glutathione reductase (c) .Lane 4represents protein bands ofarsenate reductase

5.0 Discussion

Zinc-Cadmium interaction was studied on *Ceratophyllum demersum* after day 2, day 4, day 6, day 8, day 10 and day12. It was observed from the results that with increasing concentration of Zinc along with the Cadmium, there was reduction in growth of the plant. Cd-Zn interaction clearly identified the potential toxicity of Cd and the role of Zn in antagonizing Cd toxicity. Similarly**Aravind and Prasad, (2005)** reported that the interaction between an essential micronutrient, Zn and a toxic non-essential element, Cd has been comprehensively reviewed and concluded that the antioxidant property of Zn play an important role in counteracting Cd toxicity.

In the present study, the level of thiols and activity of related enzymes were investigated in *Ceratophyllumdemersum* plants to analyze their role in combating the stress caused upon exposure tocadmium (Cd; 0–10μM) for duration up to 7 d. Plants showed the maximum accumulation after 7 d. Significant increases in the level of total non-protein thiols (NPSH) as well as upstream metabolites of the PC biosynthetic pathway, cysteine and glutathione (GSH) were observed. In addition, significant increases in the activities of cysteinesynthase (CS), glutathione-S-transferase (GST), glutathione reductase (GR), were noticed in response to Cd. In conclusion, under Cd stress, plants adapted to a new metabolic equilibrium of thiols through coordinated synthesis and consumption tocombat Cd toxicity and to accumulate it. Similarly**Mishra *et al., (*2009)** reported thatthe level of thiols and activity of related enzymes were investigated in conetail (*Ceratophyllum demersum L.*) plants to analyze their role in combating the stress caused upon exposure tocadmium (Cd; 0–10μM) for a duration up to 7 d. Plants showed the maximum accumulation of 1293μg Cd g^{-1} dw after 7 d at 10μM.

Ceratophyllum demersum L. is known to be a potential accumulator of arsenic (As), in the present study; it was analyzed that biochemical responses of *Ceratophyllum* plants to arsenate exposure toexplore the underlying mechanisms of As detoxification. Plants efficiently tolerated As toxicity up to concentrations and durationsof 7d. Significant increases were observed in the levels of various thiols including the activities of enzymes ofthiolic metabolism as well as arsenate reductase (AR). These primary responses probably enabled plants to detoxify at least some part of AsVthrough its reduction and subsequent complication. Simultaneously, a significant increase in the activities of antioxidant enzymes was observed and hence plants did not experience oxidativestress when exposed to 50μMAsV for 4 d. Exposure of plants to higher

concentrations (250μMAsV) and/or for longer durations (7 d) resulted in a significant increase in the level of As and an inverse relationship between As accumulation andvarious detoxification strategies was observed that lead to enhanced oxidative stress and hampered growth. Similarly **Mishra et al, (2008)** reported that *Ceratophyllum demersum* L. is known to be a potential accumulator of arsenic (As), but mechanisms of As detoxification have not beeninvestigated so far. In the present study, biochemical responses of *Ceratophyllum* plants to arsenate (AsV; 0–250μM) exposure were analyzed toexplore the underlying mechanisms of As detoxification. Plants efficiently tolerated As toxicity up to concentrations of 50μMAsV and durationsof 4 d with no significant effect on growth by modulating various pathways in a coordinated and complementary manner and accumulated about76μgAs g^{-1} dw. Exposure of plants to higher concentrations (250 μMAsV) and/or for longer durations (7 d) resulted in a significant increase in the level of As (maximum 525μg g^{-1}dw at 250μM after 7 d)and an inverse relationship between As accumulation andvarious detoxification strategies was observed that lead to enhanced oxidative stress and hampered growth.

6.0 Summary and Conclusion

The increased loading of heavy metals in the aquatic ecosystems furnished imbalance state and threatened the health of the native biota growing under such abnormal habitat conditions consequently, accumulation of high concentration of heavy metals such as Cd, Cu, Co, Cr, Hg, Ni, Pb and Zn has been recorded which in turn are being assimilated and transferred within food chains by the processes of bioaccumulation and bio magnifications. In the present study plants of *Certophyllum demersum* was collectedand grown for six months in large hydrophobic tubes.The effect of zinc ion concentration was studied on plant; the concentrations used for the study were 10µg/ml, 50µg/ml, 100µg/ml, 150µg/ml, 200µg/ml and 300µg/ml for 7 days in 10% Hoagland media. After day 1 there is no significant effect was observed on plant for all concentration of zinc. After day 2, 3 and 4 change in colour from green to yellow was observed with different colour intensity.It was observed that after day 5, 6 and 7 the leaves of *Certophyllum demersum* become black in colour the intensity of blackening in colour was increased as concentration of zinc ion increased.

Accumulation of Cd by *C. demersum* plants was found to be correlated to both concentration and duration of the treatment. The plant showed maximum accumulation of cadmium after 7 day at 20µM concentration.

The effect of Zinc-Cadmium interaction was studied on *Ceratophyllumdemersum* after day 2, day 4, day 6, day 8, day 10 and day12. It was observed from the results that with increasing concentration of Zinc along with the Cadmium, there was reduction in growth of the plant.

The level of thiol compounds increased significantly at all the cadmium exposure concentrations. The maximum level of thiol compound was observed at 10µM after 3 days.The levels of cysteine synthetase compounds increased significantly at all the cadmium exposure concentrations. The maximum level of cysteine synthetase was observed at 10µM after 3 days.

The level of glutathione S-transferase compounds increased significantly at all the cadmium exposure concentrations. The maximum level of Glutathione-S-transferase was observed at 10µM after 4 days.

The level of glutathione reductase compounds increased significantly at all the cadmium exposure concentrations. The maximum level of glutathione reductase was observed at 10µM after 4 days.

Accumulation of arsenic was found to depend on both concentration and duration of exposure and showed a linear increase. The plant showed maximum accumulation of arsenic after 7 day at 20µM concentration.

The level of cysteine compounds increased significantly at all the arsenate exposure concentrations. The maximum level of cysteine concentration was observed at 15µM after 4 days.

The level of reduced and oxidized glutathione compounds increased significantly at all the arsenate exposure concentrations. The maximum reduced glutathione concentration was observed at 10µM and 20µM respectively.The level of enzyme extracted from plant treated with arsenate increased significantly at all the concentrations. The maximum level of arsenate reductase was observed at 15µM on 3rdday.

The enzymes extracted from *Ceratophyllum demersum* were treated with cadmium and arsenic were cysteine synthetase, glutathione S-transferase, glutathione reductase and arsenate reductase were identified by SDS-PAGE. The molecular weight determined was found to be 42kD, 75kD, 86kD and 45kD respectively.

It can be concluded that Cd-Zn interaction clearly identified the potential toxicity of Cd and the role of Zn in antagonizing Cd toxicity. The inhibitory effect of Zn on Cd was reflected directly on analysis by accumulation by*Ceratophyllum demersum.* Plants possessed high potentiality to accumulate Cd with adequate capacity for its detoxification up tomoderate exposure concentrations. Exposure to high Cd concentration for longerdurations disturbed this coordination owing to which plants experiencedtoxicity. Thus C. demersum plants may be utilized for theremediation of moderately Cd contaminated water bodies. Further, the mechanistic details of Cd detoxification may provide tool togenetically engineer these plants to improve their tolerance and to develop an efficient phytoremediation technology.

However, from the present study increase in Asconcentration for duration (7 d) lead to disturbance in coordinationof various defense strategies and consequently toxicityto plants. These insights into the mechanistic details of Asdetoxification in *Ceratophyllum* plants may prove helpful togenetically engineer these plants to improve their tolerance toAs and enhance phytoremediation potential.

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APPENDIX

1.0 List of Media and their composition

1.1 Hoagland media composition

- 2M KNO_3 — 202 g/L
- 2.5M Ca $(NO_3)_2 \cdot 4H_2O$ — 236 g/0.5L
- 2.5Iron (Sprint 138 iron chelate) — 15 g/L
- 1.5 M $MgSO_4 \cdot 7H_2O$ — 493 g/L
- 1M NH_4NO_3 — 80 g/L
- H_3BO_3 — 2.86 g/L
- $MnCl_2 \cdot 4H_2O$ — 1.81 g/L
- $ZnSO_4 \cdot 7H_2O$ — 0.22 g/L
- $CuSO_4 \cdot 5H_2O$ — 0.05 g/L
- $H_3MoO_4 \cdot H_2O$ — 0.09 g/L
- $Na_2MoO_4 \cdot 2H_2O$ — 0.12 g/L
- 1M KH_2PO_4 (pH to 6.0) — 136 g/L

1.2 Acid ninhydrin reagent

- **Reagent 1.** 250mg ninhydrin+ 6ml acetic acid+4ml 0.6M-phosphoric acid
- **Reagent 2** 250mg ninhydrin+ 6ml acetic acid+4ml conc. HCL

1.2 Bradford reagents

- 2.5% Coomassie blue — 2.5ml CBB + 97.5ml d.w.
- 95% Ethyl alcohol — 95ml ethanol + 5ml d.w.
- 85% Hydrogen phosphate — 85ml H_3PO_4 + 15ml d.w.

1.3 List of buffers

1.3.1 Sodium Phosphate-NaCl buffer (1:1)

- Buffer A — 1M NaCl (pH 7)
- Buffer B — {0.1M Sodium Phosphate Dibasic
  0.1M Sodium Dihydrogen Orthophosphate}
  (pH 8.0)

1. 4 Determination of Cadmium

- Cadmium chloride 1μM - 20μM
- $HCLO_4$:HNO_3 1:3(v/v)

1.5 Hoagland media composition

- 2M KNO_3 202 g/L
- 2.5M Ca $(NO_3)_2$•$4H_2O$ 236 g/0.5L
- 2.5Iron (Sprint 138 iron chelate) 15 g/L
- 1.5 M $MgSO_4$•$7H_2O$ 493 g/L
- 1M NH_4NO_3 80 g/L
- H_3BO_3 2.86 g/L
- $MnCl_2$•$4H_2O$ 1.81 g/L
- $ZnSO_4$•$7H_2O$ 0.22 g/L
- $CuSO_4$•$5H_2O$ 0.05 g/L
- H_3MoO_4•H_2O 0.09 g/L
- Na_2MoO_4•$2H_2O$ 0.12 g/L
- 1M KH_2PO_4 (pH to 6.0) 136 g/L

1.6 Quantification of Cadmium

- 16mM Nitric acid 10ml
- Hydrogen peroxide 1ml

1.7 Estimation of thiol compound

- Ninhydrin 250mg
- Acetic acid 6ml
- 0.6M phosphoric acid 4ml
- Conc. HCl 4ml
- Aq. Cysteine HCL 0.5ml
- Ethanol 95%

1.8 Assay of Cysteine Synthetase

- 50mM Phosphate buffer 2ml
- 1mM EDTA 2ml
- 2mM dithiothreitol 2ml
- Triton X-100 0.1%
- PMSF 0.5mM
- O-acetyl L-serine 12.5mM
- 7.5% trichloroacetic acid 0.1ml

1.9 Assay of Glutathione S- transferase

- 100mM Phosphate buffer 2ml
- 0.1mM EDTA 2ml
- 1% polyvinyl pyrrolidone 2ml
- 0.1mM 1-chloro 2,4-dinitro benzene 2ml

1.10 Assay of Glutathione reductase

- 100mM Potassium Phosphate buffer 1ml
- 0.5mM EDTA 1ml
- 3mM 5,5 dithiobis (2-nitrobenzoic acid) 0.5ml
- H_2O_2 0.5ml
- 2mM NADPH 0.1ml

1.11 Effect of Arsenic on plant

- Na_2HAsO_4 1-20µM
- HNO_3 1ml
- 10%HCl 10ml
- 10%KI 10ml
- 5% ascorbic acid 10ml

1.12 Assay of arsenate reductase

- 50mM MOPS 1ml
- 50mM MES 1ml
- 375µM NADPH 1ml
- Yeast 0.2µM
- 1mM GSH 0.5ml
- 0.5µM glutardixin-2
- 10mM MAsV

1.13 Bradford reagents

- Crude sample
- Distilled water
- Water bath
- Spectrophotometer
- Coomassie blue
- 95% Ethyl alcohol
- 85% Hydrogen phosphate

1.14 SDS-PAGE

- ❖ 1.5M Tris HCl pH 8.8
- ❖ 0.5M Tris HCl pH 6.8
- ❖ 10% SDS Buffer
- ❖ Acrylamide Solution
  Acrylamide:Bisacrylamide (40%)
- ❖ APS(Ammonium Persulphate) 1%

1.14.1 Tank Buffer(1 liter)

- ❖ Glycine 72 gm
- ❖ SDS 5 gm
- ❖ Tris-HCL 15 gm
- ❖ Water 1000ml

1.14.2 Resolving Gel (For 10 ml)

- ❖ Distilled water 4.0 ml
- ❖ Acrylamide solution 3.330 ml
- ❖ 1.5M Tris HCl 2.5 ml
- ❖ 10% SDS 100µL
- ❖ 10% APS 50µL
- ❖ TEMED 15µL

1.14.3 Stacking Gel (For 3 ml)

- ❖ Distilled Water 1.7 ml
- ❖ Acrylamide solution 500µL
- ❖ 0.5M Tris-HCl 750µL
- ❖ 10% SDS 30µL
- ❖ 10% APS 20µL
- ❖ TEMED 8µL

1.14.4 Loading Dye

- ❖ 0.5M Tris HCl 125µL
- ❖ Distilled Water 125µL
- ❖ Glycerol 400µL
- ❖ 10%SDS 300µL
- ❖ β-mercaptoethanol 100µL
- ❖ Bromophenol Blue 600µL

1.14.5 Staining Solution (100 ml)

- ❖ Methanol 45 ml
- ❖ Acetic Acid 10 ml
- ❖ Water 45 ml
- ❖ Comassie Brilliant Blue 0.5gm

1.14.6 Destaining Solution

- ❖ Methanol 45 ml
- ❖ Acetic Acid 10 ml
- ❖ Water 45 ml