EFFECTS OF HEAVY METALS AND NaCl ON GROWTH AND ALDH EXPRESSION IN LYCOPERSICUM ESCULENTUM VAR SRIJANA

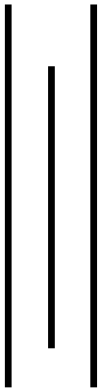
A

Dissertation

In Partial Fulfillment of the Requirements for the Award of

The Degree of Master of Science in Biotechnology

2012



Submitted to

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

By

Padam Shekhar Bhatt

Roll No: BT 016/066

Regd NO: 5-1-61-128-2003

EFFECTS OF HEAVY METALS AND NaCl ON GROWTH AND ALDH EXPRESSION IN LYCOPERSICUM ESCULENTUM VAR SRIJANA

MSc Thesis

2012

Submitted to

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

By

Padam Shekhar Bhatt

Supervisors:

Dr. Tribikram Bhattarai
Assoc. Professor
Central Department of Biotechnology,
Tribhuvan University,
Kirtipur, Nepal

Dr. Lakshmaiah Sreerama
Professor
Department of Chemistry And Physics
St. Cloud State University
Minnesota, USA

Central Department of Biotechnology

TRIBHUVAN UNIVERSITY,

Kirtipur, Kathmandu, Nepal

Date: 29-11-2012

RECOMMENDATION

This is to certify that Mr. **PadamShekhar Bhatt** has completed this dissertation work entitled "**ALDH Expression and Heavy Metal Accumulation by *Lycopersicumesculentum* Var Srijana Growing in Soil Supplimented with NaCl, $Pb(NO_3)_2$ and $CdCl_2$"** under our supervision**.** This Thesis work was performed for thepartial fulfillment for award of M.Sc. Degree in Biotechnology under the course code BT 621. The result presented here is his original findings. I, hereby, recommend this thesis for final evaluation.

Supervisors:

Dr. Tribikram Bhattarai
Assoc. Professor
Central Department of Biotechnology,
Tribhuvan University,
Kirtipur, Nepal

Dr. Lakshmaiah Sreerama
Professor and Chairperson
Department of Chemistry And Physics
St. Cloud State University
Minnesota, USA

Central Department of Biotechnology

TRIBHUVAN UNIVERSITY

Kirtipur, Kathmandu, Nepal

Tel. No.: +977-1-4336221

Date: 29-11-2012

CERTIFICATE OF EVALUATION

This is to certify that this thesis entitled **"ALDH Expression and Heavy Metal Accumulation by *Lycopersicumesculentum* Var Srijana Growing in Soil Supplimented with NaCl, $Pb(NO_3)_2$ and $CdCl_2$"** is new of its kind in Nepal and presented to evaluation committee by Mr. **Padam Shekhar Bhatt** is found satisfactory for the partial fulfillment of Master of Science in Biotechnology.

……

Dr. Tribikram Bhattarai
Supervisor
Head of Department
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

……

External Examiner

……..

Prof. Dr. Lakshmaiah Sreerama
Supervisor (External)
Dept. of Chemistry and Physics
St. Cloud State University
St Cloud, MN, USA

……

Internal Examiner
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

This thesis is dedicated to my Parents

and beloved Elder Sisters

Acknowledgement

I am glad that I can convert my research work in this format. And to come up with this success there is profound role of some good soles to whom I want to acknowledge.

First of all I would like to express my deepest gratitude to my principal supervisor **Dr. Tribikram Bhattarai**, Central Department of Biotechnology, TU for his generous support, motivation and guidance during the research period. I would like thank especially for his creative suggestion, enthusiastic comments and assessing me to relevant literature concerning the research study that make convert the work into concrete solid form.

I'm indebted to supervisor **Prof.Dr. Lakshmaiah Sreerama,** Dept. of Chemistry and Physics, St. Cloud State University, St Cloud, MN, USA for his invaluable advice, suggestions and motivation. His visit to Nepal as Fulbright scholar is proved as the golden arena of our life. The sensation of generosity, the true meaning of research and everlasting encouragement are being boomed to us by such great personality I have ever met in my life.

I express my gratitude to **Dr. Kumudini Shakya, Dr. Baby Sharma and Dr. Mukesh Kumar Chettri**, Botany Department, Amrit Science Campus, TU for their invaluable suggestions, Support and guidance for completing a part of the research in Amrit Science Campus. I would like to particularly extent my fervent gratitude to Dr. Kumudini Shakya for her insight support and guidance.

I'm also obliged to**Dr. Rajani Malla,Dr. Mohan Kharel, Dr. Deepak Raj Pant, Dr. Giri Raj Tripathi, Dr. Krishna Das Manandhar and Dr.** all the teachers and staffs of Central Department of Biotechnology for supporting me directly and indirectly.

I want to express my heartfelt thanks to my colleague Mr. Raju Pandey for his cooperative help and encouraging study with me throughout the research work. My thanks go next to my friends of my batch and juniors who helped and supported me during my work.

Finally I would like to thanks all good souls and well-wishers who are missed here to mention. Last but above of all, I would like to express my deep gratitudeto my father Jay Dev Bhatt and My mother Chandra Devi Bhatt for their overwhelming support of all spheres.

Padam Shekhar Bhatt

Abstract

A greenhouse pot experiment was conducted to determine the effects of heavy metals (Cd, Pb) and NaCl on growth morphology, accumulation, and ALDH expression in *Lycopersicumesculentum* Var Srijana. The experiments were performed to examine the effects on the seed germination, root elongation, shoot elongation, Al dehyde dehydrogenase (ALDH) expression and accumulation of Pb, Cd and Na in the selected tomato variety grown on soil-verm icompost substrate supplemented with different concentration of $Pb(NO_3)_2$, $CdCl_2$and NaCl. The selected heavy metals were treated in different concentrations ranging from 0.1, 0.3, 0.5, 1 mM and salt (NaCl) in 25, 50, 75, 100 mM separately in soil – vermicom post media (2:1) in plastic pots. The seed germination, root and shoot growth were affected by these treatments at higher concentrations. The increasing concentrations of heavy metals and salt treatments have in hibitory effects on germination, root length (RL), shoot length (SL)thoughthe plants seemto germinate and grow efficiently at all treatments.Treatments at lower concentration of $Pb(NO_3)_2$(at 0.3, 0.5mM) and of salt (at 50 mM) doses, were found to be stimulating the root and shoot elongation whereasat higher concentration of heavy metals(at 1 mM of $Pb(NO_3)_2$, $CdCl_2$) andNaCl (at 75mM, 100mM) causes the delayed germination and retardationin root and shoot length.Aldehyde dehydro genases (ALDHs) play a major role inthe detoxification processes of aldehydes generated inplants in various metabolism and when exposed to abiotic stress. ALDH catalyzes the oxidation of the chemically toxic aldehydes intermediates into corresponding carboxylic acids, reducing pyridine nucleotide. The simplest method of ALDH assay is spectrophotometric method and is used in this study. The spectrophotometric reading showed to have highest activity (693.39 mIU/mg proteins) at 0.3 mM $CdCl_2$ treatment after2ndweek of stress exposure. It was also found that the lower doses of stresses are more efficient at inducing greater ALDH responses than higher doses which shows lower ALDH activity. NATIVE-PAGE of the same prepared samples showed some bandswhich confirmed that the spectrophotometric measurements was of ALDH. The number, type and character of the isotypes of ALDH produced but couldn't be characterized.Some additional bandsin treatment plant tissue homogenate samplescan be regarded as produced due to stress exposure. The metal accumulation experiments performedunder stress of$Pb(NO_3)_2$, $CdCl_2$and NaCl suggests that their uptake takes place at all treatments. The metals were determined by using atomic absorption spectrophometry (AAS). Highest uptake of heavy metal occurs at 1mM$Pb(NO_3)_2$ and Na at 100mMNaCltreatments and least accumulation occurs at 0.1 mM$Pb(NO_3)_2$ and 25 mMNaCl.From this study, it can also be concluded that the amount of accumulation increases as the concentration of heavy metals and Na increases in the substrate.

Aldehyde dehydrogenase, *Lycopersicumesculentum* var Srijana, Abiotic stress, Heavy metals, Uptake

CONTENTS

Page No.

Glossary Acronyms

ALDH	Aldehyde dehydrogenase
ALDH-SF	Aldehyde dehydrogenase superfamily
As	Arsenic
Cd	Cadmium
$CdCl_2$	Cadmium chloride
Co	Cobalt
Cr	Cromium
Cu	Copper
DNA	Deoxyribonucleic Acid
Fe	Iron
H_2O_2	Hydrogen peroxide
Hg	Mercury
LEA	Late embryogenesis abundant protein
mg	milligram
mIU	Milliinternational units
mM	Millimolar
MN	Minnesota
Mn	Manganese
Mo	Molybdenum
mRNA	Messenger ribonucleic acid
Na	Sodium
NaCl	Sodium Chloride
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
Ni	Nickel
Pb	lead
$Pb(NO_3)_2$	Lead Nitrate
ROS	Reactive oxygen species
Sb	Antimony
TU	Tribhuvan University
U	Uranium
USA	United states of America
UV	Ultraviolet
V	Vanadium
W	Tungsten
Zn	Zinc

ABA	Absicic acid
GMPs	Genetically modified plants
MDA	Malondialdehyde
KO	Knockout
T-DNA	transfer Deoxyribonucleic acid
WT	Wild type
RL	Root length
SL	Shoot length
GF	Germination frequency
NATIVE-PAGE	NATIVE-polyacrylamide gel electrophoresis
AAS	atomic absorption spectrophotometry
WHO	World Health Organization
gm	Gram
cm	Centimeter
USEPA	United States of Environmental Protection Agency
PC	Phytochelatins
MT	Metalothionins
HMS	Heavy metals
Cys	Cysteine
SOD	Supeoxide Dismutase
APX	Ascorbate peroxidase
POX	Peroxidase
CAT	Catalase
EC	Enzyme commision
CoA	Coenzyme A
TCA	Tricarboxylic acid
NCBI	National Centre of Biotechnology Information
AGNC	ALDH Gene Nomenclature Committee
MIPS	Molecular Imprint Polymerse
EST	Expressed Sequence Tag
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
BADH	Betaine Aldehyde Dehydrogenase
CMO	Choline Monoxygenase
GSH	Glutathione
NARC	National Agricultural Research Council
EDTA	Ethylene Diaminetetraacetic acid
µl	Microlitre
nM	Nanomolar
M	Molar
D/W	Distilled water
SDS-PAGE	Sodium Dodecyl Sulphate- Polyacrylamide Gel electrophoresis
$(NH_4)_2S_2O_8$	Ammonium per Sulphate
MS	Murashige and Skoog
NPK	Nitrogen Phosphorus and Potassium
TEMED	Tetramethylethylenediamine
H_2O	Water

HNO_3	Nitric Acid
ppm	Parts per Million
ANOVA	Analysis of Variance
cDNA	Complementary Deoxyribonucleic acid
Pro	Proline
HPLC	High Performance Liquid Chromatography
ELIZA	Enzyme Linked Immunosorbant assay

CHAPTER 1.

INTRODUCTION

1.1. Background

The stresses exposed to plants are divided into two types; abiotic and biotic. Biotic stresses include the stresses imposed by the living beings such as pathogens, viruses, human and plants themselves.Biotic stresses are not the subject under study in current research. Abiotic stresses are diverse environmental factors. There are various environmental stresses some of them include salinity, water deficit, drought, UV, heavy metals, presence of hazardous chemicals etc. Since the plants are immobile, they cannot escape the stresses that environment exposes to them as animals and humans can do. So some sort of special physiological and other specific mechanisms hasevolved in them to remain viable and mitigate the effects of stresses. Stress features in plants have been studied widely in number of plant species (Zhang *et al*., 2003). Many mysteries have been understood and revealed to know the adaptation process of plants exposed to stresses (Bartel and Salamini, 2001). And these stresses must be studied very precisely since the meaningful stresses bring the crops and other plants to disastrous consequences and stresses in combination has more intense and life threatening effects which determine plant distribution and the productivity of the species.

Effects of the major stresses were studied in the present dissertation research.Heavy metals are so intimately mixed up with the biotic factors in the environment that make it the inseparable part of the biosphere. Among fifty three heavy metals in nature only seventeen are needed to living cells (Weast, 1984). Among these also fe, Mo, Mn are micronutrients; Zn, Ni, cu, V, Co, W and Cr are the elements of low importance called trace elements. And these elements are toxic beyond limits. Heavy metals like Cd, Pb, As, Ag, Sb, U and Hg have no biological functions and are regarded as toxic to living organisms (Godbold and Huttermann 1985;Nies, 1999). Heavy metals once accumulated can be never destroyed and nor can be altered by chemical or physical means and are circulated in the ecosystem. Heavy metals present in atmosphere are ultimately accumulated in the soil through precipitation and fall out (Bachaver 1973). Vascular plants are exposed very readily to the

shower of potentially toxic heavy metals from the air and equally absorb heavy metals from soil viaroot system. Toxic metal tolerance in plants occurs either by detoxification mechanism or by accumulation in special tissue and cell organelle (Bringezu*et al*., 1999) or in vacuolesor in cell wall (Chettri *et al*., 2000).Soil salinity is a major constraint to crop production because it limits crop yield and restricts use of land previously uncultivated. The United Nations Environment Program estimates that approximately 20% of agricultural land and 50% of cropland in the world is salt-stressed (Flowers and Yeo, 1995). High salinity affects plants in several ways: water stress, ion toxicity, nutritional disorders, oxidative stress, alteration of metabolic processes, membrane disorganization, reduction of cell division and expansion, genotoxicity (Zhu, 2007). Together, these effects reduce plant growth, development, reproduction and survival. During the onset and development of salt stress within a plant, all the major processes such as photosynthesis, protein synthesis and energy and lipid metabolism are affected (Paridaand Das, 2005). The osmotic effects imposed by salinity stress can be observed immediately after salt application and are believed to continue for the duration of exposure, resulting in inhibited cell expansion and cell division, as well as stomatal closure (Flowers, 2004; Munns, 2002). During long-term exposure to salinity, plants experience ionic stress, which can lead to premature senescence of adult leaves, and thus a reduction in the photosynthetic area available to support continued growth (Cramer and Nowak, 1992). In fact, excess sodium and more importantly chloride has the potential to affect plant enzymes and cause cell swelling, resulting in reduced energy production and other physiological changes (Kawasaki *et al*., 2001). Ionic stress results in premature senescence of older leaves and in toxicity symptoms (chlorosis, necrosis) in mature leaves due to high Na^+ which affects plants by disrupting protein synthesis and interfering with enzyme activity (Hasegawa*et al*.,2000;Munns, 2002). Many plants have evolved several mechanisms either to exclude salt from their cells or to tolerate its presence within the cells for their survival in the particular environment.

Plants have their inherent mechanism to cope with the stresses and adverse environmental conditions in order to adjustin the particular environmental condition for their growth and reproduction(Kheir*et al*., 2012). Firstly, they have some sort of morphological adjustment like induction of deep root system, stomatal closure, and reduction in leaf size to reduce up the water loss etc.(Nahar and Gretzmacher, 2011). Some physiological strategies of plants

may include ion exclusion, ion transport and cell wall modification by an increased flexibility, osmotic adjustments and osmoprotection (Ingram and Bartels, 1996;Pastori and Foyer, 2002; Yamaguchi-Shinozaki *et al*., 2002). Similarly,there aresome sorts of proteins responsible for the metal sequestration in certain part of the plant or cell organelle for eg. Metallothionins and Phytochelatins. In addition, late embryogenesis abundant (LEA) protein and chaperon like proteins are also induced to protect plant from water stress and are involved in protecting macromolecules like enzymes, lipids and mRNA (Ingram and Bartels, 1996; Yamaguchi- Shinozaki *et al*., 2002). LEA proteins accumulate mainly in embryos during seed desiccation and in response to water stress. Specific antioxidant molecules are also produced in stressed plants which include ascorbate peroxidases, glutathione peroxidases and gluthationereductase (Mittler, 2002). Recent studies have undisclosed some new facts and mysteries regarding the expression of some stress responsive genes like ALDH, even though more than 1300 geneof such characteristics are found to be produced inthe experimental plant *Arabidopsis thaliana*(Hoth*et al*., 2002).

Aldehyde molecules are most common and long lived intermediates in most cellular pathways such as carbohydrate, amino acid, protein, lipid or steroid metabolism (Yoshida *et al*., 1998; Kirch*et al*., 2004). However, when produced in excessive amounts, they can have detrimental effects on cellular metabolism because of their chemical reactivity (Lindahl, 1992). Aldehydes can cause genotoxic effects (i.e. chromosomal aberrations and DNA adducts) (Wacker*et al*., 2001), lipid peroxidation resulting in the loss of membrane integrity or modification of proteins subsequently causing cellular and developmental arrest (Chen and Murata, 2002). On the other hand, recent findings suggest that some aldehydes may also have some signaling functions (Weber *et al*., 2004). Therefore, maintaining the concentration of reactive aldehydes at well-balanced, non-toxic levels is crucial for the living organisms. One of the major detoxification pathways of aldehyde molecules is the oxidation of their carbonyl groups into carboxylic acids by $NAD(P)^+$-dependent aldehyde dehydrogenase (ALDH) [enzyme class (EC) 1.2.1.3] (Perozich*et al*., 1999; Vasiliou*et al*., 1999). The aldehyde dehydrogenase superfamily (ALDH-SF) of $NAD(P)^+$-dependent enzymes, in general, oxidize a wide range of endogenous and exogenous aliphatic and aromatic aldehydes to their corresponding carboxylic acids with concomitant conversion of $NAD(P)^+$ into NAD(P)H and play an important role in detoxification (Kirch*et al*., 2004). Important roles

for ALDHs have been reported for all organisms. Various plant ALDHgenes have been reported to be activated by environmental stress such as dehydration, salinity or excessive light (Sunkar*et al*., 2003). The accumulation of aldehydes above the biological needs leads to the production of reactive oxygen species (ROS) such as singlet oxygen, hydroxyl radical, superoxide and H_2O_2 (Bolwell, 1999). Under excessive generation of ROS, the capacity of the electron transport chain exceeds the consumption of reduction equivalents delivered to the stroma side of the chloroplastic thylakoid membranes leading to oxidative stress/damage (Niyogi*et al*., 1997). The ALDH proteins are represented by enzymes which share a number of highly conserved residues necessary for catalysis and cofactor binding and they include such proteins as retinal dehydrogenase, 10-formyltetrahydrofolate dehydrogenase, non-phosphorylating glyceraldehyde 3-phosphate dehydrogenase, delta(1)-pyrroline-5-carboxylate dehydrogenases, alpha-ketoglutaricsemialdehyde dehydrogenase, alpha-aminoadipicsemialdehyde dehydrogenase, coniferyl aldehyde dehydrogenase and succinate-semialdehyde dehydrogenase.The reference plant that has been studied completely for the molecular relationship of the entire genome is *Arabidopsis thaliana. A. thaliana*contains 14 ALDH genes and corresponding protein belongs to nine ALDH protein families (Kirch*et al*., 2004). These genes are expressed in response to different types of stresses in the environment. Members ofALDH family 3 and 7of *A. thaliana,* ALDH3I1 and ALDH7B4 is found to show the strong transcriptional activation in osmotic stress, ABA, high salinity, heavy metal stressesetc. (Kirch*et al*., 2005).

Over centuries, human industrial, mining and military activities as well as farming and waste practices have contaminated large areas with high concentrations of heavy metals, salts and organic pollutants (Peuke and Rennenberg, 2005). In addition to their negative effects on ecosystems and other natural resources, these sites pose a great danger to public health, because pollutants can enter food chain through agricultural products or leach into drinking water (Peuke and Rennenberg, 2005). Cleaning up contaminated soil is a costly enterprise, the overall cost to remediate affected sites may reaches billions of dollers. Furthermore, current methods of soil remediation do not really solve the problem. In Germany, for instance, only 30% of soils from contaminated sites are cleaned up in soil remediation facilities (SRU, 2004); the remaining soil must be stored in waste disposal facilities. This does not solve the problem but merely transfers it to future generations. Obviously, there is an

urgent need for alternative, cheap and efficient methods to clean up heavily polluted industrial areas.Phytoremediation uses wild or genetically modified plants (GMPs) to extract a wide range of heavy metals and organic pollutants from the soil, achieved as a new technology using plants to remove pollutants from the environment.Phytoextraction removes metals or organics from soils by accumulating them in the biomass of plants. The technique of phytoremediation exploits the use of either naturally occurring metal hyperaccumulator plants or genetically engineered plants (Cunningham *et al*., 1997). Phytoremediation, the use of plants for environmental restoration, consists of four different strategies for the remediation of polluted soils, sediments or waters namely phytoextraction, phytostabilization, rhizofiltration and phytovolatilization.

1.2.Current studies

Plantshave developed various strategies to acquire stress tolerance. Among them expression of ALDH protein during stress condition is the subject of research in the present study. Aldehyde dehydrogenase represents a group of enzymes, which may play a role in stress relevant detoxification processes. Most of the studies yet done are mainly concerned with the stress mitigating aspect of plant ALDH genes under stress conditions and their relative functions associated with abiotic stress tolerance. The major stress molecule is Aldehyde and their intermediates which are common by products of number of metabolic pathways (Bartels *et al*., 2001). Therefore, the removal of aldehydes and their intermediates is essential for cellular survival. ALDH catalyze the oxidation of toxic aldehydes to their corresponding carboxylic acid (Sreerama, 2000). Various studies regarding characterization of ALDH have been performed extensively in animals and humans but onlyALDH studies have been done in limited plants. Recently, it has been reported that various plant-ALDH transcripts accumulate in response to environmental stresses (Kirch*et al*., 2001). Current studies in ALDH have been mainly focused on the up-regulation and overexpression of ALDH genes under abioticstress and understanding their mechanism to improve stress tolerance. One of the recent studies conducted by Sunkar*et al*., 2003 involves the preparation of Transgenic plant in which the over expression of a stress-inducible ALDH genesimproves the stress tolerance. In the same study, the isolation of dehydration inducible genes encoding aldehyde dehydrogenase was done from resurrection plant *Craterostigmaplantagineum* and

dessication tolerant plant *Arabidopsis thaliana.* Then these scientists made transgenic plants by transformation Ath-ALDH gene from *Arabidopsis thaliana* making the appropriate construct for the over expression of the particular genes. Analysis of transcript accumulationexperiment revealed that Ath-ALDH3 is induced in response to NaCl, heavy metals, and chemicals inducing oxidative stress (Sunkar*et al*., 2003). This enhanced production of ALDH protein makes the plant more tolerant to stresses than the non-transformed or control plants.

Another studyconducted by Rodrigues*et al*., 2006 was concerned with the purpose to compare the potential of a cytoplasmic and a chloroplastic stress inducible ALDHin conferring stress tolerance under different stress conditions. In the study,it has been demonstrated that constitutive or stress-inducible expression of both the chloroplasticALDH3I1and the cytoplasmic ALDH7B4confers tolerance to osmotic and oxidative stress. Kotchoni*et al*., 2006 showed that stress tolerance in ALDH-transgenic plants is accompanied by a reduction of H_2O_2 and malondialdehyde (MDA) derived from cellular lipid peroxidation.In the same studythe involvement of ALDHs in stress tolerance was shown by the analysis of ALDH3I1and ALDH7B4T-DNA knockout (KO) mutants. Both mutant lines exhibited higher sensitivity to dehydration and salt than wild-type (WT) plants. The results indicate that ALDH3I1 and ALDH7B4 not only function as aldehydedetoxifying enzymes, but also as efficient reactive oxygen species (ROS) scavengers and lipid peroxidation-inhibiting enzymes (Kotchoni*et al*., 2006).

Since decades, plants are being used as the tool to uptake and eradicate the contaminants and pollutants from the soil. Using plants as a tool for the uptake of pollutants from soil is a cheap, labor intensive, efficient,easy, can be carried outin situ, environment friendly and use up the solar energy(Peuke and Rennenberg, 2005) is a technique called as phytoremediation. Scientists are involved in researchesin search of the plant acting as hyperaccumulator that can accumulate the pollutants more efficiently and fast.For phytoremediation it is needed theplant which can accumulate high concentrations of the pollutants and the mechanism ofpollutants accumulationin plants should be studied before the application of the technique.

In one of the studies conducted by Hussein *et al*. 2008, transgenic tobacco plants engineered with bacterial *mer*A and *mer*B genes via the chloroplast genome were investigated to study the uptake, translocation of different forms of mercury (Hg) from roots to shoots, and their volatilization.Volatilization of elemental mercury is the final step in the detoxification pathway of organic-Hg. The results of this study showthat insertion of *mer*A and *mer*B genes into the tobacco plastid genome resulted in significant volatilization of Hg (Hussein *et al*., 2008). Similarly, another recent finding on Arsenic research suggest that genetically engineered plants can transport arsenic in above ground tissues, reduce it to arsenite and sequester it in thiol-phosphate complexes (Kotchoni, 2006). Some of the recent studies found out that the physiology of plant hyperaccumulatorsalso works in radioactive isotopes like Uranium(Li *et al*., 2011).

1.3. Hypothesis

Tomatoes are cultivated as the major crop plants in most parts of globe and almost in all parts of Nepal. Various abiotic stresses affect the growth and development of plants differently. Pb, Cd and NaCl in soil when exceed certain limit cause stress to plants and in turn plant react by different physiological responses. One of the hypotheses of present research work was the analysis of the effects of heavy metal and NaCl stresses on seed germination and growth of tomato. Such stresses in other plants had increased ALDH activity in order to tolerate the stress effects. Therefore tomato plant should also show higher ALDH activity during stress to tolerate the stresses.

Plants show the incredible behavior to accumulate heavy metals of soil, thus helping to remediate the soil pollution. Thus, the present study hypothesized that tomato as one of the fast growing plants and should be efficient accumulator of heavy metal of soil.

1.4. Objectives

- To find out the effects of heavy metals and NaCl on germination frequency, shoot and root growth of Srijana variety of tomato.
- To determine of ALDH activity and confirmation of the activity by Native PAGE.
- To quantify the accumulation amount of Cd, Pb and Na within the plant tissues grown on the $Pb(NO_3)_2$, $CdCl_2$, and NaCl spiked soil-vermicompost substrate.

1.5. Rationale

Normal physiology of plant would be changed when plants areex posed to metals and salt. As morphology is the manifestation of physiology, heavy metals and salt would affect morphological characteristics like alteration in RL, SL. Root growth will be highly affected, since root is considered as the primary absorbing organ. If root cannot develop well then the entire plant would be misbalanced and overall expected yield of the plant would obviously decrease. Therefore, the effects of Pb, Cd and Na on growth morphology of tomato plant arestudied.

In the present study, ALDH gene expression analysis is taken into account to find out the trend of the stress responsive ALDH expression in stressed plants in comparison to control plant. The study is experimentally designed so as to measure the ALDH expression level and confirmation through the NATIVE polyacrylamide gel electrophoresis (NATIVE-PAGE). Induction of ALDH enzymes responding to the contaminants would postulate the fact of presence of inherent ALDH genes in Srijana variety of tomato and their possible role in cellular toxicity remediation.

The tomato variety selected for the study is one of the most expensive tomato seeds ever found in market of Kathmandu valley. Such tomato variety developed in our own country calls for the study including the aspects of its growth, physiology and biochemical effects.Soil pollution from heavy metal and NaCl has increased to the point that it endangers human life in some areas, and the reduction and eventual elimination of pollution in these areas is immensely urgent. The experiments on accumulation of metals by the variety would ascertain its potential to uptake the pollutants. The result of these experiments would predict whether the particular variety of plant is hyperaccumulator or metal indicator or metal inhibitor.

1.5. Scope

The scope of the study is diverse in terms of finding out the physiological behavior of plant in response to stresses. Basics of such stress biology open the gate forfurther searching the effect of more complex factors and aspects regarding the stress biology.

The physiochemical and nutritional features of the soil andvermicompost gives the sufficient pool of data and could be useful for any further work to be done using the soil of TU garden and vermicompost of Horticulture department, Kirtipur. The morphological features of the experimental plant such as GF,RLand SL with response to heavy metals and NaClcan be collected and the information can be used further in cultivation purpose.

Finding of stress induced ALDH genes may be used for biomonitoring of heavy metal pollution in soil. Increased ALDH activity if found responsible for the tolerance of heavy metal effects in plants, such ALDH gene can be used in genetic engineering of crops and horticulture plants for heavy metal tolerance.The variety of tomato tested if found to be good in accumulation of heavy metals the plant can be recommended as the phytoremediator plant.

Chapter 2.

Literature Review

Plants are sessile and sensitive organisms that encounter a variety of environmental stresses throughout the life cycle. Plant development and productivity are negatively affected by environmental stresses. During the last decade, cultivated land in several regions of the world has been affected by environmental stresses like salt, cold, drought and UV, which hinders crop cultivation and yield. Every year countries lose a substantial amount of money from reductions in crop productivity caused by abiotic stresses. It is predicted that these environmental stresses will become more intense and frequent with climate change, especially global warming.The abiotic factors determine plant distribution and the productivity of crop species.

Plants have tendency to cope with the environment in which they live and must acclimatize in order to ascertain their viability and perpetuation. Environmental stresses are the external stimuli that are responsible for imposing some sort of detrimental effects (Kim *et al*., 2003). For plants, environmental stresses are grouped into two categories; biotic stress (pathogen attack) and abiotic stress. The abiotic stresses includes water deficit, heavy metal pollution, chilling and freezing, heat stress, UV irradiation, salinity, oxygen deficiency and nutrient deficiency (Yamaguchi and Kamiya 2001). Abiotic stresses differentially affectthe various developmental stages (seed germination, seedling development, seed maturation and senescence) (Ingram and Bartels 1996).

2.1. Heavy Metals and salinity: two types of detrimental abiotic Stresses

Heavy metals are so mixed with air, water, soil, and biota that have become the inseparable part of the biosphere. Density of metals more than 5gm/cm^3 are defined as heavy metals. Altogether 53 heavy metals are present in nature. Among them only 17 are available for the living cells, basedon their solubility under physiological condition. Some heavy metals like Fe, Mg, Mn are important as micronutrients and some metals like Zn, Ni, Cu, V, Co and Au are of low demand and trace elements but cause the toxic effects on exceeding dose. Elements V, Cr, Mo, Mn, Fe, Cu, Ni, Zn, Sn,and Se are enzymatic elements and have catalytic function(Markert, 1994). Heavy metals like Cd, Pb, As, Hg, Ag, Sb, and U have no any biological significance as nutrients and seem to be toxic to living beings(Godbold and Hutternann, 1985).Heavy metals once accumulated as contaminants can neither be destroyed nor be altered by chemical and physical means, and are circulated in the ecosystem. The heavy metals present in the atmosphere get ultimately mixed with soil

through precipitation and fall out(Jagrati*etal*., 2012).Contaminated air and water are the source of heavy metal in soil. In agriculture the practice of using the inorganic fertilizer in increased amount is another source of heavy metal contamination in soil. So in summary, the sources of heavy metal contamination can be divided into natural sources and anthropogenic sources. Natural sources of heavy metal contamination include volcanic eruptions, continental dusts, earthquake like phenomenonand anthropogenic sources include various industrial activities like metal smelters, blast furnace, electrolysis, cement industry, iron and steel production, vehicular traffic, household waste, sewage sludge, agricultural wastes(food additives, phosphate fertilizers, pesticides, herbicides), petroleum combustion, nuclear power plant, high-tension lines, heating systems, waste burning etc. (Morselli*et al*. 1999). These heavy metals have very disastrous effects to human health (Rosenstock, 2003). United States of environmental Protection agency (USEPA) has stated as-Pb, Cd are potent carcinogen.

Cadmium can cause cardiovascular diseases, hypertension, hypercaciurea, proteinuria, renal tubular dysfunction, glycosuria and osteoporosis (Sabath*et al*., 2012). Lead is one of the top 10 pollutants.Lead concentration of 1.4 µg/ltr in blood shows neurological effectsand cause adverse effects to the human reproductive systems and may cause cancer (Canfield *et al*., 2003). Lead present in blood, in excess amount enters the soft tissues (brain) and ultimately deposits in bone by replacement with ca^{2+} ion.

Salinity is one of the most serious factors limiting the productivity of agricultural crops, with adverse effects on germination, plant vigor and crop yield (Fowers, 2004). Salinization affects many irrigated areas mainly due to the use of salty water. Worldwide, Salinity affects 7% of the world's land area, which amounts to 930 million ha (Szabolcs 1994; based on FAO 1989 data). The area is increasing; a global study of land use over 45 years found that 6% had become saline. High salinity affects plants in several ways: water stress, ion toxicity, nutritional disorders, oxidative stress, alteration of metabolic processes, membrane disorganization, reduction of cell division and expansion, genotoxicity (Zhu, 2007). Together, these effects reduce plant growth development and survival. During the onset and development of salt stress within a plant, all the major processes such as photosynthesis, protein synthesis and lipid metabolism are affected (Parida and Das, 2005). During initial exposure to salinity, plants experience water stress, which in turn reduces leaf expansion. The osmotic effects of salinity stress can be observed immediately after salt application and are believed to continue for the duration of exposure, resulting in inhibited cell expansion and cell division, as well as stomatal closure (Flowers, 2004; Munns, 2002). During long-term exposure to salinity, plants experience ionic stress, which can lead to premature senescence of adult leaves, and thus a reduction in the photosynthetic area available to support continued growth (Amirjani, 2011). Ionic stress results in premature senescence of older

leaves and in toxicity symptoms (chlorosis, necrosis) in mature leaves due to high Na^+ which affects plants by disrupting protein synthesis and interfering with enzyme activity (Munns, 2002). Many plants have evolved several mechanisms either to exclude salt from their cells or to tolerate its presence within the cells.

2.2. Effects of salt stresses in plants

Soil salinity affects the plant growth by various physiological and biochemical processes. Plants suffer from salt stresses by following ways:

1. Osmotically induced water stress: The ability of plant to take up water is reduced by the salt stress called osmotic stressaffecting growth and morphology (khalib*et al*, 2010). Salt don't accommodate in growing tissues at concentrations that inhibit growth, as the rapidly elongating cells can store the salt arrives in the xylem within their expanding vacuoles. So, the salt taken up by the plant does not directly inhibit the growth of new leaves (Munns, 2005). The degree to which the salt causes the damage to the pant depends upon the time scale of response and whether slow or abrupt the stress is imposed (Munns*et al*., 2000).
2. Specific ion toxicity: Toxic ions such as, Na^+, Cl^- uptake and accumulation from the soil have some detrimental functions. The cause of injury is probably due to excessive salt load in the vacuole. Rapidly salt begin building up in cytoplasm and thus inhibits enzyme action and disrupts the normal metabolic function. Alternatively they might build up in the cell walls and dehydrate the cell (Munns, 2005).
3. Nutritional imbalance: The resultant osmotic stress may disturb the uptake and utilization of essential nutrients and also in toxic ion accumulation. As a result of these changes, the activities of various enzymes and the plant metabolism are affected (Munns, 2002; Lacerda*et al*., 2003). The interactions of salts with mineral nutrients may result in considerable nutrient imbalances and deficiencies. Ionic imbalance occurs in the cells due to excessive accumulation of Na^+ and $Cl^-$and reduces uptake of other mineral nutrients, such as K^+, Ca^{2+}, and Mn^{2+} (Karimi*et al*., 2005). This k^+ deficiency may cause chlorosis and then necrosis (Gopa and Dube, 2003). Salt stress decreases the Ca^{2+}/Na^+ ratio in the root zone, which affects membrane properties, due to displacement of membrane-associated Ca^{2+} by Na^+, leading to dissolution of membrane integrity and selectivity (Kinraide, 1998), diminishing plant growth.
4. Reactive oxygen species: Exposure of plants to salt stress can up-regulate the production of reactive oxygen species (ROS) such as H_2O_2 (hydrogen peroxide), $O_2^{\cdot -}$ (superoxide), $^1O_2$ (singlet oxygen) and $^{\cdot}OH$ (hydroxyl radical)(Pitzschke*et al*., 2006). These ROS which are generated in cytosol, chloroplast, mitochondria and the apoplasticspace, causes various fatal phytotoxic reactions such as lipid peroxidation, protein degradation and DNA mutation (Pitzschke*et al*., 2006). Membrane injury induced by salt stress is related to an enhanced

production of highly toxic ROS (Shalata*et al*., 2001). Rise in ROS production may result stomatal closure, causing a decrease in CO_2 concentration inside the chloroplasts. This in turn causes a decrease in $NADP^+$ concentration with the concomitant generation of ROS (Foyer and Noctor, 2003). Photosynthesis damage is among the most affected by ROS.

2.3. Effects of heavy metal stresses in plants

High levels of metals in soil can be phytotoxic. Heavy metals interferes with several metabolic reactions of the plant, causing toxicity manifested as the reduced seed germination, root and shoots growth and phytomass, chlorosis, photosynthetic impairing, stunting and finally plant death (Gardea-Torresdey*et al*., 2004). Plant roots participate primarily in the heavy metal cation uptake. The toxic effect of heavy metal activity is connected to their concentration in cells. The abnormal concentration cause disturbances in cell membrane functioning in the photosynthetic and mitochondrial electron transport and in the inactivation of many enzymes active in the basic cell metabolism regulation, which as the result leads to diminishing energy balance and disturbances in cell mineral nutrition (Gondek and Filipek-Mazur, 2003).

2.4. Plant defense mechanism against stresses

Plants use various morphological and physiological strategies to cope with the stress conditions. Morphological approaches of plant responses to abiotic stress (water deficit) include: development of deep root systems, stomatal closure, and reduction of surface proportionally to the entire volume of the plant by dropping-off of leaves and retention of water molecules in specific water tissues especially in succulent plants (Ingram and Bartels 1996, Holmstrom*et al* 2000). The physiological strategies of plant adaptation to stress include: ion exclusion, ion transport and cell wall modification by an increased flexibility, osmotic adjustments and osmoprotection (Ingram and Bartels 1996, Pastori and Foyer 2002). Osmotic adjustment is a physiologically efficient mechanism by which plants produce osmoprotectants, therefore protecting cells by turgor maintenance of roots and shoots in response to water deficit as well as salt stress (Yamaguchi-Shinozaki *et al*., 2002). Osmoprotectants such as proline, glycine betaine and sugars (mannitol, fructans, saccharose) are well documented and known to help plants to overcome the effects of water deficit and salt stress (Bartels, 2001). One of the current findings suggests that some metal transporter proteins are present such as phytochelatins (PCs) and metallothinins (MTs). These ligands bind metals forming ligand-metal complexes and subsequent compartmentalization of these complexes either in the cell wall or vacuoles, making the heavy metals less toxic to cells.

2.4.1. Phytochelatins (PCs)

Phytochelatins are the family of cys-rich polypeptides with several repetitions of the gamma- Glu-cys dipeptide followed by terminal glu. Phytochelatins have been isolated from variety of plant species and some microorganisms and are responsible for the detoxification of heavy metals such as Cd,Zn, Pb, Cu,Hg and metalloids (As, and Se) (Vivares*et al*., 2005). Studies performed on various plants suggest that PCs play central role in homeostasis of HMs in plant that they regulate cations availability in plant cells (Guo, 2008). Thus PCs is involved in detoxification of HMs and more functionally mitigation of oxidative stress.

2.4.2. Metallothionins (MTs)

Metallothionins represent superfamily containing low molecular weight and large content of cysteine residues. These proteins are specialized for metal binding, HMs and their intracellular sequestration. In animals and fungi, MTs form complexes with HMs and the transcription of MT genes is regulated by heavy metals (Thiele, 1992). These polypeptides present distinct differences from members of the other MT families, such as the three Cys-rich regions as well as the longer linker sequences (Freisinger, 2008). In plant, MTs are induced in response to oxidative stress, abscisic acid (ABA), heat/cold shock, wounding, viral infection, senescence, salt stress and sucrose starvation (Chyan, 2005). Several data demonstrated the role of MTs in HM detoxification and homeostasis.In recent years, it has been investigated and shown that MTs may play a role in HM detoxification either because they bind HMs, or because they function as antioxidants (Akashi, 2004). The evidence is largely based on MT gene expression studies, and on yeast complementation experiments with plant MT genes (Kohler*,* 2004; Hassinen, 2009). Gene expression studies were performed to quantify mRNA levels in different tissues, at different developmental stages and under stress conditions such as HM exposure. MT genes appear to be differentially regulated in a tissue-specific manner and in relation to developmental stage and also in response to a number of stimuli, including heavy metals (Castiglione, 2007).

2.4.3. Specific proteins

Plants accumulate specific proteins such as LEA proteins (late embryogenesis abundant proteins) and chaperones (heat-stress induced proteins) in response to abiotic stress (Ingram and Bartels, 1996). LEA proteins and chaperones have been reported to be involved in protecting macromolecules like enzymes, lipids and mRNA (Ingram and Bartels, 1996; Yamaguchi- Shinozaki *et al*., 2002). LEA proteins accumulate mainly in embryos during seed desiccation and in response to water stress. The correlation between LEA gene expression and/or LEA protein accumulation and stress tolerance in a number of plant species provided

evidence for the role of the LEA proteins in stress tolerance mechanisms (Ingram and Bartels, 1996).

On the other hand, abiotic stress leads to the production of reactive oxygen species (ROS), which cause extensive damage to cells and inhibit photosynthetic reactions (Kaiser, 1979).The damaging phenomenon referred to as oxidative damage is the consequence of oxidative stress, the stress resulting from almost all abiotic stresses (Ramanjulu and Bartels, 2002; Schutzendubel, 2002). Stressed plants increase the production of specific antioxidants, which are needed for the reduction of active oxygen species.Plants use a diverse array of antioxidative enzymes, including superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POX) and catalase (CAT), in addition to low molecular weight antioxidants, such as reduced glutathione and ascorbic acid (Mittler,2002). Recently, Aldehyde dehydrogenases have been reported to display a probable antioxidative ability in *Arabidopsis thaliana* (Sunkar*et al*., 2003). Molecular studies have revealed that protein phosphorylation and dephosphorylation via kinases and phosphorylases are important signal transduction mechanisms used by plants to sense the external stimuli and to program an adequate response based on extremely fine and highly coordinated regulatory system to express stress-related gene and/or repress several other set of genes whose functions disrupt the stress adaptation mechanism (Kovtun*et al* 2000). A genome-wide survey of gene expression in *Arabidopsis thaliana* revealed that about 1354 genes whose expression was either enhanced or suppressed after ABA treatment (Hoth*et al* 2002, He and Gan 2004), were related to abiotic stress tolerance mechanisms.

2.5. Aldehyde dehydrogenase: Importance in cellular metabolism

Aldehydes are common and long lived intermediates in most cellular pathways generated from various endogenous sources such as carbohydrates, amino acids, proteins, lipid or steroid metabolism (Kirch*et al*. 2004) and exogenous sources such as abiotic and biotic stress (Sunkar*et al*. 2003, Sophos and Vasilio 2003). Aldehydes can cause genotoxic effects i.e. chromosomal aberration and DNA adducts (Wacker*et al*., 2001), protein inhibition,lipid peroxidation, resulting in the loss of membrane integrity or modification of proteins subsequently causing cellular and developmental arrest (Lindahl 1992; Chen *et al.* 1998; Chen and Murata 2002).These stress molecules are the major cause of cellular damage and cell death. One of the major detoxification pathways of aldehyde molecules is the oxidation of their carbonyl groups into carboxylic acids by $NAD(P)^+$-dependent aldehyde dehydrogenase (ALDH)[enzyme class (EC)1.2.1.3S](Yoshida *et al*., 1998; Perozich*et al*., 1999; Vasiliou*et al.,* 1999). Important roles for

ALDHs have been reported for all organisms. Various plant ALDHgenes have been reported to be activated by environmental stress such as dehydration, salinity or excessive light, heavy metals (Kirch*et al*., 2001; Sunkar*et al*., 2003). The accumulation of aldehydes above their normal biological needs leads to the production of reactive oxygen species (ROS) such as singlet oxygen, hydroxyl radical, superoxide and H_2O_2, which under excessive generation, disturbs the system so as the electron transport chain exceedingly consume reduction equivalents delivered to the stroma side of the chloroplastic thylakoid membranes leading to oxidative stress/damage.

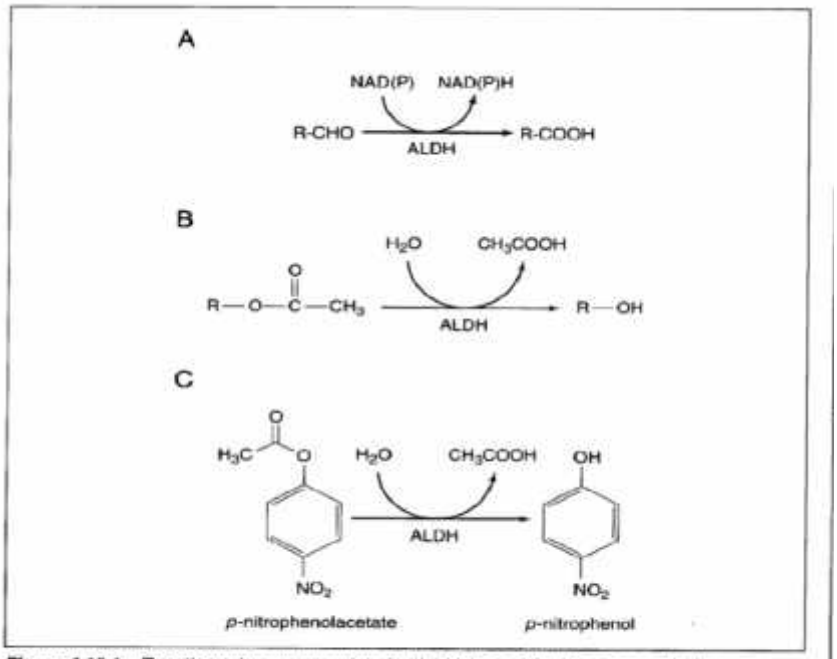


Figure 4.18.1 Reaction schemes associated with aldehyde dehydrogenase activities.

Contributed by Lakshmaiah Sreerama and Norman E. Sládek
Current Protocols in Toxicology (2005) 4.18.1–4.18.59
Copyright © 2005 by John Wiley & Sons, Inc.

Source: LakshmaiahSreerama and Norman E. Sladek, 2005.

ALDHsare widely expressed in tissues and subcellular compartments and are important in cell defense against exogenous and endogenous aldehydes such as those derived from lipid peroxidation (Vasiliou*et al*., 1996). Cytosolic class 3 ALDH enzymes are found to oxidize medium (6 to 9)-chain-length saturated and unsaturated aldehydes but not short chain aliphatic aldehydes, such as malondialdehyde and 4 –hydroxyalkenals (Lindahl and Petersen, 1991), while class 2 ALDHsare largely expressed in cytosol and transported into the mitochondrial matrix space where they are mainly responsible for the oxidation of acetaldehyde generated during *in vivo*oxidation of ethanol coupled with energy production through the use of acetyl-CoA in the TCA cycle (Canuto*et al*.,2001, Tsuji *et al*., 2003). Another possible role of ALDHs is to supply NAD(P)H for respiration. The oxidation of aldehydes into corresponding carboxylic acids by ALDHs concomitantly converts NAD^+ into

NADH in the mitochondria (Kirch*et al*., 2001). This irreversible oxidative reaction contributes to a rapid recovery of respiration and ATP synthesis. Aldehyde dehydrogenases are therefore considered crucial for cellular metabolism due to their multifunctional properties (Vasiliou*et al* 1996, Kirch*et al*., 2001).When such cell treatment resulted in the complete inhibition of the class 3 ALDH, protein synthesis is inhibited followed by cell death. However, class 3 ALDHs oxidizes relatively small amounts of saturated and unsaturated aldehyde molecules. A large number of the other unsaturated, saturated and hydroxylated aldehydes generated during peroxidation of cellular lipids are therefore oxidized by different ranges of other ALDHs that are equally important in cellular metabolism.

Sequence comparisons among ALDH genes from bacteria, animals, and plants have identified three diagnostic amino acid motifs (Kirch*et al*., 2004).

1. The ALDH glutamic acid active site signature sequence MELGGNA (PROSITE PS00687);
2. The Rossmann fold GxGxxG coenzyme binding site; and
3. The catalytic thiol (PROSITE PS00070)

The active ALDH enzyme is homodimeric or homotetrameric in structure. The enzyme has three conserved domains, a $NAD(P)^+$ cofactor-binding domain, a catalytic domain, and a bridging domain; and the active enzyme is generally either homodimeric or homotetrameric. The catalytic mechanism of ALDH enzyme include binding of aldehyde substrate to cofactor binding site and cysteine nucleophile activation takes place forming a oxaniumthiohemiacetal intermediate resulting in hydride transfer to the thioacylenzyme and the release of carboxylic acid eventually. Therefore, ALDH enzymes share a number of highly conserved domains necessary for catalysis and cofactor binding site. They include proteins as retinal dehydrogenase, 10-formyltetrahydrofolate dehydrogenase, non-phosphorylating glyceraldehyde 3-phosphate dehydrogenase, delta(1)-pyrroline-5-carboxylate dehydrogenases, alpha-ketoglutaricsemialdehyde dehydrogenase, alpha-aminoadipicsemialdehyde dehydrogenase, coniferyl aldehyde dehydrogenase and succinate-semialdehyde dehydrogenase. (Ncbi.nlm.nih.gov).

2.6.Aldehyde dehydrogenase: Nomenclature and classification

Currently, 555 independent ALDH genes encoding ALDH proteins or protein like activity have been identified throughout all taxa (Sophos and Vasiliou, 2003).The classification and nomenclature for ALDH proteins has been established by ALDH gene nomenclature committee (AGNC). The committee has deduced and formulated the special criteria and cataloguing system that make it easy to analyze the ALDH genes and protein sequences

(Vasiliou, 1999). Protein sequences that are more than 40% identical to other previously identified ALDH sequences compose a family, and sequences more than 60% identical compose a protein subfamily. Protein sequences that are less than 40% identical would describe a new ALDH protein family. Previous classifications of the ALDH gene superfamily in eukaryotes have identified 21 protein families based upon sequence identity (Skibbe, 2002). To date, plant enzymes are represented in 11 ALDH families: ALDH2, ALDH3, ALDH5, ALDH6, ALDH7, ALDH10, ALDH11, ALDH12, ALDH18, ALDH19 and ALDH21. Three protein families are unique to plants (ALDH11, ALDH19 and ALDH21) (Wood*et al*., 2002) and one of these is apparently unique to mosses (ALDH21)(Chen *et al*., 2002). The Arabidopsis genome is the one of the completely sequenced plant genome, and this allows us for the first time to examine the phylogenetic and molecular relationship of all the ALDH genes in a plant species. Based upon nomenclature developed by the AGNC (Yoshida *et al*., 1999), a revised and unified nomenclature for the Arabidopsis ALDH genes is given and describes the most important structural and functional features of the corresponding ALDH protein families. In addition, the unified nomenclature enables a critical view of a functional classification of ALDH enzymatic activities.

Since all the database and sequence analysis is completely done in *Arabidopsis thaliana*, so for ALDH analysis the database of Arabidopsis is taken as reference (Kirch*et al*., 1998).The database and information of ALDH have been retrieved from the Arabidopsis information centre (TAIR) (www.arabidopsis.org). ALDH genes contained in the Arabidopsis genome encode members of nine ALDH protein families – eight previously identified protein families (ALDH2, ALDH3, ALDH5, ALDH6, ALDH7, ALDH10, ALDH11, ALDH12) and one novel protein family (ALDH22) (Table 2.1). For the classification of each gene family, the root symbol ALDH is followed by the family designation number (1–22), a subfamily designator if required (A,Betc.) and, finally, the individual gene number. For instance, the betaine aldehyde dehydrogenase (BADH) is named as ALDH10A7 in which ALDH represents the superfamily, 10 represents the family designation number, A represents the subfamily designation number and last number 7 represents the gene number. Three of the nine ALDH families in Arabidopsis are represented by more than one gene [family 10 (two members) and families 2 and 3 (three members each)], whereas the remaining six families are encoded by singlecopy genes. It is therefore interesting that no more than four ALDH genes (ALDH6B2, ALDH22A1 and two members of the ALDH3 gene family) are located in non-duplicated regions of the Arabidopsis genome (Figure 2.1). In addition, only three genes (ALDH2B4, ALDH10A9 and ALDH12A1) are found within the same duplicated region and thus might represent ‘direct’ gene duplications, whereas the respective counterparts of the other seven ALDH genes have been lost. Although the three ALDHs show a high protein sequence divergence and even belong to different families, the gene contexts around the ALDH genes

are highly conserved between the duplicated genomic regions of chromosomes III and V. This implies that functional constraints are responsible for the rapid evolution and sequence divergence of these ALDH genes. A phylogenetic tree of the Arabidopsis ALDH sequences and other putative plant ALDHs available in the TAIR, MIPS, NCBI genome and non-redundant EST databases. The root of the tree was placed at the phylogenetically most distantly related ALDH12 family, which shows the least sequence conservation to the rest of the plant ALDHs. The phylogenetic analysis demonstrates that the plant ALDHs split up into ten protein families and confirms the assignment of the Arabidopsis sequences. Although evolutionary relationships could not be clarified for all the different families, the analysis reveals some interesting observations. Family 21, for example, is represented by just one gene from the moss *Tortularuralis*, yet it shows a close relationship to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) family. Similarly, ALDH families 5, 2 and 10 seem to cluster together, and the BADHs (family 10) probably directly diverged from ALDH family 2. Finally, the predicted cytosolic and mitochondrial ALDH forms in family 2 can be clearly separated from each other, which are in accordance with results of a recent characterization of ALDH2 genes from maize and Arabidopsis (Skibbe*et al*., 2002). A complete list of all ALDH sequences known to date along with some annoted data along with evolutionary analysis in eukaryotes is presented by Sophos *et al*. 2001.

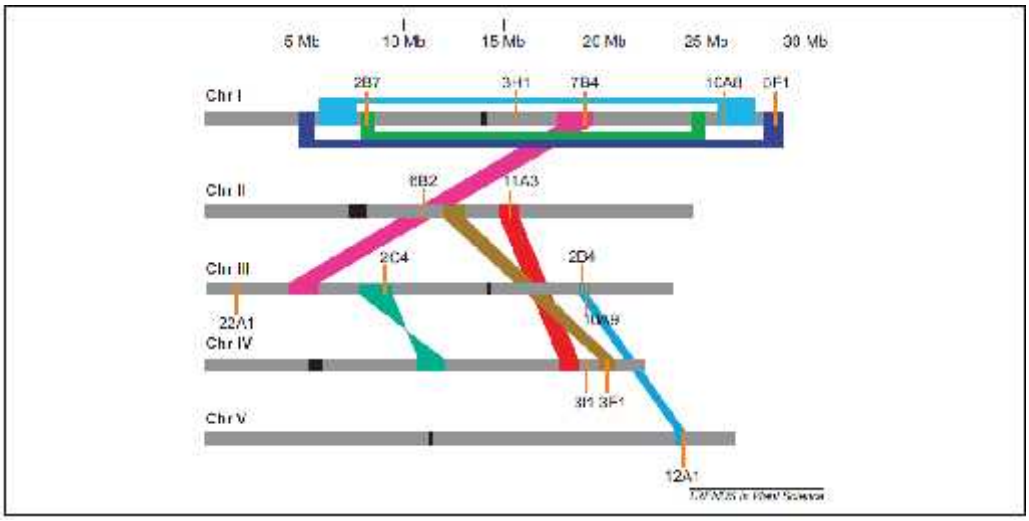


Figure 2.1. Chromosomal positions of Aldehyde dehydrogenase (ALDH) genes in *Arabidopsisthaliana*. Chromosomes I–V (Chr I–V) is depicted as horizontal gray bars with centromeres as black bars. ALDH genes are indicated by vertical orange lines and annotated using the proposed unified ALDH nomenclature. Colored bars denote duplicated regions of the Arabidopsis genome; the twisted colored bar indicates that the duplicated regions are in reverse orientation.

Source: Kirch*et al.,* 2004

Table 2.1. Arabidopsis thaliana Aldehyde dehydrogenase gene superfamily

Gene annotation	Locus	GenBank Accession number	Short informational summary			Putative function or pathway
			position	Length(Kb)	Location(Chromosome)	
ALDH2B4	At3g48000	AF349447	17716867-17720030	3.15	3,Mitochondria	Response to cadmium ion, mitochondrial ALDH
ALDH2B7	At1g23800	AF348418	8412051-8414879	2.83	1, Mitochondria	Mitrochondrial ALDH
ALDH2C4	At1g24503	AF349448	8919560-8923073	3.51	3,Mitochondria	Cytosolic ALDH, produces sinapaldehyde catalyzing both oxidation of conifer aldehyde and sinapaldehyde from ferulic acid and sinapic acid respectively
ALDH3F1	At4g36250	AJ584644	17150869-17153578	2.71	4, Endoplasmic reticulum	Variable substrate ALDH, constituitively expressing
ALDH3H1	At1g44170	AY072122	16796400-168--321	3.92	1, Endoplasmic reticulum, Vacuole membrane, Plastids	Responsive to dessication, salt stress and absisic acid stimulus, constituitively expressing
ALDH3I1	At4g34240	AJ306961	16389620-16392824	3.2	1,chloroplast	Stress responsive(dehydration and ABA)
ALDH5F1	At1g79440	AF117335	29882210-29887912	5.2	1,Mitochondria,Chloroplast	Encodes a mitochondrial succinic semialdehyde dehydrogenase(SSADH)
ALDH6B2	At2g14170	NM_126989	5977356-5981899	4.54	2	Methyl malonatr-semialdehydedehydragenase
ALDH7B4	At1g14170	AJ584645	20195264-20199136	3.87	1	Responsive to dessication, salt stress, ABA stimulus
ALDH10A8(BADH)	At1g74920	AY093071	28139009-28142744	3.74	1	BADH
ALDH10A9	At3g48170	AF370333	17786140-17790008	3.87	3	Mitochondrial BADH
ALDH11A3	At2g24270	AY037205	10327050-10329941	2.89	2	GAPN
ALDH12A1	At5g62530	AY039787	25099003-25103298	4.3	5	Responsive to salt stress, protection from proline toxicity
ALDH22A1	At3g66658	AJ584648	2095105-2099151		3, Endoplasmic reticulum	Novel ALDH, Constituitevely expressed

Source: Kirch et al., 2004

2.7. Plant ALDHs responsive to abiotic stresses

Plants, since sessile organism, have several induction mechanisms for mitigating the abiotic stresses, one by expressing specific set of genes. Different ALDHs are expressed in different parts of plant tissues differently with response to adaptation of plant to abiotic tissues. Different experiments were performed for searching the fact of ALDH expression in stress condition. Different study regarding the search for stress responsive ALDH expression has been performed includes transcript accumulation in plants analyzed under salt, H_2O_2, paraquat, draught, heavy metals (Kirch*et al*., 2001). ALDH3I1 ALDH3F1, ALDH7B4 genes were differently induced in shoot and root by ABA (SchlingenSiepen, 2003). Similarly, Cp-ALDH genes were differently induced in shoot and roots by stress treatments (Kirch*et al*., 2003). From these findings, it can be found that, ALDH3I1 and Cp-ALDH genes are induced in response to different stress treatments, indicating their relation between ALDH protein accumulation and ROS biosynthesis. This hypothesis is made confirmed with ALDH accumulation after stress treatments. Cp-ALDH shows the increased accumulation from 6hrs to 72 hrs of ABA treatment, while the ALDH3I1 proteins was found to progressively accumulate from 24 hrs to 72 hrs of ABA, paraquat and H_2O_2 treatments (Kirch*et al*., 2001). Recently it has been also found that signaling transduction pathway produced by stresses is correlated with root hair elongation, stomatal closure (Foremann*et al*., 2003). Such kind of signal transduction modulated by stress lead to the up regulation of stress inducible ALDH genes in plants. So, ROS confers the beneficial action in plants as it induces the expression of defense related genes sush a GST (glutathione S transferase) and PAL (Phenylalanine- ammonia lyase) (Grant *et al*., 2000). The study conducted by kirch*et al*. (2001) showed that ALDH gene family responds to various regulatory pathways confirming the hypothesis of ALDH response to abiotic stresses. Aldehyde dehydrogenase (ALDH) gene expression is observed through the transcript accumulation analysis in various plants in response to several types of abiotic stresses (Kotchoni*et al*., 2006). Up regulation of these genes under abiotic stress conditions is the main focus for its utilization for improving the stress tolerance in plants. ALDHs represent a group of enzymes divided in diverse super families with diverse function including detoxification, intermediately metabolism, osmotic protection and NADPH generation (Perozich*et al*. 1999). ALDH proteins can also be divided on the basis of the substrate specificity basis. Some ALDHs are known as non-specific ALDHs when they react with wide range of substrates and oxidize variety of aliphatic and aromatic aldehydes. This group includes the cytosolic and mitochondrial tetrameric class 1 and 2 ALDHs and the dimeric class 3 ALDHs and these are reported to be associated to carcinogenesis and genetic disorders in case of Humans (Yoshidae*et al*., 1998). Substrate specific ALDHs include the semi aldehyde dehydrogenases (semi ALDHs) such asglutamate semi ALDH,succinate semi ALDHs, aspartate semi ALDHs, 2-

Amino-adipate-6 semi ALDHs and others such as betaine ALDHs (BALDH) or phenylacetaldehyde dehydrogenase (Perozich*et al*. 1999; Sophos *et al*., 2001).

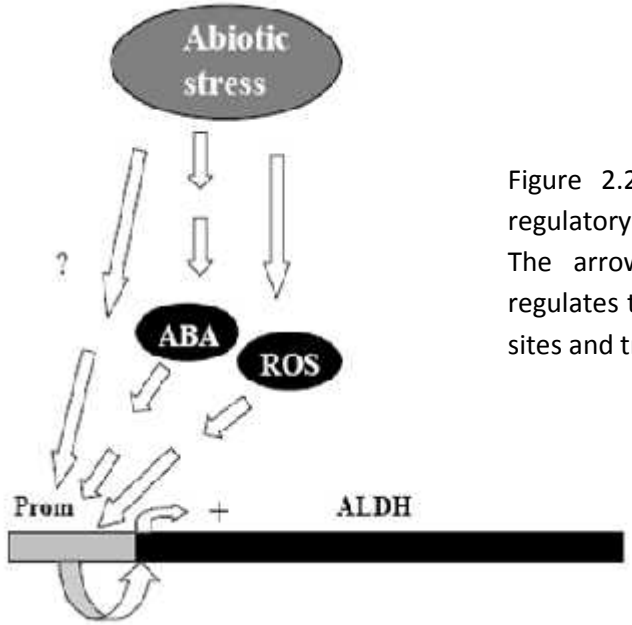


Figure 2.2. A diagrammatic sketch showing the regulatory pathway of stress inducible ALDH gene. The arrow shows the signal transduction which regulates the transcriptional factor binding to specific sites and triggers the up regulation of gene.

Source:Kotchoni, 2004

Arrows indicate the signal transduction cascade leading to the expression of the gene under stressexposure. As a result of the signal transduction, transcription factors bind to the promoter at specificstress responsive sequence elements and trigger the upregulation of the gene(+).ALDHs are one of the subjects of interesting study in various organisms; the molecular and physiological involvement of these enzymes in plant stress tolerance has to be elucidated. They are reported to be involved in ROS scavenging processes (Sunkar*et al*., 2003). Due to continuous stress, many chemically reactive aldehydes are produced most common being Malondialdehyde (MDA), a highly toxic messengers for ROS-induced damage (Kirch*et al*., 2003). So continuous detoxification of such aldehydes by corresponding ALDHs would reduce the oxidative damage. Among the different types of strategies carried by plant to cope with stress condition, osmotic adjustment is an effective mechanism used by plants in such conditions with the accumulation of compatible solutes known as osmoprotectants such as glycine betaine and proline.Glycine betaine is synthesized through the oxidation of choline. This is a two-step reaction where choline is oxidized by choline monoxygenase (CMO) to betaine aldehyde which is converted in glycine betaine by BADH. Therefore, the up regulation of plant BADH genes and production of BADH protein during stress is among the target pathway to acquire stress tolerance (Wood *et al*., 1996). Many plants also accumulate free proline in response to osmotic stress which serves as the hydroxyl radical scavenger (Smirnoff and Cumbes, 1989).In the biosynthesis pathway of proline, glutamate is phosphorylatyed to gamma-glutamyl phosphate which is then reduced subsequently

toproline and the enzyme glutamic-gamma-semialdehyde (GSA) dehydrogenase is required. The resurrection plant *Craterostigmaplantagineum* and the desiccation tolerant moss *Tortularulasis* are important experimental pants for studying the molecular basis of stress tolerance (Bartels, 2001). Cp-ALDH from *Craterostigmaplantagineum*and Ath-ALDH3 from *Arabidopsis thaliana* shows 70% similarity to each other and has been identified and reported to be responsible for stresses response and characterized to be the novel class plant-ALDHs (Kirch*et al*., 2004). So, the new molecular strategy has been developed in recent years that ALDH proteins are being engineered in crop and vegetable plants to get the stress responsive character in the normal wild type plant varieties (Bartel*et al*. 2006). For e.g. *Arabidopsis thaliana* ALDH3I1 gene is transcriptionally activated by abiotic stress and overexpression of the genetically modified gene ALDH3I1 confers tolerance in transgenic plants (Kotchoni*et al*. 2006). Cp-ALDH has been engineered in many experimental plants to get the toxic aldehydes oxidized in their corresponding carboxylic acids (Chen *et al*., 2002). ALDH21A1 gene is described as novel eukaryotic ALDH protein family found in Torula. Transcripts of ALDH21A1 accumulate in response to desiccation, ABA, UV, and NaCl. Molecular study suggests that ALDH21A1 plays an important role in detoxification of aldehyde generated in response to stress and its expression represents the unique stress tolerance mechanism (Chen *et al*., 2002).Table 2.2 summarizes the different ALDH genes isolated from different plants and their involvement in environmental stress responses.

2.8. Reactive oxygen species and ROS-scavenging function of ALDHs

Under extreme adverse conditions, molecular oxygen undergo sequential reduction (Lzumi and Schroedar, 2004) leading to production of superoxide anions, hydrogen peroxide, hydroxyl radical and hydroperoxyl radicals, commonly called Reactive oxygen species (ROS). ROS generally inactivate enzymes and damage important cellular components. Plants deploy antioxidativedefence system for limiting the oxidative damage to plants due to excess production of ROS. Thus, an equilibrium condition must be maintained between ROS level and anti-oxidative capacities under given stress (Bartels, 2001). ROS scavenging system include superoxide dismutase (SOD), which catalyzes the disproportionation of superoxides into molecular oxygen and H_2O_2 (Scandalios, 1993), ascorbate peroxidase responsible for the removal of H_2O_2, and glutathione reductase that is responsible for the protection of thiol groups on enzymes (Goel and Sheoran 2003). The effects of abiotic stress in plants are coupled with the generation of toxic by-products such aldehyde molecule. One of the most important pathways of the aldehyde metabolism is their oxidation into carboxylic acids with concomitant production of NAD(P)H. The oxidation of the carbonyl group is considered

Table 2.2. Improved stress tolerance in transgenic plants carrying ALDH cDNA constructs

Gene name	Identity	Source	Stress regulation and putative function	References
BADH(ALDH10A4)	Betaine aldehyde dehydragenase	Amaranthaushypochondriacus	Induced under osmatic stress and ABA	Legaria el al.,1995
BADH(ALDH10A3)	Betaine aldehyde dehydragenase	Atripleshortensis	Enhanced osmotic stress tolerance	Xiao *et al*.,1995
N/A(ALDH10A11)	Betaine aldehyde dehydragenase	Avicennia marina	Induced under osmotic tolerance	Hibino *et al*., 2001
BADH(ALDH10A2)	Betaine aldehyde dehydragenase	Beta vulgaris	Improved salt and osmotic stress tolerance	McCue and Hanse,1992
BADH(ALDH10A6)	Betaine aldehyde dehydragenase	Hordeumvulgare	Response to osmotic stress and ABA	Ishitani*et al*.,1995
SBALDH(ALDH10A1)	Betaine aldehyde dehydragenase	Sorghum bicolor	Improved draught tolerance	Whitsitt *et al*.,1997
BADH15(ALDH10A)	Betaine aldehyde dehydragenase	Sorghum bicolor	Improved draught tolerance	Wood *et al*.,1996
BADH(ALDH10A7)	Betaine aldehyde dehydragenase	Spinaciaoleracia	Improved draught salt stress tolerance	Weretilnyk and Hanson,1990
BNBTG26(ALDH7B3)	Turgor ALDH like protein	Brassica napus	Improved draught tolerance	Stroeher*et al*.,1995
PSCC26G(ALDH7B1)	Turgor Aldehyde dehydragenase	Pisumsativum	Increased water deficit and osmotic stress tolerance	Guerrero *et al*.,1990
P5Cs-1(ALDH18B1)	Delta 1-pyrroline-5-carboxylate synthase	Medicago sativa	Induces salt stress toleance	Ginzberg*et al*.,1990
P5cS(ALDH18B1)	Delta 1-pyrolline-5-carbolylate synthase	Oryza sativa	Improved salt stressed	Igarashi *et al*.,1997
Pro2(ALDH18B1)	Delta 1-pyrolline-5-carbolylate synthase	Solenumesculentum	Regulation of proline biosynthesis	Maggio *et al*.,1996a
CAtP5CS((ALDH18)	Delta 1-pyrolline-5-carbolylate synthase	Arabidopsis thaliana	Proline synthesis under osmotic stress	Yoshida *et al*.,1995
tompro 1(ALDH19)	Gamma- glutamyl-phosphate reductase	Solenumesculentum	Regulation of proline biosynthesis	Maggio *et al*.,1996b
N/A(ALDH11)	Glyceraldehyde-3-p-dehydragenase	Apiumgraveolens	NADPH supply and manitol biosynthesis	Goa *et al*.,2000
GrapC-Crat(ALDH11)	Cytosolic Glyceraldehyde-3-p dehydragenase	Craterostigmaplantagineum	Induced under desiccation and salt stress	Velasco *et al*.,1994
Cp-ALDH	Aldehyde dehydragenase	Craterostigmaplantagineum	Induced under desiccation and salt stress	Kirch*et al*.,2001
ALDH21A1	Aldehyde dehydragenase	Torularuralis	Indu stress	

Source: Kotchoni and Bartels, 2003

as a general detoxification process and is very crucial in avoiding molecular attack. Therefore, the bonus of NAD(P)H production during ALDH activity indirectly reduces the accumulation of ROS in the cell and promotes thereby the ALDHs as one of the potential ROS-scavenging enzymes. Therefore, ALDHs enhance the efficiency of reducing oxidative damage in cells undergoing environmental stress by detoxifying the cytotoxic products of lipid peroxidation and reducing the accumulation of ROS. Further molecular and biochemical analyses on plant ALDHs are worthy to be accomplished to fully understand the functions of these genes responses to environmental stress.

2.9. Introduction to phytoremediation

Environmental pollution is making the biosphere unsuitable for living beings day by day. So, status is exponentially degrading. Human activities, in search of getting more return from the environment using as many resourcesas they can found, arecreating conditions of life unfavorable for themselves. The sewage sluges from restaurants, industrial work, household wastes, chemical activities and salt used in deicing the road in mountains are making the land around saline. Salinity pollution is increasing and affecting the growth and physiology of plants. The increasing heavy metal concentrations in agricultural lands due to various industrial activities adversely affect crop growth and metabolism consequently lowering yields with concomitant quality degradation. Agricultural areas around industrialized places are contaminated by heavy metals that may originate due to burning of fossil fuels, industrial manufacturing's, and municipal wastes and application of pesticides, fertilizers and sewagesluges to land. This will directly and indirectly harm and cause loss to human beings. The excessive salt and heavy metals would be accumulated by the plants inexceeding limits which would subsequently enter in food chain causing many health disorders in human (Bala and Setia, 1990; Hall, 2002).

Plants, in general, are highly sensitive and show various physiological symptoms in response to toxic heavy metals. As far as the uptake strategies of heavy metals by plants are concerned, generally plants can be considered as metal excluders, metal indicators or metal accumulators (Baker and Walker, 1990). Metal excluders prevent metal uptake from polluted soil however, they may still contain large amounts of metals in their roots. Metal indicator species indicates the presence of metal contamination in the soil by accumulating only low amount of metals in their tissues (Peuke and rennenberg, 2005). Metal accumulator group includes plants that concentrate metals in their above-ground tissues to levels far exceeding those present in the soil. Usually, this latter category includes hyperaccumulator plants capable of accumulating potentially phytotoxic metals to concentrations more than 100 times those found in nonaccumulators. However, some

plants, considered as tolerant, can grow in heavy metals contaminated soil but cannot uptake must pollutants in them(Peuke and Rennenberg, 2005).

2.10. Heavy Metal and Salt Toxicity in Plants and Phytoremediation

Heavy metal stress affects various biochemicals, physiological and structural aspects of plant responses. Toxic levels of heavy metals affect the membrane structural and permeability characteristics, cause inhibition of enzymatic activities, nutrient imbalances, decrease in rates of photosynthesis and transpiration, ROS production, suppress seed germination and yield quality (Kirch*et al*., 2001).High salt concentrations inhibit the growth of tomato plants (National Research Council, USA, 1991). Salt stress inhibits the uptake and transport of potassium, calcium and phosphorus (Flores *et al*. 2001). Salt also causes the oxidative damage to membrane lipids, proteins and nucleic acids (Chinnusamy*et al*., 2005).

Phytoremediation is the biological process of using plants to clean up the pollution in the environment. Phytoremediation is composed of two Greek words phytos means plant and remediare means to remedy. Thus the word phytoremediation is generally used to describe any system where plants are introduced and employed to remove contaminants from in the environment. Phytoremediation (defined as the use of plants for the extraction, immobilization, containment and/or degradation of contaminants) is an emerging technology that holds great promise for the decontamination of a broad range of environmental pollutants, including salt and heavy metals (Glick, 2003). Phytoremediation, the use of plants for remediation of soils and waters contaminated with heavy metals, has gained acceptance in the past ten years as a cost effective and non-invasive technology. This approach is emerging as an innovative tool with greater potential that is most useful when contaminants are within the root zone of the plants (top three to six feet). Further, phytoremediation is energy efficient, cost-effective, aesthetically pleasing method of remediating sites with low to moderate levels of contamination (Schnoor, 1997; Salt *et al*., 1998). The technique of phytoremediation exploits the use of either naturally occurring metal hyperaccumulator plants or genetically engineered plants (Cunningham *et al*., 1997). Phytoremediation, reduce contaminants from the soil, sediments and water by four namely phytoextraction, phytostabilization, rhizofiltration and phytovolatilization. These different phytoremediation technologies are reviewed here with their respective advantages and limitations. An attempt has been made to review plant-based mechanisms which allow metal uptake, accumulation and translocation in plants whose better understanding is needed to further enhance the efficiency of phytoremediation.

Phytoextraction and phytoaccumulation: The extraction, uptake and translocation of heavy metals, in soluble form, from shallow contaminated soil to plant tissues, especially in the

harvestable region of plant like stem and roots. Although the heavy metals are not destroyed, this approach results in considerable reduction in heavy metal mobility. Depending on the type and concentration of the heavy metals, extraction from the plant ashes for recycling purposes might be feasible. This technique is generally used for metals such as nickel, zinc, copper, lead, chromium and cadmium.

The best long-term strategy for improving phytoextraction is to know and use the biological processes involved in metal acquisition, transport and shoot accumulation. In combination with the continuous search for novel phytoextracting plants, this understanding will enable improvements in phytoextraction efficiency. Recent advances in plant biotechnology should provide the means for the efficient mechanistic action of phytoremediation. Unfortunately, we know very little about the biological mechanisms involved in phytoremediation. Roots, which account for 20–50% of plant biomass, extract from the soil and deliver to the shoots most of the elements composing plant tissues, with the exception of carbon. These studies produced some understanding of the processes involved in the acquisition of these essential elements. However, little is known about the mechanisms of mobilization, uptake and transport of most environmentally hazardous heavy metals, such as Pb, Cd, Cu, Zn, U, Sr, and Cs. It is clear that a large proportion of these metals remain adsorbed to solid soil constituents. To acquire these 'soil-bound' metals, phytoextracting plants have to mobilize them into the soil solution. This so-called mobilization of 'soil-bound' metal can be accomplished in a number of ways:

1. Metal-chelating molecules can be secreted into the rhizosphere to chelate and solubilize 'soil-bound' metal. Until now, the major successes in phytoextraction were achieved by applying synthetic chelates to the soil; however, there is a distinct advantage in using natural root exudate compounds for this purpose. Only iron-chelating compounds, termed phytosiderophores, have been studied well in plants. These phytosiderophores are released in response to iron deficiency and can, in principle, mobilize Cu, Zn and Mn from soil. Metal-chelating proteins, perhaps related to metallothioneinsor phytochelatins (Rauser, 1999), may also function as siderophores in plants, although this has never been demonstrated; however, the contribution of phytosiderophores in toxic metal acquisition by the roots of phytoextracting plants remains largely unexplored. It has been recently reported that a Ni hyperaccumulator, Alyssum lesbiacum, may use histidine, an excellent Ni chelator, to acquire and transport Ni (Kramer*et al*., 1996).

2. Roots can reduce 'soil-bound' metal ions by specific plasma membrane bound metal reductases, which may increase metal availability. Pea plants deficient in Fe or Cu have an increased ability to reduce Fe^{3+} and $Cu^{2+,}$ which is coupled with an increased uptake of Cu, Mn, Fe and Mg (Welch, *et al*. 1993).

3. Plant roots can solubilize soil-bound toxic metals by acidifying their soil environment with protons extruded from the roots. A similar mechanism has been observed for Fe mobilization in some Fe-deficient dicotyledonous plants (Crowley*et al*., 1991).

4. Roots can employ rhizospheric organisms (mycorrhizal fungi or root-colonizing bacteria) to increase the bioavailability of metals. However, the significance of microorganisms in the phytoremediation of metals remains largely unknown. It is believed that plant uptake of certain mineral nutrients such as Fe,Mn, Cd and possibly Zn may be facilitated by rhizospheric microorganisms. Mobilized metals are taken up by plant roots from the soil solution and exported to the shoots. Very little is known about toxic metal transport into roots and their subsequent movement within the plant; however, some information is available on the transporter systems involved in the uptake of free and chelated Fe. A putative iron transporter has recently been cloned from Arabidopsis (Eide*et al*., 1996). Ca^{2+} and Mg^{2+} ions, which are present at high concentrations in soil solution and may not require mobilization, may enter the root via either extracellular (apoplastic) or intracellular (symplastic) pathways. These metal ions enter plant cells by an energy-dependent, saturable process via specific or generic metal ion carriers or channels (Clarksonand Luttge, 1989). Theoretically, toxic metals may compete for the same trans-membrane carriers as those used by Ca and Mg; however, the high concentrations of these ions in soil solution make this unlikely. Most environmentally hazardous metals are too insoluble to move freely in the vascular system of the plant. Many form sulfate, carbonate or phosphate precipitates immobilizing these metals in apoplastic and symplastic compartments. Apoplastic transport of these metals is further limited by the high cation-exchange capacity of cell walls, unless the metal ion is transported as a noncationic metal chelate. Earlier studies showed that in hyperaccumulating and non hyperaccumulating plant species, some toxic metals may be transported to the shoot complexed to organic acids, mainly citrate (Senden, 1992). Recent studies of Cd movement in B. juncea, a good Cd accumulator, showed that, in roots, Cd was present as a CdS4 complex, which may contain phytochelatins (Salt*et al*., 1995). In the xylem sap, Cd was coordinated predominantly with oxygen or nitrogen ligands, consistent with the involvement of organic acids (Salt*et al*., 1995).In the leaves, Cd preferentially accumulated in trichomes.

Phytostabilization: Phytostabilization is one of the mostly applied strategies of phytoremediation. This process mainly aims for the prevention of dispersal of contaminated sediments and soil by using plants (mainly grasses) to minimize erosion by wind or rain action. Plants are used to reduce the bioavailability of environmental pollutants. Conditions for this strategy to have maximum advantage are with plants vigorously growing roots; hydrophobic and immobile chemicals.

Rhizofiltration: This mechanism refers to the use of aquatic plants in wetlands or hydroponic reactors. Generally, plants with large root systems are used. The submerged roots of such plants act as filters for the adsorption and absorption of a wide variety of contaminants. This mechanism is commonly used for treatment of industrial discharge, agricultural runoff, metals and radioactive contamination, with the plant densities of 200- 1000 g m-2 and hydraulic detention time of several days. Although the technology is more suitable for decontamination of polluted water or removal of organic and inorganic impurities from wastewater by the use of aquatic plants, terrestrial species can also be used by growing hydroponically or on floating platforms in treatment ponds.

Phytovolatilization: Phytovolatilization is a mechanism by which plants convert a contaminant into volatile form and transpire the detoxified vapor through their aerial organs. The natural ability of a plant to volatilize a contaminant that has been taken up through its roots can be exploited as a natural air-stripping pump system. Volatile pollutants diffuse from the plant into the atmosphere through open stomata in leaves where gas exchange occurs e.g. mercury volatilization.

Table. 2.3. Phytoremediation of heavy metal

Sl. No	Mechanism	Process Goal	Media	Contaminants	Plants	Status of Research
1.	Phytoextraction	Hyper-accumulation, Contaminant extraction and capture	Soil, sediment, sludges	Inorganics: Metals: Ag, Cd, Co, Cr, Cu, Hg, Mn, Mo, Ni, Pb, Zn Radionuclides: ^{90}Sr, ^{137}Cs, ^{239}Pb, $^{238,234}U$	Indian mustard, pennycress, alyssum, sunflower, hybrid poplars	Laboratory, pilot and field applications
2.	Rhizofiltration	Rhizosphere accumulation, Contaminant extraction and capture	Groundwater, surface water	Organics/Inorganics: Metals, radionuclides	Sunflowers, Indian mustard, water hyacinth	Laboratory, Pilot scale
3.	Phytostabilization	Complexation, Contaminant destruction	Soil, sediment, sludges	Inorganics: As, Cd, Cr, Cu, Hg, Pb, Zn	Indian mustard, hybrid poplars, grasses	Field application
4.	Rhizodegradation	Contaminant destruction	Soil, sediment, sludges,groundwater	Organic compounds (TPH, PAHs, pesticides, chlorinated solvents, PCBs)	Red mulberry, grasses, hybrid poplars, cattail, rice	Field application
5.	Phytodegradation	Contaminant destruction	Soil, sediment, sludges,groundwater, surface water	Organic compounds, chlorinated solvents, phenols, herbicides, munitions	Algae, stonewort, hybrid poplar, black willow, bald cypress	Field demonstration
6.	Phytovolatilization	Volatilization by leaves, Contaminants extraction from media and release into air	Groundwater, soil, sediment, sludges	Organics/Inorganics: Chlorinated solvents, some inorganics (Se, Hg, As)	Poplars, alfalfa, black locust, Indian mustard	Laboratory and field application
7.	Hydraulic Control (plume control)	Contaminant degradation or containment	Groundwater, Surface water	Water-soluble organics and Inorganics	Hybrid poplar, cottonwood willow	Field demonstration
8.	Vegetative cover (evapotranspiration cover)	Containment erosion control	Soil, sediment, sludges	Organic and inorganics compounds	Poplars, grasses	Field application
9.	Riparian corridors	Containment destruction	Surface water, groundwater	Water soluble organics and inorganics	Poplars	Field application

Ghosh and Singh, 2005

2.11. Advantages and disadvantages of phytoremediation

Advantages of phytoremediation:

1. Phytoremediation method of pollution removal from the contaminated land is environment friendly, safe, cheap way to clean up contaminants. Calculating the cost estimation shows that plants could do that job with one tenth of cost that any other methods employed. The soil and water needn't to be gathered in and stored as hazardous wastes requiring large amount of land, money and manpower.

2. Phytoremediation technique is adaptable to removal of variety of heavy metal and salt compounds.

3. It is the technique of pollution removal applicable with effluent/ soil substance respectively.

4. In situ application method would decrease the amount of soil disturbance and relatively low cost compared to conventional methods.

5. This technique reduces the amount of waste that has to be sent to landfill (up to 95%), and this can be further utilized as bio-ore of heavy metals.

6. In situ application decreases the spread of contaminant via air and water.

7. Does not require expensive equipment or highly specialized personnel, and easy to implement and maintained.

8. Phytoremediation uses plant and it's aesthetically pleasant.

Disadvantages of phytoremediation:

1. Phytoremediation is restricted to sites with shallow contamination within rooting zone of remediative sites.
2. It is restricted in sites where there is low contaminant concentration.
3. It may take longer time even years to remediate a contaminated sites.
4. The air could be contaminated by the burning of leaves or limbs of plants containing dangerous chemicals.
5. Harvested plant biomass from phytoextraction may be classified as hazardous wastes so it must be disposed properly and carefully.
6. Phytoremediation can be adversely affected by the adverse climatic condition. So Climatic condition are the limiting factor.
7. Introduction of nonnative species may affect biodiversity. Effects to food web and ultimate contaminant fates might be unknown.
8. Consumption/utilization of contaminated plant biomass is a cause of concern.

Chapter 3.

Materials and Methods

3.1.Tomato seed selection

At various geographical distribution and locality, different varieties of tomatoes are cultivated in Nepal. Nepal Agricultural research council (NARC) has developed the different improved variety of tomatoes, among them most rigorous, improved and high yielding variety is Srijana variety. This variety is very efficiently acclimatized to give maximum yield, disease resistance and better quality. This tomato variety is taken as the experimental plant for the present research study. Tomato seeds were collected from Kalimati, Kathmandu in the month of September. The tomato seed of Srijana variety was selected as it is a popular cultivar and as of high demand in Kathmandu valley.The plant selected for the experimental purpose in this research is the variety developed through rigorous efforts and selection *Lycopersicumesculentum*varSrijana. This variety has no different history of development than mass hybrid selection method. The female line (HRD1) and male line (HRD17) were imported from India and then mass selected for few years. This has been done by private sector first and then NARC, Khumaltar, Kathmandu, Nepal has taken it into account for its improvement and seed distribution (Nepal Agriculture Research Council). The variety is made suitable for stress survival, disease resistance and better yield and quality. Yield experiments done in NARC have shown that about 1 viable seed growing into one plant can yield 10kg of tomato. Moreover, maximum of the farmers are attracted toward Srijana commercial cultivation and buy seeds in heavy expenses of Rs. 250/2gm seeds. This variety has gone so popular that in almost all districts of Nepal, this variety is being cultivated and become center for attraction among the commercial farmers.

3.2. Soil Vermicompost Substrate

Soil was collected from the depth of about 0-15 cm from Garden area of Tribhuwan University. Stones and plant tissues were carefully removed from the soil before drying process. The soil was crushed finely as possible and all the bigger soil particles are crushed into small fine particles and then stored in plastic bags in room temperature until use. Background level concentration of lead (Pb), cadmium (Cd) and sodium (Na) were analyzed by atomic absorption spectrophotometry (APHA, 1998). The physiochemical characteristics were analyzed in Soil Management Directorate, Nepal government, Hariharbhawan, Kathmandu, Nepal by standard methods. Vermicompost developed in Horticulture department of Nepal Government, was used in the present research. Concentration of lead (Pb), cadmium (Cd) and sodium (Na) in vermicompost sample were analyzed by atomic absorption spectrophotometry [AAS, Perkin Elmer] (APHA, 1998). Chemical and nutritional status of vermicompost was done in Soil Management Directorate, Nepal government,

Hariharbhawan, Kathmandu, Nepal by standard methods. And concentration of heavy metals and Na is determined using atomic absorption spectrophotometry.The collected soil was mixed thoroughly with vemicompost in 2:1 ratio. Thus prepared soil-vermicompost mixture would be called as soil-vermicompost substrate. Then 1 kg packs of prepared substrate were made in plastic pots.

3.3. *In-vitro* experiments: Effects of heavy metal and NaCl stress on tomatoes

3.3.1. Media preparation

Half MS (Murashige and skoog) media was prepared according to (Bhattarai, 2000) with slight modifications. All the stock solution was mixed and then pH of the solution was maintained at 5.8 and final volume was lastly maintained.The media was contaminated with 0.1, 0.3, 0.5 and 1 mMconcentration of heavy metals [$Pb(NO_3)_2$ or $CdCl_2$] and 25, 50, 75 and 100 mM concentration of NaCl in 20ml volume in each petri dishes. Heavy metals and Nacl were mixed in the half MS media before sterilization and then autoclaved at 121^0c for 15 minutes. Simple half MS media is used as control medium. About 20 ml of sterile media was dispensed in one sterile petriplate and cooled and stored at 4^0c.

3.3.2. Seed sterilization

Sterilization of selected tomato seeds were carried out by treating with 1.5% sodium hypochlorite for 5 minutes followed by several rinses with sterile distilled water. The surface sterilized seeds were used for further experiments.

3.3.3. Experimental design

Before the media was autoclaved, the heavy metals and NaCl were mixed up in the media to maintain the concentration of treatment in each plate required. Six seeds of tomato were inoculated in each platewith medium of different treatment.The experiments were performed in triplicates. The tomato seeds in media were kept at 28^0cfirst 2 days in dark and then in 12 hours light period for 15 days.

3.3.4. Effects of stresses on various morphological parameters in *in-vitro* grown tomatoes

After 15 days of inoculation of the sterilized tomato seeds, the plantlets developed were analyzed. The numbers of seeds germinated in each treatment were counted and germination frequency was calculated.The seedlings were uprooted from the media and then root length and shoot length were measured using centimeter scale.

3.4. *In-Vivo* experiments

3.4.1. Effects of heavy metals and NaCl on tomato growth

3.4.1.1. Seedling development

The tomato seeds are sown on a tray with soil-vermicompost media.Tomato in the artificially prepared media grew to fine and healthy seedlings after 3 days. The soil and vermicompostmix prepared by mixing up in 2:1 ratio was found to be perfectly good for the tomato culture. The soil and vermicompost used for tomato growth were analyzed for physical and chemical characteristics.

3.4.1.2. Treatment of Substrate with $Pb(NO_3)_2$, $CdCl_2$ and NaCl

The different treatments were prepared by mixing the substrate with various concentrations of heavy metals and salt. Heavy metals and NaClweretreatedto the substrate as follows: 0.1, 0.3, 0.5 and 1 mM$Pb(NO_3)_2$ concentrations; 0.1, 0.3, 0.5, 1mM $CdCl_2$ concentrations or with 25, 50, 75 and 100mMNaClconcentrations. These are mixed thoroughly with the substrate.

3.4.1.3. Experimental design

The plastic bags with different treatments and control were arranged in random block design with three replicas for each treatment. One healthy seedling in one bag with 1 kg soil-vermicompost mix was planted and let grow at normal light and temperature of laboratory with intermittent irrigation with optimum amount of water just to keep moist but avoid flooding and leaching.

3.4.2. Sample preparation and preservation

Leaf sampling of *in vivo* grown plant was collected every week for 3 consecutive weeks. The leaves collected were washed2-3 timeswith distilled water and then crushed in chilled sodium pyrophosphate buffer(pH 8.1) in mortar and pestle. All crushing were performed in chilled condition. The crude homogenate was then centrifuged for 8000 rpm for 5 minutes in chilled condition. The supernatant was saved and maintained at 4^0c until use.

3.4.3. Protein concentration determination by Bradford method

The total protein in plant extract was estimated by Bradford assay (Bradford, 1997). 450µl of Phosphate buffer (0.1M, pH 7.5) and 50 µl of test sample was added to test tube and mixed well. 2.5ml of Bradford reagent was added to the same tube and after 15min of incubation at room temperature, absorbance reading was taken at 595nm by calibrating with blank containing all components but sample replaced by distilled water.

Linear regression was performed using bovine serum albumin (BSA) as the standard to obtain standard graph. BSA standard curve was made using known concentrations of standard BSA solutions of 20, 40, 80, 120, 160, and 200µg/ml and a blank. From the standard curve, an equation was obtained for the relationship between protein concentration and the absorbance given by the equation:

$$Y = 0.005x + 0.128 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(i)}$$

Where, y= protein concentration (µg/ml)

X = absorbance (at 595nm)

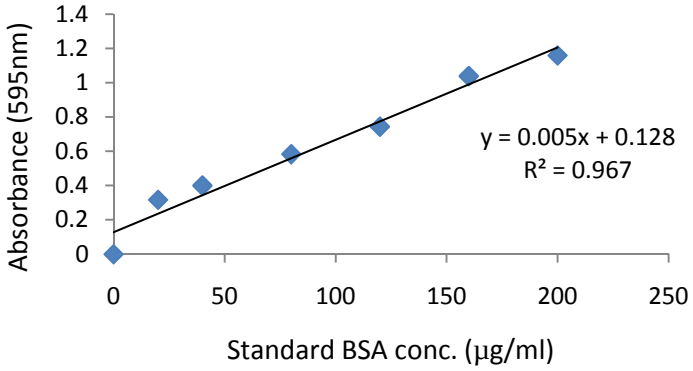


Fig 3.1. Standard BSA calibration curve. The curve was used to calculate the protein concentration in the plant sample. Protein concentration was then used to calculate the specific ALDH activity of the samples.

3.4.4. ALDH assay by spectrophotometric method

ALDH activity was determined for the control and all the treatments for 3 consecutive weeks. Samples,to measure the ALDH activity were prepared as follows: plant sample were first washed in running distilled water to remove any soil debris and dust. Then, they were cut into small pieces. The tissue was grinded in mortar and pestle by adding about 5ml of ice cold Assay buffer according to Sreerama and Sldek(2005). The crushed samples were sonicated at 20 kHz. The protein extract was centrifuged at 5000rpm for 8min at 4^{o}C in a microfuge and was kept in ice bath during the experiment. Supernatant obtained was transferred to a fresh ependroff (EP) tube. ALDH assay was done using the protocol according to Sreerama and Sladek (2005). The kinetic assay program option on spectrophotometer was run. The spectrophotometer was calibrated at 340nm using assay buffer diluted with 1:1 water as reference. The following components were added to an EP tube.

- 500µl assay buffer (32mM sodium pyrophosphate and 1mM EDTA final conc.)

- 50µl 100mM NAD (4mM final concentration)
- 50µl 100mM GSH (5mM final concentration)
- 50 µl 2mMpyrazole(0.1mM final concentration)
- 100µl test sample
- D/W to final volume of 950µl.

A negative control (blank) reaction (50 µl substrate replaced by 50µl of water) was prepared for each test preparation. The ependroff tube containing reaction mixture and another tube containing the substrate (80mM acetaldehyde) was placed in 37^{o}C water bath for 5 min. 50µl of substrate was added to the mixture (final concentration 4mM) and placed immediately into a quartz cuvette. The cuvette was placed in the spectrophotometer and the increasing absorbance/min was monitored at 340nm for 5-10min. The gross rate of reaction (dA/min) was determined by the linear regression analysis of linear portion of the graph. The negative control (blank) rate was subtracted from the gross rate to obtain the net (enzyme-mediated) rate of reaction. The net rate was multiplied by factor 1639, because the test sample taken was 100ul.The gross rate of reaction (ΔA/min) was given by the spectrophotometer along with the linear slope curve. The ALDH activity (mIU/ml) was divided by the concentration of protein in test preparation (mg/ml) (calculated from Bradford assay) to calculate the specific ALDH activity (mIU/mg protein) i.e. the nanomoles of NADH formed per min per mg protein.

3.4.5. Native PAGE and ALDH staining

Native PAGE was carried out by using modified protocol ofSreerama and Sladek (2005). The one vertical electrophoresis chamber was set up and 10ml of resolving gel was added to the gel chamber. D/W was overlaid to generate a flat top to the gel. After 1hr of polymerization, the D/W was removed with a clean filter paper. Now, 2ml of the stacking gel was poured to the chamber after the comb was inserted. The normal acrylamide concentration of SDS-PAGE of 30% was replaced by 23% to make the pore bigger in the gel to allow convenient mobility of the enzyme molecules. After 1hr of incubation, comb was removed and the apparatus was filled with running buffer. 15µl of the protein extract was mixed with same amount of the gel loading dye. No, prior heating was done to the sample as the activity of intact enzyme was supposed to be visualized. Electrophoresis was carried out at 125volts and 20mA current for 3.5 hrs. Then, the gel was removed and stained with ALDH staining solution. Gel was overfilled with 10ml of ALDH staining solution and incubated at 25^{0}C for overnight by gentle shaking at 100rpm. The photograph of gel bands were taken and saved for the analysis.

Constituents of resolving solution

Solution	5ml	10ml
H2O	1.6	3.3
23% Acrylamide	2.0	4.0
0.5 Tris (PH 6.8)	1.3	2.5
10% $(NH_4)_2S_2O_8$	0.05	0.1
TEMED	0.002	0.004

Solution for preparing stacking solution

Solution	1ml	2ml
H2O	0.68	1.4
23% Acrylamide	0.17	0.33
0.5 Tris (PH 8.8)	0.13	0.25
10% $(NH_4)_2S_2O_8$	0.01	0.02
TEMED	0.001	0.002

3.5.Phytoremediation analysis

3.5.1. Sample preparation for metal analysis in soil, vermicompost and plant sample

3.5.1.1. Grinding and packing

After 60 days the tomato plant were uprooted from the bag and washed thoroughly with tap water. Then air dried for few hours and the oven dried 40-50^{0}c until the constant dry weight. Similarly, soil and vermicompost collected in 3 replicates were sieved, dried in hot air oven at 50^{0}c until constant weight and homogenized and kept sealed in plastic bag until use. For preparation of plant powder entire plant was crushed.

3.5.1.2.Digestion of the prepared samples

Open acid digestion or Hot Plate digestions of the samples were carried out according to Katz and Jennes, 1983. Samples (1gm) were dipped in 5ml concentrated HNO_3 (Merck) in quartz digestion tubes and were left overnight at room temperature. The following day, 5ml of conc. HNO_3 was further added and heated to 50-60^{O}C in a sand bath for 2hrs. Slowly the temperature was raised to about 150-170^{O}C and digested for about 4hr till dark reddish brown fumes started to clear out. The digest was then cooled to room temperature and filtered through ashless filter paper (Whatman no 40 filter paper). During filtration, the

digest was diluted with D/W to make final volume 25ml. The filtrate solution was used for the lead, cadmium and sodium concentration determination.

3.5.2. Metal analysis by Atomic Absorption Spectrophotometry

The analysis was performed by flame Atomic Absorption Spectrometry (FAAS) with Perkin Elmer VMAA240FS instrument using APHA 21st Edition 3111B test method. The absorbance readings were then used to determine the heavy metal concentration in the soil, vermicompost and plant samples.

3.6. Statistical analysis

Significant metal uptake analysis between treatments and control and between treatments were analyzed statistically using one way ANOVA followed by duncans multiple range test (with the help of SPSS 17.0 version). All the calculation, graph presentation and data analysis were performed using windows 7 microsoft excel, 2010.

Chapter 4.

RESULTS

The present research study has been carried out in green house in plastic pots. Experiments were conducted in *in-vitro* conditions to study the effects on growth morphology of exposed stresses and *in-vivo*conditions for ALDH gene expression analysis and uptake of heavy metals and Na at various concentrations of treatments in soil-vermicom post media. The soil and vermicompost were also analyzed for physicochemical and nutritional characteristic. The results are presented below:

4.1. Effect of Heavy Metals and salt on Seed Germination

Both heavy metal and salt concentration affected seed germination. The effects of different treatments were shown in figure 4.1 and 4.2. Inhibition of the tomato seeds germination was concentration dependent. O.1mM Cd concentrations didn't affect germination whereas Cd at 0.3 mM and Pb at 0.1 and 0.3 mM concentration have very low toxic effects. At 0.5 and 1mM concentrations of Cd and Pb showed the massive reduction in the seed

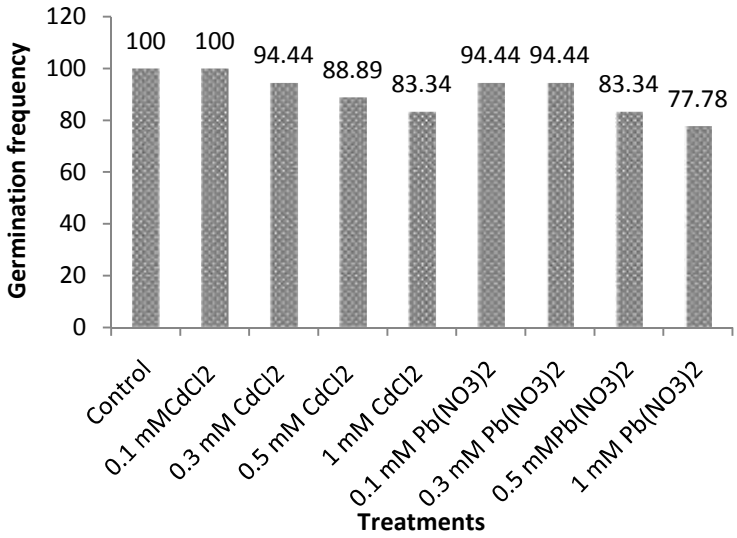


Fig 4.1: Germination frequency (%) of the tomato seeds at different treatments of heavy metals observed in 15 days after inoculation in half MS media contaminated with different concentration of $Pb(NO_3)_2$ and $CdCl_2$.

germination. In NaCl stressed treatments, 25 and 100mMNaCl do not show any effects on germination of tomato seeds and germination frequency remain same as that of control plants in NaCl treatments. In case of 75 and100mMNaCl stress treatments seed germination decrease and numerate 94.44 and 83.34 % respectively. Treatments with 75 and

100mMNaClshowed slightly less germination rate. Least germination was found to be at 1mM$Pb(NO_3)_2$ treatments which was found to be only 77 %. Delayed germination was also observed at higher concentrated treatments of heavy metals and NaCl. The resulting rank order of toxicity for metals on seed germination was Pb> Cd > Na.

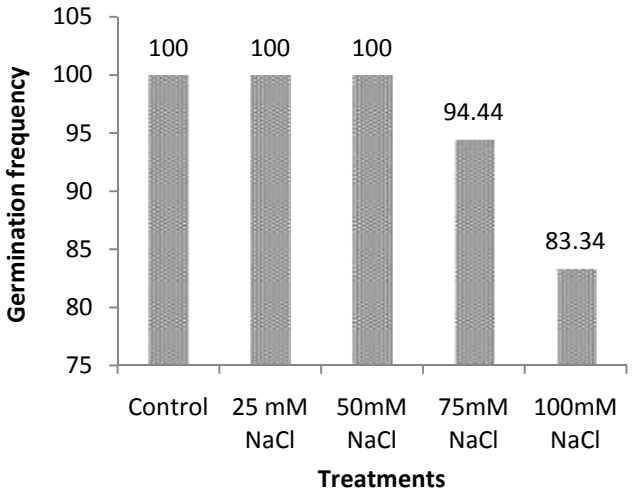


Fig 4.2: Germination frequency (%) of the tomato seeds at different treatments of NaCl observed in 15 days after inoculation in half MS media contaminated with different concentration of NaCl.

4.2. Effects of heavy metals and salt in root growth

Increase in the heavy metal concentration in the soil-vermicompostsubstrate caused root length decrease with stunt growth of roots having some obvious speculative results. The dose of 0.1mM, 0.3mM of $CdCl_2$ and 0.1mM $Pb(NO_3)_2$ did not show any remarkable difference in root length of the plants as compared to the root length of the control plants. Increase in root length in comparison to control was observed in case of 0.3 and 0.5mM $Pb(NO_3)_2$treatment. Plants given at 0.3 and 0.5 mM$Pb(NO_3)_2$ showed the highest increment in root lengths.However, at lower concentration Cd showed no effects on root size when compared with control. Cd at 0.5 and 1 mMand Pbat 1 mM treatments demonstrated inhibition of root growth. In case of salt treatment, 25 mMNaCldemonstrated the increment in root length. Plates treated with 100 mMNaCl affected root growth. The treatment of NaCl at 50, 75mM showed no remarkable effects in root length. Lateral roots were observed in all treatments of Cd, Pb and Na. The growth of roots was almost completely inhibited at 1 mM Cd and Pb and 100 mMNaCl. Highest percentage of inhibition of RL occurs at 1 mM $CdCl_2$ treatment in case of heavy metals (88.2 %) and at 100 mM in case of NaCl treatments (69.6 %). Stimulation of the RL takes place at 0.3 mM $CdCl_2$and at 0.3, 0.5 mM$Pb(NO_3)_2$in case of heavy metals and at 25 mM and 50 mM in case of NaCl treatments.

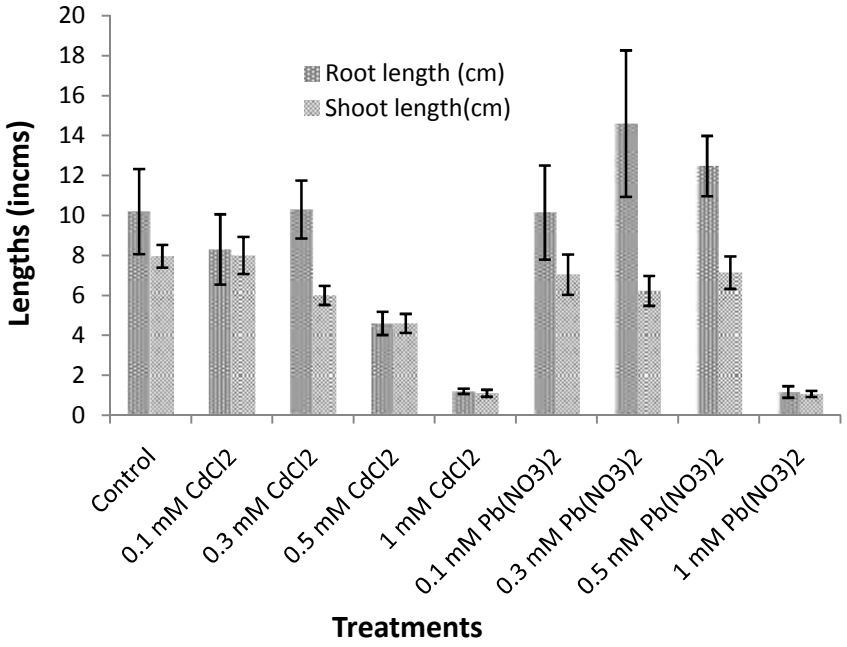


Fig 4.3: Root and shoot length of *Lycopersicum esculentum* var Srijana after 15 days of exposure to different concentration of heavy metals [$Pb(NO_3)_2$ and $CdCl_2$].

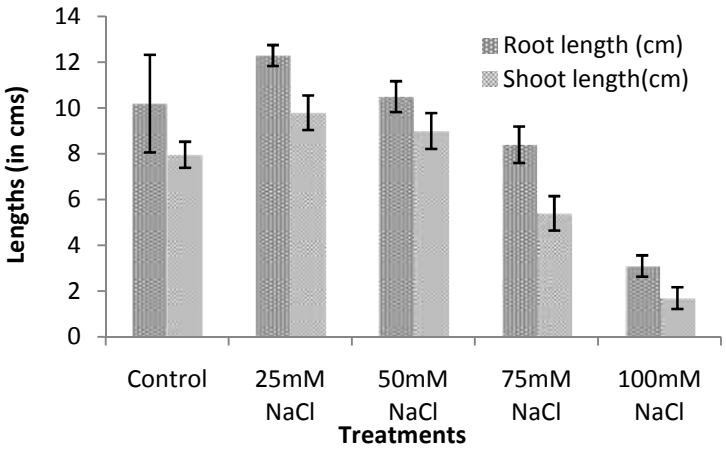


Fig 4.4: Root and shoot length of *Lycopersicum esculentum* var Srijana after 15 days of exposure to different concentration of NaCl.

Table: 4.1. Percentage inhibition or stimulation of RL and SL of tomato plant in comparison to control plants

Compound having metals	Doses (mM)	Percentage inhibition or stimulation of RL	Percentage inhibition or stimulation of SL
	0.1	18.6	0.5
$CdCl_2$	0.3	1*	24.6

	0.5	54.9	42.2
	1	88.2	86.2
$Pb(NO_3)_2$	0.1	0.5	11.6
	0.3	43.1*	21.8
	0.5	22.3*	10.4
	1	83.7	86.6
NaCl	25	20.6*	23.1*
	50	4.9*	13*
	75	17.6	32.2
	100	69.6	78.6

-Number followed by ***** signs represents percentage of stimulation with reference to control plant

4.3. Effect of heavy metals and salt in shoot growth of *in-vitro* grown tomato plants

The effects of heavy metals on the shoot growth were different from their effects on root growth at lower concentrations. Thoseconcentrations showing higher root length did not show same results on shoot length. It showed much different results. Plants showed larger root length at 0.3mM $Pb(NO_3)_2$ than control, whereas the shoot length decreased at this treatment. Almost all treatments of heavy metals didnot show shoot larger than control plants and the length decreasedremarkably with increasing concentration. On the other hand, 25 mM and 50mM doses of NaCl increased the shoot lengths as compared to the control treatment. These indicate that low concentrations of salt have stimulating effects on the tomato shoot length growth. All other treatments demonstrated decrease in the shoot size of the plants. The growth of shoot was highly inhibited at 1mM $CdCl_2$, 1mM $Pb(NO_3)_2$ and 100mM NaCl.Highest percentage of inhibition of SL was observed at 1 mM $Pb(NO_3)_2$ treatment in case of heavy metals (86.6 %) and at 100 mM in case of NaCl treatments (78.6 %). Stimulation of the SL also took place at 25 mM and 50 mM in case of NaCl treatments.

4.4. Soil-vermicompost substrate analysis for *in-vivo* experiments

The present research study has been carried out in greenhouse as pot culture experiments to study the accumulation of heavy metals and sodium at various concentrations in soil-vermicompost media.The soil and vermicompost were analyzed for physicochemical characteristic and nutritional properties separately. The soil was amended with various

concentration of heavy metals (Pb, and Cd) and NaCl. The results of analysis are presented below:

Table 4.2: Physicochemical properties of experimental Soil.

Soil Parameters	Values
Texture	Sandy loam
PH	6.22±0.1
Organic content %	1.71±0.035
Nitrogen %	0.09±0.035
Potassium (Kg/hec)	73.5±1.26
Phosphorus (Kg/hec)	2.08±0.054
Moisture content %	29±1.7
Na(ppm)	267±0.472
Pb(ppm)	4±0.009
Cd(ppm)	1.5±0.012

-Values are averages of three replicates ± S.D.

4.4.1. Soil Analysis

After selecting the appropriate site for the soil collection, soil was collected and then subjected to extensive analysis of various physiochemical parameters, which influence root establishment and morphological behaviors of plants in soil. The physical and chemical properties of soil are presented in table 4.2. Soil used was sandy loam. The pH of the soil was 6.22, which lies within the recommended value for proper growth and efficient uptake of nutrients and compound from soil. The percentage of organic matter and nitrogen were found to be 1.71 and 0.09 respectively. Macronutrients including metals were also present in substantial amount. Further to enhance the native stage of soil, it was spiked with vermicompost, which supplimented the nutrients needed to plant growth. Some level of Na, Pb and Cd is found in the soil indicating some salt (NaCl) and heavy metal (Cd, Pd) contamination to some extent in soil collected which could be regarded as the basal level contamination in soil.

4.4.2. Vemicompost analysis

The vermicompost used in the present study was purchased from Horticulture Department of Nepal government, Kirtipur, Kathmandu. The physical and chemical properties of the vermicompost are given in table 4.3. The vermicompost was found to be highly nutritious. This vermicompost developed by the vermiculture biotechnology was then used as a natural fertilizer for phytoremediation studies of metals. The vermicompost showed pH of 8.23. The organic matter content and nitrogen content was also found to be greater than that of the

soil as it is solely prepared from the organic biological materials. Carbon content was found to be 26.4% and nitrogen content was found to be 0.87%. In analyzing the metal content in case of vermicompost, it has been shown that sodium concentration as 1292.40ppm was found to be much greater than the soil and Cd, Pb was found to be 1.75ppm and 21.5ppm. This indicates some basal level metal and salt contamination in vermicompost too.

Table:4.3. Nutritional and chemical status of vermicompost.

Parameters	Values
P^H	8.23±0.071
Total Carbon %	26.4±0.40
Moisture content %	43±1.78
Total Nitrogen %	0.87±0.01
Total Phosphorus %	0.5±0.040
Total Potassium %	0.95±0.03
Na (ppm)	1292.5±1.462
Pb (ppm)	21.5±0.053
Cd (ppm)	1.75±0.017

-Values are average of three replicates±SD

4.5. ALDH activities in*in-vivo* grown Tomato plants

ALDH activities of control and treated plants were measured for three consecutive weeks and specific ALDH activities were calculated as shown in the table 4.4.

Table: 4.4. Aldehyde dehydrogenase activitiesin*Lycopersicum esculentum*var Srijana for three consecutive weeks, mean specific ALDH activity (mIU/mg protein) ± SD.

		Specific ALDH Activity		
Treatments	**Doses (mM)**	**Week 1st**	**Week 2nd**	**Week 3rd**
	0.1	252.799±2.364	347.54±6.321	397.429±3.657
	0.3	**483.861±4.658**	**693.39±4.268**	**539.347±5.021**
$CdCl_2$	0.5	434.153±5.616	455.151±4.654	429.582±6.235
	1	289.32±6.981	294.651±6.890	284.746±2.651
	0.1	260.396±5.612	258.435±4.279	208.039±5.102
	0.3	**339.513±9.265**	430.56±8.231	**367.291±6.321**
$Pb(NO_3)_2$	0.5	309.806±6.254	**462.69±7.921**	327.98±3.254
	1	231.17±7.125	280.03±7.256	239.576±7.265
	25	151.05±1.965	180.87±1.265	160.82±3.265

	50	**234±2.152**	**350.01±6.256**	**312±7.259**
NaCl	75	218.48±9.256	260±6.246	230.42±9.258
	100	180.64±6.265	220.47±5.325	208±4.265
Control	0	54.4±2.75	50.05±3.654	60.21±1.584

-Values are in average of three replicates ± SD

After one week of transfer in one kg substrate packets, gentle and healthy growing plants were seen. Then ALDH activity measurement experiments were performed for three consecutive weeks in three replicates for each treatment. Average values and the deviation of the observation from the average values were calculated and presented here in the table 4.4.

4.5.1. ALDH expression analysis in cadmium treatments

*Lycopersicum esculentum*var Srijana grown on four different concentrations of cadmium showed higher specific ALDH activity than in control plant grown without $CdCl_2$treatment.At 0.3 mM $CdCl_2$ ALDH activities were highest in all three week treatments.The maximum ALDH activity was seen on week 2 at 0.3mM $CdCl_2$concentration. ALDH activity increasedfrom 0.1 to 0.3mM $CdCl_2$and then activity decreased as the Cd increased in substrate.At 1mM $CdCl_2$the activity was least in almost all three consecutive weeks.

Week 1

In the first week, mean specific ALDH activities measured for the treatments 0.1, 0.3, 0.5 and 1mM $CdCl_2$were 252.799, 483.861, 434.153, 289.32 mIU/mg protein respectively. All the values were greater than that of control. Treatment with 0.3mMdoses $CdCl_2$ concentration showed the highest specific ALDH activity in week 1st.

Week 2

In week 2, mean specific ALDH activities measured for the treatments 0.1, 0.3, 0.5 and 1mM $CdCl_2$ were 347.54, 693.39, 455.151, 294.65 mIU/mg protein respectively. All the values were greater than that of control (table 4.4). Treatment with 0.3mM doses $CdCl_2$ showed the highest specific ALDH activity in week 2^{nd}.

Week 3

In week 3^{rd}mean specific ALDH activities measured for the treatments 0.1, 0.3, 0.5 and 1mM $CdCl_2$ were 397.429, 539.347, 429.582, 284.746 mIU/mg protein respectively. All the values

were greater than the controls (Table 4.4). Treatment with 0.3mMdoses $CdCl_2$ showed the highest specific ALDH activity in week 3rd but not as that of week 2nd.

4.5.2.ALDH expression analysis in lead treatments

*Lycopersicum esculentum*var Srijana grown on four different concentrations of lead showed highest specific ALDH activity at 0.5mM $Pb(NO_3)_2$ treatment in 2ndweek among all weeks measurements.At 1mMdoses $Pb(NO_3)_2$, ALDH activity showed least measurement in all weeks.

Week 1

In the first week, mean specific ALDH activities measured for the treatments 0.1, 0.3, 0.5 and 1mM $Pb(NO_3)_2$ were 260.396, 339.513, 309.806, 231.17 mIU/mg protein respectively. All the values were highercompared to control. Treatment with 0.3mMdoses $Pb(NO_3)_2$ concentration showed the highest specific ALDH activity in week 1st.

Week 2

In week 2nd, mean specific ALDH activities measured for the treatments 0.1, 0.3, 0.5 and 1mM$Pb(NO_3)_2$ were 258.435, 430.56, 462.69, 280.03 mIU/mg protein respectively. All the values were greater compared to control. Treatment with 0.5mMdoses $CdCl_2$ showed the highest specific ALDH activity in week 2nd and also among all treatments and in all weeks.

Week 3

In week 2, mean specific ALDH activities measured for the treatments 0.1, 0.3, 0.5 and 1mM $Pb(NO_3)_2$ were 208.039, 367.291,327.98, 239.576 mIU/mg protein respectively. Treatment with 0.3mMdoses $Pb(NO_3)_2$ showed the highest specific ALDH activity. All the values were greatercompared to control.

4.5.3. ALDH expression analysis in Salt treatments

Lycopersicum esculentum grown on four different concentrations of sodium chloride showed highest specific ALDH activity at 50mM NaCl concentration in all measurements of three consecutive weeks. In 2nd week 50mM NaCl concentration show the highest measurement of ALDH activity among all treatments made as shown in table 4.4. After 50mM NaCl ALDH activity decreased down.

Week 1

In the first week, mean specific ALDH activities measured for 25, 50, 75,100 mM NaCl were 151.05, 234, 218.48, 180.64 mIU/mg protein respectively. Among the treatments, greatest specific ALDH activity was shown in 50 mM dosesNaCl condition.

Week 2

In week 2nd, mean specific ALDH activities measured for 25, 50, 75,100 mM NaCl were 180.87, 350.01, 260, 220.47 mIU/mg protein respectively. Highest specific ALDH activity was shown in 50mM stressed condition. All the values were highercompared to the control.

Week 3

In week 3rd, mean specific ALDH activities measured for 25, 50, 75,100 mM NaCl were 160.82, 312, 230.42, 208 mIU/mg protein respectively. Highest specific ALDH activity was shown in 50mM stressed condition. All the values werehighercompared to the control.

4.5.4.ALDH expression confirmation by NATIVE-PAGE

For the confirmation of the activity of ALDH enzyme, NATIVE-PAGE was performed from the crude homogenate sample from different treatments of the tomato plants. The ALDH active proteins bands were obtained in gel as shown in figures in appendix. The different gel bands observed after staining of gels showed that different ALDH isozymes were induced or over-expressed when plants were exposed to cadmium, lead and sodium stresses. From the gel, without prior knowledge of how many ALDH isozymes are coded by the *Lycopersicum esculentum* genome with response to stresses, it wasn't possible to ascertain the particular ALDH isozymes.

4.6. Phytoremediation analysis

The mean accumulation of metals Cd, Pb, and Na by tomato plants increased as the concentrations of these metals in the soil-vermicompost substrate increased. The mean values are presened in table 4.5.

4.6.1. Heavy metal and Naaccumulation in tomato plants

4.6.1.1.Cadmium accumulation

Lycopersicum esculentum var. Srijanashowed the considerable amount of Cd accumulation as compared to the control tomato. The Cd contents (mg/kg dry weight) after growth of plant in artificially contaminated soil at 0.1, 0.3, 0.5 and 1mM $CdCl_2$ concentration for 60

days were measured to be 0.67, 2.88, 3.15, 4.11 ppm respectively. Comparing the mean values of cadmium uptake of different treatments statistically using ANOVA tool, it was found that there was significant difference among treatments of 0.1, 0.3, 0.5 and 1mM $CdCl_2$ concentrationwith respect to that of control (Duncan, P=0.05). However, between the treatments of 0.3 and 0.5mM $CdCl_2$ concentration there was no significant difference (Duncan, P=0.05) in their uptake values. Cadmium uptake by the plant increased with increase in contaminating $CdCl_2$ concentration in soil-vermicomposting substrate. Least cadmium uptake was found in 0.1mM $CdCl_2$ treatment and highest in 1mM $CdCl_2$ treatment.

4.6.1.2. Lead accumulation

Lead treatments also showed the considerable amount of Pb accumulation than that of control plants. The Pb contents (mg/kg dry weight) after growth of plant in artificially contaminated soil at 0.1, 0.3, 0.5 and 1mM $Pb(NO_3)_2$ concentration were measured to be 0.66, 2.93, 2.37, 8.1 ppm respectively. Comparing the mean values of Pb uptake of different treatments statistically using ANOVA tool, it has been found that there was significant difference among treatments of 0.1, 0.3, 0.5 and 1mM $Pb(NO_3)_2$ concentrationwith respect to that of control (Duncan, P=0.05). However, between the treatments of 0.3 and 0.5 mM $CdCl_2$ concentration there was no significant difference (Duncan, P=0.05) in their uptake values. Cadmium accumulation by the plant increases with increase in contaminating $CdCl_2$ concentration in soil-vermicomposting media.

Table. 4.5: Metal uptake by *Lycopersicumesculentum*varSrijana from the substrate treated with different concentration of Cd, Pb and Na after 60 days.

Treatments	Metals	Doses given(mM)	Uptake(ppm)
$CdCl_2$	Cd	0	0.05 ± 0.0125^{a}
		0.1	0.67 ± 0.322942^{b}
		0.3	2.88 ± 0.361144^{c}
		0.5	3.15 ± 0.361144^{c}
		1	4.11 ± 0.388019^{d}
$Pb(NO_3)_2$	Pb	0	0.13 ± 0.0125^{a}
		0.1	0.66 ± 0.005^{b}
		0.3	2.93 ± 0.56806^{c}
		0.5	2.37 ± 0.072572^{c}
		1	8.1 ± 0.61765^{d}
NaCl	Na	0	120.56 ± 30.82768^{a}
		25	176.5 ± 10.33199^{b}
		50	230.33 ± 10.1352^{c}
		75	235.96 ± 26.37301^{c}

100	492.47±21.63827[d]

-Numbers denoted same alphabets shows no significance among the measurements.(DMRT)

4.6.1.3.Sodium accumulation

Sodium treatments also showed the considerable amount of Na accumulation than the control plants without any stress. The Na contents (mg/kg dry weight) after growth of plant in artificially contaminated soil at 25, 50, 75 and 100mM NaCl concentration were measured to be 176.5, 230.33, 235.96, 492.47 ppm respectively. Comparing the mean values of Na uptake of different treatments statistically, it has been found that there was significant difference among treatments of 25, 50, 75 and 100mM NaCl concentrationwith respect to that of control (Duncan, P=0.05). However, between the treatments of 50 and 75mM NaCl concentration there is no significant difference (Duncan, P=0.05) in their uptake values. Sodium accumulation by the plant increased with increase in contaminating NaCl concentration in soil-vermicomposting media except 75mM NaCl treatment which show some less uptake value than 50mM NaCl treatments.

Chapter 5.

Discussion

Plantsencounter a variety of environmental stresses throughout their life cycle as they cannot escape the stresses as humans and animals can do. Plant development and productivity are negatively affected by environmental stresses. During the last decade, cultivated land in several regions of the world has been affected by environmental stresseslike salt, cold, drought, heavy metals and UV, which hinders crop cultivation and yield(Gill and Tuteja, 2010).Every year countries lose a substantial amount of money from reductions in crop productivity caused by abiotic stresses(Xiong et al., 2002). It is predicted that these environmental stresses will become more intense and frequent with climate change, especiallyglobal warming. On the other hand, the world population is estimated to reach near 10 billion by 2050, which will witness serious food shortages(Gill and Tuteja, 2010). Therefore, tolerant crops should be developed to feed the increasing world population. Maintaining crop yields under adverse environmental stresses is probably the major challenge facing modern agriculture. Plants possess efficient defense mechanisms to adjust with a variety of environmental stresses which includes metal, drought, UV, high-salinity, cold stresses and pathogen attack(Gill and Tuteja, 2010).Soil is the ultimate site for disposal of all heavy metals, salts and many other types of contaminants which need to be treated. While heavy metals andsalts are not degraded as organic materials (Salt *et al*., 1995) and their cleanup requires greater effects.

Using tomato as a model plant to study the stress responsive behavior and uptakeis the main area of study in the present research. Tomatos were also used byTaffouo*et al*.(2010)and also byRehman*et al*.(2011) for the investigation of the effects of different stresses in growth physiology, metal uptake.Several studies have been conducted in order to evaluate the effects of different heavy metal and salt concentrations on seedlings or adult plants (Raskin*et al*., 1997). In a few studies, the seeds have been exposed to the contaminants (Xiong, 1998) whereas in this study seedlingsare subjected to heavy metals and salt contaminant. *Lycopersicumesculentum*was chosen because it is a common experimental plant largely used in research (Carrara*et al*., 2001) and tomatoes are used much in Nepalese food.The importance of tomato fruits as good sources of ascorbic acid (Vitamin C), β-Carotene and mineral elements has been also acknowledged (Tindal, 1992).

In the present study it has been tried to examine the potential of tomato plant for uptake of heavy metals and salt. *Lycopersicumesculentum*is a good source of plant tissues, because it has been found to tolerate many contaminants and grow well in contaminated soils (Baligar*et al*., 1993). Gardea-Torresdey*et al*., 2000 have shown that tomato is a potential

source of biomaterials for the removal and recovery of heavy metal ions and salt ions. Overall, it had been tried to assess some effects of heavy metals and salt on tomato plant growth, metal accumulation and ALDH activity.

5.1. Soil and vermicompost analysis

The contamination of soils by heavy metals and excess salt is significant problem, which leads to negative influence on soil characteristics and thus limitation of productivity and environmental functions. The soil and vermicompost that were being used to prepare substrate for the experiment wereanalyzed for their physicochemical and nutritional properties. The amount of Cd, Pd and Na concentration already present in the experimental soil is only considered as the background level concentration. Possibly the level may be because of the drainage from different laboratories of Central Departments of TU,flowing down the garden. Normal range of Cd and Pb in soil has been found as compared to Na concentration(which is somewhat larger, enumeratingup to 267 ppm). Vermicompost has relatively large amount of sodium (more than that found in soil). Vermicompost also has some level of heavy metals too but less than that of soil. Kathmandu is one of the most polluted cities of the world with its land, air, water and almost all biosphere worsly polluted. Major rivers following through the valley are carrying sewage and contaminant of the capital down. And the major cultivation of vegetables in Kathmandu valley is done is the bank of such river andin itself contaminated land of city. This may be the reason of such a high value of Na, Cd, Pb in vermicompost which usesvegetable remainingbrought fromKalimati vegetable and fruit market. It seems as if vegetables used in vermicomposting also come from the waste remaining from restaurants and hotels. Organic and nitrogen content in soil and vermicompost seems to be much lower. Normal value of Nitrogen in vermicompost ranges from 1.7- 2.5% (Borah *et al*., 2006).

Several researches performed on vermiculture biotechnology have demonstrated that earthworm castings (vermicompost) have excellent aeration, porosity, structure, drainage and moisture-holding capacity. The vermicompost is a rich source of beneficial microorganisms and nutrients (Paul, 2000). It is used as a soil conditioner or fertilizer (Hattenschwile and Gaser, 2005). Increase in crop yield, soil nutrients status and nutrients uptake was reported due to application of vermicompost (Singh and Sharma, 2003). Peng*et al*., 2005 and Yang *et al*., 2005 have reported that application of vermicompost can increase the bioavailability and in-plant mobility of copper. Significantly more copper was found in grains and straw of oat treated with vermicompost as compared with the application of mineral fertilizers. Ma *et al*., 2003 had found that yield of a tropical leguminous woody shrub, *Leucaenaleucocephala*, growing in amended Pb–Zn mine tailings has been found to be increased by 10 to 30% in the presence of burrowing earthworms (*Pheretima*spp.).The

earthworms increased the available forms of N and P in soil, increased metal bioavailability and raised metal uptake into plants by 16 to 53%(Ma *et al*., 2003). Some evidence indicates that earthworms increase metal bioavailability in relatively low-level metal-contaminated soils with higher organic matter contents. This agrees with results of experiments done by Halim*et al*., 2003. So we have also used vermicompost for making the soil nutritious and fit for the tomato culture. And it in fact gives fine and healthy seedlings with soil-vermicompost substrate.

5.2. Effect on Plant growth

5.2.1. Salt effects analysis

The effects of salt toxicity and water stress are about same. The lack of water and the oxidative stress generated by water condition like draught (created by salt) have the same physiologic effects (Flower, 2004). This fact has been made clear in the review by flower, 2004. Chinnusamy*et al*., 2005 has done some experiments for improving salt tolerance in plants. They found salt stress can cause oxidative damage to membrane lipids, proteins and nucleic acids and these effects causes the reduction in germination efficiency and plant growth in contaminated soil.

5.2.1.1.Effects of salt in seed germination

Germinationwas decreased with increasing salinity. In NaCl treated experimental plants; the lower concentration (25 and 50 mMNaCl) treatments exert no effects in the germination frequency of tomato seeds. Almost all seeds get germinated normally.At75 mMtreatments,only 94.44% seeds germinated,again decrease in germinationwas seenfurther in 100mMtreatments. These results indicated that larger doses of sodium chloride solution inhibit the primary growth of tomato embryos. The control plant has highest germination percentage and germination decreases inthe treatments. In 100 mMNaCl treatments, germination is found to be least. So it could be said that salinity hurts seed germination. Since germination percentage determination is one of the most popular methods for determination of plant tolerance to salts, the germination frequency is calculated by exposing the seeds to salt solution (Dantas *et al*., 2005). Saltaffects seed germination via osmotic stress (Jamil*et al*., 2006). One of the studies conducted byDantas *et al*.(2005) showed that germination of various cultivar of cowpeaunder salt stress decreased, 6.1 to 25% when treated with NaCl 100mol/m. This research also shows that germination decreases with salinity increases. The most important enzyme which induces plant seed germination is α-amylase and increasing salt concentration destroys its activity thus germination hindered (Saboury and Karbassi, 2000). It is also assumed that in addition to

toxic effects of certain ions, higher concentration of salt reduces the water potential in medium and water absorption by germinating seeds and thereby inhibition of germination (Jamil*et al*., 2006). During the germination period of seeds it need much water absorbtion under salt stress due to accumulation of soluble solutes around seeds which increases osmotic pressure resulting in excessive uptake of the ions leading to toxicity in the plants (Robinson and Jones, 1986).

5.2.1.2.Effects of salt on root and shoot growth

RL and SL of plants also decreased as the concentration of the treatments increased in general but 25 mM and 50 mM treatments speculatively increased the RL and SL.The possible reason behind the stimulated RL and SL in 25 and 50 mMNaCl treatment may be due to increased cell elongation caused by loosed cell wall elasticity due to NaCltoxicity (Chettri,2000).The salt entering the cell causes rapid decrease in cell division and toxicity to protein, membrane lipid, nucleic acids andthis causes retardation in plant normal physiology and effects become more intense with increase in concentration (Nawaz *et al*., 2010).Flores *et al*., 2001 also found thatwhen plants were exposed to salinity in laboratory experiments, there was a rapid and temporary drop in growth rate followed by a gradual recovery to a new reduced rate of growth. This studysupported the fact of temporary effects due to rapid and often transient changes in plant water relationswhich subsequently causes changes in growth rate. Since Flores *et al*., 2001found that salt stress inhibit the uptake and transport of potassium, calcium and phosphorus;this may also be one of the causes of sodium chloride inhibition of tomato growth.

5.2.2. Heavy metal effect analysis

5.2.2.1. Effects of heavy metals in seed germination

Heavy metals are potentially highly toxic to all form of organisms including animals and plants. Singh and Singh, (1981) showed suppression of seed germination and plant growth responses,when grown in stressed conditions of heavy metals, which have been attributed to the establishment of higher toxic effect syndrome due to high accumulation of the metallic salts within the plant body biomass. Germination of Srijana tomato variety was found to be reduced at and above 0.3mM$Pb(NO_3)_2$ and $CdCl_2$treatments in the current study. The similar results have been reported by Peralta-Videa*et al*.(2004). They showed that the exposure of alfalfa plants to Cd, Cu and Zn had lethal effects on the alfalfa seedlingsobserved after four day germination.The study conducted by Aydinal and Marinova, (2009) also showed the loss of germination frequency of alfalfa plant grown on different concentration of heavy metals.

5.2.2.2. Effects of Heavy metal on RL and SL growth

In present study,increase in RLat 0.3and 0.5 mM$Pb(NO_3)_2$werefoundthan that at0.1mM treatment. This result indicatesthat the heavy metals in lower concentration may act as micronutrientsand thus exerts the stimulatingeffects.Fernandis*et al*.(1991)also showed thatsome heavy metals at low doses are essential micronutrients for plants, but in higher doses they may cause metabolic disorders and growth inhibition for most of the plants species. It has also been found that SL was not remarkably stimulated at anytreatments as RL. The possible reason might be the first and more intense exposure of roots to stresses since they are embedded in soil but shoot are not.

0.5 mM, 1 mM $CdCl_2$ and 1 mM$Pb(NO_3)_2$ showed abrupt reduction in RL and SL as compared to control. The possible reason behind reduction in growth could be due to inhibition of normal cell division by the metal as has been reported for *Dunaliellatertiolecta*exposed to mercury (Davies, 1976) and *Chlorella vulgaris* exposed to copper, mercury, and cadmium (Rosko and Rachlin, 1977; Sanita and Gabriella, 1999). The decrease in the rate of cell division caused by metals is primarily attributed to their binding to sulfhydryl groups which are important for regulating the plant cell division (Fisher *et al*., 1981). Pb is one of the ubiquitously distributed most abundant toxic elements in the soil. High level of Pb causes inhibition of enzyme activities, water imbalance, alterations in membrane permeability and disturbs mineral nutrition (Sharma and Dubey, 2005). Pb inhibits the activity of enzymes at cellular level by reacting with their sulfhydryl groups.Lead and cadmium are mostly localized extracellular in small concentration, but once they exceed the critical level, theyenter the cell membrane and interfere with metabolic processes (Chettri *et al*., 1997). This may be the reason for decrease of SL, RL in tomato mostly grown on high doses.Cd uptake in plants has been reported to block the entry of essential macro and micronutrients or pectin calcium binding sites such as Cd for Ca and decrease the cell wall elasticity (Barcelo*et al*., 1988). As a result it may affects growth i.e. differentiation and cell division. The study conducted by Aydinal and Marinova, (2009) also showed the reduction in alfalfa plant growth,grownon different concentration of heavy metals.Oancea*et al*., (2005) has also found the inhibitory function of heavy metals on plant growth and stated the effects as growth inhibition, structure damage, a decline in physiological and biochemical activities as well as function of plants.

5.3. ALDH expression analysis of *in-vivo* grown plants

ALDH genes are present in almost all the living organism. Plant ALDH are diverse, about nine ALDH families which includes fourteen ALDH genes are discovered to exist in *Arabidopsis thaliana* plant(Kirch et al., 2004). *Lycopersicumesculentum*varSijana is used as the

experimental organism in the present study taking *Arabidopsis thaliana* as the reference plant for the literature cited. Different transgenic and genetically modified plants have been produced with overexpression of ALDH gene constructs for their better survival, better yield and response to stresses(Kotchoni and Bartel, 2003). Thoughdifferent ALDH transformed transgenic plants are produced indicating that ALDH as widely studied plant, the spectrophotometric method used for ALDH activity determination and confirmation by NATIVE-PAGE conducted in the present study is new of its kind used in plants.Theanalysis of natural production of inherent ALDH in tomato plant under laboratory created stress environment and comparison with control plant is the new and innovative work being carried out in the present study.

A number of genes have been described that respond different abiotic stresses in plants (Zhang *et al*. 2004), and it is thought that their gene products may play important roles for acclimation of plants. Recently, 299 drought-inducible genes, 213 high-salinity stress-inducible genes, and 54 cold-inducible genes were identified using a cDNA microarray containing approximately 7000 independent full-length Arabidopsis cDNA clones. Functions of their gene products have been predicted from comparisons of sequence homology with known proteins. Genes induced during osmotic and cold stress conditions are thought to function not only in protecting cells from stress by the production of important metabolic proteins (functional proteins) but also in the regulation of genes for signal transduction in the stress response (regulatory proteins)(ncbi.nlm.nih.gov). Such examples of functional proteins include water channel proteins, chaperones, proteases, late embryogenesis-abundant (LEA) proteins, and enzymes that are involved with the synthesis of osmoprotectants [compatible solutes: sugars, proline (Pro), etc.]. Examples of stress-related regulatory proteins include transcription factors, protein kinases, and enzymes for phosphoinositide turnover, and enzymes for the synthesis of the plant hormone abscisic acid (ABA). Multiple studies have attempted to augment plant stress tolerance by overexpressing various kinds of functional proteins such as enzymes for the synthesis of osmoprotectants and ion transporters (Chen and Murata 2002, Zhang *et al*. 2004). However, it has become evident that the engineering of single enzymes is not sufficient, because multiple stress responses are necessary for plants to endure severe stress conditions.

Aldehyde molecules are common intermediate metabolites in most cellular pathways of carbohydrate, amino acids, protein, lipids or sterol metabolism (Kirch*et al*. 2004). However,the excessive production of these intermediates has detrimental effects because of their chemical reactivity (Lindahl 1992). Aldehydes can also be called as xenobiotic compounds as they have potential for chromosomal aberrations and DNA adducts (Comparti 1998). On the other hand, recently it has been found out that a constant level of aldehydes acts as signaling molecules, indicating that a constant level must be maintained.

One of the well-known and studied pathways for aldehyde detoxification is oxidation of aldehydes to carboxylic acid by ALDH. ALDH catalyzesNAD(P)$^+$-dependent the oxidation of aldehydes into carboxylic acid(Yoshida *et al*. 1998). NAD is as reducing equivalents and act as proton acceptor in this case. ALDH must not be confused with aldehyde oxidase. The latter catalyzes the pyridine nucleotide independent oxidation of aldehydes to acids and utilizes molecular oxygen as electron acceptor (Sreerama and Sladek, 2005). ALDH genes are one of mostly studied in plants especially *Arabidopsis*, same do in Humans. In plants ALDH form a large family and largely been annotated.

The most common method for ALDH quantification by spectrophotometric method is based on the measurement of increase in ΔA at 340nm as a criterion of NAD conversion to NADH by the enzyme in the presence of aldehyde substrate. Spectrophometric assay of ALDH can be used to quantify the collective catalytic presence of all or nearly all ALDH. Spectrophotometric assay is simple and straightforward and less time consuming than any other methods of analyzing enzyme function like HPLC, ELIZA etc. The accurate and estimated time planning is crucial for spectrophotometric assay of ALDH. For preparing one cycle of the reaction experiments about 2 to 2:30 hours of time set up is required. For each test samples 15 mins is required with additional 10 mins; 5 for preincubation and 5 for spectrophotometric kinetics measurement.For the accurate determination and measurement of ALDH activities (semi quantitative and quantitative) various precautions must be kept in mind. In assay involving aldehyde substrate, it is critical that the aldehyde be completely solubilized in aqueous solution and the solubilizer (DMSO) concentration be held under 5% in the final volume. DMSO under 5% concentrations is thought to be not affecting ALDH. And during reaction steps it is also necessary to perform positive and negative control reactionof each test to make sure that the assay is working as expected. Preincubation of reaction mixture and measurements of initial rates are also crucial in quantification of enzyme activities by spectrophotometry (Sreerama and Sladek, 2005).

In order to confirm ALDH activity measured through spectrophotometric method, NATIVE-PAGE was carried using crude homogenate of plant sample which showed considerable spectrophotometric activity. In NATIVE-PAGE, to obtain sharp ALDH activity bands, it is critical to focus the gels according to the parameters described. The results actually obtained in the experiments, NATIVE-PAGE did not show sharp bands except some of the bands in some samples butstill it could not be feasible to explain the numbers, type and characters of bands present. The bands are diffused or blur, exactly the number of isotypes cannot be assigned, observing bands on the gel. In fact the bands are of ALDH, but how many are being produced in particular samplecannot be proposed.Therefore, the type, number and characters of these isozymes could not be ascertained. Each band present corresponds to one ALDH isotype. Since ALDH NATIVE-PAGE Marker was not been run, the

particular type of isotype cannot be ascertained. Possibly due to less experimental set up the bands are also not clear. However, the visible bands in treated plant tissue samples are greater in number than that of control plant tissue samples in all three weeks. The greater number of bands which corresponds to a particular ALDH isotype in treatments can be said to be the bands of ALDH produced due to stress exposure since they are not present in non-stressed or control plant tissue samples. Week 2nd specially has some bands dark and distinct in treatment samples than control lane. Possibly the type of ALDH might be over expressed than control plant. The bands present in control sample lane might be due to the unknowing stress produced since the precise conditions cannot be maintained for the stress experiments.

5.4. Phytoremediation analysis

For phytoremediation analysis, tomato plants were grown in contaminated substrate in 1 kg plastic pots for about 8 weeks in present study. Jadia*et al*., 2008 in similar experiments had kept theiralfaalfa plants for 10 weeks in 2kg contaminated soil. The laboratory where the present research study was conducted do not have hydroponic techniques established so all work has been conducted in contaminated soil-vermicompost substrate. All the experiments were performed in replicate of three that could access for the statistical analysis. The treatments designed for the artificial contamination of the experimental soil vermicompost media was done according some articles'guidance. Jadia*et al*., 2008 selected metals and dosed at concentration ranging from 0, 5, 10, 20, 40 and 50 ppm separately in soil vermicompost media in pot experiment. Salt treatments were designedin the present study same as that done in Zahra *et al*., 2010. Zahra *et al*. also used NaCl at 25, 50, 75, and 100 mM concentrations to complete the experimental design. Uptake of heavy metals by plants from the soil is quite complex phenomenon as it does not follow any particular pattern and varies with respect to metals, plant species and plant parts (Barman *et al*., 2000). High accumulation may also be due to large or high affinity of ligands present in them and supplied metals (Tyler, 1990) and their cation exchange capacity.

The distribution and accumulation of heavy metals in plants is related to plant species, element species, chemical and bioavailability, and a number of environmental conditions such as redox, pH, cation exchange capacity, dissolved oxygen and temperature (Cheng, 2003; Weis and Weis, 2004). The tolerance of plants to heavy metals and the accumulation are dependent on various physiological factors such as uptake and leakage of metal ions by roots, root cation exchange capacity (CEC), phytochelatin production, antioxidative stress, carbohydrate production and utilization (Suresh and Ravishankar, 2004). Generally, the transportation of heavy metals is related to the chemical status in plants. Heavymetals can combine with inorganic substances (e.g. sulphides), and some small-molecular organic

substances such as glutathione (GSH), oxalic acid, histidine, citrate and metal-binding proteins in the plants (Cheng, 2003). In addition, metal ion interactions might be responsible for the regulation of metal uptake and translocation, for example, free proline acts as an antioxidant in Cd stressed cells, increasing phytochelator synthesis and sequestration of Cd, ultimately leading to hyperacculation (Suresh and Ravishankar, 2004). The responses of plants to the exposure to heavy metals are complicated due to variable tolerance as well as multivariate relationship between concentration of metals in soils and plant metal (Kabata-Pendais*et al*., 1993).

5.4.1. Lead uptake

Among the metal stresses given, tomato has been found to accumulate highest amount of Pb at 1 mM$Pb(NO_3)_2$treatment. Possible reason for this may be due to presence of organo-sulfur compounds like cysteine disulfide, methionine, glutamine, which forms complex with Pb because of being a border line metal (Kuiper 2005). Lower accumulation of all supplied heavy metals in tomato plants is indicated due to the presence of some avoidance mechanism to overcome heavy metal toxicity.

5.4.2. Cadmium uptake

Accumulation of cadmium depending on treatments ranged from 0.67 ppm to 4.11 ppm in tomato which is within the critical tissue concentration 5-30 ppm as given by Kabata-Pendias and Pendias, 1992. Cd is easily taken up by plants both actively and passively through simple diffusion and transported in aerial portion particularly binds to sulfhydryl groups (Hinsley, 1989).

5.4.3. Sodium uptake

As mentioned above, salinity commonly reduces growth and production of many vegetable crops such as tomatoes (Tantawy, 2007). Many trials have been attempted to get rid of this negative effect with the obtained research results (Li, 2000). The results of this study have a big impact in the applied field of agriculture where simple possible applications can be deployed where it's individual and/or their interactions remove the negative effects of salinity and improve optimal tomato growth and production.

NaCl at 100mM treatments showed the highest accumulation of Na in their tissues that numerates to 492.47 ppm. This is really high amount of sodium. The high accumulation of Na is also shown by the experiment by Verdez and Menendez, 2001. They showed that tomato plants can tolerate high levels of salt and in highly excessive concentrations were shown to have detrimental effects on the plants. The uptake value between 50mM and 75

mM treatments do not show statistical significance (p=0.05) in their uptake values. About same uptake value was measured. Possibly the experimental tomato plant cannot sense the difference in the treatments and that much difference in concentration cannot account for the difference in significant uptake values.

Sodium accumulation in tomato plant found to increase with the increase in concentration of metal in soil and seems to be accumulated in really large amount. The possible reason behind the larger accumulation may be due to larger amount present in the substrate media already. The soil and vermicompost itself has larger amount initially and then artificially contaminated with such larger amount lead to concentration dependent larger accumulation of Na. The concentration dependent larger accumulation may be due to the presence of more affinity of legends for salt present in the plant.Salt uptake in 1 mMNaCl treatment is found to be the highest. The plants in such treatment show some negligibly visible morphological retardation than the control plant. The effect may be due to disruption of several physiological processes in plants leading to reduction in growth and yield (Flowers *et al*., 1977). As far as the salt tolerance is concerned,plants have the ability to restrict the uptake and transport of Na ions from roots to shoots (Fernandez-Garcia *et al*, 2004).

The entry of the sodium into plant cell is still unclear. Although it is generally thought that sodium is 'mistaken' for potassium by potassium carriers or channels, it is also possible that sodium enters cells through non-selective cation channels, particularly those activated by glutamate (Maser *et al*., 2002). It is also apparent that in some plants, ions can reach the leaves through pathways that bypass the controls that normally force ions through a specialized layer of cells in the roots known as the endodermis. If there are breaks in the endodermis, water and dissolved solutes can flow to the leaves without encountering the selective barriers of cell membranes. This mechanism may be the reason for the greater accumulation of sodium ions in plant parts as potassium is essential ion and Na^+ is taken as other and thus accumulated unknowingly.

Salinity affects all dicotyledonous crops, but basic research cannot address all these species. The tomato is advocated as a model crop to test the accumulation efficiency and to study some stress responsive results of plant to overcome stresses. At the same time, the physiology of the tomato in salty and non-salty conditions has been extensively studied providing an invaluable base to understand the responses of the plants to cultural practices.

The differential accumulation of Pb, Cd and Na in tomato plants is because of their capacity of binding to ligands. The difference may also be due to metal species as Cd, Pb, and Na able

to pass through the cell membrane more readily, which are not essential for metabolic activity, are mostly retained between cell wall and cell membrane (Chettri *et al*., 2000).

CHAPTER 7: CONCLUSION

The present research work deals with ALDH expression and phytoremediation analysis by *Lycopersicumesculentum*varSrijana with response to different concentration of NaCl, $CdCl_2$ and $Pb(NO_3)_2$ in soil vermicompost media.

The effects of heavy metals and salt in plant is resulted in decrease in germination, growth inhibition, showed structure damage, change in physiological and biochemical activities, as well as the function of plants. Present study showed that both germination and plant growth are affected by the presence of heavy metals and salt in soil substrate. The low doses of heavy metals and salt applied stimulated the root and shoot elongation of tomato plants in some extents. At higher concentrations both germination and growth decreased and growth also decreased which is observed after 15 days of seed inoculation in heavy metal and salt contaminated half MS media.This conclude that the presence of stress like heavy metals and salt are deleterious to the productivity of crops since the plant can neither germinate properly nor can growth with normal RL and SL. Analyzing the expression of ALDH in response to the stresses subjected, enhanced expression of ALDH was found in stressed plants but in low level. This contributes to draw conclusion that tomato plant has inherent ALDH which functions to mitigate the environmental stresses. It would be interesting and beneficial for the identification, investigation and molecular characterization of ALDH genes in agronomical important crops with aim of improving crop tolerance to multiple environmental stresses. This finding suggests that ALDH genes are also present in our indigenously developed improved variety.Heavymetals are not significantly up taken at all concentration but it seems as if Na is efficiently up taken by the experimental plant and the uptake was increased along the increasing concentration in soil in all treatments.Therefore, experimental plant *Lycopersicumesculentum*var. Srijana cannot be assigned a good accumulator for the phytoremediation purpose. Rather, it could be stated that this plant has some features inherent that could resist the uptake of contaminants in them as hyperaccumulator plants can do. So, Srijana tomato can be cultivated in any land and harvest can be done with less contaminant entering the food chain.

Chapter 8.

References

Akashi K, Nishimura N, IshidaY and Yokota, A (2004) Potent hydroxyl radical-scavenging activity of drought-induced type-2 metallothionein in wild watermelon. *Biochemical and Biophysical Research Communications.***323:**72-78

American Public Health Association (APHA) (1998) Standard methods for the examination of water and waste water, 20th ed. Washington D.C., U.S.A

Amirjani MR (2011) Effects of salinity stress on growth,sugar content, pigments and enzyme activity of rice. *International Journal of Botany*.**7**(1): 73-81

Aydinal and Marinova (2009) The effects of heavy metals on seed germination and plant growth on alfalfa plant (*Medicago Sativa*). *Bulgarian Journal of Agricultural Sciences*.**15**(4): 347-350

Barcelo J and PoschenriederCh (1990) Plant water relation as affected by heavy metal stress: a review*. Journal of Plant Nutrition*.**13**: 1-37

Baker AJM and Walker, PL (1990) Eco-physiology of metal uptake by tolerant plants. *CRC Press.*155-178

Baligar VC, Campbell TA and Wright, RJ (1993) Differential responses of alfalfa clones to aluminum-toxic acid soil. *J. Plant Nutrition*. **16**: 219-233

BarmannSc, Sahu RK, Bhargava SK and Chetergee, C (2000) Distribution of heavy metals in wheat, mustard and weed grown in field irrigated with industrial effluents. *Bulletin Environ. Contam.Toxicol.***64**(4): 489-496

Bartels D (2001) Molecular mechanisms of desiccation tolerance in plants. Molecular Mechanisms of Metabolic Arrest: life in limbo. *BIOS Scientific.* 187-196

Bartels D (2001) Targeting detoxification pathways: an efficient approach to obtain plants with multiple stress tolerance.*Trends Plant Sci*. **6**: 284–286

Bartels D (2005) Dessication tolerance studied in the resurrection plant Craterostigmaplantagineum. *Integrative comp. Biol.***45**: 696-701

Bartels D and Salamini, S (2001) Dessication tolerance in the resurrection plant Craterostigmaplantagineum. A contribution to the study of draught tolerance at the molecular level.*Plant Physiology*.**127**: 1346-1353

Bartels D and Sunkar, R (2005) Drought and salt tolerance in plants.*Critical Reviews in Plant Sciences.***24**:23–58

Bartels D, Schneider K, Terstappen G, Piatkowski D and Salamini, F (1990) Molecular cloning of abscisic acid-modulated genes which are induced during desiccation of the resurrection plant Craterostigmaplantagineum.*Planta*.**181**: 27-34

Bhattarai T (2000) Experimental Plant Biochemistry and Plant Biotechnology (Tissue culture). *Bhunipuran Prakashan*.**1**: 86-95

Bolwell GP(1999) Role of active oxygen species and NO in plant defense responses. *CurrOpin Plant Biol.***2:** 287–294

Borah MC, Mahanta P, Koyoty SK, Saha UK and Sahasrabudhe, AD (2006) Study of quality parameters in vermicomposting.*Indian Journal of Biotechnology.***6B**: 410-413

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.*Anal Biochem*.**72**:248-254

Bringezu k, Lichtenberger O, Leopard I and Neumann, D (1999) Heavy metal tolerance of *Silene Vulgaris*.*Journal of plant physiolog*y.**154**: 536-546

Buchaver MJ (1973) contamination of soil and vegetation near a zinc smelter by zinc , Cadmium, Copper and lead. *Environ. Sci. Technol.* **7**:131-135

Burnet M, Lafontaine PJ, and Hanson, AD (1995) Assay, Purification, and Partial Characterization of Choline Monooxygenase from Spinach.*Plant Physiol.***108**: 581-588

Canfield RL, Henderson CR, Cox C and Jusko, TA (2003) Intellectual Impairment in Children with Blood Lead.*The new England journal of medicine***.348** (16): 1517-1526

Cardon DL, Villafan SM, Tovar AR, Perez S, Jimenez SP, Zuniga LAG, Allieri MAA, Perez NO and Dorantes, AR (2010) Growth response and heavy metals tolerance of *Axonopusaffinis,* inoculated with plant growth promoting rhizobacteria. *Afrcan Journal Of Biotechnology*.**9**(51): 8772-8782

Castiglione S, Franchin C, Fossati T, Lingua G, Torrigiani P and Biondi, S (2007) High zinc concentrations reduce rooting capacity and alter metallothionein gene expression in white poplar (Populus alba L. cv. Villafranca). *Chemosphere.***67:** 1117-1126

Chen HJC, Gonzalez FJ, Shou MG and Chung, FL (1998) 2,3-Epoxy-4-hydroxynonenal, a potential lipid peroxidation product for etheno adduct formation, is not a substrate of human epoxide hydrolase. *Carcinogenesis.***19:**939–943

Chen TH and Murata, N (2002) Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. *CurrOpin Plant Biol.* **5:** 250–257

Chen TH and Murata, N (2002) Enhacement of tolerance of abiotic stress by metabolic engineering of Betaines and other compatible solutes.*Cur opin. Plant Biol*. **5**: 250-257

Chen X (2002) The stress-responsive gene ALDH21A1 describes a novel eukaryotic aldehyde dehydrogenase protein family. *J. Plant Physiol*. **159**:677–684

Cheng S (2003) Review articles: Heavy metals in plants and phytoremediation.*Environmental Science and Pollution Research.* **10**(5): 335-340

Chettri MK, Swadis T, Zahariadis GA and Stratis, JA (1997) Uptake of heavy metals by living and dead chlamydia thali.*Eniron.Exp.Bot.***37**: 39-52

Chettri MK, Thapa KB, Poudel K and Acharya, B (2000) Localization of heavy metals in lichen thali: as ultrastructural approach. *Bios.***5:** 61-75

Chettri MK, Thapa KB, Poudel K and Acharya, BD (2001) Biomonitoring of toxic heavy metal in Ktm valley, *Ecoprint.***8**(11): 69-76

Chinnusamy V, Jagendrof A and Zhu, J (2005) Understanding and improving salt tolerance in plants.*Crop science*.**45**: 437-448

Chyan CL, Lee TTT, Liu CP, YangYC, Tzen JTC and Chou, WM (2005) Cloning and expression of a seed-specific metallothionein-like protein from sesame. *Bioscience Biotechnology and Biochemistry.***69:** 2319-2325

Clarkson DT and Luttge, U (1989) Mineral nutrition: divalent cations, transport and compartmentalization. *Prog Botany*.**51:** 93–112

Comporti M (1998) Lipid peroxidation and biogenic aldehydes: from the identification of 4 – hydroxynonenal to further achievements in biopathology.*Free Rad. Res*. **28**: 623-635

Cramer GR and Nowak, RS (1992) Supplemental manganese improves the relative growth, net assimilation and photosynthetic rate of salt stressed barely. *PhysiologaePlantarum.***84**: 600-605

Crowley DE, Wang YC, Reid CPP and Szansiszlo, PJ (1991) Mechanism of iron acquisition from siderophores by microorganisms and plants. *Plant and Soil.***130**: 179-198

Cunningham SD, Shann JR, Crowley DE, and Anderson TA (1997) Phytoremediation of contaminated water and soil.2-19

Dantas BF, Ribeiro LDS and Aragao, CA (2005) Physiological response of cowpea seeds to salinity stress. *Rev Bras Sementes*. **27**: 144-148

Davies AG (1976) An assessment of the basis of mercury tolerance in *Dunaliellatertiolecta*. *J. Mar. Biol. Assoc.***56**: 9–57

Del Rio D, steward AJ and Pellegrini, N (2005) A review of recent studies on malondialdehyde as toxic molecules and biological markers of oxidative stress. *Nutr Metals Cardiovacular Dis*. **15**(4): 316-28

Eide D, Broderius M, Fett J and Guerinot, ML (1996) A novel iron regulated metal transporter from plants identified by functional expression in yeast. *ProcNatlAcad Sci.* **93**:5624–5628

Fernandes JC andHenriques, FS (1991) Biochemical, physiological, and structural effects of excess copper in plants.*Bot. Rev*. **57**: 246-27

Fernandiz-Garcia N, Carvajal m, Cerda A and Martinez, V (2004) Effects of salanity on growth, mineral composition and water relations of grafted Tomato plants.*Journal of Plant nutrition and soil sciences.***167**: 612-622

Fisher NS, Jones GJ and Nelson, DM (1981) Effect of copper and zinc on growth, morphology and metabolism of Asterionella japonica (Cleve).*J. Exp. Biol. Ecol*. **51**: 37–56

Flores P, Carvajal m, Cerda A and Martinez, V (2001) Salinity and Ammonium/ Nitrate Interaction in Tomato plant development,Nutrition and metabolites.*Journal of plant nutrition.***24:** 1561-1573

Flowers TJ (2004) Improving crop salt tolerance.*Journal Exp Bot*. **55**: 307–319

Flowers TJ and Yeo AR (1995) Breeding for salinity resistance in crop plants: where next?.*Australian Journal of Plant Physiology*.**22**: 875–884

Foreman J, Demidchik V, Bothwell JHF, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, Brownlee C and Jones, JDG (2003) Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature.***422:** 422-446

Foyer CH and Noctor, G (2003) Redox sensing and signaling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. *Physiol. Plant*. **119**: 355-364

Freisinger E (2008) Plant MTs-long neglected members of the metallothionein superfamily. *Dalton Transactions.*6663-6675

Gardea-Torresdey JL, Peralta-Videa JR, Montes M, Rosa GDL and Corral–Diaz, B (2004) Bioaccumulation of cadmium, chromium and copper by *Convolvulus arvensis*L. impact on plant growth and uptake of nutritional elements. *Biores. Technol.* **92**:229-235

Ghosh M and Singh SP (2005) A Review on Phytoremediation of Heavy Metals and Utilization of its Byproducts. *Applied Ecology and Environmental Research.***3**(1): 1-18

Gill PK, Sharma AD, Singh P andBhullar, SS (2003) Changes in germination growth and soluble sugar contents of *Sorghum bicolor* (L.) Moench seeds under various abiotic stresses. *Plant Growth Regulation.***40**:157-162

Gill SS and Tuteja N (2010) Polyamines and abiotic stress tolerance in plants. *Plant Signaling & Behavior.***5**(1): 26-33

Gill T, Kumar S, Ahuja PS and Sreenivasulu, Y (2010) Over-expression of *Potentilla*superoxide dismutase improves salt stress tolerance during germination and growth in *Arabidopsis thaliana.***1**(1): 1-10

Glick BR (2003) Phytoremediation: Synergistic Use of Plants and Bacteria to Clean Up the Environment. *Biotechnology Advances.***21**: 383-393

Godbold DL and Hutternann, A (1985) Effect of Zinc, cadmium and mercury on root elongation of Piceaabies (Karst) seedlings and the significance of these metals to forest die back. *Environ, Poll.ser. A.***38**: 375-381

Goel A andSheoran, IS (2003) Lipid peroxidation and peroxide-scavenging enzymes in
Goel A, Sheoran I S (2003) Lipid peroxidation and peroxide-scavenging enzymes in cotton seeds under natural ageing. *Biol. Plant.***46**(3): 429-434

Gondek K and Filipek-Mazur B (2003) Biomass yields of shoots and roots of plants cultivated in soil amended by vermicomposts based on tannery sludge and content of heavy metals in plant tissues. *Plant Soil Environ*. **49**(9): 402-409

Gopa R and Dubem, BK (2003) Influence of variable potassium on barley metabolism. *Ann. Agric. Res.***24**: 73-77

Goyer RA (1997) Toxic and essential metal interactions.*Ann. Rev. Nutr*. **17**: 37–50

Grant JJ, Yun BW and Loake GJ (2000) Oxidative burst and cognate redox signaling reported by luciferase imaging: identification of a signal network that functions independently of ethylene, SA and Me-JA but is dependent on MAPKK activity. *Plant J.* **24**: 569-582

Halim M, Conte P and Piccolo, A (2003) Potential availability of heavy metals to phytoextraction from contaminated soils induced by exogenoushumic substances.*Chemosphere.***52**: 265

Hall JL (2002) cellular mechanism for heavy metal detoxification and tolerance.*Journal of Experimental Botany.***53**(366): 1-11

Hasegawa PM, Versan RAJ, Zhu K and Bonhert, HJ (2000) Plant cellular and molecularresponses to high salinity. *Ann. Rev. Plant Physiol. and Plant Mol. Biol.* **51**: 463-499

Hassinen V, Vallinkoski VM, Issakainen S, Tervahauta A, Karenlampi S and Servomaa, K (2009) Correlation of foliar MT2b expression with Cd and Zn concentrations in hybrid aspen (Populustremula x tremuloides) grown in contaminated soil. *Environmental Pollution.***157**: 922-930

Hattenschwile S and Gaser, P (2005) Soil animals after plant litter diversity effects on decomposition. *PNAS*.**102**: 1519–1524

He Y and Gan, S (2004) A novel zinc-finger protein with a proline-rich domain mediates ABA-regulated seed dormancy in Arabidopsis*. Plant Mol. Biol.***54**: 1-9

Hinsley H (1978) Zinc and cadmium accumulation by corn inbreds grown on sludge amended soils. *Agron J.* **70:** 425–428

Holmstrom KO, Somersalo S, Mandal A, Palvo TE and Welin, B (2000) Improved tolerance to salinity and low temperature in transgenic tobacco producing glycine betaine. *Journal of Experimental Botany*.**51**(343): 177-185

Hoth S, Morgante M, Sanchez JP, Hanafey MK, Tingey SV and Chua, NH (2002) Genome-wide gene expression profiling in Arabidopsis thaliana reveals new targets of abscisic acid and largely impaired gene regulation in the abi1-1 mutant. *J Cell Sci*.**115**: 4891–4900

http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=212336 (assessed in 2068-3-26 6:53am)
http://www.molecularfarming.com/phyto.html (assessed in 2068-3-28 10:47 am)

http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=213126 (assessed in 2012-7-23 at 12 pm)

Hussein HS, Ruiz ON, Terry N andDaniell, H (2008) Phytoremediation of Mercury and Organomercurials in Chloroplast Transgenic Plants: Enhanced Root Uptake, Translocation to Shoots, and Volatilization.*Environ Sci Technol*. **41**(24): 8439–8446

Ingram J and Bartels, D (1996) The molecular basis of dehydration tolerance in plant. *Ann. Rev. Plant Physiol. Plant Mol. Biol.***47**: 377-403

Ishitani M, Nakamura T, Han SY andTakabe, T (1995) Expression of the betaine aldehyde dehydrogenase gene in barley in response to osmotic stress and abscisic acid. *Plant Mol Biol.***27**: 307–315

Jadia CD and Fulekar, MH (2008) Phytotoxicity and Remediation of Heavy Metals by Alfalfa (*Medicago sativa*) in Soil-vermicompost Media.*Advances in Natural and Applied Sciences*. **2**(3): 141-151

Jagrati A, Sahu R, Bharadwaj N and Kalpana, S (2012) Determination of Levels of Uptake of few Heavy Metals to the Leaves of *Menthaarvensis*Atomic Absorption Spectrophotometrically. *International Journal of Research in Chemistry and Environment***.2**(2): 63-67

Jamil M, Lee DB, Jung KY, Ashraf M, Lee SHC and Rha, ESH (2006) Effect of salt (NaCl) stress on germination and early seedling growth of four vegetables species. *Central europian agriculture*.**7**(2): 273-282

Kabata-Pendias (2001) Trace elements in Soils and Plants (3rd edn.).*CRC Press, Boca Raton, Fl*. 413

Kaiser WM (1979) Reversible inhibition of the Calvin cycle and activation of the oxidative pentose phosphate cycle in isolated chloroplast by hydrogen peroxide.*Planta*.**145**: 377-382

Kamel HA (2008) Lead Accumulation and its Effect on Photosynthesis and Free Amino Acids in *Viciafaba*Grown Hydroponically.*Australian Journal of Basic and Applied Sciences*.**2**(3): 438-446

Karademir M and Toker, MC (1988) Lead accumulation in the grasses growing on same of the crossroads of Ankara from exhausts gases.*Journal Ecological Environment*.**7**(26): 9-12

Karimi G, Ghorbanli M, Heidari H, Khavarinejad RA andAssareh, MH (2005) The effects of NaCl on growth, water relations, osmolytes and ion content in Kochia prostrate. *Biol. Plant*. **49**: 301-304

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, and Shinozaki, K(1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor.*Natl. Biotechnol.***17**:287–291

Kawasaki S, Borchert C, Deyholos M, Wang H, Brazille S, Kawai K, Galbraith D and Bohnert, H(2001) Gene expression profiles during the initial phase of salt stress in rice. *Plant Cell*.**13**:889–905

Kheir EA, Sheshshayee MS, Prasad TG, and Udayakumar, M (2012) Molecular biology and physiology.*TheJournal of Cotton Science.***16**:190–199

Khurana MPS and Singh, P (2012) Waste Water Use in Crop Production: A Review.*Resources and Environment*.**2**(4): 116-131

Kim KY, Kwon SY, Lee HS, Hur Y, Bang JW andKwak, SS (2003) A novel oxidative stress inducibleperoxidase promoter from sweetpotato: molecular cloning and characterization in transgenic tobacco plants and cultured cells. *Plant Mol Biol*. **51**: 831-838

Kinraide TB (1998) Three mechanisms for the calcium alleviations of mineral toxicity. *Plant Physiol.***118**: 513-520

Kirch HH (2001) Novel ABA- and dehydration-inducible aldehyde dehydrogenase genes isolated from the resurrection plant Craterostigmaplantagineum and Arabidopsis thaliana. *Plant J*. **28**: 555–568

Kirch HH, Schlingensiepen S, Kotchoni S, Sunkar R and Bartels, D (2005) Detailed expression analysis of selected genes of the aldehyde dehydrogenase (ALDH) gene superfamily in Arabidopsis thaliana.*Plant Molecular Biology*.**57**: 315–332

Kohler A, Blaudez D, Chalot M and Martin, F (2004) Cloning and expression of multiple metallothioneins from hybrid poplar. *New Phytologist.***164:** 83-93

Kotchoni SO, Kuhns C, Ditzer A, Kirch HH andBartel, D (2006) Over-expression of different aldehyde dehydrogenasegenes in Arabidopsis thalianaconfers tolerance to abiotic stress and

protects plants against lipid peroxidation and oxidative stress *Plant. Cell and Environment*.**29**: 1033–1048

Kovtun Y, Chiu WL, Tena G and Sheen, J (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. *Proceedings of the National Academy ofSciences*.**97**:2940-2945

Krämer U, Cotter-Howells JD, Charnock JM, Baker AJM and Smith, JAC(1996) Free histidine as a metal chelator in plants that accumulate nickel. *Nature*. **379**: 635-638

Lacerda CF, Cambraia J, Cano MAO, Ruiz HA and Prisco, JT (2003) Solute accumulation and distribution during shoot and leaf development in two sorghum genotypes under salt stress. *Environ. Exp. Bot*. **49**: 107-120

Li G, Hu N, Ding D, Zheng J, Liu Y, Wang Y and Nie, X (2011) Screening of Plant Species for Phytoremediation of Uranium, Thorium, Barium, Nickel, Strontium and Lead Contaminated Soils from a Uranium Mill Tailings Repository in South China. *Bulletin of Environmental Contamination and Toxicology*.**86**(6): 646-652

Li Y (2000) *Analysis of greenhouse tomato production in relation to salinity and shoot Environment.*Ph.D. thesis submitted inWageningen Agricultural University, UK

Lindahl R (1992) Aldehyde dehydrogenases and their role in carcinogenesis.*Crit Rev BiochemMol Biol.* **27**: 283–335

Lindahl R and Petersen, DR (1991) Lipid aldehyde oxidation as a physiological role for class 3 aldehyde dehydrogenases. *Biochem.Pharmacol.***41**: 1583-1587

Ma Y, Dickinson NM and Wong, MH (2003) Interactions between earthworms, trees, soil nutrition and metal mobility in amended Pb/Zn mine tailings from Guangdong, China. *Soil Biol. Biochem.***35**: 1369

Maser P, Gierth M and Schroeder, JI (2002) Molecular mechanisms of potassium and sodium uptake in plants.*Plant Soil*.**247**: 43–54

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

Munns R, Hare RA, James RA andRebetzke, GJ (2000) Genetic variation for salt tolerance of durum wheat.*Aust. J. Agric. Res*. **51**: 69-74

Munns R (2002) Comparative Physiology of salt and water stress. *Plant, Cell and Environment*.**25**: 239–250

Nahar K and Gretzmacher, R (2011) Response of Shoot and Root Development of SevenTomato Cultivars in Hydroponic System under Water Stress.*Academic Journal of Plant Sciences*.**4**(2): 57-63

Nawaz K, Hussain K, Majeed A, Khan F, Afghan S and Ali, K (2010) Fatality of salt stress to plants: Morphological, physiological and biochemical aspects.*African Journal of Biotechnology*.**9**(34): 5475-5480

Nies DH (1999) Microbial heavy-metal resistance.*ApplMicrobiolBiotechnol*. **51**: 730 -750

Niyogi KK, Bjorkman O and Grossman, AR (1997) The role of specific xanthophylls in photoprotection. *Proc. Natl. Acad. Sci*. **94**: 14162–14167

OanceaS, Foca N and Airinei, A (2005) Effects of heavy metal in plant growth and photosynthetic activity. AnaleleStintificeuniversity.

Parida SK and Das AB (2005) Salt tolerance and salinity effects on plants.*Ecotoxicol. Environ safety.***66**: 324-349

Pastori GM and Foyer CH (2002) Common components, networks and pathways of cross-tolerance to stress.The central role of 'redox' and abscisic-acid-mediated controls.*Plant Physiology.***129**:460–468

Paul FH (2000) Earthworms. In: Hand Book of Soil Science*. Sumner M.E. (Ed.), CRC Press.* 77-85

Peng HY, Yang XE, Jiang LY and He, ZL (2005) Copper phytoavailability and uptake by Elsholtziasplendens from contaminated soil as acted by soil amendments. *Journal of Environmental Science and Health Part AToxic/ Hazardous Substances and Environmental Engineering.* **40**: 839-856

Peralta-Videa JR, Rosa G, Gonzalez JH and Gardea-Torresdey, JL (2004) Effects of the growth stage on the heavy metal tolerance of alfalfa plants. *Advances in Environmental Research*.**8:** 679-685

Perozich J, Nicholas H, Lindahl R, and Hempel, J (1999) The big book of aldehyde dehydrogenase sequences. An overview of the extended family.*Adv. Exp. Med. Biol*. **463**: 5–

52Wood AJ, Saneoka H, Rhodes D, Joly RJ, Goldsbrough PB (1996) Betaine aldehyde dehydrogenase in sorghum. *Plant Physiol*. **110**: 1301–1308

Perry MW and Greenway, H (1973) Permeation of uncharged organic molecules and water through tomato roots.*Ann. Bot*. **37**: 225–232

Peuke AD and Rennenberg, H (2005) Phytoremediation.*European molecular biology organization.***6** (6): 497-501

Pitzschke A, Forzani C andHirt, H (2006) Reactive oxygen species signaling in plants.*Antioxidants and Redox Signaling*.**8**: 1757-1764

Ramanjulu S and Bartels D (2002).Drought- and desiccation-induced modulation of gene expression in plants*. Plant Cell Environ*. **25**: 141–151

Raskin I, Smith RD and Salt, DE (1997) Phytoremediation of metals: using plants to remove pollutants from the environment. *Current Opinion in Biotechnology*.**8**:221–226

Rauser WE (1999) Structure and function of metal chelators produced by plants: the case for organic acids, amino acids, phytin, and metallothioneins. *Cell Biochem and Biophys*.**31:**19-48

Rehman F, Khan FA, Varshney D, Naushin F andRastogi, J (2011) Effect of cadmium on the growth of tomato. *Biology and Medicine.***3**(2): 187-190

Robinson SP and Jones, GP (1986) Accumulation of glycinebetaine in chloroplasts provides osmotic

Rodrigues SM, Andrade MO, Gomes APS, DaMatta FM, Baracat-Pereira MC and Fontes, EPB (2006) Arabidopsis and tobacco plants ectopically expressing the soybean antiquitin-like ALDH7 gene display enhanced tolerance to drought, salinity, and oxidative stress. *Journal of experimental biology.***57**(9):1909–1918

Rosenstock L (2003) The Environment as a Cornerstone of Public Health, *Environmental Health Perspectives*. **111**(7): 376-377

Rosko JJ andRachlin, JW (1977) The effect of cadmium, copper, mercury, zinc and lead on cell division, grown and chlorophyll a content of the chlorophyte Chlorella vulgaris, *Bull. Torrey Bot. Club.***104:** 226–275

Sabath E, Ludirina M and Sokia MLR (2012) Renal health and environment heavy metal nephrotoxicity. *Nephrologia*. 32(2): 279-286

Salt DE, Prince RC, Pickering IJ and Rasking, I (1995) Mechanism of cadmium mobility and accumulation in Mustard. *Plant Physiology.***109**: 1427-1433

Schnoor JL (1997) Phytoremediation.*Technology Evaluation Report*

Seki M, Narusaka M, Abe H, Kasuga M, Yamaguchi- Shinozaki K, Carninic P, Hayashizaki Y, and Shinozaki, K(2001) Monitoring the expression pattern of 1300 Arabidopsis genes under drought and cold stresses by using a full-length cDNA microarray. *Plant Cell*.**13**:61–72

Senden MHMH, Van Paassen FJM, Van Der Mer AJGM and Wolterbeek, H (1992) Cadmium–citric acid–xylem cell wall intractions in tomato plants. *Plant Cell Envir*.**15**:71–79

Shalata A, Mittova V, Volokita M, Guy M and Taj, M (2001) Response of cultivated tomato and its wild salt-tolerant relative *Lycopersiconpennellii*to salt-dependent oxidative stress: the root antioxidative system. *Physiol. Plant*. **112**: 487-494

Sharma P andDubey RS (2005) Lead toxicity in plant. *Brazilian J. Plant Physiol.* **17:** 35–52

Shinozaki K, Yamaguchi-Shinozaki K and Seki,M (2003) Regulatory network of gene expression in the drought and cold stress responses.*Curr.Opin Plant Biol.***6**: 410-417

Singh A and Sharma, S (2003) Effect of microbial inocula on mixed solid waste composting, vermicomposting and plant response*. Compost Science & Utilization*. **11**: 190-199

Singh KP, Singh K (1981) Stress Physiological studies on seed germination and seedling growth of source wheat hybrids. *Indian Journal of Physiology* **24**:180-186

Skibbe D (2002) Characterization of the aldehyde dehydrogenase gene families of Zea mays and Arabidopsis.*Plant Mol. Biol*. **48**: 751–764

Smirnoff N and Cumbes OJ (1989) Hydroxyl radical scavenging activity of compatible solutes.*Phytochem.***28:** 1057–1060

Sophos NA and Vasiliou V (2003) Aldehyde dehydrogenase gene superfamily: the 2002 update. *Chem. Biol. Interact*. *143*(144): 5-22

Sreerama L, Hedge MW and Sladek, NE (1995) Identification of a class 3 aldehyde dehydrogenase in human saliva and increased levels of this enzyme, glutathione S-transferases, and DT-diaphorase in the saliva of subjects who continually ingest large quantities of coffee or broccoli. *Clin. Cancer Res.* **1**: 1153-1165

SRU (2004) *Umweltgutachten 2004: UmweltpolitischeHandlungsfähigkeitsichern.* Stuttgart, Germany: Metzler-Poeschel

Sunkar P, Bartels D andKirch, HH (2003) Overexpression of a stress-inducible aldehyde dehydrogenase gene from Arabidopsis thaliana in transgenic plants improves stress tolerance.*The Plant Journal.***35**:452–464

Sunkar R, Bartels D and Kirch, HH (2003) Overexpression of stress-inducible aldehyde dehydrogenase gene from *Arabidopsis thaliana* in transgenic plants improves stress tolerance. *Plant Journal.***35:** 452–464

Suresh B and Ravishankar, GA (2004) Phytoremediation-A novel and promising approach for environmental clean-up. *Critical reviews in biotechnology.***24:** 2-3

SzabolcsI (1994) Soils and salinization.*InHandbook of Plant and Crop Stress.* 3–11

Tantawy ASN (2007) *Effect of some mineral and organic compounds on salinity tolerance in tomato.*Ph.D. thesis.Submmited to Fac. Agric. Al-Azhar University, Cairo, Egypt.

Taylor GJ and Crowder, AA (1983) Uptake and accumulation of heavy metals by *Typhalatifolia*in wetlands of the Sudbury; Orlando region.*Can. J. Bot.* **61**: 63-73

Thiele DJ (1992) Metal regulated transcription in eukaryotes*. Nucleic Acids Res*. **20**: 1183-1186

Tindal HD (1992). Commercial Vegetables Growing.*Oxford Tropical Handbook.*129–131

Tsuji H, Meguro N, Suzuki Y, Tsutsumi N, Hirai A and Nakazono, M (2003) Induction of mitochondrial aldehyde dehydrogenase by submergence facilitates oxidation of acetaldehyde during re-aeration in rice. *FEBS Letters*.**546:** 369-373

Tyler G (1990) Bryophytes and heavy metals: A Literature Review. *Bot j Linn Soc.***104:**231-2534

Vasiliou V (1999) Eukaryotic aldehyde dehydrogenase (ALDH) genes: human polymorphisms, and recommended nomenclature based on divergent evolution and chromosomal mapping. *Pharmacogenetics.***9**: 421–434

Vasiliou V, Kozak C, Lindahl R and Nebert, DW (1996) Mouse michrosomal class 3 aldehyde dehydrogenase: ALDH3 cDNA sequence, inductibility by dioxin and chlofibrate, and genetic mapping. *Cell Biol*. **15**: 235-245

Vasiliou V, Bairoch A, Tipton K F and Norbert, DW (1999) Eukaryotic aldehyde dehydrogenase (ALDH) genes: human polymorphisms, and recommended nomenclature based on divergence evolution and chromosomal mapping. *Pharmacogenetics*.**9**: 421-434

Verdez TA and Menendez MM (2001) Tomato plants Tolerate High Salt. *Environmental Science and Technology*.**35**: 402-406

Vivares D, Arnoux P and Pignol, D (2005) A papain-like enzyme at work: Native and acyl-enzyme intermediate structures in phytochelatin synthesis. *Proceedings of the National Academy of Sciences of the United States of America.***102**:18848-18853

Wacker M, Wanek P and Eder, E (2001) Detection of 1, N-2-propanodeoxyguanosineadducts of trans-4-hydroxy-2-nonenal after gavage of trans-4-hydroxy-2-nonenal or induction of lipid-peroxidation with carbon-tetrachloride in F344 rats.*Chem.-Biol. Interact.***137**: 269-283

Weast RC (1984) CRC Handbook of Chemistry and Physics.*CRC Press*

Weber H, Chetelat A, Reymond P and Farmer, E (2004) Selective and powerful stress gene expression in *Arabidopsis* in response to malondialdehyde. *Plant Journal.***37:**877–888

Weis JS and Weis, P (2004) Metal uptake, transport and release by wetland plants: implications for phytoremediation and restoration. *Environment International.* **30**(5): 685-700

Wood AJ, Saneoka H, Saneoka, Rhodes D, Joly RJ,and Goldsbrough, PB (1996) Betaine Aldehyde Dehydrogenase in Sorghum.*Plant Physiol*. **110**: 1301-1308

Xiong AM and Jayaswal RK (1998) Molecular characterization of chromosomal determinant conferring resistance to zinc and cobalt ions in Staphylococcus aureus.*Journal of Bacteriology.***180:**4024-4029

Xiong L, Schumaker KS and Zhu, JK (2002) Cell Signaling during Cold, Drought, and Salt Stress. *The plant cell.* 165-183

Yamaguchi S and Kamiya, Y (2001) Gibberellins and light-stimulated seed germination.*J. Plant Growth Regul.* 20: 369-376

Yameguchi-shinazaki K, kasuga M, Liu Q, Nakashima K, Sakama Y, Abe H, Shinwary ZK, Seki M and Shinozaki, K (2002) Biological mechanism of draught stress response.*JIRCAS working repost*. 1-8

Yoshida A, Rzhetsky A, Hsu LC and Chang, C (1998) Human aldehyde dehydrogenase gene family. *Eur. J. Biochem.***251**: 549-557

Yusuf AA, Arowolo TA and Bamgbose, O (2003) Cadmium, copper, and nickel levels in vegetables from industrial and residential areas of Lagos City, Nigeria. *Food Chem. Toxicol*.**41**: 375-378

Zahra S, Amin B and Mehdi, Y (2010) The salicylic acid effect on the tomato (lycopersicumesculentum Mill.) germination, growth and photosynthetic pigment under salinity stress (NaCl). *Journal of Stress Physiology & Biochemistry*.**6**(3): 4-16

Zhang W, Wang C, Qin C, Wood T, Olafsdottir G, Welti R and Wang, X (2003) The oleate-stimulated phospholipase D (PLDä) and phosphatidic acid decrease H2O2-induced cell death in Arabidopsis. *Plant Cell*.**15**: 2285-2295

Zhu Y, Zhang L, Fan J and Han, S (2007) Neural basis of cultural influence on self-representation. *NeuroImage*.**34**: 1310–1316

APPENDECES

Appendix I: Reagents for ALDH activity test

Aldehyde dehydrogenase staining solution

Assay buffer	5ml
Pyrazole	0.68mg (1mM final concentration)
Nitrobluetetrazolium (NBT)	8mg (1mM final concentration)
Phenazinemethosulphate	0.4mg (130 µM final concentration)
Acetaldehyde	500 µl of 80mM stock
NAD	500 µl of 80mM stock

-Adjust final volume to 10ml by H_2O. Use immediately.

Assay buffer (1000ml)

Sodium pyrophosphate	28.6g
Disodium EDTA	744mg
d/w	1000ml

pH	8.1 (with 1M NaOH)

Destaining solution

Glacial acetic acid	100ml
Methanol	300ml
d/w	600ml

Acetaldehyde (80mM)

Procedure: Dissolve 45 µl of acetaldehyde in 955 µl of ice cold d/w to get an 80mM solution. Acetaldehyde substrate is freshly prepared each time and kept on ice all the time during the experiment.

Glutathione (GSH) (100mM)

Glutathione (GSH)	307mg
d/w	10ml
pH	8.1

Store in 0.5ml aliquots in 1.5ml microcentrifuge tubes up to 1year at -20^{O}C. Store on ice during use.

NAD (80mM)

Nicotine adenine dinucleotide (NAD)	531mg
d/w	10ml

Store in 0.5ml aliquots in 1.5ml microcentrifuge tubes up to 1year at -20^{O}C. Store on ice during use.

Pyrazole (2mM)

Pyrazole	1.4mg
d/w	10ml

Store in 1ml aliquots in 1.5ml microcentrifuge tubes up to 1year at -20^{O}C. Store on ice during use.

Appendix II: NATIVE PAGE reagents

Solution for preparing resolving gel (12%) 10ml

H_2O	3.3
23% Acrylamide	4.0
0.5 Tris (PH 6.8)	2.5
10% Ammonium persulphate (APS)	0.1
TEMED	0.004

Solution for preparing stacking gel (5%) 2ml

H2O	1.4
23% Acrylamide	0.33
0.5 Tris (PH 8.8)	0.25
10% Ammonium persulphate (APS)	0.02
TEMED	0.002

Monomer solution (30% acrylamide solution)

Acrylamide	= 29gm
Bis Acrylamide	= 1gm

Final volume100ml was maintained by triple distilled water (TDW)

Electrophoresis buffer

Tris base	15g (125mM fc)
Glycine	72g (0.96M fc)
d/w	1000ml

10% Ammonium per sulfate $(NH_4)_2S_2O_8$

Ammonium persulfate = 50mg

TDW = 500µl

TEMED (N,N,N',N'-Tetramethylenediamine)

Add 50 µl TEMED in EP tube and use.

Sample loading buffer (2x)

d/w	10.4ml
0.5M Tris-Cl	1.2ml
Glycerol	1.9ml
1% (w/v) Bromophenol blue (BMB)	1ml

Running buffer (500ml)

39mMTris	2.362gm
48mM Glycine	1.80gm

Preparation: Dissolve above chemicals in 200ml TDW and mix properly with magnetic stirrer. Maintain the volume 400ml and adjust pH to 8.4. Add 100ml TDW to make final volume 500ml.Filter the solution.

Appendix III: Bradford Assay reagents

Bradford reagent (500ml)

Comassie Brilliant ble G250 (CBB)	50mg
Absolute ethanol	25ml
Ortho Phosphoric acid (85%)	50ml
d/w	425ml

Procedure: Weigh 50mg CBB-G250 and dissolve in 25ml abs. ethanol. Add 50ml of 85% ortho phosphoric acid and make final volume 500ml by d/w.

Assay buffer (100ml)

Sodium pyrophosphate	2.86gm
Disodium EDTA	74.4mg
d/w	100ml
pH	8.1 (with 1M NaOH)

Standard BSA solution

Procedure: Weigh 50mg BSA and dissolve in 20ml Assay buffer. Stir well slowly (do not allow to form froth) and adjust the final volume to 50ml in a falcon tube.

Table: Absorbance values of standard BSA protein.

BSA standard protein conc (µg/ml)	Abs (595nm)
0	0
20	0.317
40	0.4
80	0.583
120	0.743
160	1.038
200	1.158

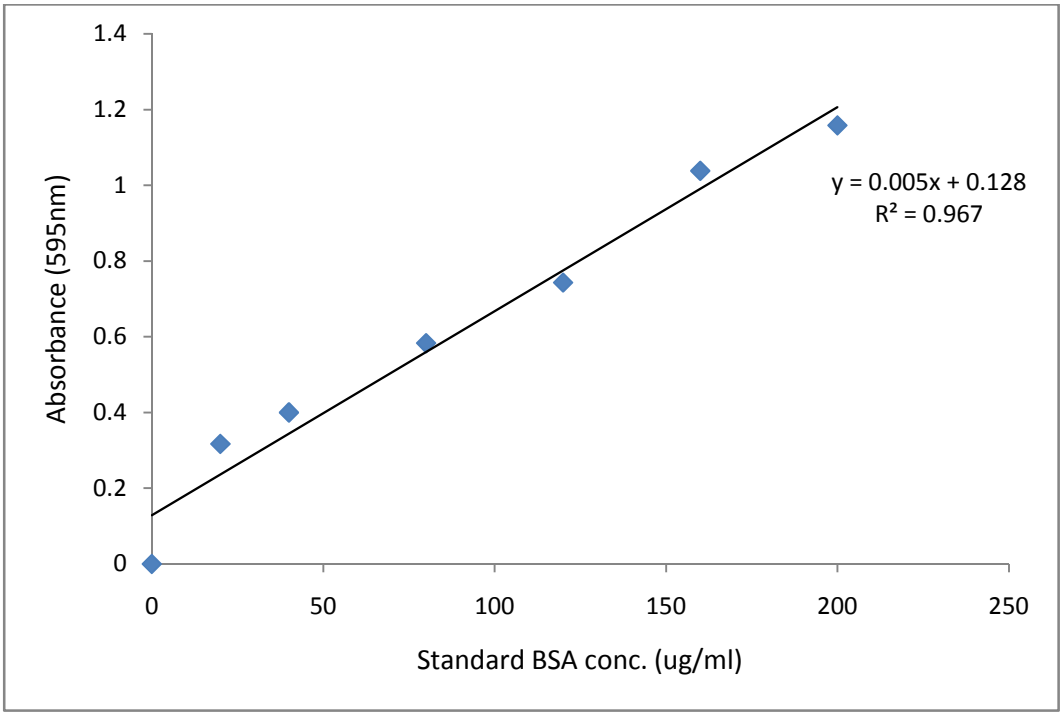


Fig: Standard BSA calibration curve. The curve was used to calculate the protein concentration in the earthworm tissue sample. Protein concentration was then used to calculate the specific ALDH activity of the samples.

Appendix IV: MS media preparation- 1ltr

MS macro Stock(10x)	100ml
MS micro Stock(10x)	1ml
MS vitamin (1000X)	1ml
Stock V	1ml
Myo-inositol	100mg
Sucrose	30gm
Agar	0.80%
pH	5.8
Final Volume	1000ml
Autoclve	121^0c for 15 mins

MS Macro Salt (10X) - 1ltr

NH_4NO_3	16.0gm
KNO_3	19.0gm
$CaCl_2.2H_2O$	4.4gm
$MgSO_4.2H_2O$	3.7gm
KH_2PO_4	1.7gm

MsMicro salt (1000X) - 100ml

$MnSO_4.4H_2O$	2.23gm
H_3BO_3	0.62gm
$ZnSO_4.7H_2O$	0.86gm
KI	0.083gm
$Na_2MoO_4.2H_2O$	0.025gm
$CuSO_4.5H_2O$	0.0025gm
$CoCl_2$	0.0025gm

MS Vitamin (1000X) – 100ml

Nicotinic acid	500mg
Pyridine HCL	50mg
Thiamine HCl	10mg
Glycine	200mg

Stock V (1000X) – Fe2 EDTA

Dissolve 27.8gm $FeSO_4.7H_2O$ + 37.2gm Na_2EDTA/ltr Distilled water

ow three band types; (viii), (ix) and (x) which are themself faint.

Fig: 4.8. NATIVE-PAGE of Aldehyde Dehydrogenases present in whole homogenates of tomato leaves in week 2. **Lane 1:** Control plant tissue sample**. Lane 2:** 25 mMNaCl contaminated soil. **Lane 3**: 50 mMNaCl plant tissue homogenate **Lane4**: 75 mMNaCl plant tissue homogenate **Lane 5**: 100mMNaCl plant tissue homogenate. **Lane 6**: 0.1 mM $CdCl_2$ plant tissue homogenate.**Lane 7, 8**: 0.3 mM $CdCl_2$ plant tissue homogenate..**Lane 9**: 0.5 mM $CdCl_2$ plant tissue homogenate.**Lane 10**: 1mM $CdCl_2$ plant tissue homogenate.**Lane 11, 12:** 0.1 mM$Pb(NO_3)_2$ plant tissue sample. **Lane 13**: 0.3mM$Pb(NO_3)_2$ plant tissue homogenate. **Lane 14**: 0.5mM$Pb(NO_3)_2$ plant tissue homogenate. **Lane 15**: 1mM$Pb(NO_3)_2$ plant tissue homogenate

Week 3rd:

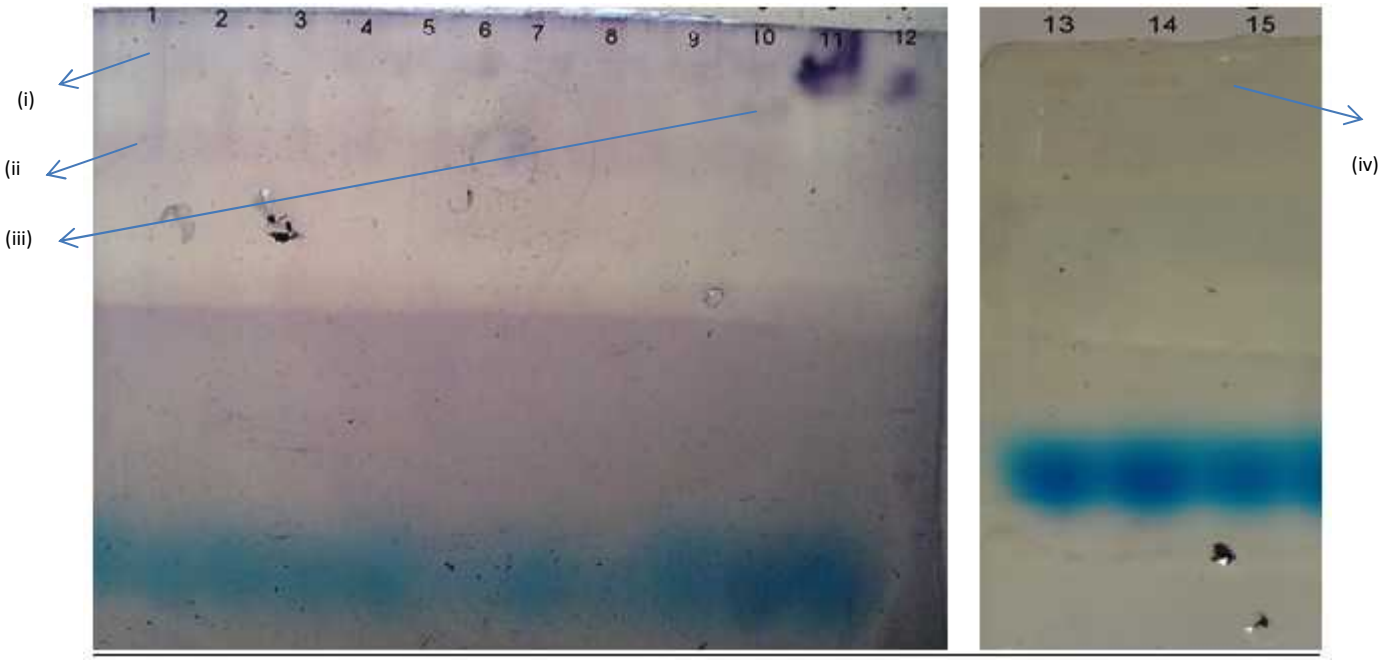


Fig: 4.9. NATIVE-PAGE of Aldehyde Dehydrogenases present in whole homogenates of tomato leaves in week 3. **Lane 1:** Control plant tissue sample**. Lane 2:** 25 mMNaCl contaminated soil. **Lane 3**: 50 mMNaCl plant tissue homogenate **Lane4**: 75 mMNaCl plant tissue homogenate **Lane 5**: 100mM NaCl plant tissue homogenate. **Lane 6**: 0.1 mM $CdCl_2$ plant tissue homogenate.**Lane 7, 8**: 0.3 mM $CdCl_2$ plant tissue homogenate. **Lane 9**: 0.5 mM $CdCl_2$ plant tissue homogenate.**Lane 10**: 1 mM $CdCl_2$ plant tissue homogenate.**Lane 11, 12:** 0.1 mM$Pb(NO_3)_2$ plant tissue sample. **Lane 13**: 0.3 mM$Pb(NO_3)_2$ plant tissue homogenate. **Lane 14**: 0.5 mM$Pb(NO_3)_2$ plant tissue homogenate. **Lane 15**: 1 mM$Pb(NO_3)_2$ plant tissue homogenate.

NATIVE-PAGE result of the samples collected after 3rd week of treatment and control are shown in figure 4.9. The bands were found very faint. All samples loaded in lane 1 to 12 show (i) and (iii) type bands and additionally lane 10 sample showed (ii) type band. In differently run gel having 13 to 15 lane samples gives only one faint (iv) band type.