INFLUENCE OF ZINC NUTRITION IN RELATION TO PLANT METABOLISM : BIOCHEMICAL AND PHYSIOLOGICAL CHANGES IN MUNGBEAN (*VIGNA RADIATA* CV. K-851) SEEDLINGS WITH PARTICULAR REFERENCE TO CELL WALL PROTEINS

A THESIS

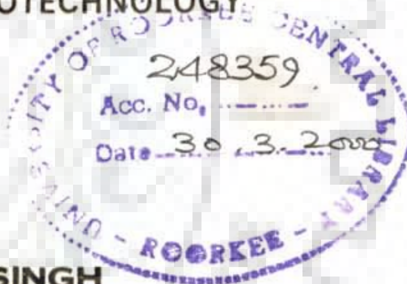
Submitted in fulfilment of the requirements for the award of the degree

of

DOCTOR OF PHILOSOPHY

in

BIOSCIENCES AND BIOTECHNOLOGY



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By

JITENDRA SINGH

DEPARTMENT OF BIOSCIENCES AND BIOTECHNOLOGY
UNIVERSITY OF ROORKEE
ROORKEE-247 667 (INDIA)

JULY, 1998

Gratis.

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"INFLUENCE OF ZINC NUTRITION IN RELATION TO PLANT METABOLISM : BIOCHEMICAL AND PHYSIOLOGICAL CHANGES IN MUNGBEAN *(VIGNA RADIATA* CV. K-851) SEEDLINGS WITH PARTICULAR REFERENCE TO CELL WALL PROTEINS"** in fulfilment of the requirement for the award of the Degree of **Doctor of Philosophy** and submitted in the **Department of Biosciences and Biotechnology** of the University is an authentic record of my own work carried out during a period from August 1991 to July 1997 under the supervision of **Dr. Vinay Sharma** and **Dr. Ben. M.J. Pereira.**

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other University.

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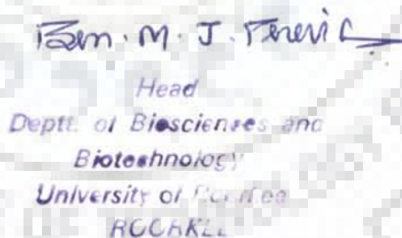

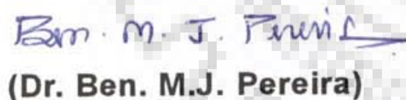

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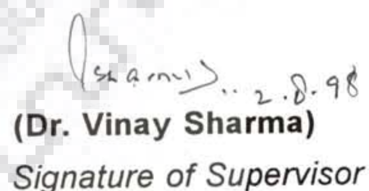

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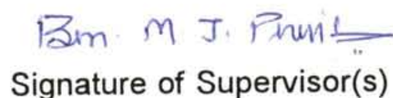

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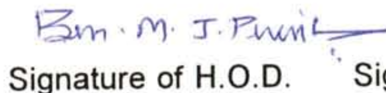

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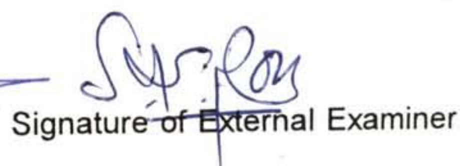

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ABSTRACT

Heavy metals, their deficiences on one hand and increased environmental burdens on the other in the soils all over the world are alarming scientists. Crop plants grown in the soils of vast Indo-Gangetic alluvial fertile plain of Ganga-Jamuna doab, particularly in green belt of western Uttar Pradesh which possess large number of sugarcane industries, commonly exhibited deficiency, tolerance and toxicity symptoms of zinc. We have surveyed the alluvial soils of Indo-Gangetic plain in and around Roorkee (District Haridwar) town for its Zn status and studied the impact of zinc in relation to various biochemical and physiological aspects of hydroponically grown 6 day old mungbean (*Vigna radiata* cv. K-851) seedlings.

All 32 soils sampled in Roorkee-Muzaffarnagar region are highly alkaline with an average pH of 7.8. Only 28% soils are deficient in total zinc content. However, more than 90% of soils contained less than 0.5 ppm DTPA-extractable (phytoavailable) Zn, which is widely considered to be the critical deficiency concentration of Zn for plants grown in clacareous soils. Soils appear to be deficient in available Zn content due to high pH values under the influence of salt Na_2CO_3.

On the basis of morphological, anatomical and biochemical parameters we have found that the optimum zinc requirement for *vigna radiata* cv. K.-851 is 0.4 ppm. It confirms the highly zinc sensitive nature of this crop, which shows toxicity beyond 15 ppm and loose tolerance at 100 ppm zinc concentration. Root was found to be most sensitive organ under zinc stress. Shoot/root length ratio was found to be minimum under deficiency and maximum under toxicity of zinc. However, due to high accumulations of ions and photoassimilates, no considerable change was recorded in dry matter of mungbean roots under zinc toxicity. Tertiary roots at low level and adventitious roots from hypocotyl at toxic (100 ppm) level of Zn were found to be developed as an alternate of checking in further increase in number of secondary roots and degenerated tap root respectively and contributes morphologically towards the tol-

erance to the plants. Deposition of blackish substance in intercellular spaces and changes in stele anatomy of the stem under zinc toxicity might be an anatomical contribution towards the tolerance to the plants. The distribution of zinc in different subcellular fractions of mungbean roots indicate the cytoplasmic sequestration of zinc and an active participation of cell wall in zinc tolerance.

Increase in cell wall hydrolases activity under low level of zinc may explain the loosening of cell wall during the cell elongation and growth of the plant. Increase in peroxidase activity under zinc toxicity is likely to promote crosslinking of cell wall molecules and this along with decreased hydrolases may be responsible for rigidity of cell wall in zinc stressed plants.

Changes in cell wall and cytoplasmic proteins of root cells were assessed under zinc stress. Both cytoplasmic and cell wall proteins increased quantitatively beyond 15 ppm zinc. Proteins profile of cell wall was not altered, however alteration in proteins profile of cytoplasm was noticed in response to zinc. Certain polypeptides like 56, 46 and 35 kDa were found to be disappear at low level and reappear at toxic level of zinc. However, 27 kDa polypeptide disappeared completely beyond 10 ppm zinc. Thus, zinc may play a role in protein synthesis at the translation level and in the formation and the suppression of some peptides at the transcription level. Low molecular weight polypeptides seemed to be greatly affected by Zn nutrition. A new 18 kDa polypeptide was found to be appear at and beyond 25 ppm Zn, aminoacid analysis of which indicates that it might be a metallothionein like polypeptide (phytochelatin) containing cysteine-rich domains. The polypeptides of approximately 30 kDa were tentatively addressed as peroxidases. However, for the other zinc-induced polypeptides, no such suggestion of functions is yet possible. This is needed to be further investigated in order to judge their possible role under zinc (heavy metal) stress.

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CONTENTS

CHAPTERS **PAGE NO.**

ABSTRACT (i)
ACKNOWLEDGEMENTS (iii)
CONTENTS (v)
LIST OF FIGURES (x)
LIST OF TABLES (xii)
ABBREVIATIONS USED (xv)

1.0 INTRODUCTION **1-3**

2.0 LITERATURE REVIEW **4-31**

2.1 Introduction 4
2.2 Zinc in soils and plant nutrition 5
2.3 Effect on photosynthesis 15
2.4 Involvement in sugar transformations 17
2.5 Growth response 19
2.6 Membrane integrity 21
2.7 Uptake, translocation and compartmentation of zinc 22
2.8 Protein synthesis and cell wall bound enzymic response 25
2.9 Phytochelatins and metal tolerance 28
2.10 Interactions with auxins and other nutrients 31

3.0 MATERIALS AND METHODS **32-57**

3.1 Materials 32
3.2 Methods 32

3.2.1 Soil analysis 32

3.2.1.1 Soil survey in and around Roorkee 32

3.2.1.1.1 Location 32
3.2.1.1.2 Climate 33
3.2.1.1.3 Sampling sites 33
3.2.1.1.4 Soil sampling 33

3.2.1.2 Soil processing 36
3.2.1.3 Measurement of soil pH 36

3.2.1.4 Estimation of zinc content 36

3.2.1.4.1 Total zinc content 37
3.2.1.4.2 Available zinc content 37

3.2.2 Germination of seeds 39

3.2.2.1 Composition of Hoagland's nutrient solution 39
3.2.2.2 Zinc treatment 40

3.2.3 Morphological studies 40

3.2.3.1 Measurement of length and width 40
3.2.3.2 Determination of the number of secondary and tertiary roots 40
3.2.3.3 Determination of fresh weights 40
3.2.3.4 Determination of dry weights 41

3.2.4 Anatomical studies 41
3.2.5 Estimation of *in vivo* level of Zn^{+} ions 41

3.2.5.1 Organwise distribution 41

3.2.5.1.1 Preparation of sample 41
3.2.5.1.2 Estimation of Zn^{+} ions 41

3.2.5.2 Subcellular distribution 42

3.2.6 Determination of activities of cell wall bound enzymes 42

3.2.6.1 Preparation of cell wall 42
3.2.6.2 Extraction of cell wall bound enzymes 43
3.2.6.3 Enzyme assays 43

3.2.6.3.1 Assay of cell wall bound glycosidase activity 43
3.2.6.3.2 Assay of cell wall bound acid phosphatase activity 43
3.2.6.3.3 Assay of cell wall bound peroxidase and polyphenoloxidase activities 45

3.2.7 Studies on cytoplasmic and cell wall proteins 45

3.2.7.1 Isolation of cytoplasmic and cell wall proteins 45
3.2.7.2 Desalting and quantitative precipitation of cell wall protein 47

3.2.7.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) 49

3.2.7.3.1 Reagents 49
3.2.7.3.2 Preparation of resolving and stacking gel 50
3.2.7.3.3 Casting of gel 50
3.2.7.3.4 Sample preparation 51
3.2.7.3.5 Electrophoresis 51

3.2.8 Determination of molecular weights 53

3.2.9 Aminoacid analysis of induced cytoplasmic proteins 53

3.2.9.1 Elution of protein bands from gel slices 53
3.2.9.2 Dialysis and lyophylization 53
3.2.9.3 Hydrolysis of proteins for analysis of aminoacid composition 55
3.2.9.4 Vacuum drying 55
3.2.9.5 Aminoacid Analysis 55

3.2.10 Other methods 55

3.2.10.1 Protein estimation 55

3.2.10.1.1 Reagents 55
3.2.10.1.2 Procedure 56

3.2.10.2 Statistical analysis 56

4.0 **RESULTS** **58-104**

4.1 Soil analysis 58

4.1.1 Zinc in soil profiles 58
4.1.2 pH Vs phytoavailable zinc 63

4.2 Zinc phytosensitivity 63

4.2.1 Zinc deficiency symptoms and plant response to zinc applications in field 63
4.2.2 Crop susceptibility to zinc phytotoxicity 63
4.2.3 Zinc-sensitivity of mungbean crop 67

4.3 Growth response at different zinc concentrations 67

4.3.1 Leaf 67
4.3.2 Epicotyl 67
4.3.3 Cotyledon 75
4.3.4 Hypocotyl 75
4.3.5 Root 75
4.3.6 Shoot/root ratio 75

4.4 Developmental response of radicle and secondary roots at different concentrations of zinc 77

4.5 Effect of different concentrations of zinc on anatonical features of various organs of mungbean seedlings 77

4.5.1 Effect of different concentrations of zinc on total section diameter of various organs 84

4.5.1.1 Changes in leaf anatomy 84
4.5.1.2 Changes in shoot anatony 84
4.5.1.3 Changes in root anatony 84

4.6 Zinc uptake by mungbean seedlings 87

4.6.1 Organwise Zn^+ ions distribution 87
4.6.2 Effect of different concentrations of zinc on sub-cellular *in-vivo* level of Zn^+ ions 87

4.6.2.1 Cytoplasmic level of Zn^+ ions 91
4.62.2 *In vivo* level of Zn^+ ions of root cell wall 91
4.6.2.3 Correlation between in vivo levels of Zn^+ ions in cytoplasmic and cell wall proteins fractions 91

4.7 Response of cell wall bound enzymes to different concentrations of zinc 93

4.8 Response of cytoplasmic and cell wall proteins to different concentrations of zinc 95

4.8.1 Quantitative changes 95

4.8.1.1 Cytoplasmic proteins at different concentrations of zinc 95
4.8.1.2 Cell wall proteins at different concentrations of zinc 95

4.8.2 Qualitative pattern 101

4.8.2.1 Cell wall protein profile at different concentrations of zinc 101

4.8.2.2 Cytoplasmic protein profile at different concentrations of zinc 101

4.9 Amino-acid analysis of zinc-induced cytoplasmic protein 104

5.0 DISCUSSION **105-115**

REFERENCES **116-130**

LIST OF FIGURES

	Titles of Figures	Page No.
Fig. 2.1	Location of sugar mills in India	8
Fig. 2.2	Conceptual model of groundwater pollution by industrial activities	11
Fig. 2.3	Index of tubewells in Roorkee region	12
Fig. 3.1	Mungbean plants in growth chamber under controlled environmental conditions	38
Fig. 3.2	Isolation of cell wall bound enzymes	44
Fig. 3.3	Isolation of cytoplasmic and cell wall proteins	46
Fig. 3.4	Desalting and quantitative recovery of protein in dilute solution	48
Fig. 3.5	Standard curve of marker protein	52
Fig. 3.6	Densitometric scan of aminoacid standards	54
Fig. 4.1	Relation between DTPA - extractable Zn^{++} concentration in the soil solution and pH at different depth of soil profile around Roorkee-Muzaffarnagar regions of western Uttar Pradesh, India.	62
Fig. 4.2	Zinc deficiency symptoms and plant response to zinc applications in field	64
Fig. 4.3	Effect of zinc on mungbean plants	65
Fig. 4.4	Optimum zinc requirement for mungbean seedlings	66

Fig. 4.5 % changes in shoot/root ratio of mungbean seedling grown at different zinc concentrations 74

Fig. 4.6 Light Micrographs showing transverse sections of roots from mungbean seedlings subjected to 6 days of treatment 78

Fig. 4.7 Light Micrographs showing transverse sections of stems from mungbean seedlings subjected to 6 days of treatment 79

Fig. 4.8 Light Micrographs showing transverse sections of a stem from mungbean seedlings grown for 6 days on 100 ppm of zinc 80

Fig. 4.9 Effect of different zinc concentrations on correlations between cell wall and cytoplasmic level of Zn^{++} ions in mungbean roots. 90

Fig. 4.10 SDS - PAGE (10%) profile of cell wall proteins of mungbean roots 99

Fig. 4.11 SDS - PAGE (10%) profile of cytoplasmic proteins of mungbean roots. 100

Fig. 4.12 HPLC chromatogram of PTH - aminoacids of 18 kDa Zn-induced cytoplasmic polypeptide 103

LIST OF TABLES

	Titles of Tables	Page No.
2.1	Composition of sugarcane molasses	9
2.2	Data of some selected physico-chemical parameters of the effluent of Mahalakshmi Sugar Mills Ltd. Iqbalpur, Haridwar (U.P)	10
2.3	Data of analysis of water	13
2.4	Contaminants reported in ground water	14
3.1	Sample site locations	34
3.2	Information sheet to accompany soil samples	35
4.1	Properties of soil samples from in and around Roorkee - Muzaffarnagar regions of western Uttar Pradesh, India	59-60
4.2	Mean concentration of zinc (ppm) in soils sampled from in and around Roorkee- Muzaffarnagar regions of western Uttar Pradesh, India.	61
4.3	Effect of different concentrations of zinc on the growth of whole mungbean seedlings subjected to 6 days of treatment	68
4.4	Effect of different concentrations of zinc on the growth of leaf in mungbean seedlings after 6 days of treatment	69
4.5	Effect of different concentrations of zinc on the growth of epicotyl in mungbean seedlings after 6 days of treatment	70
4.6	Effect of different concentrations of zinc on the growth of cotyledon in mungbean seedlings after 6 days of treatment	71

4.7 Effect of different concentrations of zinc on the growth of hypocotyl in mungbean seedlings after 6 days of treatment 72

4.8 Effect of different concentrations of zinc on the growth of root in mungbean seedlings after 6 days of treatment 73

4.9 Effect of zinc on the development on radicle and secondary roots of mungbean seedlings 76

4.10 Effect of different concentrations of zinc on total section diameter in different organs of 6 day old mungbean seedlings 81

4.11 Effect of different concentrations of zinc on number of cortical cell layers in different organs of 6 day old mungbean seedlings 82

4.12 Effect of different concentrations of zinc on cortical cell diameter in different organs of 6 day old mungbean seedlings 83

4.13 *In vivo* level of Zn^{++} ions in whole mungbean seedlings and pattern of distribution in different organs expressed per gram dry weight 85

4.14 *In vivo* level of Zn^{++} ions in whole mungbean seedling and pattern of distribution in different organs expressed per organ. 86

4.15 Effect of different zinc concentrations on *in vivo* level of Zn^{++} ions in cytoplasm of 6 day treated mungbean roots on per gram fresh tissue weight basis 88

4.16 Effect of different zinc concentrations on *in vivo* level of Zn^{++} ions in cell wall of 6 day treated mungbean roots on per gram fresh tissue weight basis. 89

4.17 Effect of different concentrations of zinc on the specific activities of some root cell wall bound enzymes of mungbean seedlings after 6 days of treatment 92

4.18 Effect of different concentrations of zinc on cytoplasmic and cell wall protein content in roots of 6 day treated mungbean seedlings 94

4.19 Effect of different concentrations of zinc on the profile of cell wall proteins in roots of 6 day treated mungbean seedlings 96

4.20 Effect of different concentrations of zinc on the profile of cytoplasmic proteins in roots of mungbean seedlings after 6 days of treatment 97-98

4.21 Aminoacid composition of zinc - induced 18 kDa cytoplasmic polypeptide 102

ABBREVIATIONS USED

AAS : Atomic Absorption Spectrophotometer

ATPase : Adenosine Tri Phosphatase

ca. : Calculated

cm : Centimeter

6d : Six day

DDW : Double Distilled Water

DTPA : Diethylene Triamine Penta Acetic acid

EDTA : Ethylene Diamine Tetra Acetic acid

Fig. : Figure

gm : Gram

h : Hour

ICP : Induced Coupled Plasma

kDa : Kilo Dalton

L : Litres

M : Molarity

mg : Milligrams

min : Minutes

mm : Millimeters

Mts : Metallothioneins

µg/gdw : Microgram per gram dry weight

µl : Micro litres

N : Normality

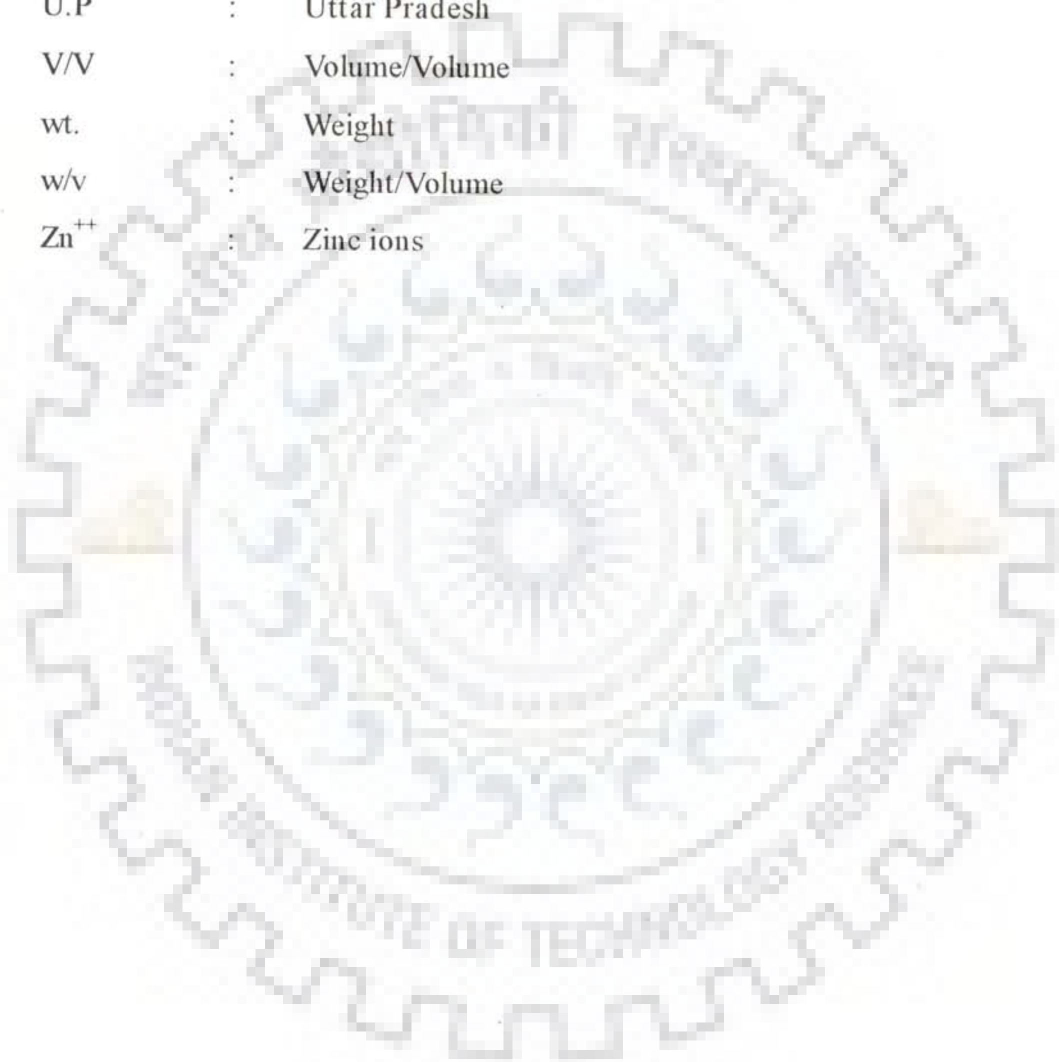
PCs : Phytochelatins

p mol : Pico Mole

ppm : Parts Per Million

rpm : Revolutions Per Minutes

s	:	Seconds
S.D	:	Standard Deviation
S.E.	:	Standard Error
SDS-PAGE	:	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TEA	:	Tri Ethanol Amine
U.P	:	Uttar Pradesh
V/V	:	Volume/Volume
wt.	:	Weight
w/v	:	Weight/Volume
Zn^{++}	:	Zinc ions



CHAPTER - 1

1.0 INTRODUCTION

Zinc, a divalent heavy metal with dual role, micronutrient on one hand and toxic environmental factor on the other, acts as a co-factor for nearly 300 different enzymes and metalloenzyme complexes. Its saturated d-orbitals and small size favour tetrahedral co-ordination for stable metalloenzyme complexes and thereby regulate various processes of plant metabolism. On account of zinc being an integral part of around 200 DNA binding proteins including reverse-transcriptase, it plays a crucial role in gene expression and is of interest to genetic engineers (Clarkson and Hanson, 1980; Vallee 1992).

Vast stretches of agricultural soil all over the world are deficient in zinc. In a global study initiated by FAO and carried out in 30 countries, it was estimated that ca. 30% of the agricultural soils of the world are Zn-deficient (Sillanpaa, 1982; Cakmak et al., 1996a). Well over 55% of Indian cultivable soil is also zinc deficient. The low organic alluvial soil of Indo-Gangetic plains is particularly deficient in zinc (about 75%) due to its high pH because of high levels of salt Na_2CO_3 (Shukla and Prasad, 1976; Katyal and Rattan, 1993). Then, there are stretches of soil which possess excess concentration of various metals including zinc that are toxic to the growth of the plants. Such type of soils have arisen mainly due to discharge of toxic effluents by the industries. Zinc stressed soils affect the yield and quality of agricultural product. It is obvious that the crop productivity could be improved several folds if the plant manages to show normal growth under such conditions.

The toxic effects of heavy metals are explained by their interaction with sulphhydryl groups and concomitant inactivation of proteins (Assche and Clijster, 1990). Therefore, it is generally accepted that tolerance towards zinc stress is based on mechanisms that maintain low free zinc concentrations in the cytoplasm. Among these mechanisms, the most important are (i) limitation of uptake by the roots (ii) specific compartmentation into the extraplasmic

compartments of the cell i.e. apoplasm and the vacuole, in particular of the epidermis (Brune *et al.*, 1994a).

The cellular basis of the differential responses of root growth to toxic metals remains largely unexplored (Francis *et al.*, 1995). Interestingly, zinc deficiency is manifested mainly by differentiation of secondary roots. However, strong inhibition of root extension occurs at toxic zinc levels. A major factor responsible for the reduction of growth is likely to be changes in the property of cell walls which in turn will determine its extensibility and ultimately cell growth. Since roots are in contact with various metals in soil longer than other organs during growth and contain more transcripts of metallothioneins (MT)-like genes, it is possible that MT-like proteins in plants play a scavanger role in chelating excess heavy metals. The responsiveness of plant MT-like genes identified only recently, to stress is a subject of new inquiries (Hsieh *et al.*, 1995).

Cereals and legumes are rich in phytate, which impairs zinc retention. Zinc is usually more bioavailable from legumes than cereal (Sandstead, 1994). Among legumes, mungbean acts as an indicator plant for zinc stress (Kanwar and Randhawa, 1981) and is widely grown in Indo-Gangetic plains. It appears an ideal system for studying zinc stress related physiological and biochemical changes. Further, the leguminous plants of Haridwar and Muzaffarnagar districts which fall in Indo-Gangetic plains, exhibit marked zinc deficiency symptoms. Also, this region has large number of sugarcane industries and the soil around them, on the contrary, shows high concentrations of zinc (Gupta, 1995; Gupta, 1996; Arora, 1997). In view of this discussion the influence of zinc nutrition in relation to certain parameters of plant metabolism with mungbean as a model plant system was undertaken under controlled laboratory conditions.

The objectives of this study were to

1. Determine the quantities of total and phytoavailable zinc in the soils in and around Roorkee town.
2. Examine the effect of zinc stress on morphological and anatomical features of seedlings.
3. Study the pattern of accumulation of Zn^{++} ions in various organs of zinc treated and control seedlings.

4. Findout the effect of zinc stress on the pattern of cytoplasmic proteins.
5. Investigate the role of the root cell wall proteins and enzymes under zinc stress conditions.
6. Determine the zinc induced changes, if any, in the overall aminoacid composition of certain key proteins.

CHAPTER -2

2.0 LITERATURE REVIEW

2.1 Introduction

Two groups of heavy metals are distinguished in respect to their metabolic functions. Microelements such as Cu and Zn are essential constituents of enzymes and necessary for the maintenance of ordinary cell structures (Marschner, 1986). Upon immobilization or withdrawal from the rooting medium, plants develop specific deficiency symptoms. At high concentrations, however, these microelements exhibit strong toxicity and affect plant growth similar to members of the second group of related heavy metals such as Cd or Hg. Members of the second group do not have a definite function in plant metabolism. Excess heavy metal concentrations mainly inhibit enzyme activities by binding to sulphhydryl groups (Assche and Clijsters, 1990). Interestingly, large variations exist between species and cultivars in their ability to grow on heavy-metal-polluted soil (Woolhouse, 1983).

Genetic and physiological approaches should merge into an unifying more comprehensive approach to breeding for zinc tolerance. The plants stress-responses vary depending upon developmental stage during which it is subjected to stress. Therefore, the various biochemical, physiological and molecular events that are associated with the preliminary stress response need to be thoroughly understood in order to exécute the strategy (Kumar et.al., 1996).

Zinc deficiency is a widespread nutritional constraint in plants, particularly in calcareous soils of arid and semi-arid regions and especially low organic alluvial soils of Indo-Gangetic alluvial plains (Cakmak et al., 1996a; Katyal and Rattan, 1993). Then there are stretches of soil which possess excess concentration of metals including zinc that are toxic to the growth of plants. Such type of soils have arisen mainly due to discharge of toxic effluents by the industries. Zinc stressed soils affect the yield and quality of agricultural product. It is obvious that the crop productivity could shoot up several folds if the plant manages to show normal growth in such conditions.

Soil or foliar application of Zn, is a widely used agronomic practice for farmers to

overcome Zn deficiency in plants. Alternatively, selection and/or breeding or biochemical manipulation of plant genotypes with higher resistance to Zn stress is a sustainable approach to over come zinc stress in plants. Realization of this approach is very resonable in view of the large genotypical differences in Zn efficiency between plant species and also genotypes of a species (Cakmak et.al., 1996a; Graham and Rengel,1993).

Zinc is an essential micronutrient for all living organisms but like all nutrient ions it can be toxic at high levels. Considerable effort has been made to understand the importance of Zn as a cofactor in several enzyme reactions (Dugas, 1989), to study Zn uptake in plants at the root level (Foy et al., 1978) and to understand mechanisms involved in the ability of some plants to grow in the presence of Zn levels that are toxic to most plants, the phenomenon known as tolerance (Woolhouse, 1983). The mechanisms underlying Zn accumulation/accommodation and Zn tolerance in plants are far from clear. A better understanding of these processes is needed because Zn deficiency, Zn tolerance, and Zn toxicity are all known to occur in agriculture.

The disturbances to metabolism caused by zinc stress result in impaired growth and are mostly or not entirely, associated with the destruction of normal enzyme activity. Zinc is involved in photosynthesis and sugar transformations, but not in respiration (Micronutrient Bureau, 1994).

2.2 Zinc in soils and plant nutrition

Heavy metals are one of the most hazardous environmental pollutants. Unlike certain organic compounds they are not degraded in biological systems. On the contrary, their concentrations rise in each trophic level in a food chain through the process of biomagnification. Heavy metals such as lead, cadmium and zinc have received considerable attention during recent years as a result of increased environmental burdens from industrial, agricultural, energy and municipal sources. Although some metals e.g. copper, zinc and magnesium, are essential for plant growth, they are generally toxic and able to interfere and inhibit plant development. They can ultimately cause the death of some plants

when present at elevated levels in soils (Kabata-Pendias and Pendias, 1984; Dudka et. al., 1996; Hanna et. al., 1997)

Heavy metals have been defined as the metallic elements with atomic number greater than 23 (except rubidium and strontium). They have also been defined as metals having density more than five times higher than that of water. On the basis of coordination chemistry of metal ions in biological systems (mostly X-ray crystallographic data), Nieboer and Richardson (1980) classified metal ions into three categories namely, class A-oxygen seeking; class B - nitrogen /sulphur seeking and borderline- considered as intermediate between class A and B. This system of classification is of significance as it reveals the biochemical basis for metal ion toxicity, and is of help in rational selection of metal ions in toxicity studies. Heavy metals cause deleterious effects in all groups of living organisms.

Understanding of zinc's many roles in metabolism came about through research that began 120 years ago with Raulin's discovery of the essentiality of zinc for *Aspergillus niger*. Fourty years later Maze described stunting of corn by zinc deficiency. Confirmatory studies by Somner and Lipman established that higher plants require zinc and provided a basis for inclusion of zinc in fertilizers. This discovery contributed to the high productivity of modern agriculture and thus contributed to human nutrition (Raulin, 1869; Maze, 1914; Somner and Lipman, 1926; Sandstead, 1994).

Depending upon the type of extractants used, available zinc content of Indian soils varied from less than 1 ppm to a few parts per million. The critical limit of DTPA - extractable plant available zinc is 0.61 ppm (Singh et. al., 1987). Based on these data, it is estimated that more than 47% soils in India are zinc deficient (Shukla and Prasad, 1976). Zinc deficiency is a constraint for successful crop production and it affects the overall yield and quality of the agricultural products. The problem is generally more acute in sandy and saline sodic soils. Zinc deficiencies are usually observed on soils with pH greater than 6.0. The soil pH is negatively correlated with the uptake of zinc by plants. The role of zinc in crop

production has been discussed by many Indian scientists. The efficiency of different plants to use zinc for dry matter production varied as rice > maize> barley> sugarcane. The relative susceptibility of various varieties of Kharif and Rubi pulses and soybean in pot culture using a zinc deficient loam soil of Pantnagar, was studied. All Kharif crops (Arhar, moong, urad, cowpea and soybean) developed characteristic zinc deficiency symptoms on the foliage. In general all varieties of moong and cowpea were very good indicator pulses as they show characteristic symptoms of zinc deficiency very early (Kanwar and Randhawa,1981; Katyal and Rattan,1993).

Relatively insoluble mineral forms account for more than 90% of the zinc in soils. The soil minerals which contain zinc are calcite, dolomite and calcium magnesite. Zinc is present in the soil in only the divalent form. Zinc that may become available for plant uptake is present as Zn^{++} in the soil solution, exchangeable zinc on the cation exchange sites, organically complexed zinc in solution and organically complexed zinc in the soil solid phase. High pH is characteristic of salt affected soils formed under the influence of Na_2CO_3. Such soils occur extensively in the Indo-Gangetic plains of India. At and below pH 5.5 zinc occurs in soil solution as Zn^{++} cations. As the pH rises above 7.0 the positively charged Zn^{++} ions may be converted into negatively charged zincate complex which reduces the solubility of zinc in alkaline soils (Barber, 1984).

Soil is used as a medium to deposit industrial and municipal solid waste containing heavy metals such as copper, zinc, chromium, lead and cadmium. After rainfall, the leachate from solid waste carrying the composite mobile organic matter moves downword through soil in the profile (Lin et.al., 1996). The distribution of Zn, Mn, Cu, Co and Ni in andosols was investigated by Rahman et. al. (1996) and found to be considerably high in the humus horizons of the profiles.

Nurain (1996) studied ground water quality of nearby villages around Roorkee region and reported heavy metals including zinc contamination in ground water. Their suggested conceptual model of groundwater pollution by industrial activities is shown

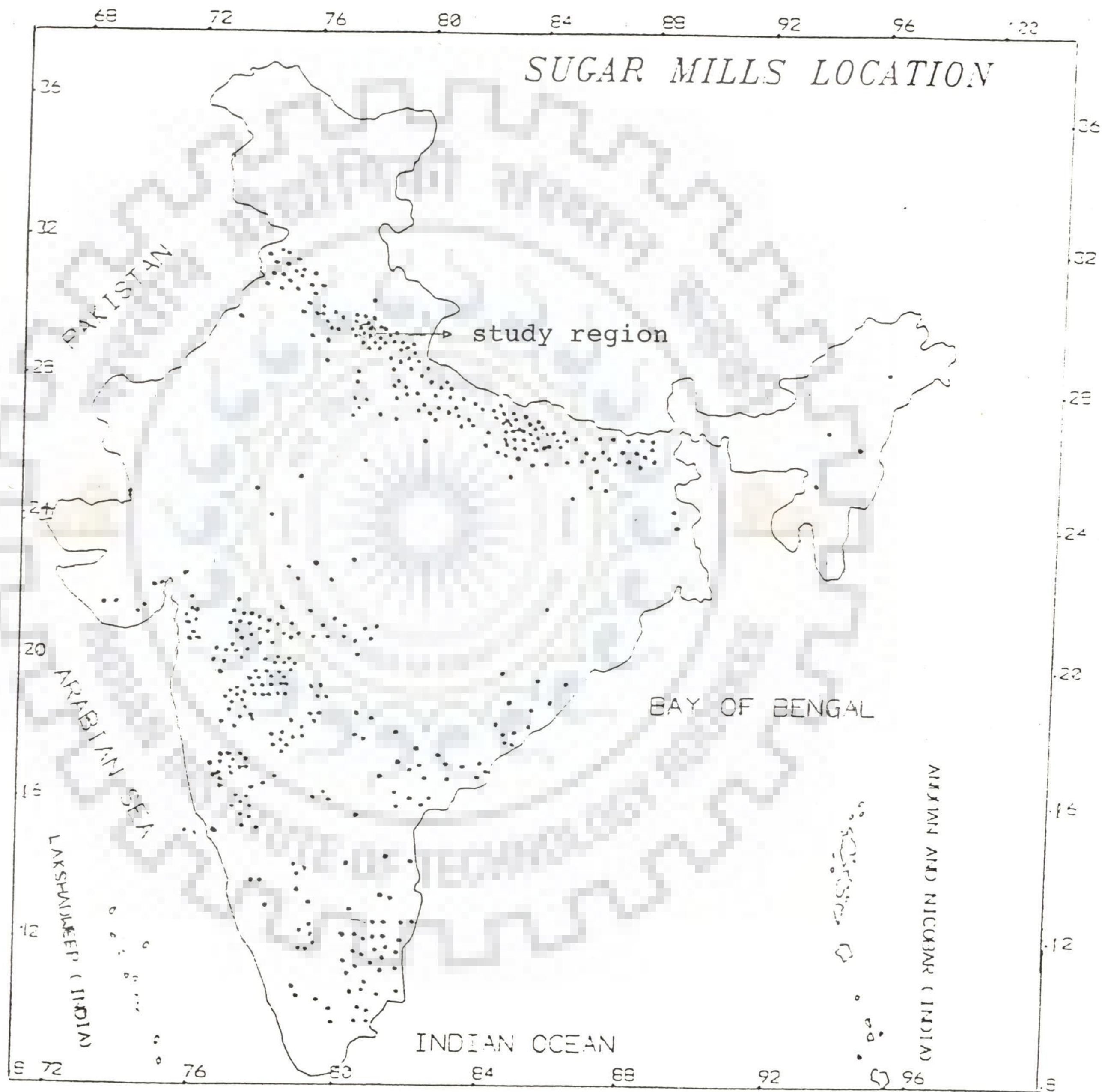


Fig. 2.1: Location of sugar mills in India

TABLE - 2.1

COMPOSITION OF SUGARCANE MOLASSES OBTAINED FROM MAHALAKSHMI SUGAR MILLS CORPORATION LTD. IQBALPUR, HARIDWAR (U.P.) INDIA (Gupta, 1995)

Component	%, (w/w)*
Total Carbohydrate	51.60±4.0
Sucrose	38.24±1.5
Protein	2.14±0.2
Sulfated ash	13.86±3.2
Iron	0.0234±0.0005
Copper	0.0015±0.0003
Zinc	0.0018±0.0003
Manganese	0.0007±0.0001
Calcium	0.7740±0.20
Phosphorous	1.4860±0.35
Potassium	2.5202±0.50
Water	27.47±4.0

* Values are average of three determinations of the pooled samples from different batch preparations.

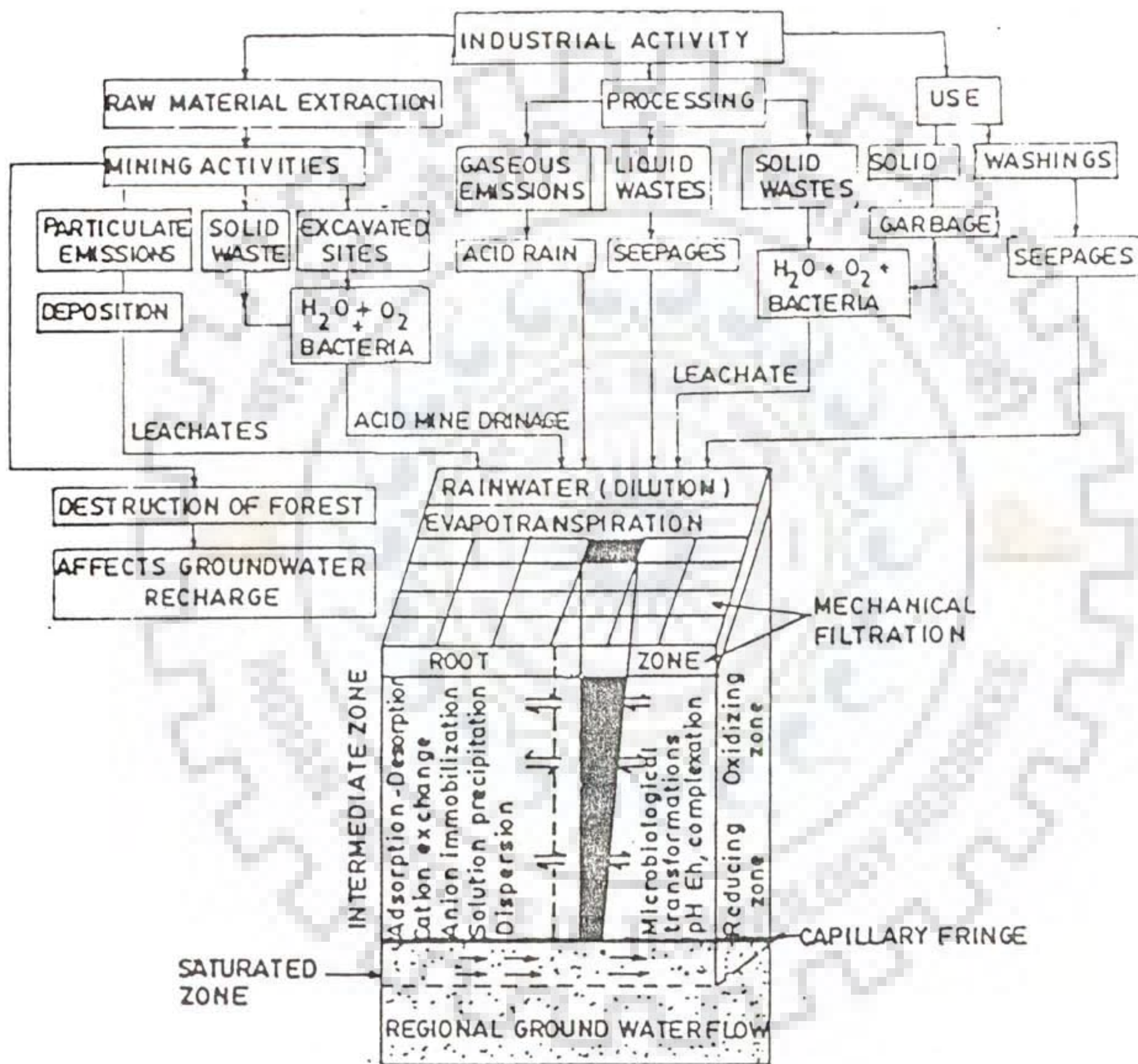
TABLE 2.2

Data of some selected physico-chemical parameters of the effluent of Mahalakshmi Sugar Mills Corporation Ltd. Iqbalpur Haridwar (U.P.) India. Values are Mean ± S.D. for six observations each. (Arora, 1997).

S. No.	Physical Parameters	Outlet Site Site Ist	At distance of hundred meters down from outlet site Site IInd	At distance of five hundred meters down from outlet site Site IIIrd
1.	Temperature (°C)	33.8±2.25	30±.05	26.0±2.25
2.	Colour	Deep blackish Brown	Deep Brownish	Light Brownish
3.	Turbidity (JTU)	280±38.50	220±3.86	140±42.31
4.	Total Dissolved Solids (TDS) (gm/L)	7.6±1.31	5.11±0.12	3.25±1.19
5.	Suspended Solids (mg/L)	610±22.30	573.3±1.15	530.83±23.40
6.	Dissolved Oxygen (mg/L)	0	0	0
7.	Biological Oxygen Demand (BOD) (mg/L)	1506.66±142.89	1270.83±6.73	1000±149.62
8.	Chemical Oxygen Demand (COD) (mg/L)	2191.66±160.31	1950±20.85	1600±181.21
9.	pH	6.22±0.17	6.44±0.05	6.93±0.2
10.	Alkalinity (mg/L)	150±19.15	114.16±1.54	86.33±17.60
11.	Salinity (mg/mL)	4.29±0.35	3.76±0.05	2.96±0.40
12.	Hardness (mg/L)	1513.33±208.6	1111.66±23.25	830.83±185.39
13.	Free CO_2 (mg/L)	63.83±2.5	59.66±0.12	54.83±2.66

Conceptual model of groundwater pollution by industrial activities

FIGURE 2.2



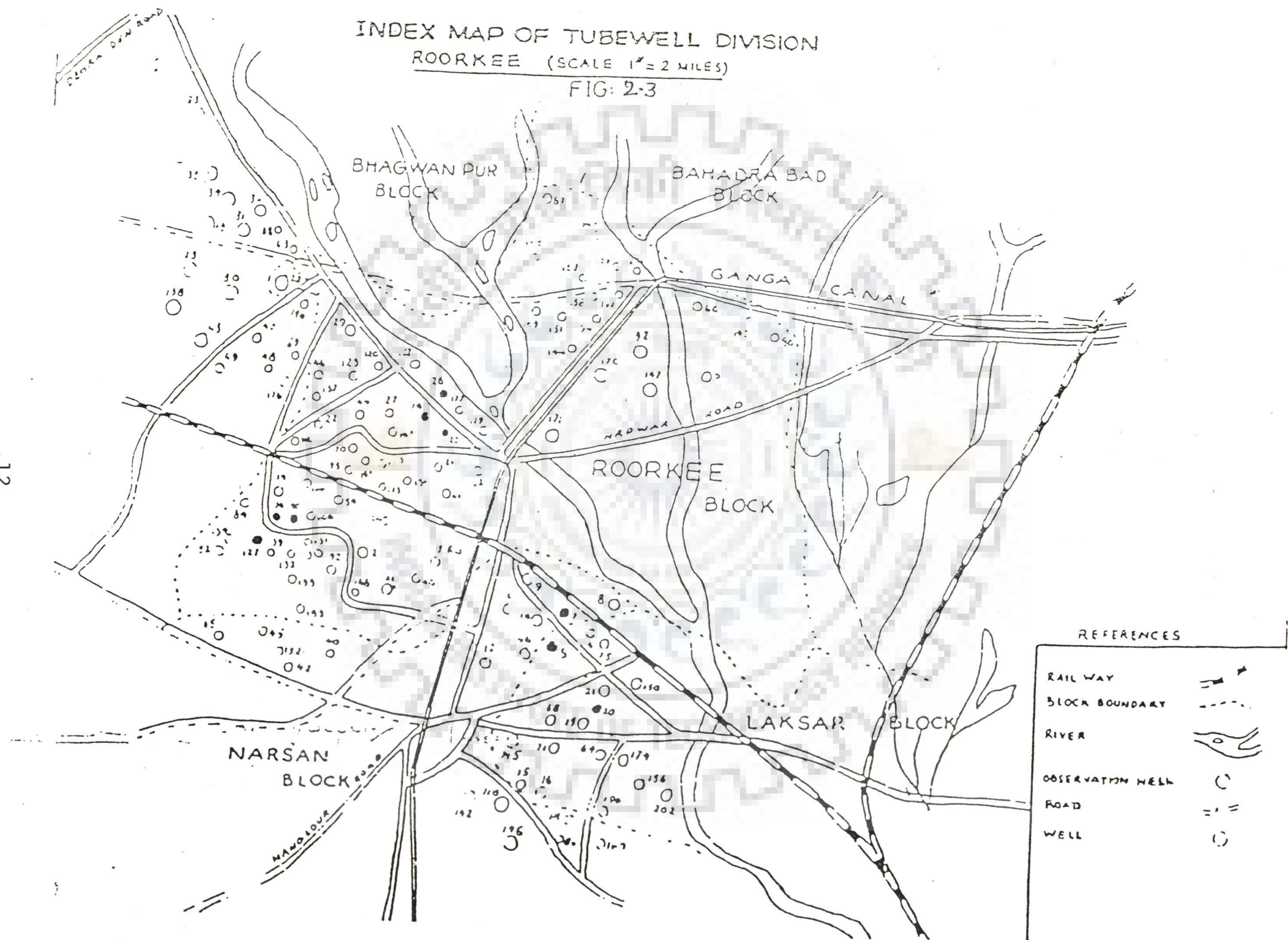

INDEX MAP OF TUBEWELL DIVISION
ROORKEE (SCALE 1" = 2 MILES)
FIG: 2-3
BHAGWAN PUR BLOCK
BAHADRA BAD BLOCK
GANGA CANAL
ROORKEE BLOCK
LAKSAR BLOCK
NARSAN BLOCK
REFERENCES
RAIL WAY
BLOCK BOUNDARY
RIVER
OBSERVATION WELL
ROAD
WELL

TABLE - 2.3
DATA OF ANALYSIS OF WATER
(Nurain, 1996)

Location : Harjauli
Date : October 9, 1996

No.	Test Source	Unit	State Tube Well No. 90	Laboratory Private Tube Well	Result Hand Pump
A.	PHYSICAL				
1.	Turbidity	N.T.U.	1.0		10
2.	Temperature	°C	27	27	27
3.	Odour		Numerous	Numerous	Numerous
4.	Colour		Clear	Clear	Clear
B.	CHEMICAL				
1.	PH		7.66	7.40	7.50
2.	Total Solid	mg/L	270	441	210
3.	Total Dissolved Solid	mg/L	2.5	432	197.5
4.	Suspended Solid	mg/L	7.5	9.0	12.50
5.	Total Hardness	mg/L	252	63	190
6.	Calcium (Ca)	mg/L	146	40	143
7.	Magnesium (Mg)	mg/L	106	23	47
8.	Nitrates (No_3)	mg/L	Numerous	Numerous	Numerous
9.	Chlorides (Cl)	mg/L	24	61	29
10.	Sulphates (SO_4)	mg/L	40	23	23
11.	Iron (Fe)	mg/L	0.7	0.8	0.6
12.	Phosphate (PO_4)	mg/L	Nil	Nil	Nil
13.	Dissolved Oxygen	mg/L	6.5	6	5.3
14.	Acidity	mg/L	50	55	58
15.	Alkalinity	mg/L	215	200	205
16.	Electro Conductivity	mhos/cm	370	719	351
17.	Potassium (K)	mg/L	3	2	2
18.	Sodium (Na)	mg/L	15	15	9
19.	Lythium (Li)	mg/L	0	0	0
C.	BACTERIOLOGICAL				
1.	Total Count	(ml per 100 ml)	1	Numerous	13
2.	Most Probable of coliforms	(Number Per 100 ml)	9	Numerous	75

TABLE 2.4
CONTAMINANTS REPORTED IN GROUND WATER
(Nurain, 1996)

Type of Contaminant				
Chemical		Microbiological	Physical	Radioactive
Inorganic	Organic			
Cr^{+3} Cd^{+2} Zn^{+2} Pb^{+2}	Detergents	Pathogenic-	Turbidity	Radium and
Fe^{+2} Ba^{+2} Mn^{+3+4} Li^{+}	Alcohols	Bacteria;	Colour	other
K^{+} Ni^{+3+4} Ag^{+} Mo^{+2}	Phenols	Sulphate	Particles	radioactive
U Se Hg^{+2} Al^{+3}	Gasoline	Reducing; Iron	Odour	nuclides
Cu^{+2} F CN^{-} Cl^{-}	Pesticides	related and	Hardness	
B^{+3} SO_4^{-2} NO_3^{-} NO_2^{-}	Dyes	Slime producing	Taste (Salinity)	

in fig. (2.2). A study of soil climatic zones of sugarcane in India done by Gupta (1996) reported that Roorkee-Muzaffarnagar region has large number of sugarcane industries (fig. 2.1). Further, Gupta (1995) and Arora (1997) analysed the molasses and effluent of Mahalakshmi Sugar Mills Corporation Ltd. Iqbalpur, situated in Roorkee region and reported that the pollution from the sugar industry influences largely the parameters like pH, Biological Oxygen demand, Chemical Oxygen demand, Total dissolved solids and suspended solids. Analysis of molasses indicates the presence of Zn^{++} ions (Table 2.1 and 2.2). Roorkee-Muzaffarnagar region is a part of the Ganga-Jamuna doab and comes under the Indo-Gangetic plains of quarternary period. Ganges water gets polluted by a number of pollutants including domestic waste which effects the physical chemical and microbiological characteristics of water (Lakkarpragada, 1989). On the other hand the impact of the waste water would depend on its characteristics and volume as well as on the self regeneration capacity of the river.

The alluvial soil is derived from the fluvial deposits formed by either braided rivers or meandering rivers. The alluvial plains of India extend over a length of about 1600 km and a width of 320 km. They comprise an area of 95, 714 km^2 in Punjab, Haryana and Rajasthan formed by the river system of Ravi, Beas, Sutlaj, Ganga and Yamuna over the ages with the sediments of Siwaliks, Himalayas and the Aravallies. Uttar Pradesh, Bihar and West Bengal have alluvial plain area of 373, 606 km^2 formed mainly by the Ganga, Yamuna, Ghagra, Gandak and Gomti river systems. Because of the shifting position of river channels, everchanging depositional velocities, source of sediments and climatic variations from humid to semiarid conditions, the nature of the alluvium varies in texture from sandy to clayey, calcareous to noncalcareous, acidic to alkaline and normal to saline (Seminar report, 1986).

2.3 Effect on photosynthesis

The role of zinc both in the biosynthesis of chlorophyll precursors and in the process of photosynthesis is of great interest. Zinc deficiency can reduce photosynthesis by over 50% depending upon plant species. It is now widely believed that zinc plays a role in photosynthesis because it is a component of carbonic anhydrase; many are the texts that say or imply this.

The truth is that the exact role of zinc in photosynthesis still remains obscure. Carbonic anhydrase catalyses the hydration of CO_2 in C_3 plants in the chloroplast and in C_4 plants in the cytoplasm. However, plants normally seem to contain a considerable excess of carbonic anhydrase which may thus serve no other purpose than that of increasing the amount of dissolved CO_2. Impaired photosynthesis caused by zinc deficiency may also be the result of a marked reduction in chlorophyll content and the derangement of the structure of chloroplasts. Such effects may be due to increased oxygen free radical production, but it would appear that impaired photosynthesis usually precedes any detectable increase in oxygen free radicals (Micronutrient Bureau, 1994). On the other side, elevated zinc induces stress on barley seedlings, which is mainly seen in a strong inhibition of root extension and a less dramatic increase in the oxidation state of Q_A, the primary electron acceptor of photosystem II (Brune et al., 1994a).

Among the chlorophyll derivatives containing metals other than Mg, only Zn containing chlorophylls have chemical features comparable to Mg chlorophylls (Watanabe and Kobayashi, 1991). Zn-porphyrin derivatives are usually more stable than Mg- derivatives, and have been widely used in the studies of artificial photosynthesis·Zn-containing bacteriochlorophyll a (Zn-BChl, which more strictly should be termed bacteriopheophytin) has even been introduced artificially into the isolated antenna proteins as replacement of light - harvesting Mg - BChl or of accessory Mg-BChl in the reaction centre complex (Scheer and Hartwich, 1995). However, photosynthesis without (Mg) chlorophylls has never been known.

Wakao et al., (1996) discovered natural Photosynthesis using Zn-containing bacteriochlorophyll *a* in an acidophilic bacterium *Acidiphilium rubrum*. Chemical analysis of the cell extracts gave a 13: 2: 1 molar ratio of Zn-bacteriochlorophyll *a* : Mg-bacterio-chlorophyll *a* : bacteriopheophytin *a*. Most of the pigments are associated with fully active reaction center and light-harvesting complexes, analogous to those in purple photosynthetic bacteria. The finding indicates an unexpectedly wide variability of photosynthesis.

Kupper et al. (1996) studied the environmental relevance of heavy metal - substituted chlorophylls using the example of water plants. It was discovered that the substitution of the central atom of chlorophyll, magnesium, by heavy metals (mercury, copper, cadmium, nickel, zinc, lead) in vivo is an important damage mechanism in stressed plants. This substitution prevents photosynthetic light - harvesting in the affected chlorophyll molecules, resulting in a breakdown of photosynthesis. The reaction varies with the intensity of light. In low light irradiance all the central atoms of the chlorophylls are accessible to heavy metals with heavy metal chlorophylls being formed, some of which are much more stable towards irradiance than Mg-chlorophyll. Consequently, plants remain green even when they are dead. In high light, however, almost all chlorophyll decays, showing that under such conditions most of the chlorophylls are inaccessible to heavy metal ions.

2.4 Involvement in sugar transformations

Zinc deficiency markedly reduces the activity of aldolase in plant tissues and is therefore likely to affect sucrose synthesis. However, conflicting reports of the effect of zinc deficiency on sucrose and starch formation have been presented, and it may be that the primary effect of zinc on sucrose and starch formation and distribution may be related to the impairment of sucrose transport by a zinc effect on the structural integrity of biomembranes (Micronutrient Bureau, 1994).

Zinc acts as a protective and stabilizing component of biomembranes against activated O_2 species in cells of both leaf (Cakmak and Marschner, 1987; Cakmak 1988) and root (Cakmak and Marschner, 1988) and thus may play a crucial role in preventing photooxidation. Under zinc deficiency photosynthetic carbon metabolism is also impaired, and concentrations of sugars especially sucrose, are increased (Sharma et al., 1982). The interactions between the Zn nutritional status of bean plants, light intensity, carbohydrate concentrations and visual deficiency symptoms were studied by Marschner and Cakmak (1989). In leaves of Zn deficient

plants the concentrations of carbohydrates (reducing sugars, sucrose, starch) increased with increasing light intensity, particularly in primary leaves. In contrast, root concentrations of carbohydrates were much lower in Zn deficient plants exposed to high light intensity. Resupply of Zinc to Zn deficient plants for upto 96 h remarkably decreased ratios of sucrose content in the older leaves. The effects of increasing light intensity the severity of chlorosis and necrosis were discussed in relation to photooxidation of thylakoid constituents by activated O_2 species. Elevated levels of these toxic O_2 species were to be expected as a result of impairment in the photosynthetic carbon turnover and electron transfer in leaves of plants deficient in Zn, K or Mg.

Stoop et. al. (1996) reviewed the mannitol metabolism in plants and suggested its role in plant responses to both biotic and abiotic stress. Reductions in growth and yield are commonly recorded symptoms of metal toxicity in plants. Exposure of white bean seedling to excess Co^{++}, Ni^{++}, or Zn^{++} ions inhibited the export of photoassimilates from unifoliate leaves and these leaves accumulated increased amounts of sucrose, reducing sugars and starch. This suggested that a primary effect of excess metal in causing growth reduction is to inhibit the export of assimilates from source regions to sink regions. Sucrose is the major translocated sugar in *Phaseolus vulgaris*. Accumulation of this carbohydrate in source leaves of metal treated seedlings indicates either an entry of sucrose into a storage pool making it unavailable for translocation, or inhibition of loading of sucrose into the phloem of minor veins. Phloem loading or vein loading is the process by which the major translocated substances are selectively and actively delivered to the sieve tubes prior to translocation from the source region. Inhibition of vein loading contributes markedly to the observed toxicological effects of reduced photoassimilate export and of accumulation of carbohydrates in fully expanded leaves of bean seedlings exposed to excess metal ions (Geiger, 1975, Rauser and Samarakoon, 1980).

2.5 Growth responses

Plants subjected to Zn deficiency exhibit symptoms consisting of leaf mottling, small leaves, and elongation repression of leaves and/or stems, etc. at the morphological level as well as decrease of protein content and accumulation of free amino acids and amides etc. at the physiological level. Further, inhibiting effects of heavy metals on plant growth have been documented by many investigators through the measurement of growth parameters like root elongation (Trivedi and Erdei, 1992; Symeonidis and Kartaglis, 1992), protein concentration (Sharma and Bisen, 1992) and fresh weight change (Greger et al., 1991). Root growth is particularly sensitive to toxic metals; rooting tests are long established methods for quantifying metal toxicity (Wilkins, 1978) and both interspecific and intraspecific variations in metal tolerance have been demonstrated in this way (Baker, 1987; Macnair, 1993). However, the cellular basis of the differential responses of root growth to toxic metals remains largely unexplored (Francis et al., 1995). Plant cell enlargement is due to the irreversible expansion of the plant cell which results from two interdependent physical processes, water absorption and cell wall yielding. As first formulated by Lockhart (1965), plant cell enlargement, elongation or relative growth rate is a linear function of turgor pressure P in excess of a critical turgor Y (the yield threshold) and cell wall extensibility. Inhibitory effects on plant growth by zinc can be demonstrated by studying the responses of the cell elongation, the effective turgor pressure (P-Y) and the cell wall extensibility of the cells of plant when subjected to zinc treatments.

Aided and Okamoto, 1993 studied the responses of elongation growth, turgor pressure and cell wall extensibility of stem cells of *Impatiens balsamina* to lead, cadmium and zinc and reported that elongation growth rate was inhibited by these heavy metals due to their suppression on cell wall extensibility. Effective turgor was also inhibited by Pb and Cd but it played secondary role in reducing the stem cell elongation growth rate. The major rate limiting factor for cell elongation growth was the cell wall extensibility. Furthermore, Cd^{++} ions was found to be more toxic than Pb^{++} ions while Pb^{++} ions was more toxic than Zn^{++} ions.

Various aspects of the experimental design and computational methods used in plant growth analysis were investigated by Poorter and Garnier (1996). Gutschick (1993) and Gutschick and Key (1995) offered an analysis of the quantitative relationship between the set of putatively adaptive responses r, V_{max}, Km, etc. and the relative growth rate. Increase of soil zinc supply resulted in the increase of shoot dry matter of *Brassica napus* plants at the rosette stage by upto 100%, at the green bud stage by upto 50% and seed yield by upto 18% (Hu et.al., 1996).

Higher sensitivity of plants to Zn deficiency is associated with higher root growth at the expense of shoot growth. Similarly a sensitive phenotypic indication of excess Zn in the rooting medium is inhibition of root elongation. This may be illustrated with the ratio of leaf to root lengths. Shoot/root dry weight ratio may, therefore, be a sensitive parameter for the evaluation of genotypes for their susceptibility to zinc stress (Brune et al., 1994a; Cakmak et al., 1996b). Lower shoot/root dry weight ratio is a well-known phenomenon in P-deficient plants (Cakmak et al., 1994) and considered as an adaptive response of plants for more efficient P acquisition from soils (Anghinoni and Barber, 1980).

Cakmak et.al. (1996b) studied the effect of varied supply of zinc on Zn deficiency symptoms in shoots,root and shoot dry matter production and distribution of Zn in roots and shoots in wheat genotypes. Despite severe decreases in shoot growth and shoot dry matter production, root growth of all genotypes was found either not affected or even increased by Zn deficiency. Correspondingly, shoot/root dry weight ratios found to be lower in Zn-deficient than in Zn-sufficient plants. On the other hand, Brune et al. (1994a) administered heavy metal zinc to barley seedlings by increasing its concentration in the hydroponic medium and reported that the most dramatic effect was a severe inhibition of root elongation with little effect on root biomass production. The growth of primary leaves was little affected although the zinc content of the primary leaves increased several-fold. An effect of zinc on M-phase and G1 of the plant cell cycle in the synchronous TBY-2 tobacco cell suspension was studied by Francis et al. (1995) and reported that once plant cells meet the requirements of late G1 check-points,

they are committed to divide, even in the presence of toxic concentrations of Zn.

Early investigations using electron microscopy on leaves of metallophytes such as *Silene cucubalus, Armeria martina ssp halleri* and *Thlaspi alpstre* found precipitates in the outer walls of epidermal cells. Light microscopic micrograph from a transverse section of a root of *Thlaspi caerulescens* exposed to 100 μM Zn show the abundance of precipitates in epidermal and sub-epidermal cells while less precipitates were seen in cortex cells (Vazquez et al., 1994). Based on solvent extraction data, it was suggested that in these species Zn was translocated in the transpiration stream to the epidermal cells, where Zn precipitation would occur in the cell walls as water is evaporated by transpiration. Zn is involved in stomatal opening and transpiration possibly as a constituent of carbonic anhydrase needed for maintaining adequate [HCO_3^-] in the guard cells, and also as a factor affecting K^+ uptake by the guard cells (Sharma et al., 1995).

There remain uncertainties concerning the structure and the chemical composition of the Zn- rich deposits. The globular deposits from *Deschampsia caespitosa* were identified as Zn phytate crystals (Van Steveninek et al., 1987). Zinc-containing electron dense deposits located in vacuoles, cell wall and intercellular spaces of epidermal and cortical cells in addition to high Zn concentrations always contained high amounts of phosphorus, potassium and calcium and a high Zn/P ratio. Storage of large amounts of Zn in a form with less P increases the proportion of P available for metabolic processes and reduces the transfer of a Zn-induced P-deficiency, which seems likely in plants using phytate as the Zn storage form. Physiological inactivation of P by high Zn concentrations in cells would be especially critical for adaptation of plants to metalliferous soils with low P availability (Vazquez et al., 1994).

2.6 Membrane integrity

Studies on root exudates have demonstrated that zinc deficiency can rapidly increase the leaking of compounds and ions across the root plasmalemma. It is currently thought that the main role of zinc in membranes is related to the protection of membrane lipids and proteins

from the damaging effects of oxygen free radicals. There is no evidence that zinc has a role in the structure of plant membranes (Micronutrient Bureau, 1994, Cakmak and Marschner, 1990; Welch and Norvell, 1993; Pinton et al., 1994). Alia et al. (1995) suggested the existence of a correlation between generation of free radicals and the accumulation of proline. They proposed that accumulation of proline is related to nonenzymatic detoxification of free radicals that are generated excessively under stress. A growing body of evidence indicates that transition metals act as catalysts in the oxidative deterioration of biological macromolecules and therefore, toxicity associated with these metals may be due, at least in part, to oxidative tissue damage. The decrease in antioxidant enzyme activities by heavy metal ions could result from the attack caused by metal ion-induced oxygen species (Stohs and Bagchi, 1995; Gallego et al. 1996).

2.7 Uptake, translocation and compartmentation of zinc

Plants roots can absorb Zn not only as a divalent cation but also in chelated form, namely as Zn-phytosiderophores. However, free Zn^{++} remains the preferential form for Zn uptake even in the presence of Zn-phytosiderophores. So far, no evidence has been found for a metabolically active uptake of chelated Zn species by plant roots (Halvorson and Lindsay, 1977). More Zn^{++} ions accumulate in zinc treated plants than control plants, which could be due to the involvement of zinc in maintaining the membrane integrity and H^+- ATPase dependent pH gradient across the plasma membrane (Pinton, 1992). Zn content (accumulation) per shoot is better correlated with the sensitivity of genotypes to Zn deficiency. Less sensitive wheat genotypes are characterized by higher Zn uptake and higher root to shoot transport capacity of Zn at deficient supply. Enhanced capacity of genotypes for Zn translocations from roots to shoot under different Zn supply markedly contributes to Zn efficiency in wheat genotypes. The reason for genotypic differences in Zn translocation to the shoot under Zn deficiency is not clear (Cakmak et al., 1996b). A number of mechanisms for Zn efficiency has been discussed which might be operated in the rhizosphere and within plants, for example difference in root morphology, mycorrhizal infection, release of Zn-mobilizing phytosiderophores (phytometallophores), uptake, translocation and compartmentation of Zn (Graham and Rengel,

1993).

There are no reports on the role of phytosiderophores in Zn translocation within the plants. Phytosiderophores are released from zinc deficient roots and are known to be effective in mobilizing by chelation sparingly soluble Zn compounds in calcareous soils leading to higher solubility and transport of Zn to the root surface (Treeby et al., 1989; Zhang et al., 1989). Phytosiderophores have also high capacity to mobilize Zn from root apoplast (Zhang et al., 1991) and are involved in inter and intracellular Fe transport in plant tissues. Phytosiderophores are also present in the xylum and phloem and might enhance mobility (physiological activity) of Zn in the plant (Mori et al., 1991; Welch, 1995; Cakmak et al., 1996c; Wiren et al., 1996).

Increase in permeability of plasma membrane and efflux of low molecular weight organic solutes as well as release of phytosiderophores in monocots, especially graminaceous species are the main responses of Zn deficient roots (Cakmak and Marschner, 1988). Cakmak et al. (1996c) reported that enhanced synthesis and release of phytosiderophores at deficient Zn is involved in Zn efficiency in wheat genotypes. They suggested that the expression of Zn efficiency mechanism is related to phytosiderophores-mediated enhanced mobilization of Zn from sparingly soluble Zn pools and from adsorption sites, both in the rhizosphere and in plants.

The main sources of trace elements to plants are their growth media (e.g. soil, air, nutrient solutions) from which trace elements are taken up by the root or the foliage. Trace element uptake by roots depends on both soil and plant factors (e.g. source and chemical form of elements in soil, pH, organic matter, plant species, plant age, etc.). Consequently, element mobility and plant availability are very important when assessing the effect of soil contamination on plant metal uptake and related phytotoxic effects (Mench et al., 1994). Interactions between elements occurring at the root surface and within the plant can affect uptake, as well as translocation and toxicity (Luo and Rimmer, 1995; Voutsa et al., 1996).

Plants exposed to excessive heavy metal concentrations in the rooting medium employ different mechanisms in order to minimize toxic effect. In principle, there are five possible mechanisms by which cytoplasmic Zn concentrations may be regulated at a low level (Clarkson

and Luttge, 1989) (a) The plasma membrane may have low permeability for Zn, thereby restricting Zn uptake into the cells. (b) After uptake into the cells, Zn could rapidly be transported into subcellular components such as the large central vacuole (c) Alternatively Zn could be precipitated as insoluble salts (d) Zn may be detoxified by complex formation or binding; for example to phytochelatins (e) cells may also reduce their heavy metal load by rapid and active extrusion across the plasma membrane into the apoplast. As a consequence of the concerted action of these different mechanisms, the cytoplasmic concentration of Zn is precisely regulated with very narrow limits in barley seedlings (Brune et al., 1994a). Computer simulation of a complex formation between organic acids and zinc in relation to their endogenously occurring vacuolar concentrations suggest that vacuolar citrate may be important in binding excess zinc in the vacuoles (Wang et al., 1992).

Early investigations pointed to cell walls as storage sites for Zn (Peterson, 1969), but a significant correlation between Zn tolerance and the Zn-binding capacity of cell walls is not a general characteristic of plants. Moreover, studies with protoplasts from Zn-tolerant and Zn-sensitive *Anthoxanthum odoratum* (Poulter et al., 1985) do not suggest an important role for cell walls in Zn-tolerance. However accumulation of Zn in the apoplast of shoot organs of barley seedlings show that high cation binding capacity of the cell wall contribute to heavy metal tolerance at least as a major component (Brune et al., 1994a).

Beside the apoplast, the epidermal cell layers also function as a compartment for preferential accumulation. The potential function of the epidermis in zinc compartmentation is related to the volume occupied by it. Storage of large amounts of Zn in a form with a relatively low proportion of P (i.e. high Zn/P ratio) may be more advantageous than storage as phytate. Storage in a form with less P increases the proportion of P available for metabolic processes and reduces the danger of a Zn-induced P-deficiency, which seems likely in plants using phytate as the Zn storage form. Physiological inactivation of P by high Zn concentrations in cells would be especially critical for adaptation of plants to metalliferous soils with low P availability (Brune et al., 1994a; Vazquez et al., 1994).

2.8 Protein synthesis and cell wall bound enzymic response

Most of leaf zinc is associated with low molecular weight complexes, storage metallo proteins, free ions and insoluble forms in the cell wall. Between 58% and 91% of plant zinc may be soluble, depending on plant species. Although the water soluble zinc fraction is considered to be the physiological active fraction and thereby a better indicator of zinc status than total zinc content, it does not seem to have found favour for diagnostic purposes. More than 70 zinc metalloenzymes in which zinc acts as a functional, structural or regulatory co-factor, have been identified in plants and represent only a small proportion of plant zinc. Soluble zinc in plants is mostly bound to low molecular weight complexes, such as aminoacids and peptides. Only a small part (5-7%) of total zinc is likely to be present as Zn^{++}. Free zinc is probably also under the control of cell walls, as such components as lignin, cellulose and hemicellulose possess a high binding affinity for zinc. Most of the zinc in roots is likely to be in the apoplast. Most of the insoluble zinc in plants (9-42%) is found in proteins (Micronutrient Bureau, 1994).

Zinc deficiency has a rapid and marked adverse effect on protein synthesis which is attributed to the sharp reduction in RNA levels, the earliest observed effect of zinc deficiency. This role of zinc in protein synthesis indicates a high demand for zinc in meristems, which is borne out by plant tissue analysis. Recent evidence indicates that zinc plays a key role in stabilizing the RNA and DNA structure, in maintaining the activity of DNA synthesizing enzymes and in controlling the activity of RNA degrading enzymes. Zinc may thus play a role in controlling gene expression. It is estimated that there are atleast about 200 DNA binding proteins which contain zinc, essentially required for their function (Vallee and Auid, 1991; Micronutrient Bureau, 1994).

It has been established unambiguously that zinc is a component of nearly 300 enzymes in different species of all phyla and therefore it will be definitely involved in almost all aspects of cell metabolism. Protein bound zinc is designated as `structural pool of zinc' and the enzyme

bound zinc is designated as" Metabolic pool of zinc" (Vallee and Auid, 1991). The findings of Clarkson and Hanson (1980) strengthen the facts mentioned above to a great extent and convey an important information that cell has need for stable metalloenzyme complexes in which co-ordination is basically tetrahedral and zinc has been reported to be most competent element to fulfil this need. Its saturated d-orbitals and small size favour tetrahedral complexes which give importance of zinc by taking part in various process of plant metabolism.

To investigate the effects of Zn deficiency on protein synthesis, proteins and peptides were extracted from meristematic tissue of rice (Kitagishi and Obata, 1986) and tobacco cells (Obata and Umebayashi, 1988; Obata et al. 1994) cultured with and without zinc, and were compared using two dimensional electrophoresis. As a result, although the electrophoretic profiles of the peptides were almost identical among the Zn treatments, the total amount of protein decreased, and clear differences were observed in some soluble cellular proteins and peptides in the Zn plots. It was concluded from these results that Zn may play a role in protein synthesis at the translation level and in the formation and suppression of some peptides at the transcription level.

In the same manner, to carryout a series of experiments in order to analyze the effects of Zn deficiency on protein synthesis in higher plants, Obata et al. (1996) used a budding yeast *Saccharomyces cerevisiae*, as model organism. Yeast cells were cultured in a chemically defined Burkholder minimum medium with and without Zn. Zinc deficiency remarkably depressed the protein content of the cells. Some soluble proteins and peptides disappeared and some proteins and peptides appeared in culture in the absence of Zn. In addition, the pI of one protein shifted from 8.0 to 7.8 under nondenaturing conditions, and the N-terminal amino acid sequence of this protein showed a 100% homology with enolase. It was concluded that Zn is one of the key elements in protein synthesis in plants and may affect peptide synthesis at the transcription and/or translation level.

It is a frequent observation, that polypeptide patterns of plants, tissues or cells change considerably in response to stress regimes. In principle, two opposite causal interpretations

of these changes are possible with respect to the physiological relevance. On the one hand, stress proteins may be essential or at least useful elements of the plants in dealing with the stress and allowing the adaptation or development of tolerance. Heat shock proteins such as HSP 70 or HSP 60 which functions as chaperones belong to this group. On the otherhand, stress may induce changes in polypeptide composition just as a consequence of disregulation and metabolic disorder. In this case, no function in stress handling will be attributed to the stress responsive proteins. As a third explanation which combines components of both extreme responses, maintenance of metabolic pathways in the presence of the stress may require up-or-down regulation of specific proteins. For example, enzymes may be inhibited under water stress by increasing ionic strength at elevated osmotic pressure (Kaiser and Heber, 1981) or under heavy metal stress by increasing concentrations of toxic ions (Tonsett and Thurman, 1988).

Proteins are, in a quantitative sense, the second component of the primary cell wall of both monocot and dicot plants (Albershein, 1965) and constitute 10 to 15% dry weight of cell wall (Lamport and Catt, 1981, Taiz, 1984), including structural proteins, enzymes and lectins (Showalter, 1993). Many soluble and or ionically bound proteins were also found in the cell wall space of expanded leaves and growing tissues. Many types of wall proteins are believed to be involved in regulating cell wall growth by participating in biochemical modifications of certain compounds in the wall. Although the kind or activity of extracellular proteins is still controversial (Kutschera, 1994), proteins such as expansins seem to play a key role in the modulation of the cell wall elongation (McQueen-Mason and Cosgrove, 1995).

Cell wall proteins are tested under number of the stresses like water deficit (Bozarth et al., 1987), development and wound stress (Rodriguez et al., 1995), elicitor treatment (Bradly et al. 1992) etc. but till today there is no report of the effect of zinc (heavy metal) stress on cell wall proteins. However, Brune et al. (1994b) reported the zinc stress induced changes in apoplasmic protein content and polypeptide composition of barley primary leaves. They

observed that excessive zinc in the rooting medium, and consequently increasing zinc content in the shoot of barley *(Hordeum vulgar)* seedlings, strongly affected the protein content and the polypeptide composition of the leaf apoplasm. In contrast, only small changes in polypeptide composition (in the IEF range of pH 4-8 and between 14 and 80 KDa) were detected in mesophyll and epidermis protoplasts. The zinc stress-induced change in apoplasmic protein content were also analysed in detail. The apoplasmic protein content increased by more than 3-fold when the zinc concentration in the medium was increased from 0.02 to about 200 μmol/l; at higher zinc concentrations no further changes occured. The increase in apoplasmic protein content was due both to a general increase in abundance of the most dominant apoplasmic polypeptides and to a pronounced increase in the abundance of specific polypeptides as monitored in electrophoretic separations or by measuring the activity of apoplasmic enzymes. The largest induction was seen for four apoplasmic polypeptides of molecular masses, 16, 23, 27 and 28 kDa with as yet unknown function.

Effects of zinc on cytoplasmic enzymes have been studied by many scientists. Zinc is positively correlated to catalase (Prakash and Iyengar, 1990) and carbonic anhydrase activity (Sharma et al., 1995). It is negatively correlated to the activity of peroxidase, ribonuclease and acid phosphatase (Mehrotra et al., 1983). Till date, with the exception of acid phosphatase there is no report on cell wall bound enzyme activity under zinc stress (Tu et al., 1988). However, report is available on the apoplasmic enzyme activities under zinc stress in barley (Brune et al. 1994b). The apoplasm is the extracellular component of the cells. Structurally, it is dominated by the polymeric structures like cellulose, pectins and lignins. Part of the proteins and enzymes associated with the apoplasm are involved in metabolism of these polymers (peroxidases).

2.9 Phytochelatins and metal tolerance

Research during last few years suggests the involvement of certain low molecular weight peptides, trivially named as phytochelatins (PCs), in the mechanism of tolerance to heavy metals

(Steffens, 1990; Rauser, 1990). These peptides were discovered by Margoches and Vallee in 1957 in horse kidney and were named as metallothioneins (MTs). The first information of plant Cd binding component was reported by Casterline and Barnett (1977). Since then, the presence of thiol containing metal binding polypeptides in plants (Phytochelatins, PCs) has been established; PCs being analogous to MTs of animal systems. The characterizing features of PCs are low molecular weight, high metal content, high cysteine content, absence of aromatic amino acids, tryptophan and histidine and abundance of Cys-X-Cys sequence (where X is an amino acid other than cysteine). The general formula for these metal binding complexes is (γ-glu-cys)n -Xa, where n =2 -11 depending upon the sources and Xa is either glycine (Gly) or ß-alanine (β-Ala). Glutamic acid was found to be the N-terminal and glycine the C-terminal amino acid of polypeptide chain (steffens, 1990; Rauser, 1990). Another important feature of PCs is that unlike other proteins they are not synthesized by ribosomes.

The original molecules defined as MTs, now termed the class I MTs, possessed arrangements of cysteine residues, as Cys-Cys and Cys-x-Cys (where x is an amino acid other than cysteine), which aligned with those of equine renal MT(the first isolate). Class II MTs are remarkably similar molecules containing Cys-Cys and Cys-x-Cys clusters but which can not be aligned easily with equine renal MT. Both class I and II MTs are gene-encoded polypeptides synthesised by transcription and translation. This is in marked contrast to a third group of polypeptide, now termed class III MTs, which are more commonly referred to as phytochelatins (PCs) or by their structural name poly[gamma-glutamylcysteinyl]glycine ([gamma EC] G). These molecules are not gene encoded: their synthesis is from glutathione (gamma ECG) by a specific enzyme gamma-glutamyl cysteine dipeptidyl transpeptidase (common name phytochelatin synthase; Loeffler et al., 1989; Kagi and Kojima, 1987).

A correlation between biosynthesis of phytochelatins and glutathione (GSH) metabolism has been established. The evidence comes mainly from the fact that buthionine sulfoximine (BSO), an inhibitor of glutathione synthesis, inhibits the biosynthesis of phytochelatins (Steffens et al., 1986). Biosynthesis of PCs from GSH is catalyzed by a specific gama-glutamylcysteine

dipeptidyl transpeptidase (phytochelatin synthase). This enzyme was purified by Grill et al. (1989) and was found to possess a molecular weight of 25×10^3 under denaturing conditions. According to the scheme suggested for PCs mediated detoxification of metals (Steffens et al., 1986), the metal ions activate the phytochelatin synthase, which in turn initiates the synthesis of PCs from glutathione. In the absence of metal the enzyme has been demonstrated to remain inactive. PC synthesis has been shown to cease immediately after addition of EDTA or metal free PCs, suggesting the existence of a feedback loop in which metal ion activates the enzyme and when sufficient phytochelatins have been synthesized to complex free metal ions, the enzyme activity ceases (Grill et al., 1989). The phytochelatin-metal complex is sequestered in the vacuole, thus lowering the metal concentration in the cytoplasm.

The above discussed plan for management of cellular metal concentrations sounds very convincing. However, it seems to have weaknesses. For example, induction of PCs and subsequent metal -PC complex formation is best supported by experimental evidences in case of Cd only (Grill et al., 1987). Recently, Tukendorf (1996) reported the phytochelatin synthesis in maize seedlings in response to excess zinc. The metals other than Cd have proved weak inducers of PCs and reports about them are inconsistent. This apparently indicates that PCs are not the exclusive means of metal detoxification in plants and that plants might possess multiple physiological and biochemical strategies for detoxification of different metals.

The plants experience a variety of stresses during their life cycle such as water deficit, temperature and salinity stress. These stresses are known to have some common features. For example, plants respond to all of them by exhibiting an accumulation of an array of metabolites. Thus, proline, an iminoacid, accumulates in the plants during water deficit stress (Aspinall and Paleg, 1981), salinity stress (Bar-num and Poljakoff-Mayber, 1977) and cold stress (Naidu et al., 1991). Thakur and Rai (1982) observed accumulation of other acids in maize cultivars in response to imposed water deficit stress. ABA, a plant growth inhibitor, has been found to accumulate in plants during water (Reid, 1990), temperature and salinity stress (Zeevaart and Creelman, 1989).

Since heavy metals can operate as stress factors, an accumulation of metabolites in response to heavy metals could be expected. Proline accumulates in plants exposed to heavy metal stress, including zinc toxicity (Alia et al., 1995). The significance, if any of the accumulated proline/total amino acids in plant tolerance to metals remains to be assessed.

2.10 Interaction with auxins and other nutrients.

Although it is over 50 years since it was reported that IAA levels were induced in tomato plants under zinc stress - a finding that has been widely confirmed, the nature of the exact relationship between IAA and zinc supply remains obscure. Early explanations related to increased IAA oxidation and to reduce synthesis of tryptophan (a precursor of IAA), have been documented; gibberellic acid which promotes the conversion of tryptophan and tryptamine to IAA, is now thought to be the primary candidate affected by zinc deficiency (Micronutrient Bureau, 1994).

The interaction of zinc with other elements is also well established. Top on this list is phosphorus. There is a huge and confusing literature on this subject. Unfortunately, the search for a significant explanation has resulted in several erroneous descriptions particularly the "P induced Zn deficiency". Interactions are even looked for where there is no reason to expect any, namely, when neither Zn or P are limiting or excessive. Even when the uncritical findings are rejected, the subject of P-Zn interaction remains a complex one involving both soils and plant phenomena (Katyal et al, 1992; Micronutrient Bureau, 1994).

CHAPTER - 3

3.0 MATERIALS AND METHODS

3.1 Materials

P-nitrophenyl β-D-galactopyranoside, p-nitrophenyl α-D-glucopyranoside, phenylmethyl sulfonyl fluoride, sephadex G-25 and molecular weight standards (Sigma VI) were obtained from Sigma Chemical Company, U.S.A. All other chemicals were obtained from either Merck (India), SRL (India) or Glaxo (India) and were of analytical grade. Certified mungbean (*Vigna radiata* cv. K-851) seeds were obtained from the local seed store of Muzaffarnagar, U.P. India.

3.2 Methods

3.2.1 Soil analysis

Crop plants from Haridwar and Muzaffarnagar districts (Uttar Pradesh), India showed characteristic zinc deficiency symptoms. This prompted us to survey the alluvial soil of these districts for its zinc content.

3.2.1.1 Soil survey in and around Roorkee

3.2.1.1.1 Location

The area around Roorkee (District Haridwar) town lies in the Northern hemisphere within the Latitude from 29^0-43'-11" to 29^0-52'-54" N and longitude from 77^0-52'-3" to 77^0-58'-30" E at an elevation of 260 m above sea level. The area is bounded by Upper Ganga Canal and is a part of the Ganga-Jamuna doab, comes under the dry subhumid agroclimatic zones of Indo-Gangetic plains of quarternary period. This vast alluvial fertile plain is made up of unconsolidated formation, comprising of sand, silt, clay and occasionally Kankar layers with sand. The soil texture is observed to vary from

sandy loam to sandy clay. The soils are grey to light grey in colour. The soil is well drained, leacheable and less erodible. The soils have gentle slope and flat topography, where erosion is not the problem.

3.2.1.1.2 Climate

The area around Roorkee has a sub-tropical monsoon climate showing extreme variations in temperature in summer and winter. The temperature sometimes rises to 38^0C in summer and decreases to 2^0C in winter.

The south west monsoon contributes a normal annual rainfall of 1037 mm, out of which about 851.91 mm rainfall occur in the monsoon period. The monsoon generally starts from mid June and is withdrawn by the end of September.

The daily evaporation rate gradually increases from 90.64 in October to around 60.40 mm/day in November and then gradually decreases by the end December.

As such, the meterological data have been collected from the Dept. of hydrology, University of Roorkee for different months during July to September in absence of such data from any where else in the study area. From the data it is evident that humidity is very high, evaporation is very low and a total of around 998.3 mm rainfall occurs during these three months indicating very small irrigation demand, during this season when mungbean is grown.

3.2.1.1.3 Sampling sites

Four villages in a radius of 10 km, north, south, east and west of Roorkee and Muzaffarnagar were chosen for soil sampling. Within a village, four crop fields (0.5-1.0 hectare) representing north, south, east and west directions were chosen as representative sites. Five soil profiles, north, south, east, west and the centre of crop field were analysed. Thus around each city 80 profile pits were exposed for soil sampling.

3.2.1.1.4 Soil sampling

TABLE - 3.1
SAMPLE SITE LOCATIONS

Sl. No.	Village Direction	Village Name	Farmer's name / Field direction: East	West	North	South
ROORKEE REGION						
1.	East	JATBAHADARPUR	Bijendra	Davendra Chauhan	Indra	Harpal Munshi
2.	West	KROUNDI	Mukanda	Harmal	Phool Singh	Hakam
3.	North	DOULATPUR	Piyara Lal	Rajpal Giri	Bija	OmGiri
4.	South	LIBBURHERI	Jugmendra Das	Neeraj Choudhary	Budh Singh	Mahak Singh
MUZAFFARNAGAR REGION						
5.	East	JATMUZHERA	Naipal Singh	Manga Saini	Kalu Ram	Mohabat Ali
6.	West	JAGAHERI	Ram Pal Singh	Harbeer Singh	Ompal Singh	Kharag Singh
7.	North	BADHAYKHURD	Satpal Singh	Dharm Pal Singh	Bhanwar Singh	Junior High School
8.	South	BHAINSI	Bhagawansa	Sukhann Singh	Nahar Singh	Ompal Singh

Rd_{12} = 12 Km away from Roorkee at Delhi Road.
V.P. = Village Prime

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UNIVERSITY OF ROORKEE
ROORKEE-247 667, INDIA

TABLE-3.2

INFORMATION SHEET TO ACCOMPANY SOIL SAMPLES

Name of farmer/owner Neeraj Kumar Chaudhary Village Bheraipur P.O. Libarheri
District Haridwar State U.P. V.P. Sh. Vinod Kumar Chaudhary.

Lab.No.	Sample No.	Depth of sampling cm	No. of samples mixed	Area (hectare)	Slope Level/ Undulating	Drainage Good/ Poor	Elevation Upper/ low land	Irrigation Irrigated/ rainfed	Salinity Saline/ alkali or not	Crop growth Good/ Poor	Recommendations Sought for which crop
1	2	3	4	5	6	7	8	9	10	11	12
Rd_{12} 1	(i)	0-15	4+1	0.5	Level	Good	Upper land	Irrigated	No	Good	The growth of mung bean plant is very good. Soil is sandy loam. Sample is taken from Sorghum field.
	(ii)	15-30	4+1	0.5	Level	Good	Upper land	Irrigated	No	Good	
	(iii)	30-45	4+1	0.5	Level	Good	Upper land	Irrigated	No.	Good	

Any other relevant information to be supplied. Zinc deficiency may be possible, since the different crops shows symptoms related to this element deficiency. When farmer used Zn as a fertilizer he found drastically change in the growth pattern of the crop. (yield will be high). The most common effected crops are Rice, Sorghum, Wheat, Sugar Cane, legumes (Black gram). The area is quite fertile.

Place: Bheraipur
Date: 14/10/91

[signature] 14/10/91
Signature

To, Jitendra Singh R/S c/o Dr. Vinay Sharma
Soil Testing Laboratory
Plant Molecular Bio-Lab.
Deptt.of Biosciences & Biotech
U.O.R., Roorkee.

V-shaped 45 cm soil profile was exposed for soil sampling using a local mechanical soil digger KHURPI. Soil samples from the top three horizons (layers) viz. 0-15, 15-30 and 30-45 cm were collected in separate polythene bags. Representative soil sample of a field (site) for each horizon was made by mixing and reducing the soils of the particular horizon collected from all five soil profile pits of the site, using quatering method. In this method (Jackson, 1969) the soil was spread uniformly over a sheet of polythene and divided into four equal portions as below :

1	2
3	4

The portions marked 1 and 4 were discarded, the remaining portions mixed together and spread out again and reduced to half by the same procedure. This process was repeated until a sample of the required bulk was obtained.

To preserve soil characteristics, each polythene bag was closed air tight with the help of rubber bands and an information chit containing all necessary information regarding village, district and depth of the soil sample was pasted on the outside of the bag and samples were processed in the laboratory.

3.2.1.2 Soil processing

The soil samples were air-dried, ground in a wooden pestle and mortar and passed through a fine muslin cloth. The physico-chemical characteristics of these soils were assessed by routine laboratory methods.

3.2.1.3 Measurement of soil pH

The pH was measured in 1:2.5 soil : water suspension after continuous shaking for half an hour.

3.2.1.4 Estimation of zinc content

The total and available zinc, using DTPA as an extractant, were extracted by the method of Oien and Gjerdingen (1977) and Lindsay and Norvell (1978), respectively. The

zinc in soil extracts was determined using an atomic absorption spectrophotometer (AAS, aa/ ae spectrophotometer, 751, Instrumentation laboratory, USA). Reagent blank and zinc standard were used for the accurate estimation of zinc.

The standard for zinc was made by dissolving 0.1044 gm of $ZnCl_2$ in 2.5 ml of 1:1 HCl and diluted quantitatively to a volume of 50 ml. Final concentration of zinc was 1000 μg/ml. AAS range was 0.25 - 1.00 μg/ml.

3.2.1.4.1 Total zinc content

Total zinc in soil was estimated by digesting the material with HNO_3 and $HClO_4$ in a long neck borosil Kjeldahl flask. Sample size was 4.0 gm. A 1:5 soil to concentrated HNO_3 mixture was first heated on a hot plate at low temperature (60-70°C) and then heated to nearly boiling for 3 to 4 h, to destroy most of the organic matter. 2 ml of conc. $HClO_4$ was added to the flask, and the contents were evaporated to dryness. The residue was dissolved in 6 ml of 2 M -HNO_3, filtered and diluted to 100 ml with distilled water. Zinc was determined directly in the filtered solution by AAS.

3.2.1.4.2 Available zinc content

Available zinc in the soil was estimated using DTPA as an extractant.

Composition of DTPA extractant

5 mM - DTPA solution

100 mM - TEA reagent

10 mM $CaCl_2 . 2H_2O$ solution

pH of the extractant solution was adjusted to 7.3 with dilute HCl.

Procedure

To 10 gm of soil in 100 ml conical flask, 20 ml DTPA extractant was added and shaken for 2 h. The extract was filtered through Whatman No 40/42 and zinc was determined directly in the filtered solution by AAS.

Fig. 3.1 : MUNGBEAN PLANTS IN GROWTH CHAMBER UNDER CONTROLLED ENVIRONMENTAL CONDITIONS

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3.2.2 Germination of seeds

Locally purchased certified seeds were surface sterilized with disinfectant savlon for 15 minutes. Seeds were then thoroughly washed with double distilled water and imbibed in it for 8 hours. Imbibed seeds were grown hydroponically in plastic pots i.e. juicers in Hoagland's nutrient solution at various zinc regimes ranging from 0-100 ppm zinc in a plant growth chamber in 14 hour photoperiod with illumination of 1000 lx at $27 \pm 2^{\circ}C$ and 80% relative humidity. In all cases seedlings exposed to zinc treatment for 6 days were harvested for the experimental work. Six day old seedlings were chosen since previous work from our laboratory showed that the morphological, physiological and biochemical characteristics were well expressed at this stage. Similarly, a control (distilled water) was also run in which no nutrient was added. Solutions were changed and adjusted to pH 5.4 by 1 M-NaOH or 1 M -H_2SO_4 to avoid ion depletion and complex formation. Non germinated seeds were discarded to avoid fungal infection.

3.2.2.1 Composition of Hoagland's nutrient solution

The hydroponic medium (a diluted and modified solution after Hoagland and Arnon, 1938) contained the following nutrients.

Stock Solution:

Concentration	Chemical	Volume
1 M	$Ca(NO_3)_2$	10 ml
1 M	KNO_3	4 ml
1 M	$MgSO_4$	2 ml
1 M	KH_2PO_4	2 ml
	NaFeEDTA*	2 ml
	micronutrients**	2 ml

NaFeEDTA* 5 mg metallic iron was chelated with 37 mg Na_2EDTA to make 42 mg per ml solution of FeEDTA.

Micronutrient** stock solution contained 2.86 g H_3BO_3, 1.81 g $MnCl_2 . 4 H_2O$, 0.11 g $ZnCl_2$, 0.05 g $CuCl_2.2H_2O$ and 0.025 Na_2MoO_4 per litre ($ZnCl_2$ was omitted from this solution for growing the seedlings used as controls and appropriate amounts of $ZnCl_2$ was included to get the desired concentration of Zn in the medium).

All these stock solutions were mixed and final volume made to 2.273 litres with double distilled water.

3.2.2.2 Zinc treatment

Seedlings were treated with different concentrations of zinc. They were grown separately in nutrient solution with 0, 0.4, 1.0, 5.0, 10.0, 15.0, 25.0, 50.0, 75.0 and 100 ppm of zinc. Seedlings grown in double distilled water alone were taken as a second control.

3.2.3 Morphological studies

3.2.3.1 Measurement of length and width

After six days of zinc treatment seedlings were harvested and dissected into roots, hypocotyls, cotyledons, epicotyls and leaves using thread. Their length and width were measured in centimeters with the help of a simple scale and screw gauge, respectively.

3.2.3.2 Determination of the number of secondary and tertiary roots

Six day old seedlings were harvested and number of secondary and tertiary roots was determined by simple counting. Nature of the reticulum formation by the roots was also noted.

3.2.3.3 Determination of fresh weight

Six day old seedlings were harvested and dissected into different organs i.e.

roots, hypocotyls, cotyledons, epicotyls and leaves. Each organ was blotted dry and weighed on electronic balance.

3.2.3.4 Determination of dry weights

Six day old mungbean seedlings were cut into different organs. Each organ was blotted dry and subsequently oven dried at 80°C for 72 hours. Then their weights were recorded with electronic balance.

3.2.4 Anatomical studies

Six day old hydroponically grown seedlings were harvested. Seedling was divided into root, stem and leaf. Sections were cut from each organ near the top. Both the tissue and razor blade were kept wet during sectioning to avoid shrinkage and distortion of the cells. The unstained sections were immediately mounted in 50% glycerine and examined with a light microscope with attached camera. Diameters of around 20 randomly chosen cortical cells in the section of each organ were recorded with the help of occulometer fitted inside the eyepiece of microscope. Each section was photographed with the help of camera attached to the microcope (2 KICEBI-1, Neovar, Austria.).

3.2.5 Estimation of *in vivo* level of Zn^{++} ions

3.2.5.1 Organwise distribution

3.2.5.1.1 Preparation of sample

Hydroponically grown zinc treated and untreated six day old seedlings were dissected into different organs i.e roots, hypocotyls, cotyledons, epicotyls and leaves and their fresh and dry weights were recorded. Dried material was ground and powdered in a stainless steel mortar and pestle.

3.2.5.1.2 Estimation of Zn^{++} ions

Powdered plant sample was acid digested using triacid mixture of HNO_3, $HClO_4$ and H_2SO_4 (10:4:1). At least 2 ml of H_2SO_4 should always be present to over come the over

heating at last stage. Zinc determination was carried out by atomic absorption spectrophotometry (Piper, 1966).

Procedure :

Powdered sample and triacid mixture in the ratio 1:15 was taken in 100 ml. flat bottomed Kjeldahl digestion flask. The contents of flask was mixed by swirling and heating gently at a low temperature until the appearence of dense brown fumes. Then the flask was removed from heating system for about 5 minutes to allow the initial vigorous reaction to subside. Then the flask was returned to the heating system and digestion was continued slowly at low temperature until the appearence of dense white fumes of H_2SO_4 appeared again. To check any bumping two small glass beads were added.

Digestion was further continued at low temperature for 5-10 minutes and 1-2 minutes at full heat. colourless when cooled indicated complete digestion. Digest was diluted by distilled water and used for the estimation of Zn^{++} ions by atomic absorption spectrophotometer.

3.2.5.2 Subcellular distribution

Different fractions of cell i.e. cytoplasm without proteins, cytoplasmic proteins, cell wall proteins and pellet of cell wall without proteins were obtained using the method of Bozarth et.al. (1987) as given in section 3.2.7.

After salting out of the proteins from the cell wall, the pellet, without protein component was mixed with 10 ml. 1N-HCl and kept at room temperature for 48 hrs. The mixture was centrifuged at 12000 rpm for 10 minutes and supernatent was considered as the cell fraction of cell wall without protein components.

Zn^{++} ions were estimated in all the fractions by ICP (Induced Coupled plasma PLASMALAB, 8440, LABTAM, AUSTRALIA).

3.2.6 Determination of activities of cell wall bound enzymes

3.2.6.1 Preparation of cell wall

The method of Masuda et al. (1988) was followed. In brief, 5 gm fresh root of 6 day

old mungbean seedlings were homogenized on the top of ice in 15 ml of 10 mM Sodium phosphate buffer (pH 7.4) using a mortar and pestle and filtered through a nylon sieve (pore diameter 42 μm). The insoluble fraction was washed thoroughly with distilled water until no protein was detectable in the filtrate and then suspended in 0.05% (w/v) sodium deoxycholate for 2 h at room temperature.

After filtration of the suspension through 42 μm nylon sieve, the residue was washed thoroughly with distilled water. The purified cell wall thus obtained was used for extraction of cell wall bound enzymes.

3.2.6.2 Extraction of cell wall bound enzymes

For extraction of enzymes, the cell wall suspension (0.5 gm wet weight) in 10 ml solution of 1.0 M-NaCl was incubated at room temperature for two hours. The suspension was filtered through filter paper and filtrate was considered as an enzyme extract for the estimation of wall bound enzyme activity.

3.2.6.3 Enzyme assays

3.2.6.3.1 Assay of cell wall bound glycosidase activities

Glycosidase activities were assayed by following the release of p-nitrophenol from its glycosides. The reaction mixture contained 0.5 ml of 5 mM substrate (p-nitrophenyl-β-galactopyranoside, p-nitrophenyl α-glucopyranoside for β-galactosidase and α-glucosidase, respectively), 0.5 ml of Mellvaine buffer (citrate-phosphate buffer) pH 4.4 and 0.5 ml of enzyme extract. The reaction was allowed to proceed at 37°C for 20 min and was terminated by addition of 1.5 ml of 0.5 M-Na_2CO_3. The concentration of p-nitrophenol liberated in the enzymic reaction was determined by recording the absorbance at 410 nm and comparing with known concentration of standard (p-nitrophenol), run simultaneously.

3.2.6.3.2 Assay of cell wall bound acid phosphatase activity

Acid phosphatase activity was measured by the method described by Odds and Hierholzer (1973) with slight modifications using p-nitrophenyl phosphate as

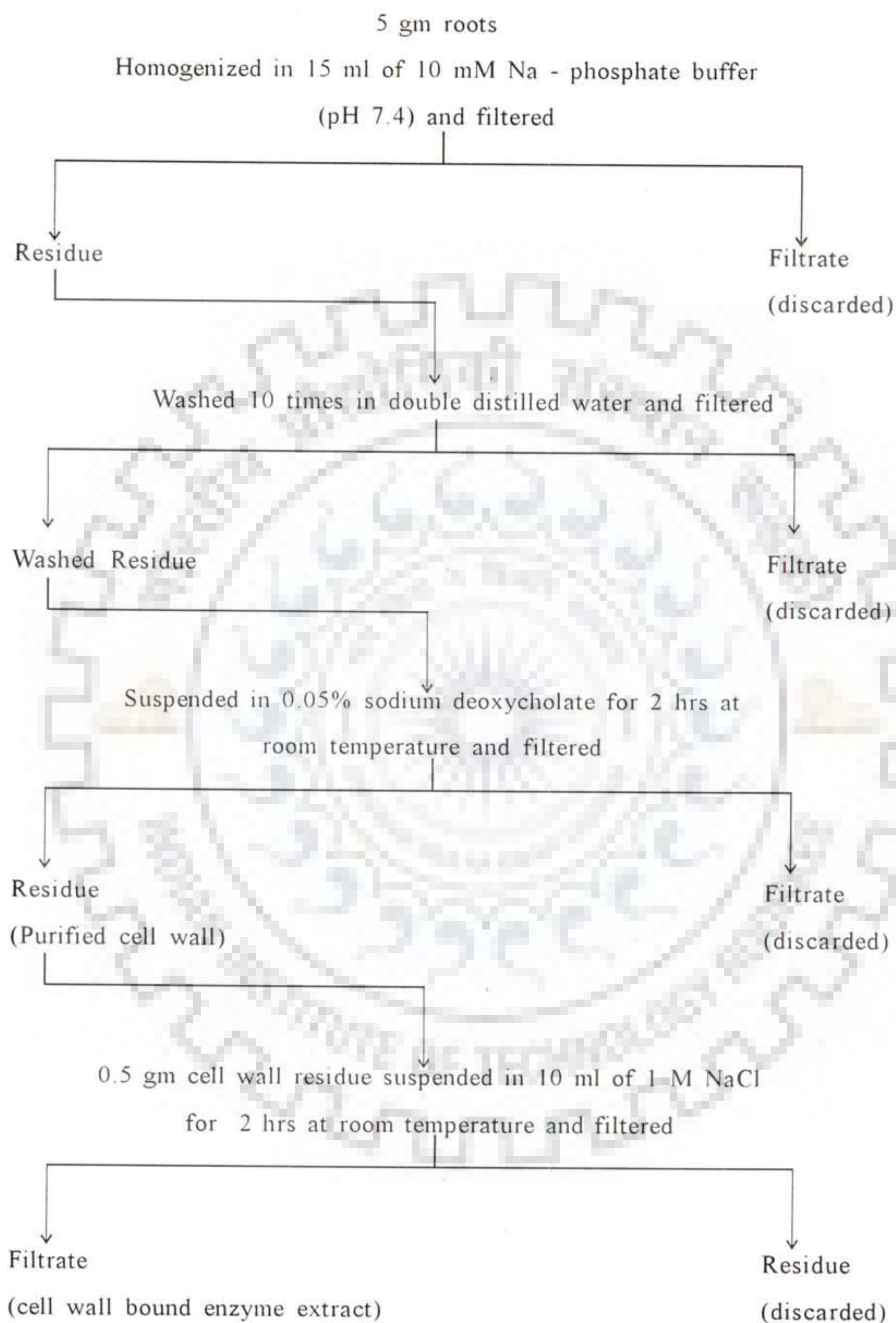


Fig. 3.2: Isolation of cell wall bound enzymes

substrate. The reaction mixture contained 0.5 ml of 50 mM sodium acetate buffer (pH 5.0) and 0.5 ml of 5 mM p-nitrophenyl phosphate. Assay was started by addition of 0.5 ml of enzyme extract. Blank and Standards were also run simultaneously. Standard curve was plotted using different concentrations of p-nitrophenol vs absorbance. The reaction was allowed to proceed at 37°C for 20 min and was terminated by addition of 1.5 ml of 0.5 M-Na_2CO_3.

3.2.6.3.3 Assay of cell wall bound peroxidase and polyphenoloxidase activities.

Cell wall bound peroxidase and polyphenoloxidase activities were assayed by the method of Kar and Mishra (1976) with slight modifications. Assay mixture for peroxidase contained 2.0 ml 0.1M-phosphate buffer pH 7.0, 1 ml 0.01 M-pyragallol, 1 ml 0.005 M-H_2O_2 and 1 ml diluted enzyme extract. Reaction mixture for polyphenoloxidase contained similar ingredients except H_2O_2. In both, the reaction was allowed to proceed for 5 min at 25°C and then stopped by adding 1 ml 2.5 N-H_2SO_4. Amount of purpurogallin formed was estimated by measuring the absorbance at 420 nm. The enzyme activities were expressed in absorbancy units/min/mg protein.

3.2.7 Studies on cytoplasmic and cell wall proteins

3.2.7.1 Isolation of cytoplasmic and cell wall proteins

Cytoplasmic and cell wall proteins were isolated according to the method of Bozarth et al. (1987) with slight modifications.

Solutions

Buffer A : 62.5 mM Tris -HCl (pH 7.2) containing 1% β-mercaptoethanol and 0.5 mM PMSF.

Buffer B: 62.5 mM Tris -HCl (pH 7.2) containing 0.5 M $CaCl_2$ and 5 mM DTT.

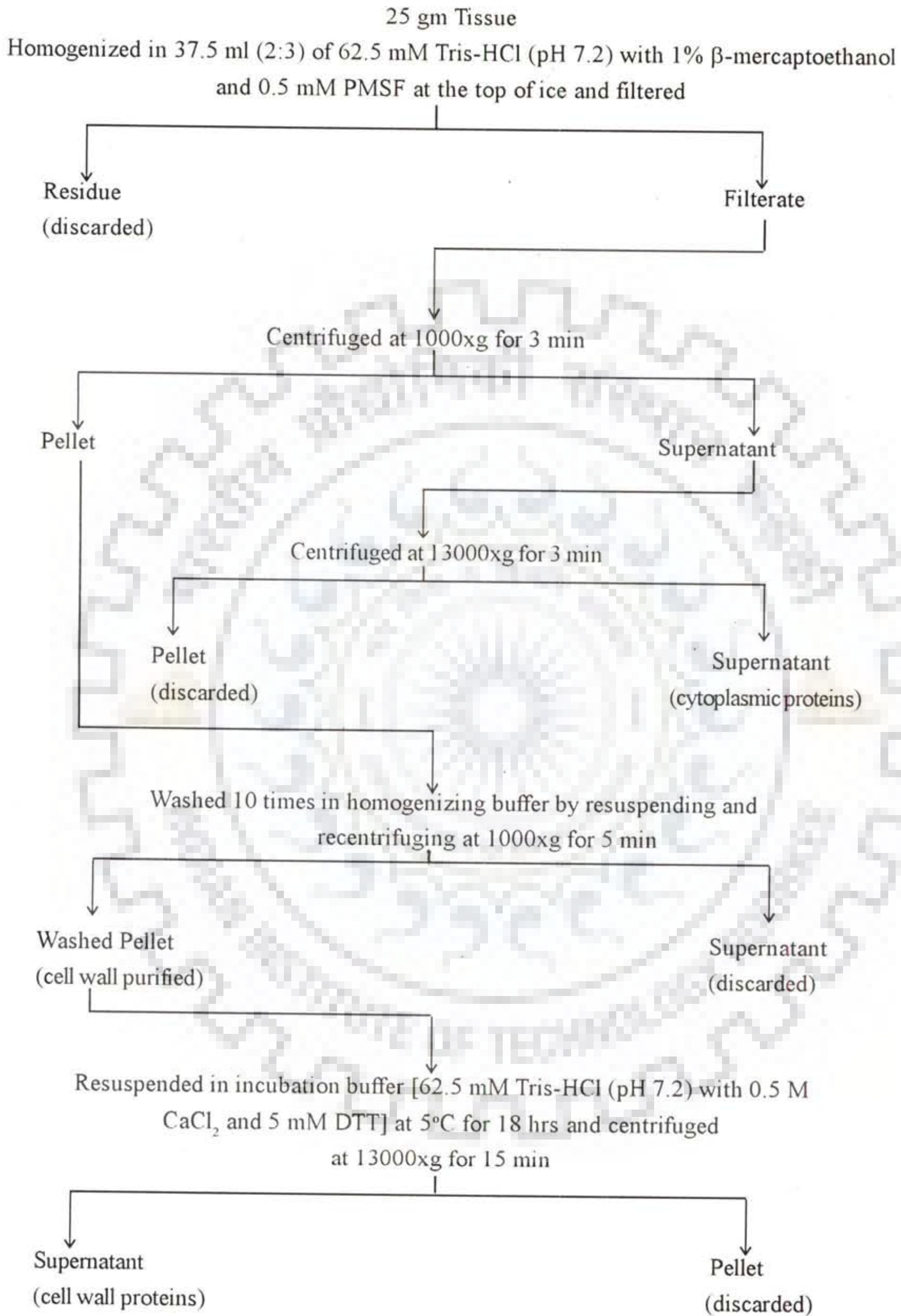


Fig. 3.3: Isolation of cytoplasmic and cell wall proteins

Procedure :

25 gm roots of 6 day old mungbean seedlings were ground in 37.5 ml homogenizing buffer (buffer A) at 4°C in a mortar and pestle. The ratio of tissue and homogenizing buffer was kept 2:3 in all the sample. Homogenate was filtered through four layers of cheese cloth and then centrifuged at 1000 x g for 3 min using RA-228 rotor in a cooling centrifuge (Kubota, 1300, Japan). The supernatant was decanted and recentrifuged at 13000 xg for 3 min. The supernatant constituted the cytoplasmic extract. The pellet from the 1000 x g centrifugation was rinsed 10 times by resuspending in fresh homogenizing buffer, recentrifuging and discarding the supernatant. The washed pellet was cell wall fraction. After the final rinse the pellet was suspended in 3.0 ml of buffer B and was incubated at 5°C for 18 hrs with occasional stirring. The suspension was centrifuged at 13000 x g for 15 min, and the supernatant (cell wall extract) was decanted from the cell wall pellet and used for further study.

3.2.7.2 Desalting and quantitative precipitation of cell wall protein

This was achieved by the rapid method of Wessel and Flugge (1984) based on a defined methanol-chloroform-water mixture for the quantitative precipitation of proteins from dilute solutions.

Procedure :

4 ml methanol was added to 1 ml of sample and the mixutre was vortexed and centrifuged at 9000 xg for 10s using an ependroff rotor in a cooling centrifuge (Kubota, 1300, Japan) for total collection of the protein from the sample. Then 1 ml chloroform was added and the samples were vortexed and centrifuged again at 9000 x g for 10s. A phase separation was achieved by the addition of 3 ml water and the samples were vortexed vigorously and centrifuged for 1 min at 9000 xg. The protein was precipitated at the chloroform-methanol-water interphase. The upper phase was carefully removed with the help of pasture pipette and discarded. The lower phase and the interphase with the precipitated protein were transfered

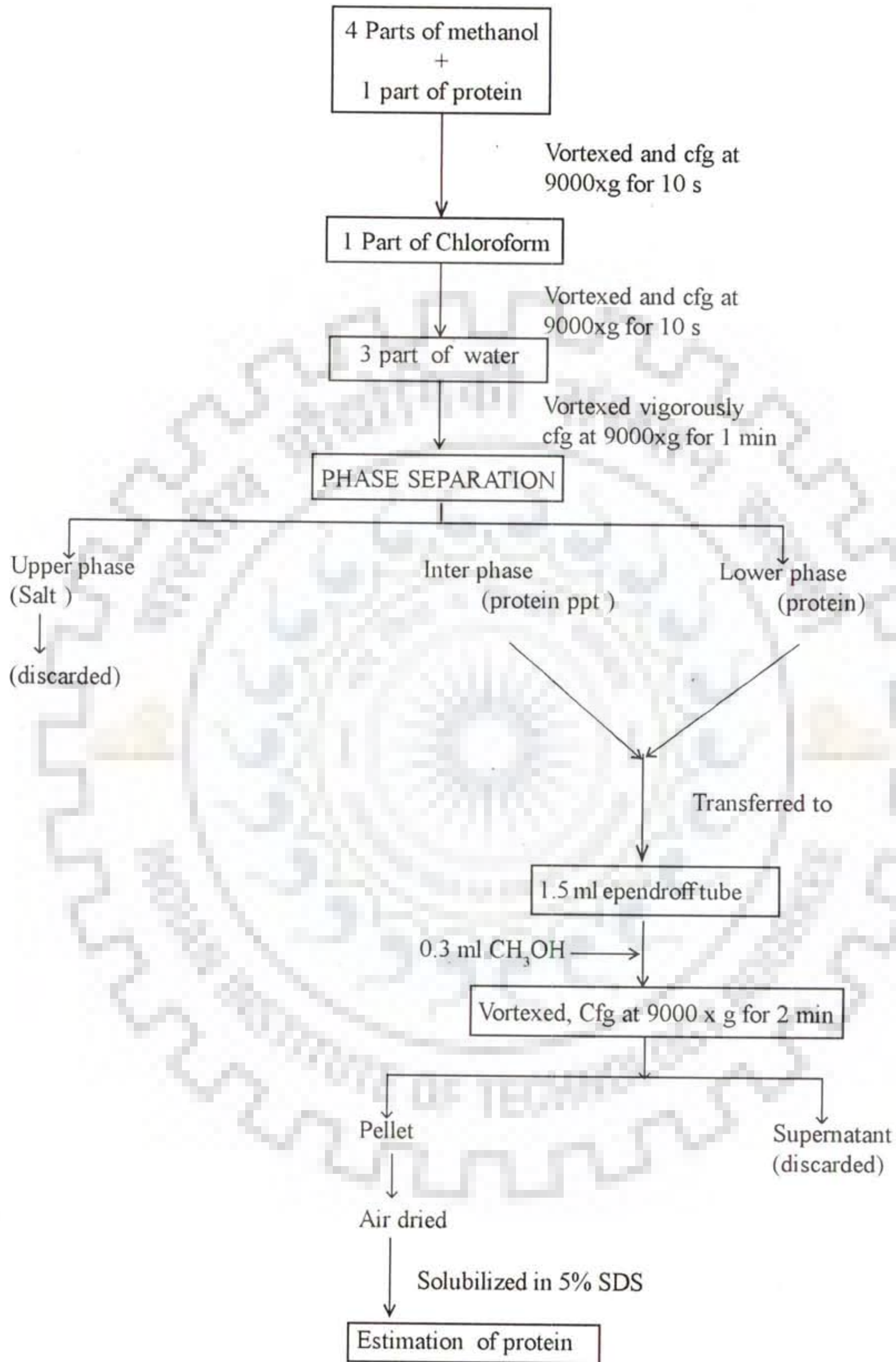


Fig. 3.4: Desalting and quantitative recovery of protein in dilute solution

to 1.5 ml ependroff tubes. A further 0.3 ml methanol was added and the samples were mixed and centrifuged again for 2 min at 9000 xg to pellet the protein. The supernatant was removed and the protein pellet was dried under a stream of air. The dried-pellet was stored frozen until use. Now the protein pellet was completely free of interfering substances like salt, β-mercaptoethanol, or detergents. The presence of the anionic detergent SDS upto 5% does not affect the recovery as well as quantitative and qualitative determination of the protein. The method has no adverse effect on molecular weight cutoffs and avoids heating or acid conditions for the precipitation of protein and is equally useful for the removal of protein from extracts which are to be analyzed for various substrates labile to extreme pH or heat.

For protein determination the protein pellets were solubilized by the addition of 0.4 ml of 5% SDS and assayed according to the Lowry procedure. For SDS gel electrophoresis the pellets can be dissolved directly in electrophoretic sample solublizing buffer containing 5% SDS.

3.2.7.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on the protein samples (cytoplasmic and cell wall proteins) according to Laemmli (1970) on 10% gels (16.0 x 18.0 x 0.3 cm) of 1.5 mm thickness with 15 wells using Hoeffer scientific electrophoresis unit. All reagents were prepared in double distilled water. In this technique, the separation of proteins occurs on the basis of molecular size.

3.2.7.3.1 Reagents

Stock solutions :

Solution A : 30% (w/v) Acrylamide solution containing 0.8% (w/v) bis (N,N-methyl ene-bisacrylamide).

Solution B : Resolving gel buffer - 3.0 M Tris HCl (pH 8.8)

Solution C : Stacking gel buffer - 0.5 M Tris HCl (pH 6.8)

Solution D : 10% (w/v) SDS

Solution E : 1.5% (w/v) freshly prepared ammonium per sulphate

Solution F : TEMED

Electrophoresis buffer : 0.25 M-Tris, 1.92 M-glycine, 1% SDS (pH 8.3)

3.2.7.3.2 Preparation of resolving and stacking gel

Stock solutions	Resolving gel (10%) (ml)	Stacking gel (ml)
Solution A	10.000	2.500
Soultion B	3.750	—
Solution C	—	5.000
Solution D	0.300	0.200
Solution E	1.500	1.000
Solution F	0.015	0.015
H_2O	14.450	11.300

3.2.7.3.3 Casting of gel

Gel was mounted in a sandwich by using (16.0 x 18.0 x 0.3 cm) glass plates. Two thin plastic spacers of 1.5 mm thickness were placed between the glass plates to form gel sandwich of uniform thickness. Plates were held together by plastic clamps. Base of gel mould was sealed by pressing the mould against a silicon rubber jacket in a casting stand. Resolving gel mixture was prepared by mixing all the components (except TEMED). This mixture was degassed for 1 min and correct volume of TEMED was added to it. Then it was gently mixed and poured between the plates leaving sufficient space at the top for a stacking gel to be polymerized later and sample wells to be formed. Now gel was overlayered with

240359

resolving gel buffer. After polymerization of the resolving gel the assembly was tilted to pour off the over layer. Then stacking gel mixture was over layered on resolving gel and immediately comb was inserted into the mixture to form the wells. After polymerization of stacking gel, comb was carefully removed to expose the sample wells. Wells were then rinsed with reservoir buffer and the sandwich was used for electrophoresis.

3.2.7.3.4 Sample preparation

Desalted and pelleted protein samples were dissolved in the sample solubilizing buffer [0.0625M-tris-HCl (pH 6.8) with 2% SDS (w/v), 10% (v/v) glycerol and 5% (v/v) β–mercaptoethanol]. Samples were then heated in a boiling water bath for 3 min. Molecular weight standards were also treated in the same manner. After heating, samples were allowed to cool at room temperature.

3.2.7.3.5 Electrophoresis

Before loading the samples, 10 µl of 0.002% (w/v) tracking dye bromophenol blue with 10% (v/v) glycerol was loaded into the wells. Then 100µl samples containing 80 µg protein were loaded in the wells on the gel surface using a micro pipette. Electrophoresis was then carried out at constant voltage. The values of constant voltage for stacking and resolution of the applied samples were 120 volts and 150 volts, respectively. Run was continued until the tracking dye reached close to the base (1 cm from bottom) of the gel. The direction of the run was from cathode to anode. After completion of the run, gel was removed from plates and stained by leaving it overnight in 0.1% coomassie brilliant blue R-250 in water : methanol : glacial acetic acid [5:5:2 (v/v/v)] at room temperature. Gel was destained by washing it in 12.5% isopropanol and 10% acetic acid. Destaining solution was renewed as stain leaches out of the gel over a period of 48 hours. Finally, gel was stored in 7% acetic acid and photographed.

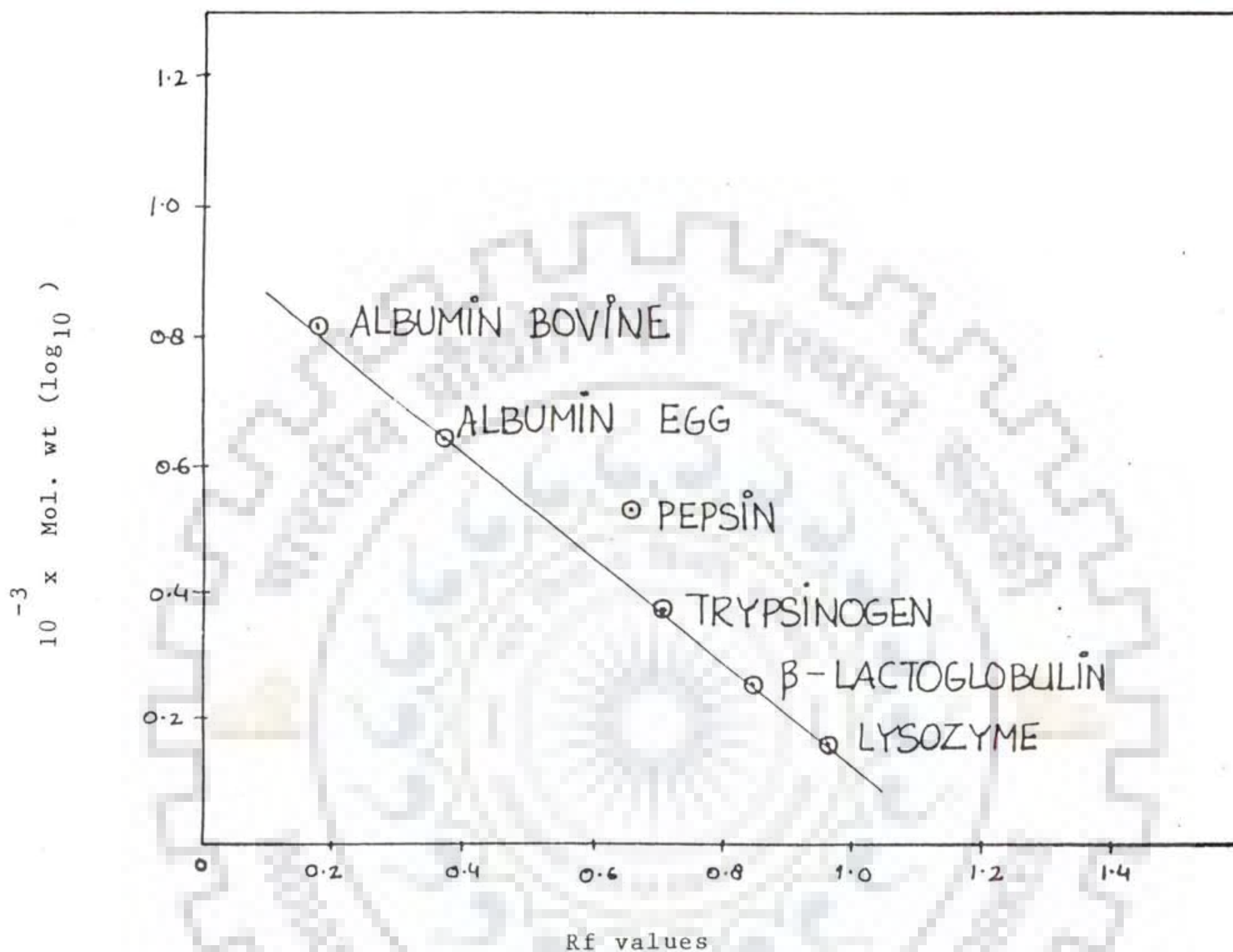


Fig. 3.5 Standard curve of marker protein, DALTON MARK VI on 10% SDS - PAGE.

3.2.8 Determination of molecular weights

Molecular weight of different protein bands were determined by constructing a plot of $\log_{10}$ polypeptide molecular mass versus relative mobility (Rf) (fig. 3.5). Relative mobilities of different bands were calculated by using the following formula :

$$\text{Relative mobility (RF)} = \frac{\text{distance travelled by protein bands}}{\text{distance travelled by tracking dye}}$$

3.2.9 Aminoacid analysis of induced cytoplasmic proteins

The coomassie protein - SDS complex extracted from the gel was dialyzed, lyopholized and subjected to aminoacid analysis after hydrolysis with constant boiling HCl according to the method of Sreekrishna et al. (1979).

3.2.9.1 Elution of protein bands from gel slices

The stained protein band was excised with a razor blade from the gel and washed with double distilled water. The coomassie - protein SDS complex present in the gel was eluted by homogenization of the gel in 2 ml of 0.05 M-NH_4HCO_3 containing 0.05% SDS followed by incubation at 37°C for 10 h on a shaking water bath. The gel suspension was centrifuged at 12000 xg for 15 min in a cooling centrifuge (Kubota, 1300, Japan) using RA-150 AM rotor. The clear blue supernatant solution containing the coomassie-protein-SDS complex was carefully separated from the pellet. The pellet was vortexed vigorously with another 1 ml of 0.05 M NH_4HCO_3 containing 0.05% SDS and recentrifuged. The supernatants were pooled and their protein content was quantified by the method of Lowry et. al. (1951).

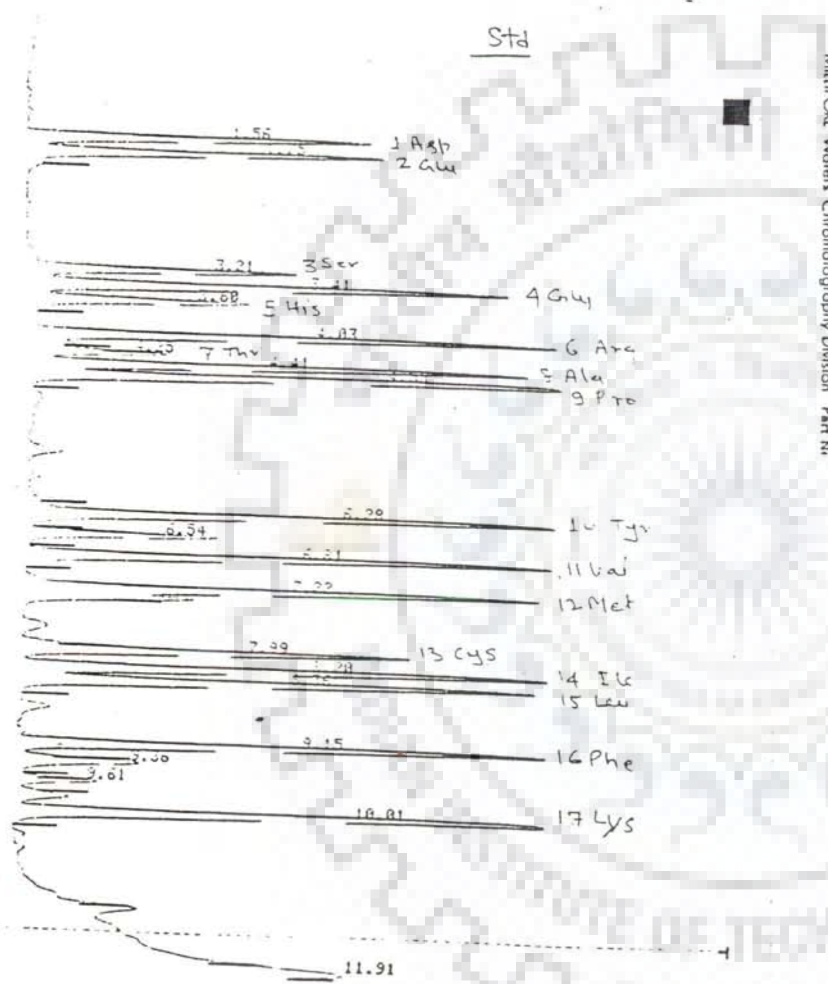
3.2.9.2 Dialysis and Lyophilization

The pooled supernatant solution (about 3 ml) was dialyzed extensively at 4°C against 0.005% SDS. The dialyzed material was lyophilized repeatedly to remove NH_4HCO_3.

	215.64500	9.26	215645 L	
	92.92400	9.48	92924 L	0.000000E0
	1142.36000	9.86	1142361 L	0.000000E0
TOTAL	13857.70000			0.000000E0

INJECT

MILLIPORE Waters Chromatography Division Part No.



JAN. 15, 1997 10:59:06 CHART 2.00 CM/MIN
COLUMN RUN #3
SOLVENT CALC #0
OPR ID 6

13	125.00000	7.99	711217 F	0.175700E0
14	250.00000	8.20	928226 F	0.269300E0
15	250.00000	8.36	894421 L	0.279500E0
16	250.00000	9.15	760340 L	0.328800E0
UNK		9.36	265849 L	
UNK		9.61	82274 L	
17	250.00000	10.01	1199087 L	0.208400E0
UNK		11.91	510600 HL	

MILLIPORE Waters Chromatography Division Part No.

PEAK#	RT	AMOUNT	RF
1 Asp	1.56	250.00000	0.389500E0
2 Glu	1.75	250.00000	0.376100E0
3 Ser	3.21	250.00000	0.526500E0
4 Gly	3.41	250.00000	0.277100E0
5 His	3.60	250.00000	0.592100E0
6 Arg	4.03	250.00000	0.315300E0
7 Thr	4.25	250.00000	0.175700E1
8 Ala	4.41	250.00000	0.331100E0
9 Pro	4.55	250.00000	0.228700E0
10 Tyr	6.28	250.00000	0.285900E0
11 Val	6.91	250.00000	0.263000E0
12 Met	7.22	250.00000	0.295600E0
13 Cys	7.99	125.00000	0.175700E0
14 Ile	8.20	250.00000	0.269300E0
15 Leu	8.36	250.00000	0.279500E0
16 Phe	9.15	250.00000	0.328800E0
17 Lys	10.01	250.00000	0.208400E0

Fig. 3.6 Densitometric scan of amino acid standards

037

3.2.9.3 Hydrolysis of proteins for analysis of aminoacid composition

Lyophilized dry samples were hydrolyzed in sealed evacuated tubes with 5.7 N-HCl for 24 h at 110°C.

3.2.9.4 Vacuum drying

Sealed evacuated tubes containing hydrolyzed samples were broken and samples were collected in 25 ml beakers. These samples were then vacuum dried. Dried samples were redissolved in 62.5 mM Tris-HCl (pH 7.2) and used for the analysis of aminoacid composition.

3.2.9.5 Aminoacid analysis

Aminoacid analysis was carried out by loading the samples into C_{18} column of Waters 440 HPLC aminoacid analyzer. Aminoacid composition of samples were determined through the standards in absorbance spectrophotometer.

3.2.10 Other methods

3.2.10.1 Protein estimation

Protein estimation was done according to the method of Lowry et. al. (1951). The final colour developed is a result of :

1) Biuret reaction of protein with copper ion in alkali.
2) Reduction of the phosphotungstic reagent phosphomolybdic reagent with tyrosine and tryptophan present in the treated protein.

3.2.10.1.1 Reagents

Reagent A : 2% Na_2CO_3 in 0.1 N-NaOH

Reagent B : 0.5% $CuSO_4$ in 1% Na-K-tartrate
(prepared fresh just befor use)

Reagent C : 50 ml of reagent A mixed with 1 ml of reagent B.

Reagent D : 1 N Folin and Ciocalteu's phenol reagent.

Reagent E : 1 mg/ml BSA (Bovine serum albumin).

3.2.10.1.2 Procedure

0.1 ml of protein samples were taken into test tubes. Reagent E was diluted to make 200 μg/ml BSA stock. Standards of 20-100 μg/ml were prepared from 200 μg/ml BSA stock by taking different aliquots. Then 0.1 N-NaOH was added to standards and samples to make upto the volume 1.5 ml. Blank was also prepared by taking 1.5 ml 0.1 N-NaOH. 1.5 ml of reagent C was added to all the tubes and vortexed immediately. Then, reaction mixture was incubated for 15 min at room temperature. Subsequently 0.15 ml of reagent D was added in all test tubes and vortexed immediately. Mixture was then incubated at room temperature in the dark for 45 min. Absorbance of the samples was recorded at 690nm. Protein content was calculated by plotting a standard curve.

3.2.10.2 Statistical analysis

The data were subjected to statistical analysis and expressed as Mean ± S.E.M. (standard error of mean). The S.E.M. was calculated by the following formula.

$$S.E.M. = \frac{\sqrt{\varepsilon x^2 - \frac{(\varepsilon x)^2}{n}}}{n(n-1)}$$

where x = individual observations

n = number of observations

Students `t' test was used to calculate the degree of significance by the formula given below:

$$t = \frac{x_1 - x_2}{S.\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

Where $S = \sqrt{\dfrac{\varepsilon x_1^2 - \dfrac{(\varepsilon x_1)^2}{n_1} + \varepsilon x_2^2 - \dfrac{(\varepsilon x_2)^2}{n_2}}{n_1 + n_2 - 2}}$

n_1 and n_2 denote the number of obsrvations in the two classes being compared (Ostle, 1954). Based on the degree of freedom (n_1+n_2-2), value of probability was obtained from the standard table given by Fischer andYates (1948). If the calculated value was less than the table value it is significant at that probability level.

CHAPTER - 4

4.0 RESULTS

4.1 Soil analysis

4.1.1 Zinc in soil profiles

The physico-chemical properties of the soil profiles in agricultural fields in and around two cities of western Uttar Pradesh are presented in Table 4.1 and the mean concentrations of phytoavailable and total zinc are shown in Table 4.2.

In surface soil samples (0-15 cm) of Roorkee and Muzaffarnagar regions, total zinc content varied from 3.75 to 94.74 and 12.88 to 52.25 with the mean values of 40.30 and 30.90 ppm, respectively. However, the concentration of DTPA-extractable (Phytoavailable) Zn in surface soil around the same town varied from 0.198 to 1.20 and 0.207 to 0.614 with the mean values of 0.396 and 0.328 ppm, respectively.

Out of the 16 representative sampled soils around each city (Roorkee and Muzaffarnagar), more than 18% and 37% respectively contained less than critical limit of total zinc content (25-250 ppm) and 87% and 93% respectively contained less then 0.5 ppm DTPA-extractable Zn, which is widely considered to be in the range of the critical deficiency concentration of Zn for plants grown in calcareous soils (Cakmak et al., 1996a).

The pattern of total zinc accumulation with the depth of soil was nearly same. However, in Doulatpur (north-east of Roorkee) and south-west of Muzaffarnagar, zinc levels increased with the depth of soil and was found to be higher at 30-45 cm depth than the top soil.On the other hand, in all soil samples without exception the available zinc content decreased with the depth of the soil profile.

Statistical analysis (Coefficient of variation) of the sampled soils indicate that Muzaffarnagar region has more variation than Roorkee region with respect to total Zn content while the reverse is the case for available Zn content (Table 4.2).

TABLE 4.1

PROPERTIES OF SOIL SAMPLES FROM IN AND AROUND ROORKEE - MUZAFFARNAGAR REGIONS OF WESTERN UTTAR PRADESH, INDIA.

Profile location (Village)	Profile depth (cm)	Textural class	Soil pH (1:2.5)		Zinc content (ppm)			
					Total		Available	
			Range	Mean	Range	Mean	Range	Mean
ROORKEE REGION								
	0-15		6.80-7.20	6.95	35.88-43.63	40.50	0.204-0.502	0.328
JATBAHADARPUR	15-30	Sandy	6.68-7.18	6.90	30.13-50.63	37.85	0.200-0.410	0.295
	30-45	loam	6.85-7.30	7.08	28.25-62.88	46.69	0.189-0.300	0.223
	0-15		7.35-8.15	7.67	3.75-60.25	27.97	0.198-0.380	0.274
KROUNDI	15-30	Sandy	7.54-8.45	7.83	7.38-55.75	28.00	0.115-0.350	0.233
	30-45	loam	7.50-8.68	7.95	5.50-44.75	30.47	0.100-0.330	0.225
	0-15		7.00-7.65	7.36	18.38-62.13	38.53	0.383-1.200	0.602
DOULATPUR	15-30	Silty	7.40-7.60	7.51	17.13-54.50	38.94	0.348-0.978	0.549
	30-45	loam	7.45-7.65	7.58	36.75-62.75	51.31	0.325-0.623	0.418
	0-15		6.81-7.85	7.41	25.75-94.74	54.19	0.345-0.420	0.380
LIBBURHERI	15-30	Loam	7.20-8.85	7.71	19.00-60.75	44.25	0.210-0.280	0.246
	30-45		6.70-8.95	7.51	17.00-64.00	43.32	0.200-0.287	0.240

MUZAFFARNAGAR REGION							
	0-15	7.35-8.05	7.84	22.00-32.63	26.88	0.236-0.416	0.328
JATMUZHERA	15-30 Sandy	7.54-8.25	7.97	15.25-39.38	25.85	0.200-0.400	0.299
	30-45 loam	7.98-8.37	8.23	13.50-41.38	24.25	0.131-0.375	0.209
	0-15	8.25-8.60	8.36	12.88-43-00	23.63	0.209-0.322	0.247
JAGAHERI	15-30 Loam	8.37-8.68	8.53	17.00-54.88	33.57	0.188-0.221	0.207
	30-45	8.45-8.79	8.66	16.13-43.25	30.04	0.131-0.197	0.162
	0-15	8.40-8.73	8.62	46.75-52.25	49.60	0.207-0.38	0.284
BADHAY KHURD	15-30 Loam	8.52-8.79	8.71	45.88-62.38	57.47	0.203-0.303	0.250
	30-45	8.45-9.00	8.79	36.50-58.13	44.81	0.131-0.286	0.216
	0-15	7.65-8.43	8.19	17.00-28.25	23.50	0.351-0.614	0.454
BHAINSI	15-30 Loam	7.50-8.42	8.20	14.50-38.75	25.31	0.309-0.430	0.359
	30-45	7.53-8.55	8.26	11.25-52.50	27.94	0.287-0.400	0.327

Upper cropland low organic alluvial soils of dry subhumid agroclimatic zones of Indo-Gangetic plains lying at an elevation of 260 m above sea level were surveyed for their zinc content. Four villages in 10 km radius representing four main directions around each city were chosen. Four samples were collected from within the boundary of each village. Each samples comprised of soil collected from 5 pits. The soil collected at the same depth from each of these five pits were mixed and subjected to analysis.

TABLE 4.2

MEAN CONCENTRATION OF ZINC (ppm = mg/kg) IN SOILS SAMPLED FROM IN AND AROUND ROORKEE - MUZAFFARNAGAR REGIONS OF WESTERN UTTAR PRADESH, INDIA

Sampling location (Town)	No. of samples	No. of Profiles	Profile depth	Zinc content							
				Total				Available			
				Range	Mean	SD[a]	CV[b]	Range	Mean	SD[a]	CV[b]
ROORKEE	16	80	0-15	27.97-54.19	40.30	9.33	23.15	0.274-0.602	0.396	0.125	31.57
			15-30	28.50-4425	37.39	5.67	15.16	0.233-0.549	0.331	0.128	38.67
			30-45	30.47-5131	42.92	7.74	18.03	0.223-0.418	0.277	0.082	29.60
MUZAFFAR-NAGAR	16	80	0-15	23.50-49.60	30.90	10.18	35.19	0.247-0.454	0.328	0.078	23.78
			15-30	25.31-57.47	35.55	13.10	36.85	0.207-0.359	0.279	0.057	20.43
			30-45	24.25-44.81	31.76	7.81	24.59	0.162-0.327	0.229	0.061	26.64

SD[a] = Standard deviation
CV[b] = Coefficient of variation

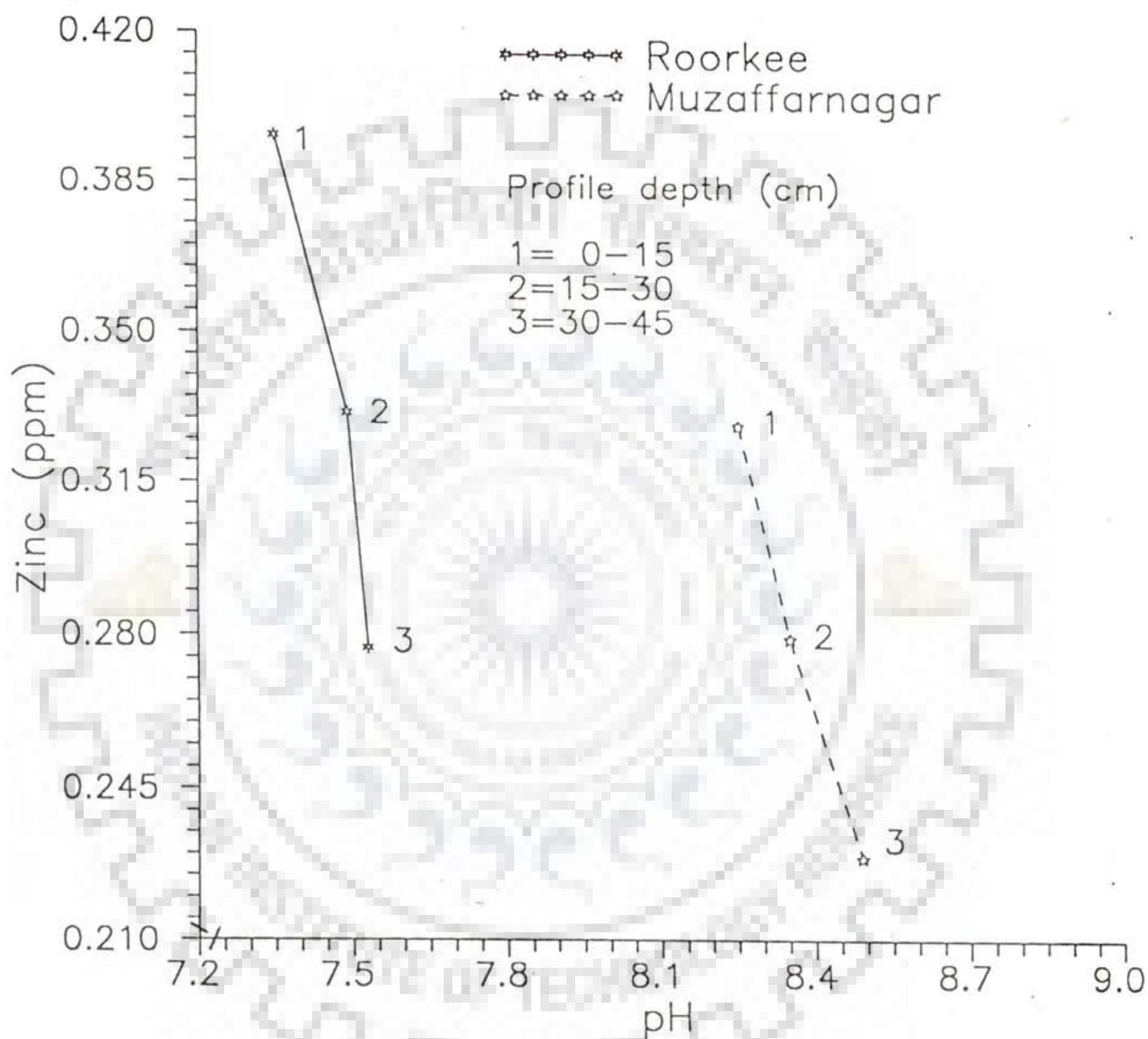


Fig.4.1 Relation between DTPA-Extractable Zn^{++} concentration in the soil solution and pH at different depth of soil profile around Roorkee -Muzaffarnagar regions of western Uttar Pradesh, India

4.1.2 pH VS Phytoavailable zinc

Soils sampled around Roorkee and Muzaffarnagar regions had high pH values ranging between 7.00 to 8.15 and 7.35 to 8.73 with a mean of 7.35 and 8.25, respectively. In all samples, the pH was found to increase with the depth of soil profile. Fig. 4.1 shows the negative correlation between pH and available zinc content of the sampled soils. With the depth of soil, pH increases and available zinc content decreases. Around both towns a one percent decrease per cm depth of soil was recorded in available zinc content. Further, the available zinc content declined by 1/10 for each unit increase in pH.

4.2 Zinc phytosensitivity

4.2.1 Zinc deficiency symptoms and plant response to Zn application in field

According to field observations in Roorkee and Muzaffarnagar regions, typical symptoms of Zn deficiency were: inhibition in plant height and development of whitish-brown patches on leaf blades, predominantly in the adult and older leaves. With the severity of Zn deficiency whitish-brown lesions spread on the leaves, and they appeared burnt. The existence of zinc deficiency can be clearly seen in wheat, a crop that is grown extensively in this region (Fig. 4.2).

Plants were found to be highly responsive to Zn, since application of zinc to soil or leaves of Zn deficient plants resulted in very rapid growth and regreening within 2-3 weeks.

4.2.2 Crop susceptibility to zinc phytotoxicity

Around the industrial area in Roorkee and Muzaffarnagar regions soils were polluted due to toxic effluents and showed zinc phytotoxicity. Crops varied widely in their susceptibility to excess soil zinc. Most grasses were more tolerant than most dicots. Among dicots, the leafy vegetable crops and the beet family were particularly sensitive as were many legumes.

Leaf chlorosis, stunting, purple coloration of the main stem and petioles, stem splitting, root inhibition and premature necrosis were the main symptoms.

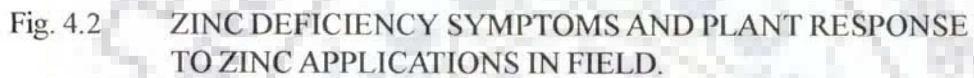


Fig. 4.2 ZINC DEFICIENCY SYMPTOMS AND PLANT RESPONSE TO ZINC APPLICATIONS IN FIELD.

Marked differences are seen in the growth and development of wheat in (A) Zn sufficient and (B) Zn deficient areas. The response of plants grown in Zn deficient areas C-1 to 15 Kg./hectare Zn sufficient C-2 are compared.

A

B

C1
C2

Fig. 4.3 EFFECT OF ZINC ON MUNGBEAN SEEDLINGS

Seeds were grown hydroponically in nutrient medium in the presence of 0-100 ppm concentration of Zn for 6 days. A control grown in double distilled water alone was also included.

Zinc treatment (ppm.)
Left to right DDW, 0.0, 0.4, 1.0, 10, 15, 20, 25, 50, 75 and 100.

Top panel - shows germination response
Middle panel - shows root growth response
Bottom panel - shows growth pattern

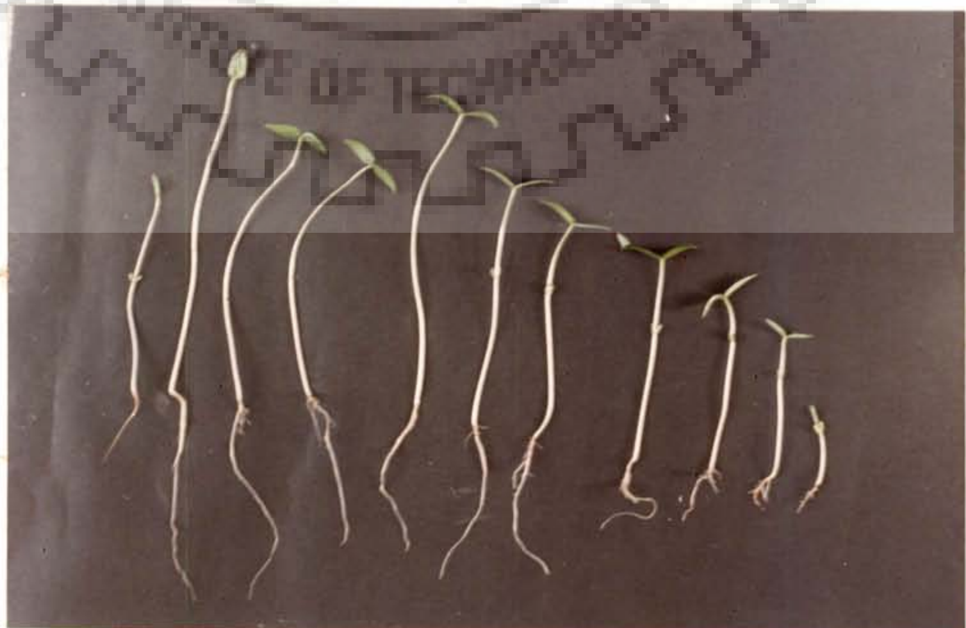


Fig. 4.4 OPTIMUM ZINC REQUIREMENT FOR MUNGBEAN SEEDLINGS

Seeds of mungbean were grown hydroponically in Hoagland's nutrient medium for 6 days with presence (C) and absence of Zn (B). Controls grown in double distilled water alone (A) was also included. Seedlings were found to grow best in Hoagland's medium with 0.4 ppm (Left to right - DDW, 0.0 and 0.4 ppm Zn).

A
B
C

4.2.3 Zinc-sensitivity of mungbean crop

Fig. 4.3 shows that mungbean seedlings could tolerate zinc concentrations only upto100 ppm. On the basis of morphological, anatomical and biochemical parameters as given in subsequent pages it is observed that optimum zinc requirement for the *Vigna radiata* cv. K-851 is 0.4 ppm, which confirms the highly zinc sensitive nature of this crop (Fig. 4.4).

4.3 Growth response at different zinc concentrations

Fig 4.3 shows the effect of zinc stress on the overall growth of mungbean seedlings after 6 days of treatment. The growth response of seedlings was found to be substantially higher in plants grown in Hoagland's nutrient medium than those grown in DDW even in the absence of zinc. Inclusion of optimal levels of zinc in the nutrient medium were able to sustain this growth. However, exposure to excess zinc produced marked reduction in growth of mungbean seedlings. Growth parameters i.e. fresh weight, dry weight and length of seedling decreased with high zinc concentrations. In comparison to the length and fresh weight, excess zinc caused small change in dry weight of the seedlings (Table 4.3).

Effect of zinc stress on fresh weight, dry weight and length of individual organs of 6 d treated mungbean seedlings was recorded and results are described below :

4.3.1 Leaf

Table 4.4 shows an increase in all three growth parameters of leaves of 6 d old mungbean seedlings only at 0.4 ppm of zinc which appears to be optimal. At all other concentrations upto 75 ppm Zn there was no substantial change. A reduction in the fresh and dry weights and length of leaves was observed at 100 ppm Zn implying that Zn becomes toxic at this concentration.

4.3.2 Epicotyl

The growth of the epicotyl was better in seedlings grown in nutrient medium than on distilled water alone (Table 4.5). The inclusion of small amounts of zinc in the nutrient medium

TABLE 4.3

EFFECT OF DIFFERENT CONCENTRATIONS OF ZINC ON THE GROWTH OF WHOLE MUNGBEAN SEEDLINGS SUBJECTED TO 6 DAYS OF TREATMENT

Concentration of zinc (ppm) in nutrient media	Changes in growth of whole mungbean seedlings		
	Fresh weight (mg /plant)	Dry weight (mg /plant)	Length (cm)
DDW	172.40±21.5*	20.40±4.10	18.78±1.76*
0.0	462.00±17.0	24.60±4.10	35.25±3.99
0.4	470.00±17.0	25.00±4.10	36.81±4.18
1.0	365.40±21.5*	21.80±8.20	35.66±3.89
5.0	352.40±23.3*	22.40±8.20	33.54±3.46
10	390.40±27.7*	24.60±4.50	35.80±3.96
15	389.60±21.5*	24.60±5.70	35.59±2.80
25	318.40±23.3*	21.40±4.10	29.48±4.11
50	265.00±27.7*	21.40±4.10	26.22±2.66*
75	240.40±32.5*	20.20±5.70	18.58±3.68*
100	158.00±37.4*	19.40±8.20	9.02 ±2.52*

Mungbean seeds were grown hydroponically on nutrient media with varying concentrations of zinc. About 20 seedlings were chosen randomly for measurements. Results are mean ± SE of 5 experiments. All comparisons have been made with seedlings grown in Hoagland's nutrient media in the absence of zinc (* significant at 1% level - Students `t' test).

TABLE 4.4

EFFECT OF DIFFERENT CONCENTRATIONS OF ZINC ON THE GROWTH OF LEAF IN MUNGBEAN SEEDLINGS AFTER 6 DAYS OF TREATMENT

Concentration of zinc (ppm) in nutrient media	Changes in growth of mungbean leaf		
	Fresh weight (mg /organ)	Dry weight (mg /plant)	Length (cm)
DDW	11.0±2.0*	4.00±0.20*	1.83±0.07*
0.0	23.0±2.0	5.40±0.20	2.65±0.15
0.4	28.4±2.0	6.60±0.10*	3.02±0.18*
1.0	23.3±1.0	5.60±0.19	2.51±0.23
5.0	24.5±1.0	5.80±0.10	2.70±0.26
10	24.5±1.0	6.00±0.20	2.60±0.22
15	21.9±2.0	5.60±0.18	2.64±0.12
25	23.0±2.0	5.20±0.20	2.60±0.23
50	17.7±1.0*	5.10±0.10	2.32±0.08
75	18.7±2.0*	5.00±0.10	2.14±0.27*
100	8.9±2.0*	3.20±0.20*	1.60±0.26*

Mungbean seeds were grown hydroponically on nutrient media with varying concentrations of zinc. About 20 seedlings were chosen randomly for measurements and leaves were separated from them. Results are mean ± SE of 5 experiments. All comparisons have been made with seedlings grown in Hoagland's nutrient media in the absence of zinc (* significant at 1% level - Students 't' test).

TABLE 4.5

EFFECT OF DIFFERENT CONCENTRATIONS OF ZINC ON THE GROWTH OF EPICOTYL IN MUNGBEAN SEEDLINGS AFTER 6 DAYS OF TREATMENT

Concentration of zinc (ppm) in nutrient media	Changes in growth of mungbean epicotyl		
	Fresh weight (mg /organ)	Dry weight (mg /plant)	Length (cm)
DDW	35.40±1.23*	3.80±0.26*	5.20±0.30*
0.0	105.20±5.00	4.80±0.13	9.35±1.85
0.4	90.00±9.00	4.40±0.13	9.45±1.45
1.0	97.40±5.00	4.60±0.19	10.35±0.65
5.0	99.80±2.25	5.00±0.15	9.15±1.45
10.0	106.40±2.46	5.00±0.15	9.65±1.35
15.0	117.60±2.46	5.60±0.16	11.20±0.68
25.0	74.80±2.13*	3.40±0.20*	8.08±1.28
50.0	57.80±4.45*	3.80±0.25*	7.40±1.24
75.0	62.40±1.23*	4.00±0.27*	6.16±1.70
100.0	11.0±3.33*	1.20±0.13*	1.50±0.54*

Mungbean seeds were grown hydroponically on nutrient media with varying concentrations of zinc. After six days of treatment about 20 seedlings were chosen randomly from each group for measurements and epicotyl were separated from them. Results are mean ± S.E. of 5 experiments. All comparisons have been made with seedlings grown in Hoagland's nutrient media in the absence of zinc (*significant at 1% level - Students `t' test).

TABLE 4.6

EFFECT OF DIFFERENT CONCENTRATIONS OF ZINC ON THE GROWTH OF COTYLEDON IN MUNGBEAN SEEDLINGS AFTER 6 DAYS OF TREATMENT

Concentration of zinc (ppm) in nutrient media	Changes in growth of mungbean cotyledon		
	Fresh weight (mg /organ)	Dry weight (mg /plant)	Width (cm)
DDW	9.8±1.5*	4.2±1.0*	0.215±0.005*
0.0	2.2±1.0	1.2±0.5	0.125±0.025
0.4	9.8±1.5*	1.6±0.3	0.120±0.030
1.0	8.7±1.5*	2.6±0.5*	0.155±0.005
5.0	4.3±1.0	1.2±0.3	0.165±0.005*
10.0	3.4±0.5	1.2±0.3	0.170±0.030*
15.0	8.0±1.5*	2.0±0.5	0.200±0.020*
25.0	5.6±0.5*	1.8±0.3	0.200±0.010*
50.0	10.9±1.5*	3.4±1.0*	0.200±0.000*
75.0	11.1±1.0*	4.0±1.2*	0.200±0.000*
100	18.4±1.5*	8.6±1.5*	0.270±0.080*

Mungbean seeds were grown hydroponically on nutrient media with varying concentrations of zinc. After six days of treatment 20 seedlings were chosen randomly from each group for measurements and cotyledons were separated from them. Results are mean ± SE of 5 experiments.All comparisons have been made with seedlings grown in Hoagland's nutrient media in the absence of zinc (*significant at1% level - Students `t' test).

TABLE 4.7

EFFECT OF DIFFERENT CONCENTRATIONS OF ZINC ON THE GROWTH OF HYPOCOTYL IN MUNGBEAN SEEDLINGS AFTER 6 DAYS OF TREATMENT

Concentration of zinc (ppm) in nutrient media	Changes in growth of mungbean hypocotyl		
	Fresh weight (mg /organ)	Dry weight (mg /plant)	Length (cm)
DDW	74.20±10.00*	4.80±1.30	6.65±0.05*
0.0	138.20±17.00	6.80±1.30	9.45±0.55
0.4	140.00±21.00	7.00±1.60	9.54±0.41
1.0	145.60±17.00	8.60±1.60	9.80±0.20
5.0	143.60±19.00	7.80±1.90	9.64±0.24
10.0	143.40±19.00	7.40±1.90	9.50±0.10
15.0	155.00±21.00	7.60±1.30	9.12±0.62
25.0	130.40±16.00	7.20±1.30	8.20±0.75
50.0	117.00±11.00	7.40±1.70	8.00±0.63
75.0	114.60±11.00	8.40±1.90	6.98±1.31
100	75.40±9.00*	9.00±1.90	4.30±1.24*

Mungbean seeds were grown hydroponically on nutrient media with varying concentrations of zinc. After six days of treatment 20 seedlings were chosen randomly from each group for measurements and hypocotyls were separated from them. Results are mean ± SE of 5 experiments. All comparisons have been made with seedlings grown in Hoagland's nutrient media in the absence of zinc (* significant at 1% level - Students `t' test).

TABLE 4.8

EFFECT OF DIFFERENT CONCENTRATIONS OF ZINC ON THE GROWTH OF ROOT IN MUNGBEAN SEEDLINGS AFTER 6 DAYS OF TREATMENT

Concentration of zinc (ppm) in nutrient media	Changes in growth of mungbean root		
	Fresh weight (mg /organ)	Dry weight (mg /plant)	Length (cm)
DDW	28.20±3.5*	1.00±0.13*	5.10±0.40*
0.0	57.60±3.5	2.20±0.13	13.80±0.20
0.4	83.40±4.5*	3.60±0.16*	14.80±0.51
1.0	64.00±3.6	2.2±0.19	13.00±0.60
5.0	55.20±3.5	2.6±0.16	12.05±0.65
10.0	71.20±3.6	2.80±0.13*	13.85±0.35
15.0	85.60±3.6*	3.40±0.19*	12.63±1.38
25.0	65.00±2.2	2.60±0.19	10.60±1.85*
50.0	42.30±2.8*	2.40±0.20	8.50±0.71*
75.0	34.60±2.0*	2.00±0.17	3.30±0.40*
100	10.00±2.0*	1.80±0.15*	1.62±0.48*

Mungbean seeds were grown hydroponically on nutrient media with varying concentrations of zinc. After six days of treatment 20 seedlings were chosen randomly from each group for measurements and roots were separated from them. Results are mean ± SE of 6 experiments. All comparisons have been made with seedlings grown in Hoagland's nutrient media in the absence of zinc (* significant at 1% level - Students 't' test).

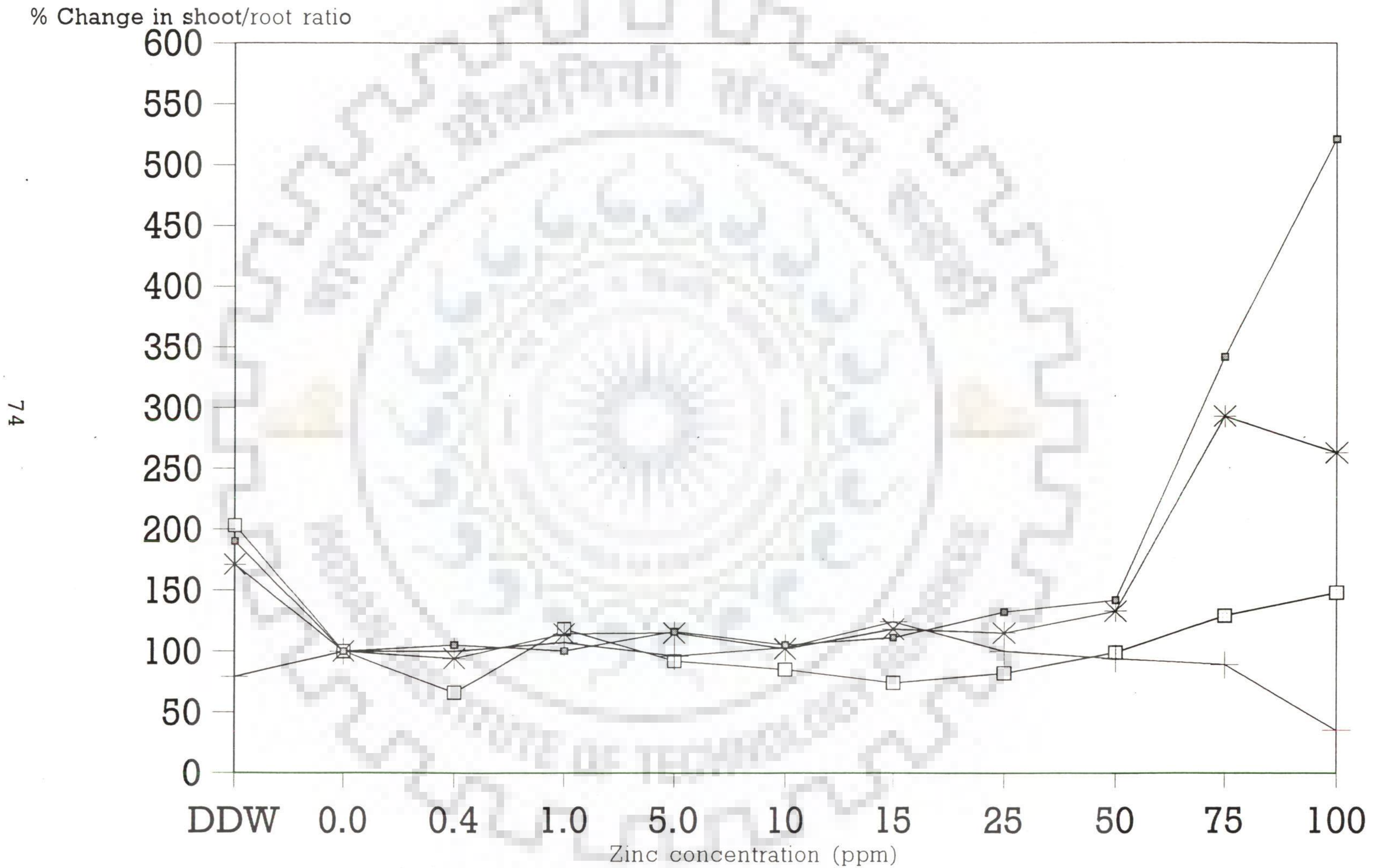


Fig. 4.5 % Changes in shoot/root ratio of mungbean seedling grown at different zinc concentrations

did not affect the epicotyl. Higher than 15 ppm Zn progressively inhibited the growth parameters. At 100 ppm Zn, the inhibition was maximum.

4.3.3 Cotyledon

Effect of zinc is evident visually in cotyledons of 6 d old mungbean seedlings. Table 4.6 shows that fresh and dry weight of cotyledon was higher in 0.4 ppm Zn treated plants than in controls grown in the same medium but in the absence of zinc. Increasing the concentration of zinc to 10 ppm did not favour cotyledon growth. However, toxic levels of zinc lead to further increase in cotyledon growth. Width of cotyledon reduced to nearly half in control over distilled water grown seedling then increased with zinc treatment, remained constant between 15 to 75 ppm and reached a maximum at 100 ppm zinc regime.

4.3.4 Hypocotyl

Table 4.7 shows the changes in growth parameters of hypocotyl from mungbean seedlings subjected to 6 days of zinc stress. No significant changes were recorded in the hypocotyl of Zn treated mungbean seedling upto 75 ppm Zn. However, at 100 ppm Zn concentration fresh weight and hypocotyl length were substantially inhibited while dry weight was not altered.

4.3.5 Root

Table 4.8 shows the growth response of root under zinc stress. Fresh and dry weight of root increased at 10 and 15 ppm zinc levels and thereafter represented an inverse relation with increase in zinc treatment.

4.3.6 Shoot / root ratio

Fig. 4.5 shows the changes in (leaf/root, epicotyl/hypocotyl, shoot/root) lengths and (shoot/root) dry matter ratios. It is clear from the data that plants grown in distilled water alone have a disturbed shoot/root ratios. However, seedlings grown in Hoagland's nutrient medium with zinc concentrations upto 50 ppm are able to maintain a constant shoot/root ratio. Beyond this concentration of zinc, the plants are unable to regulate/maintain this relationship.

TABLE 4.9

EFFECT OF ZINC ON THE DEVELOPMENT ON RADICLE AND SECONDARY ROOTS IN MUNGBEAN SEEDLINGS :

Age of seedling (days)	Length of radicle (mm)					Development of secondary roots (number)				
	Control		Zinc treated (ppm)			Control		Zinc treated (ppm)		
	DOW	0.0	0.4	10	100	DOW	0.0	0.4	10	100
1.	12.50 ±0.32	10.93 ±0.75	12.23 ±0.30	15.00 ±0.32	9.98 ±0.34	none	none	none	none	none[a]
2.	41.50 ±5.72	46.50 ±4.50	45.25 ±4.87	43.50 ±5.27	10.90 ±1.22	5.40 ±1.50	3.65 ±0.48	2.85 ±0.57	2.45 ±0.50	none
3.	60.75 ±4.27	51.00 ±3.39	48.50 ±2.78	59.50 ±5.22	—a	11.30 ±2.33	8.85 ±1.59	9.55 ± 0.50	11.35 ±2.01	none
4.	68.50 ±4.50	65.00 ±3.87	56.25 ±4.15	72.50 ±2.50	—a	—b	16.20 ±2.34	16.80 ±1.47	18.40 ±1.02	none
5.	77.00 ±2.45	82.50 ±4.87	96.25 ±6.10	92.75 ±5.36	—a	—b	—b	33.25 ±2.01	—b	2.5[d]
6.	—a	130.50 ±4.72	142.75 ±5.59	133.75 ±4.15	—a	—b	—b	—c	—c	3.55[d]

a = degeneration starts from root tip
b = elongation and reticulum formation takes place by secondary roots without affecting their number.
c = secondary roots split into tertiary roots.
d = emergence of adventitious roots from hypocotyl.

Mungbean seedlings were grown in water culture medium (Hoagland's solution) containing varying zinc levels for periods upto 6 days at 28°C. Twenty seedlings were randomly picked up from each group to monitor the stage of development and length of radicle and number of secondary roots. Two controls were used (one grown on double distilled water alone and the other in Hoagland's solution without zinc). Results are mean ±SE of three different experiments carried out under identical conditions.

4.4 Developmental response of radicle and secondary roots at different concentrations of zinc

Table 4.9 shows the effect of zinc on different stages of development, length of radicle and number of secondary roots. In both controls length of radicle was found to increase progressively with the advancing stage of the seedlings. While degeneration of root tip commenced on 6th day in the controls grown in DDW, the length of radicle continued to increase in controls grown in Hoagland's media in the absence of zinc. Zinc treated seedlings showed normal growth of radicle at 0.4 and 10 ppm zinc concentration. But seedlings grown on 100 ppm zinc concentration showed degeneration in radicle from the third day onwards.

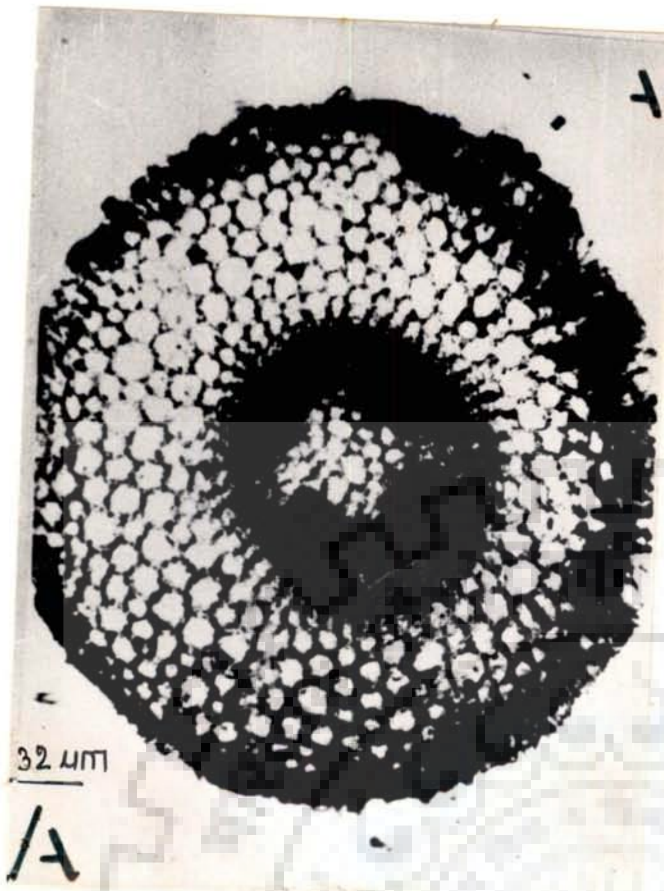
Observation on the development of secondary roots was also unique. While the elongation and reticulum formation resulting in secondary roots started on the 4th day in DDW control, the same was found to occur on 5th day in controls grown on Hoagland's solution without zinc. Zinc treatment of 0.4 and 10 ppm caused secondary roots to split into tertiary roots on the 6th day. Concentration of 100 ppm Zn inhibited the development of secondary roots upto four days and adventitious roots began to emerge from the hypocotyl from 5th day onwards.

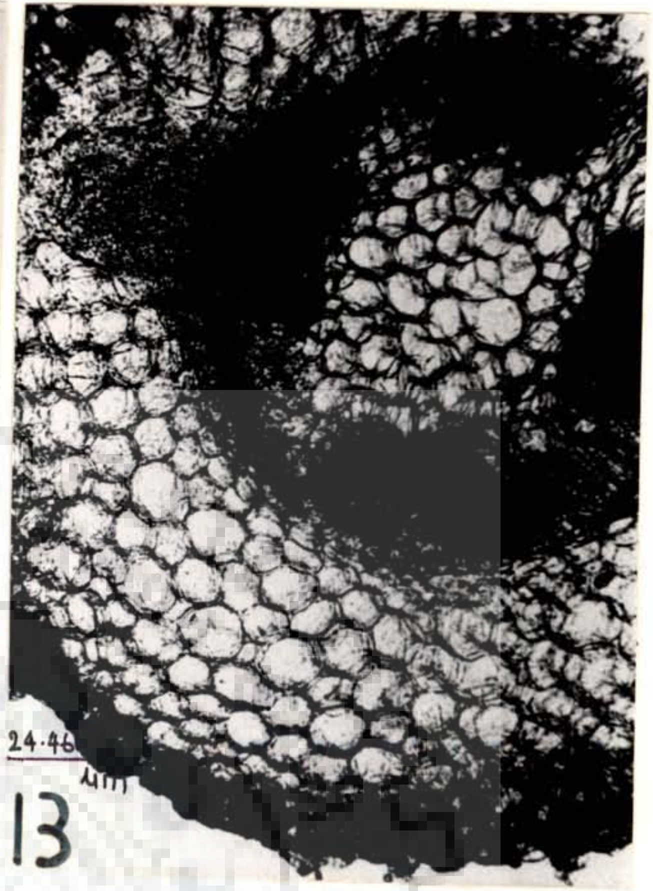
4.5 Effect of different concentrations of zinc on anatomical features of various organs of mungbean seedlings

The effect of zinc on anatomy of root, shoot and leaf of 6 d old mungbean seedlings were studied. While the low level of zinc affect considerably only the root anatomy, its toxic level affects the anatomy of all organs. The effect is seen in terms of total cell diameter, cortical cell layers, cell size and number, presence or absence of deposition of substances and anatomy of vascular bundles. The photographs of the section of different organs of seedling under zinc stress are given in fig. 4.6 to 4.8. The data for the same is presented in Table 4.10, 4.11 and 4.12.

Fig. 4.6 LIGHT MICROGRAPHS SHOWING TRANSVERSE SECTIONS OF ROOTS FROM MUNGBEAN SEEDLINGS SUBJECTED TO 6 DAYS OF TREATMENT.

A-represents control (0.0 ppm Zn, Magnification 312.5X). B - represents 0.4 ppm of zinc treatment (Magnification 408.75X). C-represents 25 ppm of zinc treatment (Magnification 408.75X) and D-represents 75 ppm zinc (Magnification 408.75X). The black deposits (Dpt.) and precipitated material (Ppt) seen in roots of mungbean grown in the presence of zinc are indicated.


32 um
A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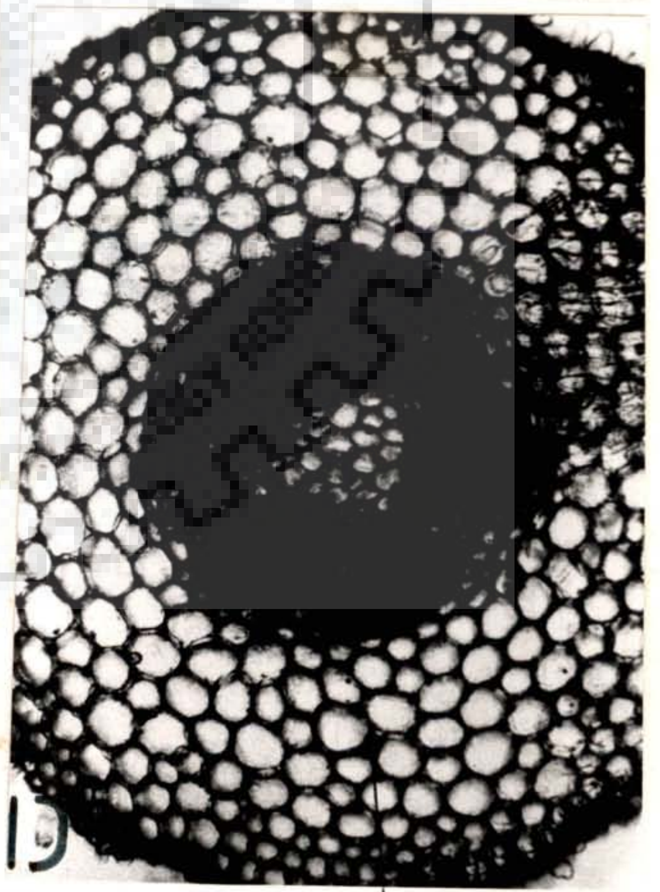
B

C

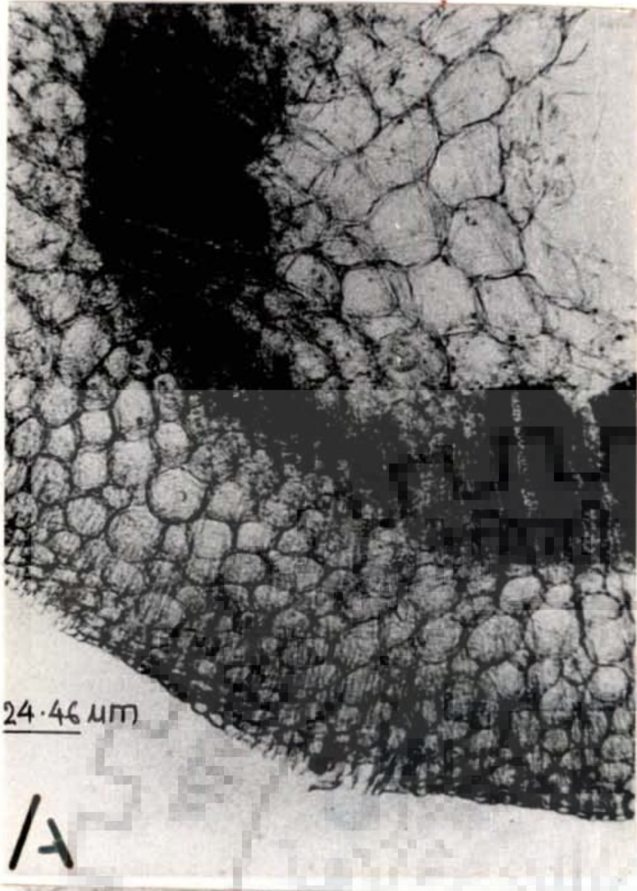

D

Ppt
Dpt

Fig. 4.7 LIGHT MICROGRAPHS SHOWING TRANSVERSE SECTIONS OF STEMS FROM MUNGBEAN SEEDLINGS SUBJECTED TO 6 DAYS OF TREATMENT.

A-represents control (0.0 ppm Zn, Magnification 408.75X). B-represents 0.4 ppm of zinc treatment (Magnification 408.75X). C-represents 25 ppm of zinc treatment (Magnification 408.75X) and D-represents 75 ppm zinc (Magnification 408.75X). 1 cm bar represents 24.46 μm.

Note the abundance of granular deposits (Grn) in the intracellular region of cortical cells in zinc treated plants. The boundaries of the cells in the cortical, hypodermal and epidermal regions of stem are also laden with dark deposits particularly in seedlings grown at higher concentrations of zinc.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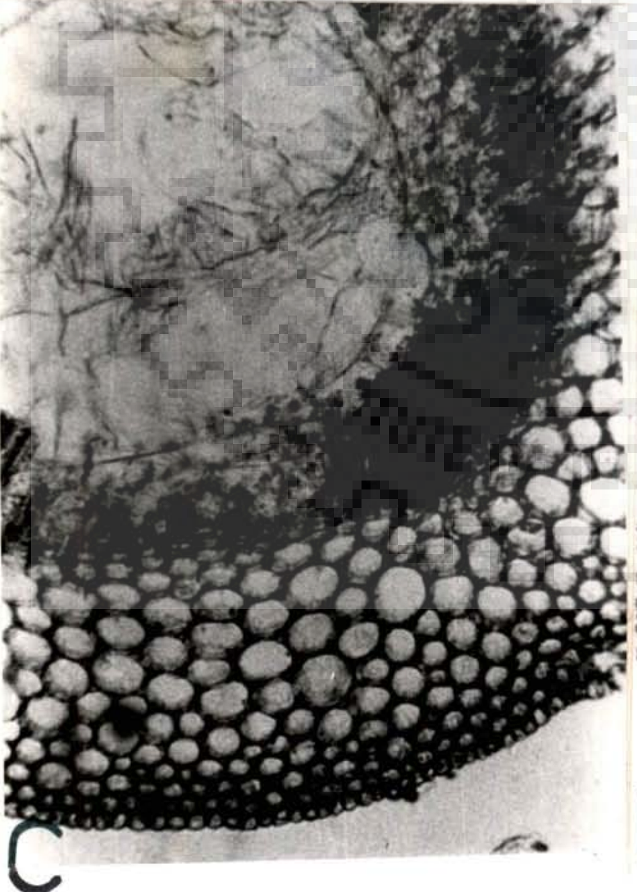
A

B


C

Grn
D

Fig. 4.8 LIGHT MICROGRAPH SHOWING TRANSVERSE SECTION OF A STEM FROM MUNGBEAN SEEDLINGS GROWN FOR 6 DAYS ON 100 PPM OF ZINC (MAGNIFICATION 408.75X; 1 CM BAR = 24.46 μM).

While the roots of these plants were degenerated, the stem was found to take over their function. The emergence of adventitious roots and increase in the number of vascular bundles are some of the modifications that are clearly seen in the steler region.

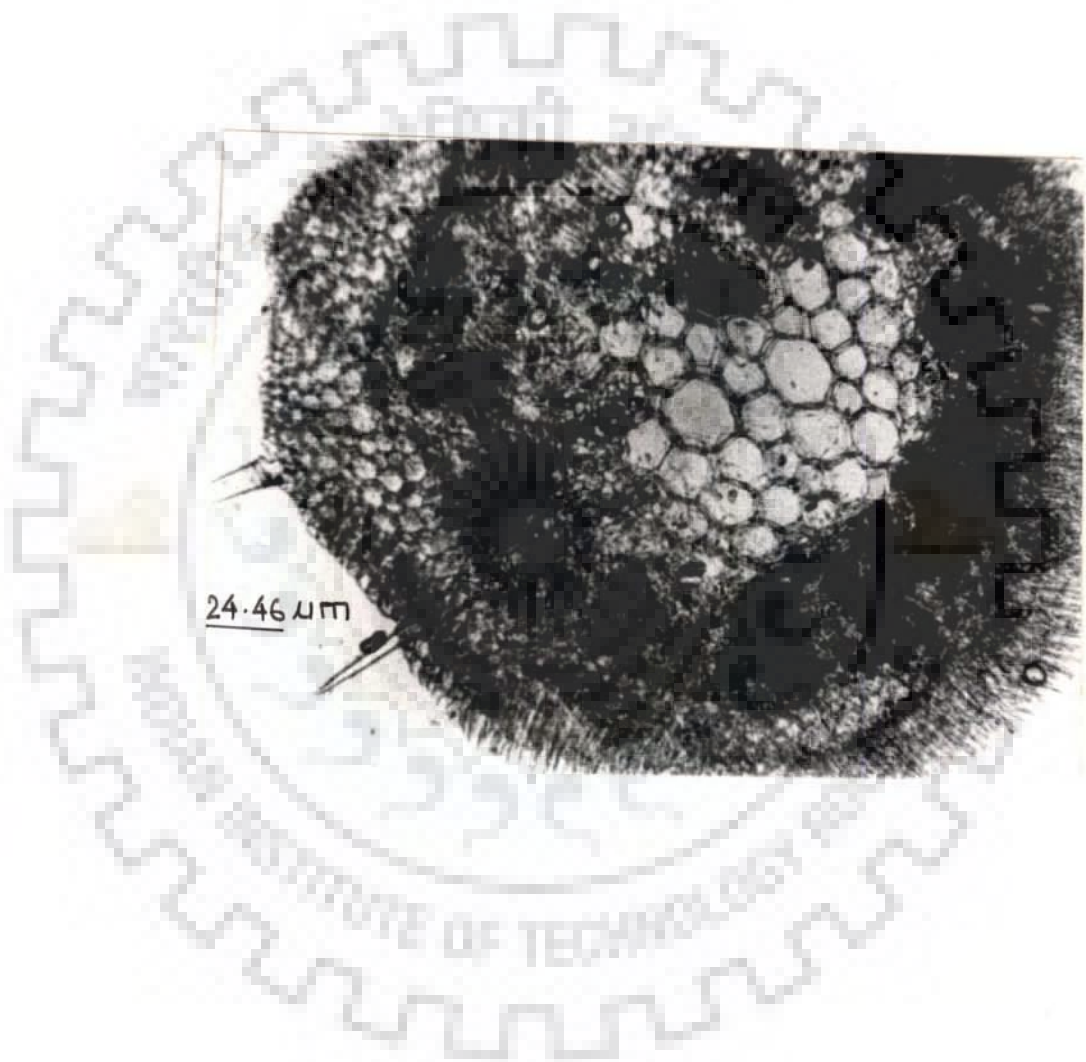

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TABLE 4.10

EFFECT OF DIFFERENT CONCENTRATIONS OF ZINC ON TOTAL SECTION DIAMETER IN DIFFERENT ORGANS OF 6 DAY OLD MUNGBEAN SEEDLINGS

Concentration of zinc (ppm) in nutrient media	Diameter of total section (µm) of different seedling parts		
	Leaf (Midrib region)	Shoot	Root
DDW	250.67±24.51*	644.72±113.72	237.00±57.48*
0.0	361.33±32.72	534.32±135.84	272.00±73.12
0.4	362.67±31.04	534.32±135.84	348.60±39.80
1.0	348.00±19.87	534.32±135.84	355.28±48.56
10.0	362.40±26.75	472.72±99.00	345.16±49.52
15.0	334.00±22.09	473.12±99.52	261.60±44.72
25.0	291.33±20.42*	421.76±75.52	251.24±64.20
50.0	257.33±16.76*	397.40±69.84	218.68±56.76
75.0	221.33±16.44*	341.00±71.92*	204.40±69.88
100.0	192.00±8.64*	284.04±65.56*	143.48±46.24*

Mungbean seedlings were grown hydroponically on nutrient media with varying concentrations of zinc. Seedlings were chosen randomly for estimations and separated into various organs. Their sections were cut and diameters were measured. Values are mean ± SE of 3 experiments (* significant at 1% level - Students `t' test)

TABLE 4.11

EFFECT OF DIFFERENT CONCENTRATIONS OF ZINC ON NUMBER OF CORTICAL CELL LAYERS IN DIFFERENT ORGANS OF 6 DAY OLD MUNGBEAN SEEDLINGS

Concentration of zinc (ppm) in nutrient media	Number of cortical cell layers in different seedling parts		
	Leaf (Mesophyll cell layers)	Shoot	Root
DDW	4±0.82	6±0.47	6±0.00
0.0	5±0.82	7±0.00	6±0.00
0.4	6±0.47	7±0.00	7±0.47
1.0	6±0.82	7±0.47	6±0.47
10.0	6 ±0.47	7±0.47	7±0.47
15.0	5±0.47	7±0.47	6±0.47
25.0	4±0.00	6±0.47	6±0.47
50.0	4±0.82	6±0.47	6±0.82
75.0	4±0.00	6±0.47	6±0.82
10.00	4± 0.82	6±0.00	3±0.47*

Mungbean seedlings were grown hydroponically on nutrient media with varying concentrations of zinc. Seedlings were chosen randomly for estimations and separated into various organs. Their sections were cut and diameters were measured. Results are mean ± SE of 3 experiments (* significant at 1% level - Students 't' test).

TABLE 4.12

EFFECT OF DIFFERENT CONCENTRATIONS OF ZINC ON CORTICAL CELL DIAMETER IN DIFFERENT ORGANS OF 6 DAY OLD MUNGBEAN SEEDLINGS

Concentration of zinc (ppm) in nutrient media	Diamerter of Leaf Vein cells	Diameter of Cortical Cells (μm)	
		Shoot	Root
DDW	0.28± .02*	17.1±1.7	13.3±2.1
0.0	0.39± .01	19.5±2.2	15.4±3.0
0.4	0.39± .01	19.8±1.9	17.5±3.9
1.0	0.39± .01	19.5±2.2	17.5±3.9
10.0	0.28± .02*	19.8±1.9	17.5±3.9
15.0	0.28± .02*	19.5±2.2	17.5±3.9
25.0	0.28± .02*	19.8±1.9	17.5±3.9
50.0	0.20± .01*	17.1±1.7	15.4±3.0
75.0	0.20± .01*	14.7±1.3*	15.4±3.0
10.00	0.18± .01*	8.2±1.1*	13.3±2.1

Mungbean seedlings were grown hydroponically on nutrient media with varying concentrations of zinc. Seedlings were chosen randomly for estimations and separated into various organs. Their sections were cut and diameter of cortical cells were measured. Results are mean ± SE of 3 experiments (* significant at 1% level - Students `t' test).

4.5.1 Effect of different concentrations of zinc on total section diameter of various organs

Table 4.10 shows that optimum level range of zinc affects only root section diameter where around 30% increase is found over control. In contrast, toxic concentrations of zinc decrease the total section diameter in all organs and a constant 47% decrease over control is noticed in all organs at 100 ppm zinc treatment.

4.5.1.1 Changes in leaf anatomy

Results show that there are only minor changes in leaf anatomy in terms of cell size at low level of zinc. However at higher than 10 ppm zinc, there is continuous decrease in vein diameter and its cell size which decreases upto 47% and 54% respectively at 100 ppm zinc level (Table 4.12).

4.5.1.2 Changes in shoot anatomy

Results show that upto 10 ppm zinc level there is no change in shoot anatomy. However, at higher concentrations total section diameter, cortical cell size decrease. Further, deposits of blackish substances also appear in the inter and intracellular spaces (Fig. 4.7). One very interesting feature is change in stele anatomy of shoot at 100 ppm zinc level. At such a high dose, shoot section shows presence of vascular bundles in pith region and inside stele. This confirms the possible emergence of adventitious roots from hypocotyl region which may contribute towards the tolerance of the seedlings to such a toxic level of zinc (fig. 4.8).

4.5.1.3 Changes in root anatomy

Root is affected most by zinc in terms of vascular bundle development, deposition of blackish substance in intracelluler spaces and coating like structure around cell wall (fig. 4.6).

In total section diameter there is ca. 30% increase at optimum level of zinc while with higher dose of zinc there is decline. Only at 100 ppm dose ca. 50% reduction occurs in

TABLE 4.13

IN VIVO LEVEL OF Zn^{++} IONS IN WHOLE MUNGBEAN SEEDLINGS AND PATTERN OF DISTRIBUTION IN DIFFERENT ORGANS EXPRESSED PER GRAM DRY WEIGHT

Organ	*In vivo* level of Zn^{++} ions (µg/gdw)		
	Control		
	DDW	0.0	0.4 ppm Zn
WHOLE SEEDLING	34.27 ±4.67	34.47 ±4.83	59.99* ±6.84
ROOT	55.47* ±4.23	37.73 ±4.46	109.13* ±2.02
HYPOCOTYL	35.63 ±3.14	29.05 ±4.76	68.03* ±1.38
COTYLEDON	12.01 ±2.29	17.17 ±2.36	20.99 ±2.94
EPICOTYL	16.94* ±2.56	32.95 ±4.18	50.73* ±1.42
LEAF	51.40 ±4.00	55.47 ±2.87	71.76* ±1.43

Mungbean seeds were grown hydroponically on nutrient media with and without zinc for six days. Seedlings were chosen randomly for estimation and separated into its various parts, oven dried and acid digested as described in "Materials and methods". Zn^{++} ions content was determined by atomic absorption spectrophotometer.Results are mean ± SE of 5 experiments. All comparisons have been made with seedlings grown in nutrient midia without zinc. (* Significant at 1% level - Students `t' test)

TABLE 4.14

IN VIVO LEVEL OF Zn^{++} IONS IN WHOLE MUNGBEAN SEEDLING AND PATTERN OF DISTRIBUTION IN DIFFERENT ORGANS EXPRESSED PER ORGAN

Organ	*In vivo* level of Zn^{++} ions (µg/organ)		
	Control		
	DDW	0.0	0.4 ppm Zn
WHOLE SEEDLING	0.568 ±0.019	0.613 ±0.031	1.437* ±0.035
ROOT	0.080 ±0.004	0.088 ±0.013	0.478* ±0.028
HYPOCOTYL	0.132 ±0.014	0.146 ±0.012	0.437* ±0.006
COTYLEDON	0.075* ±0.004	0.018 ±0.005	0.020 ±0.003
EPICOTYL	0.056* ±0.004	0.133 ±0.012	0.187* ±0.012
LEAF	0.075 ±0.009	0.106 ±0.013	0.157* ±0.006

Mungbean seeds were grown hydroponically on nutrient media with and without zinc for six days. Seedlings were chosen randomly for estimation and separated into its various parts, oven dried and acid digested as described in "Materials and methods". Zn^{++} ions content was determined by atomic absorption spectrophotometer Results are mean ± SE of 5 experiments.All comparisons have been made with seedlings grown in nutrient midia without zinc. (* Significant at 1% level - Students `t' test)

number of cortical cell layer. Upto 25 ppm zinc level there is the ca. 14% increase in cortical cell size which is reduced at higher zinc concentration accomponied by deposition of blackish substance in interacellular space and around the cell wall. Cortical cell number and development of vascular bundle is also enhanced with zinc concentrations (Table 4.10, 4.11 and 4.12).

4.6 Zinc uptake by mungbean seedlings

Zinc is absorbed by the plants in the form of Zn^{++} ions. Organwise and sub-cellular distribution of Zn^{++} ions in 6 d old mungbean seedlings was studied.

4.6.1 Organwise Zn^{++} ions distribution

Table 4.13 and 4.14 show the pattern of accumulation of the Zn^{++} ions in whole seedlings and also at the individual organ level. Whole seedling data shows that in plants grown in DDW and 0.0. ppm Zn there is no overall change in Zn level. However, there are strong relative differences between the two controls when the data on organs are compared. Results also show that more Zn^{++} ions accumulate in zinc treated plants than controls. Organwise Zn^{++} ions distribution on per gram tissue dry weight basis is found in the order of root > leaf > hypocotyl > epicotyl > cotyledon.A different pattern of Zn accumulation in the order root > hypocotyl > epicotyl > leaf >cotyledon is seen when the data is considered on per organ basis.

4.6.2 Effect of different concentrations of zinc on sub-cellular *in vivo* level of Zn^{++} ions

In vivo levels of Zn^{++} ions in protein and non protein components of cell cytoplasm and cell wall in root cells of mungbean seedlings that were subjected to 6 days of treatment are presented.

TABLE 4.15

EFFECT OF DIFFERENT ZINC CONCENTRATIONS ON *IN VIVO* LEVEL OF Zn^{++} IONS IN CYTOPLASM OF 6 DAY TREATED MUNGBEAN ROOTS ON PER GRAM FRESH TISSUE WEIGHT BASIS

Concentration of zinc (ppm) in nutrient media	Cytoplasmic level of Zn^{++} ions (ppm)		
	Cytoplasmic protein fraction	Cytoplasm without protein fraction	Total cytoplasm
DDW	3.31	0.13	3.26
0.0	2.64	0.08	2.72
0.4	2.66	0.09	2.75
1.0	5.14	0.18	5.32
10.0	40.80	2.47	43.27
15.0	68.00	2.70	70.70
25.0	108.36	3.99	112.35
50.0	144.00	6.64	150.64
75.0	190.93	10.59	201.52
100.0	233.71	43.54	277.25

Mungbean seeds were grown hydroponically on nutrient media with varying concentrations of zinc for six days. Roots of the seedings were used for the experiment. Total cytoplasm, cytoplasmic proteins and cytoplasm without proteins fractions were isolated as described in "Materials and methods". Zinc content in the fractions was estimated by ICP. Values are the average of 10 readings measured by ICP.

TABLE 4.16

EFFECT OF DIFFERENT ZINC CONCENTRATIONS ON *IN VIVO* LEVEL OF Zn^{++} IONS IN CELL WALL OF 6 DAY TREATED MUNGBEAN ROOTS ON PER GRAM FRESH TISSUE WEIGHT BASIS

Concentration of zinc (ppm) in nutrient media	*In vivo* level of Zn^{++} ions (ppm) in cell wall		
	Cell wall Protein fraction	Cell wall without Protein fraction	Total Cell wall
DDW	0.151	0.137	0.29
0.0	0.029	0.084	0.11
0.4	0.008	0.099	0.11
1.0	0.000	0.107	0.11
10.0	0.076	1.068	1.14
15.0	0.184	2.464	2.65
25.0	0.768	8.920	9.69
50.0	0.739	11.000	11.74
75.0	0.656	11.443	12.10
100.0	1.723	8.457	10.18

Mungbean seeds were grown hydroponically on nutrient media with varying concentrations of zinc for six days. Roots of the seedlings were used for the experiment. Cell wall and cell wall proteins were isolated as described in "Materials and methods". The concentration of Zn^{++} ions in the fractions were estimated by ICP. Values are the average of 10 readings measured by ICP.

A B C

A = Cytoplasmic protein/Cytoplasm without protein

B = Cell wall without protein/Cell wall protein

C = Cytoplasmic protein/Cell wall protein

Ratio of Zn++ ions levels of different fractions

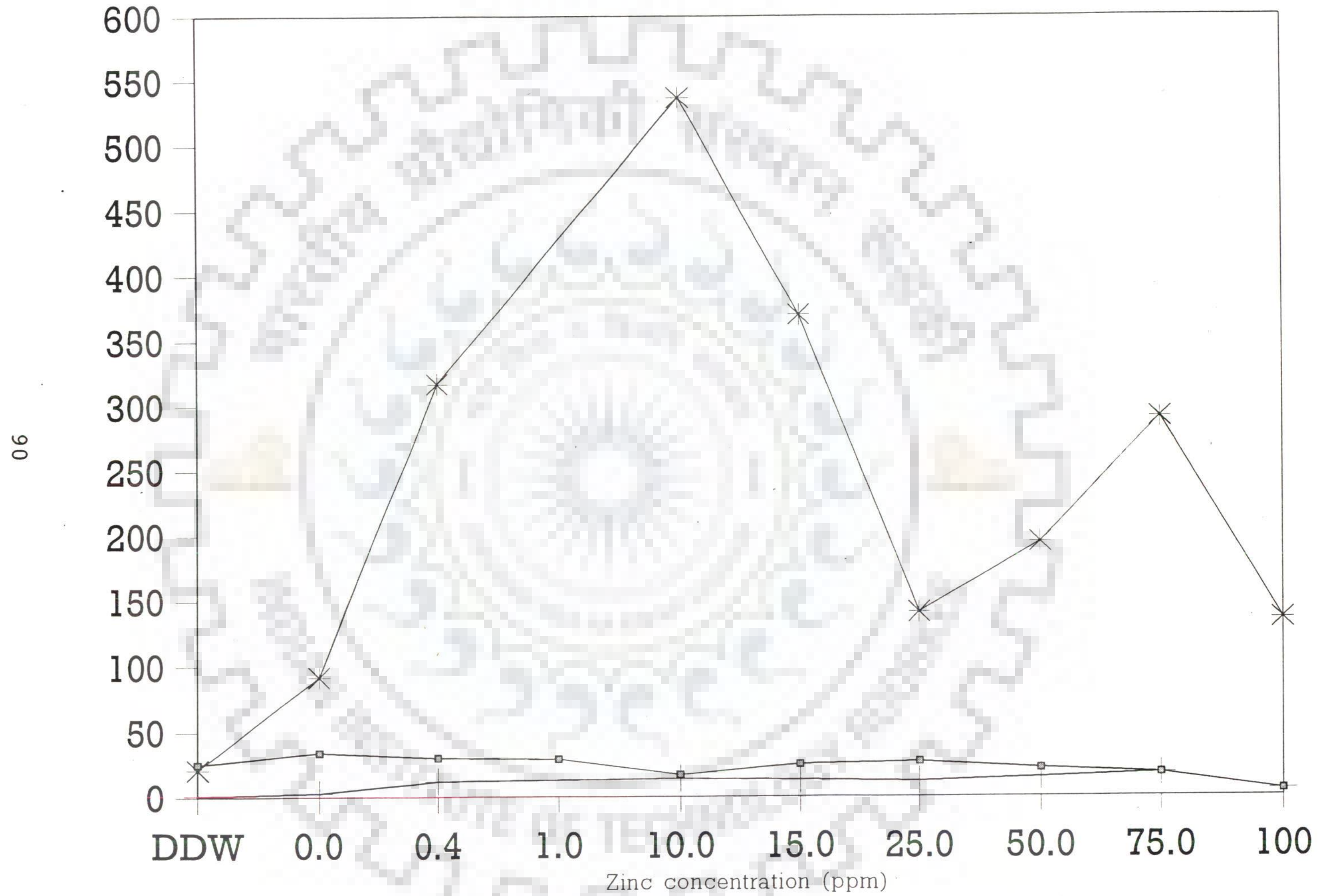


Fig. 4.9 Effect of different zinc concentrations on co-relations between cell wall and cytoplasmic level of Zn++ ions in mungbean roots

4.6.2.1 Cytoplasmic level of Zn^{++} ions

Table 4.15 shows pattern of in vivo level of Zn^{++} ions in cytoplasm of 6 d old mungbean root cells at different zinc concentrations. From the table it is obvious that irrespective of the type of treatment, zinc is associated with the protein rather than the non-protein fractions in the cytoplasm of root cells. It is also clear that with the increase in Zn content of the nutrient medium in which the mungbean seeds were grown, there was greater uptake by the root cells which ultimately accumulated in the cytoplasmic protein fractions. Further, it is also observed that with the increase in zinc concentrations in growth media, there is a concomittant increase in the level of zinc in the non protein fraction of root cytoplasm as well. Perhaps, the toxic levels of the zinc present slows down the incorporation of Zn into cytoplasmic proteins.

4.6.2.2 *In vivo* level of Zn^{++} ions of root cell wall

Table 4.16 shows the pattern of *in vivo* level of Zn^{++} ions in cell wall of root cells of 6 d treated mungbean seedlings at different zinc concentrations. Results indicate that in comparison to control grown on nutrient media without zinc there is no change in *in vivo* level of Zn^{++} ions of total root cell wall at 0.4 and 1.0 ppm zinc concentrations. However, the zinc concentration in cell wall increased continuously with higher levels of zinc treatment. In contrast to the results on zinc accumulation in cytoplasmic proteins, the cell wall proteins did not appreciably capture zinc. In fact, the non protein components of cell wall showed accumulation of zinc which suggests that zinc may be incorporated into the molecules that make up the structural component of cell wall

4.6.2.3 Correlation between *in vivo* levels of Zn^{++} ions in cytoplasmic and cell wall protein fractions.

Table 4.15 and 4.16 show that there is continuous negative correlation upto 1.0 ppm zinc, thereafter positive correlation upto 25 ppm zinc then again negative correlation upto 75 ppm zinc and finally positive correlation at 100 ppm zinc dose, between levels of Zn^{++} ions in cytoplasmic and cell wall proteins fractions of root cells of 6 d old mungbean seedlings grown at different concentrations of zinc.

TABLE 4.17

EFFECT OF DIFFERENT CONCENTRATIONS OF ZINC ON THE SPECIFIC ACTIVITIES OF SOME ROOT CELL WALL BOUND ENZYMES OF MUNGBEAN SEEDLINGS AFTER 6 DAYS OF TREATMENT.

Concentration of zinc (ppm) in nutrient media	Specific activity of cell wall bound enzymes				
	ß-gal[1]	α−glu[1]	Acid phos[1]	Perox[2]	Polyphenox[2]
DDW	187.6* ± 15.0	62.0 ±11.9	62.7 ±13.2	0.266* ±0.006	0.181* ±0.006
0.0	132.6 ±17.3	61.8 ±11.1	77.0 ±14.0	0.173 ±0.005	0.127 ±0.005
0.4	111.5 ±16.6	82.5 +11.6	112.0 ±14.3	0.183 ±0.007	0.142* ±0.005
1.0	107.1 ±13.7	74.9 ±12.0	101.0 ±14.9	0.198 * ±0.007	0.142* ±0.005
10	170.1 ±17.1	66.3 ±12.0	193.8* ±18.6	0.194* ±0.005	0.127 ±0.005
25	189.7* ±17.1	62.5 ±8.8	244.4* ±17.2	0.205* ±0.005	0.126 ±0.007
50	180.7* ±13.4	62.6 ±11.7	221.8* ±13.7	0.369* ±0.007	0.104* ±0.005
75	107.0 ±15.3	42.0 ±12.0	199.8* ±16.2	0.456* ±0.005	0.104* ±0.005

1 = PNP liberated min^{-1} mg cell wall $protein^{-1}$
2 = abs λ 420 min^{-1} mg cell wall $protein^{-1}$
β−gal = β-galactosidase
α−glu = α−glucosidase
Acid phos = Acid phosphatase
Perox = Peroxidases

Polyphenox= Polyphenoloxidase

Mungbean seedlings were grown hydroponically on nutrient media with varying concentrations of zinc. 6 d treated seedlings were used for monitoring enzyme activity. Cell wall bound enzymes were extracted and estimated as described in "Materials and methods". Values are mean ± SE of 5 experiments. All comparisons have been made with seedlings grown on nutrient media without zinc (* significant at 1% level)

Zn^{++} ion levels ratio of cytoplasmic to cell wall protein fraction varies with zinc treatment and is ca. 3.45, ∞, 5.84, 1.53, 3.16 and 1.48 folds over control at 0.4, 1.0, 10, 25, 75 and 100 ppm zinc concentrations respectively (fig. 4.9). The same ratio is ca. 2.27, ∞, 3.85, 0.99, 2.33 and 1.22 folds over protein contents ratio of cytoplasm to cell wall of control at same zinc concentrations as mentioned above. This indicates more affinity of Zn^{++} ions towards cytoplasmic proteins than cell wall proteins.

4.7 Response of cell wall bound enzymes to different concentrations of zinc

Table 4.17 shows the effect of different concentrations of zinc on enzyme activities of cell wall bound hydrolases and peroxidases in roots of mungbean seedlings given 6 days of treatment. The activity of five important enzymes: β-glactosidase, α-glucosidase, acid phosphatase, peroxidase and polyphenoloxidase extracted from the cell wall of roots from zinc treated mungbean seedlings have been studied with appropriate controls. It was found that the activity of β-galactosidase, peroxidase and polyphenol oxidase were significanlty higher in seedlings grown in distilled water then in seedlings grown on nutrient media without zinc. In the case of β-galactosidase, the enzyme activity of seedlings grown on media with zinc concentrations upto 1.0 ppm did not show any appreciable change when compared to seedlings grown on medium without zinc. However, at higher concentration of 25 and 50 ppm zinc the enzyme activity was significantly high. α-glucosidase remained unaffected by zinc treatment. The activity of acid phosphatase and peroxidase showed a similar pattern of response to zinc treatment. At lower concentrations, the activities of these two enzymes remained unaffected but an enhancement of activity was seen as the concentration of zinc in the growth medium was increased. A some what unique trend was seen in the activity of polyphenol oxidase in seedlings supplied with different concentration of zinc. At concentrations of upto 1 ppm zinc the enzyme activity was enhanced, while at high concentrations of 50 and 75 ppm zinc, the activity of enzyme seemed to be inhibited. There was no change in the activity of enzymes in seedlings given 10 and 25 ppm of zinc. Thus, the response of individual enzymes to varying concentration of zinc was found to be different.

TABLE 4.18

EFFECT OF DIFFERENT CONCENTRATIONS OF ZINC ON CYTOPLASMIC AND CELL WALL PROTEIN CONTENT IN ROOTS OF 6 DAY TREATED MUNGBEAN SEEDLINGS.

Concentration of zinc (ppm) in nutrient media	Protein content in mungbean roots		
	Cytoplasmic (mg/gfw)	Cell wall (μg/gfw)	Ratio (cytoplasmic/cell wall)
DDW	1.67 ± 0.09*	10.1 ± 0.8*	165
0.0	1.37 ± 0.03	6.3 ± 0.4	218
0.4	1.16 ± 0.02	8.3 ± 1.0	140
1.0	1.34 ± 0.03	5.9 ± 0.7	227
10	1.03 ± 0.04	7.4 ± 0.8	139
15	0.91 ± 0.05	7.9 ± 0.8	115
25	1.27 ± 0.05	8.9 ± 0.7	143
50	1.57 ± 0.03*	10.1 ± 1.0*	155
75	1.78 ± 0.07*	14.2 ± 0.7*	125
100	2.14 ± 0.10*	19.2 ± 3.2*	112

Mungbean seedlings were grown hydroponically on nutrient media with varying concentration of zinc. Roots of six day zinc treated seedlings were used for the experiment. Cytoplasmic and cell wall proteins were isolated and their content was estimated as described in "Materials and methods". Values are mean ± SE of five experiments.All comparisons have been made with seedlings grown on nutrient media without zinc (* significant at 1% level)

From the data it may be generalized that at 0.4 ppm zinc which is considered to be optimal the activity of enzymes studied remained unaffected. However, at higher concentrations of zinc the activity of the very same enzymes were disturbed, perhaps due to the stress and the seedling response to manage this stress.

4.8 Response of cytoplasmic and cell wall proteins to different concentrations of zinc

Table 4.18, 4.19, 4.20 and fig. 4.10 and 4.11 show the significant changes in cytoplasmic and cell wall proteins including their ratio at different concentrations of zinc. Results indicate quantitative change in both cases and change in qualitative pattern of only cytoplasmic proteins due to zinc treatment. Due to degeneration and poor formation of roots, changes in cell wall proteins could not be studied at 75 and 100 ppm zinc levels.

4.8.1 Quantitative changes

4.8.1.1 Cytoplasmic proteins at different concentrations of zinc

Table 4.18 shows that the protein content declines upto 15 ppm zinc and increases at higher zinc concentrations, when compared to seedlings grown nutrient media minus zinc. Cytoplasmic protein content was higher in roots of mungbean seedlings grown in distilled water alone.

4.8.1.2 Cell wall proteins at different concentrations of zinc

Table 4.18 shows that the content of cell wall proteins increases continuously with the zinc concentration in the medium. Cell wall proteins were significantly increased in distilled water grown seedlings compared to the seedlings grown in nutrient medium without zinc.

When the data on the cytoplasmic/cell wall protein ratio is analysed there does not seem to be any particular dose related pattern in response to zinc treatment.

TABLE 4.19

EFFECT OF DIFFERENT CONCENTRATIONS OF ZINC ON THE PROFILE OF CELL WALL PROTEINS IN ROOTS OF 6 DAY TREATED MUNGBEAN SEEDLINGS.

Concentration of zinc (ppm) in nutrient media	Molecular weights of polypeptides of cell wall proteins (kDa)					
DDW	42**	40	38.4	22	19
0.0		40	38.4	22	19
0.4		40	38.4	22	19
1.0		40	38.4	22	19
10		40	38.4	22	19
15		40	38.4	22	19
25		40	38.4	22	19
50		40	38.4	22	19

** Only appeared in DDW

Mungbean seeds were grown hydroponically on nutrient media with varying concentrations of zinc Roots of 6 d treated seedlings were used for experiments. Cell wall proteins were isolated and separated through SDS-PAGE on 10% gel.

TABLE 4.20

EFFECT OF DIFFERENT CONCENTRATIONS OF ZINC ON THE PROFILE OF CYTOPLASMIC PROTEINS IN ROOTS OF MUNGBEAN SEEDLINGS AFTER 6 DAYS OF TREATMENT.

Concentration of zinc (ppm) in nutrient media	Molecular weights of polypeptides of cytoplasmic proteins (kDa)
DDW	81, 78, 71^{*}, 69^{*}, 56^{**}, $51^{\#*}$, $46^{\sim***}$, 43, 41, 39, 38, $35^{\bullet}$, 33^{****}, 29.5, 28, $27^{\#}$, 20, $18^{\bullet\bullet}$, 16, 15.
0.0	81, 78, 56^{**}, $51^{\#*}$, $46^{\sim***}$, 43, 41, 39, 38, $35^{\bullet}$, 29.5, 28, $27^{\#}$, 20, 18^{**}, 16, 15.
0.4	81, 78, 56^{**}, $51^{\#*}$, 46^{***}, 43, 41, 39, 38, $35^{\bullet}$, 29.5, 28, $27^{\#}$, 20, 18^{**}, 16, 15.
1.0	81, 78, 56^{**}, $51^{\#*}$, 43, 41, 39, 38, $35^{\bullet}$, 29.5, 28, $27^{\#}$, 20, 18^{**}, 16, 15.
10	81, 78, 56^{**}, $51^{\#*}$, 43, 41, 39, 38, $35^{\bullet}$, 33^{****}, 29.5, 28, $27^{\#}$, 20, 18^{**}, 16, 15.
15	81, 78, $51^{\#*}$, 43, 41, 39, 38, $35^{\bullet}$, 33^{****}, 29.5, 28, 20, 18^{**}, 16, 15.
25	81, 78, $51^{\#*}$, 43, 41, 39, 38, $35^{\bullet}$, 33^{****}, 29.5, 28, 20, 18^{**}, 16, 15.
50	81, 78, 56^{**}, 46^{***}, 43, 41, 39, 38, $35^{\bullet}$, 33^{****}, 29.5, 28, 20, $18^{\bullet\bullet}$, 16, 15.
75	81, 78, 56^{**}, 46^{***}, 43, 41, 39, 38, $35^{\bullet}$, 33^{****}, 29.5, 28, 20, $18^{\bullet\bullet}$, 16, 15.
100	81, 78, 56^{**}, 46^{***}, 43, 41, 39, 38, $35^{\bullet}$, 33^{****}, 29.5, 28, 20, $18^{\bullet\bullet}$, 16, 15.

Contd...

*   Only in DDW

~   Induced only in controls

**   Disappeared after 10 ppm and reappeared at 50,75and 100 ppm zinc concentrations

***   Disappeared after 0.4 ppm and reappeared at 50,75 and 100 ppm zinc concentrations.

**** Disappeared after DDW and reappeared after 1 ppm

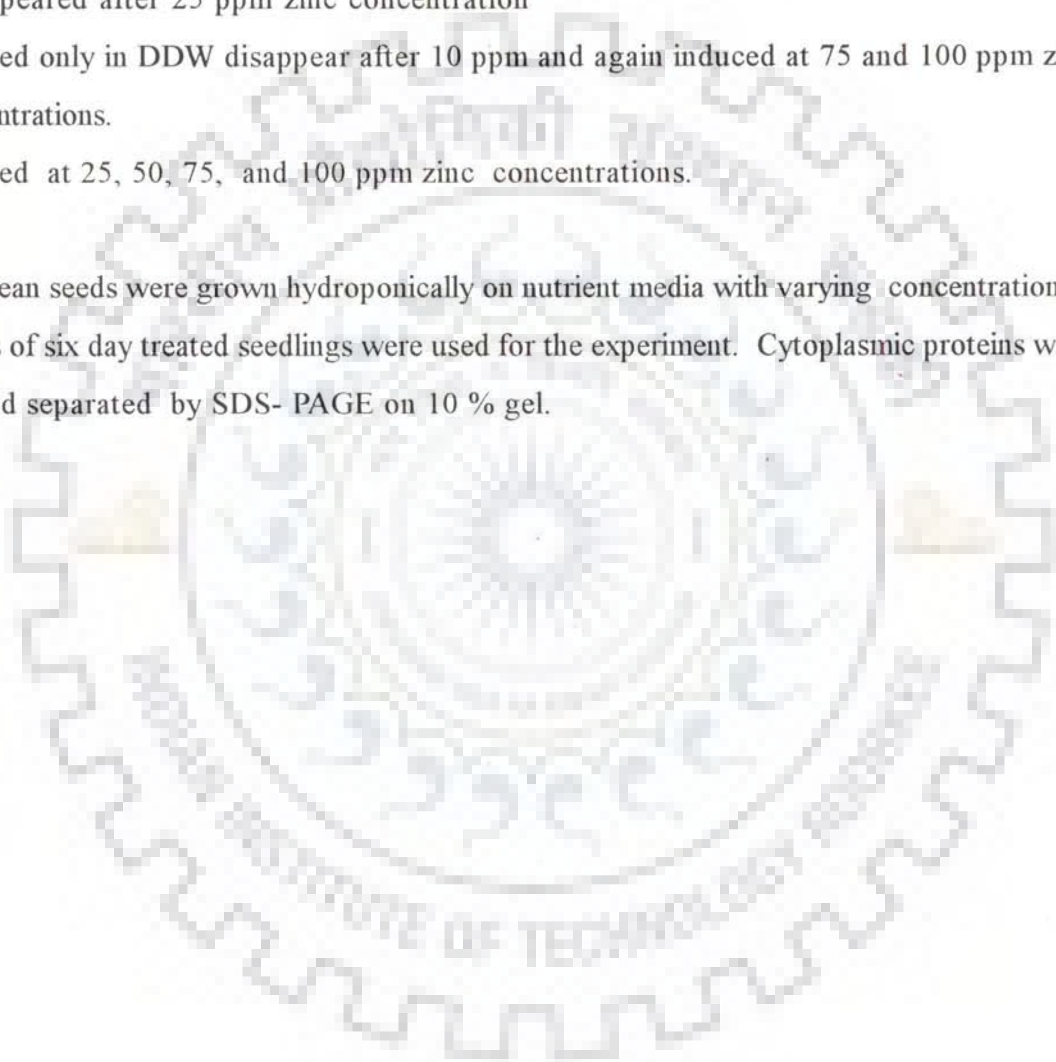
Disappeared after 10 ppm zinc concentration

#*   Disappeared after 25 ppm zinc concentration

●   Induced only in DDW disappear after 10 ppm and again induced at 75 and 100 ppm zinc concentrations.

●●   Induced at 25, 50, 75, and 100 ppm zinc concentrations.

Mungbean seeds were grown hydroponically on nutrient media with varying concentration of zinc. Roots of six day treated seedlings were used for the experiment. Cytoplasmic proteins were isolated and separated by SDS- PAGE on 10 % gel.



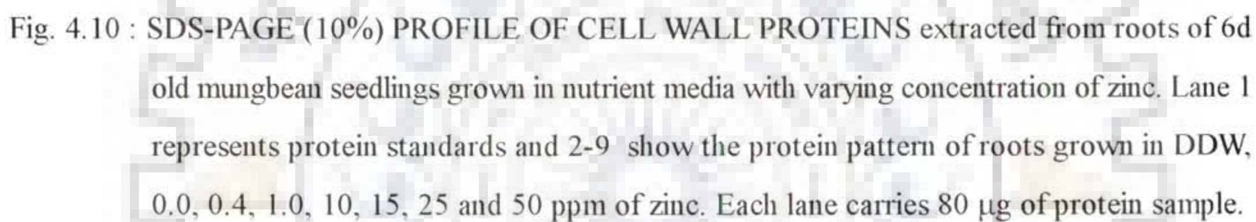
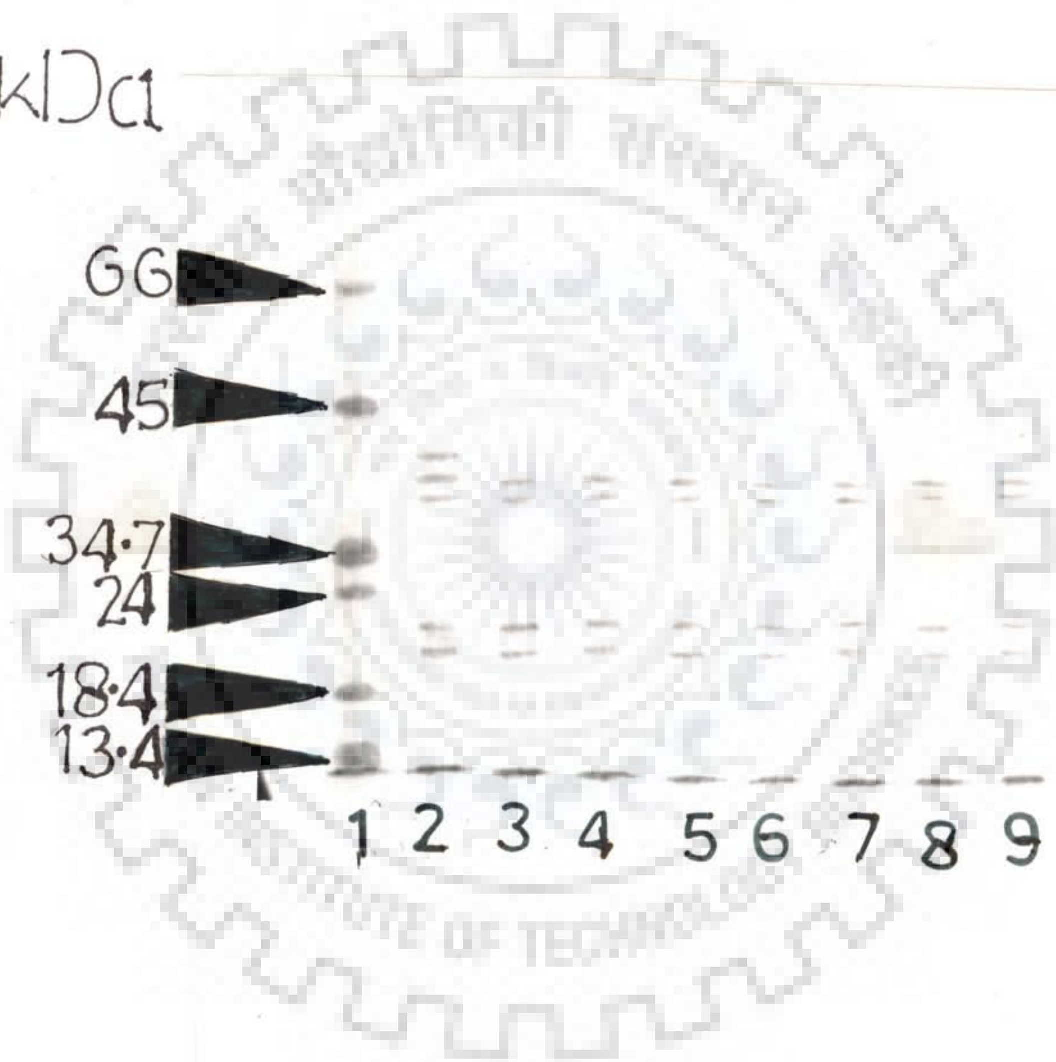


Fig. 4.10 : SDS-PAGE (10%) PROFILE OF CELL WALL PROTEINS extracted from roots of 6d old mungbean seedlings grown in nutrient media with varying concentration of zinc. Lane 1 represents protein standards and 2-9 show the protein pattern of roots grown in DDW, 0.0, 0.4, 1.0, 10, 15, 25 and 50 ppm of zinc. Each lane carries 80 μg of protein sample.


kDa
66
45
34.7
24
18.4
13.4
1 2 3 4 5 6 7 8 9

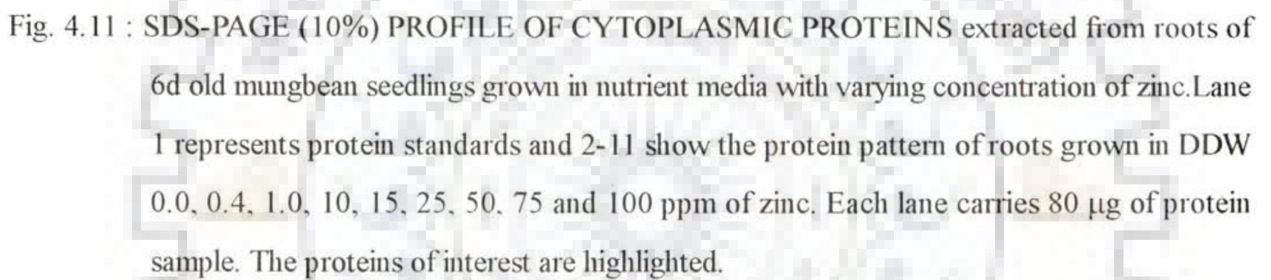
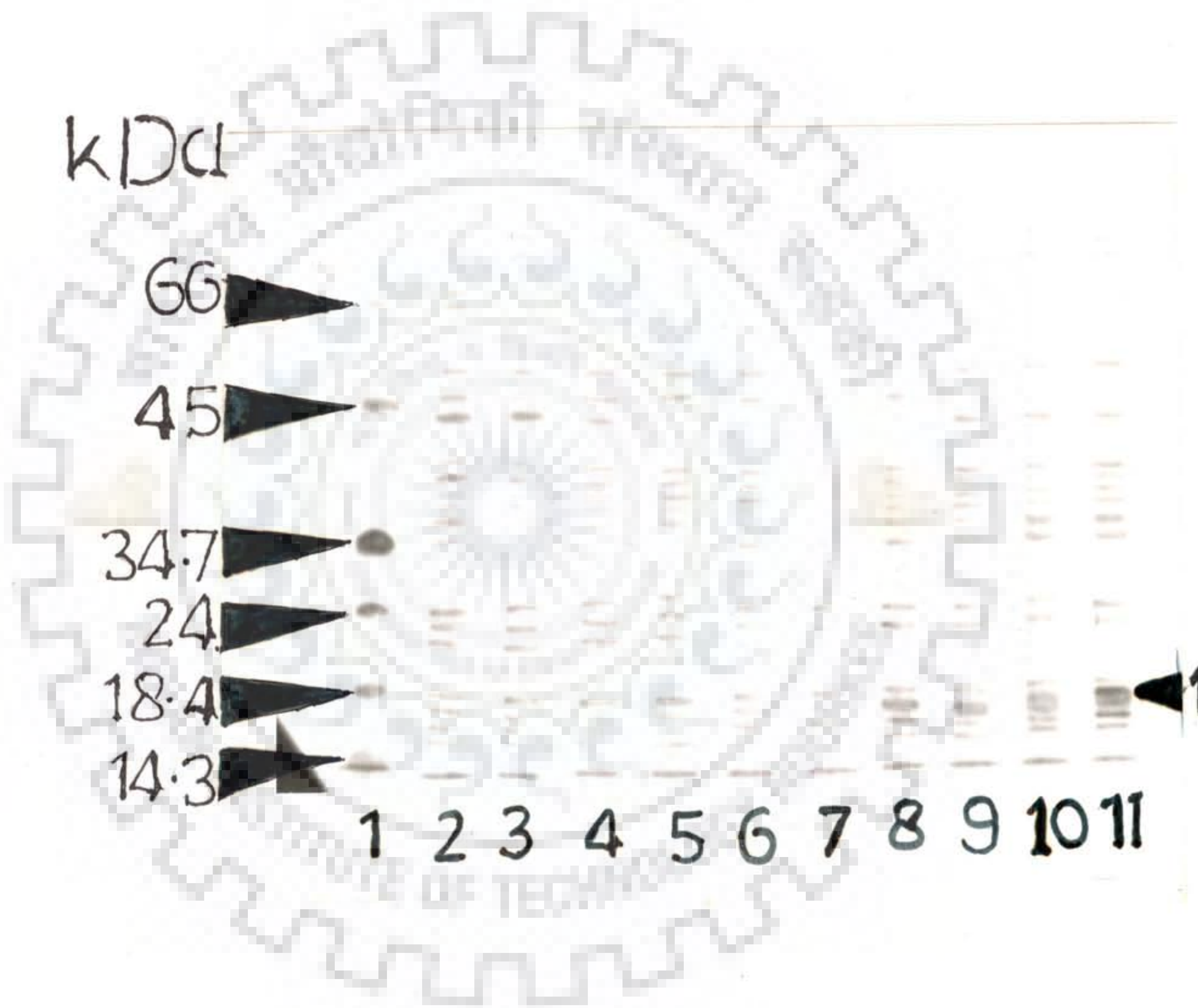


Fig. 4.11 : SDS-PAGE (10%) PROFILE OF CYTOPLASMIC PROTEINS extracted from roots of 6d old mungbean seedlings grown in nutrient media with varying concentration of zinc.Lane 1 represents protein standards and 2-11 show the protein pattern of roots grown in DDW 0.0, 0.4, 1.0, 10, 15, 25, 50, 75 and 100 ppm of zinc. Each lane carries 80 μg of protein sample. The proteins of interest are highlighted.



kDa
66
45
34.7
24
18.4
14.3
1 2 3 4 5 6 7 8 9 10 11

4.8.2 Qualitative pattern

4.8.2.1 Cell wall Protein profile at different concentrations of zinc

Fig. 4.10 shows SDS- PAGE profile of cell wall proteins and table 4.19 summarize the molecular weights of cell wall polypeptides in control and 6 day zinc treated roots of mungbean seedlings. On exposure to different concentrations of zinc, there is no alteration in protein profile of cell wall. Cell wall proteins contain 4 polypeptides of 19, 22, 38.4 and 40 kDa at all zinc concentrations including control. One 42 kDa additional band is also observed in case of distilled water grown seedlings.

4.8.2.2 Cytoplasmic protein profile at different concentrations of zinc

Fig. 4.11 shows SDS-PAGE profile of cytoplasmic proteins and Table 4.20 summarizes the data on molecular weights of cytoplasmic polypeptides in control and zinc treated roots of 6 d old mungbean seedlings. In control seedlings grown on nutrient media without zinc, 18 proteins were resolved on 10% SDS-polyacrylamide gels. When seedlings were grown on distilled water alone two additional proteins, 71 kDa and 69 kDa were seen. The protein patterns were analysed in seedlings grown at different zinc concentrations and the following are the highlights. A protein of 56 kDa was found to be present upto 10 ppm zinc treatment, was not detectable at 15 and 25 ppm concentrations but was present in seedlings grown at 50, 75 and 100 ppm of zinc. Similarly, another protein (46 kDa) was not detected in seedlings grown above 0.4 ppm zinc but made its appearance at 50, 75 and 100 ppm zinc concentrations. The most unique observation was that of a 33 kDa protein which was not present in seedlings grown at optimum zinc levels (0-1 ppm) but was present in samples of seedlings grown at all other higher concentrations of zinc. This protein was also present in seedlings grown in distilled water alone, implying that this could be a stress protein. Two other proteins (27 kDa and 51 kDa) were found to be absent from samples grown at zinc concentrations above 10 ppm and 25 ppm, respectively. They were found in distilled water grown seedlings too. It appears that higher concentrations of zinc inhibits the expression of this protein. Interestingly, a protein with molecular weight of 18 kDa was found to be pre-

TABLE 4.21

AMINO ACID COMPOSITION OF ZINC - INDUCED 18 kDa CYTOPLASMIC POLYPEPTIDE

Amino acids	Concentration of amino acids (p mol)
LYSINE	676.81
TYROSINE	175.58
LEUCINE	154.25
ARGININE	120.53
PHENYLALANINE	54.23
CYSTEINE	23.89

PEAK#	AMOUNT	RT	AREA		RF
6	118.52000	3.93	375897	L	0.315300E0
UNK		5.80	126328	F	
10	204.49200	5.96	715256	L	0.285900E0
12	34.53350	7.47	116825	L	0.295600E0
15	141.32500	8.36	505635	F	0.279500E0
UNK		8.53	92878	F	
16	50.62200	8.67	153960	L	0.328800E0
UNK		9.83	3194081	L	
17	124.47000	10.15	597265	L	0.208400E0
UNK		11.48	433902	F	
UNK		11.66	197476	L	
TOTAL	673.96300				

-ion Part No. 74701

INJECT

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JAN. 15, 1997 14:27:34 CHART 2.00 CM/MIN
RUN #9 CALC #0
COLUMN SOLVENT OPR ID: 6

EXTERNAL STANDARD QUANTITATION

PEAK#	AMOUNT	RT	EXP RT	AREA		RF
UNK	Picomoles	0.97		102364	F	
UNK		1.04		141863	L	
Arg 6	120.53100	3.90		382274	L	0.315300E0
Tyr 10	175.57700	6.04		614119	L	0.285900E0
Cys 13	23.89030	7.65		135972	L	0.175700E0
Leu 15	154.24900	8.50		551874	F	0.279500E0
UNK		8.68		101588	F	
Phe 16	54.22830	8.80		164928	L	0.328800E0
Lys 17	676.81400	9.92		3247686	L	0.208400E0
UNK		10.22		675898	L	
UNK		11.49		439190	F	
UNK		11.67		208827	L	
TOTAL	1205.28000					

046

Fig. 4.12 HPLC chromatogram of PTH-aminoacids of 18kDa Zn-induced cytoplasmic polypeptide

dominant at high concentrations of zinc. There was therefore a need to further characterize this protein.

4.9 Amino acid analysis of zinc-induced cytoplasmic protein

A typical HPLC data sheet of amino acid analysis is presented in Fig. 4.12. Table 4.21 summarizes the amino acid composition of zinc - induced 18 kDa cytoplasmic protein. This polypeptide is rich in lysine (676.81 p mol) tyrosine (175.58 p mol) and leucine (154.25 p mol) with less amount of arginine (120.53 p mol), phenylalanine (54.23 p mol) and cysteine (23.89 p mol).

CHAPTER - 5

5.0 DISCUSSION

This chapter incorporates the discussion of the results on studies on zinc status of the natural habitat of plants and its impact on various biochemical and physiological aspects of hydroponically grown mungbean seedlings.

The divalent heavy metal zinc was selected for investigations for the following regions. First, zinc is considered as an essential micronutrient for plant growth and development Second, several pockets of the Indo-Gangetic plains were found to be deficient in zinc and are routenly supplemented with zinc as well when symptoms are expressed while raising crops. Finally , municipal and industrial effluents in the Indo-Gangetic area make the level of zinc in agricultural fields toxic to plants. It would therefore, be of interest to ascertain how the plants cope up with the conditions of zinc stress. Mungbean is widely grown in Indo-Gangetic alluvial plains of western Uttar Pradesh and was chosen as a model for the present study since it acts as a good indicator of zinc stress (Kanwar and Randhawa, 1981).

In around 28% and 90% of the soils sampled in Indo-Gangetic alluvial plains of Roorkee-Muzaffarnagar region, concentration of total and DTPA-extractable (Phytoavailable) zinc respectively is lower than the widely accepted critical zinc concentrations of 25-250 ppm and 0.5 ppm respectively (Cakmak et al., 1996a). However in India, based on field and greenhouse experiments about 0.6 ppm DTPA-extractable Zn has been suggested as a critical concentration for calcareous soils of arid region. (Singh et al., 1987). Various soil samples collected from different region are highly alkaline with an average pH of 7.8 and thus may negatively alter the status of phytoavailable Zn in surface horizon as well as its pattern in subsoils. Solubility of Zn highly depends on pH and a 100 fold decrease in solubility occurs with each unit increase in pH. Zinc deficiencies are usually observed in soils with pH greater than 6.0 (Lindsay, 1972; Kanwar and Randhawa, 1981; Katyal and Rattan, 1993). The soil

pH is negatively correlated with the uptake of zinc by plants (Massey, 1957; Cakmak et al., 1996a).

A low Zn content in soils of Indo-Gangetic alluvial plains of Roorkee- Muzaffarnagar region as a reason for Zn deficiency can be excluded, as the total content of Zn in soils used is fairly high with an average of 35.6 ppm Zn. Thus the soil analysis conducted strongly suggest that Zn deficiency is a serious nutritional problem limiting crop production in western Uttar Pradesh, India. The combination of high pH, Na_2CO_3 and clay in soils together with low annual precipitation are considered to be the major factors causing Zn deficiency in plants grown in Indo-Gangetic alluvial plains of Haridwar and Muzaffarnagar disticts. Just as in this study, similar observations have been made by Katyal and Rattan (1993) and Cakmak et al., (1996a). Zinc moves to plant roots in soils mainly by diffusion (Marschner, 1993; Wilkinson et al., 1968). Since tortuosity of the diffusion path is largely enhanced by decreases in soil water status (Warncke and Barber, 1972). Zinc nutrition of plants is often threatened in arid soils having low plant available concentration of Zn. In alkaline calcareous soils, diffusion coefficient for Zn is about 50 times lower than in acid soils (Melton et al., 1973).

On the other hand data available on physico-chemical analysis of ground water of nearby villages and effluent as well as molasses of Mahalakshmi Sugar Mills Iqbalpur Ltd. of Roorkee region (Gupta, 1995; Nurain, 1996; Gupta, 1996; Arora, 1997) suggest that the large number of sugarcane industries of Indo-Gangetic alluvial plains of Roorkee-Muzaffarnagar region may contribute to the toxic levels of zinc found in this region. Thus, the Indo-Gangetic plain is represented by regions of Zn deficiency as well as toxic concentration making it necessary to take up Zn related studies.

Mungbean seedlings do not tolerate zinc concentrations higher than 100 ppm and hence the present studies were restricted to this limit. On the basis of morphological, anatomical and biochemical parameters as discussed below, it may be considered that optimum zinc requirement for the *Vigna radiata* cv K-851 is 0.4 ppm and concentrations beyond 15 ppm zinc are toxic. This confirms the highly Zn sensitive nature of this crop.

Inhibitory effects of heavy (toxic) metals on plant growth are well documented (Pahlsson, 1990) and have been demonstrated by many investigators through the measurement of growth parameters like root elongation (Trivedi and Erdei, 1992; Symeonidis and Karataglis, 1992), protein concentration (Sharma and Bisen, 1992) and fresh weight change (Greger et al., 1991). Marked changes in growth of mungbean seedlings are observed at different zinc regimes. It is well established that zinc stimulates growth at low concentrations by regulating a number of important metabolic processes responsible for growth and development of the plant (Alia et.al., 1995). The reduction in growth observed at higher concentrations of zinc is probably a consequence of its interference with certain essential metabolic events. Inspite of considerable literature available, the fundamental biochemical mechanism of zinc phytotoxicity has not yet been identified for any plant (Chaney, 1993).

Fluctuation in growth between 0.4 and 10 ppm zinc might be due to enzymic adjustment (adaptation process) in response to change in the optimum zinc level (Brune et al., 1994b; Thiyagarajah et al., 1996). It is a frequent observation that polypeptide patterns of plants, tissues or cells change considerably in response to stress regimes. In other words, maintenance of metabolic pathways in the presence of the stress may require up or down regulation of specific proteins.

Stunted growth in plants caused by heavy metals could be due to inhibition of cell elongation growth which might be controlled mainly by cell wall extensibility, although cell turgor plays an important role (Lockhart, 1965). Zinc has an inhibitory effect on cell elongation by suppressing cell wall extensibility (Aidid and Okamoto, 1993). It is possible that zinc may be extremely deterimental to the proton pump operating at the plasmalemma thereby affecting cell wall extensibility. Heavy metal zinc is toxic to plant growth. However, the degree of toxicity to a particular species of plants is quite different and depend on Zn^{++} ions accumulation which could ultimately contribute to the regulation of tissue dry weight and inhibition of enzymes (Aidid and Okamoto, 1993; Brune et al., 1994b).

A sensitive phenotypic indication of excess Zn in the rooting medium is inhibition of root elongation (Brune et al., 1994a). This may be illustrated with the ratio of leaf to root

lengths which increases after 15 ppm zinc. Interestingly, elevated Zn-concentrations cause low reduction in root biomass production probably due to high carbohydrate content in the roots (Cakmak et al., 1994). In contrast to root development, expansion of leaves is hardly affected. Similar findings were reported by Brune et al. (1994) in barley seedlings with zinc treatment. Tertiary roots at low level and adventitious roots from hypocotyl at toxic level (100 ppm) of zinc are developed as an alternate route for checking further increase in number of secondary roots and degenerated tap root respectively. This might be visualized as an adaptation toward the development of zinc tolerance in concentration of both deficiency and phytotoxicity.

Shoot/root ratios of lengths and dry matters are minimum at 0.4 and 10 to 15 ppm zinc levels. In between this and beyond 15 ppm zinc, these ratios are enhanced. Higher sensitivity of mungbean to zinc deficiency is associated with higher root growth at the expense of shoot growth. Similar observation has been made by Rengel and Graham (1995). Shoot/root dry weight ratio may therefore be a sensitive parameter for the evaluation of genotypes for their susceptibility to Zn deficiency. Lower shoot/root dry weight ratio is a well known phenomenon in P-deficient plants (Cakmak et al., 1994) and is considered as an adaptive response of plants for more efficient P acquisition from soils (Anghinoni and Barber, 1980). Also soil grown mungbean plants show higher sensitivity to Zn-deficiency (Kanwar and Randhawa, 1981). Thus, Zn - deficiency-induced enhancement of root growth may not be interpreted as an adaptive mechanism for Zn-acquisition in Zn-deficient genotypes. More likely, lower shoot /root dry weight ratio under zinc deficiency, in sensitive genotypes in particular, might be a reflection of Zn deficiency -induced photooxidative damage in shoots leading to lower shoot growth. Further, roots should become more competitive for photosynthates than the shoots, leading to lower shoot/root dry weight ratios in Zn deficiency. Similar observation has been made by Cakmak et al. (1996b) with wheat genotypes. Due to the root inhibition under zinc toxicity, shoot/root ratios are enhanced.

Low level of Zn affect considerably only the root anatomy while its toxic level affect the anatomy of all organs. Root is highly affected by zinc in terms of vascular bundle development,

deposition of blackish substance in intercellular spaces and a coating around cell wall. There is no considerable change in shoot anatomy on exposure to zinc. The change in the stele anatomy i.e. presence of numerous vascular bundles in pith and inside stele at 100 ppm Zn could be due to the emergence of adventitious roots from the stem which may contribute towards the tolerance to the plants in such a toxic level of zinc. No change is observed in total diameter, cortical cell layers and cortical cell size upto 10 ppm, however at higher concentrations there is decline. Decrease in cell diameter could be due to low water availability and formation of some blackish complex in the cytoplasm or zinc precipitation in the cell walls under the influence of zinc toxicity (Vazquez et al., 1994).

There remain uncertainties concerning the structure and the chemical composition of the Zn-rich deposits. The globular deposits from *Deschampsia caespitosa* were identified as Zn phytate crystals. Sequestration of Zn as phytate in vacuoles of root cortex cell has been proposed as a Zn-detoxification mechanism implicated in Zn tolerance (Van Steveninck et al., 1987). S-containing compounds such as glucosinolates are involved in Zn tolerance in cruciferae (Mathys, 1977). Zinc-containing electron dense deposits located in vacuoles, cell wall and intercellular spaces of epidermal and cortical cells in addition to high Zn concentrations always contained high amounts of P, K and Ca and a high Zn/P ratio (Vazquez et al., 1994). In this study accumulation of black deposits in the intercellular spaces is observed only in those mungbean seedlings that are provided with macro and micronutrients. Since these observations were made in 6 day old seedlings which at such a young age are expected to be self sufficient in the essential metabolites and ions that are needed to sustain growth, it is possible that these particles result from the complexing of the ions-which are in excess amount, with some other metabolites.

Due to the exogenous absence of zinc in case of DDW and 0.0 control there is no overall change in Zn level of whole seedlings. However, strong relative differences in organs could be due to differences in vascular bundles development and because of the fact that in DDW no nutrients are present whereas all nutrients minus zinc are present in 0.0 control. Further, the uptake of the specific mineral is known to be influenced by the presence of

other nutrients in many ways, e.g. Zn-P antagonism (Hale and Orcutt, 1987; Singh, 1991).

More Zn^{++} ions accumulate in zinc treated plants than control plants, which could be due to the involvement of zinc in maintaining the membrane integrity and H^{+}-ATPase dependent pH gradient accross the plasma membrane (Pinton, 1993). The organwise pattern of accumulation of zinc in 6 day old mungbean seedlings is found in the order of root> leaf> hypocotyl> stem> cotyledon on tissue dry weight basis. The order is changed to root> hypocotyl> stem > leaf >cotyledon on per organ basis. This may be attributed to faster delivery of excess zinc accumulated in roots by the transpiration stream to the leaves where it is mobilized in the phloem and may form organic complexes with citrate or malate or may be precipitated in the epidermal cell walls during evaporation of water by transpiration (Longnecker and Robson, 1993; Vazquez et al., 1994).

In the present study around 50% and 70% increase is found in shoot/root and leaf/root ratios of accumulated zinc on per gram tissue dry weight basis and per organ basis respectively in zinc deficient seedlings over optimum zinc grown seedlings. Similar results were reported by Brune et al. (1994a) in barley seedlings. There are no reports on the role of phytosiderophores in Zn translocation within the plants. Phytosiderophores are released from Zn deficient roots and are known to be effective in mobilizing by chelation sparingly soluble Zn compounds in calcareous soils leading to higher solubility and transport of Zn to the root surface (Treeby et al., 1989; Zhang et al., 1989). Phytosiderophores have also high capacity to mobilize Zn from root apoplast (Zhang et al., 1991) and are involved in inter and intracellular Fe transport in plant tissues (Mori et al., 1991). In a similar manner, Zn might be transported in plants as phytosiderophore complexes. Phytosiderophores (phytometallophores) might form stable complexes with Zn in the xylum sap and thereby contribute to Zn translocation to shoot (Welch, 1995).

For the tolerance towards high concentrations of Zn, plants are known to maintain low free Zn^{++} ions concentration in cytoplasm by specific compartmentation into the extraplasmic compartments of the cell i.e. cell wall and the vacuole (Brune et al., 1994a). In this study as well, the amount of zinc in cytoplasm is higher than cell wall. Zinc content in

different cell fractions i.e. total cytoplasm, cytoplasmic protein, cytoplasm without proteins and cell wall without proteins increases with zinc treatment. However, the level of zinc in total cell wall fraction remains unchanged upto 1 ppm then increases upto 75 ppm and decreases at 100 ppm Zn regime. There is more affinity of Zn^{++} ions towards cytoplasmic proteins than cell wall proteins. It appears that upto 1 ppm, Zn is transported to the cytoplasm then in order to maintain Zn homeostasis, cell wall chelates excess Zn with its proteins and the other components and thus a considerable increase in cell wall chelated extracellular matrix is 100 times faster than Zn uptake into the cells. Under low Zn nutrition highest Zn contents are observed in the cytoplasm while at inhibitory Zn concentrations in the hydroponic medium, Zn levels dramatically and preferentially increased in the apoplastic space. Thus the results suggest both cytoplasmic sequestration of Zn and an active participation of cell wall in Zn tolerance (Grill et al., 1985; Brune et al., 1994a; Hsieh et al., 1995).

Several functionally identifiable enzymes are found in the primary cell walls including peroxidases, glycosidases and phosphatases. Presumably these enzymes are important in cell wall metabolism and participate directly or indirectly in cell wall loosening and tightening, nutrient transport, recognition and disease resistance.

Activities of cell wall bound hydrolases and peroxidases in roots of 6 day zinc treated mungbean seedlings were studied. β-galactosidase was responsive only to high levels of zinc treatment and α-glucosidase showed no response at all. Although the galactosidases and glucosidases are not consistently correlated with growth, they are known to hydrolyse wall polymers which can cause structural changes in the cell wall (Dopico et al., 1989; Munoz et al., 1993). Significance of the acid phosphatase in cell wall is still unknown (Kiba et al., 1996). However, activation of this enzyme with Zn upto 25 ppm Zn treatment in root tissue may be related with its function in hydrolyzing and mobilizing phosphorus from organic compounds in the soil for plant nutrition. This view is supported by the observation of an elevated root cell wall phosphatase activity under phosphate deficiency conditions. In the structure of cell walls, the acid phosphatase acitivity has been found to be concentrated on the negatively charged exterior surface of the cell wall. Thus it is

expected that the acid phosphatase activity may be modified by the electrical status of cell walls. The induced stimulation or inhibition of bound acid phosphatases is quantitatively related to cation binding in the cell wall structure and has physiological significance in Zn-P antagonism (Tu et al., 1988).

Peroxidase activity increases with Zn concentration while low effect of Zn is recorded in the activity of cell wall bound polyphenoloxidase. Results provide evidence showing that cell wall bound peroxidase activity is inversely related to the growth of seedlings and roots especially at toxic Zn regimes and are in agreement with the findings of Goldberg et al. (1987)and Zheng et al. (1992). The cell wall bound peroxidase is a haemoprotein and is involved in the regulation of cell wall lignification, the cross linking between extensin and feruloylated polysaccharides and the conversion of ferulic acid to diferulic acid on polysaccharides (fry, 1986; Van Hystee, 1987). It is known that the diferulic acid formation by peroxidase catalysis is negatively related to cell wall extensibility and restrict the cell expansion. Another function of peroxidase in respect of cell elongation at low level of Zn is the lowering of isodityrosine formation or lignification of cell wall. It is known that cell wall bound peroxidase can catalyze the insolubilization of hydroxyproline-rich glycoprotein in the wall via crosslinking of isodityrosine and the level of insoluble hydroxyproline-rich glycoprotein is inversely related to cell wall extensibility (Cooper and Varner, 1983; Iiyma et al., 1994; Lee and Lin, 1995). Peroxidase and polyphenoloxidase have functional similarities. It has been suggested that plants contain either peroxidase or polyphenoloxidase but mungbean hypocotyl cell walls contain both enzymes (Chabnet et al., 1993). In our study enzyme shows stability and low sensitivity towards Zn treatments. Hence the enzyme can not be linked with Zn tolerance and its change in activity at low level of Zn may be attributed to other physiological factors.

Ionic tolerance is a general property of enzymes of the cell wall compartment, and the present study suggests that this tolerance permits the enzymes of Zn stressed seedlings to function normally in the conditions prevailing at their cell walls, and is not an adaptation. Although the conditions likely to occur in the walls of seedlings grown at toxic Zn concentrations are extreme in terms of ionic concentration, conditions in the apoplast will generally be much more variable than within the membrane-bound compartments of the cell.

On exposure to increasing concentrations of Zn, significant elevation in the amount of cell wall proteins are noted. Increased cell wall protein content at higher Zn dose may be explained by their increased synthesis and vesicular transport, decreased degradation or improved extractability of the apoplasm due to structural changes of the cell wall under heavy metal stress. However, there exists no convincing evidence yet to support any of these hypothesis (Brune et al., 1994b).

There is no alteration in the qualitative protein profile of cell wall under Zn stress as seen by SDS-PAGE. These results are in agreement with the findings of Obata et al. (1996). A 40 kDa cell wall polypeptide responsible for elongation growth in higher plants was identified by Reinard et al., (1994). In the present sutdy this cell wall protein was not influenced by zinc treatment.

On exposure to Zn, changes in quantitative and qualitative pattern of cytoplasmic proteins are observed. Content of cytoplasmic proteins declines with low levels of Zn and increases at toxic Zn concentrations. Since Zn functions as an essential element, uptake, metabolization and compartmentation are normal reactions taking place under unstressed conditions (Brune et al. 1994b).

Cytoplasmic contain 20 polypeptides ranging from 15 to 81 kDa in distilled water grown seedlings. Out of these two polypeptides of 69 and 71 kDa are the characteristic of distilled water grown seedlings only. Other polypeptides like 56, 51, 46, 35, 33 and 27 kDa exist only at low level of Zn then disappear at slightly higher concentrations and then reappear at toxic Zn concentrations. These proteins may be involved in regulating the tolerance to zinc. Thus, Zn may play a role in protein synthesis at the translation level and in the formation and suppression of some peptides at the transcription level (Obata et al., 1996).

The polypeptides of approximately 30 kDa are tentatively addressed as peroxidases. The increase in Zn concentration in the rooting medium coincides with the stimulation of peroxidase activity in cytoplasm (Brune et al., 1994b). A number of peroxidases have been characterized on a molecular basis after purification of the proteins or after molecular cloning. The molecular masses of these peroxidases fall into the same range of size. Increase in activities of peroxidase isoforms are frequently observed under stress conditions, for example, upon infection with mildew (Kerby and Somerville, 1992). Van Assche and Clijsters (1990) observed that

peroxidase activities increase in *Phaseolus vulgaris* upon heavy metal stress. It is likely that the 33 kDa protein detected in the present studies at toxic concentration of zinc is a member of this peroxidase family which function in conditions of stress. A low molecular weight 18 kDa polypeptide appear at higher Zn doses i.e. 25, 50, 75 and 100 ppm Zn concentrations. This low molecular weight polypeptide is perhaps concerned with the regulation of zinc stress. For the other Zn-induced polypeptides, no such suggestion of functions is yet possible.

Recent research implicates certain peptides (phytochelatins, PCs) in metal tolerance. The proposed PC mediated metal detoxification involves induction of PC synthesis in the presence of a metal, metal-PC complex formation and its sequestration in the vacuole (Steffens, 1990; Rauser, 1990). Different steps of this scheme have been experimentally proven in case of Cd only (Tukendorf, 1993). Reports about other metals are inconsistent. There is a single report about phytochelatin synthesis in plants i.e. maize seedlings in response to excess Zn (Tukendorf, 1996). In this study aminoacid analysis of Zn-induced 18 kDa polypeptide shows that it is rich in lysine and tyrosine besides leucine, arginine, phenylalanine and cysteine. Thus it is possible that it is a metallothionein like polypeptide (Phytochelatin) containing cysteine-rich domains (Kagi and Schaffer, 1988; Hsieh et al., 1995). PCs can be synthesised very rapidly (within 5 min.) in response to metals entering the cells, even when protein synthesis is totally inhibited (Scheller et al., 1987). The ability to bind Zn might be anticipated in any protein containing so many SH groups. It should be noted that phytochelatins with molecular masses of 4 - 10 kDa (Grill et al., 1987) which are known to be induced by heavy metals are not resolved in the 10% SDS - PAGE. Therefore Zn-induced 18 kDa polypeptide might be MT-like (class I or II) protein as aminoacid analysis of this does not show the presence of glutamic acid and glycine and matches with the sequence of MT-like protein i.e. Cys- x - Cys, where x is an aminoacid other than cysteine.

Since roots are in contact with various metals in soil earlier and longer than other organs during growth and contain more transcripts of MT-like genes, it is possible that MT-like proteins in plants play a scavanger role in chelating excess heavy metals (Hsieh et al., 1995). Obviously PCs are not the only means available to plants for managing internal metal

concentrations. There could be multiple strategies involved in tolerating different metals. Proline accumulation has been linked with stress (particularly water deficit) resistance and has been ascribed a variety of functions (Aspinall and Paleg, 1981). Absence of the proline in Zn-induced 18 kDa polypeptide in this study ruled out its adaptive significance against zinc toxicity. However, proline accumulation under zinc toxicity in plants has been reported (Alia et al., 1995). Rigorous research, however, is needed to establish the suggested involvement of individual domains of proteins.

"It must be mentioned that the effect of zinc reported in this thesis pertains to very young seedlings. Therefore, caution would have to be exercised while extrapolating these results in older plants".

REFERENCES

1. Aidid, S.B. and Okamoto, H. (1993). Responses of elongation growth rate, turgor pressure and cell wall extensibility of stem cells of *Impatiens balsamina* to lead, cadmium and zinc. **BioMetals 6:** 245-246.
2. Albersheim, P. (1965). Biogenesis of the cell wall. In: *Plant Biochemistry* (Bonner, J. and Varner, J.E. eds.), Academic Press, New York, pp. 298-321.
3. Alia; Prasad, K.V.S.K. and Saradhi, P.P. (1995). Effect of zinc on free radicals and proline in Brassica and Cajanus. **Phytochemistry 39:** 45-47.
4. Anghinoni, I. and Barber, S.A. (1980). Phosphorus influx and growth characteristics of corn roots as influenced by phosphorus supply. **Agron. J. 72:** 685-688.
5. Arora, S. (1997). Study of some selected physico-chemical parameters of the effluent of Mahalakshmi Sugar Mills Ltd. Iqbalpur, Haridwar (U.P.), **MSc. Project Report,** Gurukul Kangri University, Haridwar, India.
6. Aspinall, D. and Paleg, L.G. (1981). Proline accumulation: Physiological aspects In: *The Physiology and Biochemistry of Drought Resistance in Plants* (Paleg, L.g. and Aspinall, D. eds.), Academic Press, Australia.
7. Assche, F. Van and Clijsters, H. (1990). Effects of metals on enzyme activity in plants. **Plant cell Environ. 13:** 195-206.
8. Baker, A.J.M. (1987). Metal tolerance. **New Phytol. 106** (Suppl.): 93-111.
9. Barber, S.A. (1984). In : *Soil Nutrient Bioavailability,* a mechanistic approach, a Wiley Interscience publication, John Wiley and Sons, USA.
10. Bar-Num, N. and Poljakoff-Mayber, A. (1977). Salinity stress and the content of proline in roots of *Pisum sativum* and *Tamerix tetraguna*. **Ann. Bot. 41**: 173-179.
11. Bozarth, C.; Mullet, J.E. and Boyer, J.S. (1987). Cell wall proteins at low water potentials. **Plant Physiol. 85:** 261-267.

12. Bradly, D.J.; Kjellbom, P. and Lamb, C.J. (1992). Elicitor and wound - induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defence response. **Cell 70:** 21-30.

13. Brune, A.; Urbach, W. and Dietz, K.J. (1994 a). Compartmentation and transport of zinc in barley primary leaves as basic mechanism involved in zinc tolerance. **Plant Cell Environ. 17 :** 153 - 62.

14. Brune, A.; Urbach, W. and Dietz, K.J. (1994 b). Zinc stress induces changes in apoplasmic protein content and polypeptide composition of barley primary leaves. **J. Exp. Biol. 45:** 1189 - 1196.

15*. Cakmak, I . (1988). Morphologische and physiologische veranderungen bei Zink mangelpflanzen. **Ph.D. thesis,** University Hohenheim.

16. Cakmak, I; Hengeler, C. and Marschner, H. (1994). Partitioning of shoot and root dry matter and carbohydrates in bean plants suffering from phosphorus, potassium and magnesium deficiency. **J. Exp. Bot. 45 :** 1245 - 1250.

17. Cakmak, I and Marschner, H. (1987). Mechanism of phosphorus induced zinc deficiency in cotton. III. Changes in physiological availablity of zinc in plants. **Physiol. Plant. 70:** 13-20.

18 Cakmak, I. and Marschner, H. (1988). Increase in membrane permeability and exudation in roots of zinc deficient plants. **J. Plant Physiol. 132 :** 361 - 365.

19 Cakmak, I. and Marschner, H. (1990) . Decrease in nitrate uptake and increase in proton release in Zn-deficient cotton, sunflower and buckwheat plants. **Plant and Soil 129:** 261 - 268.

20. Cakmak, I.; Yilmaz, A.; Kalayci, M.; Ekiz, H.; Torun, B.; Erenoglu, B. and Braun, H.J. (1996 a). Zinc deficiency as a critical problem in wheat production in central Anatolia. **Plant and Soil 180 :** 165 - 172.

21. Cakmak, I.; Sari, N.; Marschner, H.; Kalayci, M.; Yilmaz, A.; Eker , S. and Gulut, K.Y. (1996 b). Dry matter production and distribution of zinc in bread and durum wheat genotypes differing in zinc efficiency. **Plant and Soil 180 :** 173 - 181.

22. Cakmak, I.; Sari, N.; Marschner , H.; Ekiz, H.; Kalayci, M.; Yilmez, A. and Braun, H.J. (1996 c) Phytosiderophore release in bread and durum wheat genotypes differing in zinc efficiency. **Plant and Soil 180:** 183 - 189.

23. Casterline, J.L. Jr. and Barnett, N.M. (1977). Isolation and characteristics of cadmium-binding components in soyabean plant. **Plant Physiol. 59** (suppl.):673.

24. Chabnet, A.; Catesson, A.M. and Goldberg, R. (1993). Peroxidase and phenoloxidase activities in mungbean hypocotyl cell walls. **Phytochemistry 33:** 759-763.

25*. Chaney, R.L. (1993). *Zinc in Soils and Plants* (Robson, A.D. ed.), Kluwer, Academic Publishers, Dordrecht. The Netherlands, pp. 135.

26*. Clarkson, T. and Hanson, B.J. (1980). **Ann. Rev. Plant Physiol. 31:** 239-298.

27*. Clarkson, D.T. and Luttge, U. (!989). Mineral Nutrition: Divalent cations, transport and compartmentation. **Progress in Botany 51:** Berlin, Springer-Verlag, 93-112.

28. Cooper, J.B. and Varner, J.E. (1983). Insolubilization of hydroxyproline-rich cell wall glycoprotein in aerated carrot root slices. **Biochem. Biophys. Res. Commun. 112:** 161-167.

29. Dopico, B.: Nicolas, G. and Labrador, E. (1989). Partial purification of cell wall β-galactosidases from *Cicer arietinum* epicotyls. Relationship with cell wall autolytic process. **Physiol. Plant. 75:** 458-64.

30 Dudka, S.; Piotrowska, M. and Terelak, H. (1996). Transfer of Cd, Pb, and Zn from industrially contaminated soil to crop plants: A field study. **Environ. Pollut. 94:** 181-188.

31. Dugas, H. (1989). Metal ions. In: *Bioorganic Chemistry* (Cantor, C.R. ed.), Springer-Verlag, New York, pp. 398-488.

32. * Fischer, R.A. and Yates, F. (1948). In: *Statistical Tables for biological, agricultural and medical research*, Oliver and Boyd, London.

33. Foy, C.D.; Chaney, R.L. and White, M.C. (1978). The physiology of metal toxicity in plants. **Ann. Rev. Plant Physiol. 29:** 511-566.

34. Francis, D.; Davies, M.S.; Braybrook, C.; James, N.C. and Herbert, R.J. (1995). An effect of zinc on M-phase and G1 of the plant cell cycle in the synchronous TBY—2 tobacco cell suspension. **J. Exp. Bot. 46:** 1887-1894.

35. Fry, S.C. (1986). Cross-linkage of matrix polymers in the growing cell walls of angiosperms. **Ann. Rev. Plant Physiol. 38:** 205-219.

36. Gallego, S.M.; Benavides, M.P. and Tomaro, M.L. (1996). Effect of heavy metal ion excess on sunflower leaves: evidence for involvement of oxidative stress. **Plant Science 121:** 151-159.

37. Geiger, D.R. (1975). Phloem loading. In: *Encyclopedia of Plant Physiology* (Zimmerman, M.H. and Milbern, J.A. eds.), New series, Vol. 1, Chap. 17, Springer-Verlag, Berlin, pp. 395-431.

38. Goldberg, H.; Liberman, M.; Mathieu, C.; Pierron, M. and Catesson, A.M. (1987). Development of epidermal cell wall peroxidase along the mungbean hypocotyl: possible involvement in the cell wall stiffening process. **J. Exp. Bot. 38:** 1378-1390.

39. Graham, R.D. and Rengel, Z. (1993). Genotypic variation in zinc uptake and utilization by plants. In: *Zinc in Soils and Plants* (Robson, A.D. ed.), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 107-118.

*40. Greger, M.; Brammer, E.; Lindberg, S. et al. (1991). Uptake and physiological effects of cadmium in sugar beet *(Beta vulgaris)* related to mineral provision. **J. Exp. Bot. 42:** 729-737.

41. Grill, E.; Loeffler, S.; Winnacker, E.L. and Zenk, M.H. (1989). Phytochelatins, the heavy metal binding peptides of plants are synthesised from glutathione by a specific gamma-glutamyl cysteine dipeptidyl transpeptidase (phytochelatin synthase), **Proc. Natl. Acad. Sci. (USA) 86:** 6838-42.

42. Grill, E.; Winnacker, E.L. and Zenk, M.H. (1985). Phytochelatins: The principal heavy-metal complexing peptides of higher plants. **Science 230:** 674-694.

43. Grill, E.; Winnacker, E.L. and Zenk, M.H. (1987). Phytochelatins, a class of heavy metal binding peptides from plants are functionally analogous to metallothioneins. **Proc. Natl. Acad. Sci. (USA) 84:** 439-443.

44. Gupta, S. (1995). Bioprocess development of citric acid production. **Ph.D. Thesis,** University of Roorkee, India.

45. Gupta, R. (1996). Study of soil climatic zones of sugarcane in India. **M.E. Project Report, WRDTC,** University of Roorkee, India.

46. Gutschick, V.P. (1993). Nutrient-limited growth rates: Roles of nutrient-use efficiency and of adaptation to increase nutrient uptake. **J.Exp.Bot.44:** 41-51.

47. Gutschick, V.P. and Key, L.E. (1995). Nutrient-limited growth rates: Quantitative benifits of stress responses and some aspects of regulation. **J.Exp.Bot.46:** 995-1009.

48. Hale and Orcutt (1987). *The Physiology of Plants Under Stress.* John Wiley and Sons, USA.

49. Halvorson, A.D. and Lindsay, W.L. (1977). The critical Zn^{++} concentration for corn and the nonabsorption of chelated zinc. **Soil Sci. Soc. Am. J. 41:** 531-534.

50. Hanna, L.A.; Peters, J.M.; Wiley, L.M.; Clegg, M.S. and Keen, C.L. (1997). Comparative effects of essential and nonessential metals on preimplantation mouse embryo development in vitro. **Toxicology 116:** 123-131.

51. Hoagland, D.R. and Arnon, D.I. (1938). The water culture method for growing plants without soil. University of California Agricultural Experiment Station **Circular 347.**

52. Hsieh, H.M; Liu, W-K. and Huang, P.C. (1995). A novel stress-inducible metallothionein-like gene from rice. **Plant Mol. Biol. 28:** 381-389.

53. Hu,D.; Bell, R.W. and Xie, Z. (1996). Zinc and phosphorus responses in transplanted oilseed rape *(Brassica napus).* **Soil Sci. Plant Nutr. 12:** 333-344.

54. Iiyama, K.; Lam, T.B.T. and Stone, B.A. (1994). Covalent crosslinks in the cell wall. **Plant Physiol. 104:** 315-320.

55. Jackson, M.L. (1969). *Soil Chemical Analysis* Advanced Course, 2nd ed. Madison, Wisconsin, USA.

56. Kabata - Pendias, A. and Pendias, H. (1984). *Trace Elements in Soils and Plants.* Boca Ratan, FL.CRC Press, U.S.A.

57. Kagi J.H.R and Kojima, Y. (1987). Chemistry and biochemistry of metallothionein. In : *Metallothionein II.* (Kagi, J.H.R. and Kojima. Y. eds.), Basel : Birkhauser verlag, pp 25 - 61.

58. Kagi, J.H.R. and Schaffer, A . (1988). Biochemistry of metallothionein. **Biochemistry 27:** 8509 - 15.

59. Kaiser, W.M. and Heber, U. (1981). Photosynthesis under osmotic stress. **Planta 153:** 423 - 31.

60. Kanwar, J.S. and Randhawa, N.S. (1981). Micronutrient research in soils and plants in India. **ICAR Technical Bulletin (Agric) No. 50 :** 115.

61*. Kar, M. and Mishra, D. (1976). **Plant Physiol. 57:** 315.

62. Katyal , J.C.; Das, S.K.; Sharma, K. L. and Saharan, N. (1992). Interactions of zinc in soil and their management. **Fert. News 37** : 27 - 33.

63. Katyal, J.C. and Rattan, R.K. (1993). Distribution of zinc in Indian soils. **Fert. News 38:** 15 - 26.

64. Kerby, K. and Somerville, S.C. (1992). Purification of an infection-related, extracellular peroxidase from barley. **Plant Physiol. 100:** 397 - 402.

65 Kiba, A; Toyoda, K.; Ichinose, Y.; Yamada, T. and Shiraishi, T. (1996). Specific response of partially purified cell wall - bound ATPases to fungal suppressor. **Plant Cell Physiol. 37:** 207 - 214.

66. Kitagishi, K. and Obata, H. (1986). Effects of zinc deficiency on the nitrogen metabolism of meristematic tissues of rice plants with reference to protein synthesis. **Soil Sci. Plant Nutr. 32 :** 397 - 405.

67. Kumar, C.S.V.R.; Karunasree, B. and Reddy, A.R. (1996). *Molecular approaches to abiotic stress tolerance.* 5th Ann. meeting Natl. Rice Biotech network Abstracts, IARI, New Delhi, Nov. 13 -16, 1996.

68. Kupper, H.; Kupper, F. and Spiller, M. (1996). Environmental relevance of heavy metal - substituted chlorophylls using the example of water plants. **J. Exp. Bot. 47 :** 259 - 266.

69. Kustchera, U. (1994). The current status of acid growth hypothesis. **New Phytol. 126 :** 549 - 569.

70. Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. **Nature 227 :** 680 - 685.

71. Lakkarpragada, S.N.K. (1989). Mineral load and transport in Upper Ganga basin. **Ph.D. Thesis,** University of Roorkee, India.

72. Lamport, D.T.A and catt, J.W. (1981). Glycoproteins and enzymes of the cell wall. In: *Plant Carbohydrates II* (Tanner, W. and Loewus, F.A eds.), Springer - verlag , Berlin, pp. 133 - 165.

73. Lee, T.M. and Lin, Y.H. (1995). Changes in soluble and cell wall- bound peroxidase activities with growth in anoxia - treated rice *(Oryza sativa* L.*)* coleoptiles and roots. **Plant Science 106:** 1-7.

74. Lin , K.Y.; Chang, B.V. and Wang, Y.S. (1996). Mobility of copper, zinc and chromium with municipal solid waste leachate in soils. **Proc. Natl. Sci. Council ROC, Life Sciences 20:** 19-25.

75. Lindsay (1972). Zinc in soils and plant nutrition. **Adv. in Agron. 24 :** 147-186.

76. Lindsay, W.L. and Norvell, W.A. (1978). Development of a DTPA soil test for zinc, iron, manganese and copper. **Soil Sci. Soc. Am. J. 42 :** 421-428.

77. Lockhart, J.A. (1965). An analysis of irreversible plant cell elongation. **J. Theoret. Biol. 8 :** 264-275.

78. Loeffler, S. ; Hochberger, A.; Grill, E.; Winnacker, E.L. and Zenk, M.H. (1989). Termination of the phytochelatin synthase reaction through sequestration of heavy metals by the reaction product. **FEBS Letters 258:** 42-47.

79. Longnecker, N.E. and Robson, A.D. (1993). Distribution and transport of zinc in plants. In : *Zinc in Soils and Plants* (Robson, A.D., eds.), Kluwer Academic Publishers. Dordrecht, pp. 79-91.

80. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L. and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. **J. Biol. Chem. 193 :** 265-275.

81. Luo, Y. and Rimmer, D.L. (1995). Zinc-Copper interaction affecting plant growth on a metal - contaminated soil. **Environ. Pollut. 88 :** 79-83.

82. Macnair, M.R. (1993). The genetics of metal tolerance in vascular plants. **New Phytol. 124 :** 541-59.

83. Margoches, M. and Vallee, B.L. (1957). A cadmium protein from Equine kidney cortex. **J. Am. Chem. Soc. 79 :** 4813-4814.

84. Marschner, H. (1986). *Mineral Nutrition in Higher Plants.* Academic Press, Harcourt Brace Jovanovich Publishers, London.

85. Marschner, H. (1993). Zinc uptake from soils. In : Zinc in Soils and Plants (Robson, A.D. ed.). Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 59-77.

86. Marschner, H. and Cakmak, I. (1989). High light intensity enhances chlorosis and necrosis in leaves of zinc, potassium and magnesium deficient bean *(Phaseolus vulgaris)* plants. **J. Plant Physiol. 134:** 308-315.

87. Massay, H.F. (1957). Relation between diathiozone extractable Zn in the soil and Zn uptake by corn plants. **Soil Science 83:** 123-129.

88. Masuda, H.; Komiyana, S. and Sugawara, S. (1988). Extraction of enzymes from cell walls of sugar beet cells grown in suspension culture. **Plant Cell Physiol. 29:** 623-627.

89. Mathys, W. (1977). The role of malate oxalate and mustard oil glucosides in the evolution of zinc-resistance in herbage plants. **Physiol. Plant. 40 :** 130-136.

90*. Maze, P. (1914). Respectives des elements de solution minerals sur le development du mais. **Ann. Inst. Pasteur. 28:** 21-69.

91. McQueen - Mason, S.J. and Cosgrove, D.J. (1995). Expansin mode of action on cell walls. Analysis of wall hydrolysis, stress relaxation, and binding. **Plant Physiol. 107**: 87-100.

92. Mehrotra, N.K.; Dube, B.K. and Agarwala. S.C. (1983). Effect of Zn deficiency on uptake of Zn, Mn and P and activity of some enzymes in four pigeonpea genotypes. **Ind. J. Agri. Sci. 53.**

93. Melton, J.R.; Mahtab, S.K. and Swoboda, A.R. (1973). Diffusion of zinc in soils as a function of applied zinc, phosphorus and soil pH. **Soil Sci. Soc. Am. Proc. 37** : 379-381.

94. Mench, M.; Vangronsveld, J.; Didier, V. and Clijster, H. (1994). Evaluation of metal mobility, plant availability and immobilization by chemical agents in a limed - silty soil. **Environ. Pollut. 86**: 279-286.

95. Micronutrient Bureau (1994). Zinc - the most important micronutrient. Perth symposium - part 2. **Micronutrient news and information. 14(3)**, Perth, Western Australia.

96. Mori, S.; Nishizawa, N., Hayashi, H.; Chino, M., Yoshimura, E. and Ishihara, J. (1991). Why are young rice plants highly susceptible to iron deficiency? **Plant and Soil 130:** 143-156.

97. Munoz, F.J.; Labrador, E. and Dopico, B. (1993). Effects of osmotic stress on the growth of epicotyls of ***Cicer arietinum*** in relation to changes in the autolytic process and glycan hydrolytic cell wall enzymes. **Physiol. Plant. 87** : 544-51.

98. Naidu, B.P.; Paleg, L.G., Aspinall, D.; Jennings, A.C. and Jones, G.P. (1991). Aminoacid and glycine betaine accumulation in cold stressed wheat seedlings. **Phytochemistry 30** : 407-409.

99. Neiboer, E. and Richardson, D.H.S. (1980). The development of nondescriptive term heavy metal by a biologically and chemically significant classification of metal ions. **Environ. Pollut. Ser. B.I.** : 3-26.

100. Nurain, N. (1996). Ground water quality - a case study. **M.E. Project Report,** WRDTC, University of Roorkee, India.

101. Obata, H.; Hayashi, A.; Toda. T. and Umebayashi, M. (1996). Effects of zinc deficiency on the growth proteins and other constituents of yeast *Saccharomyces cerevisiae* cells. **Soil Sci. Plant Nutr. 12** : 147-154.

102. Obata, H.; Toda, T. and Umebayashi, M. (1994). Effects of zinc deficiency on peptide synthesis in cultured tobacco plant cells. **Soil Sci. Plant Nutr. 40** : 149-154.

103. Obata, H. and Umebayashi, M. (1988). Effect of zinc deficiency on protein synthesis in cultured tobacco plant cells. **Soil Sci. Plant Nutr. 34 :** 351-357.

104. Odds, F.C. and Hierholzer, J.C. (1973). Purification and properties of a glycoprotein acid phosphatase from *Candida albicans*. **J. Bact. 114 :** 257-266.

105. Oien, A and Gjerdingen, K. (1977). Determination of cadmium and lead in soils by means of solvent extraction and atomic absorption. **Acta Agric. 27 :** 67-70.

106. Ostle, V. (1954). *Statistics in Research.* Oxford and IBH Publications, New Delhi.

107. Pahlsson, A.M.B. (1990). Toxicity of heavy metals (Zn, Cu, Cd, Pb) to vascular plants. **Water Air and Soil Pollution 47 :** 287-319.

108. Parkash, R. and Iyenger, E.R.R. (1990). Effect of presowing soaking treatment with zinc and cobalt on growth, catalase activity and total sugars in Jojoba (*Simmondsia chinensis* L.). **Ind. J. Plant Physiol. 34 :** 246-249.

109. Peterson, P.J. (1969). The distribution of zinc - 65 in *Agrostis tenuis sibth* and A. *Stolonifera* L. tissues. **J. Exp. Bot. 20** : 863-875.

110. Pinton, R.; Cakmak, I. and Marschner, H. (1993). Effect of zinc deficiency on proton fluxes in plasma membrane - enriched vesicles isolated from bean roots. **J. Exp. Bot. 44 :** 623-630.

111. Pinton, R.; Cakmak, I. and Marschner, H. (1994). Zinc deficiency enhanced NAD (P) H-dependent superoxide radical production in plasma membrane vesicles isolated from roots of bean plants. **J. Exp. Bot. 45 :** 45-50.

112. Piper, C.S. (1966). *Soil and Plant Analysis.* Indian Reprint, Hans Publishers, Bombay.

113. Poorter, H. and Garnier, E. (1996). Plant growth analysis: an evaluation of experimental design and computational method. **J. Exp. Bot. 47 :** 1343-1351.

114. Poulter, A.; Collin, H.A.; Thurman, D.A. and Hardwick, K. (1985). The role of the cell wall in the mechanism of lead and zinc tolerance in *Anthoxanthum odoratum* L. **Plant Science 42 :** 61-66.

115. Rahman, S.; Takaki, H.; Tamai, M. and Nagatomo, Y. (1996). Distribution of zinc manganese, copper, cobalt and nickel in andosole profiles. **Soil Sci. Plant Nutr. 42 :** 881-891.

116. Raman Rao, T.C.; Bhagyalakshmi, K.V. and Thuljaram Rao, J. (!979). *Indian Atlas of Sugarcane.* SBI, Coimbatore.

117*. Raulin, J. (1869). Etudes cliniques sur la vegetation. **Ann. Sci. Nat. Bot. Biol. Veg. 11** : 93.

118. Rauser, W.E. (1990). Phytochelatins. **Ann. Rev. Biochem. 59** : 61-86.

119. Rauser, W.E. and Samarakoon, A.B. (1980). Vein loading in seedlings of *Phaseolus vulgaris* exposed to excess cobalt, nickel and zinc. **Plant Physiol. 65** : 578-584.

120. Reid, J.B. (1990). Phytohormone mutants in plant research. **J. Plant Growth Reg. 9:** 97-111.

121. Reinard, T.; Sprunck S.; Altherr, S. and Jacobsen, H.J. (1994). Biochemical properties of a novel cell wall protein associated with elongation growth in higher plants. **J. Exp. Bot. 45 :** 1593-1601.

122. Rengel, Z. and Graham, R.D. (1995). Wheat genotypes differ in zinc efficiency when grown in the chelate-buffered nutrient solution I. **Growth Plant and Soil 176 :** 307-316.

123. Rengel, Z. and Graham, R.D. (1996). Uptake of zinc from chelate-buffered nutrient solutions by wheat genotypes differing in zinc efficiency. **J. Exp. Bot. 47 :** 217-226.

124. Rodriguez, J.G. and Cardemil, L. (1995). Tissue specific expression of cell wall proteins of seedlings of *Prosopis chilensis* during the development and wound stress. **Physiol. Plant. 93 :** 457-463.

125. Sandstead, H.H. (1994). Understanding zinc : Recent observations and interpretations. **J. Lab. Clin. Med. 124** : 322-327.

126. Scheer, H. and Hartwich, G. (1995). Bacterial reaction centers with modified tetrapyrrol chromophores. In : *Anoxygenic Photosynthetic Bacteria* (Blankenship, R.E., Madiagan, M.T. and Bauer, C.E. eds.), Kluwer Academic Publishers, Dordrecht. pp 649-663.

127. Scheller, H.V.; Haung, B.; Hatch, E. and Goldsbrough, P.B. (1987). Phytochelatin synthesis and glutathione levels in response to heavy metals in tomato cells. **Plant Physiol. 85**: 1131-35.

128. Seminar report (1986). *Problems of water logging and salinity in agricultural soils.* New Delhi, May 2-4, 1986, sponsored by Govt. of India, Ministry of water resources and Govt. of Netherlands.

129. Sharma, C.P.; Sharma, P.N.; Bisht, S.S. and Nautiyal, B.D. (1982). Zinc deficiency induces changes in cabbage. In : *Proceeding of the Ninth Plant Nutrition Colloquium* (Scaife A. ed.). *Commonw, Agric. Bur*, Farnham Royal, Bucks, pp. 601-606.

130. Sharma, P.N.; Tripathi, A. and Bisht, S.S. (1995). Zinc requirement for stomatal opening in cauliflower. **Plant Physiol. 107 :** 751-756.

131. Sharma, S.K. and Bisen, P.S. (1992). Hg^{++} and Cd^{++} induced inhibition of light induced proton efflux in the cyanobacterium, *Anabaena flos-aquae.* **Bio Metals 5 :** 163-167.

132. Showalter, A.M. (1993). Structure and function of plant cell wall proteins. **The Plant cell 5** : 9-23.

133*. Shukla, U.C. and Prasad, K.G. (1976). **Ind. J. Agric. Chem. 9 :** 31.

134. Sillanpaa, M. (1982). Micronutrients and the nutrient status of soils. A global study. **FAO Soils Bulletin, No.** 48, FAO, Rome, Italy.

135. Singh, J. (1991). Influence of mineral nutrition on RNA metabolism in groundnut *(Arachis hypogea)* seedlings. **M.Sc. Project Report**, University of Roorkee, India

136. Singh, K.; Shukla, U.C. and Karwasra, S.P.S. (1987). Chemical assessment of the zinc status of some soils of the semi-arid region of India. **Fert. Res. 13 :** 191-197.

137*. Somner, A.I. and Lipman, C.B. (1926). Evidence on the indispensable nature of zinc and boron for higher green plants. **Plant Physiol. 1:** 231-244.

138. Sreekrishna, K.; Jones, C.E.; Guetzow, A.; Prasad, M.R. and Joshi, V.C. (1979). A modified method for aminoacid analysis with ninhydrin of coomassie-stained proteins from polyacrylamide gels. **Anal. Biochem. 103 :** 55-57.

139. Steffens, J.C. (1990). The heavy metal binding peptides of plants. **Ann. Rev. Plant Physiol. Plant Mol. Biol. 41 :** 553-575.

140. Steffens, J.C.; Hunt, D.F. and Williams, B.G. (1986). Accumulation of non-protein metal-binding polypeptides (gamma-glutamylcysteinyl)$_n$ -glycine in selected cadmium resistant tomato cells. **J. Biochem. 261 :** 13879-82.

141. Stohs, S.J. and Bagchi, D. (1995). Oxidative mechanisms in the toxicity of metal ions. **Free Rad. Biol. Med. 18 :** 321-326.

142. Stoop, J.M.H.; Williamson, J.D. and Pharr, D.M. (1996). Mannitol metabolism in plants: a method for coping with stress. **Trends in Plant Science 1 :** 139-144.

143. Symeonidis, L. and Karataglis, S. (1992). Interactive effects of cadmium, lead and zinc on root growth of two metal tolerant genotypes of *Holcus lanatus* L. **Bio Metals 5 :** 173-178.

144. Taiz, L. (1984). Plant cell expansion : regulation of cell wall mechanical properties. **Ann. Rev. Plant Physiol. 35 :** 585-657.

145. Thakur, P.S. and Rai, V.K. (1982). Dynamics of aminoacid accumulation of two differentially drought resistant *Zea mays* cultivars in response to osmotic stress. **Env. Exp. Bot. 22 :** 221-226.

146. Thiyagarajah, M.; Fry, S.C. and Yeo, A.R. (1996). In vitro salt tolerance of cell wall enzymes from halophytes and glycophytes. **J. Exp. Bot. 47** : 1717-1724.

147. Tomsett, A.B. and Thurman, D.A (1988). Molecular biology of metal tolerance of plants. **Plant Cell and Environ. 11 :** 383-94.

148. Treeby, M.; Marschner, H. and Romheld, V. (1989). Mobilization of iron and other micronutrient cations from a calcareous soil by plantborne, microbial and synthetic metal chelators. **Plant and Soil 114** : 217-226.

149. Trivedi, S. and Erdei, L. (1992). Effects of cadmium and lead on the accumulation of Ca^{++} and K^+ and on the influx and translation of K^+ in wheat of low and high K^+ status. **Physiol. Plant. 84 :** 94-100.

150. Tu, S-I.; Brouillette, J.N.; Nagahashi G. and Kumosinski, T.F. (1988). Effects of multivalent cations on cell wall associated acid phosphatase activity. **Plant Physiol. 88 :** 61-68.

152. Tukendorf, A. (1993). The response of spinach plants to excess of copper and cadmium. **Photosynthetica 28 :** 573-575.

152. Tukendorf, A. (1996). Phytochelatin synthesis in maize seedlings in response to excess zinc. **Biol. Plant. 38 :** 137-140.

153. Vallee, B.L. and Auid, D.S. (1991). *Matrix metalloproteinases and inhibitors* (Birkedal Hesen, H; Werb 2; Welgus, H. and Vanward, H. E. eds.), Guster Fischer Verlag, Stuffgart.

154. VanHuystee, R.B. (1987). Some molecular aspects of plant peroxidase biosynthetic studies. **Ann. Rev. Plant Physiol. 37 :** 165-186.

155. Van Steveninck, R.F.M.; Van steveninck, M.E.; Fernando, D.R.; Godbold, D.L.; Horst, W.J. and Marschner, H. (1987). Identification of zinc-containing globules in roots of a zinc tolerant ecotype of *Deschampsia caespitosa.* **J. Plant Nutr. 10:** 1239-1246.

156. Vazquez, M.D.; Poschenrieder, Ch.; Barcelo, J.; Baker, A.J.M.; Hatton, P. and Cope, G.H. (1994). Compartmentation of zinc in roots and leaves of the zinc hyperaccumulator *Thalaspi caerulescens* J and C Presp. **Bot. Acta 107** : 243-250.

157. Voutsa, A.; Grimanis, A. and Samara, C. (1996). Trace elements in vegetables grown in an industrial area in relation to soil and air particulate matter. **Environ. Pollut. 94:** 325-335.

158. Wakao, N.; Yokoi, N.; Isoyama, N.; Hiraishi, A.; Shimada, K.; Kobayashi, M; Kise, H.; Iwaki, M.; Itoh, S.; Takaichi, S. and Sakurai, Y. (1996). Discovery of natural photosynthesis using Zn - Containing Bacteriochlorophyll in an aerobic bacterium *Acidiphilium rubrum*. **Plant cell Physiol. 37 :** 889 - 893.

159. Wang J.; Evangelou, B.P.; Nielsen, M.T. and Wagner, G.J. (1992). Computer - simulated evaluation of possible mechanisms for sequestering metal ion activity in plant vacuoles. **Plant Physiol. 99 :** 621-626.

160. Warncke. D.D. and Barber, S.A. (1972). Diffusion of zinc in soil : I. The influence of soil moisture. **Soil Sci. Soc. Am. Proc. 36 :** 39-42.

161. Watanabe, T. and Kobayashi M. (1991). Electrochemistry of chlorophylls. In : *Chlorophylls* (Scheer H. ed.), CRC Press, Boca Ratan. pp. 287-315.

162. Welch, R.M. (1995). Micronutrient nutrition of Plants. **Crit. Rev. Plant Sci. 14 :** 49-82.

163. Welch, R.M. and Norvell, W.A. (1993). Growth and nutrient uptake by barley (*Hordium vulgare* L. cv. Herta) : Studies using an N (2-hydroxyethyl) ethylenedinitrilotriacetic acid - buffered nutrient solution technique. II. Role of zinc in the uptake and root leakage of mineral nutrients. **Plant Physiol. 101 :** 627-631.

164. Wessel, D. and Flugge, U.I. (1984). A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. **Anal. Biochem. 138 :** 141-143.

165. Wilkins, D.A. (1978). The measurement of tolerance to edaphic factors by means of root growth. **New Phytol. 106 :** 679-688.

166. Wilkinson, H.F. Loneragan, J.F. and Quirk, J.P. (1968). The movement of zinc to plant roots. **Soil Sci. Soc. Am. Proc. 32 :** 831-833.

167. Wiren, N.V.; Marschner, H. and Romheld, V. (1996). Roots of iron - efficient maize also absorb phytosiderophore - chelated zinc. **Plant Physiol. 111 :** 1119-1125.

168. Woolhouse, H.W. (1983). Toxicity and tolerance in the response of plants to metals. In : *Encyclopedia of Plant Physiology* (Lange O.L.; Nobel, P.S.; Osmond, C.B. and Ziegler, H. eds.). Springer - Verlag, Berlin. Vol. 12 C, pp. 245-300.

169. Zeevaart, J.A.D. and Creelman, R.A. (1988). Metabolism and physiology of abscisic acid. **Ann. Rev. Plant Physiol. Plant Mol. Biol. 39** : 439-473.

170*. Zhang, F.; Romheld, V. and Marschner, H. (1989). Effect of zinc deficiency in wheat on the release of zinc and iron mobilizing exudates. **Z. Pflanzenernaehr. Bodenkd. 152 :** 205-210.

171. Zhang, F.; Romheld, V. and Marschner, H. (1991). Role of the root apoplasm for iron acquisition by wheat plants. **Plant Physiol. 97 :** 1302-1305.

172. Zheng, X. and VanHuystee, R.B. (1992). Peroxidase - regulated elongation of segments from peanut hypocotyls. **Plant Science 81 :** 47-56.

* *Due to nonavailability of literature, various review articles were consulted to seek the information and used as cross reference.*