INFLUENCE OF SALINITY ON PLANT METABOLISM : SOME PHYSIOLOGICAL AND BIOCHEMICAL CHANGES IN PEANUT SEEDLINGS (*ARACHIS HYPOGEA L.*) WITH PARTICULAR REFERENCE TO CELL WALL PROTEINS

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled "INFLUENCE OF SALINITY ON PLANT METABOLISM: SOME PHYSIOLOGICAL AND BIOCHEMICAL CHANGES IN PEANUT SEEDLINGS (*ARACHIS HYPOGEA L*) 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 October, 1996 under the supervision of Dr. Vinay Sharma and Dr. R. P. Singh.

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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ABSTRACT

There are vast stretches of wasteland all over the world due to high concentration of salts in the soil. The saline soil is wide spread in various parts of India as well. Salinity creates problems due to its effects on crop species which are predominantly salt-sensitive.

Peanut seedlings were used in present investigations to determine the effect of saline stress on cell wall proteins and certain other biochemical parameters. Young peanut seedlings do not tolerate salt concentration higher than 100 mM NaCl which confirms its salt sensitive nature.

There is marked reduction in seedling growth under salt stress. Decrease in length and fresh weight of all parts of seedlings as well as whole seedling is observed on exposure to salt. In contrast, dry weight of whole seedling and its various parts is increased. The poor growth in salts may be ascribed either to low water uptake or to high internal salt concentration. Further marked difference were also noted in anatomical features under salt stress. For example, in roots, while the number of cortical cell layers increase, their diameter decreases with increase in NaCl concentration. Reduction in cell size could be due to low water availability and increase in cell layer could be for accommodating higher ion concentration. However, in shoot anatomy no significant change was observed under salt stress.

Salt treatment leads to accumulation of Na^+ and Cl^- ions in various plant parts which could be due to large passive leakage in membranes due to which ion entry will rapidly increase the needs of osmotic adjustment and direct toxicity will result from metabolic interference leading to growth reduction and a positive feedback cycle. Organwise distribution shows that Na^+ and Cl^- ions accumulated more in roots than the other organs of the seedlings. The reason for this type of organ wise distribution is that majority of glycophytes are leaf excluders and accumulate high levels of ions mainly in their roots.

With increase in concentration of salt, decrease in both plasma membrane ATPase and tonoplast ATPase activity is also observed. The reduction in plasma membrane activity may be one of the physiological factors involved in the delay of the normal plant development described under saline conditions. While decrease in tonoplast activity due to the damage of proton pump of the tonoplast by a toxic level of salts can be fatal to the plant cells.

Activity of various cell wall glycosidases and acid phosphatase decreases under salt stress. In contrast, there is about two fold increase in cell wall peroxidase activity at 100 mM salt concentration. The decreased activity of various hydrolases and increased activity of peroxidases under salt stress prevents the cell wall damage and contribute rigidity to the wall.

Significant increase in cytoplasmic protein content under salt stress is observed. On exposure to salinity, change in

cytoplasmic protein profile is also observed. At 75 and 100 mM salt concentrations 13 new polypeptides are formed, of which 3 also appear at 50 mM salt concentration. The functional importance of these stress responsive proteins is not yet established.

Under salt stress, increase in cell wall protein content is also observed. There is alteration in cell wall protein profile also under salt stress. At 50 mM salt concentration, a new polypeptide of 19 kDa is appeared. It is not a glycoprotein and is rich in proline and tyrosine. Besides these two, phenylalanine, glycine and lysine are also present in significant amount. This proline and tyrosine rich protein may be specific for the adaptation of the cell wall to salt stress and may cause changes in the cell wall that allow cells to tolerate salt stress.

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ABBREVIATIONS

A	Adenine
ATP	Adenosine 5'-triphosphate
ATPase	Adenosine triphosphatase
BADH	Betaine aldehyde dehydrogenase
bP	Basepairs
BSA	Bovine serum Albumin
CAM	Crassulacean acid metabolism
cm	centimeter
Con A	Concanavalin A
d	Day
Da	Daltons
DRE	Dehydration responsive element
DTT	Dithiotreithol
DW	Distilled water
EDTA	Ethylene diamine tetraacetic acid
EF-1 alpha	Elongation factor-1 alpha
g	Gram
gdw	Gram dry weight
gfw	Gram fresh weight
hr	hour
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
IEF	Isoelectric focussing
kb	Kilobase
kDa	kilodaltons
LEA	Late embryogenesis abundant
M	Molar
MES	2-(N-morpholino)-ethanesulphonic acid
mg	miligram
min	minute
ml	mililiter

mm	milimeter
mM	milimolar
mPa	milipascal
mRNA	messenger RNA
MW	Molecular weight
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
µmol	micromole
N	Normal
NADP	Nicotinamide adenine dinucleotide phosphate
nm	nanometer
nmol	nanomoles
PAS	Periodic-acid schiff
PEPcase	Phosphoenolpyruvate carboxylase
PEPC-PK	Phosphoenol pyruvate carboxykinase
Pi	inorganic phosphate
pI	isoelectric point
PM	plasmamembrane
Pmol	picomole
PMSF	Phenylmethylsulfonylfluoride
PNP	p-nitrophenol
PVN 1	Plant vibronectin like 1
RuBP	Ribulose-1,5-bisphosphate
RuBPcase	Ribulose-1,5-biphosphate carboxylase
SE	standard error
SDS	Sodium dodecyl sulphate
ssp.	subspecies
T	Thymine
TEMED	N, N, N', N'-tetramethylethylenediamine

Tris	tris (hydroxymethyl) aminomethane
UV	Ultraviolet light
v/v	volume/volume
v/v/v	volume/volume/volume
w/v	weight/volume

CHAPTER-1

1.0 INTRODUCTION

There are vast stretches of wasteland all over the world due to high concentrations of salt in the soil. The saline soil is widespread in various parts of India as well. It has been estimated that as large as 7 million hectare area is covered by such soil in our country .About 25 % of saline soil is located in Uttar Pradesh only, due to which large areas of land in this state have gone out of cultivation [124].

If such wastelands can be used for cultivation by evolving high salt tolerant crop varieties, the productivity may increase several folds. Unfortunately the mechanism(s) that impart salt tolerance to plants are still not fully understood. Generally glycophytic crop species initiate a growth reduction response on exposure to salt. This can be attributed to the fact that glycophytic species fail to distinguish dessication environments from salt environments [141]. The reduced growth rate in the saline environment not only results in drastic decrease in the crop yield but may even endanger the survival of the plant. A genetic alteration which removes the growth reduction response might allow plants under saline stress to adapt to the environment. Elimination of the growth reduction response in glycophytic plants by genetic manipulation would be greatly facilitated by a more complete understanding of the molecular basis of this response. The growth reduction response to high salt may involve altered extensibility of the cell wall. The cell wall

extensibility amongst other factors is determined by the composition of cell wall structural and enzymic proteins. By identifying the factors responsible for the changed pattern of cell wall structural and enzymic proteins, the mechanism of salt tolerance can be understood.

Peanut is an important crop species of tropical climate and is widely grown in our country. Peanut seeds store about 12% carbohydrates, 31% proteins and 48% fats [8]. It is moderately salt sensitive crop species [18], so it is an ideal system for studying the salt stress responses.

In view of the above, following objectives were undertaken for present study.

1. To examine the effect of salt stress on morphological and anatomical features of seedlings.
2. To study the pattern of accumulation of Na^+ and Cl^- ions in various organs of salt treated and control seedlings.
3. To study the effect of salt stress on the activities of some cell wall bound enzymes and certain membrane ATPases.
4. To find out the effect of salt stress on the pattern of structural proteins of cell wall.

CHAPTER-2

2.0 LITERATURE REVIEW

2.1 Introduction

Soil salinity is an important world wide problem in crop production. Salinity creates problems due to its effects on crop species which are predominantly sensitive to presence of high salt concentrations in the soil. Approximately one-third of the developed agricultural land in the arid and semi-arid regions reflect some degree of salinity accumulation [18]. It has been estimated that as large as seven million hectare area is covered by saline soils in India [124]. The problems of secondary salinization are more serious, since they usually represent losses of once agricultural land. Approximately 30 to 50% of the irrigated land world wide is affected by salinity and the problem is becoming increasingly severe in many areas [62]. According to Rengel [129] increasing demands for quality water due to both population rise and industrial development is likely to increase usage of low quality, brackish water (even sea water) in agriculture, thus aggravating the salinity problem. More and more of the marginal lands (including salt affected soils) will have to be used for crop production because the best lands are already farmed. Traditional means of ameliorating salt affected soils through reclamations, drainage and use of excess irrigation water to leach salts below the root zone have to be complemented with the genetic approach through the screening germplasm and breeding crops for higher salt tolerance. Genetic and physiological approaches should merge into

the unifying more comprehensive approach to breeding for salt tolerance. Extending our knowledge on physiological mechanism of salt-tolerance is of utmost importance in developing plants better adapted to saline soils. However, information on mechanism of salt-tolerance is lacking [81].

Much of the physiological research into salinity has concentrated on three topics: water relations, photosynthesis and the accumulation of a particular metabolite assuming that one or more of these process would limit growth in saline conditions. Most of these studies have been descriptive and have established patterns of responses in many crop species, but they have not elucidated mechanism at either the biochemical or whole plant level [108].

2.2 Water relations

Water relations are integral to any study of salt tolerance. There is a prevailing dogma that turgor regulates stomatal conductance and cell expansion, and hence plant growth in soils of low water potential. This has been the basis of hundreds of studies on water relations and osmotic adjustment. Yet, there is no evidence that stomatal conductance or cell expansion is regulated by turgor or that osmotic adjustment has any direct effect on these processes [108].

Flowers et al. [46] suggested that halophytes are able to cope with high electrolyte concentrations in the nutrient medium due to their capability of taking up large amounts of ions and sequestering them into the vacuoles where they contribute to the osmotic potential. Halophytes even need an excess of salts for

attaining osmotic potentials as high as -2.0 to -5.0 Mpa. According to Greenway and Munns [58] some non-halophytic species (glycophytes) may posses the same mechanism to a less developed degree, but there are other species which prevent excess salt uptake and thus protect the cells against too high ion concentrations. In such species however, a lack of solutes may result in poor turgor, so that water deficiency rather than salt toxicity may be the growth limiting factor. Cachorro et al. [26] reported that despite the fact that plant growth was inhibited, water relations seems unlikely to be the limiting factor. This also suggest that the achievement of osmotic adjustment would thus not imply an increase in salt tolerance. There is substantial evidence that glycophytic as well as halophytic plant species adjust to high salt concentrations by lowering tissue osmotic potentials with an increase in dissolved solutes or a combination of both. According to them,this generalization appears to hold for *Phaseolus vulgaris* because accumulation of high levels of inorganic ions predominantly Na^+, K^+ and Cl^- into its tissue as well as a decrease in water content with salinity observed. The effects of saline condition on the water relations of cells in intact leaf tissue of the facultative CAM plant *Mesembryanthemum crystallinum* were studied by Rygol et al. [132]. They have reported that during a 12 hr light/dark regime a maximum in turgor pressure was recorded for the mesophyll cells of salt treated CAM plants at the beginning of the light period followed by 6 hour later by a pressure maximum in the bladder cells of the upper epidermis. In contrast, the turgor pressure in the bladder cells of

the upper epidermis remained constant during light/dark regime. Turgor pressure maxima were not observed in untreated (C_3)plants. High levels of salinity decreased leaf water potential and bulk leaf osmotic potential of leaf tissue, but had no effect on calculated bulk leaf turgor potential [102, 109, 147]. According to Munns [108], turgor does not control growth and osmotic adjustment and have little value in predicting or explaining growth rates of salt affected plants. Of course, turgor is essential for growth, without turgor there would be no expanding force acting on the cell wall and it could not expand. But, the rate of cell wall expansion is controlled by rheological properties of the cell wall and not directly by turgor.

2.3 Effect on photosynthesis

A lot of work has been done on effect of salt stress on photosynthesis in halophytes and glycophytes. Mostly workers assumed that growth in saline environment is limited by the rate of photosynthesis. This view is based upon the frequent observations that photosynthesis is reducing in salt affected plants.

Seeman and Sharky [138] reported that salinity (100 mM NaCl) reduces photosynthetic capacity and this reduction is independent of stomatal closure in *Phaseolus vulgaris*. This reduction was shown to be a consequence of a reduction in the efficiency of RuBPcase rather than a reduction in the leaf content of photosynthetic machinery. They have shown that salinity reduces the photosynthetic capacity of leaves by a) reducing the pool of RuBP by an effect on RuBP regeneration capacity and b) reducing the activity of RuBPcase by an unknown

mechanism when RuBP is in limiting supply. Over the range of 0-400 mM NaCl, net rate of CO_2 uptake (P_n) declined 51% in *Alternanthera philoxeroides*, an alligator weed. Stomatal conductance declined in parallel with P_n and as a result, there was no reduction in intracellular CO_2 concentration and therefore no reduction in amount of CO_2 available for photosynthesis. The CO_2 compensation point did not change with salt stress [91]. Lin and Sternberg [90] suggested that in general fluctuating salinity has significant negative effects on photosynthesis and plant growth of red mangrove (*Rhizophora mangle L.*). While Bloom and Epstein [13] reported that shoot photosynthesis did not vary significantly with salt treatments in *Arivat* and *California Mariout* barley which are salt sensitive and salt tolerant varieties respectively. Nocturnal CO_2 uptake in cactus *Cerus validus*, an obligate CAM plant was inhibited 67% upon treatment with 400 mM NaCl for 14 days [114]. By applying NaCl to intact plants of *Mesembryanthemum crystallinum*, a shift in the mode of carbon assimilation from that typical of C_3 plants to that of CAM plants is observed which exhibit net carbon gain at night. During the induction period, activity of PEPcase, an enzyme associated with CAM increased dramatically [65, 149 and 152] . According to Brugnoli and Bjorkman [25], in cotton plants partial stomatal closure accounted for nearly all of the fall in the photosynthetic rate in 26% seawater, but in 55% seawater much of the fall also can be attributed to non-stomatal factors. Salinity induced reduction in non-stomatal photosynthetic capacity was not caused by any detrimental effect on the

photosynthetic apparatus, but reflects a decreased allocation to enzymes of carbon fixation. Bethke and Drew [7] reported that leaves of young bell pepper (*Capsicum annuum L.*) exposed to 50 moles per cubic meter NaCl after 10 to 14 days of salinization showed little change in photosynthetic ability, whereas those treated with 100 or 150 moles per cubic meter NaCl had up to 85% inhibition with increase in CO_2 compensation point. Partial stomatal closure occurred with salinization, but reduction in photosynthesis was primarily non-stomatal in origin. Meinzer et al. [105] reported decrease in CO_2 assimilation rate and stomatal conductance with high saline environment. Carbon isotope discrimination in tissue obtained from the uppermost fully expanded leaf increased with salinity. It is now frequently noted that the reduction of growth is greater than the decrease in realized or potential photosynthesis and the reduction of shoot growth is much greater than root growth reductions. So, such single point comparisons should be interpreted with caution [31].

2.4 Accumulation of metabolites

The third area which underlies much salinity research is that excessive uptake of salts into cells affects the production of a particular metabolite which directly affects growth. This idea is hard to prove or disprove, since it is unclear what metabolite this would be, what enzyme would be controlling its synthesis or whether it would be located in growing or fully expanded tissues [108].

Greenway and Munns [58] suggested that high concentrations of organic solutes in cytoplasm can have the following roles, a) a

contribution to the osmotic balance when electrolytes are lower in the cytoplasm than in the vacuole and b) a protective effect on enzymes in the presence of high electrolytes in the cytoplasm. Solutes which increase at high salinity in various species reported by many workers include glycinebetaine, proline and sucrose. These compounds at concentrations up to 500 mM do not appreciably inhibit *in vitro* enzyme activity, but there is less evidence for their protective roles. The accumulation of various organic compounds is reported within cells of halophytic organisms to concentrations high enough to significantly affect their osmotic and ionic balance. The accumulation of such materials (primarily organic acids, nitrogen compounds and carbohydrates) is not unique to halophytes and the response is apparently not solely to salinity [46].

While the anion/cation balance in halophytes is generally maintained by high Cl^- uptake, there is evidence that in some species Na^+ is balanced at least in part by organic acid production [28]. For example, there are reports of high oxalate levels in saline condition in many plant species [46 and 150]. Accumulation of malic acid which is produced via the CAM pathway on exposure to salt stress has also been reported in *Mesembryanthemum crystallinum* and in some other plants [114, 122, 132 and 152].

Although malate is the predominant acid formed in non-halophytes and also in some halophytic species including *Mesembryanthemum crystallinum* under salt stress , various studies suggest that at least among halophytes some amino acids (mainly

asparate, glutamate and proline) are accumulating metabolites [46]. Cachorro et al. [26] reported that presence of 100 mM NaCl in the external solution significantly increase proline concentration in *Phaseolus vulgaris* plants. Nabil and Coudret [109] reported that an increase in proline content is observed for 2 *Accacia nilotica* subspecies (ssp. *Cupressiformis* and ssp. *Tomentosa*) grown on saline media. Bertli et al. [6] reported that there is increase in ferredoxin-dependent glutamate synthase activity under salt stress which may provide the glutamate required for proline synthesis a common response to salt stress. Many other workers also reported increase in proline content in stressed plants [12, 15, 28, 36, 81, 92, 126, 148, 155 and 157].

In some species where proline accumulation is not correlated with the external salt concentration, betain, choline or some other quarternary ammonium compounds are accumulated [46 and 58].

The significance of the part played by various carbohydrates in the adjustment of higher plants to salinity is unclear [46]. Some workers reported significant increase in total sugar at higher NaCl in external solution [26, 28 and 84].

2.5 Transpiration under salt stress

A few attempts have also been made to study the effect of salt stress on transpiration in higher plants. According to Flowers and Yeo [47], an increase in the external salinity decreases the rate of transpiration per unit leaf area regardless of the effect of that salinity on the growth. They suggested That this reduction in

transpiration rate under salt stress could be due to the stomatal closure and reduced leaf area as it mimicks water deficit. Even though leaf area may increase in halophytes, transpiration per unit area does not increase because the number of stomata per unit area declines with succulence. Bloom and Epstein [13] reported that under salt stress there is no significant change in transpiration in barley. Tattini et al. [147] reported that transpiration rate diminished under salt stress in olive plants. They have observed that transpiration rate increased during the first stress relief cycle. Perera et al. [118] reported partial stomatal closure, reduction in transpiration rate and increase in water use efficiency with increasing NaCl concentration in *Aster tripolium*. Vaadia [150] observed reduction in transpiration in salt stressed *Lycopersicon penellii* and *Lycopersicon esculentum*.

2.6 Growth responses

2.6.1 Morphological changes under salt stress

As far as growth is concerned, there is a plethora of data. It relates to every degree of tolerance, from the most sensitive glycophyte to the most resistant halophyte and plants are normally categorized on the basis of their growth response to salt [58].

In glycophytic species, growth will be reduced by any significant increase in salinity, while for halophytes this will depend on the external salinity in relation to the growth optimum. Above the growth optimum, halophytes also show similar behaviour to glycophytes [47]. In halophytes at lower concentrations of salt growth rate increased, but after certain limit of salt concentration it

decreased [23, 39, 99, 100, 109 and 117]. In cotton, relative growth rate fell by 46% in 26% seawater and 83% in 55% seawater [25]. Lin and Sternberg [90] reported growth reduction in red mangrove in fluctuating and continuous salinity. Growth reduction in seedlings is more in fluctuating salinity than in continuous salinity. Meinzer et al. [105] reported decline in shoot growth rate in sugar cane. In olive plants growth was inhibited at all salt levels, but most growth parameters of plants treated with 50 or 100 mM NaCl returned to control levels after 4 weeks of relief [147]. Gersani et al. [52] reported reduction in root development as salinity increased. Reduction in dry weights was 40% by 30 mM NaCl and 93% by 100 mM in *Opuntia ficus-indica*. Matsuda and Riazi [102] reported that rapid growth responses occur in barley seedlings by low concentration of salt and at higher NaCl concentrations growth resumption rate is decreasing. In *Altenanthera philoxeroides (Mart) Griseb* an alligator weed, leaf area per unit dry weight is decreasing with increasing salinity [91]. Many other workers also reported decrease in growth rate under salt stress (2, 5, 12, 85, 92 and 141]. Gulati and Jaiwal [61] reported inhibitory effect of more than 150 mM NaCl concentration on shoot formation in cultured explants of *Vigna radiata (L.)*.

2.6.2 Anatomical changes under salt stress

Some workers also reported changes in the anatomy of plant organs under salt stress. Kurth et al. [80] and Lauchli and Schubert [85] observed that the cortical cells of the cotton roots were longer and narrower in salt treated plants than those of control plants. They

have also observed that cell production declined with increasing salinity. Binzel et al. [12] and Singh et.al. [141] observed that adaptation of cultured tobacco cells to NaCl is associated with reduced cell expansion even though turgor is maintained, a result similar to that commonly reported for whole plants exposed to salinity. Cells adapted to 428 mM NaCl expanded only one fifth the volume of unadapted cells. Sodium induced cell enlargement is observed in sugarbeet leaf discs, which is accompanied by proton efflux [115]. Longstreth et al. [91] observed that in alligator weed *Alternanthera philoxeroide*, increasing NaCl concentration from 0-400 mM resulted in an approximately doubling of leaf mesophyll thickness. Increase in lengths of palisade cells as well as the increase in the number of cell layers contributed to the thickness changes in alligator weed. Although NaCl may reduce cell size in some species (e.g. bean palisade cells), the effect on alligator weed was opposite. Weimberg [156] observed that at the cellular level even though the total surface area of a leaf from a salt damaged bean or radish plant is about one-half that of a normal leaf, the number of cells per unit area is nearly the same under the both conditions. Thus, salinity has somehow depressed cell division but has no effect on cellular expansion in the plane parallel to the leaf surface.

2.7 Ions accumulation

It is reported that sodium is an essential micronutreint element for higher plants having C_4 pathway [21 and 22]. Survival of angiospermic halophytes at high salinity is invariably accompanied by

high ion content. Glycophytes on the other hand, respond to high salinity basically by ion exclusion. The majority of these species in practice are leaf excluders and may accumulate high levels of Na^+ in their roots and stems. There is in fact increasing evidence for the control of Na^+ levels in the xylem of glycophytes by reabsorption in proximal root regions. Generally, in sensitive species an increase in shoot Na^+ results when control fails and this failure is correlated with reduction in growth. Since unlike halophytes, Na^+ is excluded from the shoots, these glycophytes do not show dramatic growth increase in NaCl [46].

A lot of work has been done on the accumulation of Na^+, Cl^-, Ca^{++} and K^+ and Mg^{++} ions under salt stress. There are lot of reports on accumulation of these stress related ions under salt stress in halophytes as well as in glycophytes. Some workers reported that in halophytes along with the increase in the NaCl concentration in the medium, Na^+ and Cl^- contents increased and K^+, Ca^{++} and Mg^{++} contents fell [39, 52, 100, 109, and 117]. Refoufi and Larhar [126] observed that salt tolerance in *Medicago* species is partly related to lower level of sodium accumulation and partly to their ability to maintain a high internal potassium level. Some workers found increase in K^+ with Na^+ and Cl^- ions [53, 114 and 134]. Adams et al. [1] reported that major inorganic ions in bladder cells of salt stressed *Mesembryanthemum crystallinum* are Na^+, Cl^-, K^+, Ca^{++} and Mg^{++}. Shah et al. [140] observed that NaCl-tolerant callus of *Medicago sativa L.* was considerably more tolerant to the chlorides of all alkali metals than

the non-selected callus, as shown by good growth at concentrations of alkali metals that inhibited growth of non-selected cells. This tolerance was toward the alkali metals and not to the Cl^- ions and shows that cotolerance to other alkali metals is imposed by the selection of tolerance to Na^+.

There are also some reports which suggested that Na/K selectivity mechanism could contribute to salt-tolerance [46, 53 and 63]. Perera et al. [118] suggested that probably Na^+ ions interfere directly with the influx of K^+ into the guard cell, because by increasing salt concentration there was suppression of stomatal opening.

Many workers reported the compartmentalization of ions in the vacuoles in halophytes [31, 38, 46, and 101]. Kuchitsu et al. [79] reported that during the adaptation to the salt stress both cytoplasmic and vacuolar pH increased. They assumed that vacuolar alkalization is due to the passive H^+ leakage through the tonoplast which is enhanced by the cytoplasmic alkalization which could be due to intracellular compartmentalization of ions.

There is evidence that the salt sensitivity of certain varieties of glycophytic species is due to the absorption of relatively high amounts of Cl^- and/or Na^+ i.e. these varieties suffer from ion excess in their expanded leaves. Both the varietal and species comparisons show that sensitivity towards high Cl^- and/or Na^+ concentration in leaves is much greater for nonhalophytes. This difference is almost certainly based on inadequate cellular

compartmentation of ions in the leaves of non halophytes [7, 58, 84 and 147]. Cachorro et al. [26] reported that *Phaseolus vulgaris L.* seedlings accumulate very high amounts of Na^+, Cl^- and K^+ in salt stressed conditions, but Ca^{++} ion content remain same.

Many workers reported that some salt-tolerant glycophytic species accumulate less amount of Na^+ and Cl^- ions and relatively more K^+ in the shoots and their K^+/Na^+ ratio was also higher, while in salt-sensitive varieties it was opposite [5, 12, 57, 62, 136, 137, 144, 146, 150, 155 and 157]. While Kwon et al. [81] observed low level of K^+ and the high amount of Ca^{++} in the salt resistant cell line of rice, Nunes et al. [115] reported decrease in K^+ and increase in Na^+ during salt stimulated cell expansion in sugarbeet leaf discs. They reported that there is a sodium stimulated proton pump which is active during cell enlargement.

Some work has also been done on Na^+/Ca^{++} interaction in salt stressed plants. Martinez and Lauchli [97] observed that high Ca^{++} partially mitigated the salt-induced increase in Na^+ content and decrease in K^+ content of the barley roots. High salt appears to decrease the Pi content of the vacuole, while high Ca^{++} increased this content irrespective of the salt treatment. Sodium reduces binding of Ca^{++} to the plasma membrane, inhibits influx while increasing efflux of Ca^{++} and depletes the internal stores of Ca^{++} from endomembranes. Ameliorating effects of supplemental Ca^{++} on salt stress are exerted through preventing Na^+-related changes in the cell by Ca^{++} homeostasis [80 and 129]. Reid et al. [127] reported doubling of Ca^{++} influx at

the plasmalemma by increasing salinity in *Chara*. They suggested that most probably the principal role of Ca^{++} under these conditions is exerted externally through the control of membrane voltage and permeability. Galvez et al. [49] argued that the greater tolerance of salinity at elevated Ca^{++} concentration is due to more efficient transduction of the signal. Chaudhary et al. [30] reported that regenerated salt tolerant plants of Lucerne (*Medicago media Pers*) from salt-selected suspension cultures were more salt tolerant than original plants, but this was dependent on high concentration of Ca^{++} in the nutrient medium. Chung and Matsumoto [33] reported lower levels of Ca^{++} in root microsomal membranes of cucumber after the treatment with the NaCl.

Ahmad et al. [2] reported that silicon significantly lowered the Na^{+} content in the flag leaves and roots of wheat under saline conditions. Concentration of silicon in roots increased with increasing salinity and silicon levels.

2.8 Enzyme responses

2.8.1 ATPase activity under salt stress

The majority of research on Na^{+} metabolism in plants, has been concerned with initial uptake across the root cell plasmalemma. An enzyme considered to play an important role in regulating ion transport at this interface is plasmalemma ATPase [128]. The H^{+} pump in the plasmalemma is a P-type ATPase. Uptake of Na^{+} and Ca^{++} occurs passively across the plasma membrane and efflux is presumably due to activities of a Na^{+}/H^{+} antiporter and a Ca^{++}-ATPase respectively. Cl^{-}

uptake is assumed to be coupled to a H^+ symporter because of the large inside negative membrane potential. Extrusion of Cl^- takes place by electrophoretic flux [113 and 153].

Bruggemann and Janiesch [24] observed that salt treatment does not affect the properties of the plasma membrane ATPase in *Plantago maritima L.* plants and that the role of this enzyme in ion flux control in the halophyte is merely limited to the energization of the plasma membrane for secondary active transport. Iwano [72] observed by comparing the staining profiles of salt-stressed cells of *Nostoc muscorum* with those of control cells that a high salt environment activates the ouabain-sensitive Na^+, K^+-ATPase, which seems likely to be involved in the efflux of Na^+ ions. Gronwald et al. [60] and Wilson et al. [160] reported reduction in V_{max} of Mg^{++}-ATPase activity of plasma membrane in salt stressed tomato roots and mature leaves. Suhayda et.al.[145] observed salinity induced changes in plasma membrane electrostatic properties in tomato roots which may influence ion transport across the plasma membrane. In cyanobacteria *Spirulina subsalsa* increase in plasma membrane H^+-ATPase activity is observed under hypersaline conditions which is involved in extrusion of Na^+ and in conferring resistance to salt stress [48]. Fisher et al. [45] and Perez-Prat et al.[120] observed induction of plasma membrane protein and H^+-ATPase mRNA in alga *Dunaliella* and tobacco cells which could play a role in ionic homeostasis in conditions of high salinity. A significant reduction in K^+, Mg^{++}-ATPase activity is observed under salt stress [33, 40 and 135].

Beyond the initial uptake, the aspect of Na^+ metabolism which has received the most attention is cellular level compartmentation and it is widely accepted that Na^+ must be excluded from the bulk cytoplasm [31 and 63]. Salt tolerant varieties are able to cope with high electrolyte concentrations in the nutrient medium due to their capability of taking up large amount of ions and sequestering them into the vacuoles where they contribute to the osmotic potential [46, 78 and 96]. Kliemchen et al. [77] observed that shift from the C_3 to the CAM mode of photosynthesis upon salinity stress in *Mesembryanthemum crystallinum* leads to an increase in the rigidity of the tonoplast. Similar to the plasmalemma, though much distinct in its properties tonoplast membrane also posses a H^+-translocating ATPase. This enzyme has been found to show a positive correlation with increasing salt levels [128]. Matoh et al. [101] suggested that the vacuoles are the major sequestration site of NaCl in leaves of *Atriplex gmelini* plants grown under saline stress. Kuchitsu et al. [79] reported increase in pH values of both cytoplasm and vacuoles in alga *Dunaliella salina* under salt stress. They assumed that the vacuolar alkalization is due to the passive H^+ leakage through the tonoplast which is enhanced by the cytoplasmic alkalization. Under normal conditions, the H^+ leakage out of the vacuoles is compensated by the H^+ pump in the tonoplast. Under the salt stress, however, increased Pi in the cytoplasm inhibits the H^+ pump, resulting in the long lasting vacuolar alkalization. Leigh and Tomos [86] reported that between 53% and 90% of Na^+ was located in the vacuole and the vacuolar

concentration of Na^+ ranged between 4 and 45 mol m^{-3} in red beet. There is induction of Na^+/H^+ antiport activity in the tonoplast vesicles under salt stress in salt tolerant species [14, 50, 51, 103 and 154]. Narsimhan et al. [111] reported that transcription of the 70 kilodalton subunit gene or mRNA stability of tobacco tonoplast ATPase was induced by short-term NaCl treatment in NaCl adapted cells. Low et al.[93] studied early effects of salt exposure on vacuolar H^+-ATPase gene expression in *Mesmbryanthemum crystallinum*. They have analyzed mRNA levels for the vacuolar H^+-ATPase subunits A, B and c and found higher transcript levels in young roots and leaves. Whereas in fully expanded leaves subunit c mRNA was specifically up-regulated, although this increase appeared to be transient. Sanchez-Agayo et al. [135] reported increase in the tonoplast ATPase activity in *Lycopersicon esculentum* under salt stress. Katsuhara et al. [76] reported increase in vacuolar pH under salt stress in *Nitellopsis obtusa* cells which results from the inibition of the H^+-translocating pyrophosphate in the vacuolar membrane, since this H^+-translocating system is sensitive to salt induced increase in the cytoplasmic (Na^+) and simultaneous decrease in the cytoplasmic (K^+).

There is also a report of induction of putative endoplasmic reticulum Ca^{++}-ATPase mRNA in NaCl-adapted tobacco cells [119].

2.8.2 Some other Enzyme responses under salt stress

Effects of salt stress on many enzymes are studied by lot of workers. *In Vitro* studies have shown that soluble enzymes from halophytes and nonhalophytes have similar sensitivities to

electrolytes. Growth of many salt tolerant species is much less sensitive to high internal concentration of electrolytes than would be expected from the response of isolated enzymes. Additionally, enzymes may be less sensitive to electrolytes *in vivo* than *in vitro*. For most nonhalophytes , there is no discrepancy between the salt sensitivity of enzyme activities *in vitro* and the growth response [58]. Thus, enzyme of halophytes are neither remarkably salt resistant nor salt requiring. Indeed, they are very similar to the enzymes of other plants. Their activity is generally inhibited by high concentration of electrolytes, although the inhibition may be overcome to some extent by increasing the substrate concentration and by acclimatization to the growth conditions [46]. Greenway and Osmond [59] reported that malate dehydrognase, asparate transaminase, glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase extracted from *Atriplex spongiosa*, *Salicornia australis* and *Phaseolus vulgaris* show similar *in vitro* NaCl sensitivity despite great difference in salt tolerance of the plants. Weimberg [156] determined level of 18 enzymes in peanut seedling under salt stress. Specific activities of the enzymes were the same in all the plants. The electrophoretic pattern of isozymes of malate dehydrogenase was also not altered, but the isozyme pattern of peroxidase from roots was altered. Bertli et al. [6] reported two-fold increase in the activity of ferredoxin-dependent glutamate synthase in the leaves of tomato under salt stress. Preiss et al. [122] reported appearance of the $NADP^+$-malic enzyme in *zea mays* variety (*cv.Pirat*) under salt stress. Salinity reduces the RuBPcase activity in *Phaseolus*

vulgaris [138]. Many workers reported increase in PEPcase and PEPC-PK activity under salt stress in *Mesembryanthemum crystallinum* [15, 35, 65, 89, 148, 149 and 152]. Botella et al. [16] characterized salt-induced tomato peroxidase mRNA. They reported enhanced accumulation of transcripts of TPX1 a cDNA clone isolated from a library of NaCl-treated tomato plants using peroxidase-specific oligonucleotide probe. Transcripts that hybridized to TPX1 were detected only in epidermal and sub epidermal cell layers. IEF of root extracts showed 2 major bands of peroxidase activity. Both activities increased with salt treatment. Some workers reported two- to four-fold increase in the activity of BADH in leaves and roots of sugarbeet and spinach under salt stress [104, 158 and 159].

However, till date there is no report on the effect of salt stress on any cell wall bound enzyme.

2.9 Changes in total proteins under salt stress

A lot of work has been done to identify the polypeptides which are involved in the mechanism of salinity tolerance in cell cultures and whole plants. In these studies, a few polypeptides thought to be important for the tolerance/resistance of the plants, have been isolated and sequenced, but as yet most of them do not have any assigned function.

Lopez et al. [92] compared patterns of total protein extracted from the leaves of control and stressed plants of *Raphanus sativus* and observed a new 22 kDa (pI 7.5) polypeptide and its mRNA which accumulated after exposure of the plants to NaCl. Intensity of a

22 kDa double polypeptide was increased by salinity in *Brassica napus* leaves also [130]. Golldack et al. [56] reported that polypeptides of 36 and 170 kDa were typically expressed in *Dunaliella parva* adapted to 1.5 M NaCl. These polypeptides were absent or synthesized at very low rates in 0.75 M NaCl adapted cells. Polypeptides of 23, 24.5 and 37.5 kDa showed their highest rate of expression in the low salt algae. Hurkman and Tanaka [70] and Hurkman et al. [71] showed that salt stress caused increase and decrease in the synthesis of a number of polypeptides in barley roots. Most significant increase occurred in a set of polypeptides having MW of 26 and 27 kDa and pI of 6.3 and 6.5. Six specific or enhanced polypeptide bands (SAP-1,-2,-3,-4,-5 and-6) of 84, 57, 21, 19 and 17 kDa were observed in salt adapted winged bean cells. SAP-1 with molecular weight of 84 kDa was abundantly secreted at 1 and 1.5% NaCl [41 and 42]. Nine proteins were induced in potato under salt stress [133]. Godoy et al. [54 and 55] reported that 5-12.5 g/l NaCl induce dehydrin TAS14 protein in NaCl-treated tomato seedlings. In salt-stressed mature plants TAS14 was expressed abundantly and continuously in aerial parts, but only slightly and transiently in roots. Ben-Hayyim et al.[4] reported a significant increase in the amount of a polypeptide of 23-25 kDa and pI 6.1 in adapted cultured cells of salt-tolerant citrus. Two predominant polypeptides of 70 and 23 kDa respectively were induced under salt stress in germinating seeds of rice, of which the 23 kDa is the more abundant polypeptide. Two other polypeptides of 15 and 26 kDa were found to be specifically induced in shoots of NaCl-treated rice

seedlings [123]. Moons et al. [106] reported that abundance of ABA-induced proteins was highest in the most tolerant indica rice variety *pokkali*. Three ABA-responsive proteins of 40, 26 and 24 kDa were present in roots of tolerant and sensitive rice varieties. Protein changes induced by salt stress were also investigated in the roots of the salt-sensitive rice cultivar Taichung native 1 by Claes et al. [34]. They observed 8 induced proteins under salt stress in this cultivar. Lane et al. [83] observed that the amount of germin-like proteins and coding elements undergo conspicuous change when salt-tolerant higher plants are subjected to salt stress. According to Xu et al. [162] late embryogenesis abundant proteins play an important role in the protection of plants under water or salt stress condition.

2.10 Changes in cell wall properties

Reduced growth rate in the saline environment could be due to altered properties of the cell wall or wall loosening mechanism which would cause reduced extensibility in response to stress [11 and 19]. These changes will be in the composition of matrix polysaccharide and in protein constituents of the cell wall as well [141]. Zhong and Lauchli [167] observed a significant increase in the cell wall uronic acid content on per unit dry weight basis in salt treated cotton seedlings. The neutral sugar composition of the cell wall showed no changes. An increase in the cell wall polysaccharide of intermediate molecular size and a decrease in that of small size in the hemicellulose 1 fraction is also observed under high salinity which

indicates a possible inhibition of polysaccharide degradation. Singh et al. [141] observed that salt adapted tobacco cells release more protein into the medium than unadapted cells and some of these proteins including 29 kDa protein are detectable at very low levels in medium of unadapted cells. Jones and Mullet [74] reported increase in Cyp15a mRNA level in salt treated seedlings which encodes a cell wall protein. Deutch and Winicov [37] reported accumulation of MsPRP2 gene which encodes a chimeric 40,569 Da cell wall protein in salt-tolerant alfalfa cells grown in the presence of salt. Hurkman et al. [71] reported increase in polypeptides of 26 and 27 kDa that were differentially associated with endoplasmic reticulum, tonoplast, plasma membrane and cell wall fractions. Yen et al. [164] reported reduction in a Con A-binding 24 kDa polypeptide (SRgp 24) in *Mesembryanthemum crystallinum* which was associated with the cell wall region. Zhu et al. [168] isolated PVN 1 protein from 428 mM NaCl-adapted tobacco cells. It was detected in all tissues but was most abundant in roots and salt-adapted cultured cells. This protein was localized in the cell wall of cortical and transmitting tissue cells of pollinated mature styles. It was observed that PVN 1 is related to the translational elongation factor-1 alpha.

2.11 Identification of induced cell wall proteins

Some workers identified salt-induced cell wall proteins as glycoproteins. Esaka et al. [42] reported that salt induce 84 kDa polypeptide (SAP 1) in winged bean cells is a hydroxyproline rich

glycoprotein. Yen et al. [164] reported that salt-responsive 24 kDa polypeptide in *Mesembryanthemum crystallinum* is a glycoprotein.

2.12 Changes in amino acid composition

Some workers also studied changes in the amino acid composition of salt induced polypeptides. Singh et al. [141] reported that amino acid composition of total ionically wall bound protein differs between salt adapted and unadapted tobacco cells. Most striking was the dramatic reduction of hydroxyproline in ionically bound wall protein of adapted cells and this protein was having high alanine content. Moons et al. [106] reported that ABA responsive salt induced 40 kDa protein in rice is histidine rich. Specific secretion of 5 proline rich and 1 hyroxyproline rich protein was observed in salt-adapted winged bean cells [41 and 42].

2.13 cDNA clones and mRNA level

Many workers identified cDNA clones and observed increase in mRNA levels under salt stress. Claes et al. [34] characterized a rice gene showing organ-specific expression in response to salt stress. They observed induction of 8 proteins in the roots of the salt-sensitive rice cultivar Taichung native 1 under salt stress. A cDNA clone Sal T was found to encode one 15 kDa protein out of the 8 induced proteins. Sal T mRNA accumulates very rapidly in sheaths and roots of mature plants and seedlings upon treatment with NaCl. The organ-specific responses of Sal T is correlated with the pattern of Na^+ accumulation during salt stress. In sugarbeet and spinach the

activity of BADH was found to increase two-to four-fold in both leaves and roots under salt stress. This increase in activity of BADH was paralleled by an increase in the level of translatable BADH mRNA. Several cDNAs encoding BADH were cloned from a lambda gt10 library representing poly (A^+) RNA from salinized leaves of sugarbeet plants by hybridization with a spinach BADH cDNA [104, 158 and 159]. Winicov [161] identified cDNA encoding putative zinc finger motif from salt tolerant Alfalfa (*Medicago sativa L.*) cells. NaCl stress causes the accumulation of several mRNAs in the tomato seedlings. An up regulated cDNA clone SAM 1 was found to encode a AdoMet synthetase enzyme whose activity and level is increasing under salt stress [45]. A gene encoding for a citrus salt associated protein Cit-SAP was cloned from salt treated *Citrus sinensis* cell suspension. The gene CSa was isolated from a cDNA expression library. This protein was homologous to mammalian glutathione peroxidase [66]. A cDNA clone with high homology to cotton Lea5 gene which induced in response to salt stress was isolated from citrus cell suspension. This gene C-Lea5 is expressed in citrus leaves as well as in cell suspension that is grown in 0.2 M NaCl [110]. Lopez et al. [92] isolated a cDNA clone corresponding to the 22 kDa radish protein from salt stressed plants. Salt stress induces a large accumulation of this mRNA in the leaves of radish. Hurkman et al. [69] obtained a single full length cDNA-encoding barley germin subunit which most likely encodes one of the 26 or 27 kDa polypeptides whose level changed during salt stress. Chen et al. [32] isolated a cDNA clone pcz1, with a 1.1 Kb insert from

a NaCl-adapted tobacco cell cDNA library that encodes an approximately full-length 29 kDa protein (251 amino acids) with a calculated pI of 5.7. The encoded peptide had a high amino acid sequence identity with brain protein 14-3-3. Botella et al. [16] reported that NaCl treatment of tomato plants in hydroponic culture at concentrations as low as 50 mM resulted in enhanced accumulation of transcripts of TPX1, a full length cDNA clone which they isolated from a library of NaCl-treated tomato plants, using a peroxidase-specific oligonucleotide probe. Yamaguchi-Shinozaki and Shinozaki [163] reported that 2 closely located genes rd29A and rd29B of the *Arabidopsis* genome are differentially induced under condition of high salinity. They analyzed rd29A promoter in both transgenic *Arabidopsis* and tobacco and identified a novel cis-acting, dehydration responsive element (DRE) containing 9bp, TACCGACAT which is involved in the first rapid response of rd29A to conditions of dehydration or high salt.

Galvez et al. [49] compared the accumulation of early salt induced mRNAs in salt tolerant *Lophopyrum elongatum*, salt sensitive wheat and their amphiploid. Amitai-Zeigerson et al. [3] reported an elevation of the steady state levels of both ASr1 mRNA and protein under salt stress in tomato seedlings. Perez-Prat et al. [119] isolated a cDNA clone that encodes the partial sequence of a putative endoplasmic reticulum Ca^{++}-ATPase of tobacco. The level of the transcripts of this was induced by salt treatment. Narsimhan et al. [111] and Perez-Prat et al. [120] reported that transcription of the 70 kDa subunit gene of tobacco tonoplast ATPase or mRNA stability and

plasma membrane H^+-ATPase mRNA accumulation were induced by NaCl. Botella et al. [16] characterized a salt-induced tomato peroxidase mRNA. Vernon et al. [151] observed that a number of mRNAs which encodes proteins involved in different biochemical pathways accumulate in *Mesembryanthemum crystallinum* leaf tissue under salt stress. Low et al. [93] analyzed mRNA levels for the vacuolar H^+-ATPase subunit A, B and c in *Mesembryanthemum crystallinum* under salt stress. They observed that in roots and young leaves, mRNA levels for all 3 subunits increased about two-fold compared to control plants, whereas in fully expanded leaves only subunit c mRNA responded to salt.

2.14 Development of mutant cell lines

The study of stage-specific variabilities in response to saline stress may result in the identification of the heritable components of salt resistance. Isolation and characterization of mutants with stable salt resistant properties at given growth stages should provide valuable insight into salt-tolerant systems and ultimately into the characterization of relevant genes. Three mutant strains of *Arabidopsis thaliana var columbia* were selected by Saleki et al. [134] for their ability to germinate in elevated concentration of NaCl. They were not more tolerant than wild type at subsequent developmental stages. Genetic analysis of F_1 and F_2 progeny of out crosses suggest that the salt-tolerant mutations are recessive and they define three complementation groups. Kwon et al. [81] reported enhanced saline stress resistance in threonine and methionine rich overproducing mutant cell line from protoplast culture of rice (*Oryza*

sativa L.). Zhang et al. [166] obtained a salt tolerant rice mutant (M-20) through selection *in vitro*. Its tolerance was stably inherited over 8 generations and most traits between M-20 and its sensitive original 77-170 were very similar. Lutts et al. [95] studied changes in plant responses in NaCl during development of rice varieties differing in salinity resistance which is very essential to improve the understanding of the effects of salt stress upon phenology and to elaborate further breeding programs. Xu et al. [162] introduced a LEA protein gene HVA1 from barley (*Hordeum vulgare L.*) into rice (*Oryza sativa L.*) plants. Expression of the barley HVA1 gene regulated by rice actin 1 gene promoter led to high level, constitutive accumulation of the HVA1 protein in both leaves and roots of transgenic rice plants. The extent of salt tolerance is correlated with the level of HVA1 protein accumulation. Second generation transgenic rice plants showed significantly increased tolerance to water deficit and salinity. Gulati and Jaiwal [61] reported *in vitro* selection of salt resistant *Vigna radiata* plants by adventitious shoot formation from cultured cotyledon explants.

2.15 Some other responses

Cushman and Bohnert [35] reported that salt stress alters A/T-rich DNA-binding factor interactions within the PEPcase promoter from *Mesembryanthemum crystallinum*.

It has been shown that at least in some herbaceous mono- and dicotyledon species frost hardening can be induced by treating plants with NaCl at otherwise non-hardening temperature. Hincha [64] observed

rapid induction of frost hardiness in spinach seedlings under salt stress. This rapid *in vivo* increase in hardiness was reflected in reduced *in vitro* freeze-thaw damage to thylakoides isolated from seedlings after only one hour of salt stress.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Materials

P-nitrophenyl β-D-galactopyranoside, p-nitrophenyl α-D -galactopyranoside, p-nitrophenyl β-D-glucopyranoside, sodium orthovanadate, phenylmethylsulfonyl fluoride, sephadex G-25, and molecular weight standards (sigma VI) were obtained from Sigma Chemical Co., U.S.A. All other chemicals were obtained from either Merck (India) or SRL (India) or Glaxo (India) and were of analytical grade. Peanut (*Arachis hypogea*) seeds were obtained from the local market.

3.2 Methods

3.2.1 Germination of seeds

Locally purchased seeds were surface sterilized with disinfectant savlon for 15 min. Seeds were then thoroughly washed with double distilled water and imbibed in it for six hours. Imbibed seeds were grown hydroponically in plastic trays in nutrient solution at various salinities ranging from 0-100 mM NaCl in a plant growth chamber in dark at 30±2 °C and 80% relative humidity [50]. In some experiments one more control was set up by growing seeds in distilled water only without adding any nutrient. Solutions were changed after every 48 hours to avoid ion depletion. Non-germinated seeds were discarded to avoid fungal infection. Seedlings were harvested when required for the experimental work.

3.2.1.1 Composition of nutrient solution

Nutrient solution was prepared according to Moore [107] with the following composition:

Stock Solutions :

Concentration	Chemical	Volume
1 M	$Ca(NO_3)_2$	10 ml
1 M	KNO_3	10 ml
1 M	$MgSO_4$	4 ml
1 M	KH_2PO_4	2 ml
	FeEDTA*	2 ml
	micro nutrients**	2 ml

* = 5 mg metallic iron was chelated with 37 mg Na_2 EDTA to make 42 mg per ml solution of FeEDTA.

** = micronutrient stock solution contains per liter 2.86 g H_3BO_3, 1.81 g $MnCl_2.4H_2O$, 0.11 g $ZnCl_2$, 0.05 g $CuCl_2.2H_2O$ and 0.025 g Na_2MoO_4.

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

3.2.1.2 Salt treatment

Seedlings were treated with different concentrations of NaCl. They were grown separately in nutrient solution with 0, 25, 50, 75, and 100 mM of NaCl. In some experiments one more control was set up by growing seedlings in double distilled water alone.

3.2.2 Measurement of length

Seeds were harvested on tenth day for measurement of length. They were separated into roots, shoots, and leaves . Their length was then measured in centimeters with the help of a scale.

3.2.3 Determination of fresh weight

10 day old seedlings were harvested for determination of fresh weight. Seedlings were dissected into roots, shoots and leaves. They were blotted dry and their fresh weights were measured [99 and 100].

3.2.4 Determination of dry weights

10 d old peanut seedlings were cut into its parts i.e. into leaves, shoots and roots. Each part was blotted dry and subsequently was oven dried at 70°C for 16 hours. Then their weights were recorded [99 and 100].

3.2.5 Anatomical studies

Anatomical studies were done as described by Kurth et al. [80]. Hydroponically grown seedlings were harvested on tenth day. Plants were divided into its various parts. Sections were cut from roots and shoots near the tip. 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% glycerin and examined under a light microscope (2 KICEBI-1, Neovar, Austria). The diameter of around 20 randomly chosen cortical cells in roots and shoots were measured with the help of occulometer fitted inside the

eye piece of microscope. Then photographs of the sections were taken from the camera attached with the microscope (Reichert Jung, Austria).

3.2.6. Estimation of *in vivo* level of Na^+ and Cl^- ions

3.2.6.1 Preparation of samples for estimating ions

Hydroponically grown treated and untreated plants were divided into roots, shoots and leaves and their fresh and dry weights were measured. Dried material was finally grounded with the help of a stainless steel mortar and pestle [99 and 100].

3.2.6.2 Estimation of Na^+ ions

For estimating sodium ions method of Humphries [67] was followed. 10 g finely grounded powdered material was taken into 100 ml Kjeldahl flask. 10 ml sulphuric acid and 10 ml nitric acid was added to it. If necessary, more nitric acid was added to keep the sample fluid. Very gentle heat was applied and excessive frothing was avoided. When all the nitric acid fumes came out, flask was allowed to cool and more nitric acid was added to it if necessary. When all the organic matter has been oxidized the temperature was increased for 15 minutes and then flask was allowed to cool. Final volume of the sample made to 100 ml. Sodium ions were estimated in the samples by atomic absorption spectrophotometer (aa/ae spectrophotometer, 751, Instrumentation laboratory, USA).

3.2.6.3 Estimation of Cl^- ions

1 g dried powdered material was extracted in 100 ml boiling water for the colorimetric determination of chloride ions. The method

of Iwasaki et al. [73] was followed with slight modifications for the determination of Cl^- ions.

Procedure :

10 ml aliquot of the chloride solution was placed in a test tube. 1 ml saturated solution of mercuric thiocyanate in ethanol was added to it. Then 2 ml 0.25 M ferric ammonium sulphate in 9 M nitric acid was added. After 10 minutes, the absorbance of the sample and also of the blank was recorded in DU-6 Beckman spectrophotometer at 460 nm against water in the reference cell. The amount of chloride ion in the sample corresponds to the difference between the two absorbance and is obtained from a calibration curve which was constructed by using a standard sodium chloride solution containing 10 to 100 μg Cl^- per ml.

3.2.7 Determination of tonoplast and plasma membrane ATPase activity

3.2.7.1 Isolation of tonoplast and plasma membrane fractions

For isolation of tonoplast and plasma membrane fractions, methods of Sen and Sharma [139] and Bhushan and Sharma [10] were essentially followed. All operations were carried out at 4°C unless stated otherwise. 5 g 7 d old seedlings were homogenized in a mortar in 20 ml 20 mM HEPES-Tris (pH 7.8) containing 250 mM sucrose, 1 mM Na_2 EDTA, 10 mM $MgCl_2$ and 5 mM β-mercaptoethanol. The homogenate was filtered through an ordinary nylon sieve (pore diameter 500 μm approximately) and subsequently centrifuged at 13000xg for 20 minutes

using RA-228 rotor in a cooling centrifuge (Kubota, 1300). The pellet was discarded and the supernatant (microsomal fraction) was used for estimating enzymatic activities.

3.2.7.2 ATPase assay

Following buffers were used for the assay of plasma membrane and tonoplast ATPases.

Assay buffer: 50 mM Tris-Mes (pH 8.0), 3 mM $MgSO_4$, 3 mM ATP, 50 mM KCl and 100 μM ammonium molybdate.

Standard Solution: 10 mM KH_2PO_4, diluted 1:10 to obtain 1 mM concentration.

Stopping reagent: 10% SDS

Colouring reagent: 6 part of 3.6 mM ammonium molybdate in 0.5 M H_2SO_4 + 1 part 10% ascorbic acid.

Assay procedure:

The tonoplast ATPase activity was analyzed by monitoring the release of Pi in the presence and absence of 50 mM NO_3^- ions. Inhibitors of other ATPases i.e. sodium azide (0.5 mM) for mitochondria and vanadate (0.2 mM) for plasmalemma were routinely added to reaction mixture. 100 μM ammonium molybdate was added to inhibit acid phosphatase activity [87]. While the plasma membrane ATPase activity was determined by monitoring the release of Pi in the presence and absence of 0.2 mM sodium orthovanadate. 0.5 mM sodium azide, 50 mM KNO_3 and 100 μM ammonium molybdate were also added in this reaction mixture to inhibit other ATPases and acid phosphatase.

The assay was started by addition of membrane fractions (0.1 ml with about 2.0-2.5 μg protein) to reaction medium (0.45 ml) as given below.

Reaction mixture:

	Probe	Standard	Blank
Assay buffer	300 μl	300 μl	300 μl
Water	50 μl	25 μl	50 μl
Standard	0 μl	25 μl	0 μl
Membrane extract	100 μl	100 μl	100 μl

After mixing all the reagents, mixture was immediately vortexed and incubated for 45 minutes at 37°C. The reaction was stopped by adding 0.5 ml of 10% SDS. Blank and standards (with Pi) were also run simultaneously where 10% SDS was added prior to the addition of membrane fractions. The amount of Pi released was determined by the method of Leigh and Walker [88]. For this 0.6 ml of colouring reagent was added in the probe, standards and blanks and incubation was carried out at 37°C for 45 min. After this, tubes were kept in ice for 5 minutes and their absorbance was read at 820 nm.

3.2.8 Determination of activities of cell wall bound enzymes

3.2.8.1 Preparation of cell wall

For preparation of cell wall method of Masuda et al. [98] was followed. In brief, 5 gram of 6 day old peanut seedlings were homogenized at 0°C in 15 ml of 10 mM Na-phosphate buffer (pH 7.4) using a mortar and pestle and filtered through an ordinary 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 hour 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.

3.2.8.2 Extraction of cell wall bound enzymes

The extraction of enzymes with NaCl was conducted by the method of Masuda et al. [98] by suspending the cell wall (0.5 g wet weight) in 10 ml solution of NaCl at various concentrations (0.5 M for β-galactosidase, β- glucosidase and acid phosphatase, 1 M for peroxidase and 1.5 M for α-galactosidase) and incubation at room temperature for two hours. The enzymatic activity in the extracts from the wall was measured after filtration of the suspension.

3.2.8.3 Enzyme assays

3.2.8.3.1 Assay of cell wall bound glycosidase activity

Glycosidase activity was assayed by following the release of p-nitrophenol from its glycosides. The reaction mixture contained 0.5 ml 5 mM substrate (p-nitrophenyl β-glucopyranoside, p-nitrophenyl β-galactopyranosidase and p-nirophenyl α-galactopyranoside for β-glucosidase, β-galactosidase and α-galactosidase respectively), 0.5 ml of McIlvaine buffer (citrate-phosphate buffer) and 0.5 ml of the enzyme extract. The pH of the reaction was 4.4 for β-glucosidase and

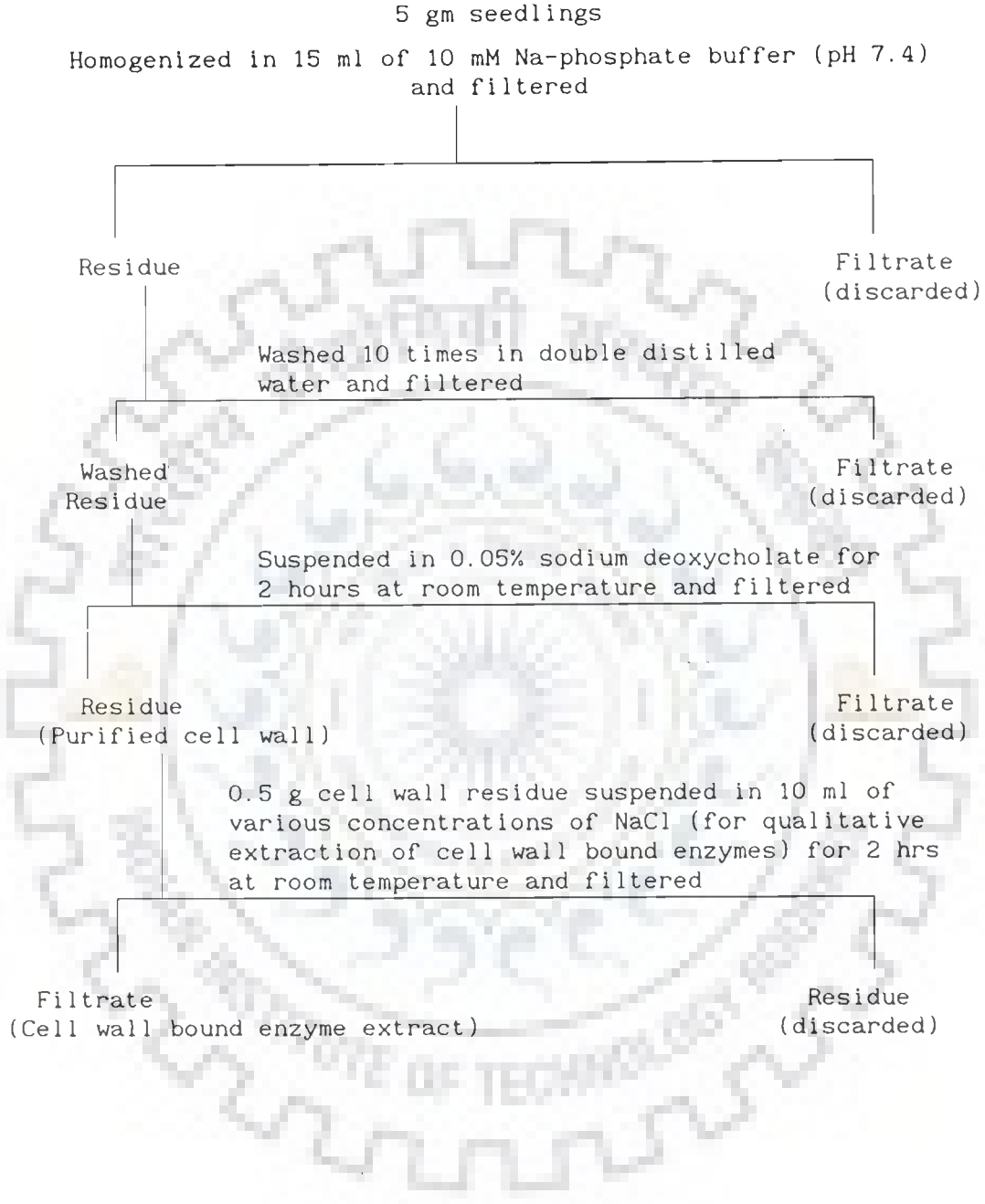


Fig.3.1: Isolation of cell wall bound enzymes

β-galactosidase and 5.6 for α-galactosidase. The reaction was allowed to proceed at 37°C for 10 min and was terminated by addition of 1.5 ml of 0.5 M sodium carbonate. The concentration of p-nitrophenol liberated in the enzymatic reaction was determined by recording the absorbance at 410 nm and comparing with the standard with known concentration of p-nitrophenol which was run simultaneously.

3.2.8.3.2 Assay of cell wall bound acid phosphatase activity

Acid phosphatase activity was measured by the method described by Odds and Hierholzer [116] with slight modifications using p-nitrophenyl phosphate as substrate. The reaction mixture contained 0.5 ml of 50 mM sodium acetate buffer (pH 5.0) and 0.5 ml p-nitrophenyl phosphate. The assay was started by addition of 0.5 ml of enzyme extract. Blank and standards were also run simultaneously. Standards were prepared by taking different concentrations of p-nitrophenol in the place of enzyme solution. The reaction was allowed to proceed at 37° C for 10 min and was terminated by addition of 1.5 ml 0.5 M sodium carbonate.

3.2.8.3.3 Assay of cell wall bound peroxidase activity

Cell wall peroxidase activity was assayed by the method of Kar and Mishra [75] with slight modifications. Reaction mixture contained 2 ml of 0.1 M phosphate buffer (pH 7.0),1 ml of 0.01 M pyrogallol, 1 ml of 0.005 M H_2O_2 and 1 ml well diluted enzyme extract. 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 activity was expressed in absorbancy units/min/mg protein.

3.2.9. Studies on cytoplasmic and cell wall proteins

3.2.9.1 Isolation of cytoplasmic and cell wall proteins

Cytoplasmic and cell wall proteins were isolated according to the method of Bozarth et al. [17] with slight modifications. Following buffers were used for isolating cytoplasmic and cell wall proteins.

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

Procedure:

25 g of 6 day old peanut seedlings were grounded in 25 ml homogenizing buffer (buffer A) at 0°C in a mortar and pestle. The homogenate was filtered through nylon sieve (pore diameter 500 μm approximately) and then centrifuged at 1000xg for 5 min using RA-228 rotor in a cooling centrifuge (Kubota, 1300, Japan). The supernatant was decanted and recentrifuged at 13000xg for 5 min. The supernatant constituted the cytoplasmic extract. The pellet from the 1000xg 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 1 ml buffer B and was incubated at 5°C for 16

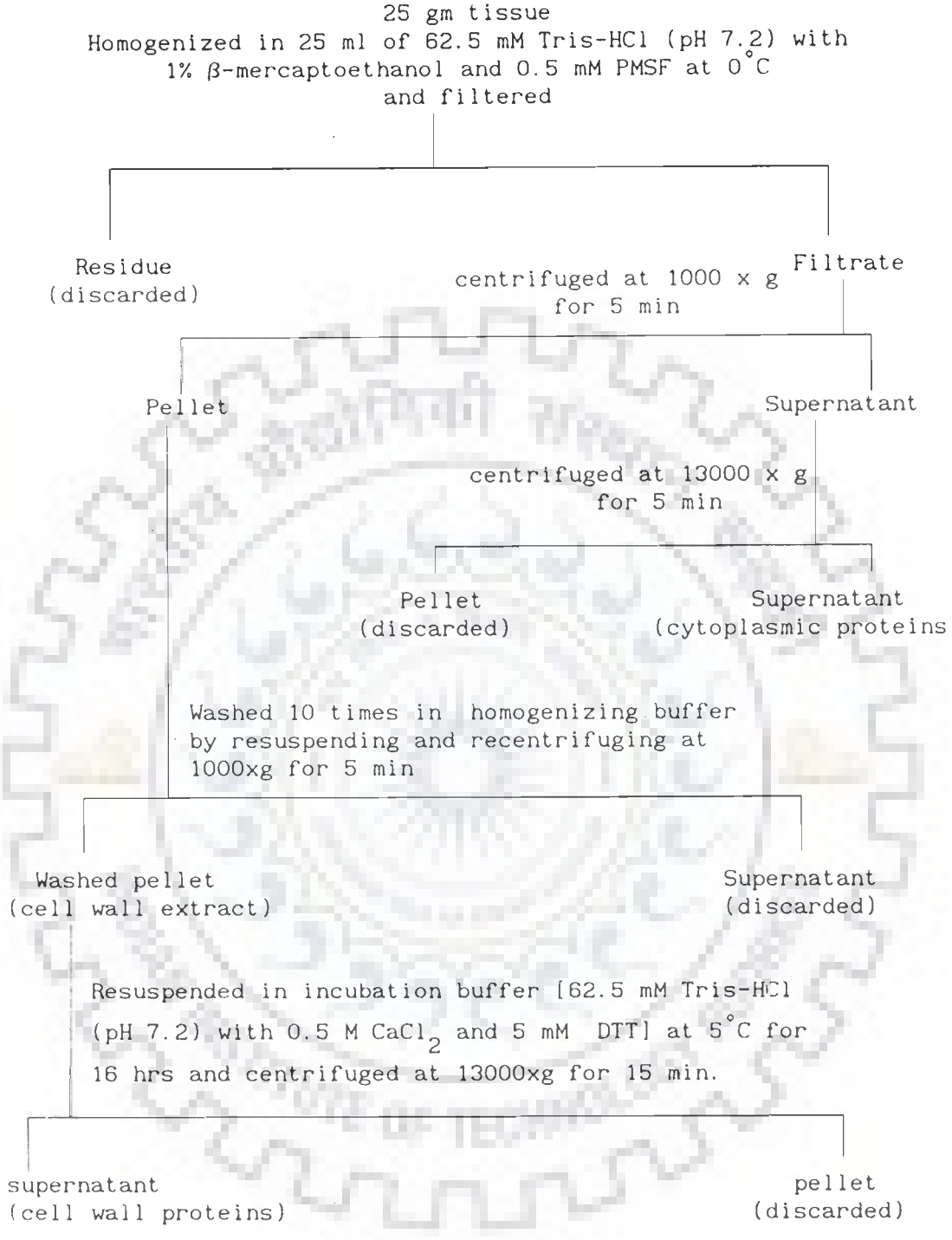


Fig.3.2: Isolation of cytoplasmic and cell wall proteins

hours with occasional stirring. The suspension was centrifuged at 13000xg for 15 min and the supernatant (cell wall extract) was decanted from the cell wall pellet and used for further experiments.

3.2.9.2 Desalting of cell wall protein by gel filtration

Desalting of cell wall proteins was carried out by the method of Porath and Flodin [121].

3.2.9.2.1 Elution buffer

62.5 mM Tris-HCl (pH 7.2) was used as elution buffer for gel filtration chromatography.

3.2.9.2.2 Gel filtration

5 gm Sephadex G-25 (superfine) was swollen in 30 ml of elution buffer by leaving it overnight at room temperature. Gel and buffer were degassed subsequently. The gel was poured into the vertically mounted column (1.3 x 15 cm) using a glass rod. Then three column volume of elution buffer was passed through the bed to stabilize it.The flow rate was kept 45 ml/hour. After that 1 ml protein sample was applied to the bed of column through the syringe and run was started.The flow rate was kept 40 ml/hour.Fractions of 1 ml eluate were collected and the spot tests were done to sort out protein samples from salt solutions. Concentration of protein samples was estimated at 280 nm (in UV) through Beckman DU-6 spectrophotometer.

3.2.9.3 Lyophilization

Desalted samples were concentrated by lyophilization. For this, samples were deep freezed at -70°C and then lyophilized through lyophilizer. These concentrated samples were used for SDS-PAGE.

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

SDS -PAGE was performed on the protein samples according to Laemmli [82] 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.

3.2.9.4.1 Reagents

Solution A: 30% (w/v) Acrylamide solution containing 0.8% (w/v) bisacrylamide (N, N-methylene-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 persulphate

Solution F: TEMED (N, N, N', N'-tetramethylene diamene)

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

3.2.9.4.2 Preparation of resolving and stacking gel

Solutions	Resolving gel (10%) (ml)	Stacking gel (ml)
Solution A	10.000	2.500
Solution 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
Water	14.450	11.300

3.2.9.4.3 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. Then plates were held together by plastic clamps which run down each side of the gel mould. The base of gel mould was sealed by pressing the mould against a silicon rubber gasket in a casting stand. Resolving gel mixture was prepared by mixing all the components (except TEMED). This mixture was degassed for 1 min and 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. After this gel was overlayered with resolving gel buffer. After polymerization of the resolving gel,the assembly was tilted to pour off the overlayer.Then stacking gel mixture was overlayered 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.

3.2.9.4.4 Sample preparation

Desalted protein samples were concentrated and dissolved in the sample solubilizing buffer [0.0625 M 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.

3.2.9.4.5. Electrophoresis

Before loading the samples, 10 µl 0.002% (w/v) bromophenol blue (tracking dye) was loaded into the wells. Then 100 µl of samples containing 100 µg protein were loaded in the wells on the gel surface using a micropipette. Electrophoresis was then carried out at constant voltage. Stacking was done at 120 volts and resolving was done at 150 volts constant voltage. Run was carried out until the tracking dye reached close to the base (1 cm above from bottom) of the gel. The direction of the current 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 then destained by washing it in 12.5% isopropanol and 10% acetic acid. Destaining was done over a period of 48 hours. Solution was renewed as stain leaches out of the gel. Then gel was stored in 7% acetic acid and photographed.

3.2.10. Determination of molecular weights

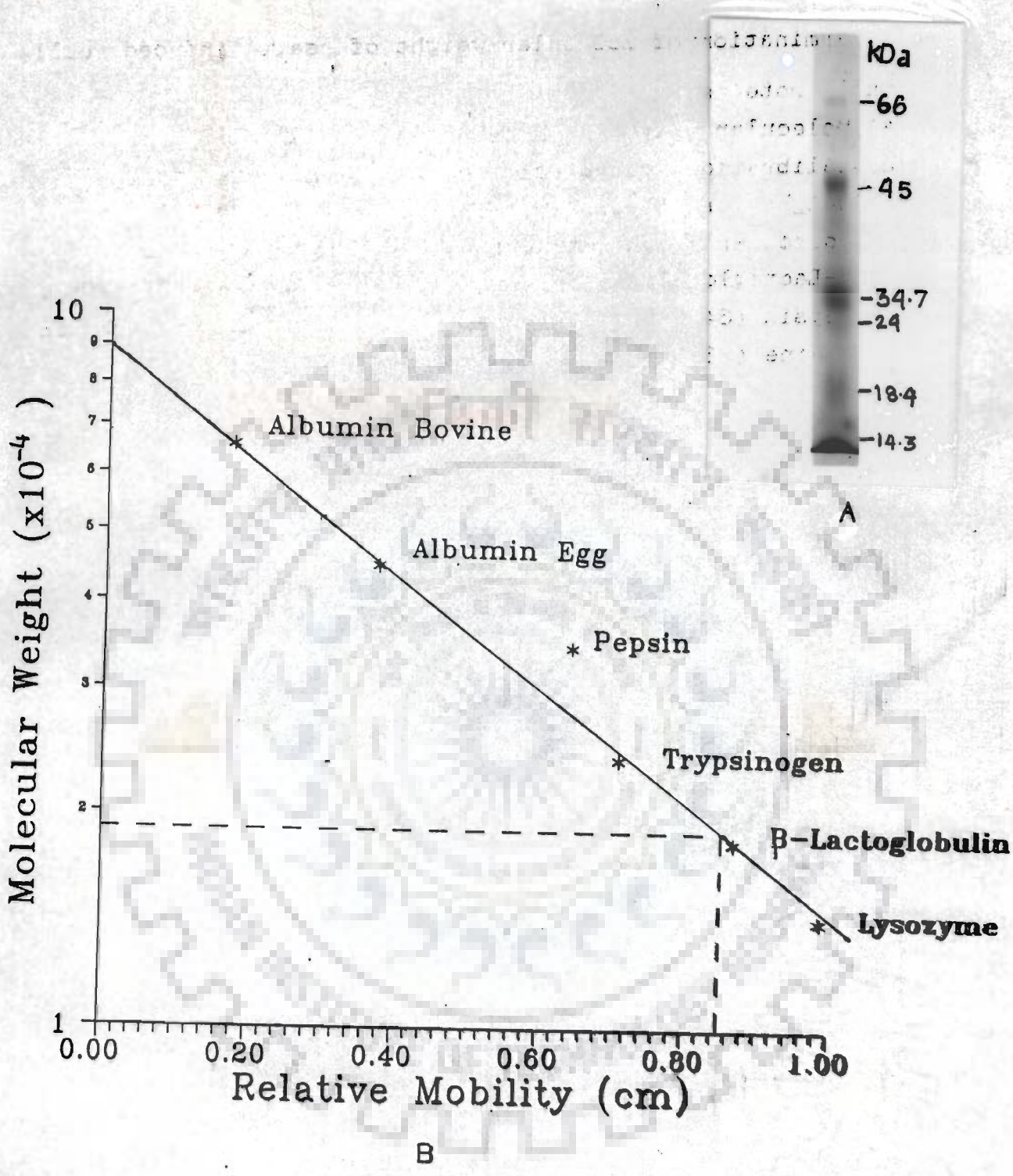
Molecular weight of different protein bands were determined by constructing a plot of $\log_{10}$ polypeptide molecular mass versus relative mobility (R_f) [Fig.3.3]. Relative mobilities of different bands were calculated by using following equation :

$$\text{Relative mobility} = \frac{\text{distance travelled by protein band}}{\text{distance travelled by tracking dye}}$$

Fig. 3.3 Determination of molecular weight of salt induced cell wall proteins

A) Molecular weight markers run on 10% SDS-PAGE

B) Calibration curve of $\log_{10}$ polypeptide molecular weight versus relative mobility on 10% SDS-PAGE. The molecular weight markers used are Lysozyme (14.3 kDa), β-Lactoglobulin (18.4 kDa), Trypsinogen (24 kDa), Pepsin (34.7 kDa), Albumin Egg (45 kDa) and Albumin Bovine (66kDa).


kDa
-66
-45
-34.7
-24
-18.4
-14.3
A
Albumin Bovine
Albumin Egg
Pepsin
Trypsinogen
β-Lactoglobulin
Lysozyme
Molecular Weight (x10⁻⁴)
10
9
8
7
6
5
4
3
2
1
0.00
0.20
0.40
0.60
0.80
1.00
Relative Mobility (cm)
B

3.2.11 Identification of 19 kDa cell wall protein

3.2.11.1 electrophoresis

Cell wall proteins were separated on 10% gel using the method of Laemmli [82]. Constant amount of proteins (100 µg/lane) were loaded in the wells. Electrophoresis was done at constant voltage by stacking it at 120 volts and resolving at 150 volts. After the run, the gel was removed from the plates and stained by the periodic acid-Schiff procedure.

3.2.11.2 Glycoprotein staining

For detection of glycoproteins, PAS staining was done according to the method of Fairbanks et al. [44]. Gel was fixed overnight in 500 ml fixing solution (25% isopropanol and 10% acetic acid). Then, it was kept in 500 ml solution of 10% isopropanol and 10% acetic acid for 6-9 hours. After this, gel was left overnight in 10% acetic acid. It was then transferred to fresh solution of 10% acetic acid and kept in it for several hours, until the background was clear. Fixed gel was left for 2 hours in 0.5% periodic acid solution and then transferred to 500 ml of 0.5% sodium arsenite and 5% acetic acid for 30-60 min. Then, it was kept in the solution of 0.1% sodium arsenite and 5% acetic acid for 20 minutes which was repeated twice. This fixed gel was rinsed in acetic acid for 10-20 min and transferred to Schiff reagent which was prepared by dissolving 2.5 g of basic fuchsin in 500 ml of water, then adding 5 g of sodium metabisulphite and 50 ml of 1 N HCl. The solution was stirred for several hours, and decolourized with about 2 g of activated charcoal. Gel was left overnight in Schiff

reagent and then returned to large bath for incubation in 0.1% sodium metabisulphite and 0.01 N HCl for several hours. This was repeated until the rinse solution failed to turn pink upon addition of formaldehyde. After this, stained gel was photographed.

3.2.12 Characterization of 19 kDa cell wall protein

3.2.12.1 Electrophoresis

Cell wall proteins were separated on 10% gel using the method of Laemmli [82]. Constant amount of proteins (100 µg/lane) were loaded in the wells. Electrophoresis was done at constant voltage by stacking it at 120 volts and resolving at 150 volts.

3.2.12.2 Staining and destaining

After electrophoresis, the gel was stained and destained rapidly according to the method described by Hunkapiller et al. [68]. The staining was done in 0.5% coomassie brilliant blue R-250 in acetic acid:isopropanol:water [1:3:6 (v:v:v)] for 15 min with gentle shaking at room temperature. Destaining was done in acetic acid: methanol: water [50:165:785 (v:v:v)] for 2 to 3 hours with gentle shaking. Then gel was rinsed with water to remove excess fixative.

3.2.12.3 Elution of protein bands from gel slices

Elution of protein bands from gel slices was done according to Bhown and Bennett [9]. Bands were cut from the gel with a clean razor blade. The gel slices were then macerated by chopping finely with a scalpel. This was done to increase the gel surface area so that when a suitable buffer is added elution can occur more readily [143].

Gel pieces were placed into an eppendorf tube. Then 100 µl of elution buffer [50 mM Tris-HCl (pH 8.0) containing 0.1 mM EDTA, 0.1% SDS and 150 mM NaCl] was added and the gel pieces were incubated overnight at 37°C. After incubation the gel containing mixture was centrifuged at 12000xg for 15 min in a cooling centrifuge (Kubota, 1300) using RA-150 AM rotor. The gel pieces were extracted a second time for a shorter period and again centrifuged at 12000xg for 15 min. The supernatants were pooled and their protein content was measured by the method of Lowry et al. [94].

3.2.12.4 Hydrolysis of protein bands for analysis of amino acid composition

Protein bands which were eluted from the gel slices were suspended in 6 N HCl as described by Brown and Howard [20] and hydrolyzed for 24 hours at 110°C in sealed test tubes.

3.2.12.5 Vacuum drying

Sealed test tubes containing hydrolyzed samples were then broken and samples were collected in flasks. These samples were then vacuum dried. Dried samples were redissolved in 62.5 mM Tris-HCl (pH 7.2) and used for analysis of amino acid composition.

3.2.12.6 Amino acid analysis

Samples were loaded into C_{18} column in Waters 440 High performance liquid chromatography (HPLC) system (Millipore Co., MA, USA) for amino acid analysis. Amino acid composition of samples were determined through the standards in absorbance spectrophotometer.

3.2.13 Other methods

3.2.13.1 Protein estimation

Protein estimation was done according to the method of Lowry et al. [94]. The final colour developed as a result of: 1) Biuret reaction of protein with copper ions in alkali. 2) Reduction of the phosphotungstic phosphomolybdic reagent with tyrosine and tryptophan present in the treated protein.

3.2.13.1.1 Reagents

Reagent A: 2% Na_2CO_3 in 0.1 N NaOH

Reagent B: Freshly prepared 0.5% $CuSO_4$ in 1% Na-K-tartrate

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

3.2.13.1.2 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 aliquotes. After this 0.1 N NaOH was added to standards and samples to make the volume 1.5 ml. Blank was also prepared by taking 1.5 ml 0.1 N NaOH. Then 1.5 ml of reagent C was added and vortexed immediately. After this, reaction mixture was incubated for 15 min at room temperature. Subsequently, 150 µl of reagent D was added in test tubes and mixed immediately by vortex. Mixture was then incubated at room

temperature in the dark for 45 min. The absorbance was recorded at 690 nm. Protein content was calculated by plotting a standard curve.

3.2.13.2 Statistical Analysis

One way analysis of variance (ANOVA) was done to test the significance of biochemical and physiological changes in peanut seedlings under salt stress using the method of Rao [125]:

Let there be k samples of sizes n_1, ..., n_k from k populations with unknown means μ_1, ... , μ_k and with a common unknown variance σ^2. The hypothesis which is desired to be tested is:

$$\mu_1 = \mu_2 = \ldots = \mu_k. \qquad (1.1)$$

The observational equations, $n_1 + \ldots + n_k = n$ in number, are given next:

First sample		...	kth sample	
Variable	Expectation	...	Variable	Expectation
y_{11}	μ_1	...	y_{k1}	μ_k
.	.	...	.	.
y_{1n_1}	μ_1	...	y_{kn_k}	μ_k
Total T_1		...	T_k	

The computation is set out in Table 3.1. The quantities marked by * are obtained by subtraction. The F statistic is constructed using the mean sum of squares derived from Table 3.1.

TABLE 3.1 Analysis of Variance, One-Way Classification

Source of variation	D.F.	S.S.	MSS	F
Due to treatment	$k - 1$	$\sum \frac{T_i^2}{n_i} - \frac{T^2}{n}$	MSt =	MSS/MSE
		= SSt	SSt/ k-1	
Error	$n - k^*$	SSE*	MSE= SSE/ n-k	
Total	$n - 1$	$\sum \sum y_{ij}^2 - \frac{T^2}{n}$		

Where D.F. denotes degrees of freedom, S.S. denotes sum of squares, and MSS denotes mean sum of squares.

CHAPTER-4

4.0 RESULTS

4.1 Salt-sensitivity of crop

Fig. 4.1 shows that peanut seedlings do not tolerate salt concentrations above 100 mM NaCl. Growth of seedlings is totally stopped at 150 and 200 mM NaCl. It confirms salt sensitive nature of the crop as it is susceptible to even very low amount of salt concentration.

4.2 Effect of salt stress on the growth of peanut seedling

4.2.1 Morphological changes

Fig. 4.1 and 4.2 shows the effect of salt stress on growth of peanut seedlings. On exposure to salt, marked reduction in growth of peanut seedlings is observed. With increase in the concentration of salt, decrease in growth is observed. There is decrease in length and fresh weight of 10 d old peanut seedlings under salt stress, while dry weight increases (Table 1 and Fig. 4.2). Effect of salt stress on fresh and dry weight and length of various organs is recorded in 10 d old seedlings. Ten day old seedlings are used for the experiments as they have well-differentiated organs. The results are described below:

4.2.1.1 Leaf

Table 2 shows reduction in length and fresh weight of leaves of 10 d old peanut seedlings under salt stress. There is about 11%, 28%, 51% and 75% reduction in length of leaves at 25, 50, 75 and 100 mM salt concentrations respectively (Fig. 4.3). While reduction in

TABLE 1

EFFECT OF SALT STRESS ON THE GROWTH IN 10 DAY OLD WHOLE PEANUT SEEDLINGS

Concentration of NaCl (mM) in nutrient media	CHANGES IN GROWTH OF WHOLE PEANUT SEEDLINGS		
	FRESH WEIGHT (g/organ)	DRY WEIGHT*** (g/gdw)	LENGTH*** (cm)
0	2.55±0.41	0.13±0.07	25.01±1.86
25	2.12±0.41	0.15±0.02	23.51±2.09
50	2.04±0.82	0.15±0.04	22.29±2.21
75	2.11±0.57	0.16±0.04	16.98±3.00
100	1.49±0.45	0.30±0.10	11.93±1.26

*** = significant at 1% level

Peanut seedlings were grown hydroponically on nutrient media with varying concentrations of NaCl. About 20 seedlings were chosen randomly for measurements. Results are mean±SE of 6 observations.

TABLE 2

EFFECT OF SALT STRESS ON THE GROWTH OF LEAF IN 10 DAY OLD WHOLE PEANUT SEEDLINGS

Concentration of NaCl (mM) in nutrient media	CHANGES IN GROWTH OF PEANUT LEAF		
	FRESH WEIGHT*** (g/organ)	DRY WEIGHT*** (g/gdw)	LENGTH*** (cm)
0	0.022±0.002	0.130±0.020	1.550±0.060
25	0.019±0.002	0.120±0.010	1.380±0.060
50	0.019±0.002	0.130±0.010	1.120±0.120
75	0.015±0.001	0.140±0.010	0.760±0.060
100	0.011±0.002	0.290±0.190	0.380±0.030

*** = significant at 1% level

Peanut seedlings were grown hydroponically on nutrient media with varying concentrations of NaCl. About 20 seedlings were chosen randomly for measurements and leaves were separated from them. Results are mean±SE of 6 observations.

Fig. 4.1 Growth pattern of the peanut seedling under salt stress Seedlings were hydroponically grown in (1) distilled water, (2) 0 mM, (3) 25 mM, (4) 50 mM, (5) 75 mM (6) 100 mM (7) 150 mM and (8) 200 mM NaCl concentrations.

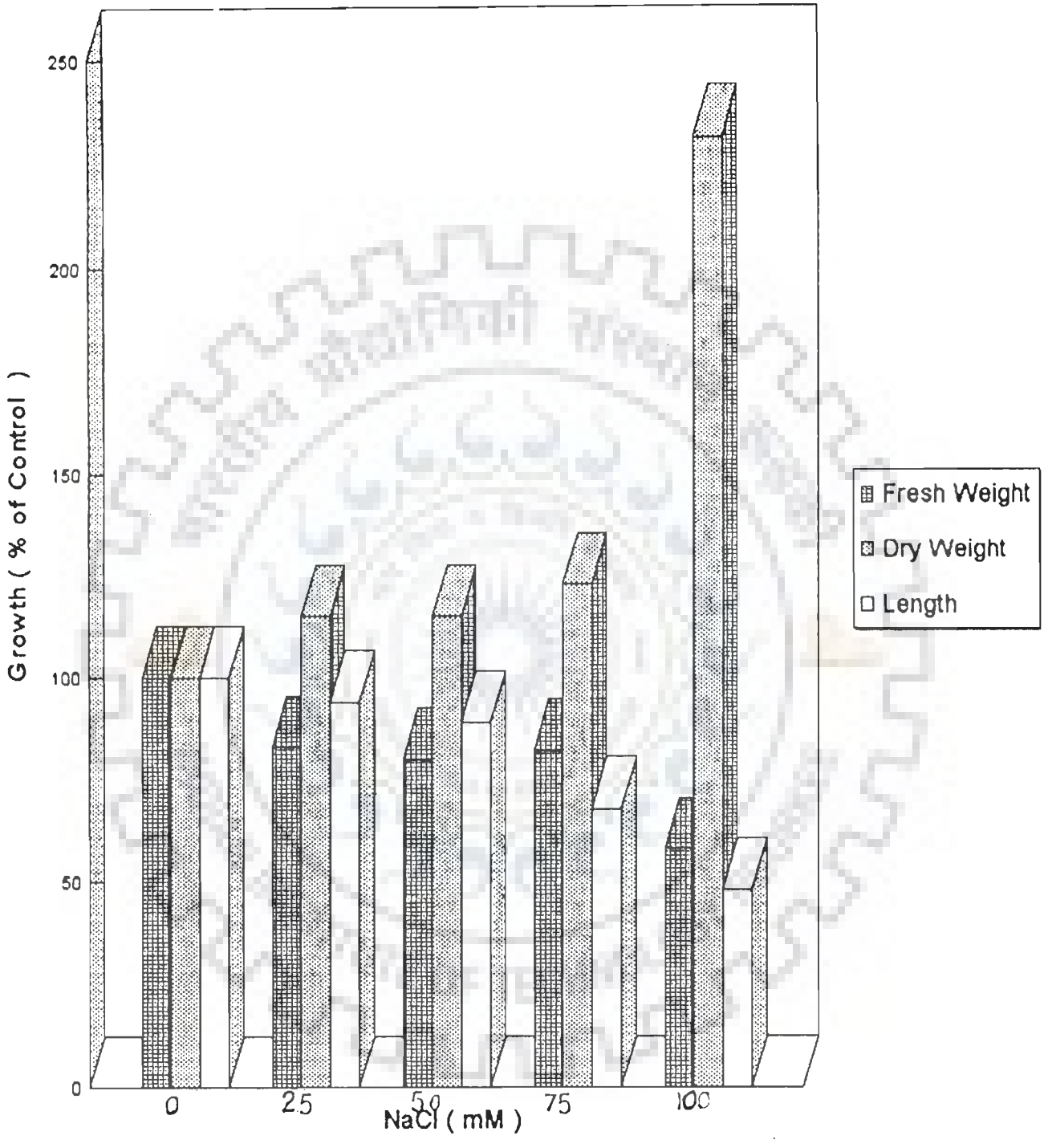


Fig. 4.2 Effect of salt stress on the growth of peanut seedling

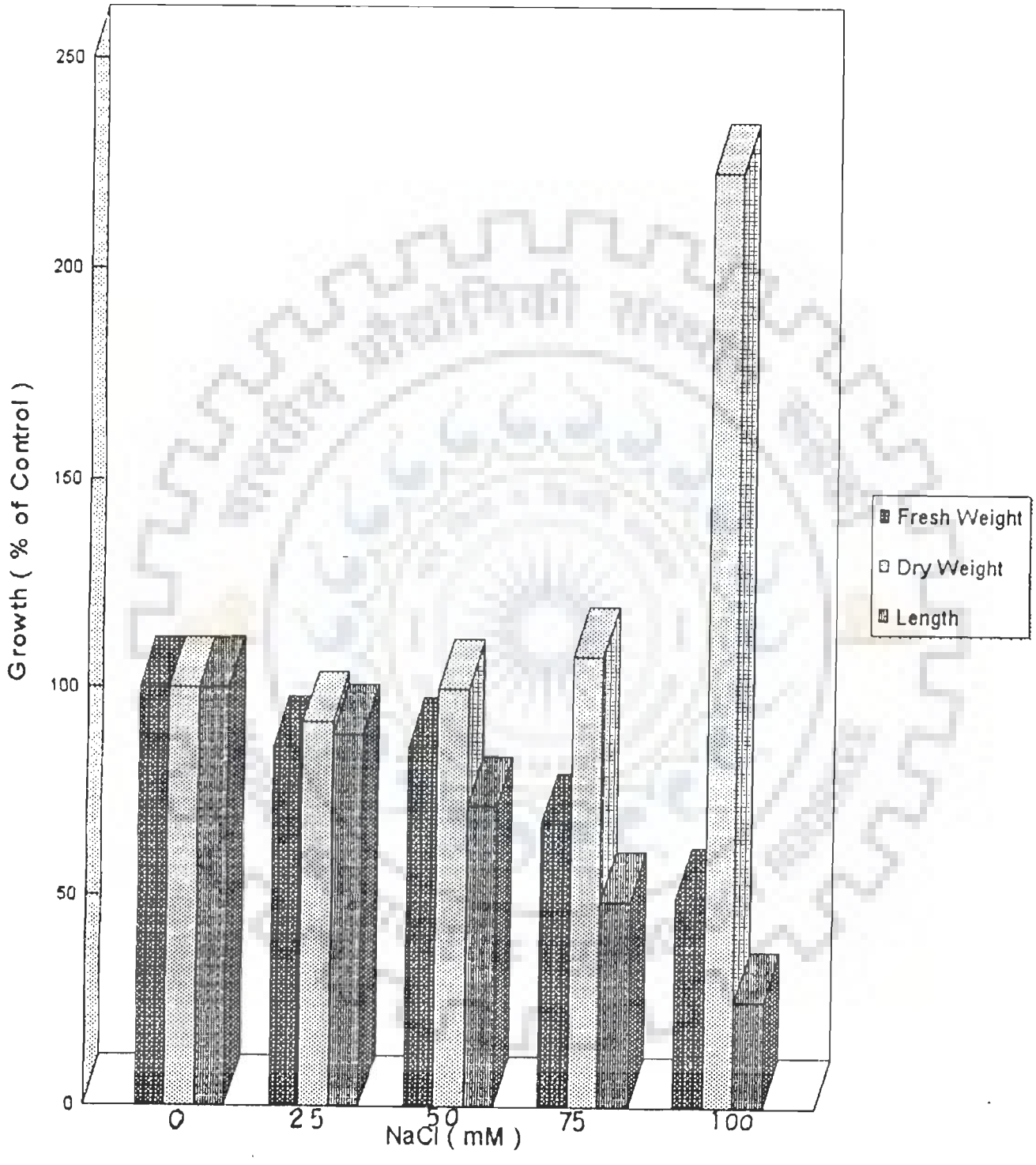


Fig. 4.3 Effect of salt stress on the growth of peanut leaf

fresh weight is 14% in 25 and 50 mM salt treated seedlings and 32% and 50% in 75 and 100 mM salt treated seedlings. There is no marked difference in dry weight of leaves at all salt concentrations except 100 mM where increase is 2.23 folds over controls.

4.2.1.2 Shoot

There is marked reduction in length and fresh weight of shoot under salt stress. Table 3 and Fig. 4.4 shows that length of shoot is reducing ca. 2%, 25%, 72% and 92% in 25, 50, 75 and 100 mM salt treated seedlings respectively. There is ca. 15%, 28%, 40% and 69% reduction in fresh weight at 25, 50, 75 and 100 mM salt concentrations respectively. Slight increase in dry weight of shoots is also observed. Increase in dry weight is 1.13 folds over the controls in 25 and 50 mM salt treated seedlings, whereas it is 1.25 and 1.50 folds higher in 75 and 100 mM salt treated seedlings.

4.2.1.3 Root

It can be seen from Table 4 that there is change in growth of roots under salt stress. About 7%, 6%, 20% and 39% reduction in root length of 25, 50, 75 and 100 mM salt treated seedlings is observed. Fresh weight of root is also reducing with increase in concentration of salt. There is ca. 3%, 9%, 22% and 48% reduction in fresh weight at 25, 50, 75 and 100 mM salt concentrations respectively. Dry weight of roots is 1.17 folds higher at 50 and 75 mM and 1.33 folds higher at 100 mM salt concentration than controls (Fig. 4.5).

TABLE 3

EFFECT OF SALT STRESS ON THE GROWTH OF SHOOT IN 10 DAY OLD WHOLE PEANUT SEEDLINGS

Concentration of NaCl (mM) in nutrient media	CHANGES IN GROWTH OF PEANUT SHOOT		
	FRESH WEIGHT*** (g/organ)	DRY WEIGHT (g/gdw)	LENGTH*** (cm)
0	1.04±0.13	0.08±0.02	5.02±0.63
25	0.88±0.19	0.09±0.01	4.93±0.66
50	0.75±0.19	0.09±0.02	3.76±0.65
75	0.62±0.13	0.10±0.02	1.41±0.89
100	0.32±0.16	0.12±0.05	0.40±0.06

*** = significant at 1% level

Peanut seedlings were grown hydroponically on nutrient media with varying concentrations of NaCl. About 20 seedlings were chosen randomly for measurements and shoots were separated from them. Results are mean±SE of 6 observations.

TABLE 4

EFFECT OF SALT STRESS ON THE GROWTH OF ROOT IN 10 DAY OLD WHOLE PEANUT SEEDLINGS

Concentration of NaCl (mM) in nutrient media	CHANGES IN GROWTH OF PEANUT ROOT		
	FRESH WEIGHT*** (g/organ)	DRY WEIGHT (g/gdw)	LENGTH*** (cm)
0	0.92±0.18	0.06±0.01	18.44±1.74
25	0.89±0.11	0.06±0.01	17.20±2.27
50	0.84±0.14	0.07±0.01	17.41±1.69
75	0.72±0.14	0.07±0.01	14.81±2.46
100	0.48±0.10	0.08±0.03	11.16±1.29

*** = significant at 1% level

Peanut seedlings were grown hydroponically on nutrient media with varying concentrations of NaCl. About 20 seedlings were chosen randomly for measurements and roots were separated from them. Results are mean±SE of 6 observations.

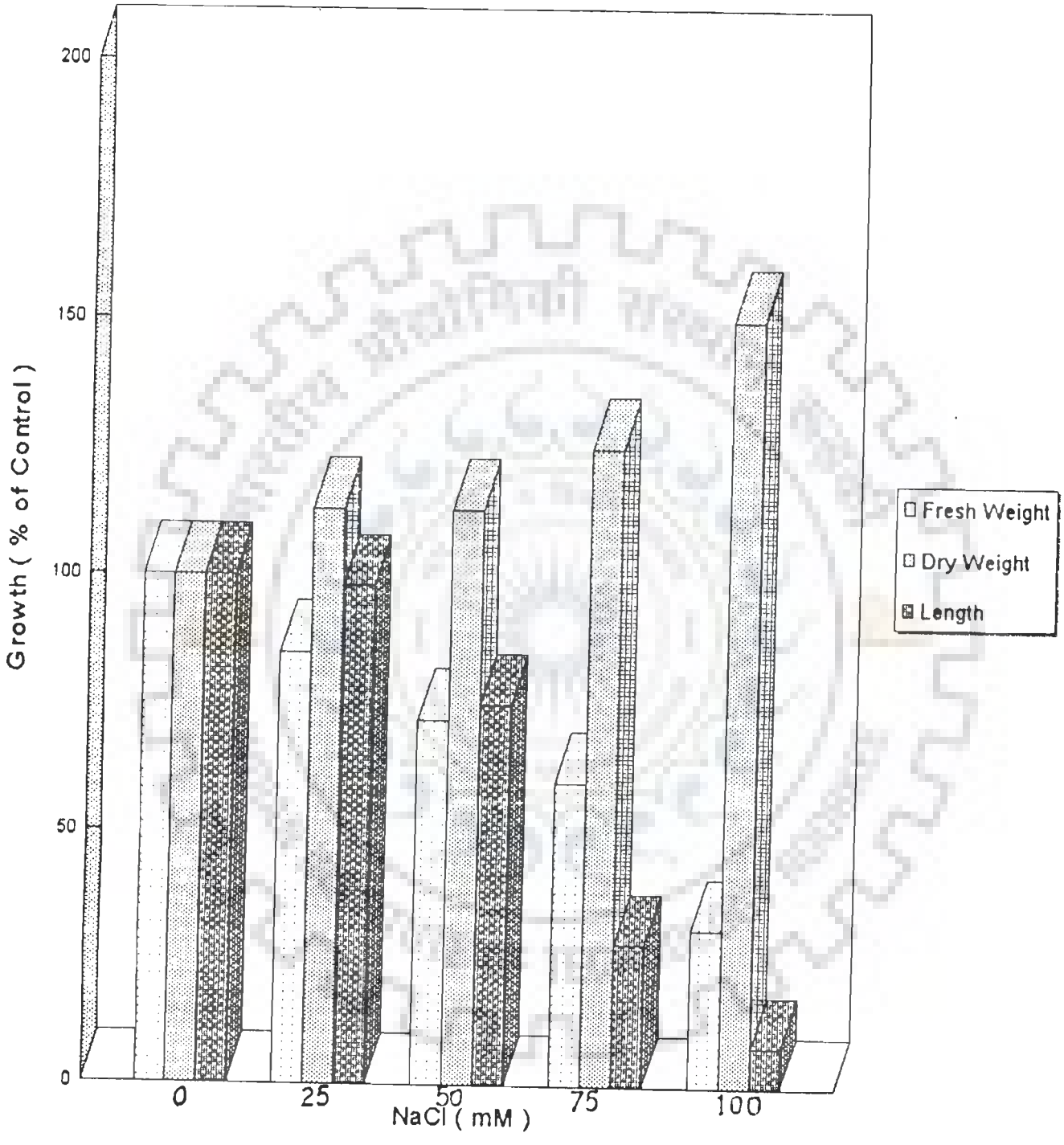


Fig. 4.4 Effect of salt stress on the growth of peanut shoot

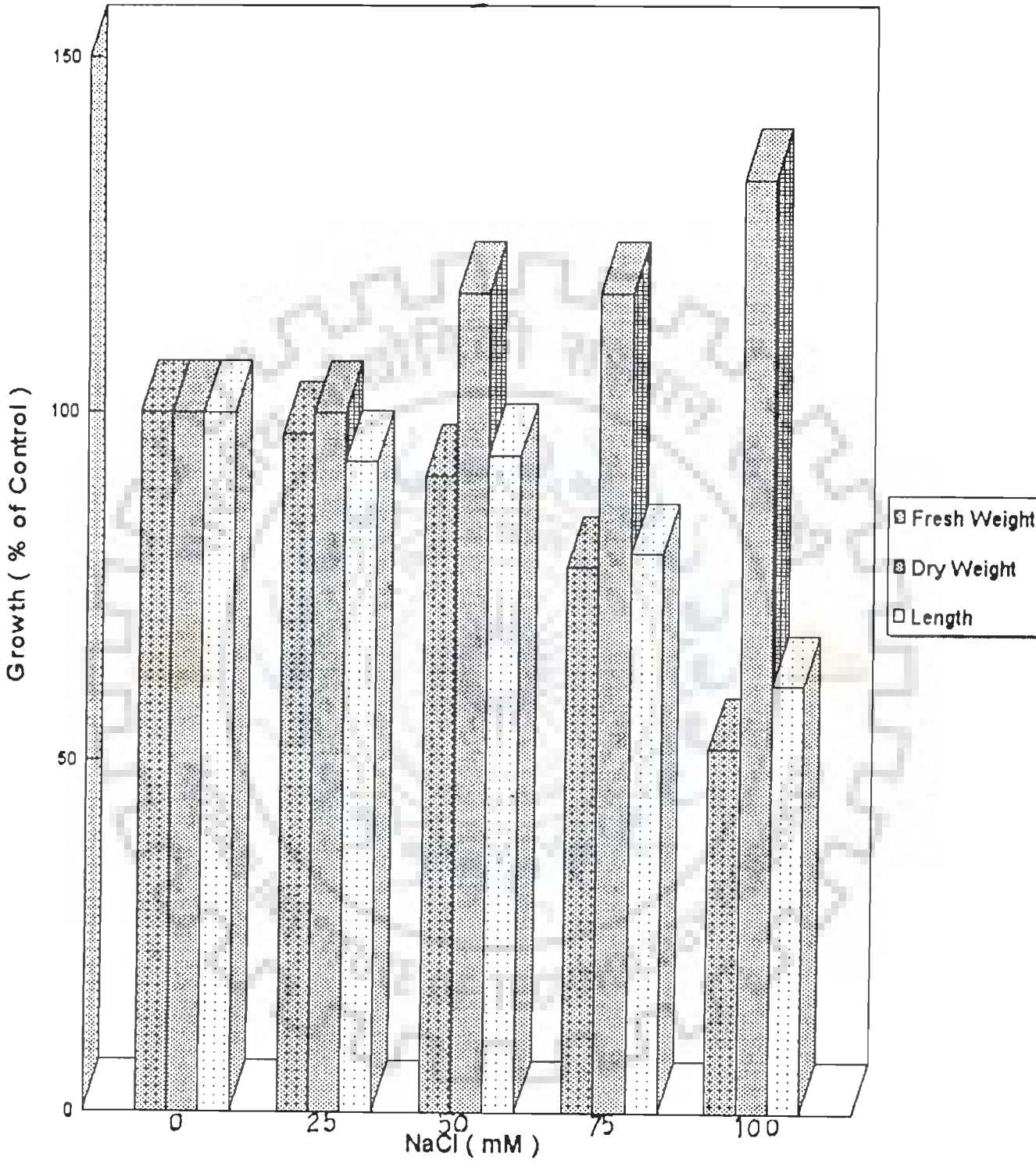


Fig. 4.5 Effect of salt stress on the growth of peanut root

4.2.2 Anatomical changes

The effect of salt stress on the anatomy of roots and shoots of peanut seedlings is also investigated. While root anatomy changes under salt stress, there is no substantial change in shoot anatomy. The results are given in Tables 5, 6 and Fig. 4.6-4.17.

4.2.2.1 Root

The cortical cells of the root register a reduction in diameter by 1%, 2%, 21% and 26% at 25, 50, 75 and 100 mM salt concentrations. However, the number of cortical cell layers increase from 12 in controls to 15, 16, 17 and 17 at at 25, 50, 75 and 100 mM salt concentrations respectively. While there is ca. 25% and 33% increase in cortical cell layers at 25 and 50 mM salt concentrations, 42% increase is observed at 75 and 100 mM salt concentrations (Fig. 4.6).

4.2.2.2 Shoot

On exposure to salt stress, no marked difference in cortical cell size is observed (Fig. 4.7, 4.13-4.17). The number of cortical cell layers also remain same in control as well as in different salt treated seedlings (Fig. 4.6).

4.3 Ion uptake by peanut seedlings under salt stress

Influence of salinity on Na^+ and Cl^- ions accumulation pattern in peanut seedlings is also studied. For this hydroponically grown 10 d old salt treated and control plants which have well-differentiated roots, shoots and leaves are used.

TABLE 5

EFFECT OF SALT STRESS ON NUMBER OF CORTICAL CELL LAYERS IN ROOTS AND SHOOTS OF 10 DAY OLD PEANUT SEEDLINGS

Concentration of NaCl (mM) in nutrient media	NUMBER OF CORTICAL CELL LAYERS IN ROOTS AND SHOOTS OF PEANUT SEEDLING	
	ROOT***	SHOOT
0	12±0.00	10±0.40
25	15±0.88	10±0.49
50	16±0.90	10±0.63
75	17±0.50	10±0.40
100	17±0.76	10±0.00

*** = significant at 1% level

Peanut seedlings were grown hydroponically on nutrient media with varying concentrations of NaCl. Seedlings were chosen randomly for estimations, and separated into roots and shoots. Their sections were cut and diameters were estimated. Values are mean±SE of 4 observations.

TABLE 6

EFFECT OF SALT STRESS ON CORTICAL CELL DIAMETER IN DIFFERENT ORGANS OF 10 DAY OLD PEANUT SEEDLINGS.

Concentration of NaCl (mM) in nutrient media	DIAMETER OF ROOT AND SHOOT CORTICAL CELLS (μM)	
	ROOT***	SHOOT
0	10.75±1.50	10.60±1.50
25	10.70±1.90	14.25±3.30
50	10.64±2.60	13.65±3.00
75	8.48±1.00	10.10±2.20
100	7.95±2.00	10.80±1.70

*** = significant at 1% level

Peanut seedlings were grown hydroponically on nutrient media with varying concentrations of NaCl. Seedlings were chosen randomly for estimations, and separated into roots and shoots. Their sections were cut and diameter were measured. Values are mean±SE of 4 observations.

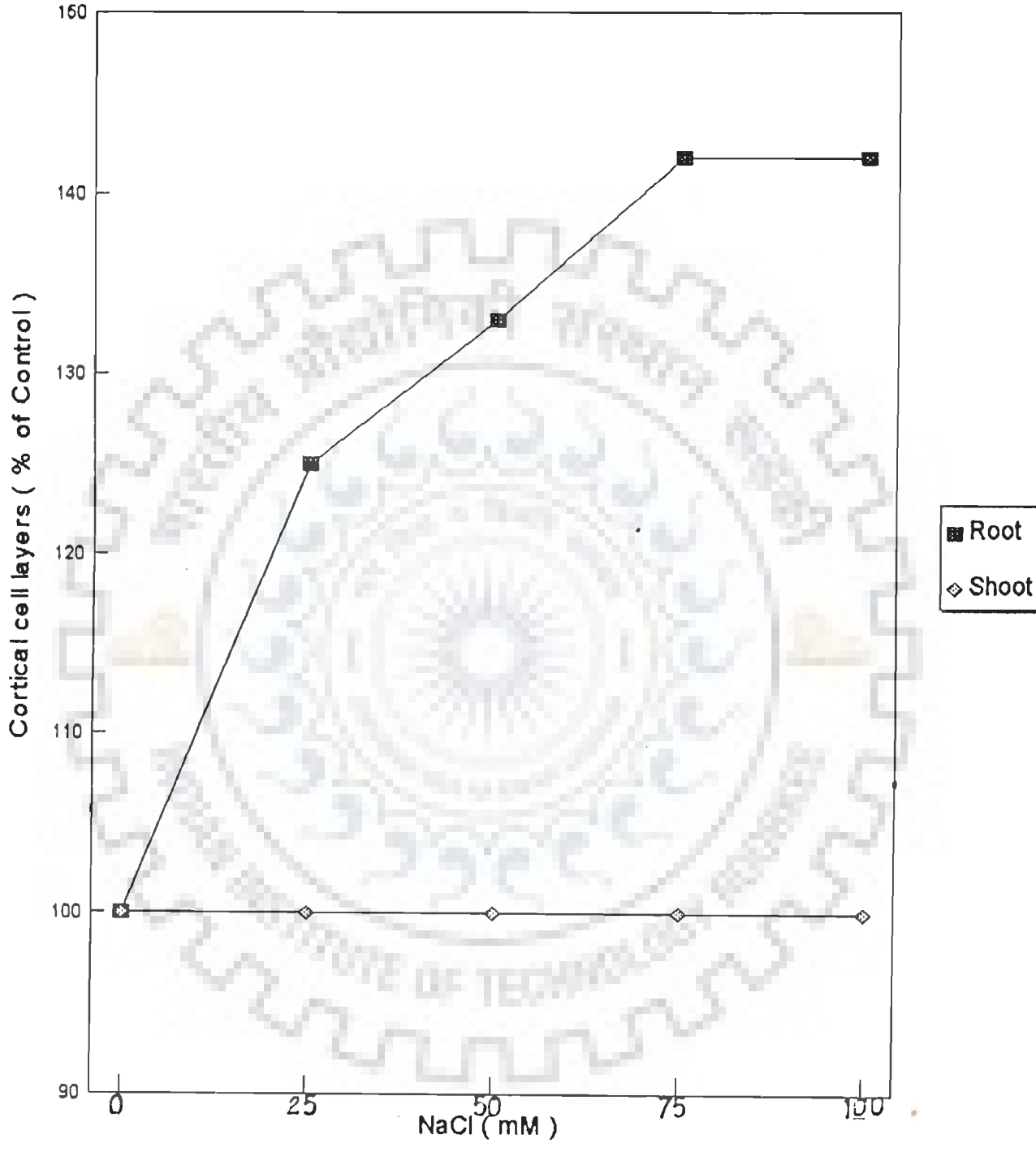


Fig. 4.6 Effect of salt stress on the number of cortical cell layers in peanut root and shoot

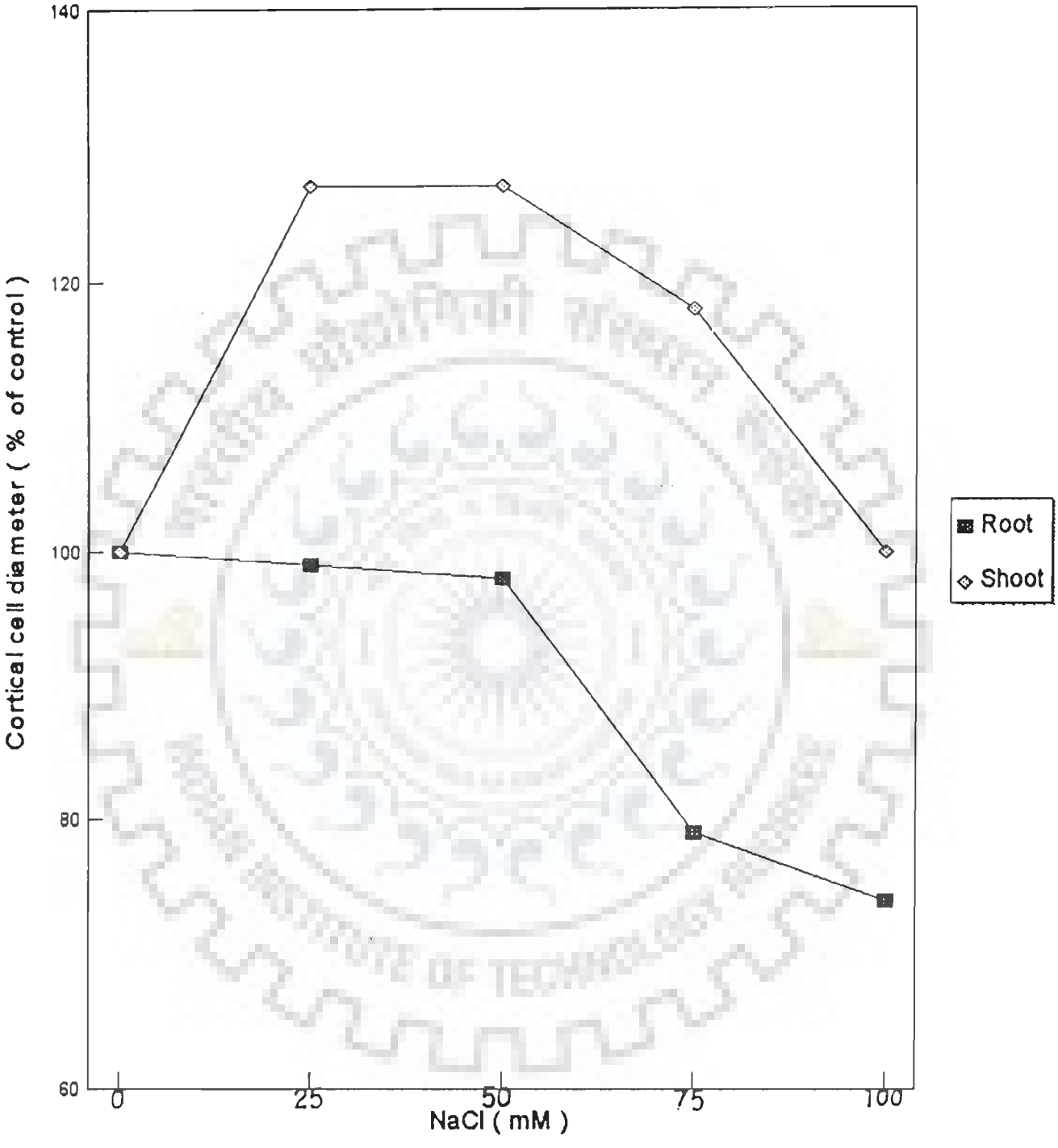


Fig. 4.7 Effect of salt stress on the cortical cell diameter in peanut root and shoot

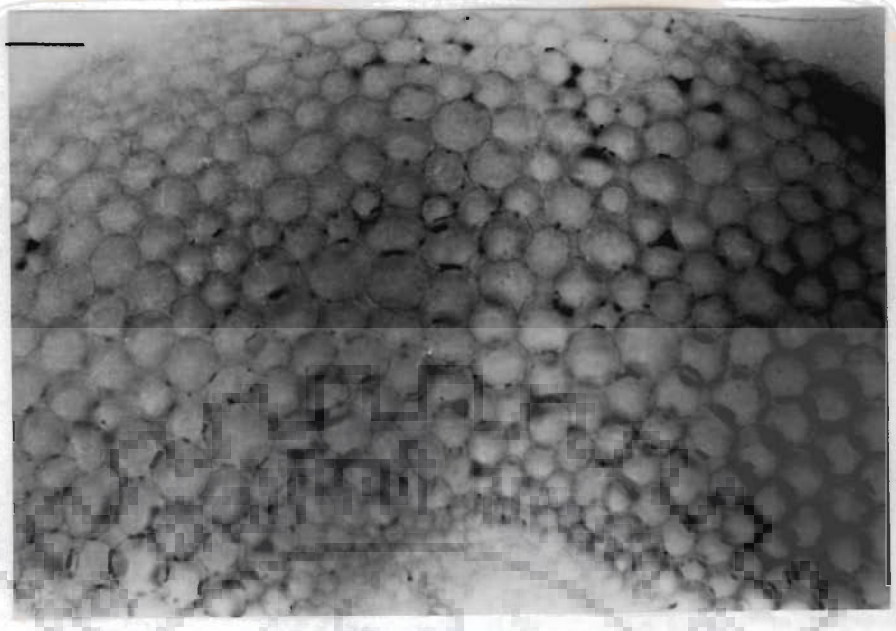


Fig. 4.8 Light micrograph of root section of control peanut seedling (X600). Bar = 16μm.

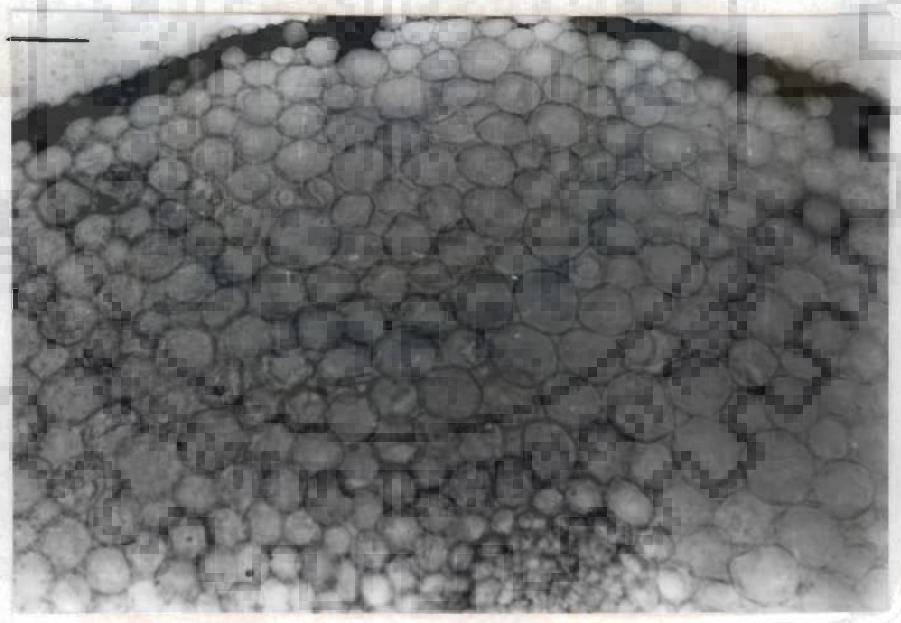


Fig. 4.9 Light micrograph of root section of 25 mM NaCl treated peanut seedling. (X600). Bar = 16 μm.

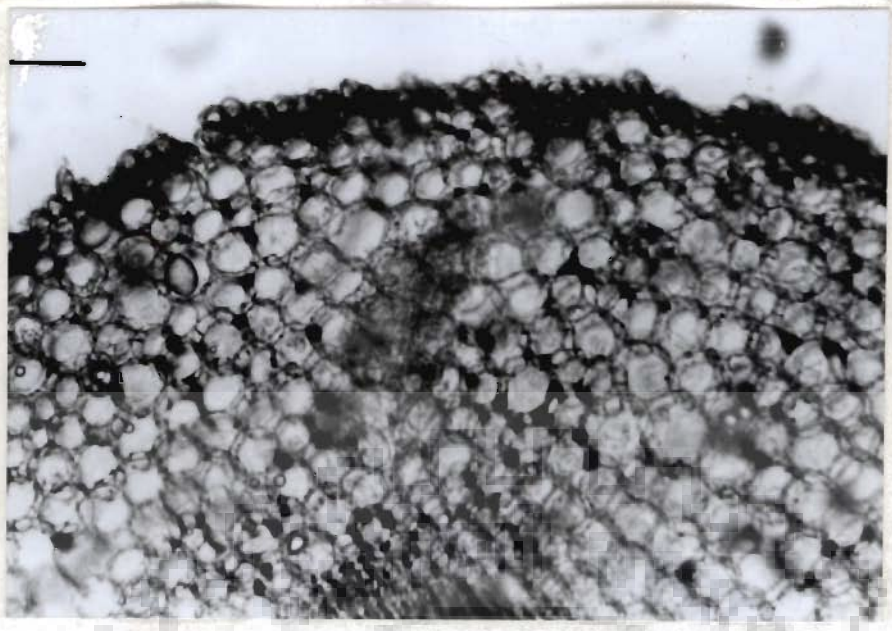


Fig. 4.10 Light micrograph of root section of 50 mM NaCl treated peanut seedling. (X312.5). Bar = 32 μm.

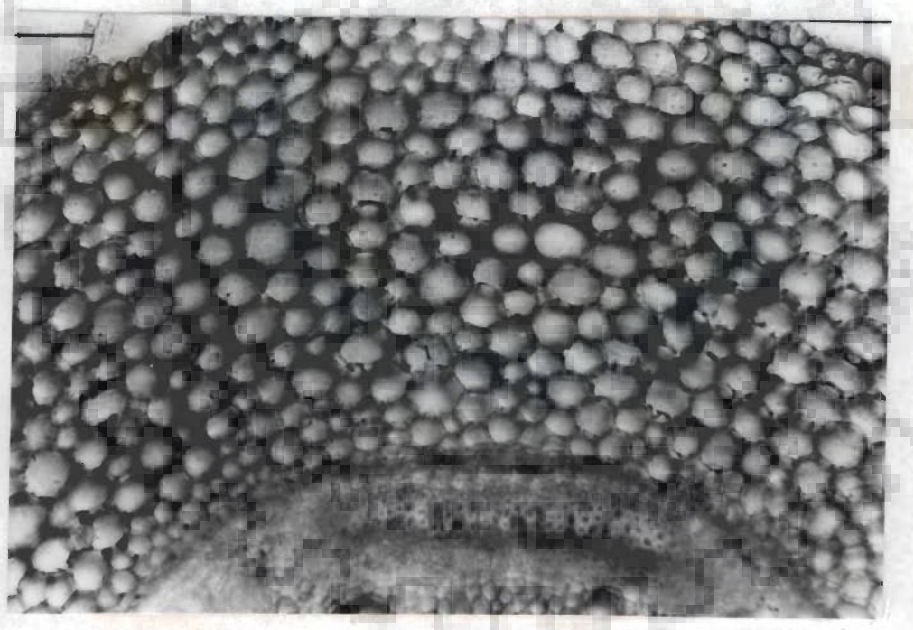


Fig. 4.11 Light micrograph of root section of 75 mM NaCl treated peanut seedling. (X600). Bar = 16 μm.

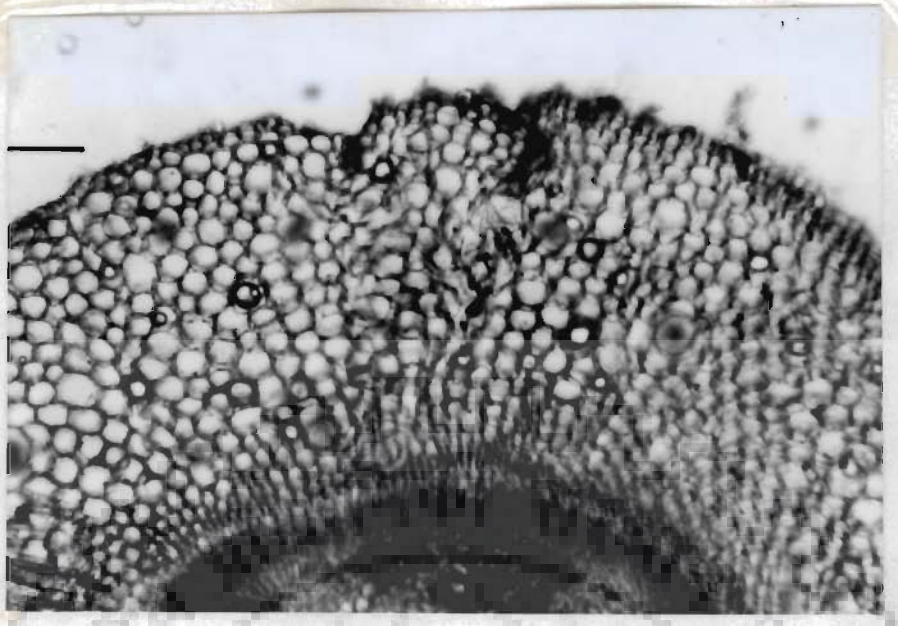


Fig. 4.12 Light micrograph of root section of 100 mM NaCl treated peanut seedling. (X312.5). Bar = 32 μm.

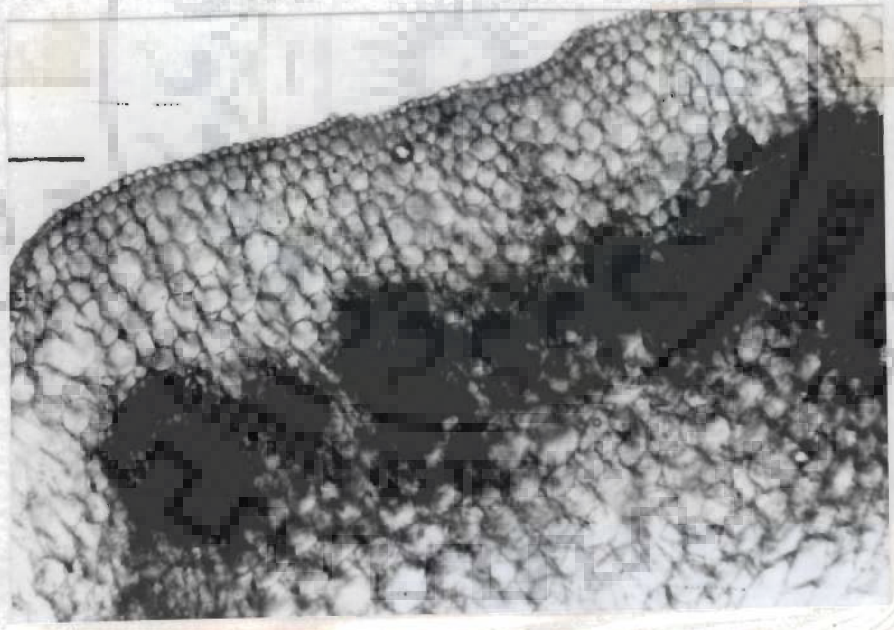


Fig. 4.13 Light micrograph of shoot section of control peanut seedling. (X312.5). Bar = 32 μm.

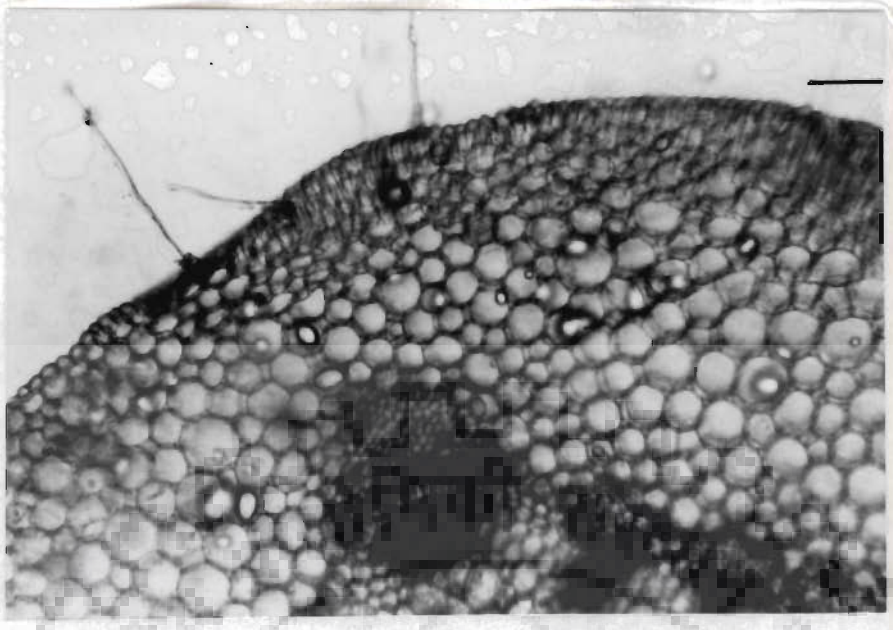


Fig. 4.14 Light micrograph of shoot section of 25 mM NaCl treated peanut seedling. (X312.5). Bar = 32 μm.

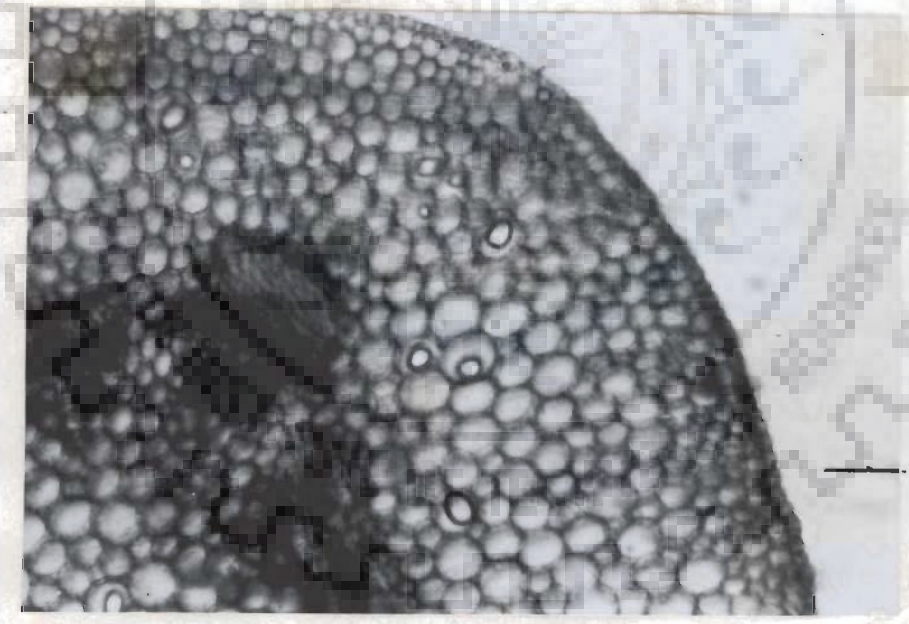


Fig. 4.15 Light micrograph of shoot section of 50 mM NaCl treated peanut seedling. (X312.5). Bar = 32 μm.

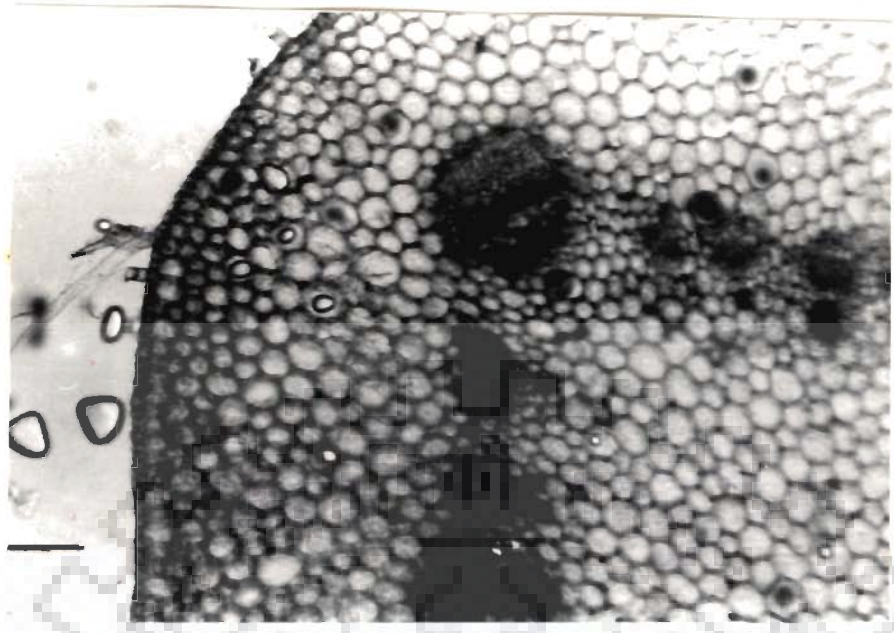


Fig. 4.16 Light micrograph of shoot section of 75 mM NaCl treated peanut seedling. (X312.5). Bar = 32 μm.

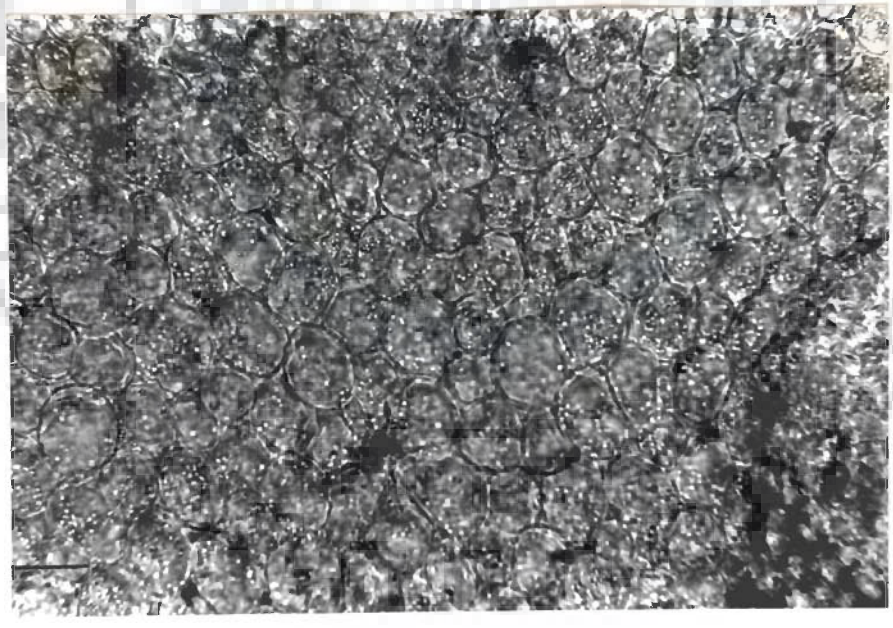


Fig. 4.17 Light micrograph of shoot section of 100 mM NaCl treated peanut seedling. (X750). Bar = 13 μm.

4.3.1 Effect of salt stress on Na^+ ion accumulation in seedlings

Table 7 and 8 summarize the effect of salt stress on *in vivo* level of Na^+ ions in whole seedlings as well as in its various organs. Results show that Na^+ ions accumulate in very high amount in salt treated plants than controls. Organwise distribution shows that roots accumulate more Na^+ ions than the other organs of the seedlings.

4.3.1.1 Whole seedlings

Our results show that on per organ as well as on per gdw basis more Na^+ ions accumulate in salt treated seedlings than control plants. The *in vivo* level of Na^+ ions increase up to 75 mM salt concentration before it slightly decrease at 100 mM salt concentration. It is observed that there is ca. 2.05, 2.35, 3.73 and 2.64 fold increase in Na^+ ions level in 25, 50, 75 and 100 mM salt treated seedlings respectively on per seedling basis (Fig. 4.19). In contrast, on per gdw basis increase in Na^+ ion levels is less pronounced. Here, the increase in Na^+ ions level is ca. 1.51, 2.09, 2.52 and 2.00 folds over the controls in 25, 50, 75 and 100 mM salt treated seedlings (Fig. 4.18).

4.3.1.2 Roots

Highly pronounced effect of salt treatment on *in vivo* level of Na^+ ions in roots is observed. Results show that on per gram dry weight basis *in vivo* level of Na^+ ions increase from 3.93 mg/gdw in

TABLE 7

EFFECT OF SALT STRESS ON THE *IN VIVO* LEVEL OF Na^+ IONS IN 10 DAY OLD WHOLE SEEDLING AND DIFFERENT ORGANS OF PEANUT ON PER GRAM DRY WEIGHT BASIS.

Concentration of NaCl (mM) in nutrient media	*In vivo* level of Na^+ ions (mg/gdw)			
	WHOLE** SEEDLING	SEEDLING PARTS		
		LEAF	SHOOT	ROOT***
0	5.51±2.37	4.75±2.31	4.39±2.34	3.93±2.06
25	8.34±4.97	5.15±2.00	4.49±1.75	17.58±2.52
50	11.53±2.64	5.04±1.33	5.20±2.51	29.90±7.61
75	13.89±2.48	6.06±1.41	6.10±3.12	31.53±11.61
100	11.03±2.44	6.71±2.76	7.23±3.69	32.27±9.04

*** = significant at 1% level
** = significant at 5% level

Peanut seedlings were grown hydroponically on nutrient media with varying concentrations of NaCl. Seedlings were chosen randomly for estimations, and separated into its various parts, oven-dried and acid digested as described in" Materials and Methods". Na^+ content was determined by atomic absorption spectrophotometer. Results are mean±SE of 5 observations.

TABLE 8

EFFECT OF SALT STRESS ON THE *IN VIVO* LEVEL OF Na^+ IONS IN 10 DAY OLD WHOLE SEEDLING AND DIFFERENT ORGANS OF PEANUT ON PER ORGAN BASIS.

Concentration of NaCl (mM) in nutrient media	*In vivo* level of Na^+ ions (mg/organ)			
	WHOLE** SEEDLING	SEEDLING PARTS		
		LEAF	SHOOT	ROOT**
0	1.37±0.44	0.01±0.005	0.36±0.19	0.38±0.28
25	2.81±1.90	0.01±0.005	0.33±0.17	0.99±0.37
50	3.22±0.84	0.01±0.003	0.31±0.12	1.46±0.65
75	5.10±1.72	0.01±0.003	0.26±0.12	1.79±0.76
100	3.62±1.12	0.02±0.012	0.19±0.08	1.25±0.44

** = significant at 5% level

Peanut seedlings were grown hydroponically on nutrient media with varying concentrations of NaCl. Seedlings were chosen randomly for estimations, and separated into its various parts, oven-dried and acid digested as described in" Materials and Methods". Na^+ content was determined by atomic absorption spectrophotometer. Results are mean±SE of 5 observations.

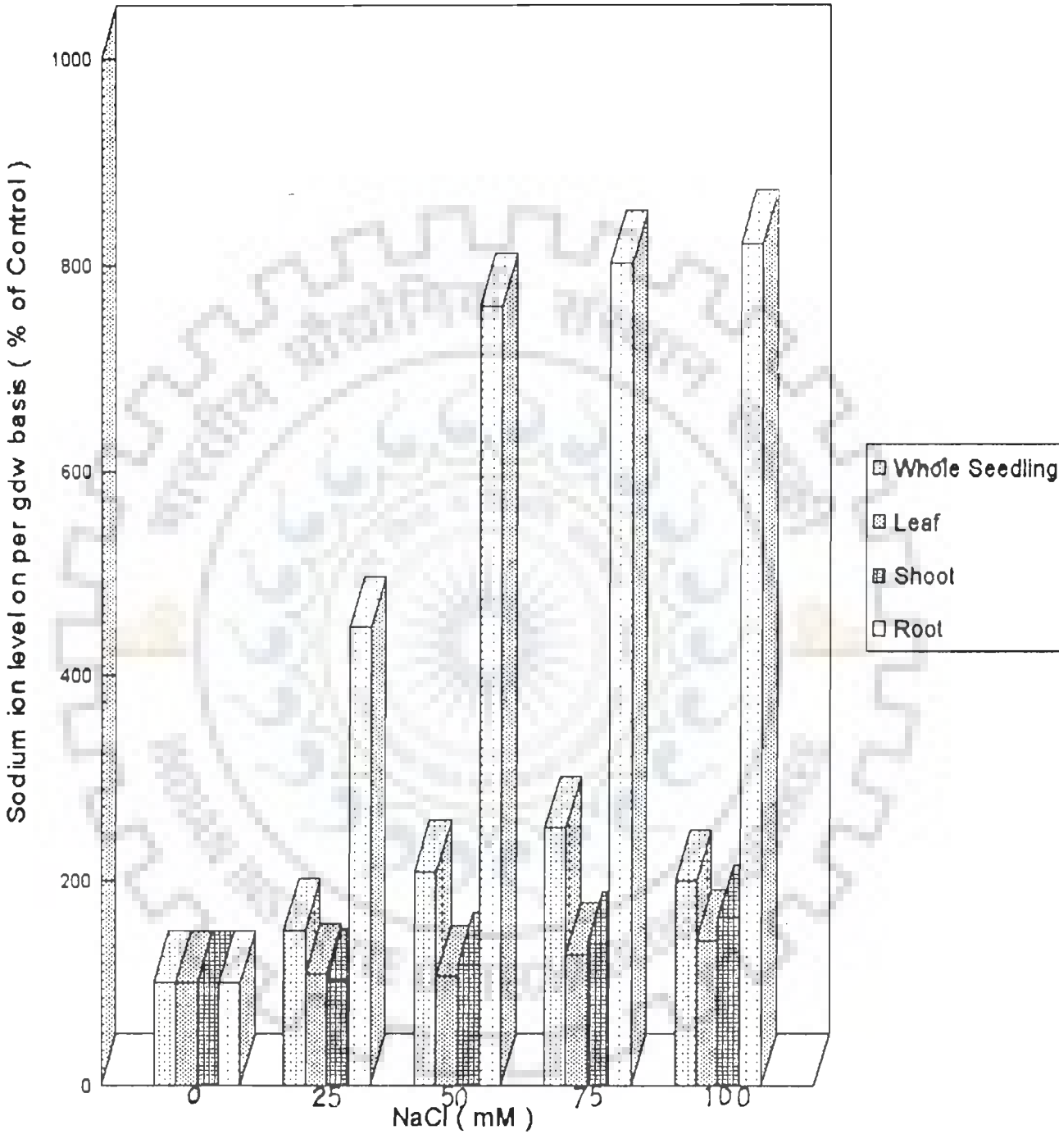


Fig. 4.18 Sodium ions accumulation on per gdw basis in peanut seedling under salt stress

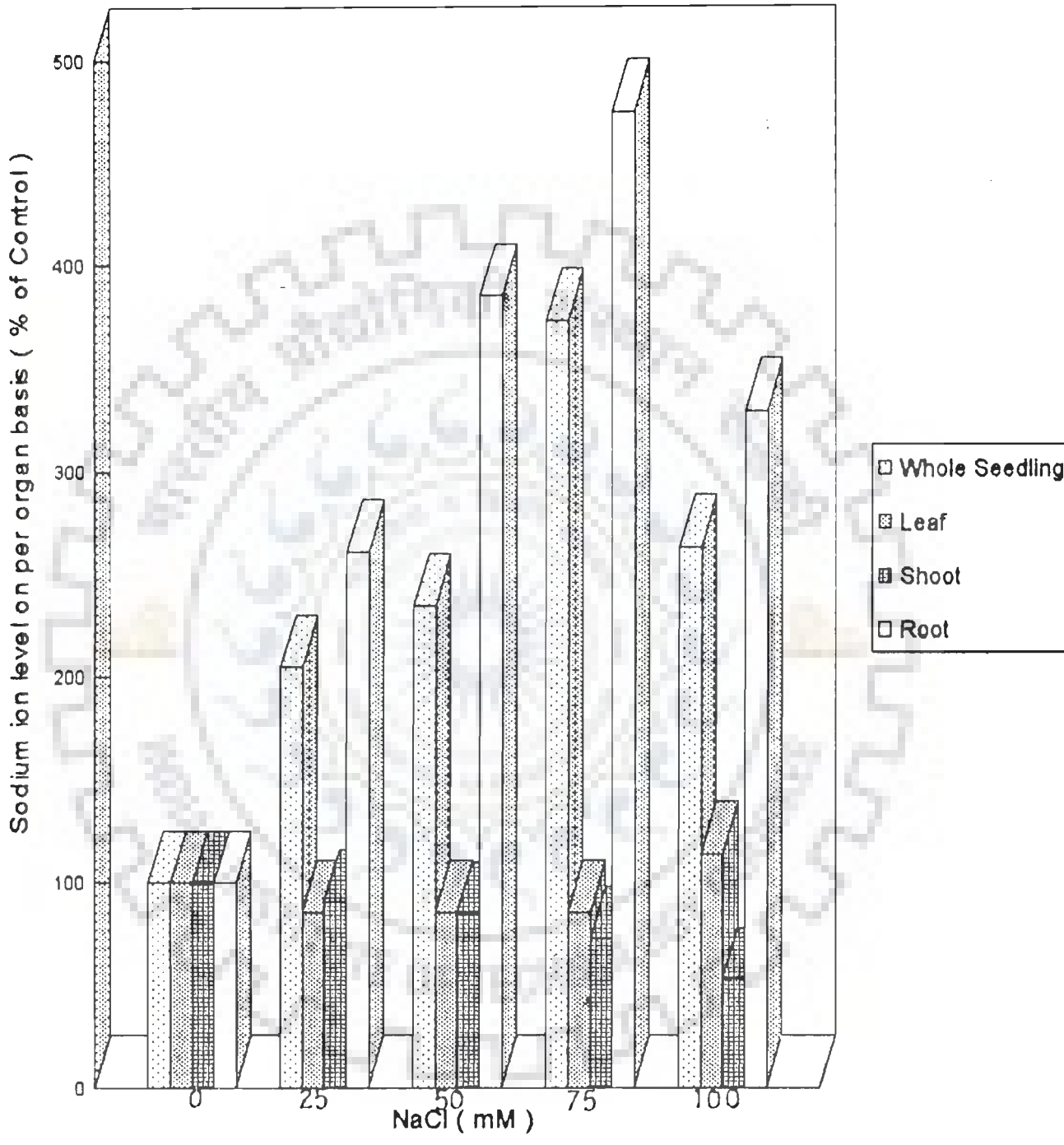


Fig. 4.19 Sodium ions accumulation on per organ basis in peanut seedling under salt stress

control to 17.58 mg/gdw (4.47 fold increase)at 25 mM, 29.90 mg/gdw (7.61 fold increase) at 50 mM, 31.53 mg/gdw (8.02 fold increase) at 75 mM and 32.27 mg/gdw (8.21 fold increase) at 100 mM salt concentration. On per organ basis roots accumulate about 2.61, 3.85, 4.75 and 3.30 folds higher Na^+ ions than controls.

4.3.1.3 Leaves

Table 7 show that leaves accumulated 4.75 mg/gdw Na^+ ions in controls and 5.15, 5.04, 6.06 and 6.71 mg/gdw Na^+ ions in 25, 50, 75, and 100 mM salt treated seedlings respectively. There is about 1.08, 1.06, 1.28 and 1.41 fold increase in Na^+ accumulation in 25, 50, 75 and 100 mM NaCl treated seedlings (Fig. 4.18). In contrast, on per organ basis, sodium ion concentration remain almost same upto 75 mM salt treatment and subsequently is doubled at 100 mM salt concentration (Fig. 4.19).

4.3.1.4. Shoots

Results show that on per gram dry weight basis, there is slight increase in sodium ion levels in shoot with increase in concentration of salt. Thus on per gdw basis, there is about 1.02, 1.18, 1.39 and 1.65 fold increase in sodium ion levels in peanut shoot when exposed to 25, 50, 75 and 100 mM salt concentrations respectively (Fig. 4.18). This pattern is reversed on per organ basis where sodium ion levels constantly decrease with increase in salt amounts. There is about 9%, 15%, 27% and 47% decrease in sodium ions level in shoots at 25, 50, 75 and 100 mM salt concentrations respectively (fig 4.19).

4.3.2 Effect of salt stress on Cl^- ion accumulation in peanut seedlings

Effect of salt exposure on *in vivo* level of Cl^- ions in whole seedlings and its various organs is given in Table 9 and 10. Seedlings growing in excess amount of salts accumulation more Cl^- ions in comparison to controls. Organwise distribution shows that Cl^- ions accumulate more in roots followed by shoots and leaves. While there is increase in Cl^- ions accumulation in roots and shoots on per organ as well as on per gram dry weight basis, in leaves the concentration of Cl^- ions remain similar to controls on per organ basis. Further while there is reduction in Cl^- ions accumulation at higher salt concentrations in leaves on gdw basis, their content increase at lower concentrations.

4.3.2.1 Whole seedlings

A dramatic increase in Cl^- ions accumulation is observed in salt treated seedlings in comparison to controls. On gdw basis, with increase in concentrations of salt Cl^- ions accumulation increase up to 75 mM and then declines. The increase in Cl^- ion levels over the controls is 1.47, 1.85, 2.27 and 1.62 folds at 25, 50, 75 and 100 mM salt concentrations (Fig. 4.20). In contrast, on per seedling basis the increase in Cl^- ions accumulation over the controls is about 1.48, 1.62, 1.99 and 2.16 fold at 25, 50, 75 and 100 mM salt concentrations respectively (Fig. 4.21).

4.3.2.2 Roots

Table 9 and Fig. 4.20 shows that on per gdw basis *in vivo* level of Cl^- ions increases from 538 mg/gdw in controls to 695, 1290,

TABLE 9

EFFECT OF SALT STRESS ON THE *IN VIVO* LEVEL OF Cl^- IONS IN 10 DAY OLD WHOLE SEEDLINGS AND DIFFERENT ORGANS OF PEANUT ON PER GRAM DRY WEIGHT BASIS.

Concentration of NaCl (mM) in nutrient media	*In vivo* level of Cl^- ions (mg/gdw)			
	WHOLE SEEDLING	SEEDLING PARTS		
		LEAF	SHOOT	ROOT***
0	343±197	300±230	437±146	538±326
25	505±090	323±160	600±241	695±390
50	633±260	263±200	600±100	1290±310
75	780±080	196±085	625±101	1748±310
100	557±180	143±049	697±180	1608±240

*** = significant at 1% level

Peanut seedlings were grown hydroponically on nutrient media with varying concentrations of NaCl. Seedlings were chosen randomly for estimations, and separated into its various parts, oven-dried and acid digested as described in" Materials and Methods". Cl^- content was determined colorometrically by Beckman DU-6 spectrophotometer. Results are mean±SE of 4 observations.

TABLE 10

EFFECT OF SALT STRESS ON THE *IN VIVO* LEVEL OF Cl^- IONS IN 10 DAY OLD WHOLE SEEDLING AND DIFFERENT ORGANS OF PEANUT ON PER ORGAN BASIS.

Concentration of NaCl (mM) in nutrient media	*In vivo* level of Cl^- ions (mg/organ)			
	WHOLE SEEDLING	SEEDLING PARTS		
		LEAF	SHOOT	ROOT*
0	117.0±099.8	1.0±0.8	25.0±20.0	33.0±15.0
25	173.0±040.0	1.0±0.3	45.0±19.0	43.0±20.0
50	190.0±090.0	1.0±0.7	38.0±10.0	70.0±20.0
75	233.0±120.0	1.0±0.3	38.0±17.0	80.0±10.0
100	253.0±140.0	1.0±0.3	30.0±20.0	77.0±30.0

* = significant at 10% level

Peanut seedlings were grown hydroponically on nutrient media with varying concentrations of NaCl. Seedlings were chosen randomly for estimations, and separated into its various parts, oven-dried and acid digested as described in" Materials and Methods". Cl^- content was determined colorometrically by Beckman DU-6 spectrophotometer. Results are mean±SE of 4 observations.

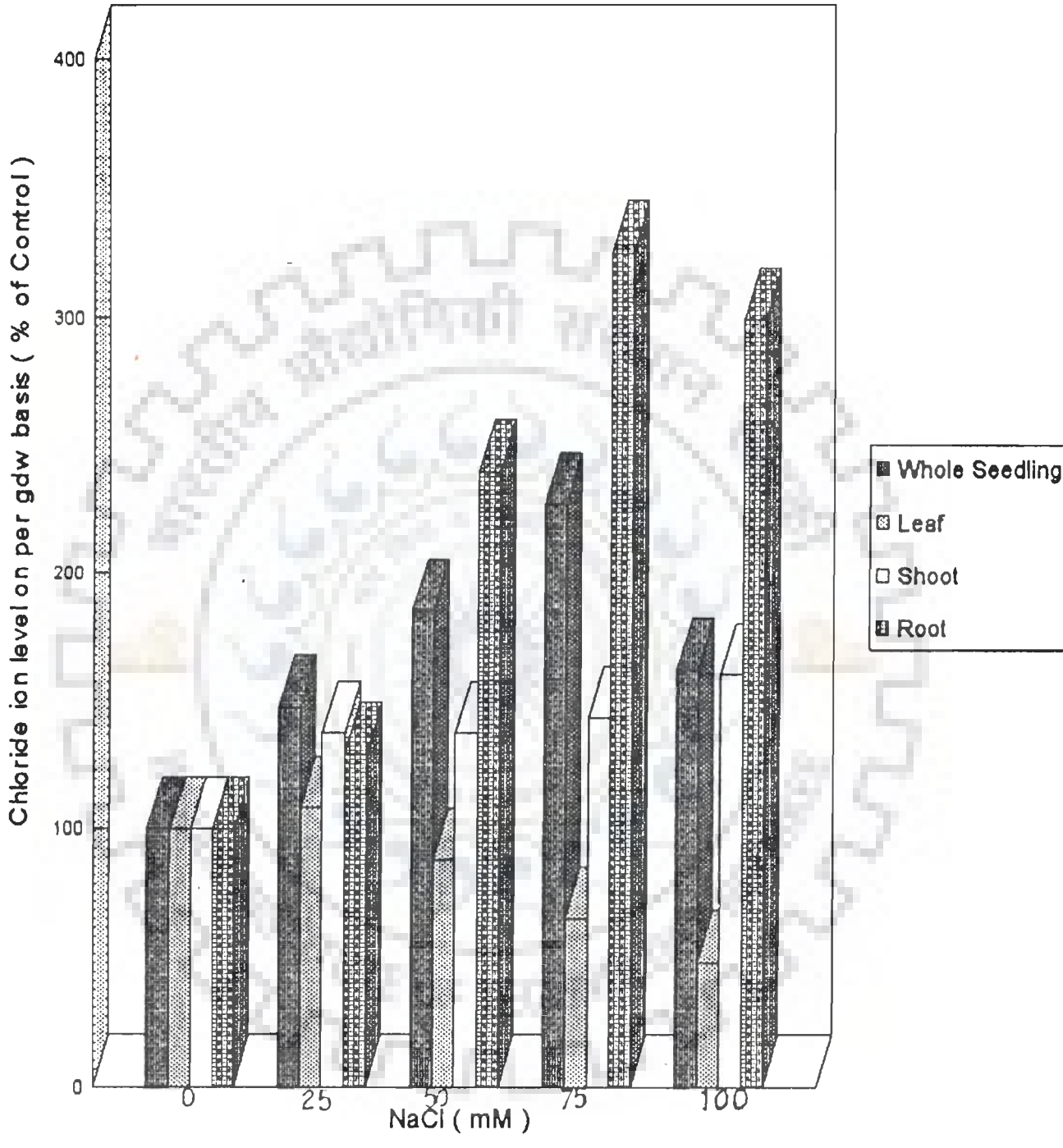


Fig. 4.20 Chloride ions accumulation on per gdw basis in peanut seedling under salt stress

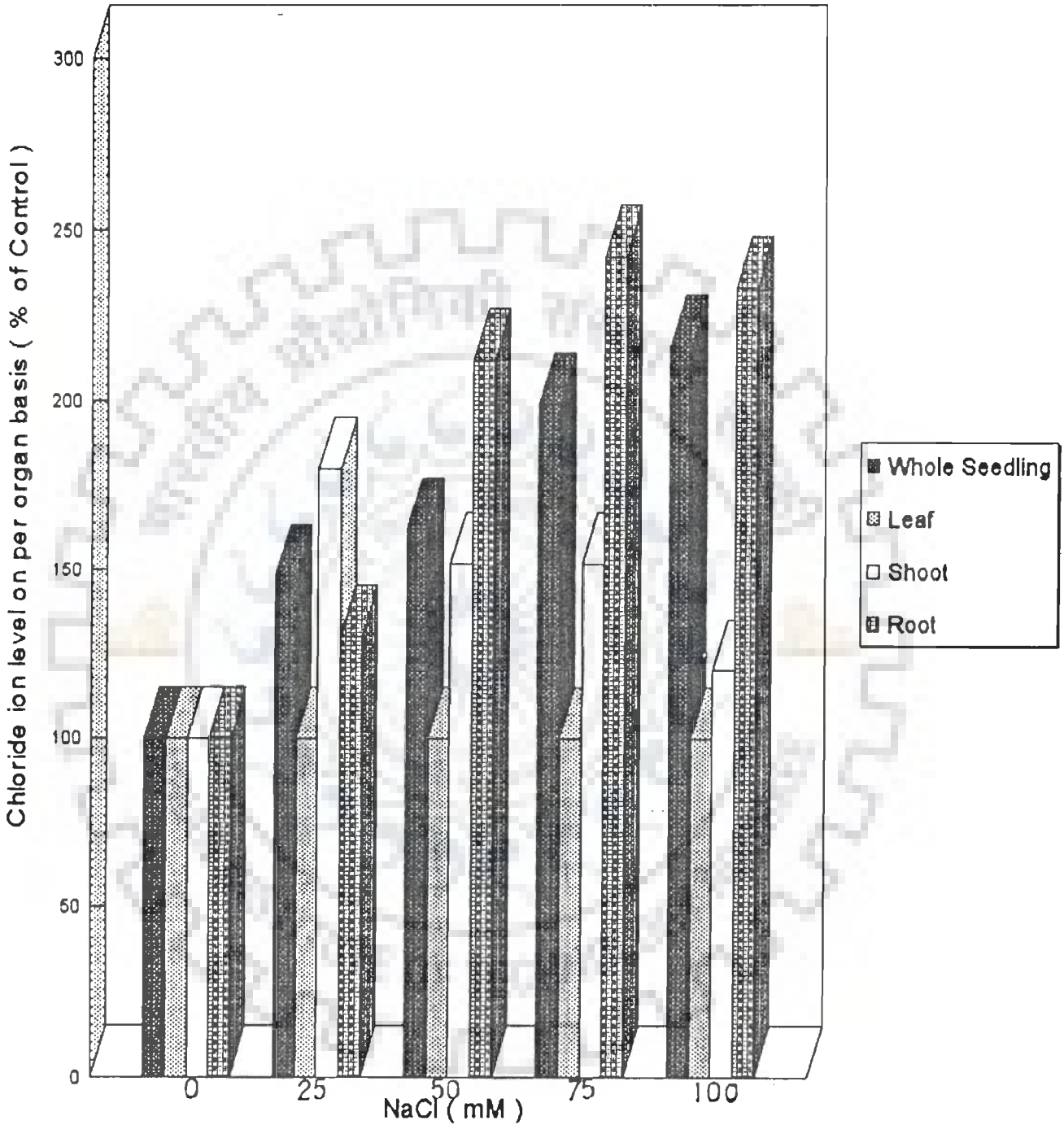


Fig. 4.21 Chloride ions accumulation on per organ basis in peanut seedling under salt stress

1748 and 1608 mg/gdw (about 1.29, 2.40, 3.25 and 2.99 fold increase) at 25, 50, 75 and 100 mM salt concentrations respectively. On per organ basis accumulation of Cl^- ions is changed to 1.30, 2.12, 2.42 and 2.33 folds in 25, 50, 75 and 100 mM NaCl treated seedlings respectively (Fig. 4.21).

4.3.2.3 Shoots

With increase in concentration of salt, *in vivo* level of Cl^- ions in shoots is increase slightly. Whereas the increase in Cl^- ions concentration over the controls is 1.37, 1.37, 1.43 and 1.60 folds at 25, 50, 75 and 100 mM salt concentrations respectively on per gdw basis (Fig. 4.20), on per organ basis it is changed to 1.80, 1.52, 1.52 and 1.20 folds in 25, 50, 75 and 100 mM salt treated seedlings (Fig. 4.21).

4.3.2.4. Leaves

In leaves on gdw basis although at 25 mM salt concentration there is slight increase in the amount of Cl^- ions, over the controls, at higher salt concentrations, there is decline. In contrast, on per organ basis, there is no effect of salt treatment on the content of Cl^- ions in leaves.

4.4 Effect of salt stress on some enzymes

4.4.1 Effect of salt stress on specific activities of plasma membrane and tonoplast ATPase

Table 11 summarize the changes in plasma membrane and tonoplast ATPase activities in relation to salt stress. Results show

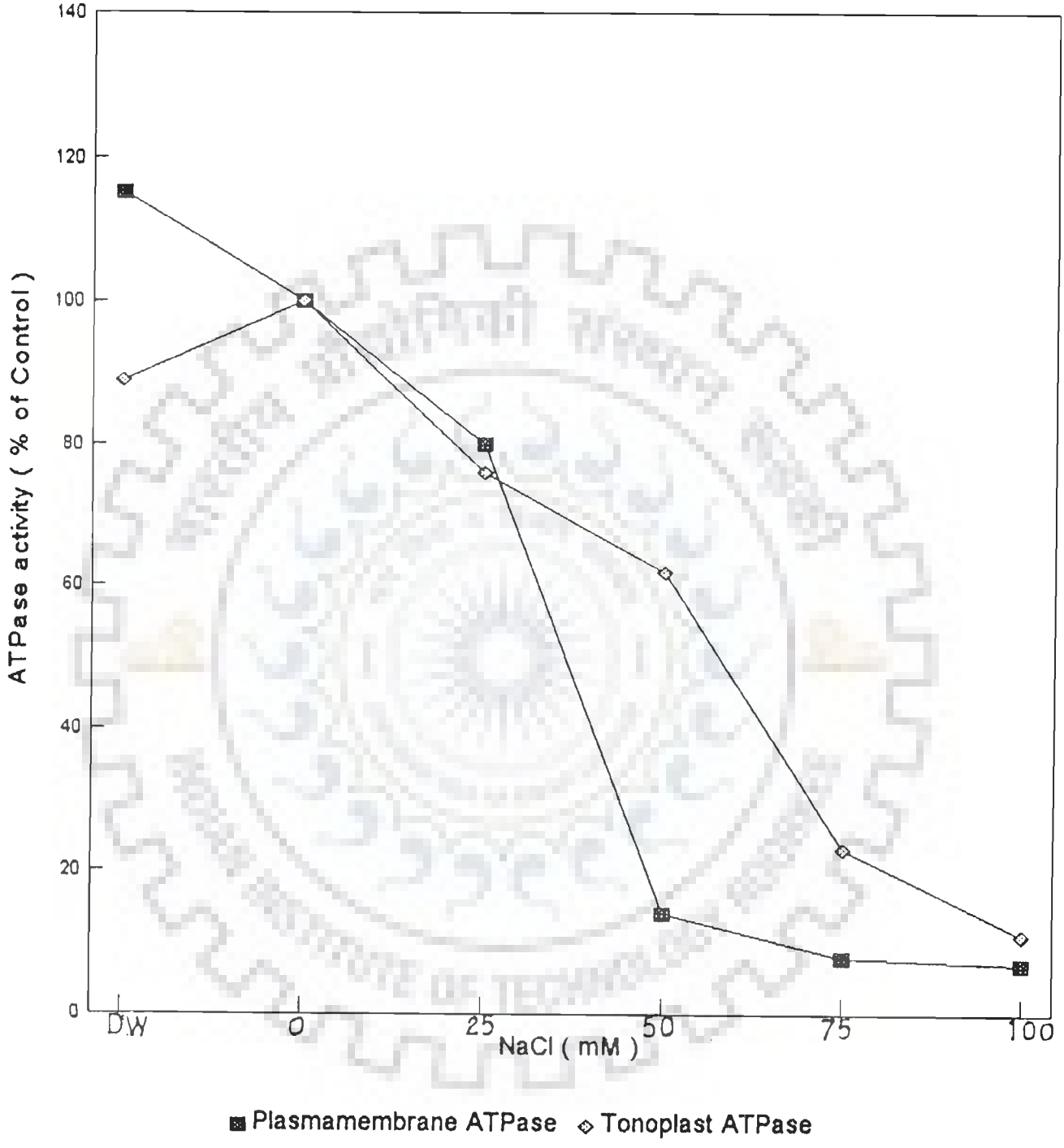


Fig. 4.22 Plasmamembrane and tonoplast ATPase activities under salt stress

that salt exposure of seedlings causes drastic reduction in vanadate-sensitive ATPase activity. The PM ATPase activity is only 80%, 14%, 8% and 7% in 25, 50, 75 and 100 mM salt treated seedlings respectively (Fig. 4.22). Interestingly the PM ATPase activity in distilled water grown seedlings is 15% more than controls.

Similar to PM ATPase, vacuolar membrane ATPase activity of seedlings also declines on exposure to salt. Thus there is 24%, 38%, 77% and 89% reduction in tonoplast ATPase activity when the seedlings are grown in 25, 50, 75 and 100 mM salt concentrations respectively (Fig. 4.22).

4.4.2 Effect of salt stress on some cell wall bound enzymes

There is slight difference in cell wall hydrolases and peroxidases activities in salt treated seedlings. On exposure to saline conditions, reduction in cell wall bound hydrolases activity and increase in cell wall bound peroxidases activity is observed (Table 12 and Fig. 4.23).

4.4.2.1 β-Glucosidase

Cell wall bound β-glucosidase activity decreases in salt treated seedlings in comparison to controls. Results show that there is about 43%, 67%, 56% and 16% reduction in cell wall bound β-glucosidase activity in 25, 50, 75 and 100 mM salt treated seedlings.

4.4.2.2 β-Galactosidase

Cell wall bound β-galactosidase activity also declines on exposure of seedlings to salt. Thus the enzyme activity is reduced from 126 nmol min^{-1} mg $protein^{-1}$ in controls to 124 nmol min^{-1} mg

TABLE 12

EFFECT OF SALT STRESS ON THE SPECIFIC ACTIVITIES OF SOME CELL WALL BOUND ENZYMES IN 6 d OLD PEANUT SEEDLINGS

Concentration of Nacl (mM) in nutrient media	Specific activity of some cell wall bound enzymes				
	β-glu[1]	β-gal[1]	α-gal[1]	acid phos[1]	peroxidases[2]
DW	450.0 ±250.0	174.0 ±090.0	116.0 ±110.0	1362.0 ±1000.0	50.0 ±39.0
0	457.5 ±110.0	126.0 ±110.0	118.0 ±100.0	1200.0 ±0900.0	60.0 ±41.0
25	262.5 ±060.0	124.0 ±050.0	100.0 ±080.0	1242.0 ±0900.0	72.5 ±60.0
50	152.5 ±010.0	088.0 ±040.0	084.0 ±030.0	0366.0 ±0104.0	80.0 ±47.0
75	202.5 ±040.0	105.0 ±019.2	082.0 ±030.0	0492.0 ±0192.0	97.5 ±35.0
100	385.0 ±270.0	096.7 ±020.8	068.0 ±020.0	0574.0 ±0200.0	85.0 ±50.0

1 = nmol min^{-1} mg $protein^{-1}$

2 = abs λ_{420} min^{-1} mg $protein^{-1}$

β-glu = β-glucosidase

β-gal = β-galactosidase

α-gal = α-galactosidase

acid phos = acid phosphatase

Peanut seedlings were grown hydroponically on nutrient media with varying concentrations of NaCl. 6 d old seedlings were used for experiments. Cell wall bound enzymes were extracted and estimated as described in " materials and methods ". Values are mean±SE of 5 observations.

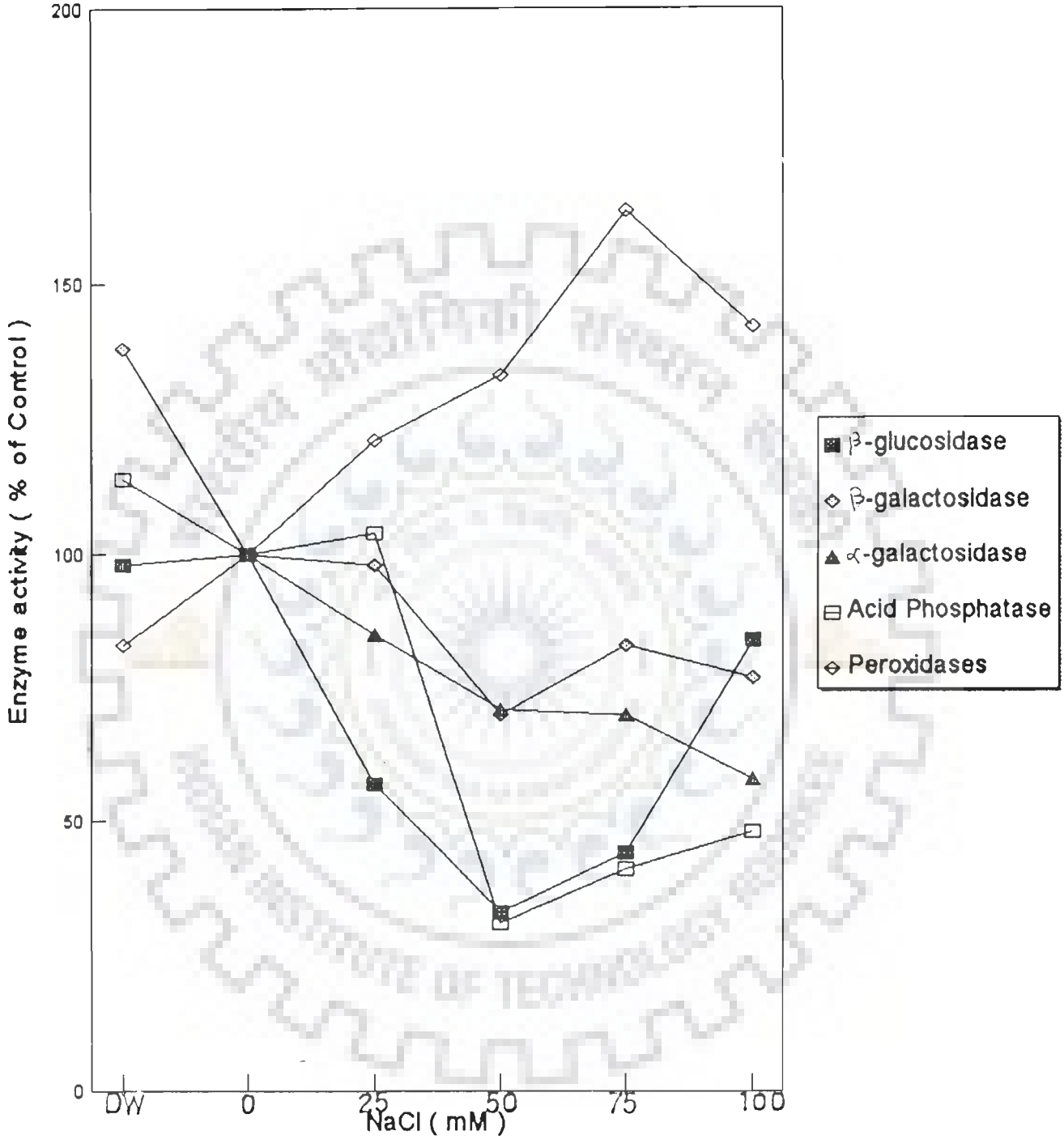


Fig. 4.23 Effect of salt stress on the specific activities of some cell wall bound enzymes

protein^{-1} (2% reduction) in 25 mM, 88 nmol min^{-1}mg protein^{-1}(30% reduction) in 50 mM, 105 nmol min^{-1}mg protein^{-1}(17 % reduction) in 75 mM and 96.7 nmol min^{-1}mg protein^{-1}(23% reduction) in 100 mM NaCl treated seedlings. On the contrary, β-galactosidase activity is 1.38 times more in DW grown seedlings (Fig. 4.23).

4.4.2.3 α-Galactosidase

Results show that there is constant decrease in α-galactosidase activity in the cell wall with increase in concentration of salt. The enzyme activity declines by ca. 15%, 29%, 30% and 42% over the controls at 25, 50, 75 and 100 mM salt concentrations respectively (Fig. 4.45).

4.4.2.4 Acid phosphatase

Acid phosphatase activity changes from 1200 nmol min^{-1}mg protein^{-1}in control to 1242 (4% increase), 366 (69% reduction), 492 (59% reduction) and 574 nmol min^{-1}mg protein^{-1}(52% reduction) at 25, 50, 75 and 100 mM salt concentrations respectively. Cell wall bound acid phosphatase activity is 1362 nmol min^{-1}mg protein^{-1} in distilled water grown seedlings (Fig.4.23).

4.4.2.5 Peroxidase

Quite in contrast to hydrolases, salt exposure causes increase in peroxidase activity. There is about 1.21, 1.33, 1.63 and 1.42 fold increase in cell wall bound peroxidase activity at 25, 50, 75 and 100 mM salt concentrations respectively (Fig. 4.23).

4.5 Effect of salt stress on the proteins in peanut seedlings

4.5.1 Cytoplasmic proteins

It can be seen from Table 13, 14 and Fig. 4.24 and 4.25 that there are significant changes in cytoplasmic proteins under salt stress. Results show that cytoplasmic protein level increases with increase in concentration of salt. Some new polypeptides are also appeared in cytoplasmic protein profile of salt treated seedlings.

4.5.1.1 Effect of salt stress on cytoplasmic protein level in peanut seedlings

Table 13 shows that on exposure to salt stress, cytoplasmic protein content increase from 18.12 mg/gfw in controls to 21.64, 22.07, 26.01 and 28.75 mg/gfw at 25, 50, 75 and 100 mM salt concentrations respectively. Thus there is about 19%, 22%, 44% and 59% increase in cytoplasmic protein content in 25, 50, 75 and 100 mM NaCl treated seedlings respectively (Fig. 4.24).

4.5.1.2 Changes in the cytoplasmic protein profile with relation to salt stress

On exposure to salt stress, changes in the profile of cytoplasmic proteins are observed (Fig. 4.25). Table 14 summarize the molecular weights of polypeptides of cytoplasmic proteins in control as well as salt treated seedlings. Cytoplasmic protein profile contains 3 polypeptides of 14, 21 and 28 kDa in DW, control and 25 mM salt treated seedlings. At 50 mM salt concentration total 6 bands of 14, 17, 20, 21, 28 and 29 kDa are present. Thus three new polypeptides

TABLE 11

EFFECT OF SALT STRESS ON THE SPECIFIC ACTIVITY OF PLASMA MEMBRANE AND TONOPLAST ATPase IN 6 DAY OLD PEANUT SEEDLINGS

Concentration of NaCl (mM) in nutrient media	Plasma membrane and tonoplast ATPase activity (μ mol Pi mg^{-1} protein h^{-1})			
	PM* ATPase ACTIVITY	% PM ATPase ACTIVITY	TONOPLAST*** ATPase ACTIVITY	% TONOPLAST ATPase ACTIVITY
DW	4.97±2.58	115	5.06±1.65	89
0 (control)	4.34±3.96	100	5.71±1.67	100
25	3.48±2.91	80	4.32±1.34	76
50	0.69±0.53	14	3.53±2.25	62
75	0.36±0.19	8	1.32±0.80	23
100	0.32±0.28	7	0.63±0.48	11

*** = significant at 1% level
* = significant at 10% level

Peanut seedlings were grown hydroponically on nutrient media with varying concentrations of NaCl. 6 d old seedlings were used for experiments. ATPase activity was estimated as described in "materials and methods". Values are mean±SE of 4 observations.

TABLE 13

EFFECT OF SALT STRESS ON CYTOPLASMIC PROTEIN CONTENT IN 6 DAY OLD PEANUT SEEDLINGS

Concentration of NaCl (mM) in nutrient media	CYTOPLASMIC PROTEIN CONTENT IN PEANUT SEEDLINGS (Mg/gfw)
DW	18.92±11.20
0	18.12±08.80
25	21.64±09.90
50	22.07±08.96
75	26.01±08.50
100	28.75±11.30

Peanut seedlings were grown hydroponically on nutrient media with varying concentrations of NaCl. 6 d old seedlings were used for experiments. Cytoplasmic proteins were isolated and their content was estimated as described in " materials and methods ". Values are mean±SE of 11 observations.

TABLE 14

EFFECT OF SALT STRESS ON THE PROFILE OF CYTOPLASMIC PROTEINS IN 6 d OLD PEANUT SEEDLINGS

Concentration of NaCl(mM) in nutrient media	MOLECULAR WEIGHTS OF POLYPEPTIDES OF CYTOPLASMIC PROEINS (kDa)
DW	28, 21 & 14
0	28, 21 & 14
25	28, 21 & 14
50	29*, 28, 21, 20*, 17* & 14
75	71#, 55#, 45#, 43#, 38#, 35#, 33#, 29*, 28, 26#, 23#, 21, 20*, 18#, 17*, 14
100	71#, 55#, 45#, 43#, 38#, 35#, 33#, 29*, 28, 26#, 23#, 21, 20*, 18#, 17*, 14

* = induced at 50,75 and 100 mM salt concentrations.
# = induced at 75 and 100 mM salt concentrations.

Peanut seedlings were grown hydroponically on nutrient media with varying concentrations of NaCl.6 d old seedlings were used for experiments. Cytoplasmic proteins were isolated and separated through SDS-PAGE on 10 % gel, as described in "materials and methods".

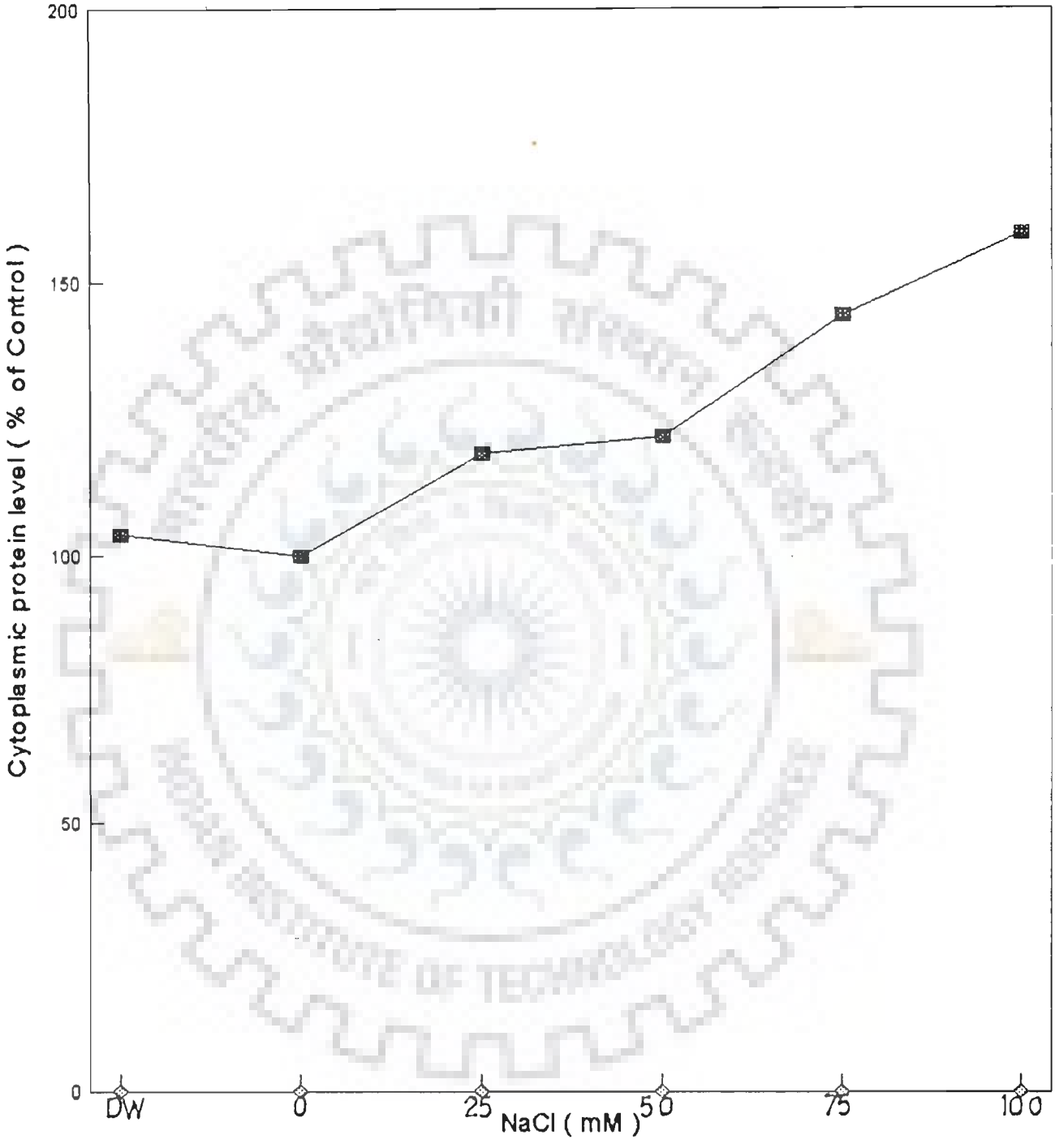


Fig. 4.24 Influence of salinity stress on the cytoplasmic protein content

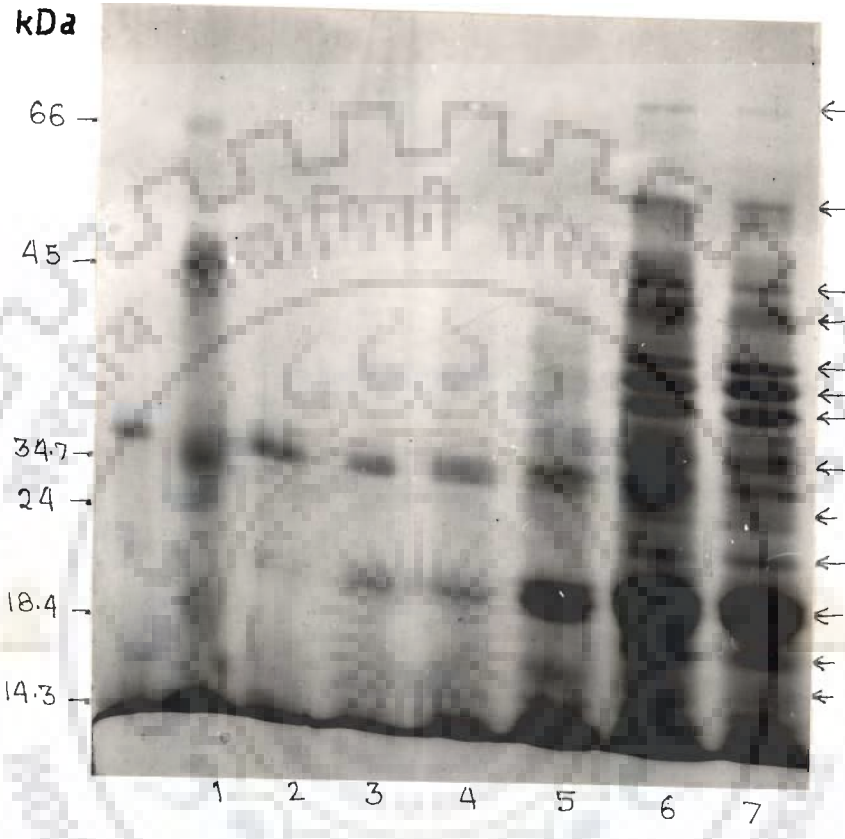


Fig. 4.25 SDS-PAGE (10%) profile of cytoplasmic proteins under salt stress.
Lane 2-7 carry equal amounts of cytoplasmic proteins isolated from DW (lane 2), 0 mM (lane 3), 25 mM (lane 4), 50 mM (lane 5), 75 mM (lane 6) and 100mM (lane 7) grown peanut seedlings. Molecular weight markers were loaded in lane 1.

of 17, 20 and 29 kDa appears. At 75 and 100 mM salt concentrations, there are total 16 polypeptides of which 10 i.e. of 18, 23, 26, 33, 35, 38, 43, 45, 55 and 71 kDa are new. Further, the intensity of 17 and 20 kDa also increase.

4.5.2 cell wall proteins

4.5.2.1 Effect of salt stress on cell wall protein level in peanut seedlings

The data on quantitative and qualitative changes in cell wall proteins on salt exposure of seedlings is given in Tables 15, 16 and Fig. 4.26 and 4.27. It is observed that salt treatment cause changes in both total protein levels and the type of polypeptides present. There is increase in cell wall protein content with increase in salt concentration. There is about 2 folds increase in protein content at 50 mM salt concentration, while at 75 and 100 mM salt concentrations the increase is 4 fold.

4.5.2.2 Effect of salt stress on cell wall protein profile in peanut seedings

Results show that on exposure to salt stress, cell wall protein pattern (Fig. 4.27) changes. Table 16 summarize the molecular weights of polypeptides of cell wall proteins of control as well as salt treated plants. A total of 12 polypeptides are present in DW grown , control and 25 mM salt treated seedlings. The molecular weights of these polypeptides are 55, 50, 39, 36, 32, 30, 29, 27, 25, 24, 18 and 16 kDa respectively. In these seedlings high molecular

TABLE 15

EFFECT OF SALT STRESS ON CELL WALL PROTEIN CONTENT IN 6 DAY OLD PEANUT SEEDLINGS

Concentration of NaCl (mM) in nutrient media	CELL WALL PROTEIN CONTENT (Mg/gfw)***
DW	0.01±0.00
0 (control)	0.01±0.00
25	0.01±0.00
50	0.02±0.01
75	0.04±0.02
100	0.04±0.02

*** = significant at 1% level

Peanut seedlings were grown hydroponically on nutrient media with varying concentrations of NaCl. 6 d old seedlings were used for experiments. Cell wall proteins were isolated and their content was estimated as described in " materials and methods." Values are mean±SE of 7 observations.

TABLE 16

EFFECT OF SALT STRESS ON THE PROFILE OF CELL WALL PROTEIN IN 6 DAY OLD PEANUT SEEDLINGS

Concentration of NaCl(mM) in nutrient media	MOLECULAR WEIGHTS OF POLYPEPTIDES OF CELL WALL PROTEINS (kDa)
DW	55, 50, 39, 36, 32, 30, 29, 27, 25, 24, 18 & 16
0	55, 50, 39, 36, 32, 30, 29, 27, 25, 24, 18 & 16
25	55, 50, 39, 36, 32, 30, 29, 27, 25, 24, 18 & 16
50	55, 50, 39, 36, 32, 30, 29, 27, 25, 24, 19*, 18 & 16
75	55, 50, 34, 36, 32, 30, 29, 27, 25, 24 & 19*
100	55, 50, 39, 36, 32, 30, 29, 27, 25, 24 & 19*

* = induced at 50,75 and 100 mM salt concentrations.

Peanut seedlings were grown hydroponically on nutrient media with varying concentrations of NaCl.6 d old seedlings were used for experiments. Cell wall proteins were isolated and separated through SDS-PAGE on 10 % gel, as described in "materials and methods".

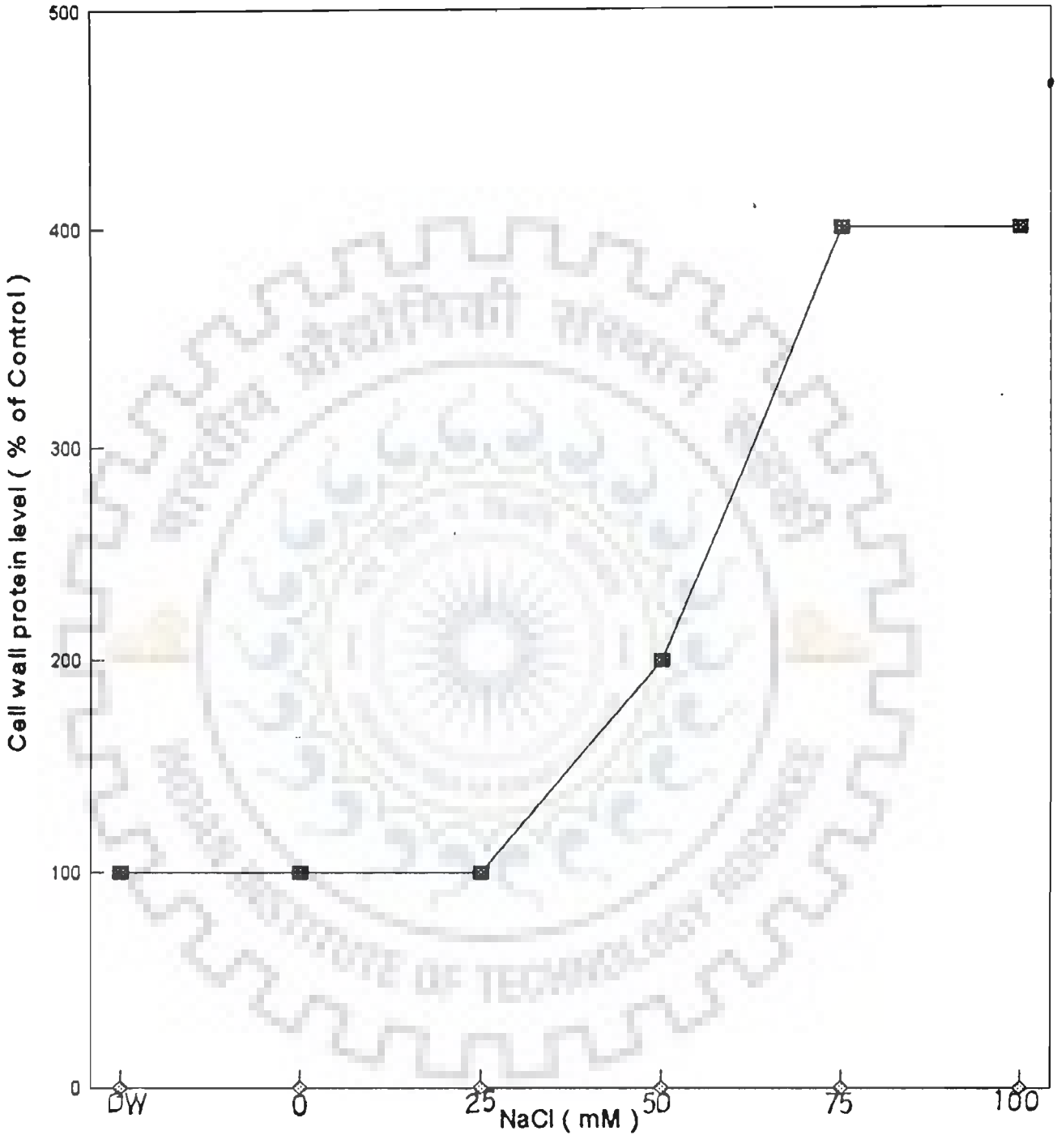


Fig. 4.26 Influence of salinity stress on the cell wall protein content

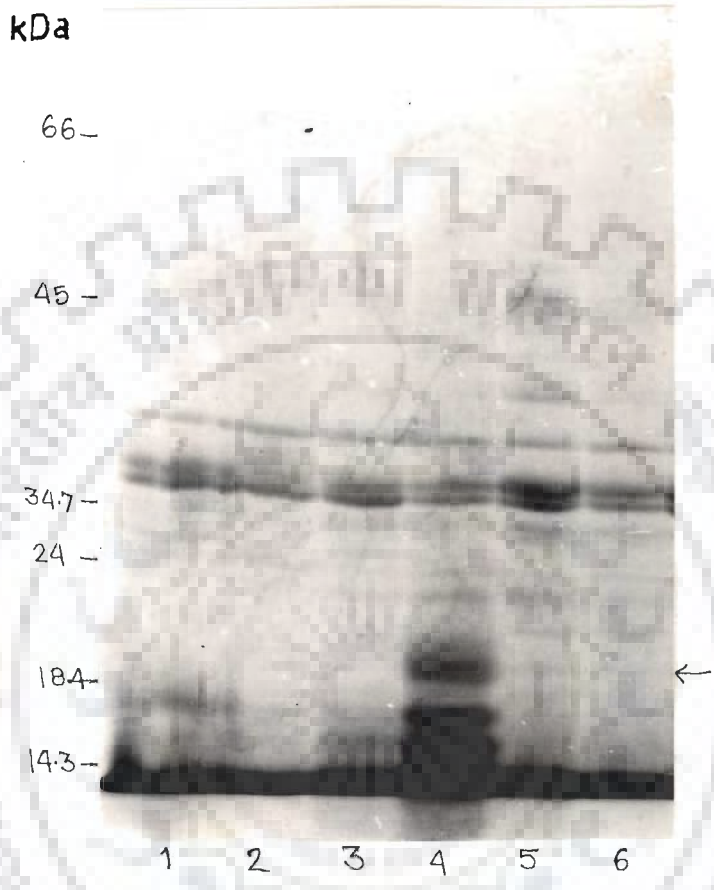


Fig. 4.27 SDS-PAGE (10%) profile of cell wall proteins under salt stress.
Lane 1-6 carry equal amounts of protein extracted from DW (lane 1), 0 mM (lane 2), 25 mM (lane 3), 50 mM (lane 4), 75 mM (lane 5) and 100 mM (lane 6) salt treated seedlings.

weight polypeptides are less intense than 75 and 100 mM salt treated seedlings. At 50 mM salt concentration of NaCl total 13 polypeptides are present in cell wall protein profile of which 12 are common with controls. Thus, only one polypeptide of 19 kDa is new at this salt concentration. The intensity of 16 and 18 kDa polypeptide also increases at 50 mM salt concentration. At 75 and 100 mM salt concentrations number of polypeptides in cell wall protein profile reduce from 12 in controls to 10. While 16 and 18 kDa bands disappear totally, the intensity of 19 kDa band decrease in 75 and 100 mM salt treated seedlings.

4.5.2.3 Identification of salt associated cell wall protein

The non-glycoproteinic nature of salt-induced 19 kDa band is demonstrated by staining the gel after SDS-PAGE with peiodic acid-Schiff stain. Fig. 4.28 show that only one 24 kDa cell wall polypeptide is taking PAS stain. Thus, the 19 kDa cell wall protein induced by 50 mM NaCl is not a glycoprotein.

4.5.2.4 Amino acid analysis of salt associated cell wall protein

Table 17 and Fig. 4.29 summarize the amino acid composition of salt-induced 19 kDa cell wall protein. This polypeptide is rich in Tyrosine (24.39 nmol/mg protein) and proline (15.8 nmol/mg protein) with less amount of Phenylalanine (1.50 nmol/mg protein), glycine (1.27 nmol/mg protein) and lysine (0.40 nmol/mg protein).

Fig. 4.28 Identification of 19 kDa cell wall protein.
Cell wall proteins were seperated via SDS-PAGE on 10% gel and for detection of glycoproteins PAS staining was used. Lane 1-6 carry 100 μg protein extracted from DW (lane 1), 0 mM (lane 2), 25 mM (lane 3), 50 mM (lane 4), 75 mM (lane 5) and 100 mM (lane 6) NaCl treated peanut seedlings

Fig. 4.29 Chromatogram showing the separation of amino acids of 19 kDa cell wall protein.
1, 2, 3, 4 and 5 denotes the peaks of Gly, Pro, Tyr, Phe and Lys.

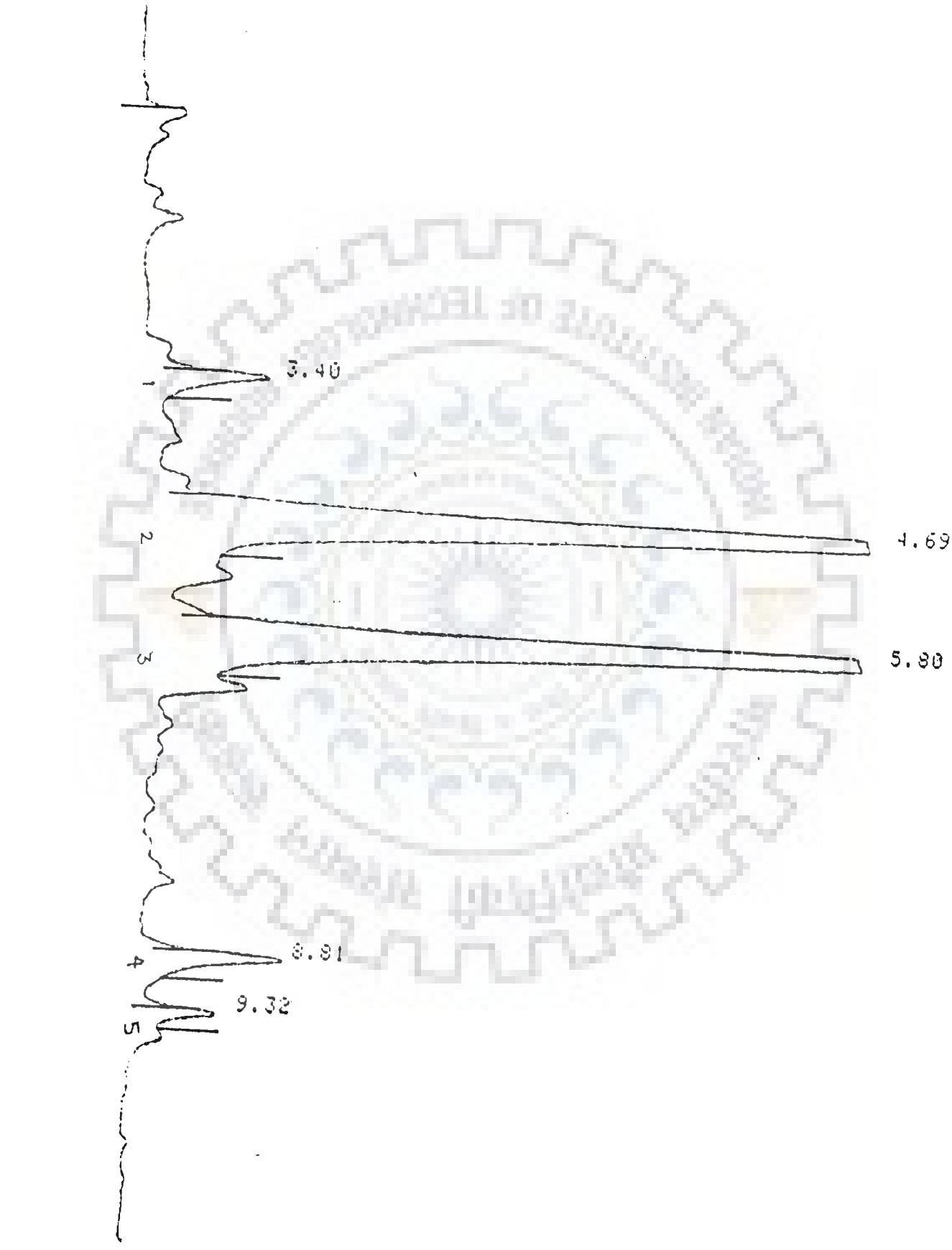

INJECT
3.40
4.69
5.80
8.91
9.32
1
2
3
4
5

TABLE 17

AMINO ACID COMPOSITION OF SALT-ASSOCIATED 19 kDa CELL WALL PROTEIN

AMINO ACIDS	CONCENTRATION OF AMINO ACID (n mol/mg protein)
TYROSINE	24.39
PROLINE	15.80
PHENYLALANINE	1.50
GLYCINE	1.27
LYSINE	0.40

The amino acid composition of salt associated protein was obtained after hydrolysis of the protein band which was eluted from polyacrylamide gel after SDS-PAGE. HPLC was performed in Waters 440 system on C_{18} column.

CHAPTER-5

5.0 DISCUSSION

In the present study influences of salt stress on certain morphological and biochemical aspects of peanut seedlings were investigated.

For giving salt treatment NaCl was chosen, as it is the most common cause of salinity [47]. Peanut is considered as a salt sensitive crop [18]. Its seedlings do not tolerate salt concentrations above 100 mM NaCl, which confirms its salt sensitive nature.

To study the effect of salt stress on growth of peanut seedlings length, fresh weight and dry weight of whole seedlings and of its various parts were measured in salt treated and control plants. Results show that with increase in concentration of salt, growth of seedlings decreases. Poor growth in salts may be ascribed either to low water uptake or to internal salt concentrations [58 and 100] and also could be due to the toxic effect of salts [26].

Reduction in length of whole seedlings and in its various organs was observed on exposure to salt stress. These results are in good agreement with other reports. Snapp and Shennan [142] and Gersani et al. [52] reported reduction in root length in *Lycopersicon esculentum* and *Opuntia ficus-indica* respectively under salt stress. Erdei and Kuiper [39] also reported growth reduction in roots and shoots. This stress induced growth inhibition could be due to small undetectable loss of water [102].

With increase in concentration of salt, decrease in fresh weight of whole seedling as well as of various parts of peanut seedlings was observed. This decrease in fresh weight is attributed to loss of water in stressed condition by many workers [11, 26 and 102]. However according to Singh et al. [141], it could also be due to altered cell wall properties or wall loosening mechanism as stress induced reduction of turgor is insufficient to explain reduced growth rate caused by salt stress. The alteration in the protein constituents of the cell wall may be involved in the reduced cell expansion rates of osmotically adapted cells .

On exposure to salt stress, dry weight of whole seedlings as well as of various organs increases. This increase in dry weight can be attributed to high internal content of Na^+ and Cl^- ions [39 and 46]. This is supported by the present study also.

There is decrease in cortical cell diameter and increase in the number of cortical cell layers in the roots under salt stress, while no change in shoot anatomy is observed. Actually, plants respond to salt stress in many ways including reduced cell expansion [12 and 58]. The reduction in cortical cell size in roots under salt stress might be due to low water availability . Gersani et al. [52] suggested that cell expansion may have been metabolically limited by the accumulated sodium. According to Flowers and Yeo [47] salt-tolerance would be expected to involve mechanisms which limit the accumulation of these ions in leaves. Given that there is a maximum acceptable limit on cellular ion concentration, an increase in delivery can be

accommodated by an increase in size or an increase in cell number (a branched habit).

A dramatic increase in Na^+ and Cl^- ions levels was observed in whole seedlings as well as in different parts of peanut seedlings. This increase in ions accumulation may be due to large passive leakage in membranes due to which ion entry will rapidly increase the needs of osmotic adjustment and direct toxicity will result from metabolic interference leading to growth reduction and a positive feedback cycle [165]. Organwise distribution shows that roots accumulate more Na^+ and Cl^- ions than the other organs of the seedlings. Generally the amount of these ions is much less in leaves. The reason for this type of organwise distribution is that majority of glycophytes are leaf excluders and accumulate high levels of ions chiefly in their roots [46]. Glycophytes are salt-sensitive due to their inability to translocate Na^+ from roots to shoots. As a consequence of an insufficient operation of this transport system (i.e. root-shoot transport) which is present in some halophytic species like *Plantago*, the Na^+ ion content of the roots increase above a level acceptable for the cytoplasm resulting in overloading of the vacuole and damage to the cells [39 and 84]. Greenway and Munns [58] observed that sensitivity toward Cl^- and/or Na^+ concentration in leaves is much greater for non-halophytes than for halophytes. On the contrary, few workers have also reported substantial increase in Na^+ and Cl^- ions in the leaves of salt-sensitive species [7, 30 and 150]. It is possible that some other mechanisms may operate in these species.

As H^+-translocating ATPases are expected to play a role in the transport of Na^+ and Cl^- ions, the effect of salt stress on the activity of these enzymes has also been investigated. There is a decline in plasma membrane and tonoplast ATPase under salt stress. The reduction in plasma membrane ATPase activity under salt stress could be due to the decrease in levels of phospholipids and calcium in membrane vesicles [33]. Suhayda et al. [145] suggested that modulation of the electrostatic properties of the plasma membrane may represent a mechanism by which the cell limits the stimulation of ATPase activity by monovalent cations when potentially high concentrations of these ions have accumulated intracellularly during salt stress. Thus, NaCl could inhibit the ATPase activity associated with plasma membrane or can delay the maturation process of the enzyme. Since, the plasma membrane ATPase seems to regulate plant growth, the lack of this activity may be one of the physiological factors involved in the delay of the normal plant development described under saline conditions [135]. While, decrease in tonoplast ATPase activity under salt stress could be either due to the lack of mechanism of vacuolar compartmentalization of ions in glycophytes or due to the damage of proton pump of tonoplast by toxic level of salts [103]. Vacuolar compartmentation of Na^+ and Cl^- is an essential mechanism for salt tolerance in halophytes, since it results in lower cytosolic ion levels and facilitates osmotic adjustment required for cell expansion and maintenance of turgor [113]. The sequestration of the salts into the vacuoles is carried out by the proton pump associated with the

tonoplast. Therefore in salt sensitive plants, decrease in tonoplast ATPase activity due to the damage of proton pump of the tonoplast by a toxic level of salts would be fatal to the plant cells [103].

Many workers investigated the effect of salt stress on the various enzymes extracted from cytoplasm as well as from other cell organelles [6, 15, 16, 35, 59, 65, 89, 104, 122, 138, 148, 149, 152, 156, 158 and 159]. But, there is no report on the effect of salt stress on cell wall bound enzymes. Actually, the enzymes bound to plant cell walls have been studied for more than three decades, but the nature of their binding, their localization in the cell wall and their functions have not yet been completely elucidated [98]. In the present study, changes in cell wall bound enzymes in relation to salt stress were observed. While there is decrease in specific activities of cell wall bound β-galactosidase, α-galactosidase, β-glucosidase and acid phosphatase, cell wall bound peroxidase activity increases significantly under salt stress. The precise role of these cell wall bound hydrolases and peroxidases is not clear. But, as glycosidases catalyze degradation of sugar chains, the decrease in their activities may result in decreased wall elasticity, thereby making them more rigid. Consequently, it will also prevent cell wall damage under salt stress. The significance of the presence of acid phosphatase in the cell wall is not known. But, most probably it is involved in the defense mechanism [27]. Decrease in acid phosphatase activity may also imply less amount of free PO_4^- ions in cell wall under saline stress. Peroxidases catalyze phenolic cross-links during the formation of

lignins and between macro molecules such as lignin, protein, hemicellulose and ferulic acid [29 and 131]. High wall peroxidase levels in the present study may imply increased lignification in cell walls, which in turn may restrict the cell expansion by affecting cell wall extensibility. Under salt stress increased peroxidase activity may also contribute to prevention of cell wall damage. Thus both i.e. decreased activity of glycosidases and increased activity of peroxidases on salt exposure of peanut seedlings are likely to make its cell wall more rigid.

On exposure to salt stress, total cytoplasmic proteins register an increase. Moreover 13 new polypeptides were formed at 75 and 100 mM salt concentrations. Many other workers have reported changes in protein profile of salt stressed plants [3, 4, 6, 56, 66, 69, 70, 71, 92, 123, 130 and 133]. The functional importance of these stress responsive proteins is not yet unequivocally established. Whether, these salt stress responsive polypeptides contribute to salt tolerance mechanism of plants remain to be established. This salt induced modification of proteins in higher plants is mostly restricted to molecular mass ranging from approximately 20 to 35 kDa [56]. However in peanut seedlings several polypeptides of more than 35 size were also formed. But, these appear as minor bands only. It is generally assumed that these stress induced proteins might play a role in tolerance, but direct evidence is still lacking, and the functions of many stress responsive genes are unknown.

On exposure to salt stress, changes in cell wall protein

content and profile were also observed. There was rise in cell wall protein content with increase in concentration of salt. Moreover, a new polypeptide of 19 kDa appears and intensity of 16 and 18 kDa polypeptides increase at 50 mM salt concentration. Interestingly these polypeptides disappear at 75 and 100 mM salt concentration. Therefore, it is clear that this 19 kDa polypeptide appears at low salt concentrations. Some other workers also reported synthesis of salt stress related cell wall polypeptides. [42 and 106]. Hurkman et al. [71] reported increase in several polypeptides of 26- and 27-kDa on salt exposure in barley roots that were differentially associated with endoplasmic reticulum, tonoplast, plasma membrane and cell wall fractions. Yen et al. [164] reported a salt stress responsive 24 kDa cell wall glycoprotein in *Mesembryanthemum crystallinum*. Singh et al. [141] observed that the protein constituent of the cell wall of NaCl adapted tobacco cells has altered and suggested that such alteration may be involved in the reduced cell wall expansion rates of osmotically adapted cells.

Further characterization of this low salt induced 19 kDa cell wall polypeptide was also done. It is observed that this polypeptide is not a glycoprotein as it fails to take PAS stain. Only 24 kDa cell wall polypeptide in distilled water seedlings is taking this stain. Actually, intensity of this polypeptide decrease under salt stress as shown by coomassiae blue stained polyacrylamide gel. Therefore, due to low quantity of this 24 kDa polypeptide in other seedlings, it could not be detected by PAS stain.

Amino acid analysis of the 19 kDa polypeptide shows that this is rich in proline and tyrosine and has a good amount of phenylalanine, glycine and lysine. The amount of other amino acids could not be quantified by analyzer as these are present in very low amounts. Some workers reported induction of proline and hydroxyproline rich cell wall proteins under salt stress. Esaka and Hayakawa [41] reported specific secretion of proline-rich proteins by salt adapted winged bean cells. According to them, proline-rich proteins may be specific for the adaptation of the cell wall to salt stress and may cause changes in the cell wall that allow cells to tolerate salt stress. A novel hydroxyproline-rich cell wall glycoprotein SAP1 is specifically secreted by salt-adapted winged bean cells [42]. Thus, cell wall proteins may play an important role in salt-adaptation of plants. In peanut seedlings 19 kDa protein may be such a protein.

CHAPTER SIX

6.0 SUMMARY AND CONCLUSIONS

Peanut seedlings do not tolerate salt concentrations higher than 100 mM NaCl, which confirms the moderately salt sensitive nature of this crop.

On exposure to salt stress, there was marked growth reduction in peanut seedlings which could be due to the toxic effect of salt stress. With increase in concentration of salt, length and fresh weight of all parts of seedlings as well as of whole seedlings decreases which could be either due to loss of water or due to the altered properties of the cell wall. In contrast, the increase in dry weight of whole peanut seedlings as well as of its various parts observed which may be attributed to high internal content of ions.

Changes in root anatomy were observed in peanut seedlings under salt stress. But, there is no change in shoot anatomy on exposure to salt stress. It is observed that cortical cell diameter of roots decreases with increase in salt concentration, while cortical cell layers increase. Decrease in cell diameter could be due to low water availability and increase in cell layers could be for accommodating higher ion concentration.

More Na^+ and Cl^- ions accumulate in salt treated plants than control plants, which may be due to large passive leakage in membranes due to which ion entry rapidly increase the needs of osmotic adjustment and direct toxicity will result from metabolic interference

leading to growth reduction and a positive feedback cycle. Organ wise distribution shows that Na^+ and Cl^- ions accumulate more in roots followed by shoots and leaves. The reason for this could be that majority of glycophytes are leaf excluders and accumulate high levels of ions chiefly in their roots.

With increase in concentration of salt, decrease in both plasma membrane ATPase and tonoplast ATPase activity is observed. The reduction in plasma membrane ATPase and tonoplast ATPase activity is 93 % and 89 % respectively at 100 mM salt concentration. Reduction in plasma membrane ATPase activity could be due to disruption of membrane integrity which can ultimately delay the normal plant development. While decline in tonoplast ATPase activity may be attributed to the lack of mechanism of vacuolar compartmentalization of ions in glycophytes or to the damage of proton pump of tonoplast by toxic level of salt which would be fatal to the plant cells.

Under salt stress, activity of some cell wall enzymes except peroxidase decreases. While activity of various cell wall glycosidases and acid phosphatase decreases under salt stress, peroxidase activity increases about two folds. Reduction in cell wall hydrolases activity under salt stress might be for prevention of cell wall damage by reducing its elasticity. Increase in peroxidase activity under saline stress is likely to promote cross linking of cell wall molecules and thus making cell wall more rigid.

There is significant increase in cytoplasmic protein content under salt stress. On exposure to salt stress, cytoplasmic protein profile also changed. At 75 and 100 mM salt concentration, 13 new polypeptides of 71, 55, 45, 43, 38, 35, 33, 29, 26, 23, 20, 18 and 17 kDa appears, of which 3 polypeptides of 29, 20 and 17 kDa are also present at 50 mM salt concentration. The functional importance of these stress responsive proteins is not yet fully established. It is generally assumed that these stress responsive proteins might play a role in tolerance, but direct evidence is still lacking.

With increase in salt concentration, increase in cell wall protein content is also observed. On exposure to salt stress, there is alteration in protein profile of cell wall also. At 50 mM salt concentration, a new polypeptide of 19 kDa appears, which again disappears at 75 and 100 mM salt concentration. Further, characterization of this 19 kDa polypeptide has also been done, which shows it not to be a glycoprotein. The amino acid analysis of this polypeptide shows that it is rich in proline and tyrosine, besides phenylalanine, glycine and lysine are also present in significant amount.

Therefore, it can be concluded from present study that salt stress leads to marked reduction in the growth of peanut seedlings. Further there are alterations in anatomical features specially in roots. In peanut, tonoplast ATPase do not seem to be involved in sequestration of Na^+ or Cl^- ions into the vacuoles. Interestingly salt

exposure drastically changes the pattern of structural proteins of the cell wall. A new cell wall bound polypeptide appears which may be involved in salt stress tolerance. The enzymic proteins are also altered in a manner so that they may make cell wall more rigid under saline stress. The information derived from the present study will be helpful in furthering our knowledge on biochemistry of salt stress specially the role of cell wall proteins and enzymes in peanut.

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