INVESTIGATIONS ON THE MECHANISMS OF SALT TOLERANCE USING *Arachis hypogaea* L. CULTURES.

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CERTIFICATE

The research work embodied in this dissertation has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi. The work is original and has not been submitted so far in part or full for any other degree or diploma of any other University.

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ABBREVIATIONS

A_{595}	absorbance at 595nm
ABA	abscisic acid
% acrylamide	polyacrylamide gel concentration expressed in terms of total monomer
BAP	benzylaminopurine
BADH	betaine aldehyde dehydrogenase
BSA	bovine serum albumin
^{o}C	degree centigrade
CAM	crassulacean acid metabolism
DMSO	dimethyl sulphoxide
g	gram
kD	kilodalton
l	litre
mg	milligram
ml	millilitre
mm	millimeter
MS media	Murashige and Skoog media
NAA	napthalene acetic acid
NaCl	sodium chloride
Na_2 EDTA	ethylene diamine tetra acetic acid
Na_2SO_4	sodium sulphate
nm	nanometer

PAGE	polyacrylamide gel electrophoresis
PEPCase	phosphoenol pyruvate carboxylase
PPO	2.5 diphenyloxazole
TEMED	N,N,N' tetramethyl-ethylene diamine
Tris-HCl	tris (hydroxy methyl) amino methane hydrochloride
v/v	volume by volume
w/v	weight by volume

CONTENTS

INTRODUCTION

Plants are organised to function under a set of optimum environmental conditions like water, temperature, light intensity, nutrients and soil which vary from species to species. Any substantial deviation from these optimal conditions would cause detrimental effects on plant growth and development and is referred to as 'stress'. This stress in turn affects the yield of useful agricultural products. However, many plants adapt to stress by changing quantitatively and qualitatively some molecules of the cells. This may help a plant to adapt to suitable alternate pathways or change their morphology and developmental pattern in order to cope up with stress.

This investigation was carried out to understand the effect of salt stress on plants, since vast stretches of lands in India have been rendered non-cultivable because of over accumulation of salt. Due to the ever increasing population pressure on the agricultural lands, utilisation of such lands will become necessary in future. To put these lands to use, either the plants should be modified to enable them to

withstand the unfavourable concentration of salts in the soil or the lands should be cleared off the excess salts. Since the desalinisation of such large areas is neither practical nor feasible, the only way out is to engineer plants to make them capable of withstanding the adverse environmental conditions.

Plants exposed to saline environments encounter three basic problems:

1. A reduction in the water potential in the surrounding environment resulting in water becoming less available.
2. Interference of toxic ions with physiochemical and biochemical processes of the organism.
3. Limitation of essential nutrients due to predominance of these toxic ions.

It has been observed that higher plants have evolved different mechanisms to cope up with a saline environment. Accordingly they are categorised as follows:

Salt Excluders: These plants have an enhanced ability to exclude salt, either from the entire plant or from particular organs. This is accomplished by cell membranes with high ion selectivity. Such plants are more prone to moisture deficits in saline conditions and

produce organic solutes for osmotic adjustments which involves high metabolic costs.(Stavarek and Rains, 1985). These types of plants are characterised by low levels of sodium and chloride in the plant tissues.

Salt Accumulators: These plants are able to cope up with high uptake of salt in several ways:

1. Tolerance to high levels of intracellular salt levels: Halophytes and some glycophytes, for example, sugarbeet can tolerate high levels of intracellular salt levels. In these plants, cell metabolism is relatively unimpaired by high internal salt concentrations and plant tissues maintain high Na^+/K^+ ratio.

2. Removal of excess salt: Plants freely take up salts but high intracellular concentrations are avoided by (i)compartmentation of salts into various cell organelles or tissues such as vacuoles (eg. in barley) or stem (eg. in broad-bean) (ii) extrusion of salts from the plant surface by salt glands (*Atriplex* spp.)

3. Succulence: The ability of the plant to vastly increase the cell volume with reserved water for maintaining an appropriate osmotic potential is called succulence. This is observed in cactus.

Understanding of the processes related to salt tolerance could lead to the identification of potential

biological markers and characteristics. Elucidation of the mechanism by which plants adjust to a saline environment and the identification of plant genotypes capable of increased tolerance to salt, and incorporation of these desirable traits in the economically important crop plants is important in programmes attempting to establish salt tolerant crop species. Cell culture system offers various advantages for these studies, because,

1. It allows the elimination of all stress responses except those that are operating at the cellular level.

2. It provides an opportunity to rigorously control the physical environment and nutritional status of the cells, parameters that are difficult to control in studies with intact plants.

3. It provides a fairly homogeneous (developmentally and metabolically) population of cells compared with the heterogeneous population constituting the whole plant.

4. Cell lines varying in their levels of salt tolerance in the same genotype can be developed thus avoiding comparison between tolerant and nontolerant plants of different genotypes.

Arachis hypogaea (groundnut) was chosen as the experimental material because of its economic importance

as the second largest source of vegetable oils in India. Its shelled nuts contain 26% protein and 45% oil. The kernels are also a rich source of phosphorus and vitamins particularly thiamine, riboflavin and niacin. Groundnut seeds have a very high calorific value (549 cal./100g, which is nearly five times that of beef) and are easily digestible. The biological value of groundnuts is among the highest of the vegetable proteins. In India a very high percentage of the total production of groundnut oil is used for the manufacture of "vegetable ghee" by hydrogenation (Kochhar S.L. 1981). Since *A. hypogaea* is very sensitive to salinity, it was thought to be worthwhile to develop salt tolerance in it and understand its mechanism. The present investigation was taken up with the following objectives:

1. To study the responses at the cellular level by comparing the polypeptide pattern using SDS-PAGE of

   a. salt-tolerant and salt sensitive cell lines

   b. salt-sensitive cells subjected to a high salt shock.

   c. salt tolerant cells shocked by transfer on salt free medium

2. To study the response at the whole plant level on exposure to salt.

3. To study the pattern of *de novo* synthesis of proteins in different tissues on exposure to high salt.

Study of the changes in the polypeptide profile of the salt tolerant and salt shocked cell lines and plants has been the focal point of many investigations including ours, because it is well known that a plant responds to its environment by altering the proteins qualitatively and quantitatively as discussed in the literature review.

LITERATURE REVIEW

I Development of salt tolerant cell lines:

The first successful report of the development of a salt tolerant cell line was by Zenk (1974). He obtained *Nicotiana sylvestris* haploid cell line which could survive and grow in 0.17M NaCl at half the rates of control cultures. Nabors et al. (1975) developed tobacco cell lines from chemically mutagenised and nonmutagenised cultures resistant to 0.09 M NaCl. The explanation forwarded for the fact that salt tolerant cell lines could be derived even from nonmutagenised cells was the appearance of a spontaneous dominant mutation.

Dix and Street(1975) showed that the Nicotiana sylvestris cell lines that could withstand upto 2% NaCl did not lose the character of salinity tolerance even after several passages through the NaCl lacking media. However, Hasegawa et al(1980), reported the loss of salinity tolerance of tobacco cell lines tolerant to 1% NaCl when subcultured on NaCl lacking media for five cell mass doublings. Apart from the use of model plants like tobacco, salt tolerant cell lines have also been developed from other plant species. Croughan et al. (1978) isolated NaCl resistant *Medicago sativa* (alfalfa) cell line. This cell line was resistant to 0.17M NaCl.

Gosal and Bajaj (1984) reported isolation of NaCl tolerant cell lines in the crop legumes *Cicer arietinum*, Pisum sativum and *Vigna radiata*.

Jain et al (1987) raised salt tolerant cell lines of egg plant. Proline level studies has been done and possible role for it in salt tolerance has been suggested. Role of proline has aslo been studied by Chandler and Thorpe (1987) in salt tolerance studies of cell lines of *Brassica napus*. Muralitharan et al(1990) report selection of callus lines of *Vaccinium corymbosum*, tolerant up to 100mM NaCl, and the physiology of salt tolerance with respect to ionic regulation has been reported.

II Regeneration of cell lines:

Reports of regeneration of the salt tolerant lines are few. Sexual transmission of the salt tolerance character was reported by Nabors et al (1980). They showed that the regenerated plants could tolerate higher levels of NaCl as compared to the cell cultures. Some plants in the F1 generation also showed this character. McCoy (1987), reported the regeneration of NaCl tolerant *Medicago sativa*. The regenerated plants could not be multiplied as they showed chromosomal aberrations.

Smith and McComb (1983) reported the regeneration of alfalfa plants from NaCl tolerant cell cultures. But the regenerated plants did not retain salt tolerance character. Kochba et al (1982) developed embryos from salt tolerant citrus cell lines. The regeneration of plants from these embryos was not reported. Wong et al (1983) reported the regeneration of rice plants from anthers. The salt tolerant plants thus obtained were partly sterile. Mechanism of tolerance has not been reported.

Vajrabahya et al (1989) used embryogenic callus of rice to raise cell lines resistant to 2% salt in the media. Seedlings regenerated from these lines could tolerate 0.5% NaCl in the media, and this salt tolerance character was found to be heritable for three generations that were tested. It was concluded that salt tolerance in rice operating at cellular level was retained in the regenerants as well. Similarly salt tolerant sugarbeet progeny has been obtained by Freytag et al (1990), using petiole explants which regenerated by organogenesis by challenging with different salts for three generations in culture. The mechanism has not been studied in both rice and sugarbeet reported above.

III Identification and characterisation of proteins:

a. Studies with cell lines:

All of the biological responses to environment are mediated directly or indirectly by the proteins. Therefore some attention has been focussed on the studies concerning polypeptide patterns to elucidate the mechanism of salt tolerance in cell lines as well as in whole plants. These studies could provide a clue to the genes which are induced under stress conditions and facilitate their identification, cloning and possible transfer to economically important crops to improve their tolerance to salt stress.

One of the first reports on the investigation of the polypeptide profiles of salt tolerant cell lines was made by Ericson and Alfinito (1984), who compared the protein patterns of a NaCl adapted line of *Nicotiana tabacum* and a non NaCl- adapted line. The quantitative and qualitative changes in the specific proteins which occurred when the cells become resistant to salt stress as well as the stability of these changes upon return of the cells to NaCl free medium have been described. They reported an increase in 20, 26 and 32 kD proteins in salt adapted cells. The 20 kD protein increased 50 fold in salt adapted cells. Also a marked decrease in the

levels of 20 and 32 kD protein within 4-6 days of transfer to NaCl free medium was observed which was comparable to the levels found in control cells within 12 days. However, the level of 26 kD protein remained constant throughout the experiment but was undetectable after 2-3 passages in control medium. From this experiment it was concluded that 26kD protein is salt inducible. The fact that 26 kD protein behaved differently from the other two proteins pointed to a possibility of the former being under a separate control.

Singh et al. (1985), showed enhancement in the levels of some proteins after adaptation to both water and salt stress(43 and 26 kD). However, a few proteins were specifically enhanced after adaptation only to salt stress (58, 37, 35.5, 34,21,19.5 and 18 kD) or water stress (17.5, 16.5, and 11 kD). A correlation between increased levels of NaCl tolerance and the levels of certain polypeptides suggested an altered expression of the genes for these proteins which in turn could be involved in the cell's ability to grow in salt containing medium. The differences between water stress and salt stress induced proteins could be explained by the differences in the mechanism of tolerance. Their results also suggested the involvement of 26kD

polypeptide in the adaptation of cultured tobacco cells to NaCl and water stress.

King et al (1986) characterised the 26 kD protein which accumulated in *Nicotiana tabacum* cell suspensions growing on media containing 10-35 g/l NaCl. Antibodies raised against the protein were used to examine the protein accumulation in both suspension cultured cells and whole plants by western blots. In the suspension culture the 26 kD protein accumulated in the log phase irrespective of the presence or absence of NaCl. The accumulation of this protein was also seen at whole plant level and was tissue specific, being more in roots than in the stems of green house grown plants. An interesting observation was that this protein accumulated under osmotic stress and not under non-osmotic stresses. The presence of this protein has also been reported in *Medicago sativa*, and *Phaseolus vulgaris*. The fact that antibodies raised against the 26 kD protein of tobacco cross-reacted with the 26 kD protein of *Medicago* and *Phaseolus* indicated that this protein was fairly conserved among different plant species. Bressan et al (1987) showed hormonal induction of the 26 kD protein. ABA (a plant growth regulator) induced 26kD protein was immunologically similar in

different plant species. On examining the expression of this protein in differentiated plant tissues they found that much more immunoprecipitable 26 kD protein was synthesised in outer stem than in leaf or root of tobacco which is contrast with the report of King et al (1986). They also showed that the synthesis of 26kD protein during or after adaptation to salt always followed an increase in endogenous ABA levels. Therefore, it appears that ABA might be some how involved in the regulation of expression of 26 kD protein. Singh, et al. (1987b) also characterised Osmotin from cultured tobacco cells adapted to NaCl. In these cells they could detect two forms of Osmotin, an aqueous soluble form (Osmotin I) and a detergent soluble form (Osmotin II). The first twenty two N-terminal aminoacids in both being identical. Osmotin II cross reacted with anti-Osmotin I. Immunocytochemical detection of Osmotin revealed that Osmotin is concentrated in dense inclusion bodies within the vacuole. The same authors reported the molecular cloning of Osmotin in 1989.

Ben-Hayyim et al (1989) compared the proteins associated with salt adaptation in citrus and tomato cells. Stress induced changes in 25 and 27 kD proteins were reported for NaCl adapted cells of citrus and

tomato respectively. The enhancement of the 25 kD protein in citrus was constitutive, but the NaCl induced changes in 27 kD protein in tomato was affected by the presence or absence of NaCl in the nutrient medium. A significant observation was the suppression of expression of most of the proteins in citrus, and enhancement in tomato under identical conditions i.e. in the presence of salt. Based on these observations the authors concluded that the salt induced changes might be species specific. However, more research needs to be done to support their work.

Winicov et al (1989) developed and analysed a salt tolerant cell line of alfalfa. On analysing the differential gene expression of the two cell lines at the mRNA level by *in vitro* translation of the mRNA they found high translational activities of mRNA in salt tolerant cell lines specifying polypeptides of 115, 92, 82, 75, 72 and 61 kD and a number of polypeptides in the lower molecular weight range from 42 to 17 kD. Some of these new or enhanced mRNAs were expressed irrespective of the presence of NaCl in the medium in which salt tolerant cell lines were growing while others needed NaCl for their induction. Similar results were obtained when cellular polypeptides were analysed.

b. Studies with intact plants:

Hurkman et al (1988) characterised the polypeptide patterns of membrane fractions enriched in endoplasmic reticulum (ER), tonoplast and plasma membrane (PM) to identify the salt induced polypeptide changes in the membrane fractions. They found that salt stress caused quantitative changes in a number of polypeptides which were induced by salt. They reported that 26 and 27 kD proteins in ER and tonoplast greatly increased and this increase was specific to roots. Other plant parts did not show any change in the level of these proteins.

Reviron et al. (1992) studied the molecular changes in the differentiated leaves of *Brassica napus* under salt and drought stresses. They found that ten percent of the polypeptides had altered abundance and others were unique to drought stressed plants. No polypeptide unique to salt stress could be visualised. 22 kD double polypeptide was increased under water as well as salinity stress but disappeared upon rehydration. They appeared to be synthesised as a 24 kD precursor, and their expression was, to some extent, post transcriptionally regulated, because the transcripts for these proteins were present in the control well-watered leaves, where as the polypeptides were never detected.

Although the effects of salinity on protein synthesis have been discussed extensively in all the papers reported, the identity and function of these proteins remain unknown. Since salinity tolerance is a complex trait controlled by polygenic system (Tal, M. 1985), the molecular biology of salinity stress in higher plants is not well understood Genetic variability of this trait has been recognised in a wide range of crops but the genes regulating salt tolerance are yet to be identified.

Ramagopal (1987), analysed the total proteins and mRNAs in roots, shoots and embryos of two genotypes of barley before and after salt treatment during seed germination and early seedling growth. They found that at least 2% NaCl was required to trigger the changes in proteins, while salt treatment did not alter the steady state protein level (as revealed by staining the gels with coomassie blue or by silver stain) in either genotype. The pattern of newly synthesised proteins (as analysed by *in vivo* labeling with ^{35}S methionine and fluorograpy) was altered and the two genotypes responded differently. A set of proteins were either repressed or enhanced while some were newly synthesised . During germination new proteins were induced only in roots but

during seedling growth new proteins were induced both in roots and shoots.

Ramgopal, S (1987b) also demonstrated the regulation of gene expression in the salt tolerant (CM 72) and salt sensitive genotype (Prato) of barley. On analysing the root tissue, it was found that 12 new root mRNAs were induced out of which nine were found in CM 72 and five in Prato. Two of these (coding for 34.0 and 21.0 kD peptides) were common to both genotypes, but were more strongly repressed in Prato than in CM 72. Seven were unique to CM 72 and three were unique to Prato. Shoots responded differently to salinity. Nine new shoot mRNAs were induced by stress, out of which four were induced in CM 72 and six in Prato. Three of these nine were unique to CM 72 and five were unique to Prato, while one (coding for 27.5 kD peptide) was common to both genotypes. These new mRNAs encode proteins ranging from 18.0 to 50.5 kD. The unstressed seedlings of both genotypes expressed qualitatively similar mRNAs yielding identical translational products except for few differences in roots and shoots. Transcriptional regulation in response to salt stress was also done by Gulick and Dvarak (1987) who compared the salinity sensitive bread wheat and salinity-tolerant

amphidiploid (bread wheat X *Elytrigia elongata*). They could detect altered transcription of 18 transcripts on autoradiograms of the *in vitro* translation products from mRNA fractions isolated from the roots of salt treated and control amphidiploid plants. Five transcripts were induced by the salt treatment and 13 transcripts showed quantitative changes in their expression. Bread wheat plants responded differently to salinity stress, and the expression of 34 transcripts was affected. No difference was observed in the mRNA fraction of salt treated and control plants of both genotypes. It was postulated that the repression or altered expression of so many transcripts in the salt treated bread wheat plant may not have anything to do with salt tolerance but may be an effect of salt stress on the overall metabolism of the plant.

Ostrem et al (1987) examined the effect of salt stress on the level of translatable mRNA for phosphoenol pyruvic acid carboxylase (PEPCase) a key enzyme in crassulacean metabolism (CAM)pathway in *Mesembryanthemum crystallinum*. This plant responds to salt stress by switching from C3 photosynthesis to CAM. The authors showed that the age of the plant influenced the response to salt stress. PEPCase enzyme activity did not increase earlier than six weeks from the onset of

germination, though the transcripts of this enzyme accumulated as early as four weeks from the onset of germination (Cushman et al. 1989). Together with the increase in PEPCase activity there was an increase in the levels of translatable mRNA of this enzyme. This suggested that PEPCase synthesis was under transcriptional control at the germination stage though, it is possible that salt stress might have affected the stability of PEPCase transcripts *in vivo*. That the increased levels of translatable mRNA, protein and enzyme activity are reversible was demonstrated by Daniel M. Vernon (1988). He found that when salt was thoroughly flushed from the soil PEPCase mRNA levels dropped by 77% within 2.5 hours after salt removal but PEPCase activity and polypeptide levels declined more slowly, with a half-life of 2-3 days.

Cushman et al (1989) characterised two distinct PEPCase genes that differ markedly in their expression during CAM induction. One of the genes Ppc 1, encodes a form of the enzyme whose expression is induced by salt stress. The second gene Ppc 2 encodes an alternate form of the enzyme whose expression is not enhanced by stress. The steady state transcript levels of Ppc 1 increased by about 30 fold while levels of Ppc 2

decreased after five days of salt stress. By nuclear run on assays Cushman et al confirmed that increased levels of Ppc 1 resulted from transcriptional activation resulting in increased transcriptional rates which were much higher as compared to that of Ppc 2. This transcriptional induction could be brought about by simply withholding water from the plants.

Claes et al (1990) investigated the changes in proteins in the roots of salt sensitive rice cultivars when exposed to 2.2 times the normal concentration of MS salts for 4 days. They found the induction of eight new proteins in these as compared to the unexposed cell lines and obtained partial sequence of one with a molecular mass of 15kD. Using an oligonucleotide probe based on this information, a cDNA clone sal T was selected. This clone has an open reading frame coding for a protein of 195 aminoacid residues. Sal T mRNA accumulated very rapidly in leaf sheath and roots from mature plants and seedlings of rice on treatment with salt, PEG or ABA. No induction was however seen in the leaf lamina. This organ specific response of sal T could be correlated to the pattern of Na^+ accumulation in different tissues during salt stress.

Godoy et al (1990) characterised a new cDNA clone

TAS 14 from tomato which was inducible by salt as well as ABA. From nucleotide sequence it was predicted to be having an open reading frame coding for a highly hydrophobic and glycine rich protein of 130 amino acids and of molecular weight 13.948 kD. Tas 14 mRNA accumulated in tomato seedlings upon treatment with NaCl, ABA or mannitol suggesting that osmotic component of salt stress was sufficient to induce the expression of this mRNA. Other stresses like cold and wounding could not elicit the expression of this particular mRNA.

Weretilnyk et al (1990) cloned the gene for betaine-aldehyde dehydrogenase (BADH) from salt stressed spinach (*Spinacea oleracea* L.). It was a nuclear encoded chloroplastic enzyme which catalyses the last step in betaine synthesis. Betaine, is a non-toxic protective osmolyte which accumulates under saline or drought conditions. This is a rare finding where a link between salt or water stress metabolism and gene expression corresponding to a known protein with known biochemical and physiological function has been demonstrated.

Thus from the survey of the literature it can be seen that efforts were mainly put on isolating salt tolerance cell lines and study of their physiological

mechanism in the seventies and early eighties. Certain c-DNA clones which might be responsible for conferring salt tolerance have been isolated and regulation is being studied. The next step in future would be to engineer the expression of these into crop plants.

MATERIALS AND METHODS

PLANT MATERIAL

Arachis hypogaea (Groundnut) var. JL-24 was obtained from ICRISAT, Hyderabad and National Research centre for Groundnut, Junagadh, India.

GERMINATION STUDIES ON SALT MEDIUM

Seeds of different varieties of groundnut were subjected to varying concentration of NaCl ranging from 50mM to 200mM in semi solid agar medium, after surface sterilisation with 0.1% mercuric chloride for 5min., rinsing thoroughly with sterile water and soaking in sterile water overnight before implanting.

INDUCTION OF CALLUS

Callus was initiated from explants (hypocotyls and leaves) of 10-12 day old, *in vitro* grown groundnut seedlings on MS (Murashige and Skoog, 1962) medium supplemented with different auxin and cytokinin concentrations (table 1). In another set of experiments, the hypocotyls were excised and cultured on MS medium supplemented with auxins and cytokinins as well as different concentrations of salt (50, 100, 150 and 200mM) respectively.

SELECTION FOR SALT-TOLERANCE

Both single step and multistep approaches were used for selection of salt tolerant callus lines:

Single Step Selection

Calli were directly cultured on high levels (up to 200 mM) of NaCl to pick up somaclonal variants/ mutants.

Multistep Selection

The callus was initially multiplied on a lower level (50 mM) of NaCl and then transferred stepwise on medium containing higher levels of salt (up to 200 mM).

TESTING FOR THE STABILITY OF SELECTED TRAIT

Cell lines selected after single step and multistep selection methods were maintained for 5-6 generations on medium minus salt before transferring them back to the selection medium (containing high level of NaCl). The cell lines which continued to grow on this medium were referred to as the 'tolerant lines'.

GROWTH RESPONSES OF SALT-TOLERANT LINES

Fresh weight of the control and the tolerant cell lines was recorded after three weeks. Tolerant cell lines were also transferred to lower as well as

higher levels of NaCl to record the percentage increase or decrease in fresh weight against the controls.

SHOCK TREATMENT

Callus which was growing on media minus NaCl as well as seven day old seedlings were transferred to MS media containing 200 mM NaCl for two days and seven days respectively before analysing the polypeptides.

ANALYSIS OF POLYPEPTIDE PATTERNS

a. Extraction of cellular proteins: 0.3-0.5 g of the tissue was ground with 0.3-0.5 ml of the extraction buffer (composition given below) using pestle and mortar at room temperature. The homogenate was centrifuged in an eppendorf tube at 13,000 rpm for 5 min. using a table top micro centrifuge. The supernatant was carefully decanted in a fresh eppendorf tube and was used for subsequent experiments. The concentration of protein in the supernatant was estimated by the Bradford's (1976) method The supernatant was mixed with sample buffer (see composition below) in 1:1 ratio and the proteins were completely denatured by boiling the samples at 100^{o}C for 3 min., before loading into the wells of SDS-PAGE gels for carrying out electrophoresis.

Extraction Buffer

sodium hydrogen phosphate (pH 7.0)	50mM
2-mercaptoethanol	10mM
ethylene diamine tetra acetic acid disodium salt (Na_2 EDTA)	1mM
triton X-100	0.1%
phenyl methyl sulfonyl fluoride (PMSF)	2mM

Sample buffer

tris HCl (pH 6.8)	0.125 M
sodium dodecyl sulphate	4% (w/v)
glycerol	20%(v/v)
2-mercaptoethanol	10% (v/v)

b. Estimation of protein content: The total protein content was estimated according to the Bradford (1976) method. Bovine serum albumin (BSA) was used as standard protein for calibration. 5 ml of the Bradford reagent (0.01 % w/v coomassie brilliant blue G-250, 4.7% w/v phosphoric acid in water) was mixed by vortexing with 0.1 ml of 10-100 ug BSA in 0.15M NaCl. After 20 mins. the O.D. was measured at 595 nm using Shimadzu UV 2000. The blank sample was prepared by mixing 5 ml of Bradford

reagent with 0.1 ml of distilled water as BSA was dissolved in distilled water. A standard curve was drawn using A_{595} and different BSA concentrations. The protein concentration of the samples was estimated by comparing the A_{595} with the standard curve.

Electrophoresis of proteins: One dimensional electrophoresis using the procedure of Laemmeli (1970) was carried out. Slab gels with 6% acrylamide in stacking gel and 10-15% acrylamide in separating gel were prepared from a stock solution of 30% (w/v) of acrylamide and 0.8% (w/v) of N,N bismethylene acrylamide. The final concentration in the separating gel was as follows: 0.375 M tris-HCl (pH 8.8), 0.1% (v/v) of TEMED and 0.33% ammonium persulphate. Stacking gels of 6% acrylamide and length of 1 cm contained 0.12 M tris-HCl (pH 6.8) and 0.1% SDS and were chemically polymerised in the same manner as for the separating gel. The electrode buffer (pH 8.3) contained 0.025 M tris-HCl and 0.172 M glycine and 0.1% SDS.

Electrophoresis was carried out at 80mV till the tracking dye [0.001 (w/v) bromophenol blue in water] reached one cm less from the bottom of the gel.

Staining of protein gels: The gels were stained with

coomassie brilliant blue R-250. The gel was carefully removed from the plates, rinsed with water and fixed and stained in ten volumes of a solution containing acetic acid (10%), coomassie brilliant blue R-250 (0.025%) and methanol (50%) for 10 hours. Excess stain was removed by repeated washing with destaining solution having 50% methanol(v/v), and 10% acetic acid (v/v) in distilled water. Gels were further destained in 5% methanol and 7% acetic acid in distilled water.

Molecular weight markers: The standard molecular weight markers for SDS-PAGE used for most of the experiments were myosin, rabbit muscle (205 kD), B-galactosidase, *E. coli* (116 kD), phosphorylase b, rabbit muscle (97.4 kD), albumin bovine (66kD), albumin egg (45kD), carbonic anhydrase, bovine erythrocytes(29kD).

Labelling of proteins *in vivo* by [^{35}S]L-methionine: Hypocotyls from 4 day old seedlings as well as cell suspensions obtained by shaking the calli in liquid MS medium were used for *in vivo* labelling of proteins. The hypocotyls were sliced into one centimeter pieces and transferred immediately to a small petriplate (35 mm) containing MS medium (supplemented with NAA 1mg l^{-1} BAP 1mg l^{-1} and 2.5% sucrose) and kept shaking for a few hours at 100 rpm at 25^{o}C before transferring to 0.5ml

fresh medium containing 100uCi / 1 ml [^{35}S] L-methionine, 200mM NaCl and 2.5% sucrose. After 12 hr. of incubation in this mixture the plant material was washed thrice with distilled water and proteins extracted from it were analysed on SDS-PAGE followed by fluorography.

Fluorography: The unstained gels were fixed in 20% TCA for one hour. The gels were soaked in twenty times its volume of DMSO for 30 mins. followed by a second 30 mins. immersion in fresh DMSO to replace the water content of the gel with DMSO. Subsequently the gels were immersed in four volumes of 20% (w/w) PPO in DMSO for three hours. After impregnation of PPO the gels were drained thoroughly and soaked in twenty volumes of water for one hour. The aqueous step helps in regaining the normal size of the gels. The gels were vacuum dried in Pharmacia gel drier. The dried gels were exposed to Indu X-Ray film for 5-10 days at -70^{o}C.

RESULTS AND DISCUSSION

COMPARATIVE DATA ON THE EFFECT OF SALT ON GERMINATION OF DIFFERENT VARIETIES.

Amongst the various varieties of groundnut tested, JL-24 showed the best response of germination in salt supplemented media, although there was a drop in the percentage germination even with 50mM NaCl in the germination medium. On increasing the salt concentration in the germination media, the length of the hypocotyls decreased and browning and subsequent death of the seedling was observed. No germination was observed in the media supplemented with more than 100mM NaCl.

INDUCTION OF CALLUS

Different media were tested for the induction of callus from various explants of seedlings germinated *in vitro*. Table 1 shows the efficiency for callus induction using different media. The most suitable media for induction of callus was found to be MS supplemented with NAA (1mg l^{-1}) and BAP (1mgl^{-1}) referred to as MS 1. This medium was also suitable for the maintenance of callus. All the explants tested

Composition of media	Efficiency of callusing
1. MS + NAA (1 mg l^{-1}) + BAP (1 mg l^{-1})	+ + + +
2. MS + NAA (2 mg l^{-1}) + BAP (1 mg l^{-1})	+ + + +
3. MS + NAA (2 mg l^{-1}) + BAP (2 mg l^{-1})	+ + + +
4. MS + NAA (0.5mg l^{-1}) + BAP (3 mg l^{-1})	+ +
5. MS + NAA (1 mg l^{-1}) + BAP (3 mg l^{-1})	+ + +
6. MS + 2,4-D (1mg l^{-1}) + BAP (0.5 mg l^{-1})	+ + +

Table 1: Efficiency of callus formation on different media

(leaves, hypocotyls, epicotyls) responded very well and formed callus within 10 days, but the best response was obtained from hypocotyls and leaf explants. There was not much reduction in the induction of callus when MS 1 medium was supplemented with 50mM NaCl but on increasing the concentration of salt, the initiation and growth of callus was slow.

SELECTION FOR SALT TOLERANT CELL LINES

Both single step as well as multistep methods were found to be useful for selection of salt tolerant variants/mutants. The selected lines were tested for their salt tolerance and only those lines which were found to grow on 50 mM, 100mM, 150 mM and 200 mM NaCl supplemented media even after several passages on media minus salt were characterized further.

CHARACTERIZATION OF SALT TOLERANT LINES

Effect on growth

Effect on the growth pattern of salt tolerant and control lines is depicted in fig.1. Whereas the tolerant callus lines continued to grow on NaCl supplemented media with slight suppression of growth,

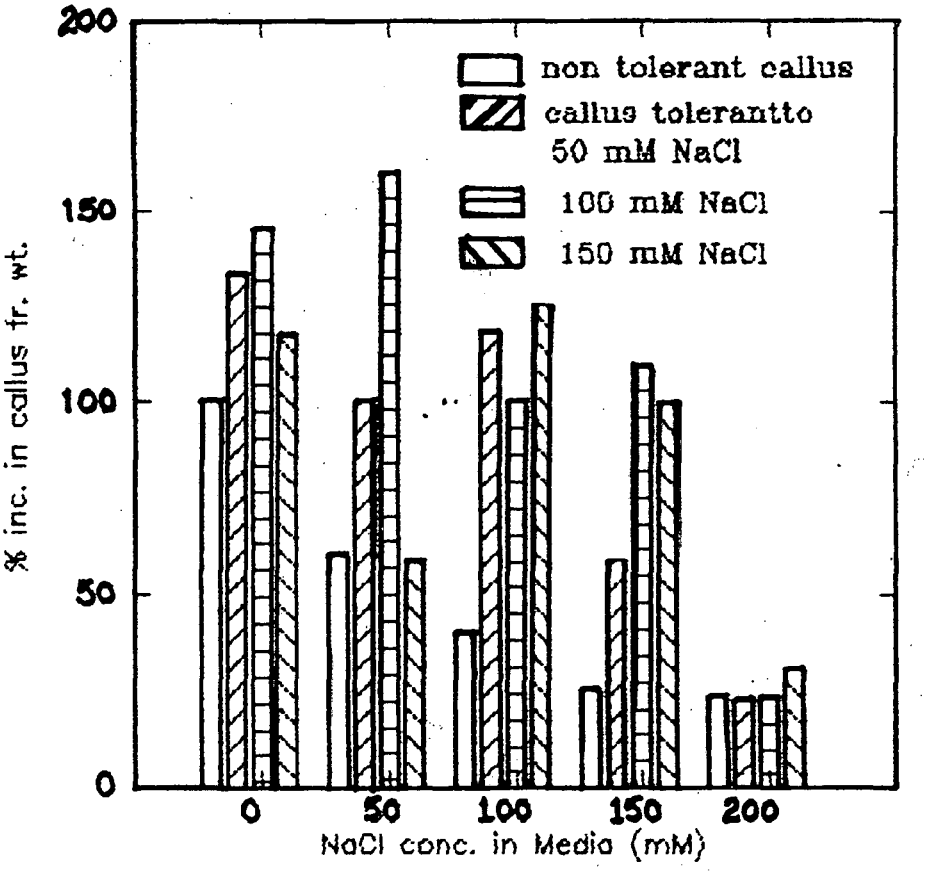


FIG 1: Growth studies in Callus (tolerant and non-tolerant to Salt)

non-tolerant lines showed a much higher reduction of growth rate on increasing concentration of salt in MS1. This was probably due to the high metabolic cost incurred by the plant in the process of overcoming the salt stress. The effect was more pronounced in non-tolerant lines which showed an inability to overcome stress leading to slowing down of growth and finally leading to cell death.

1. Investigations on polypeptide pattern of salt sensitive and salt tolerant callus lines

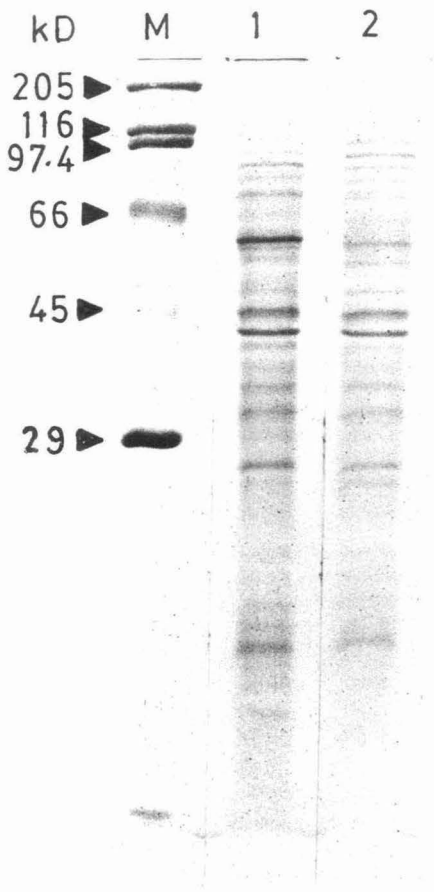
a) Comparison of salt sensitive versus salt tolerant callus lines.

Polypeptide profiles of salt sensitive callus lines maintained on MS 1 supplemented with 200 mM NaCl were compared with salt tolerant cell lines on single dimensional SDS-PAGE (fig. 2). On the coomassie blue stained gels significant differences were found in the level of accumulation of low molecular weight polypeptides viz. 13, 25, 31, 34, 58 and 69kD, which were found to be more abundant in salt tolerant cell lines as compared to the salt sensitive cell lines. A 76 kD polypeptide also showed enhanced level as indicated by the intensity of the band. In contrast to this, 80 kD polypeptide exhibited decreased level of

Fig. 2: Polypeptide profiles of salt-sensitive callus growing on NaCl free medium vs salt tolerant callus maintained on MS 1 + NaCl (200 mM).

lane 1 - salt tolerant callus maintained on MS B1 + NaCl (200mM).

lane 2 - salt sensitive callus maintained on NaCl free medium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accumulation.

Other researchers have also reported a change in the polypeptide profiles of salt tolerant cell lines as compared to the salt sensitive cell lines in some higher plants [King et al.,1986, Ramagopal 1986, Ericson and Alfinito 1984, Singh et al.1985]. Most of the reports are on tobacco salt tolerant cell lines in which an increase was found in the level of 26 kD protein in salt adapted or salt shocked cells. In the present investigation, no alteration in the level of 26 kD protein was observed. However, some changes in the level of 25 kD polypeptide were detected in the salt tolerant callus lines as compared to the sensitive lines. The level of the polypeptide decreased on withdrawal of salt from the medium but still remained higher as compared to sensitive cell lines. Ben-Hayyim et al.(1989) have also reported enhanced levels of 25 kD polypeptide in the salt tolerant cells of citrus compared to salt sensitive cells. The present investigation, with groundnut showed similar response.

Ericson and Alfinito (1984) reported an increase in the level of 32 kD polypeptide in the salt adapted tobacco cells. The present investigation showed an increased level of 31 kD polypeptide in cells tolerant

to 200mM NaCl. Considering the relatively unsophisticated method used for estimation of molecular weights of polypeptides, the possibility that the 31 kD polypeptide may be the same as 32 kD polypeptide which was reported by Singh et al.(1985) in tobacco cannot be ruled out. An increase was also observed in 34 and 58 kD polypeptides in the salt tolerant callus lines of groundnut. A general decrease in the high molecular weight polypeptides in salt adapted cells has been reported by Ericson and Alfinito (1984) a prominent decrease in the level of only 80 kD polypeptide in the high molecular weight range was observed in groundnut.

The altered levels of various polypeptides in the salt tolerant versus salt sensitive callus lines suggest either an altered expression of genes for these polypeptides or other regulatory controls at the post transcription or post translational levels. The identity of the polypeptides which showed altered expression in salt tolerant cells has not yet been established.

b) Effect of salt shock on salt sensitive callus lines:

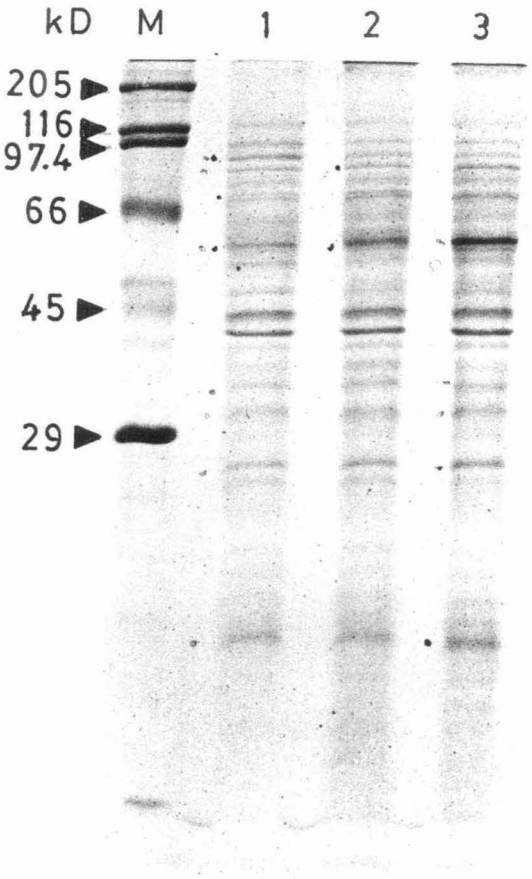
Experiments were carried out to check whether the increased/decreased levels of accumulation of polypeptides in salt tolerant cells (compared to sensitive cells) was characteristic of only salt-

Fig. 3: Polypeptide profiles of tolerant callus (maintained on 200 mM NaCl) , salt - sensitive callus on salt free, and 200 mM NaCl supplemented medium (for 48 hrs.)

lane 1 - sensitive callus maintained on NaCl free medium

lane 2 - sensitive callus exposed to 200 mM NaCl for 48 hrs.

lane 3 - tolerant callus maintained on MS 1 + NaCl (200mM)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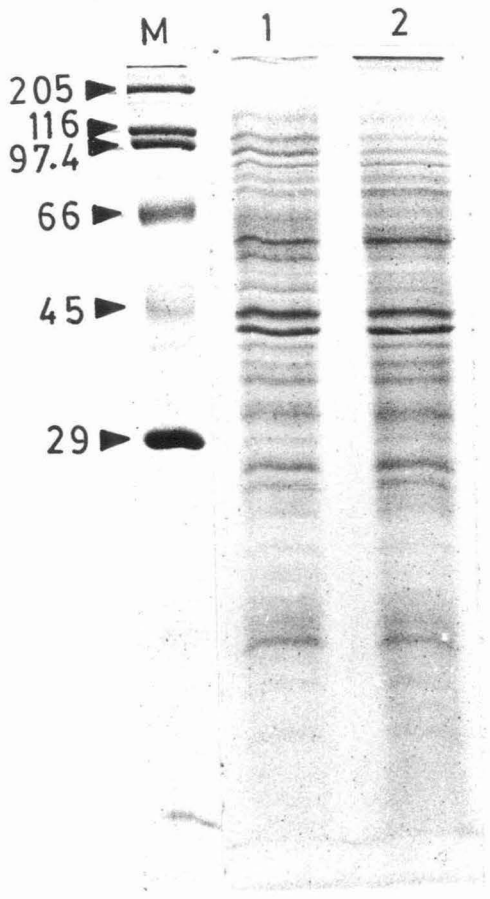
tolerant cells, or similar alterations could be brought about by exposing sensitive cells to high salt media, i.e. by giving a salt shock. No change in the polypeptide profile was detected after exposure of salt sensitive lines to NaCl for 24 hours. However, after 48 hours enhanced level of 58 and 69 kD polypeptides could be observed (fig.3) but 80 kD polypeptide band showed decreased intensity when compared to non-shocked sensitive cells. Intensity of the band corresponding to 80 and 69 kD polypeptides in salt shocked sensitive cells was comparable to that of tolerant cells, but the level of 58 kD polypeptide, though higher in salt shocked cells when compared to sensitive cells, was found to be lower when compared with salt-tolerant cells. After 7 days of salt shock the level of 58 kD and 69 kD polypeptides decreased, and was comparable to the level in non-shocked cells (fig.4). It appears that 58 and 69 kD polypeptides could be involved in adaptation to higher level of salt. In the tolerant callus lines, the enhanced level of these polypeptides was maintained, suggesting that these may be related with the 'tolerance' character. Therefore, in the non-tolerant callus lines a decrease in these polypeptide was observed after 7 days of shock treatment. Reduced level of 80 kD polypeptide was observed in salt-tolerant lines as well as after 48 hrs. and 7 days salt-shock

Fig. 4: Polypeptide profiles of salt shocked (7days) sensitive cell lines.

lane 1 - sensitive cells

lane 2 - salt shocked sensitive cells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treatment to the sensitive cell lines. It is possible that this polypeptide may be related to growth of the cells, which is comparatively suppressed under salt stress. Two other polypeptides, 55 and 82 kD showed decreased level of accumulation after 7 days of salt shock to the sensitive cell lines, though these polypeptides remained unaffected after 48 hours salt shock. It is possible that on longer exposure to NaCl, either these polypeptides might be getting degraded or their synthesis might be suppressed. Alternately this may be attributed to the general reduction in the level of some polypeptides due to their instability under high salt concentrations. Further experiments are needed to confirm or negate these possibilities although the reduced level of accumulation of polypeptides in high salt medium have also been reported by other authors. Ben-Hayyim (1989) reported considerable decrease in the level of 27 kD polypeptide when unadapted tomato cells were exposed to 1% NaCl. Ramagopal (1986) reported the disappearance of 39.5 and 39 kD polypeptides in maize callus when exposed to 2% NaCl.

c) Response of salt tolerant callus on exposure to medium lacking NaCl

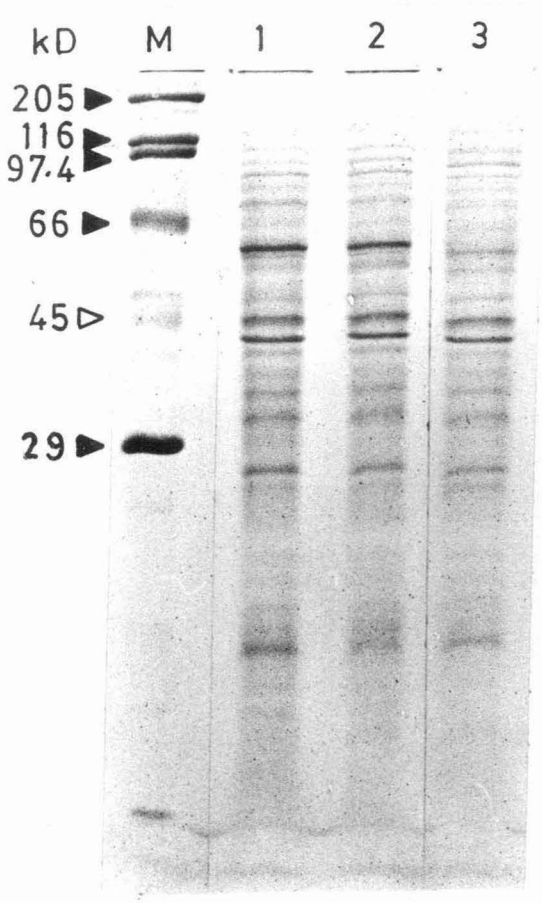
Studies were also carried out to find out the fate

Fig.5: Polypeptide profiles of tolerant callus, tolerant callus on medium minus salt maintained for 48 hrs.and sensitive callus.

lane 1 - tolerant callus

lane 2 - tolerant callus on medium minus salt

lane 3 - sensitive callus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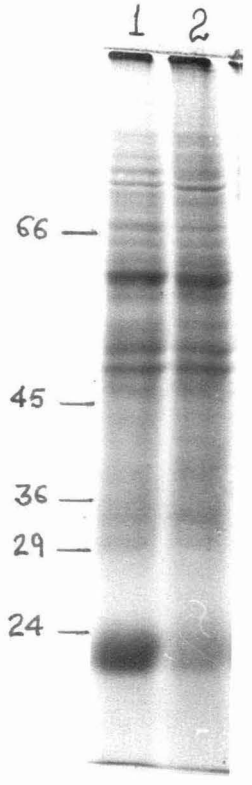
of the polypeptides which showed altered levels in the salt-tolerant cell lines, when they were grown on medium without salt. Therefore, the salt tolerant callus lines which were adapted to grow on higher salt concentration (200mM) were transferred to medium minus salt for 48 hrs. (fig. 5) as well as 7 days (fig. 6) after which the proteins were extracted and analysed on SDS-PAGE. The level of 13, 25, 31, 34 and 58 kD polypeptides, which was earlier found to be enhanced in salt-tolerant cell lines as compared to salt sensitive cell line, showed a transient drop after 48 hrs. of withdrawal of NaCl. The enhanced levels were, however, regained after 7 days. The transient drop could be due to a stage of physiological and metabolic adjustment on sudden removal of salt, leading to normalisation at a later stage. The expression of 76 kD protein, however, did not show any alteration after 48 hours or 7 days. The enhanced level of this polypeptide which was evident in salt-tolerant callus lines was observed throughout the experiment, irrespective of the presence or absence of salt. The above observations suggested that these polypeptides may be related to the salt-tolerance character of the cell lines. Similar pattern of expression of a 26 kD has been reported by Ben-Hayyim et al (1989) in citrus.

Fig. 6: Polypeptide profiles of salt tolerant cell line maintined on salt free media for 7 days.

lane 1 - tolerant cell line maintained on 200 mM NaCl

lane 2 - tolerat cell line of salt free medium for 7 days.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Another interesting observation was the enhanced level of accumulation of the 80 kD polypeptide on transfer of salt tolerant cell lines to media minus salt. As reduced level of this polypeptide was earlier observed in tolerant cell lines (maintained on 200mM NaCl) as well as in salt-sensitive cell lines exposed to 200 mM NaCl, it can be concluded that the expression of this polypeptide was inhibited in the presence of NaCl. This is contrary to the observation of Winicov et al. (1989) who reported enhanced level of a 80 kD polypeptide under salt stress in *Medicago sativa*. It is possible that the response to salt stress differ from species to species as has been shown by Ben-Hayyim et al.(1989).

Two polypeptides of 24 and 69 kD exhibited reduced intensity after 7 days of exposure of salt tolerant callus lines to medium minus salt. Whether the reduction in the level of these polypeptides was a consequence of ionic or osmotic shock needs confirmation.

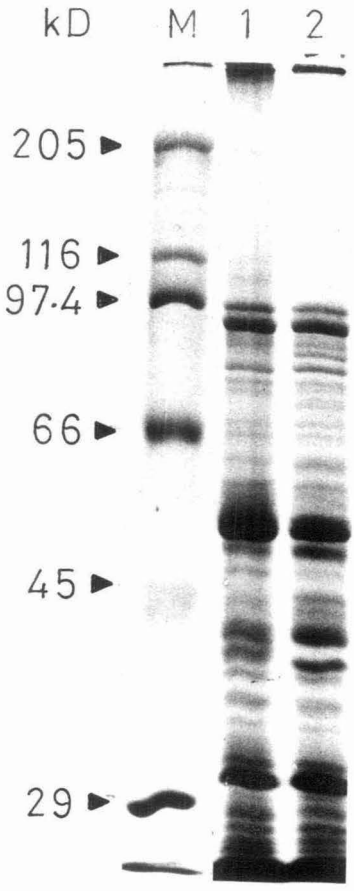
2. Response of whole plant to shock

A comparison of cellular level response to salinity was made with whole plant response to see if any correlation existed between the two. 7 day old *in vitro*

Fig. 7: Polypeptide profiles of salt shocked and non shocked plants. Plants were salt shocked by transferring them to 200 mM NaCl containing medium.

lane 1 - non-shocked plant

Lane 2 - shocked plant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grown seedlings were subjected to 200 mM NaCl stress. Proteins were extracted from the seedlings after 48 hrs. of NaCl stress and analysed on SDS-PAGE (fig. 7). While the levels of 39, 41 and 51 kD protein showed greater intensity on coomassie blue stained gels, as compared to the non-shocked seedlings, 48 kD polypeptide showed decreased intensity. Ramagopal (1987b), had also reported increased level of 39 kD polypeptide in a salt-sensitive cultivar of barley on exposure to salt stress. Further the mRNA coding for this polypeptide was identified and shown to be induced under salt stress. This 39 kD polypeptide has been localised in the shoots. In the present investigation no attempts were made to identify the mRNA for this protein. .

3. *de novo* synthesis of proteins under salt stress

To study *de novo* synthesis of proteins in salt tolerant and salt shocked calli and salt shocked germinating seedlings, cells and hypocotyls were labeled with ^{35}S methionine. On loading equal amounts of protein of all the samples on the gel, it was found that overall incorporation of ^{35}S methionine was much greater in proteins from non-shocked cells (fig. 8). The actual reduction in the incorporation of the label in the proteins could not be quantitated due to the

Fig. 8: Polypeptide profiles of *de novo* synthesised proteins in salt shocked plants, salt shocked sensitive calli and tolerant calli on medium minus salt for 48 hrs.

lane 1 - salt shocked sensitive callus

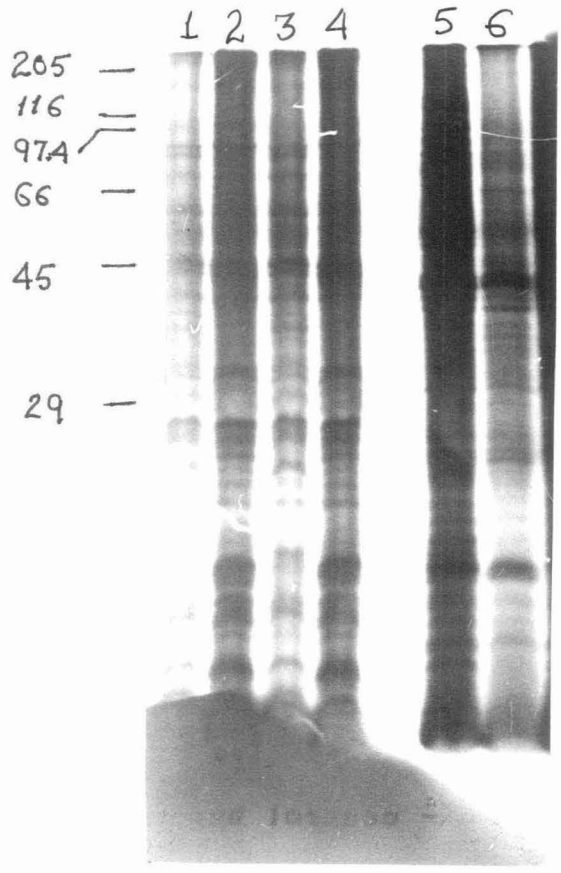
lane 2 - tolerant callus maintained on high salt medium

lane 3 - tolerant callus on medium minus salt

lane 4 - sensitive callus

lane 5 - control hypocotyls

lane 6 - shocked hypocotyls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limitation of time. The autoradiogram of gel confirmed that the overall protein synthesis is repressed under salt stress. As the cells have to divert part of the energy in overcoming stress, these results are not surprising.

SUMMARY

Optimal environmental conditions eg. water, temperature, nutrients etc. are required for the proper functioning of plants. Any substantial deviation from the optimal conditions causes detrimental effects on plant growth and development and is referred to as 'stress'. Large areas of lands in India have been rendered noncultivable because of excess accumulation of salts. Since desalinisation of such large areas of land is not feasible, the only way out is to engineer plants to make them capable of withstanding the adverse environmental conditions.

Plants are known to adapt to stress by changing qualitatively and quantitavely some molecules in the cells. The present investigation was carried out to select salt tolerant callus lines of *Arachis hypogaea* L. using in vitro cultures, and understanding the mechanism of tolerance at the cellular level. *Arachis* is the second source of vegetable oils in India and is economically very important. Tissue culture system was used because of the ease of handling complex traits like salinity tolerance at the cellular level. Salt tolerant lines of *Arachis hypogaea* L. were developed either by single step selection, where calli were directly

cultured on high levels (up to 200mM) of NaCl to pick up somaclonal variants /mutants, or by multistep selection where callus was initially multiplied on a lower level (50mM) of NaCl and then transferred step wise on medium containing higher levels of salt (up to 200mM). Callus lines which did not lose the salt tolerance character even in the absence of selection pressure (high NaCl), were referred to as 'tolerant lines'. Salt tolerant lines maintained on high NaCl media exhibited decreased growth as compared to the control callus maintained on salt free media. Analysis of protein patterns of the salt tolerant vs sensitive lines subjected to various experimental conditions revealed many interesting points. Salt treatment altered the steady state protein level in salt tolerant and salt shocked lines. Proteins that showed alteration in the levels in response to salinity stress in cell cultures can be grouped in to the following classes.

1. Proteins whose accumulation was more in tolerant cell lines but the level of which was reduced transiently on salt free media and regained to the original level after prolonged exposure to salt. eg. 13, 25,31,34,58 and 69kD proteins.

2. Proteins whose level was high in tolerant cell lines and remained unchanged even on salt free medium. eg.

76kD protein.

3. Proteins which showed reduced level in tolerant cell lines (maintained on salt media), but accumulated to higher levels when transferred on salt free medium. eg. 80kD protein.

4. Some proteins like 58 and 69 kD were common to salt shocked as well as salt tolerant cell lines. They accumulated only when the salt was present.

Although some of the cellular level responses in the salt tolerant and salt shocked cell lines were similar, there was no commonalty of response when compared with whole plants exposed to salt, except for the fact that the overall protein synthesis was inhibited in salt shocked callus lines as well as whole plants. This was demonstrated by checking the de novo synthesis by labelling the proteins with ^{35}S methionine.

As the expression of salt tolerance is associated with tissue organisation, it would be interesting to investigate, whether the regenerated plants from the salt tolerant lines are able retain the 'tolerance character' and whether similar or different mechanisms are operative in the regenerated plants.

As regards the role of various proteins whose levels were enhanced under conditions of salt shock or salt tolerance, it can only be speculated that they may

be involved in physiological and metabolic adjustments under salt stress conditions. Other proteins which showed reduced levels may be related to growth, as the growth of the callus on the salt media was also affected. To date, hardly any stress proteins have been identified with known functions and an in-depth study needs to be carried out to unravel the role of these proteins in plant systems.

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