BIOCHEMICAL AND MOLECULAR CHANGES UNDER SALINITY STRESS IN *ARACHIS HYPOGAEA*

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I hereby certify that the work which is being presented in the thesis entitled **BIOCHEMICAL AND MOLECULAR CHANGES UNDER SALINITY STRESS IN *ARACHIS HYPOGAEA*** in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy and submitted in the Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from March 2002 to August 2007 under the supervision of Dr. Ramasare Prasad, Associate Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

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

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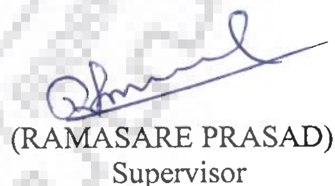

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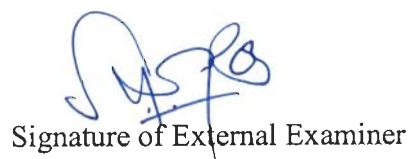

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ABSTRACT

In the present work attempts have been made to understand the biochemical and molecular basis of salt stress response in groundnut (*Arachis hypogaea)* at seedling stage. The groundnut seedlings used in all experiments were grown hydroponically under salinity stress of 50-200 mM NaCl for seven days.

The effect of NaCl stress on the growth of groundnut seedlings was studied. The remarkable reduction in the growth of root as well as shoot was seen in the seedlings under salinity stress from salt level 50 to 200 mM. The inhibition of growth was also supported by reduction in fresh weights of shoot and root of seedlings. Further, marked differences were also noted in anatomical features under salt stress. The decreased in cell size due to thickening of cell wall and disappearance of intercellular space in both roots and shoots of NaCl treated seedlings were seen. Reduction in cell size and thickening of cell wall resulted in stunted growth due to reduction in overall extensibility of cell. These are common salt stress responses shown by most of the plants.

There was increase in proline accumulation in roots and shoots of salt stressed seedlings. The accumulation of proline under salinity stress by plant is one of the major responses and it plays important role in salt tolerance by acting as osmoprotectant. Besides, proline was also reported to play protective role by stabilizing various cellular proteins and enzymes. It may be likely that the proline may play a similar role in groundnut as well.

Peroxidases and various hydrolases are found to play important role in cell growth. The effect of salt stress on peroxidases and hydrolases was studied. There was significant increase in peroxidase activity and reduction in hydrolase activity

under salt stress. The most prominent increase was found in ionically bound cell wall peroxidase fraction. The purification of ionically bound fraction resulted in identification of salt stress induced ionically bound anionic peroxidase of molecular weight 39 kDa and pI 5.5. An inverse relationship was found in peroxidase activity and growth. Peroxidases reduce cell growth by altering cell extensibility by increasing cell wall rigidity due to cross linking of cell wall polysaccharides and structural proteins like extensin. A number of cationic and anionic peroxidase isoforms cause cell wall rigidity by ferulic acid mediated lignification and suberinization and cross linking of extensin. It is likely that the salt stress induced increase in activity of ionically bound anionic peroxidase in present study may be involved in reduced growth by catalyzing ferulic acid mediated cross linking of cell wall polymers. This was confirmed by the purified peroxidase specificity for ferulic acid and ferulic acid polymer formation. A number of ionically bound anionic and cationic peroxidases induced under stress condition from various plants have been reported in earlier studies.

Effect of NaCl on the overall protein profile using SDS-PAGE and 2-D PAGE was studied. There found to be change in protein expression profile under salt stress, the expression of a number of proteins induced while a few were repressed. Several new proteins were synthesized under salt stress those were not present in normal seedling. A 31.6 kDa protein band identified to be the most prominently induced while a 48 kDa protein most prominently repressed as revealed from SDS-PAGE.

Polyclonal antibodies were generated against protein band of 31.6 and 48 kDa in rabbit and the antisera were used for expression studies. Expression profile of these two proteins under salt stress and various abiotic stresses, and ABA were studied. The 31.6 kDa salt induced protein expression increased with increasing salt concentration

while the expression of 48 kDa protein repressed with salt increase as expected. Under different abiotic stresses like NaCl, KCl and PEG expression of 31.6 kDa protein was found to be most prominent and is supposed to be major osmotic stress induced protein. However, it was induced by ABA though the level of expression was relatively low. Therefore, it seems to be ABA responsive as well.

Expression of 31.6 kDa protein was also studied in 10 cultivars of groundnut having different salt sensitivity. By comparing the expression pattern of 31.6 kDa protein in control and salt treated seedlings, it was found that the expression of this protein was more in salt treated seedlings of T-64, JAWAN, KAUSHAL, TG-1, TIRUPATI-1, KADRI-4, TIRUPATI-4 and KRG-1 cultivars of groundnut. Differences were also observed in the expression of 31.6 kDa protein among the control seedlings of different cultivars. The expression of this protein was high in control as well as in salt treated seedling of ICGS-37 cultivar which indicates its possible role in salt tolerance.

On the other hand the expression of 48 kDa protein was seen to be repressed under various abiotic stresses like NaCl, KCl, mannitol and PEG but no remarkable change was observed in presence of ABA. The expression of 48 kDa protein was also decreased significantly in salt treated seedling of TMV-7, ICGS-37, JAWAN, KRG-1, KAUSHAL, TG-1, TIRUPATI-1, KADRI-4 and TIRUPATI-4. There found to be little variations in the various cultivars. The maximum reduction was found in ICGS-37.

Tissue specific expression of 31.6 kDa and 48 kDa proteins showed that 31.6 kDa band is more intense in case of shoot treated with salt in comparison with the root, while the expression of 48 kDa protein was equally repressed in salt treated roots and shoots.

MALDI-TOF peptide mass fingerprinting studies of 31.6 kDa polypeptide showed its similarity with apple (*Malus domestica*) NADP-dependent sorbitol -6-phosphate dehydrogenase (S6PDH). Since this enzyme has a key role in the accumulation of sorbitol in the tissue which has a role of osmoprotection during salt stress. NADP-dependent sorbitol-6-phosphate dehydrogenase nature of 31.6 kDa protein was further confirmed by the sorbitol accumulation and NADP-dependent sorbitol-6-phosphate dehydrogenase activity. There was found to be a strong correlation between sorbitol accumulation and NADP-dependent sorbitol-6-phosphate dehydrogenase enzyme activity with increase in salt concentration. The NADP-dependent sorbitol-6- phosphate dehydrogenase was partially purified and analyzed on SDS-PAGE and Native PAGE. The immuno-reactivity of antibody raised against 31.6 kDa protein band recognized approximately a 31.6 kDa band in SDS-PAGE and 63 kDa in Native-PAGE, indicating the dimeric nature of the enzyme.

The immunoflourescence study using FITC labeled conjugates showed the difference in expression of 31.6 kDa protein in root and shoot of salt treated and control seedling. The protein is more abundant in shoot tissue of salt treated compared to control and it more abundant in shoot tissue than root. The protein was found to be localized in cell organelle particularly in plastids.

Therefore, the accumulation of sorbitol under salt stress may be one of the mechanisms of salt tolerance in groundnut seedling and NADP-dependent sorbitol-6-phosphate dehydrogenase plays important role in it. Role of sorbitol accumulation in salt tolerance has been reported from other plants like Japaneese persimmon (*Diospyros kaki*), *Plantago maritime* and tobacco.

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(AJEET SINGH)

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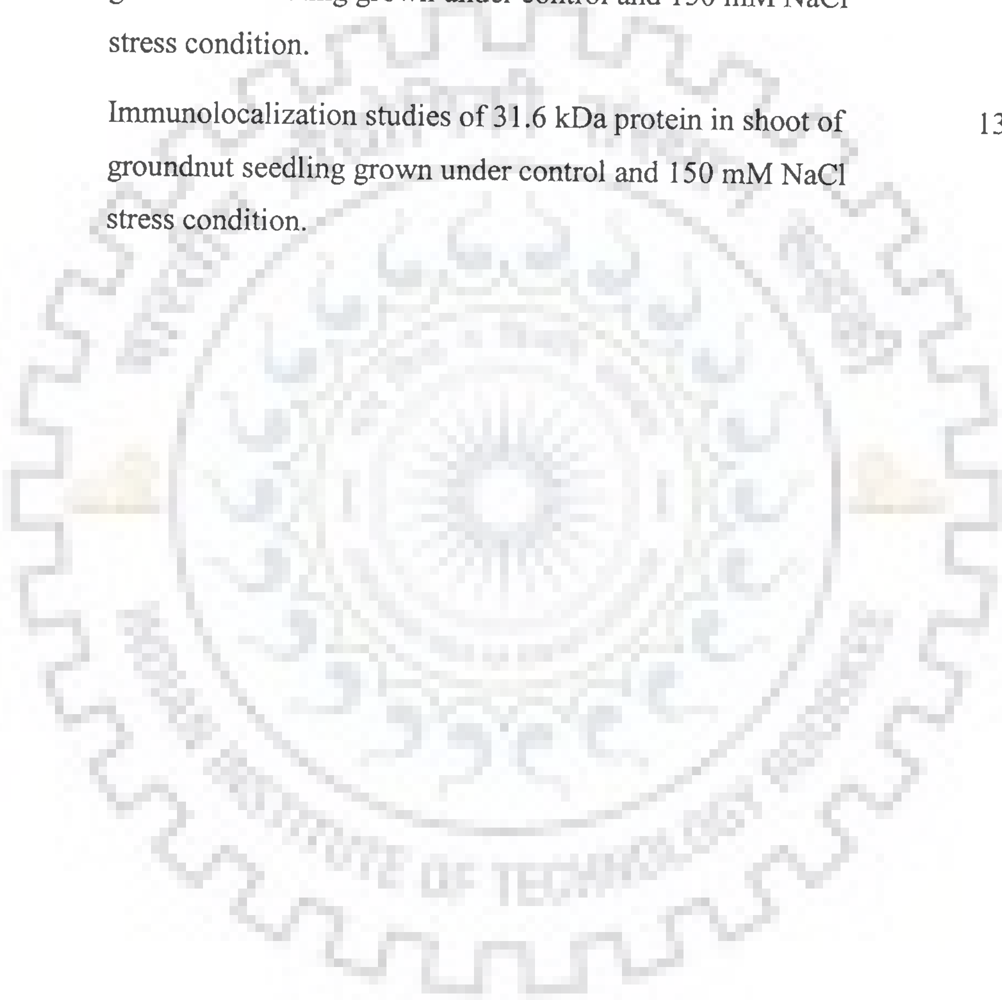

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ABBREVIATIONS

%	: Percent
°C	: Degree Celsius
APS	: Ammonium per sulphate
CFA	: Complete Freund's adjuvant
cm	: Centimeter
DAB	: 3, 3′ diaminobenzidine
DEAE	: Diethylaminoethyl
EDTA	: Ethylenediamine tetraacetic acid
Fig.	: Figure
g	: Gram
hr	: Hour
IFA	: Incomplete Freund's adjuvant
IgG-FITC	: Immunoglobulin G fluorescein isothiocyanate
IgG-HRP	: Immunoglobulin G Horse Radish Peroxidase
min	: Minutes
mL	: Milli litre
mM	: Milli molar
MT	: Metric Tons
O/N	: Over night
PAGE	: Polyacrylamide gel electrophoresis
PBS	: Phosphate buffer saline
PMSF	: Phenylmethylsulphonyl fluoride
PVDF	: Polyvinylidene Difluoride
RT	: Room temperature
SDS	: Sodium dodecyl sulfate
S6PDH	: NADP-dependent Sorbitol-6-phosphate dehydrogenase
TEMED	: N, N, N, N-tetra methylethylenediamine
w/v	: Weight per volume
µL	: Micro litre

CHAPTER-1

INTRODUCTION

CHAPTER - 1

INTRODUCTION

Salinity is one of the major abiotic stresses affecting crop growth and productivity. As per United Nations Environment Program estimate approximately 20% of agricultural land and 50 % of irrigated land in the world is salt stressed (Flowers and Yeo, 1995; Flowers and Flowers, 2005). It has been estimated that in India alone as large as seven million hectares area is covered by saline soils (Rajan and Rao, 1978). The problem becomes more severe as the area of cultivable land is decreasing day by day due to ever increasing salinization of the newer areas irrigated land (Tilman et al., 2002). The soil salinization is caused by both natural (Wanjogu et al., 2001) and human associated factors (Ponnamperuma et al., 1984). The loss of farmable land due to salinization is in direct contrast with increase of world population which is proposed to become 9.07 billion by the year 2050 (FAO 2006). Thus, there is an urgent need to sustain the food demand for the growing population. There could be two ways to achieve this; the first is to stop ever increasing salinization of the soil by proper management of irrigation systems, agricultural practices and development of better sewage systems and second is by increasing the yield of crop plants in normal soils and the cultivation on the saline lands by evolving the salt tolerant varieties.

Soil salinity has been found to have adverse affects on survival, biomass production and productivity of most of the crop plants (Khanna-Chopra et al., 1998; Grover et al., 1998; Khush et al., 1998). The processes such as seed germination, seedling growth and vigour, vegetative growth, flowering and fruiting are adversely affected by salt stress and ultimately causing poor produce and low yield. The extent of loss or

damage by salinity stress depends on: the degree of salinity, the degree of susceptibility of plant species and varieties to salinity (Bolarin et al., 1991; Ghoulam et al., 2002), environmental conditions like temperature, humidity etc. (Shannon et al., 1994) and to some extent also associated with the developmental stage of plant such as germination, seedling, and vegetative growth, flowering and fruiting (Vicente et al., 2004). It is very difficult to define soil salinity in precise term as several factors contribute for salinity effects on plants as mentioned above. However, soil salinity can be defined as soils having a high concentration of soluble salts and they are classed as saline when the EC_e is ≥ 4 dS m^{-1} (≈ 40 mM NaCl equivalent) where EC_e is the electrical conductivity of the saturated paste extract, and reflects the concentration of salts in saturated soil (Chinnusamy et al., 2005; Munns, 2005). Plants are classified as halophytes or glycophytes according to their ability to survive and grow in high salt conditions. Halophytes are those plants which can grow on relatively high saline conditions. On the other hand, most of the plants including important crops are glycophytes and either can not tolerate saline stress or have diminished growth. According to Carvajal et al., (1999); Yeo (1998); Grattan and Grieve (1999); Sreenivasulu et al., (2007) the adverse affects of high salt concentration (salt stress) on plant growth are due to: (i) reduction in the osmotic potential of the soil solution that reduces plant available water and thus creating a water stress in plants, (ii) deterioration in the physical structure of the soil such that water permeability and soil aeration are diminished, (iii) increase in the concentration of certain ions particularly Na^+ which causes severe ion toxicity, since Na^+ is not readily sequestered into vacuoles as in halophytes, and (iv) the interaction of salts with mineral nutrition may result in nutrient imbalances and deficiencies. The

consequences of these can ultimately lead to plant death as a result of growth arrest and molecular damage. Therefore, to achieve salt-tolerance the emphasis should be given either to prevent the damage, or to maintain the homeostatic conditions in the salt stress environment. This could be only possible when the molecular basis of salt stress response of plants and molecular mechanism of salt tolerance are fully understood which is a great challenge presently due to complex and multigenic traits of abiotic stress in general and salt stress in particular. Efforts to improve crop production under environmental stresses in the past had not been that fruitful because the fundamental mechanisms of stress tolerance in plants remain to be completely understood. Besides, the identification of key genetic determinants of stress tolerance is pre-requisite for the development of salt tolerant crops. The existence of salt tolerant plants (halophytes) and differences in salt tolerance among genotypes of salt sensitive plant species (glycophytes) indicate that there is a genetic basis to salt response.

Therefore, in order to develop salt tolerant plants by genetic manipulation either through molecular marker assisted selection or generation of transgenic plants by introducing novel or by altering the expression level of existing genes, it is essential to understand the molecular basis of salt stress responses of the plants. Study of salt stress response has been one of the thrust areas of research globally and attempts are being made to understand the molecular and biochemical basis of salt stress responses of various important crops and plants (Holmstrom et al., 2000; Branco et al., 2004; Cao et al., 2006; Brini et al., 2007). Ion transporter proteins, antioxidant enzymes and accumulation of compatible osmolytes like proline, glycinebetaine, mannitol, sorbitol etc are reported to play important role in salt tolerance of several plants and it is found to be

common mechanism under salt stress. Recent advances in molecular tools and technologies have been helpful to identify a number of salt stress responsive proteins and their respective genes (Sreenivasulu et al., 2004; 2007; Munns, 2005). Transgenic plants with enhanced salt tolerance have been produced using candidate genes in number of plant including *Arabidopsis* (Karakas et al., 1997; Hmida-Sayari et al., 2005; Cho et al., 2006; Chen et al., 2007a; Chen et al., 2007b).

Groundnut (*Arachis hypogaea* L.) is one of the major oilseed crops and is a member of genus *Arachis* and family Leguminaceae. *Arachis hypogeaea* is an alleotetrapoloid (2n = 4x= 40) species native to South America (Sharma and Bhatnagar-Mathur, 2006). It has been domesticated and widely cultivated around the world in tropical, subtropical and warm temperate climates and presently cultivated in more than 100 countries. According to FAO, Annual production in 2001 was 35.09 MT from 25.54 million hectare. In Asia, the crop is cultivated in more than 25 countries, which accounting for 67% of total world production. Two major producers in Asia are India with 8.2 million hectare and China with 4.6 million hectare which constitutes 55.9% and 31.6% area of Asia, respectively (Swamy et al., 2003).

The seeds of groundnut contain 44-56% oil and 22-30% protein on a dry seed basis. Oleic (O) and linoleic (L) are two major fatty acids together account for 75-80% of the total fatty acids in groundnut. Groundnut is an important commodity in many developing countries for both direct human food and oil production, particularly in India where the nitrogen (N)-rich crop residues are also used as fodder. In addition, it is a good source of minerals like P, Ca, Mg and K. Seeds also contains vitamins E, K and B group (Dwivedi et al., 1996). The production of groundnut in India needs to be increased from

the current 8 million tonnes to about 14 million tonnes by 2020 to meet the increasing demand of the oil and confectionery industry (Girdhar, 2004). This increase will have to be partially achieved by growing groundnut in lands considered so far as unsuitable for agriculture, like rice (*Oryza sativa*) fallow affected by salinity during the post-rainy season. Little is known about the salinity tolerance of groundnut and no attempt has been made to develop salt tolerant groundnut varieties.

Keeping the above facts in mind, in the present work attempts have been made to understand the molecular and biochemical basis of salt stress response in groundnut (*Arachis hypogaea)* with following main objectives:

1. Study the effect of salt stress on growth parameters.
2. Study the effect of salt stress on anatomical changes in root and shoot tissues.
3. Study the effect of salt stress on cell wall bound peroxidase and it possible role.
4. Effect of salt stress on protein profile using SDS-PAGE and 2-D PAGE.
5. Study the expression and localization of major salt stress responsive proteins.
6. Identification of the major salt responsive protein and its possible role.

CHAPTER-2

LITERATURE REVIEW

CHAPTER - 2

REVIEW OF LITERATURE

2.1 SOIL SALINITY PROBLEM AND AGRICULTURE: AN OVERVIEW

The population explosion in past few decades has raised many problems for mankind; one of them is "food for all". The population of world was approximately 2.51 billion in 1950 and will become 9.07 billions by the year 2050. Now it is approximately 6.50 billion out of which 16% is from India alone (FAO 2006). On the other side the area of arable land is decreasing day by day due to urbanization, industrialization and land degradation. Soil salinization has been identified as a major process of land degradation.

Salinity of arable land is an increasing problem of many irrigated, arid and semi-arid areas of the world where rainfall is insufficient to leach salts from the root zone and it is one of the major environmental abiotic stresses affecting plant growth and productivity (Francois and Maas, 1994). Ponnamperuma (1984) has defined saline soils are those that contain sufficient salt in the root zone to impair the growth of crop plants. Salinity adversely affects plant growth at all stages and at seedling and reproductive stages in particular, severely reducing the crop yield (Munns, 2002). Since salt injury depends on plant species and varieties suceptibility to salt, development stage of plant, environmental factors, and nature of the salts, it is difficult to define saline soils precisely. The most widely accepted definition of a saline soil has been adopted from FAO (1997) as one that has an electrical conductivity of the saturation extract (EC_e) of 4 dS m^{-1} or more, and soils with EC_e exceeding 15 dSm^{-1} are considered to be strongly saline. The common cations associated with salinity are Na^+,Ca^{2+} and Mg^{2+},

while the common anions are Cl^-, SO_4^{2-} and HCO_3^-. However, Na^+ and Cl^- ions are considered the most important, since Na^+ in particular causes deterioration of the physical structure of the soil and both Na^+ and Cl^- are toxic to plants (Dudley,1994; Hasegawa et al., 2000). The loss of farmable land due salinization is in direct conflict with the increase of world population. Thus there is an urgent need to sustain the food demand for growing population. To achieve this uphill task there is need:

(1) to understand the various possible causes of soil salinization.

(2) to stop the ever increasing salinization of the soil by proper management.

(3) to increase the productivity of normal lands and do cultivation on the saline lands by evolving the salt tolerant varieties.

The synergestic impact of the above facts may help us to sustain the future food need of world population.

2.2 CAUSES OF SALINITY

There are several factors which cause salinization of soil and can be categorized as primary and secondary causes of salinity.

2.2.1 Primary Cause

Natural geological, hydrological and pedological processes are reported to be the primary factors for development of salinized soil (Wanjogu et al., 2001). Salinization of soils may be further facilitated by climatic and weather conditions. It has been reported that in arid and semi-arid lands evapotranspiration plays important role in soil salinity. Salinization of coastal lands occurs mainly due to tides and cyclones which cause intrusion of saline water into rivers (Cyrus et al., 1997), acqufiers (Howard and Mullings, 1996) and coastal crop fields (Sultana et al., 2001).

2.2.2 Secondary Cause

Salinized soils that develop due human associated factors are called secondary salinization. These factors include:

(1) **Irrigation with poor quality water and improper irrigation practices:** This is one of the main human associated cause of soil salinity. Due to speedy urbanization and heavy industrialization, there is competition for fresh water among municipal, industrial and agricultural sectors (Tilman et al., 2002). This leads to scarcity of fresh water for agricultural applications. The result is increasing pressure to irrigate with poor quality salt containing water like ground water, drainage water and municipal treated water. The problem is more alarming in developing countries with high population growth. It has been reported that in developing country like China & Kenya more than one-third of the total area of irrigated lands are salinized due to poor irrigation and poor drainage (Qiao, 1995; Mugwanja et al., 1995). Besides, it has also been reported that improper methods of irrigation such as heavy watering and water logging can lead to salinization (Ponnamperuma, 1984).

(2) **Deforestation:** Deforestation is recognised as one of the other major cause of salinization and alkalization of soils. It has been observed that in Southeast India and Australia, vast areas of former forestland became increasingly saline and alkaline a few years after the felling of the woods (Szabolcs, 1994; Fitzpatrick et al., 1994).

(3) **Accumulation of air-borne or water-borne salts in soils:** It has been reported that chemical accumulation from industrial emission and waste water and sludge of

municipalities containing hight salt concentration cause salinization of the upper layer of soil (Bouwer, 2002; Szabolcs ,1994).

(4) **Salinization caused by contamination with chemicals:** This kind of salinization occurs more often in greenhouses and intensive farming systems and is more frequently in developed countries where intensive agriculture is common practice (Pessarakli, 1991).

(5) **Overgrazing:** Because of overgrazing, the natural vegetation becomes sparse and progressive salinization develops and this is more frequent in arid and semi arid regions (Szabolcs ,1994).

2.3 MANAGING SALINITY IN AGRICULTURAL PRODUCTION

Saline lands can be converted to more productive croplands by proper farm management practices, correcting soil toxicities and nutrient deficiencies, and leaching the salts out of the root zone. However, the major drawback of these practices is high cost of reclamation.

2.3.1 Farm Management Practices

Salinity can be restricted by proper farm management practices such as drip or microjet irrigation to optimize water use (Munns et al., 2002); reducing the amount of water passing beyond the roots by re-introducing deep rooted perennial plants like *Medicago sativa* (Ridley et al., 2001).

2.3.2 Amelioration through Fertilization

Interaction of salt with mineral nutrient result in nutrient imbalance and deficiencies, mainly by lowering concentration of micro-nutrients (N, P, Ca and K) in

plant. Therefore, the most easy way to recover the normal nutrients within the plant would be to raise their concentrations in the root zone by use of higher fertilizer dosages. There are several studies which showed that salt stress can be alleviated by an increased supply of Ca^{2+} (Rausch et al., 1996; Kaya et al., 2002), K^+ (Lopez and Satti, 1996; Kaya et al., 2001), N (Gómez et al., 1996), and P (Awad, et al., 1990; Kaya et al., 2001) in growth media. It has also been reported that tomato plants grown in saline medium supplemented with Ca^{2+} and P, enhanced the capacity of tomato plant to regulate Na^+, Cl^- and K^+ distribution, and improved plant growth (Song and Fujiyama, 1996; Kaya et al., 2001).

It was observed that raising fertilizers dosages may work well for irrigation with water containing low salt concentrations. However, in water with high salt concentrations, the concentrations of antagonist ion needed is so high that it causes a marked increase in the osmotic pressure of the soil solution and compounds the stress imposed by the salinity ions (Feigin, 1985). Furthermore some of the antagonist ions like P at high concentrations may results in severe toxicity (Grattan and Maas,1988).

2.3.3 Leaching

The one of the most effective methods to reclaim saline soil is leaching. This requires good quality irrigation water and works well in soils with good permeability. The major drawback of this approach is that it might descrease permeability and increase pH which may result in decomposition of roots as soil becomes more sodic (Dregne, 1976).

2.3.4 Uses of Salt Stress Tolerant Plants

Some areas have naturally occurring salinity and cultivation of salt-tolerant crop plants may be a better means of utilizing these resources for food production. Salinity can

possibly also be managed through genetic manipulation of plants (Shannon, 1984; Pitman and Laüchli, 2002). Identification of plant genotypes with tolerance to salt, and incorporation of desirable traits into economically useful crop plants, may reduce the effects of salinity on productivity. Development of salt tolerant crop plant will have double advantages, one it can be cultivated in saline soil and second it will permit the use of poor quality water for irrigation and thereby will control to some extent the demand for high quality water for irrigation. Much emphasis, now-a-days, are being given globally to develop salt tolerant crop plants.

2.4 EFFECTS OF SOIL SALINITY ON PLANTS

2.4.1 Effects on Plant Growth

Plants are classified as glycophytes or halophytes according to their capability to grow on saline conditions. Most of the plants are glycophytes and cannot tolerate salt-stress (Greenway and Munns, 1980). Halophytes can grow well at an optimum level of salt tolerance above which their growth get affected however, growth of glycophyte reduced significantly by any considerable increase in salt stress (Flowers et al., 1977; Matoh et al., 1986). The direct effects of salts on plant growth may be divided into three broad categories: (i) a decrease in the osmotic potential of the soil solution which lessen plant available water, (ii) a deterioration in the physical structure of the soil such that water permeability and soil aeration are reduced and (iii) increase in the concentration of certain ions that have an adverse effect on plant metabolism. (Carvajal et al., 1999; Yeo, 1998; Grattan and Grieve, 1999). These factors together or in combination have adverse effects on plant growth.

According to Dubey (1997) salt causes both ionic and osmotic effects on plants and most of the known responses of plants to salinity are linked to these effects. The

general response of plants to salinity is reduction in growth (Romero-Aranda et al., 2001; Ghoulam et al., 2002). Sodium and chloride, usually the most prevalent ions in saline soils or water, account for most of the deleterious effects that can be related to specific ion toxicities (Levitt, 1980). The degree to which growth is reduced by salinity differs greatly with species and to a lesser extent with varieties (Bolarin et al., 1991; Ghoulam et al., 2002). Though all stages of plants are affected by the salt stress but the seedling and reproductive stages are most affected (Sehmer et al., 1995; Ramoliya et al., 2004). The severity of salinity response is also mediated by environmental interactions such as relative humidity, temperature, radiation and air pollution (Shannon et al., 1994). Salt accumulation in leaves causes premature senescence, reducing the supply of assimilates to the growing regions and thus decreasing plant growth (Munns et al., 1995).

Reduction in growth under salinity has been reported in various plants species e.g. rice (Demiral and Türkan, 2006), tomato (Kaya et al., 2001; Maggio et al., 2007), cotton (Meloni et al., 2001), sugar beet (Papp et al., 1983), raphanus (Lopez et al., 1994), maize (Xinghong et al., 2005), barley (Ansari, 1990) and others. However, there are differences in tolerance to salinity among species and cultivars. It has been observed from various studies that increasing salinity is accompnied by significant reductions in root, shoot and leaf biomass, shoot and root length, plant height, number of leaves per plant and increase in root/shoot ratio in most of plants (Mohammad et al., 1998; Meloni et al., 2001; Maggio et al., 2007).

Moons et al., (1995) reported that NaCl at 50 mM significantly reduced the young seedling and root growth of the rice in salt-sensitive variety but tolerant varieties was not

inhibited. Differences in the effect of NaCl on growth rate were also observed in salt-tolerant and salt-sensitive cultivars of fox-tail millet (*Setaria italica*). The growth of salt-sensitive cultivar inhibited at salt concentrations lower than 200 mM NaCl but salt-tolerant cultivar withstood up to 250 mM but at 300 mM NaCl the growth of tolerant variety was also inhibited (Sreenivasulu et al., 2000). The optimum growth is obtained at 50% seawater and declines with further increases in salinity in *Rhizophora mucronata* (Aziz and Khan, 2001). In *Salicornia rubra* fresh and dry weights of plants increase with an increase in salinity up to 200 mM NaCl but the growth declines with a further increase in salinity (Khan et al., 2001). The salt secretor mangrove *Aegiceras corniculatum* can tolerate up to 250 mM NaCl and 300 mM is found lethal (Mishra and Das, 2003). On the other hand a non-secretor mangrove *Brugueira parviflora* can tolerate up to 100 mM NaCl under hydroponics culture, whereas further increase in NaCl concentration delayed plant growth and 500 mM NaCl is found to be lethal in this species (Parida et al., 2004). In *Alhagi pseudoalhagi* (leguminous plant), total plant weight increased at low salinity (50 mM NaCl) but decreased at high salinity (100 and 200 mM NaCl) (Kurban et al., 1999). In sugar beet (*Beta vulgaris*) leaf area, fresh and dry mass of leaves and roots were dramatically reduced at 200 mM NaCl, but leaf number was less affected (Ghoulam et al., 2002). The effect of high concentration of salinity (150 mM) resulted in decreased of leaf area of olive (*Olea europaea* L.) tree (Tabatabaei, 2006).

Effect of the salt stress on growth, at cellular level has also been studied by several groups (Binzel et al., 1989; Adams et al., 1992; Ben-Hayyim et al., 2001; Borsani et al., 2005). The growth of salt adapted callus of jojoba (*Simmondsia californica*) was faster as compare to the salt sensitive callus at 150 mM NaCl and the cells of adapted line

had thicker cell walls as compared to the sensitive one (Chretien et al., 1992). Yen et al, (1994) reported that there was optimum growth at 25 mM of NaCl but further increase in salt concentration up to 200 mM caused a progressive decline in growth of *Mesembryanthemum crystallinum* cell culture. It was seen that adaptation of cultured tobacco cells to NaCl reduced cell expansion even though turgor is maintained, a result similar to that commonly reported for whole plants exposed to salinity (Binzel, et al., 1989; Singh et al., 1989).

2.4.2 Effects on Anatomy

The reduction in growth of plant under salinity is due to changes in the anatomy of different tissues. Generally, cell shape, size and decrease of intercellular spaces are seen in the plants under salt stress. Though, anatomical changes take place at all organs levels which causes growth reduction, however, most of the studies have been focused to study the anatomical changes in leaf under salinity as it plays major a role in food synthesis and water losses (Delphine et al., 1998; Mitsuya et al., 2000; Romero-Aranda et al., 2001; Parida et al., 2004). An increase in epidermal and mesophyll cells thickness and palisade cell length have been reported from leaves of bean, cotton, and atriplex under salinity (Longstreth and Nobel, 1979). In contrast, there found to be significant reduction in epidermal and mesophyll cells thickness and interacellular spaces in NaCl treated leaves of mangrove *Brugueira parviflora* (Parida et al, 2004). Reduction in intercellular space also found in spinach leaves under salinity (Delphine et al., 1998). Salt stress is reported to cause rounding of cells, smaller intercellular spaces, and a reduction in chloroplast number in leaves of potato (Bruns and Hecht–Buchholz, 1990). Besides, changes have also been observed at organelles level under salt stress likes vacuolation,

swelling of endoplasmic reticulum and mitochondria, decrease in mitochondrial cristase, vesiculation and fragmentation of tonoplast and degradation of cytoplasm by the mixture of cytoplasmic and vacuolar matrices in leaves of sweet potato (Mitsuya et al., 2000).

In comparison to leaves there are only few studies regarding study of anatomical changes in root and shoot tissue under salinity stress (Cachorro et al., 1993; Zenoff et al., 1994; Cachorro et al., 1995; Surjus and Durand, 1996; Hilal et al., 1998). It has been observed that the cortical cells of the cotton roots were longer and narrower in salt treated plants than those of control plants and cell production declined with increasing salinity (Kurth et al, 1986; Lauchli and Schubert, 1989). In bean roots, it was reported that excess NaCl in the growth medium induces structural changes as well as leakage of ions correlated with alterations of the cell membranes (Cachorro et al., 1995). In soybean roots an NaCl-induced acceleration of the development of secondary xylem was also observed (Hilal et al., 1998).

2.4.3 Effects on Photosynthesis

Plant growth and biomass production depends on photosynthesis. Therefore, most environmental stresses inhibiting growth also have adverse effect on photosynthesis. The reduction in rate of photosynthesis under salinity have been reported from rice, jute, chick pea, guava and mangrove species under salt stress (Chaudhuri and Choudhuri, 1997; Soussi et al., 1998; AliDinar et al., 1999; Kawasaki et al., 2001; Romero-Aranda et al., 2001; Kao et al., 2001), which in turn reduce plant growth. A positive association between photosynthetic rate and plant growth under saline stress have been reported from a number of crop plants such as *Gossypium hirsutum* (Pettigrew and Meredith, 1994) and *Asparagus officinalis* (Faville et al., 1999) and *V. vinifera* (Fisarakis et al,.

2001). In contrast, there are many studies in which no or little association between growth and photosynthetic capacity is evident, as in *Triticum repens* (Rogers and Noble, 1992) and *Triticum aestivum* (Hawkins and Lewis, 1993). Reduction in photosynthesis is found to be because of decreased CO_2 assimilation into carbohydrate due to salinity through reductions in leaf area (Papp et al., 1983; Munns et al., 2000) and stomatal conductance (Brugnoli and Lauteri, 1991; Ouerghi et al., 2000; Agastian et al., 2000; Parida et al., 2003).

It is not necessary that photosynthesis is always suppressed at all salt concentrations in all plants. The effect of salinity on photosynthetic rate depends on salt concentration and plant species. There are evidences that at low salt concentration salinity may stimulate photosynthesis in plants like in *B. parviflora* (Parida et al., 2004), *Ceriops roxburghiana* (Rajesh et al., 1998), *Alhagi psuedoalhagi* (Kurban et al., 1999). It has been observed in the above studies that photosynthetic rate increased at low salinity and decreased at high salinity, whereas stomatal conductance was unchanged at low salinity and decreased at high salinity. The reduction of photosynthesis rate under salinity is reported to be result of a number of factors likes: reduction of CO_2 permeability due to dehydration of cell membrane (Iyengar and Reddy, 1996), Salt toxicity caused particularly by Na^+ and Cl^- ions (Bãnuls et al., 1990), reduction of CO_2 supply because of closure of stomata (Brugnoli and Bjorkman, 1992).

2.4.4 Effects on Ion Levels and Nutrient Contents in Plants

At high salt (NaCl) concentration, there is competition for uptake between salt ions (Na^+ & Cl^-) and other nutrient ions such as Ca^{2+}, K^+ N, and P which causes nutritional deficiency and resulting in quality and yield of plant (Grattan and Grieve,

1999). Increased NaCl concentration has been reported to induce increases in Na^+ and Cl^- and decreases in Ca^{2+}, K^+ and Mg^{2+} level in a number of plants like tomato (Perez-Afocea et al., 1996), *Haloxylon recurvum* (Khan et al., 2000) and *Phaseolus sp.* (Bayuelo-Jiménez et al., 2003). It is commonly accepted that competetion exists between Na^+ and K^+ leading to reduced level of internal K^+ at high external NaCl concentrations. This phenomenon is described in plants as well as in cultured cells (Perez-Afocea et al., 1996; Khan et al., 2000; Bayuelo-Jiménez et al., 2003). It has been reported in mangrove *B. parviflora* that a significant increase in Na^+ and Cl^- content in leaves, stem, and root was seen without any significant alteration of the endogenous level of K^+ and Fe^{2+}, with a decreases of Ca^{2+} and Mg^{2+} content in leaves (Parida et al., 2004).

Phoshphorus and Nitrogen uptake are also affected in plants under salt stress. Generally, salinity decreases the accumulation of P in plant (Sonneveld and de Kreij, 1999; Kaya et al., 2001) but some studies showed increased, or no effect on P uptake (Ansari, 1990). A number of studies have shown that salinity can reduce N accumulation in plants like lettuce and chinese cabbage (Feigin et al., 1991), celery (Pardossi et al., 1999), cowpea (Silveira et al., 2001). An increase in Cl^- uptake and accumulation has been observed to be accompanied by a decrease in shoot NO_3- concentration as in a number of plants (Savvas and Lenz, 1996; Fisarakis et al., 2001). The details about the salt stress effects on uptake of micronutrient by plants have been highlighted in recent publication (Grattan and Grieve, 1999).

2.4.5 Effects on Water Relation

The main reason for the stunted growth of plant under salinity stress is disturbance in water relations. Higher salts in root medium tend to decrease water

potential and it is difficult for plant to absorb optimum water for growth, and to maintain turgidity (Sohan et al., 1999). There are several reports which state that water and osmotic potential of plants reduce under salinity while turgor pressure increased (Meloni et al., 2001; Romero-Aranda et al., 2001; Khan et al., 2003). A significant decrease in leaf water potential and evaporation rate have being observed in the halophyte *Suaeda salsa* (Lu et al., 2002), *Brassica species* (Ashraf, 2001) and sunflower (Sohan et al., 1999) with increasing NaCl concentration. Salt treatment reported to cause significant decrease in relative water content (RWC) in sugar beet varieties (Ghoulam et al., 2002). A decrease in RWC indicates a loss of turgor that result in limited water availability for cell extension processes (Katerji et al., 1997). Please refer to recent review for details (Parida and Das, 2005; Zhu J-K 2003; Yokoi et al., 2002).

2.5 MECHANISM OF SALT STRESS RESISTANCE

Plant salt stress resistance has been defined as the inherent ability of plants to withstand the effects of high salt concentrations in the root zone or on the leaves without a significant adverse effect. Although individual responses to high salinity may differ, several lines of evidence suggest that all plants use the same general salt tolerance regulatory mechanisms, and that the differences between halophytic and glycophytic species are of a quantitative rather than qualitative nature (Greenway and Munns, 1980; Zhu, 2001). Plant sensitivity to salt levels in the soil is also highly depended on environmental factors (Shannon et al., 1994), plant species, cultivars within a species (Greenway and Munns, 1980; Ashraf, 2002), as well as the stage of plant development (Vicente et al., 2004). Plants have developed a number of mechanisms at biochemical and molecular level to deal with salt stress. Some of the biochemical approaches are:

(i) control of ions uptake and their compartmentalization, (ii) induction of antioxidative enzymes, (iii) accumulation of compatible solutes, (iv) induction of plant hormones.

2.5.1 Control of Ion Uptake and their Compartmentalization

Both glycophytes and halophytes can not tolerate high amount of salt in cytoplasm, they have to reduce the salt concentration in cytoplasm. Higher accumulation of Na^+ during salt stress condition causes toxicity to the cell, therefore, its concentration should be regulated in the cell for optimum metabolic activity. One of the common mechanism to control Na^+ ion during salinity stress conditions is its compartmentalization in different tissues. (Reddy et al., 1992; Iyengar and Reddy, 1996; Zhu, 2003). At cellular level ion sequestering in vacoules or ion exclusion at plasma membrane are proposed mechanisms which involved transport proteins like plasmamembrane ATPase, vacoular ATPase and pyrophosphate proton pumps (Ashraf and Harris, 2004). ATPases are integral transport proteins that hydrolyze ATP to pump protons across a membrane thus function to maintain the electrochemical gradients across the membrane.Na^+/H^+ antipoters are intergral transport proteins that use proton gradient established by H^+ pumps to exchange Na^+ for H^+. There are several studies of increased activity of Na^+/H^+ antiport under salinity stress (Apse et al., 1999; Yokoi et al., 2002; Mansour et al., 2003). Salt inducible enzyme Na^+/H^+ antiporter has reported to play a crucial role in removal of soduim from cytoplasm or its compartmentalization in vacoules (Apse et al., 1999). The *Arabidopsis thaliana* AtNXH1 gene encodes a vacuolar Na^+/H^+ antiporter that is important in salt tolerance , is expressed in all tissues except root tip (Shi and Zhu, 2002) and a similar antiporter is found in *Atriplex gmelini* (Hamada et al.,

2001). Ion compartmentlization of Na^+ under salinity stress and role of transporter protein described in details in recent review articles (Yokoi et al., 2002; Zhu J-K 2003; Parida and Das, 2005).

2.5.2 Induction of Antioxidative Enzymes

Exposure of plants to salt stress conditions leads to the production of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$) (Halliwell and Gutteridge, 1985; Elstner, 1987). The hydroxyl radical and other ROS can damage proteins, DNA, lipids, chlorophyll and other important macromolecules, ultimately affecting plant growth and yield (Fridovich, 1986; Sairam and Tyagi 2004). The alleviation of this oxidative damage could provide enhanced plant resistance to salt stress. Plants possess both enzymatic and non enzymatic mechanisms for scavenging of ROS. These include metalloenzyme superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT) and a variety of peroxidases (Parida and Das, 2005). Increase in activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), guaicol peroxidase (POD) under salinity have been reported and there exists correlation between level of enzyme and salt tolerance (Gossett et al., 1994; Sehmer et al., 1995; Kennedy and De Fillippis, 1999; Hernández et al., 2000; Sreenivasulu et al., 2000; Benavides et al., 2000; Lee et al., 2001; Mittova et al., 2002; 2003).

Transgenic plants have been generated to confirm the anti-oxidative enzymes role of ROS scavenging and salinity stress tolerance. Over expression of glutathione-S-transferase (GST) and glutathione peroxidase (GPX) in transgenic tobacco seedlings (Roxas et al., 2000) and yeast Mn-SOD in transgenic rice (Tanaka et al., 1999), showed

more salinity tolerance than wild type and also showed reduced lipid peroxidation under salinity. Sopory and coworkers have reported the role of glyoxylase system in stress tolerance (Singla-Pareek et al., 2003). The glyoxylase system is ubiquitous in nature and consists of two enzymes, glyoxylase I and glyoxylase II. Transgenic tobacco plants were generated by transformation with Gly I (Glyoxylase I) cDNA from *Brassica juncea*. Transgenic plants over-expressing glyoxylase I showed significant tolerance to salt-stress, which was correlated with degree of Gly I expression (Veena et al., 1999). A detailed description of antioxidative enzymes role in salt tolerance have been given in two recent reviews (Parida and Das, 2005; Sairam and Tyagi, 2004).

The reduction in plants growth under salinity is results of cessation of cell elongation and a number of factors contribute to it (Wang and Nil, 2000). Modification of cell wall structure is main factor in reduction of cell elongation thus reduction of over all growth. During salt stress conditions there was measured decrease in the plastic extensibility of cell walls of roots, shoots and leaves by hardening (Neumann, 1997; Neumann et al., 1994). This is mainly due to cell wall thickening due to lignification and suberization and cross linking of cell structure protein and polysaccharides (Degenhardt and Gimmler, 2000). Synthesis of cell walls under salinity stress on the isolated protoplasts of Colt cherry indicates the changes in the cell wall as cellular mechanism associated with salt tolerance (Ochatt and Power 1989). High rate of suberization and lignification of exodermis and endodermis cells have been reported during salt stress (Schreiber et al., 1999). Peroxidases were found to play an important role in cell wall development (De Jong, 1967). The key role of cell wall peroxidases isoforms is in stiffening of the cell wall through the formation of biphenyl bridges between wall

polymers and cessation of cell elongation (Sanchez et al., 1996). Increase in peroxidase activity under stress conditions have been reported from several plants like cotton (Meloni et al., 2003), rice (Lin and Kao, 2001) and fox-tail (Sreenivasulu et al., 1999), and correlation between cell wall thickening and peroxidase activity under stress was observed. There are direct evidences for the involvement of some apoplastic peroxidases in plant cell wall processing, including cross-linking of structural proteins and polysaccharides (Fry, 1986; Schnabelrauch et al., 1996) and lignification (Quiroga et al., 2000). A number of peroxidases both cationic and anionic isoforms have been isolated from various plants and their role in lignification and suberization have been established under stress conditions like wounding , salt stress and other stresses. Quiroga et al studied that a basic peroxidase isoenzyme of isoelectric point (pI) 9.6 is an enzyme involved in ligno-suberization of tomato roots grown in hydroponic conditions (Quiroga et al., 2000; 2001). This enzyme encoded by the peroxidase gene *TPX1* is induced in response to 100 mM NaCl treatments, Over-expression of *TPX1* in transgenic tomato plants has been related to anatomical differences in the roots that probably caused a reduction of hydraulic conductance (Botella et al., 1994; Lucena et al., 2003). The increasing activity of peroxidase against ferulic acid (FPOD) and ionically bound peroxidase was seen with progressively increasing NaCl concentration from 50-150 mM in rice seedlings (Lin and Kao, 1999; 2001). The significant increase in cell wall peroxidase activity in leaf elongation zone was seen during drought in *Lolium temulentum* L. (Bacon et al., 1997). High total peroxidase activity was seen in salt tolerant fox-tail millet as compare to sensitive one during salinity stress (Sreenivasulu et al., 1999).

2.5.3 Accumulation of Compatible Solutes

It is one of the most common mechanisms of salt tolerance in plants. Plants maintain ionic balance under salt stress conditions by accumulating low molecular mass compounds in cytoplasm of their cells. These compounds are termed as compatible solutes and include mainly proline, glycine betaine, soluble sugars, polyols (mannitol, sorbitol, pinitol etc) and polyamines. Accumulation of these solutes has been reported in various plants (Tao et al., 1995; Kavi Kishor et al., 1995; Sheveleva et al., 1997).Their accumulations do not interfere with normal biochemical reactions. These compounds act as osmolytes, shifting water potential gradients, or as osmoprotectants, maintaining a sphere of hydration around proteins (Ford, 1984). Accumulation of various compatible solutes under stress has been described in detail in recent reviews (Ashraf, and Harris, 2004; Parida and Das, 2005).

2.5.3.1 Proline accumulation under salinity stress

Proline accumulation may vary up to 80% of the amino acid pool under stress conditions like salt and drought in many plant species, say for example, in *Arabidopsis* it can account for up to 20% after NaCl stress (Kavi Kishor et al., 2005). Proline plays multifarious roles such as an osmolytes for osmotic adjustment stabilizing proteins and scavenging free radicals accumulate during stress conditions (Vanrensburg et al., 1993; Bohnert and Shen, 1999; Ashraf and Foolad, 2007). Some salt stress responsive genes were reported to be induced by proline. These genes have proline responsive elements (*PRE*) in their promoters (Satoh et al., 2002; Oono et al., 2003; Chinnusamy et al., 2005). There are many reports on accumulation of proline under salinity stress, some of them are: Three barley *(Hordeum vulgare* L.) mutants accumulate proline grew better under

salt stress conditions (Kueh and Bright, 1982). *Distichlis spicata* cultures grown continuously in the presence of 200 mM NaCl had elevated levels of free proline when compared to cultures grown in non-saline media (Daines and Gould, 1985). Handa et al., 1986 reported accumulation of proline in cultured cells of *Lycopersicum esculentum* to a low water potential environment and interpreted its high accumulation as osmotic solute. Salt stress caused an enhancement in proline up to 34 fold in seedlings and up to 16 fold in leaf tissue of *Brassica juncea* at different developmental stages (Madan, et al., 1995). Proline improves the salt-tolerance of *Pancratium maritimum* L. by protecting the protein turnover machinery against stress-damage and up-regulating stress protective proteins (Khedr, et al., 2003). Demiral and Turkan, (2005) studied the accumulation of proline in two rice cultivars differing in salt tolerance and found more accumulation in IR8, a salt sensitive cultivar, compared to Pokkali. The tolerance to salt stress was observed in transgenics developed by transferring genes associated with the biosynthetic pathway of proline (Kavi Kishor et al., 1995; Zhu et al., 1998; Han et al., 2003). Some studies on transgenics for proline over-production proved its role in abiotic stress tolelance like freezing and salinity (Kavi Kishor et al., 2005). The transgenic tobacco over-producing proline can withstand well at 200 mM NaCl and also has low level of free radicals as compared to control ones (Hong et al., 2000). Transgenic *Arabidopsis* with cDNA of antisense proline dehydrogenase can tolerate NaCl up to 600 mM (Nanjo et al., 2003).

2.5.3.2 Glycine betaine accumulation under salinity stress

Role of glycine betaine accumulation during salt stress tolerance was strongly suggested during a study on maize lines. Maize line containing gene for glycine betaine grown better as compare to deficient one (Saneoka et al., 1995). Higher concentration of

glycine betaine in *Atriplex griffithii, Suaeda fruiticosa, Haloxylon recurvum* and *Halopyrum mucronatum* was seen with increasing NaCl stress and was sufficiently high to act as an osmoticum (Khan et al., 1999). In maize (*Zea mays*, L.), exogenously applied glycine betaine improved growth, leaf water content, net photosynthesis, and the apparent quantum yield of photosynthesis of the salt-stressed plants (Yang and Lu, 2005). Exogenous applications of glycine betaine and proline to plants, before, during, or after stress exposure, have been shown to increase the internal levels of these compounds and generally enhance plant growth and final crop yield under stress conditions (Ashraf and Foolad, 2007).

2.5.3.3 Polyol accumulation under salinity stress

Other class of compatible solutes is polyols. The reduced forms of corresponding sugars are called polyols or sugar alcohols. Polyols are of two types, cyclic forms (onoitol and pinitol) and acyclic forms (mannitol, sorbitol, and glycerol). Both forms are widely distributed in plant kingdom. The most common types of polyols found in plants are mannitol, glycerol and sorbitol, believed to play several roles: as compatible solute, as scavengers of stress-induced reactive oxygen species and as low molecular weight chaperones (Ashraf and Harris, 2004; Parida and Das, 2005). Polyols function in two ways-

1. Osmotic adjustment- they act as osmolytes facilitating the retention of water in cytoplasm and allowing sodium sequestration to vacuole or apoplast.
2. Osmoprotection- they protect cellular structures by interacting with membranes, protein complexes, or enzymes (Parida and Das, 2005).

Myo-insositol derived from glucose-6-phosphate serves as precursor to a number of metabolites which are related to membrane biogenesis, cell signaling and stress protection. Ononitol, pinitol, mannitol and sorbitol are synthesized in different enzyme-catalysed steps involving myo-inositol-1-phosphate synthetase, myo-inositol-1-phosphate phosphatase, myo-inositol-*O*-methyltransferase, D-ononitol epimerase, sorbitol-6-phosphate dehydrogenase and mannitol-1-phosphate dehydrogenase (Rathinasabapathi, 2000). In whole plants of *Mesembryanthemum crystallinum* the cyclic sugar alcohol, pinitol, accumulates to amounts that approach 1 M during stress, while in suspension cells no increase in sugar alcohols was observed. The distribution of carbon to different sugars is markedly different between cells and plants under stress. Particularly obvious is the distinction between cell types in the different composition of sugars and polyols, as exemplified by the epidermal bladder cells of ice plants. Ion contents and the content of sugars and sugar alcohols of bladder cells indicate that Na^+, Cl^-, pinitol and an unknown carbohydrate compound provide osmotic pressure in these cells, while organic anion concentrations are low (Adams et al., 1992). Mannitol and its catabolic enzyme, mannitol dehydrogenase, plays an important role in salt tolerance. Gene from *Escherichia coli, mtl*D gene, encoding mannitol dehydrogenase was responsible for salt stress tolerance to tobacco plant, and similarly three rice transgenics with *mtl*D gene resist salt stress by accumulating mannitol (Ashraf and Harris, 2004).

Throughout the *Rosaceae,* sorbitol is a major photosynthetic product translocated from leaves to roots and other sink tissues. Sorbitol is synthesized in source tissues from glucose-6-phosphate via the action of NADP dependent sorbitol-6-phosphate dehydrogenase (S6PDH) and a phosphatase (Loescher et al., 1982). In sink tissues,

sorbitol phosphate is converted to fructose via the action of sorbitol dehydrogenase. Increased sorbitol in source tissues correlates with salt stress tolerance. For example, salt stressed Japanese pear, *Pyrus pyrifolia* (Burm. f.) Nakai, leaves showed increased sorbitol concentration, producing ^{14}C sorbitol when incubated with ^{14}C glucose. Glucose, fructose and sucrose showed no trace of ^{14}C, suggesting that under stress conditions sorbitol is the favored compatible solute in Japanese pear (Deguchi et al., 2002). In peach, (*Prunus persica* L., Batsch) water stress transiently increases sorbitol content of the leaves but not the roots (Cui et al., 2004). Persimmons (*Diospyros kaki* L.) transformed to express S6PDH from apple (*Malus.*) accumulated sorbitol where wild type did not. When placed under salt stress, the transformants maintained higher photosynthetic activity than untransformed controls (Gao et al., 2001).

2.5.4 Induction of Plant Hormone

The phytohormones, Abscisic acid (ABA) & Jasmonic acid (JA), are found to accumulate in plants under salinity stress. Their levels increase with high salt concentration (Aldesuquy, 1998; Vaidyanathan et al., 1999; Gomezcadenas et al., 2002), ABA and JA have antagonistic effect on salt stress inducible transcripts in rice (Moons et al., 1997b). It has been reported in citrus that increase of ABA is responsible for Ca^{2+} uptake and maintenance of membrane integrity to withstand salt stress (Chen et al., 2001). In rice, the inductions of salt stress genes are ABA dependent (Gupta et al., 1998). The treatment with ABA enhances salt tolerance (Noaman et al., 2002). Jasmonic acid was also believed to play an important role in salt tolerance; its level is higher in salt tolerant than sensitive tomato (Hilda et al., 2003).

2.5.5 Salt Stress Responsive Proteins and Genes

Improving salinity and drought tolerance of crop plants by genetic means has been an important but largely unfulfilled aim of modern agricultural development mainly due to complex nature and poor understanding of salt stress resistance mechanism. Rapid progress in understanding biochemical mechanisms that may participate in plant stress responses and salt tolerance, as well as the molecular cloning of genes involved in the various metabolic pathways that respond to salt stress, offer new approaches to solving this persistent problem (Bohnert and Jensen, 1996; Winicov and Bastola, 1997). Much emphasis has been for understanding the, molecular mechanism salt tolerance and for identification and characterization of salt stress inducible proteins and genes from various crops like citrus (Ben-Hayyim et al., 1993), *Mesembryanthemum crystallinum* (Forsthoefel et al., 1995; Bolte et al., 2000), *Brassica napus* (Srivastva et al., 2004) and finger-millet (Aarati et al., 2003). There is much variation in gene expression profiles of various plants (both transcripts and proteins level) under salt stress and control conditions (Ingram and Bartels, 1996; Bray, 1997; Liu et al., 1998). Physiologic or metabolic adaptations to salt stress at the cellular level are the main responses amenable to molecular analysis and have led to the identification of a large number of genes induced by salt (Ingram and Bartels, 1996; Bray, 1997; Yamaguchi-Shinozaki and Shinozaki, 1994). Many salt-responsive genes have been isolated and characterized during last two decades and change in their expression at the transcriptional and post-transcriptional levels were studied by analysis of protein profiles under salt stress conditions. These studies revealed both qualitative and quantitative changes in the pattern of polypeptides synthesized following salt treatment (Ericscon et al., 1984; Ramagopal, 1987; Singh et

al., 1989a; Chen and Tabaeizadeh, 1991; Moons et al., 1997c). There are several recent reviews describing the salinity induced changes in gene expression in plants (Grover et al., 2001; Sairam and Tyagi, 2004; Munns, 2005). Some important salt stress induced genes and proteins with their possible role in salt tolerance are listed in Table-1.

Several salt-induced proteins have been identified in plants species and have been classified into two distinct groups (Hurkman and Tanaka, 1988; Hurkman et al., 1988; Mansour et al., 2003); salt stress proteins, which accumulate only due to salt stress, and stress associated proteins, which also accumulate in response other stresses like heat, cold, drought, waterlogging, and high and low mineral nutrients. Proteins that accumulate in plants grown under saline conditions may play a role in osmotic adjustment by providing a storage form of nitrogen that is re-utilized when stress is over (Singh et al., 1987) and proteins may be synthesized *de novo* in response to salt stress or may be present constitutively at low concentration and increase when plants are exposed to salt stress (Pareek et al., 1997). In salt adapted cells of tobacco (*Nicotiana tabacum*) induction and repression of several polypeptides have been observed among them, most prominent a 26.0 kDa salt induced protein named as osmotin is well characterized and found to play role in adaptation of cells to NaCl stress (Singh et al., 1985).

Table 1: Salt responsive protein /gens in different plants

Plant species	Salt responsive protein (*gene*)	Characteristic feature	Reference
	-58 ,37,35.5,34 ,26 , 21,19.5 & 18 kDa polypeptide	-increased with NaCl Tolerance	Singh et al., 1985
	-Osmotin	-located in vacuoles, and also found in alfalfa, green beans	Singh et al., 1987
	-Δ-1-pyrroline-5-carboxylate synthetase (*P5CS* mod)	-turgor maintenance	Hong et al., 2000
	-mannitol-1-phosphate dehydrogenase (*mt1D*)	mannitol may be involved in hexose sensing or in mopping up hydroxyl radicals.	Tarczynski et al., 1993
Hordeum vulgare	-26 & 27 kDa protein	-salt induced polypeptide, but not water induced	Hurkman & Tanaka, 1988
	-Germin-like protein (26 kDa)	-98.5% similarity with wheat germin -protein	Hurkman et al., 1988; 1989; 1994
Citrus sinensis	-25 kDa protein, later named Cit-SAP	-expressed in presence of salt, ABA and PEG	Ben-Hayyim, G. et al., 1989; 1993; 2001;
Psophocarpus tetragonolobus	-84 kDa	-hydroxy-proline rich salt induced glycoprotein	Esaka, et al., 1992.
Raphanus sativus	-22-kDa	-belonging to Kunitz protease inhibitor family	Lopez et al., 1994.

Contd/--

Plant species	Salt responsive protein (*gene*)	Characteristic feature	Reference
Arachis hypogaea	-24 proteins spots	-most of them belong to PR 10 protien family	Jain et al., 2006
	-Nine different esterase isoenzymes,	-induced more in roots	Hassanein, 1999
	-127 & 52 kDa	- salt induced proteins	
Eleusine coracana Gaertn.	-54 kDa protein	-found in genotype GE 415	Uma et al., 1995
Oryza sativa	-22 and 31 kDa	-expression of 31 kDa varies with cultivars	Kong-ngern et al., 2005
	-14.5 kDa, SALT protein (*salT*)	-mannose-binding lectin	de Souza Filho et al., 2003
	-15 kDa	-jaclin-related mannose binding lectin	Zhang et al., 2000
	-29-kDa proteins - and two 40-kDa proteins	-recognized as OSR40 protein family	Moons et al., 1995; 1997
	-Choline oxidase (*codA*)	-possibly redox regulation, to buffer cellular redox potential as metabolic activities slow down during stress, and so to promote recovery from stress.	Sakamoto et al. 1998
Mesembryanthemum crystallinum L	-24 kDa, termed "SRgp24"	-N-terminus sequence showed 55-60% similarity with osmotin	Yen et al., 1994

Contd/--

Plant species	Salt responsive protein (*gene*)	Characteristic feature	Reference
Arabidopsis thaliana	-Na^+ antiporter (*NHX1*)	-AtNHX1 (Na^+/H^+ exchanger) is an Na^+/H^+ antiporter on the tonoplast membrane. It is expressed in roots and leaves, and selectively transports Na^+ into the vacuole, as well as K^+ in nonsaline conditions	Apse et al., 2003
	-DNA-binding (*Atmyb2*)		-Urao et al., 1993
	-Proline biosynthesis (*AtP5CS*)		-Yoshiba et al., 1995
Medicago sativa	-DNA-binding (*Alfin1*)		Bastola et al., 1998
	-Cell wall protein (*MsPRP2*)		Deutsch and Winicov, 1995

This protein is located in the vacuoles of the cell and reported to accumulate also in other members of Solanaceae, alfalfa and green beans (King et al., 1986; Singh et al, 1987). Expression of osmotin gene is regulated by at least five signals including ABA, osmotic stress, ethylene, wounding, and TMV infection. Out of the five demonstrated signals that cause large increases in osmotin mRNA accumulation, only osmotic stress and to some extent, ethylene treatment resulted in increased protein accumulation (LaRosa et al, 1985; 1987; 1989; 1992). In salt stressed *Mesembryanthemum crystallinum* it was found that osmotin-like protein was increased relative to non-stressed plants (Thomas and Bohnert, 1993). In barley, two polypeptides of 26.0 kDa (pI 6.3 and 6.5) were named as germin-like proteins, later the sequence of barley germin cDNA was found to have 98.5% similarity with wheat germin gf-2.8 (Hurkman et al.,1988; Hurkman and Tanaka, 1988). Lopez et al., (1994) found a 22 kDa protein in response to salt stress in radish. 18, 27 and 49 kDa proteins were found in callus of *Simmodsia chinensis* (Chretien, et al., 1992), Cit-SAP and an enzyme phospholipids hydroperoxide glutathione peroxidase (PHGPX) in *Citrus sinensis*, have been identified and characterized which was over expressed in presence of salt, ABA and PEG (Ben-Hayyim et al., 1993; Ben-Hayyim et al., 2001; Gueta-Dahan et al., 1997). While assessing the combined effect of salinity and boron on wheat, Wimmer et al., 2003 reported that the combined stresses induced 25 kDa and 33 kDa proteins. Agastian et al., 2000 have reported that soluble protein increases at low salinity and decreases at high salinity in mulberry (*Morus alba*). SDS–PAGE analysis of proteins in peanut (*Arachis hypogaea* L.) reveals that plants grown under NaCl show induction of 127 and 52 kDa and repression of (260 and 38

kDa) in the synthesis of a few polypeptides (Hassanein, 1999). NaCl induces accumulation of four polypeptides with molecular masses of 61, 51, 39, and 29 kDa in maize roots (Tamas et al., 2001).

While investigating the mechanisms of salt tolerance in a mangrove, *Bruguiera sexangula*, Yamada et al., (2002) found a specific protein, allene oxide cyclase (AOC) responsible for enhanced salt tolerance. They designated this protein as “mangrin”. Furthermore, expression of mangrin in *Saccharomyces cerevisiae* and tobacco cell lines also enhanced salt tolerance in these species. A higher content of soluble proteins has been observed in salt tolerant than in salt sensitive cultivars of barley (Hurkman et al., 1989), sunflower (Ashraf and Tufail, 1995), finger millet (Uma et al., 1995), and rice (Pareek et al., 1997; Lutts et al., 1996; Rains, 1989). Many studies have been done in *Oryza sativa* at proteomic as well as genomic level to understand the basis of salt stress. Two protein bands (22 and 31 kDa), whose expression was specifically increased under salt stress were identified in Thai rice cv. Leaung Anan. The expression pattern of the 31 kDa varies with the tolerance of cultivar to salt stress (Kong-ngern et al., 2005). The expression of a SALT protein of 14.5 kDa, identified as mannose-binding lectin, was reported in rice plant roots in response to NaCl stress, its expression differs in abiotic stresses (de Souza Filho et al., 2003). In wheat, Ashraf and O’Leary, 1999 reported that total soluble proteins increased due to salt stress in all cultivars tested but that increase was more marked in a salt sensitive cultivar, Potohar, and low in a salt tolerant line, S24, compared with the other lines. Patterns of polypeptides in wheat cultivars were identical and the differences between cultivars under salt stress were only quantitative. For

example, 29 and 48 kDa polypeptides were reduced significantly in salt sensitive cv. Potohar due to salt stress. However, the quantitative changes in polypeptides may be responsible for adjustments in metabolic pathways under saline conditions. The induction of salt stress responsive protein is variable in different species and in different genotypes of a species (Uma et al., 1995; Morabito et al., 1996; Kong-ngern et al., 2005). Difference in the expression of proteins with molecular weight of 70-72, 54, 52, 37, 34 and 23 kDa were found in salt responsive (GE415) and poorly responsive genotype (VL481) of finger-millet (*Eleusine coracana)* grown in 200 mM NaCl 54 kDa and 23–24 kDa proteins were expressed only in GE415 and responsible for salt or drought tolerance (Uma et al., 1995). Similarly in *Eucalyptus microtheca*, two cultivars (called clone 42 and 43) when grown with saline stress of 200 mM showed varried level of expression of number of polypeptides (Morabito et al., 1996).

In contrast, in lentil, Ashraf and Waheed, (1993) reported that leaf soluble proteins decreased due to salt stress in all lines, irrespective of their salt tolerance. Ashraf and Fatima, (1995) found that salt tolerant and salt sensitive accessions of safflower did not differ significantly in leaf soluble proteins. Similarly, comparison of salt tolerant wild populations with cultivated populations of *Melilotus indica* and *Eruca sativa* (Ashraf, 1994) showed that the salt tolerant populations did not differ from salt sensitive populations in soluble protein content of their leaves at varying salt levels of the growth medium. It has been reported that salinity causes a decrease in intensity of several protein bands of molecular weight 17, 23, 32, 33, and 34 kDa in *B. parviflora* (Parida et al., 2004) and the degree of decrease of these protein bands seems to be roughly proportional

to the external NaCl concentration. The most obvious change concerns a 23 kDa polypeptide, which disappears after treatment with 400 mM NaCl but reappears when these salinized seedlings are desalinized. These observations suggest the possible involvement of these polypeptides for osmotic adjustment under salt stress in this species (Parida et al., 2004). Although Pareek et al., 1997 suggested that stress proteins could be used as important molecular markers for improvement of salt tolerance using genetic engineering techniques; in many studies the proteins produced under salt stress are not always associated with salt tolerance. Thus using proteins as salt tolerance indicators depends on the nature of the plant species or cultivar.

The salt induced genes identified so for can be classified in various functional groups related to their physiologic or metabolic function predicted from sequence homology with known proteins and is summarized in following Table-2.

Table 2: Functional groups of genes /proteins activated in salt stress with potential for providing tolerance (Source: Winikov, 1998).

1. Carbon metabolism and energy production photosynthesis
2. Cell wall membrane structural components
3. Osmoprotectants and molecular chaperons
4. Water channel proteins
5. Ion transport
6. Oxidative stress defenses
7. Detoxifying enzymes
8. Proteinases
9. Proteins involved in signaling
10. Transcription factors

Salt tolerance is a multigenic trait and a number of genes categorized into different functional groups are responsible for encoding salt-stress proteins. These genes fall into three main broad functional groups: (1) those that control salt uptake and transport; (2) those that have an osmotic or protective function; and (3) those that could make a plant grow more quickly in saline soil. These functional groups are describes in details in some recent review articles (Sreenivasulu et al., 2007; Munns, 2005; Chinnusamy et al., 2005; Parida and Das 2005; Sairam and Tyagi, 2004; Winicov, 1998) however, these are discussed briefly below in following sections. Most of the genes in the functional groups have been identified as salt inducible under stress conditions, however, most of them also induced under other abiotic stresses such as dehydration, cold, ABA and heat etc. Some of the genes which are salt induced and also induced by under other stress condition are listed in Table-3.

Table 3: Examples of differential activation of NaCl inducible genes by dehydration, cold and ABA(Source: Winicov, 1999).

Gene		Function*	NaCl	Dehdr	Cold	ABA
Alfin1	Alfalfa	DNA-binding	+	nd	nd	-
ARSK1	Arabidopsis	Prot.Kinase	+	+	+	+
ATCDPK1	Arabidopsis	Prot.Kinase	+	+	-	-
ATCDPK2	Arabidopsis	Prot.Kinase	+	+	-	-
Atmyb2	Arabidopsis	DNA-binding	+	+	-	+
AtP5CS	Arabidopsis	Proline biosyn	+	+	+	+
AtPLC1	Arabidopsis	Phospholipase C	+	+	+	+
cor6.6	Arabidopsis	Antifreeze prot	+	+	+	+
kin1	Arabidopsis	Antifreeze prot	+	+	+	+
Mlip15	Maize	DNA-binding	+	-	+	+
MsPRP2	Alfalfa	Cell wall prot	+	nd	nd	-
OsBZ8	Rice	DNA-binding	+	+	±	+
PKABA1	Wheat	Protein kinase	+	+	+	+
Rd22	Arabidopsis	Seed protein	+	+	nd	+
Rd29A(COR78)	Arabidopsis		+	+	+	±
Rd29B	Arabidopsis		±	±	-	+

± weak or delayed response, nd: not determined: * function shown or implied by sequence similarities with protein of known functions.

2.5.5.1 Genes that control salt uptake and transport

This group includes the genes which code for the various ion transporters which play important role in ion homeostasis. Although NaCl is required in some plants, particularly halophytes, a high concentration of NaCl is toxic and affects plant growth (Glenn et al., 1999). The alteration of ion ratios in plants is due to the influx of Na^+ through pathways that function in the acquisition of K^+ (Blumwald, 1987). The sensitivity of cytosolic enzymes to salt is similar in both glycophytes and halophytes, indicating that the maintenance of a high cytosolic K^+/Na^+ concentration ratio is a key requirement for plant growth in soils with a high concentration of salt (Glenn et al., 1999). Strategies that plants could use to maintain a high K^+/Na^+ ratio in the cytosol include: (i) extrusion of NaCl ions out of the cell and (ii) vacuolar compartmentation of NaCl ions. Salt-sensitive plants restrict the uptake of salt and adjust their osmotic pressure by the synthesis of compatible solutes (e.g. proline, glycinebetaine and sugars) (Tal and Shannon, 1983). Salt-tolerant plants sequester and accumulate salt into the cell vacuoles, controlling the salt concentrations in the cytosol and maintaining a high cytosolic K^+/Na^+ ratio in their cells (Glenn et al., 1999). A number of genes involved in efflux and sequestering of Na^+ have been identified from various plants such as *SOS1* and *AtNHX1* (Munns, 2005; Yamaguchi and Blumwald, 2005). Model plant *Arabidopsis thaliana* had been used in different studies to crack the puzzel of salt stress tolerance in plants. Five salt tolerance genes, *SOS1*, *SOS2*, *SOS3*, *SOS4*, and *SOS5* ; *SOS1* encodes a putative Na^+/H^+ antiporter with a predicted molecular mass of 127 kDa (Zhu, 2001). A number of candidate genes which control Na^+ or K^+ uptake by roots and transport in

plants and play important role in salt tolerance have been clones and listed in Table-4. Transgenic plants over-expressing various ion transporter with improve salt tolerance have been developed in number of crop plants like *Arabidopsis*, tomato, brassica, rice tobacco etc. Some of the transgenic plants developed using ion transporter genes are listed in Table-5.

An antiporter gene cloned from *Arabidopsis*, *AtNHX1*, when overexpressed in that plant, increased its salt tolerance so that plants could then grow and set seed at 200 mM NaCl, whereas before they were limited to 100 mM NaCl (Apse et al., 1999). Similar results with *AtNHX1* were presented for tomato and brassica (Zhang et al., 2000; Zhang and Blumwald, 2001). Overexpression of the plasma membrane Na^+/H^+ antiporter *SOS1* in *Arabidopsis* increased the tolerance of *Arabidopsis* (Shi et al., 2003). Roots grew more quickly at 100 mM NaCl, and the percentage of plants that survived five days at 200 mM NaCl was increased from 17 to 43%. HKT transporters are probably very important in regulation of K^+ and Na^+ transport from root to shoots, (Rus et al., 2004) and transgenic plant developed using (*AtHKT1)* clearly increased salt sensitivity (Rus et al., 2004). The *HAL1* gene from yeast controls K^+/Na^+ selectivity and salt tolerance of yeast cells. Expression in tomato increased fruit yield and enhanced K^+/Na^+ selectivity in leaves (Rus et al., 2001). The exact function of this gene in higher plants is not known. More details about the various ion transporter genes and their role in salt tolerance can be seen in recent reviews (Munns, 2005; Yamaguchi and Blumwald, 2005).

Table 4: Cloned genes with likely relevance for controlling K^+ or Na^+ uptake by roots and transport within the plant, and which are candidates for overexpression studies. (Source: Munns, 2005: References there in)

Type of transporter	Gene family	Candidate genes for salt tolerance	Probable function in higher plants
K^+ channel	Shaker type (single pore, tetramer) inward channel	*AKT1, AKT2, KAT1*	*AKT1* (*Arabidopsis K^+ transporter*) is an inward-rectifying K^+ channel expressed in roots. It is highly selective for K^+ over Na^+ (*P*Na/*P*K = 0.05). However, at high salinities this channel could transport Na^+. *AKT2* and *KAT1* are related. These are expressed in leaf phloem tissue and guard cells, respectively, in *Arabidopsis*, but may function in other cell types in other species.
K^+ channel	Shaker type, outward channel	*SKOR*	*SKOR* (*stelar K^+ outward rectifier*) is important in maintaining K^+ homeostasis in both roots and shoots. *SKOR* mutants have lower K^+ concentrations in shoots but not roots, indicating that *SKOR* influences xylem loading of K^+. *SKOR* is probably located on the plasma membrane.
K^+ channel	*KCO* family (two pore channel)	*KOC1*	*KCO1* (*K^+ channel outward*) rectifier is expressed in leaf cells, probably on the tonoplast.
Nonselective cation channel	*CNGC* and *GLR* families	*CNGC1-20,GLR1-20*	Some members of the *CNGC*s (*cyclic nucleotide-gated channels*) and *GLR*s (*glutamate receptors*) families are predicted to have a similar permeability to Na^+ and K^+, and to be regulated by Ca^{2+}
K^+ antiporter	K/H antiporter	*KEA or CPA (CHA) Family*	K^+ antiporters may be important in K^+ homeostasis by loading K^+ into vacuoles. *KEA* (*K^+ exchange antiporter*) is present in the plant genome, but its function is unknown. It is possible that these could carry Na+, just as Na^+/H^+ antiporters can carry K^+.

Contd.

K^+ antiporter	*KUP/HAK/KT* family	*HAK1-10, KUP1-4*	A very large family of K^+ transporters, similar to the K^+ uptake family in bacteria and high-affinity K^+ transporters in fungi. There are many variants in all higher plants, and they are likely to be very important in control of K^+ homeostasis. Their selectivity for K^+ over Na^+ in higher plants is not known.
K^+ antiporter	*HKT* family	*HKT1*	K^+ starvation induces *HKT1* (*high-affinity K^+ transporter*) expression in wheat, indicating that it functions in high-affinity K^+ uptake, but it also transports Na^+. In *Arabidopsis* it is likely that *AtHKT1* is important in regulating Na^+ and K^+ homeostasis, as mutations that disrupt its function alter the transport of Na^+ from root to shoot, and the K^+/Na^+ ratio in the root
Cation antiporter	*CHX* family	*CHX10,15*	*Cation hydrogen exchangers* such as *CHX10* and *CHX15* regulate K^+ uptake by vacuoles. They may carry Na^+, and their expression is downregulated under salt stress
Na^+ antiporter	*NHX* family	*NHX1* *NHX2-5* *SOS1*	-*AtNHX1* (*Na^+/H^+ exchanger*) is an Na^+/H^+ antiporter on the tonoplast membrane. It is expressed in roots and leaves, and selectively transports Na+ into the vacuole, as well as K^+ in nonsaline conditions -*AtNHX2–5* are expressed in specific cell types, transport Na^+ or K^+ into the vacuole, and have a likely role in K^+ or pH regulation. -*SOS1* (*AtNHX7*) is an Na^+/H^+ antiporter on the plasma membrane. SOS1 (*salt overly sensitive*) is expressed in root cells. SOS1 would efflux Na^+ from cells and may be important in Na^+ extrusion from roots into the external medium.
Proton pump	AHA P-type H ± ATPase	*AHA2*	H^+ transport across plasma membrane.
Proton pump	H ± PPase	*AVP1*	H^+ transport across tonoplast.

Table 5: Salt tolerance in transgenic plants expressing genes involved in ion transporters (Source: Yamaguchi and Blumwald, 2005. References there in)

Gene	Gene product	Source	Cellular role(s)	Target plant	Parameter studied
AtNHX1	Vacuolar Na^+/H^+ antiport	*Arabidopsis*	Na^+vacuolar sequestration	*Arabidopsis*	Biomass
AtNHX1	Vacuolar Na^+/H^+ antiport	*Arabidopsis*	Na^+vacuolar sequestration	Tomato	Biomass
AtNHX1	Vacuolar Na^+/H^+ antiport	*Arabidopsis*	Na^+vacuolar sequestration	*Brassica napus*	Biomass,oil production
AtNHX1	Vacuolar Na^+/H^+ antiport	*Arabidopsis*	Na^+vacuolar sequestration	Maize	Biomass
AtNHX1	Vacuolar Na^+/H^+ antiport	*Arabidopsis*	Na^+vacuolar sequestration	Wheat	Biomass, grain yield
GhNX1	Vacuolar Na^+/H^+ antiport	*Gossypium hirusitum*	Na^+vacuolar sequestration	Tobacco	Biomass
AgNHX1	Vacuolar Na^+/H^+ antiport	*Atriplex gmelini*	Na^+vacuolar sequestration	Rice	Biomass
OsNHX1	Vacuolar Na^+/H^+ antiport	*Brassica napus*	Na^+vacuolar sequestration	Rice	Growth, seed yield
BnNHX1	Vacuolar Na^+/H^+ antiport	*Hordeum brevisubculatum*	Na^+vacuolar sequestration	Tobacco	Biomass
HbNHX1	Vacuolar Na^+/H^+ antiport	*Arabidopsis*	Na^+ extrusion	Tobacco	Biomass
AtSOS1	Plasma membrane Na^+/H^+ antiport	*Arabidopsis*	Na^+ extrusion	*Arabidopsis*	Biomass
SOD2	Plasma membrane Na^+/H^+ antiport	*Schizosaccharomyces pombe*	Na^+ extrusion	*Arabidopsis*	Biomass, photosynthesis
nhaA	Plasma membrane Na^+/H^+ antiport	*Escherichia coli*	Na^+ extrusion	Rice	Biomass, ion content
AVP1	Vacular H^+-pyrophosphatase	*Arabidopsis*	Vacuolar acidification	*Arabidopsis*	Biomass

Table 6: Salt stress tolerance of transgenic plants over expressing compatible osmolytes. (Source: Chinnusamy et al., 2005: References there in).

Gene and source	Transgenic plants	Stress tolerant traits
	Mannitol	
E. coli mtlD (mannitol-1-phosphate dehydrogenase)	Tobacco	Fresh weight, plant height and flowering under salinity stress
E. coli mtlD	*Arabidopsis*	germination at 400 m*M* NaCl
E. coli mtlD	Tobacco	Salt-stress tolerance; mannitol contributed only to 30-40 % of the osmotic adjustment
E. coli mtlD	Wheat (*Triticum aestivum* L.)	Only 8% biomass reduction when compared to 56% reduction in control plants in 150 mM NaCl stress.
	D-Ononitol	
IMT1 (myo-inositol *O*-methyl trans-ferase) of common ice plant	Tobacco	Drought and salinity stress
	Sorbitol	
Stpd1 (sorbitol-6phosphate dehydrogenase) of apple, driven by CaMV 35S promoter	Japanese persimmon	Tolerance in Fv/Fm ratio under NaCl stress
	Glycine betaine	
Arthrobacter globiformis CodA (choline oxidase)	*Arabidopsis*	Germination at 300 m*M* NaCl; seedling growth at 200 mM NaCl; retention of PSII activity at 400 mM NaCl
A. globiformis CodA targeted to the chloroplasts or cytosol	Rice	Faster recovery after 150 mM NaCl stress
A. globiformis CodA	*Brassica juncea* (L.) Czernj.	Germination in 100–150 mM NaCl; seedling growth in 200 mM NaCl

E. coli choline dehydrogenase (*betA*) and betaine aldehyde dehydrogenase (*betB*) genes	Tobacco	Biomass production of greenhouse grown plants under salt stress; faster recovery from photo inhibition under high light, salt stress and cold stresses
Atriplex hortensis betaine aldehyde dehydrogenase (*BADH*) gene under maize ubiquitin promoter	Wheat (*Triticum aestivum* L.)	Seedling growth in 0.7% (=120 m*M*) NaCl
Barley peroxisomal *BADH* gene	Rice	Stability in chlorophyll fluorescence under 100 mM NaCl stress; accumulates less Na^+ and Cl^- ions but maintained K^+ uptake
	Proline	
Vigna aconitifolia L. *P5CS* (Δ^1-pyrroline-5-carboxylate synthetase) gene	Tobacco	Root growth; flower development
Vigna aconitifolia L. *P5CS* gene under barley HVA22 promoter	Rice	Faster recovery after a short period of salt stress
Mutated gene of *Vigna aconitifolia* L. *P5CS* which encode P5CS enzyme that lacks end product (proline) inhibition	Tobacco	Improved seedlings tolerance and low free radical levels at 200 mM NaCl
Antisense proline dehydrogenase gene	*Arabidopsis*	Tolerant to high salinity (600 m*M* NaCl); constitutive freezing tolerance (–7°C)
	Trehalose	
E. coli otsA (Trehalose-6-phosphate synthase) and *otsB* (Trehalose-6-phosphate phosphatase) bi-functional fusion gene (*TPSP*) under the control of ABA responsive promoter or Rubisco small subunit (*rbcS*) promoter	Rice	Root and shoot growth at 4 week of 100 m*M* NaCl stress; survival under prolonged salt stress;maintenance of high K^+/Na^+ ratio; Low Na^+ accumulation in the shoot; maintained high PSII activity and soluble sugar levels
E. coli TPSP under maize ubiquitin promoter	Rice	Better seedling growth and PSII yield under salt, drought and cold stresses

2.5.5.2 Genes with an osmotic or unknown protective function

Various types of osmoprotectants and their roles in salt tolerance have been described in previous section. Though the main role of these osmolytes in osmotic adjustment during stress. In addition, certain solutes could have a metabolic protective role – they could stabilize soluble or membrane proteins, and so maintain growth at high salinity. The term 'osmoprotectant' has arisen for this function (reviewed by Rhodes et al., 2002). This section mainly described the genes which induced under stress conditions and involved in biosynthesis of these osmolytes. Genes involved in osmoprotectant biosynthesis are up-regulated under salt stress, and concentration of accumulated osmoprotectants correlate with osmotic stress tolerance (Zhu, 2002). Candidates genes involved in osmolytes biosytheis such as *P5CS* (Δ-1-pyrroline-5-carboxylate synthetase from *Vinca aconitifolia*) for proline; *codA* (choline oxidase from *Arthrobacter globiformis*) for glycine betain; *otsA* and *otsB* (trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase from *Escherichia coli*) for trehalose; *mt1D* (mannitol-1-phosphate dehydrogenase from *E. coli*) for mannitol; *S6PDH* (sorbitol-6-phosphate dehydrogenase modified from apple) for sorbitol; *imt1*(D-*myo*-inositol methyltransferase from *Mesembryanthemum crystallinum*) for ononitol, have been clones and used for developing salt tolerant transgenic plants (Hong et al., 2000; Sakamoto and Murata, 1998; Garg et al., 2002; Abebe et al., 2003; Gao et al., 2001; Sheveleva et al., 1997). Many papers have reported positive results for salt tolerance when plants are transformed with genes for osmoprotectants or protective proteins, summarized by Hare et al., (1998); Nuccio et al. (1999); Chen and Murata (2002); Gorham and Wyn Jones (2002); Rhodes et al. (2002); Flowers (2004). A few cases of transgenic plants are highlighted in Table-6.

Overexpression of genes for glycine betaine synthesis in *Arabidopsis*, rice and tobacco species enhanced salt tolerance in terms of biomass production or survival, despite the fact that the glycine betaine level in the transgenic plants was much lower than in species which naturally accumulate this compound, and too low to have a significant effect on osmotic pressure even if confined to the cytoplasm (reviewed by Chen and Murata, 2002). Thus improved salt tolerance in these transgenic must have been via a protective effect of the low levels of glycine betaine, rather than an osmotic effect bringing about more favorable water relations. All species accumulate proline under stress, and the gene for its synthesis, *P5CS* (Δ-1-pyrroline-5-carboxylate synthetase), is induced rapidly by stress in all tissues (Hong et al., 2000). However this enzyme is subject to feedback control, and overexpression with this gene increases the concentration of proline in leaves only twofold (Hong et al., 2000). Tobacco carrying a modified *P5CS* gene from *Vinca aconitifolia* to avoid feedback inhibition had a fourfold increase in proline (up to 60 mM compared with the control, with vector only, of 15 mM) in 200 mM NaCl (Hong et al., 2000). P5CS involved in proline biosynthesis from glutamate has been reported to accumulate in leaves and roots in response to salt-stress in *Pisum sativum* (Williamson and Slocum, 1992), while in *Oryza sativa* and *A. thaliana*, by salt dehydration and ABA (Yoshiba et al., 1995; Igarashi et al., 1997). Overexpression with the bacterial gene *mtlD* for mannitol synthesis has resulted in increased salinity tolerance, for example in wheat (Abebe et al., 2003). Overexpression with the bacterial genes for trehalose synthesis increased salt tolerance in rice. There was four fold greater dry weight after 4 week in 100 mM NaCl in transformed than in untransformed plants (Garg et al., 2002).

During salinity stress the cell encounters with extreme osmotic stress conditions and normal cellular activities are disrupted leading to the damage of cellular components. This cellular damage can be repaired by synthesis and accumulation of group of proteins called Late Embryogenesis Abundant (LEA)-like proteins. LEA protein have been placed in different groups based on amino acid sequence homology encoded by *lea* gene are: Group 1 LEA proteins, Group 2 LEA proteins, Group-3 and 5 LEA proteins, Group 4 LEA proteins and LEA D95 (Sairam and Tyagi, 2004). Salt stress induced expression of genes of Group-1 LEA proteins was reported in vegetative tissues of rice (Bostock et al., 1992) and of Group-2 LEA proteins in *Arabidopsis thaliana* by cold and ABA (Mundy et al., 1988; Gilmour et al, 1992). Genes encoding Group-3 LEA proteins were expressed in response to salt water deficit and ABA in soyabean and Barley, and salt and ABA in roots of rice, and Group-4 from tomato (Hong et al., 1992; Hsing et al., 1995; Moons 1997a; Cohen et al., 1991). Transgenic rice plants engineered to overexpress a barley *LEA* gene, *HVA1*, under control of the rice actin 1 promoter exhibit better stress tolerance under 200 mM NaCl and drought than wild-type plants (Xu et al., 1996). For details account of various osmolytes and protective proteins refers to recent reviews (Ashraf and Harris, 2004; Sairam and Tyagi, 2004).

2.5.5.3 Genes that involves in cell signaling and act as transcriptional factors

Genes that are involved in cell signaling play important role in cell and tissue growth and work in coordinated way. Candidate genes controlling growth are probably involved in signaling pathways that start with a sensor and involve hormones, transcription factors, protein kinases, protein phosphatases and other signalling molecules such as calmodulin binding proteins. Progress on the discovery of transcription factors

and signalling pathways is fast, and is being covered by a series of comprehensive reviews and updates (Shinozaki and Yamaguchi-Shinozaki, 2000; Knight and Knight, 2001; Zhu, 2002; Wang et al., 2003; Zhang et al., 2004). It is highly likely that such genes are common to drought stress (reviewed by Chaves et al., 2003), and are common to other stresses such as cold, ABA and soil conditions. Since the level of the plant hormone abscisic acid (ABA) increases with salt, drought and cold stress, it has been postulated to play a central role in signaling for these stress responses besides playing an important role in seed production. Exogenous ABA can activate transcription of many of the genes induced by salt/drought stress, while other salt/drought inducible genes are not activated by ABA, suggesting both ABA-dependent and ABA-independent signalling pathways (Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997).

In *A. thaliana*, the expression of receptor-like protein kinase gene is induced in response to salinity and ABA (Hong et al., 1997). A receptor protein kinase cDNA was also isolated from rice (Naot et al., 1995). These receptors transduce an extracellular signal across the membrane to activate cellular signal transduction pathways. Frandsen et al., (1996) reported an ABA, salt and desiccation-induced gene encoding a protein containing a conserved Ca^{2+} binding site, suggesting that Ca^{2+}-linked signalling occurs in osmotically stressed plants. A gene encoding a phosphatidyl inositol-specific phospholipase-C (PI-PLC) is expressed in response to salinity and desiccation (Hirayama et al., 1995). PI-PLC hydrolyses phosphatidyl inositol 4,5-biphosphate to produce inositol 1,4,5-triphosphate (IP3) and 1,2-diacyl glycerol. IP3 opens Ca^{2+} channels in ER membrane, causing Ca^{2+} efflux to the cytoplasm. This gene is also responsive to ABA, suggesting involvement of Ca^{2+}-linked signalling in the mediation of ABA and osmotic

stress responses. Mizoguchi et al. (1995; 1996) reported a gene in *A. thaliana* encoding components of signal transduction and identified it to be mitogen activated protein kinase (MAPK). Other similar genes are MAPKK kinase and a ribosomal S6 kinase, which all function in the MAPK cascade. The expression of these genes increases under salt stress.

Another attractive target category for manipulation and coordinate gene regulation is the small group of transcription factors that have been identified to bind to promoter regulatory elements in genes regulated by salt/ drought stress (Shinozaki and Yamaguchi-Shinozaki, 1997; Winicov and Bastola, 1997). Information to date on transcriptional regulation in response to salt/drought stress is relatively gene specific. A number of ABA-dependent (Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997) and ABA-independent transcriptional factors has been reported from alfalfa and other plants (Yamaguchi-Shinozaki and Shinozaki, 1994). ABA dependent *Atmyb2* from *Arabidopsis* (Urao et al., 1993) and *cpm10* and *cpm7*, encode MYB type transcription factors and are induced by dehydration stress. The *rd22BP1* gene from *Arabidopsis* has been shown to encode a *myc* type transcription factor (Abe et al., 1997). The ABA-independent salt/drought inducible DRE element (TACCGACAT) was initially identified in *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki, 1994) and is recognized by the transcription factor CBF1 (Stockinger et al., 1997). While CBF1 over expression has been shown to increase *COR* gene transcription and provide increased cold tolerance (Jaglo-Ottosen et al., 1998), no data are currently available on the salt/drought tolerance of these transgenic plants.

A putative transcriptional regulator *Alfn1* was reported from long term salt tolerance acquired in alfalfa and rice. *Alfn1* cDNA encodes a novel member of zinc finger

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family of proteins and *Alfn1* over expression in transgenic alfalfa leads to enhanced levels of *MsPRP2* transcript accumulation in callus and in roots (Bastola et al., 1998), indicating that *Alfn1* can act as transcriptional regulator on endogenous genes when transformed into alfalfa and it over expression reported to induce the *MsPRP2* gene, which is also induced by salt. Studies on over expression of 13 transcription factors have been described in a recent study (Zhang et al., 2004).

2.5.6 Genomics Approaches to Decipher the Regulatory Mechanisms of Salt Stress Tolerance in Plants

Recent adavances in functional genomic approaches triggered a major paradigm from single gene discovery to thousands of genes by using multi-parallel high through put techniques. Generation of expressed sequence tags (ESTs) from cDNA libraries prepared from abiotic stress-treated seedlings of various crops, complete genome sequence information of rice and *Arabidopsis* provided a valuable resource for gene discovery. Furthermore, employment of multi-parallel techniques such as expression profiling by microarrays, random and targeted mutagenesis, complementation and promoter-trapping strategies allow the identification of the key stress-responsive gene pools and in turn provide important clues for functional characterization of stress responsive genes and stress tolerance mechanisms. Attempts are being made to decipher the regulatory mechanisms of abiotic stress tolerance in plants by genomic approaches (Bohnert et al., 2001). New technologies are employed to advance understanding: large-scale EST preparation and expression profiling, microarray analysis identifying target genes whose expression is stress-regulated, and the generation of mutants in stress-relevant signal transduction and response pathways. Various genetic approaches use to deciphers the regulatory mechanisms of abiotic stress tolerance in plants are reviewed

(Sreenivasulu et al., 2007). Exclusively cDNA libraries generated with RNA from stressed rice (*Oryza sativa* L., Pokkali), ice plant (*Mesembryanthemum crystallinum* L.) and *Arabidopsis* (Bohnert et al., 2001). In addition, libraries and ESTs were generated for salt-stressed corn, barley, tobacco, and from the alga *Dunaliella salina* during an upshift in the medium salt concentration (Sreenivasulu et al., 2004a; Sreenivasulu et al., 2004b). Libraries are available from various tissues, mainly roots and leaves, at different developmental stages and at different times during stress (Sreenivasulu et al., 2006). Gene expressions profiling using cDNA macroarrays or microarrays (Chen et al., 2002) are novel approaches to identify higher number of transcripts and pathways related to stress tolerance mechanisms than before. There are several studies reported related to abiotic stress transcriptome profiling in model species such as *Arabidopsis* and rice that have revealed several new stress-related pathways in addition to the previously well described stress-related genes (Desikan et al., 2001; Chen et al., 2002; Kreps et al., 2002; Seki et al., 2002; Oh et al., 2005). These developments will be of great help in understanding the regulatory mechanism of salt tolerance and developing more effective tolerant plants.

2.6 THE GROUNDNUT

The groundnut (*Arachis hypogaea* L.) is a species in the Leguminaceae family native to South America. Groundnut is grown in many countries of the world. Major groundnut producers in the world are: China, India, Nigeria, USA, Indonesia and Sudan. Two major producers in Asia are India with 8.2 million hectares and China with 4.6 million hectares which constitutes 55.9% and 31.6% of Asia, respectively (Swamy et al.,

2003). In India, groundnut is grown on 6.6 million hectares with a production of 7.2 million metric tons (FAO, 2006).

2.6.1 Groundnut Utilization

Almost every part of the groundnut plant is used in some way. While the kernels are used for human consumption, vines are used as fodder for cattle in many African and Asian countries (Shankarappa et al., 2004). Groundnut roots left behind in the soil add a valuable nutrition to the soil. While groundnuts are used primarily for vegetable oil in most of the world, in the USA they are grown mainly for food including peanut butter, roasted-in-the-shell, candy, and as shelled whole seeds that are salted or dry-roasted (Isleib and Wynne, 1992). Among major peanut foods in the USA, peanut butter constituted a major item (52%) followed by salted groundnuts (24%), peanut candy (20%), and crackers/cookies (2%).Groundnut production and consumption in the period up to 2010 is likely to shift progressively more to developing countries. This boost will be seen in all regions with most rapid growth in Asia. Per capita consumption will grow sharply in Asia, slowly in sub-Saharan Africa and will decline in Latin America. Utilization will continue to shift away from groundnut oil towards groundnut meal, especially confectionery products (Freeman et al., 1999).

2.6.2 Salinity studies in Groundnut

Groundnut is a moderately salt sensitive crop and genotypic variation in salt sensitivity with EC_e of 3.2 has been reported (Chinnusamy et al., 2005). There are some study related to drought (Jain et al., 2001a), accumulation of proline and glycine betaine during salt stress (Girija et al., 2002), and jasmonic acid (Kumari et al., 2003) induced changes in groundnut. A number of cDNAs had been identified in *Arachis hypogaea* that

correspond to transcripts affected by water stress (Jain et al., 2001a). The differentially expressed transcripts were collectively named as PTRD (Peanut Transcript Responsive to Drought). Out of total 43 trancripts, 12 showed complete suppression due to prolonged drought, 2 were down regulated, and two were up regulated (Jain et al., 2001a). Except for some preliminary reports regarding changes in total sugar and lipid content (Hassanein, 1999) and increase in proline content (Jain et al., 2001b) under salinity stress, little is known about the salinity tolerance of groundnut. No efforts have been made to develop salt tolerant groundnut varieties due to poor understand of the molecular mechanism of salt stress in groundnut. Some initiative have been taken on this regards, in one of the study nine different esterase isoenzymes and two polypeptides (127 kDa and 52 kDa) were detected in embryos of seed germinated in 105 mM NaCl (Hassanein, 1999).

In salinity tolerant cell line of *Arachis hypogaea*, using proteomic approach 24 proteins showing induced expression have been reported and most of them identified to belong to PR10 protien family while one each belong to RNA binding protein and 14-3-3 protein family (Jain et al., 2006). According to one report the production of groundnut in India needs to be increased from the current 8 million tons to about 14 million tons by 2020 to meet the increasing demand of the oil and confectionery industry (Girdhar, 2004). This increase will have to be partially achieved by growing groundnut in lands considered so far as unsuitable for agriculture. Therefore, the better understanding of the molecular mechanisms for salinity tolerance in groundnut will be helpful for developing salt tolerant varieties to solve the increasing problem of salinity.

CHAPTER-3

MATERIALS & METHODS

CHAPTER - 3

MATERIAL AND METHODS

MATERIALS

All routinely used biochemicals or solvents in this study were obtained from M/s Sigma-Aldrich (U.S.A.), Pierce Chemicals (USA), Merck India, BDH (India) and SRL (India). Specific biochemicals were purchased from their respective sources such as Freund's adjuvant from Sigma-Aldrich (U.S.A.), goat anti rabbit IgG-HRP conjugate from Santa Cruz Biotech. Inc. (U.S.A) and goat anti-rabbit IgG-FITC conjugate were from Bangalore Genei (India). Groundnut (*Arachis hypogaea* cv.Kaushal) seeds were obtained from the National Seed Centre, Roorkee, Uttarakhand, India and TG-64, TMV-7, ICGS-37, Jawan, KRG-1, TG-1, Tirupati-1, Kadri-4 and Tirupati-4 cultivars of groundnut were obtained from National Research Centre for Groundnut, Junagarh, Gujrat, India.

METHODS

3.1 PRETREATMENT OF SEEDS AND GROWTH

Sterilization of seeds was done as per method described (Rohini and Rao, 2000). Seeds were surface sterilized by treatment with disinfectant 0.1% $HgCl_2$ for 5 min with continuous stirring, washed thoroughly and imbibed for 12 hr in sterile water. Imbibed seeds were placed on moist stack of blotting sheets for sprouting. After that, well sprouted seeds with uniformity were selected and transferred to plastic trays for growing hydroponically in Hoagland's medium (Hoagland and Arnon, 1950) under controlled temperature (28°C) at various salinities ranging from 0-200 mM NaCl in a plant growth chamber in dark at 80% relative humidity. Medium was changed after every 48 hr to

avoid nutrient depletion. Seedlings were harvested after seven days for the experimental work.

3.2 COMPOSITION OF NUTRIENT SOLUTION

Nutrient solution for hydroponics was prepared with the following composition:

Table 7: Ingredients of Hoagland's nutrient medium for groundnut

Ingredients	Stock Solution	Working Concentration	Volume/Liter added
KNO_3	10.1 g/100 mL	5 mM	5 mL
$Ca(NO_3)_2$	23.6 g/100 mL	5 mM	5 mL
$MgSO_4.7H_2O$	24.6 g/100 mL	2 mM	2 mL
KH_2PO_4	13.6 g/100 mL	1 mM	1 mL
H_3BO_3	0.618 g/100 mL	0.05 mM	5 mL
$MnSO_4.H_2O$	0.169 g/100 mL	9.00 μM	900 μL
$ZnSO_4.7H_2O$	0.28 g/100 mL	0.77 μM	77 μL
$CuSO_4.5H_2O$	0.249 g/100 mL	0.30 μM	30 μL
$Na_2MoO_4.2H_2O$	0.241 g/100 mL	0.10 μM	10 μL
*Fe-EDTA		0.05 mM	5 mL

* Fe-EDTA stock solution was made by adding 0.27 g of $FeCl_3.6H_2O$ and 0.29 g of EDTA to 100 mL of doubled distilled water. The nutrient solution was autoclaved and then Fe-EDTA solution was added aspetically.

3.2.3 DETERMINATION OF EFFECTS OF SALT STRESS ON GROWTH PARAMETERS

3.3.1 Measurement of Length

Seven days old seedlings were harvested for measurement of length. They were separated into roots and shoots, and lengths were measured in centimeters.

3.3.2 Determination of Fresh Weight

Seven days old seedlings were harvested and rinsed thoroughly with double distilled water (DDW). The seedlings were then blot dried on blotting sheet and cut into shoots and roots. The fresh weight of roots and shoots were measured separately.

3.4 STUDY OF ANATOMICAL CHANGES DUE TO SALT STRESS

Effects of salt stress on the anatomical changes in groundnut seedlings grown in different salt stress conditions were studied by observing stained ultra-thin transverse sections under light microscope (Johansen, 1940). Seven day's old seedlings were selected and paraffin blocks were made for microtomy. The procedure followed for block preparation and sectioning was as follows:

3.4.1 Fixation

1. Seedlings were brought into the lab shortly before fixation. Seedlings were cut off with a new single-sided razor blade and immediately placed in a glass petri dish containing ice-cold acetone. The seedlings were trimmed to desired size of tissue block.
2. The resulting tissue block was placed in a vial with 15 mL ice-cold fixative (100% acetone) and kept on ice.
3. Eight to ten seedlings were prepared in the same way and added to the same vial. Total preparation time of 8-10 seedlings per vial was kept around 5-10 min. Samples were kept in the fixative over night at 4°C.

3.4.2 Dehydration/Xylene Infiltration

1. The following morning samples were brought to room temperature (RT) and given a fresh change of acetone and then placed on a rotator for 1 hr.

2. The samples were passed through a dehydration series having 30%, 50%, 70%, 90%, 95%, and absolute ethyl alcohol in different vials. The samples were placed for 1 hr in each vial and then passed to next.
3. The samples were then kept in xylene for 15 min twice.

3.4.3 Paraplast Infiltration

1. A small amount of paraplast chips were placed into vials after the third pure xylene change and left to dissolve overnight (O/N) on a rotator at RT.
2. Next morning the vials were placed in oven at 60°C to melt any chips not yet dissolved. When paraplast was melted, vials were gently inverted back and forth to mix xylene and paraplast evenly.
3 Paraplast chips were added for melting and left for 1.5 hr. Half of this mixture was discarded and replaced with pure molten paraplast, then mixed by inversion and left overnight in the oven at 60°C.
4. Next, all of the mixture was replaced with pure paraplast. Throughout day four a total of at least three pure paraplast exchanges were done, each lasting 4 hr.
5. Next morning, paraplast was exchanged and left for another 4 hr.

3.4.4 Embedding

1. Tissues were embedded using Tissue Tek base moulds (metal) together with Tissue Tek embedding rings.
2. The embedding procedure was carried out using a "home-made" device consisting of a gradient metal warming plate (hot on one end and cold [RT] on the opposite end). Paraplast was dispensed from a 1.5 L reservoir into the assembled base moulds plus embedding rings.

3. Tissue and paraplast were poured from the vial into a metal weighing dish on the hot side of the warming plate. The tissue was scooped out with a weighing spatula, placed into the assembled base mould/ embedding ring combination, and oriented for sectioning on a rotary microtome.
4. Blocks were first cooled down to room temperature and then placed on ice for easy un-moulding. Blocks were kept in plastic bags at 4°C.

3.4.5 Sectioning and Staining

Ultra-thin sections were made with the help of rotary microtome. Before staining each section was passed through a rehydration series which included two changes of xylene (5 min each), then to absolute alcohol, 95%, 90%, 70%, 50%, 30% ethyl alcohol and finally to distilled water (5 min each) to remove paraplast. The sections were then put in 1% toludiene blue staining solution and passed through the dehydration series keeping 5 min in each to remove extra stain. Finally sections were mounted on slides for microscopic examinations in a light microscope (Axiostar plus, Zeiss , GERMANY).

3.5 DETERMINATION OF PROLINE CONTENT IN SALT STRESS SEEDLINGS

Accumulation of proline under salinity stress was studied according to the method of Bates et al. (1973). Plant material (0.5 g) was homogenized in 10 mL of 3% aqueous sulfosalicyclic acid and filtered through Whatman # 2 paper. 2 mL of filtrate was reacted with 2 mL acid-ninhydrin and 2 mL of glacial acetic acid in a test tube for 1 hr at 100°C, after that reaction was terminated in an ice bath. The reaction mixture was extracted with 4 mL toluene mixed vigorously by vortexing for 15-20 sec. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance was read at 520 nm using toluene as blank. The proline

concentration was determined from a standard curve and calculated on a fresh weight basis as follows:

[(μg proline/mL × mL toluene) / 115.5 μg/μmole]/ [(g sample)/5] = μmoles proline/ g of fresh weight material.

Acid-ninhydrin was prepared by warming 1.25 g ninhydrin in 30 mL glacial acetic acid and 20 mL 6 M phosphoric acid, with agitation, until dissolved. It was stored at 4°C.

3.6 DETERMINATION OF EFFECT OF SALT STRESS ON ACTIVITIES OF CELL WALL BOUND HYDROLASES (β-GALACTOSIDASE, α-GLUCOSIDASE AND ACID PHOSPHATASE)

3.6.1 Preparation of Cell Wall

Extraction of cell wall bound enzymes was performed according to method of Masuda et al. (1988) with minor modifications. Preparation of cell walls was performed as follows. Plant material (5 g) was homogenized in 15 mL of 10 mM Na-phosphate buffer (pH 7.4) and filtered. 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 hr at room temperature. After filtration of the suspension through 41 μm nylon screen, the residue was washed thoroughly with distilled water. The purified cell walls thus obtained were used for extraction of cell wall-bound enzymes.

3.6.2 Extraction of β-Galactosidase, α-Glucosidase and Acid Phosphatase

Extraction of β-galactosidase, α-glucosidase and acid phosphatase was done by suspending purified cell wall (1 g wet weight) in 10 mL of 0.5 M NaCl and incubation at room temperature for 2 hr. The enzyme activity was measured after filtration of the suspension.

3.6.3 Extraction of α-Galactosidase

The extraction of α-galactosidase was done by suspending purified cell wall (1 g wet weight) in 10 mL of 1.5 M NaCl and incubating at room temperature for 2 hr. The enzyme activity in the extract was measured after filtration of the suspension.

3.6.4 Assay of Glycosidase Activity (α-Galactosidase, β-Galactosidase, α-Glucosidase)

Glycosidase activity in extract was assayed by following the release of p-nitrophenol from its glycosides. The reaction mixture contained 0.5 mL of 5 mM substrate (α-galactopyranoside, p-nitrophenyl β-galactopyranoside and p-nitrophenyl α-glucopyranoside, respectively), 0.5 mL of Mcllavaine buffer (citrate phosphate buffer) and 0.5 mL of the enzyme extract. The pH of the reaction was 4.4 for α-glucosidase, β-galactosidase and 5.5 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.

3.6.5 Assay of Acid Phosphatase Activity

Acid phosphatase activity in extract was measured by the method described by Odds and Hierholzer (1973) 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. 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.

3.7 DETERMINATION OF THE EFFECT OF SALT STRESS ON PEROXIDASE ACTIVITY

3.7.1 Extraction of Peroxidase

Extraction of peroxidase was performed according to the method of Sreenivasulu et al. (1999) with some modifications. 10 g seedlings were homogenized in 10 mL of Tris-HCl buffer (50 mM, pH 7.2) at 4°C. The homogenate was centrifuged at 9000xg for 20 min. The pellet was washed with same extraction buffer and centrifuged again. The combined supernatants were assayed for peroxidase activity.

3.7.2 Extraction of Cytoplasmic, Covalently and Ionically Bound Fractions

Various peroxidase fractions were prepared from seven days old seedlings grown under different salt concentrations (0-200 mM NaCl), as per method (Zheng and Huystee, 1992). 15 g seedlings were homogenized in ice-cold McIlavine buffer (10mM citric acid and 20 mM sodium phosphate, pH 5.8). The homogenate was centrifuged at 4000xg for 10 min. The pellet was re-suspended in 10 mL of same buffer and centrifuged. The step was repeated twice. The combined supernatant fraction was referred as the cytoplasmic fraction. The pellet containing cell walls was suspended in 50 mL of ice-cold 3 M lithium chloride buffered in McIlavine buffer and stirred for 24 hr at 4 °C. The suspension was centrifuged at 4000xg for 10 min. The pellet was washed with 20 mL of more buffer. The combine supernatant and washing fluid was referred as ionically bound peroxidase fraction. The remaining pellet was treated with 10 mL of solution containing 0.5% cellulase and incubated for 12 hr with occasional shaking. The covalently bound fraction was collected by filtering the treated pellet and washing. Each of the three fractions were treated with 10% trichloroacetic acid and centrifuged. The pellets were dissolved in 10

mM sodium phosphate buffer, pH 7.4 and dialyzed against the same buffer. The protein concentration and peroxidase activity was determined in each fraction.

3.7.3 Assay of Peroxidase Activity

Peroxidase activity was determined using guaiacol as substrate as per the method (Srinivas et al, 1999) by following the formation of tetraguaiacol by measuring the absorbance at 470 nm, and using an extinction coefficient of 26.6 mM^{-1} cm^{-1} to calculate the amount of tetraguaiacol. 3.0 mL reaction mixture contained 1 mL 10 mM phosphate buffer, pH 7.4, 1 mL 5 mM 2-methoxy phenol (guaiacol), 1 mL of 0.3 % hydrogen peroxide with 50μL of enzyme extract. The reaction was carried out for 2 min. One unit of peroxidase activity represents the amount of enzyme catalysing the oxidation of 1 μmol of guaiacol in 1 min.

3.7.4 Purification of Ionically Bound Peroxidase

Purification of ionically bound peroxidase was done according to method followed by Sreenivasulu et al. (1999). Solid ammonium sulphate was added to the crude enzyme to give final 40–80% saturation. The resulting solution was centrifuged at 9000xg for 30 min and the pellet was dissolved in minimum amount of Tris–HCl buffer (pH 7.2) and dialysed against the same buffer over night with four changes.

3.7.4.1 Gel filtration chromatography

The dialyzed sample was subjected to Sephadex G-75 column (80x1.5 cm) equilibrated and eluted with Tris–HCl buffer (50 mM, pH 7.0). 2 mL fractions were collected, absorbance was read at 280 nm for total protein and total peroxidase activity was monitored for each fraction as described in section 3.7.3. The enzyme containing fractions were pooled, lyophilized and used for electrophoresis.

3.7.4.2 Ion-exchange chromatography

G-75 activity showing fractions (7–15) were pooled, and subjected to ion-exchange chromatography on DEAE-Sepharose (Sigma) column (10x2.5 cm) which was pre-equilibrated with Tris–HCl buffer (50 mM pH 7.2). The sample was eluted with Tris–HCl buffer as flow-through and step-gradient KCl (0.1, 0.5 and 1.0 M) as eluted fractions. All fractions were concentrated individually by lyophilization and used for electrophoresis.

3.7.5 Analysis of Ionically Bound Peroxidase by Native-PAGE and Peroxidase Activity Staining

The purified fraction was resolved on a 10 % Native-PAGE and gel was stained with coomassie brilliant blue and also for peroxidase activity using guaiacol. The peroxidase activity staining was performed by washing the gel with distilled water and then incubated with 5 mM substrate in dark and then 0.1 mL of 0.3% (v/v) H_2O_2 was added and appearance of color was monitored.

3.7.6 Determination of Isoelectric Point of Purified Peroxidase

The pI of purified peroxidase was determined according to the method of Gillikin and Graham (1991) with slight modifications. Analytical vertical isoelectric focusing polyacrylamide gel containing ampholines in the pH range of 3.5 to 10 (Hoeffer, USA) was used in which purified peroxidase was loaded in one lane and H_2O was mock loaded in an adjacent lane. The gel was subjected to electrophoresis for 1.5 hr at 0.125 W/cm^2 at 10°C. The lanes were then separated and the one containing peroxidase was stained with guaiacol to determine the distance which peroxidase had migrated relative to the anode. The mock loaded lane was sliced into 0.25 cm pieces. Gel slices were then kept in

eppendorf tubes with 2 mL of H_2O and incubated at room temperature overnight with shaking. The pH of the solution containing each gel slice was determined and plotted as a function of distance from the anode. By comparing the distance from the anode that the peroxidase had migrated to the pH of the gel at that position, the pI of the enzyme was determined.

3.7.7 Role of Exogenous Proline on Peroxidase Activity under Salinity Stress

Exogenous proline treatment to seedlings grown under salinity stress was followed according to Khedr et al. (2003). Seedlings were grown for seven days as described earlier with Hoagland's medium containing NaCl (0- 200 mM) with and without 5 mM proline. The ionically bound peroxidase was purified from roots and shoots of seedling as described earlier and peroxidase activity was determined both in solution and in gel assay.

3.7.8 Study of Peroxidase Activity using Ferulic Acid as Substrate

Peroxidase activity was also measured using ferulic acid as substrate as per method with slight modifications (Takahama et al., 1992). The oxidation of ferulic acid was measured spectrophotometrically following the absorbance decrease at 310 nm (extinction coefficient = 11.3 mM^{-1} cm^{-1}) in a reaction mixture (total volume 1 mL) containing 40 μM ferulic acid, 90 mM sodium-phosphate buffer, pH 7.2, 10 μL of purified enzyme and 0.5 mM hydrogen peroxide.

3.7.9 Study of Ferulic Acid Polymer Formation by using Purified Ionically Bound Peroxidase

Polymeric products were prepared as per method (Bernards et al., 1999). The product was formed by slow addition (0.8 mL h^{-1}) of H_2O_2 (50 mM, 10 mL, in 10 mM

phosphate buffer, pH 7) to a stirring solution (10 mL, 10 mM phosphate buffer, pH 7) of pure peroxidase and ferulic acid (0.1 mM) at 40°C in water bath. All solutions were bubbled with N_2 gas prior to use. After 24 hr , the reaction mixture was deep red, and the product was precipitated with the addition of a few drops of concentrated HCl, collected by centrifugation (1250xg) for 10 min at RT, and washed with water (twice), collecting the precipitate by centrifugation as above. The final pellet was freeze-dried to yield a dark orange powder, reconstituted in 1 mL of 0.1 M NaOH, loaded onto a 1.5x25 cm Sephadex G25-M column (Pharmacia) pre-equilibrated with 0.1 M NaOH and eluted with 0.1 M NaOH at 1.8 mL min^{-1}. The product formation was monitored by observing the absorbance of eluted fractions at 310 nm.

3.8 STUDY THE EFFECT OF SALT STRESS ON PROTEIN PROFILE

3.8.1 Extraction of Total Protein

The total protein extraction of seven days old control and seedlings grown under different NaCl concentrations (50-200 mM) were performed as per methods with slight modifications (Aarati et al., 2003). Seedlings were washed with double distilled water, cut into small pieces and powdered in liquid nitrogen using mortar pestle. The powder was homogenized in (1:1 w/v) extraction buffer (Tris–HCl buffer (50 mM, pH 8.0), 1 mM PMSF, 5 mM β-mercaptoethanol, 1 mM EDTA) at 4 °C and centrifuged at 7,000xg for 40 min at 4 °C. Supernatant was collected for further experiments.

3.8.2 SDS-PAGE

SDS-PAGE was carried out according to Laemmli (1970) with little modifications.

3.8.2.1 Preparation of reagents

The stock solutions of various gel components were prepared as mentioned below.

1. **Acrylamide solution (30%):** 30% w/v acrylamide solution containing 0.8% w/v N, N-methylene-bis-acrylamide: 29.2 g of acrylamide and 0.8 g of bisacrylamide was dissolved in 70 mL of deionised water. When acrylamide was completely dissolved, the water was added to make a final volume of 100 mL, solution was filtered with a Whatman No.1 paper and stored at 4 °C in a dark bottle.
2. **Resolving buffer (1.5 M Tris-Cl, pH 8.8):** 18.2 g of Tris base was dissolved in 80 mL of water and the pH was adjusted to 8.8 with HCl and water was added to make a final volume of 100 mL and stored at 4 °C.
3. **Stacking buffer (0.5 M Tris-Cl, pH 6.8):** 6.1 g of Tris base was dissolved in 80 mL of water and pH was adjusted to 6.8 with HCl and water was added to make a final volume of 100 mL and stored at 4 °C.
4. **Sodium dodecyl sulphate (SDS, 10% w/v):** 10 g dissolved in 60 mL of water and kept at room temperature overnight without shaking. Then the volume was leveled at 100 mL by distilled water and stored at room temperature.
5. **Catalyst: 10% ammonium per sulphate (APS):** 10 mg APS was dissolved in 100 µL of water (Freshly prepared solution was used).
6. **TEMED (N, N, N, N-tetra methylethylenediamine):** It was used undiluted from the bottle stored at cool, dry and dark place.

7. **Electrode buffer:** (0.025 M Tris, 0.192 M glycine, 0.1%w/v SDS, pH 8.3) 0.3 g Tris base, 1.4 g glycine, 1 mL 10% SDS/100 mL electrode buffer. Electrode buffer was also prepared as stock solution 5X concentration, consisting of 15 g Tris base, 72 g glycine and 5 g SDS/liter. It was stored at room temperature and was diluted to 5 times by adding 4 parts of distilled water before use.

8. **Stock sample buffer (2X)** (0.125 M Tris pH 6.8, 2.5% SDS, 20% glycerol, 0.002% Bromophenol blue, 10% β-mercaptoethanol).

   The sample buffer (2X) was prepared by mixing the stock solutions as per given composition.

Preparation of sample buffer

Ingredient	Volume
Water	3.0 mL
0.5M Tris-Cl, pH 6.8	2.5 mL
10% SDS	2.5 mL
Glycerol	2.0 mL
0.5% Bromophenol blue (w/v)	0.5 mg

Stored at room temperature, SDS-reducing sample buffer was prepared by adding 100 µl of β-mercaptoethanol to each 0.9 mL of stock sample buffer, before use.

3.8.2.2 Casting of gel

10% Denaturing discontinuous gel was prepared by mixing gel stock solutions as per given composition. The monomer solution was prepared for resolving gel by mixing all of the reagents given below except the ammonium per sulfate and TEMED.

Recipe for resolving gel (10%): (10 mL)

Ingredient	Volume
Acrylamide solution 30%	3.3 mL
1.5 M Tris-Cl pH 8.8	2.5 mL
10% SDS	1.0 mL
10% APS	0.1 mL
Water	4.0 mL
TEMED	5.0 µL

Recipe for stacking gel (5%): (5 mL)

Ingredient	Volume
Acrylamide solution 30%	0.83 mL
0.5 M Tris-Cl pH 6.8	0.63 mL
10% SDS	0.05 mL
10% APS	0.05 mL
Water	3.40 mL
TEMED	5.00 µL

The solution was degassed for at least 3-5 min. The APS and TEMED were gently mixed into the degassed monomer solution. The solution was well mixed uniformly and poured gently in between the plates. The resolving gel was cast up to 2/3 height on pre marked plates followed by layering of 200 µL butanol overlaying solution. After 15 min the demarcation occurred between the acrylamide layer and butanol layer, indicated the complete polymerization of gel. Butanol was decanted and the space was washed with distilled water. Similarly 5% stacking gel was also layered on top of the resolving gel. The well was caste in a stacking gel by placing the teflon comb in between and at the top of the two plates after 15-20 min the comb was removed carefully.

3.8.2.3 Sample preparation

Protein concentration 150µg/50µL were mixed with 1 volume of sample treatment buffer (0.125 M Tris pH 6.8, 2.5% SDS, 20% glycerol, 0.002% Bromophenol blue, 10% β-mercaptoethanol) and heated to 95°C to boiling point for 5 min.

3.8.2.4 Electrophoresis

Electrophoretic separation was done by using BIO-RAD Mini-PROTEAN® 3 Cell electrophoresis unit. The prepared samples were loaded into the wells with a protein concentration of 30µg of each sample and electrophoresed at 80 V through stacking gel. Once the sample was concentrated at the interface of the stacking and separating gel as sharp blue line, the voltage was increased to 120 V and the electrophoresis was continued until the tracking dye reached at the bottom of the gel.

3.8.2.5 Staining and destaining of gel

After the run, the gel was removed from plates and put in staining solution (0.1% Coomassie brilliant blue R-250 w/v in 40% methanol, 10% Acetic acid w/w) for 4-6 hr with mild shaking at room temperature. Then, the gel was destained with several changes of destaining solution I (40% methanol, 10% glacial acetic acid) and finally kept in destain II (10% glacial acetic acid).

3.8.2.6 Imaging and densitometeric analysis of gel

The well destained gel was photographed using BIO-RAD gel documentation system and analyzed for the changes in protein profiles. The molecular weight of desired protein bands were determined by using Quantity one software. The quantitative densitometry analysis of desired protein bands was done.

3.8.3 Two-Dimensional Gel Electrophoresis

2-D electrophoresis is a powerful analytical technique, widely used to separate complex mixture of proteins. The resolving power of this technique is much more than that of one dimensional electrophoresis. Commonly, the mixture is first resolved on the basis of net charge (by isoelectric focusing). Subsequently SDS-PAGE was used to separate molecules on the basis of size.

3.8.3.1 Buffers and solutions

1. **Isoelectric focusing sample buffer:** (9.5 M urea ultra pure, 2% Triton X-100, 2% ampholyte, and 5% 2-β mercaptoethanol).

   IEF sample buffer was prepared by mixing ingredients given below.

   Sample buffer for IEF (5 mL)

Ingredient	Volume
Urea	2.7 g
10% Triton X-100	1.0 mL
Ampholyte pH 5-7	0.2 mL
Ampholyte pH 3-10	0.05 mL
2-β mercaptoethanol	0.25 mL

   Made up volume to 5 mL with deionised distilled water and 200 μL aliquots were frozen at -70 °C.

2. **Sample overlay buffer (5.0 mL):** The sample overlay buffer was prepared as per the table given below:

   Sample overlay buffer (5.0 mL)

Ingredient	Volume
Urea	2.4 g
Ampholyte pH 5-7	0.1 mL
Ampholyte pH 3-10	0.025 mL

Dissolved urea well and made up volume to 5.0 mL using deionised water. 200 μL aliquots were frozen at -70 °C.

3. **Equilibration buffer:** (10 % glycerol, 2.3% SDS, 5% 2-β mercaptoethanol, 62 mM Tris-Cl, pH 6.8):

The equilibration buffer was prepared as per the table given below:

Preparation of equilibration buffer

Ingredient	Volume
4X Tris-Cl pH 6.8	12.5 mL
10% SDS	20.0 mL
Glycerol	10.0 mL
2-β mercaptoethanol	5.00 mL
Bromophenol blue	50 mg

Made up volume to 100 mL using deionised distilled water. 10 mL aliquots were made and frozen at -70 °C.

4. **Agar solution (1%) in equilibration buffer without bromophenol blue:**

1 g of agar dissolved in 100 mL of equilibration buffer. 4 mL aliquots were made and frozen at -20 °C.

5. **NaOH cathode solution (0.02 M NaOH):**

Boiled for about 5 min, degassed and allowed to cool.

6. **Phosphoric acid anode solution (10 mM phosphoric acid):**

0.5 mL of phosphoric acid in 730 mL of deionised distilled water.

7. **Stock acrylamide (for IEF):**

Acrylamide - 28.4 g

Bisacrylamide - 1.62 g

Dissolved in 100 mL and filtered.

3.8.3.2 Sample Preparation

The homogenates of control and salt stress grown seedlings were first lyophilized and then reconstituted in appropriate volume of IEF sample buffer to give protein concentration approximately in the range of at 10 mg/mL. Usually 40 µL (150 µg) of sample was used for each 2-D gel.

3.8.3.3 First Dimension

Isoelectric focusing gels were made in glass tubes (150X 3.5 mm inside diameter) sealed at bottom with parafilm. Since the length of the gel tube and the length of the gel itself affect the reproducibility, care was taken to carry out all 2-D analysis using identical set of conditions. To make 10 mL gel mixture, 5.5 g of urea, 1.33 mL of acrylamide (30 % stock), 2 mL of Triton X-100, 1.97 mL of H_2O, 0.4 mL of ampholine pH range 5-7, 0.1 mL pH 3-10 (to make 2% ampholine) were mixed in a 100 mL side arm flask. 10 µL of 10% ammonium persulphate and 7 µL of TEMED were used to polymerize the acrylamide solution. Immediately after addition it was loaded into gel tubes. To avoid bubble trapping Pasteur pipette was used to fill the tubes with gel mixture till the marked position. The gels were overlaid with small amount of water and after 1-2 hr this overlaid water was removed and replaced with 20µL of IEF sample buffer and small amount of water till further use. IEF was carried out using BRL (USA) tube gel apparatus. The tubes and upper chamber were filled with 0.02 M NaOH and the lower chamber with 0.01 M H_3PO_4, respectively. The gels were then electrophoresed without samples according to (i) 200 V for 15 min (ii) 300 V for 30 min and (iii) 400 V for 30 min. After pre-electrophoresis, the samples were layered on the top of IEF gels and

further electrophoresed first at 350 V for 14 hr and then at 800 V for 1 hr, a total of 4800 V-h.

After electrophoresis was over gels were take out carefully from the tubes and were either equilibrated and immediately loaded on the second dimension gel or loaded on a second dimension gel without any treatments. The equilibration of IEF gels were carried out by incubating with 5 mL of 1X SDS sample buffer at room temperature for 2 hr. Equilibrated gels were stored at -20 °C till further use.

3.8.3.4 Second Dimension

The second dimension gels were 10% discontinuous SDS-PAGE as described previously in section (3.8.2). After polymerization of stacking gel, IEF gel was placed carefully on the top of stacking gel. The gel was kept in place by using agar overlay buffer. Electrophoresis was started after allowing 5-10 min for solidification of agar solution.

3.8.3.5 Gel staining and destaining

The gels were stained and destained as described previously in section (3.8.2.5).

3.8.3.6 Measurement of pH gradient

The IEF gels, without any sample were run along with gels containing samples. Five mm sections were cut and placed in vials containing 10 mL of degassed water. After 45 min the pH of water in each vial was determined.

3.9 GENERATION OF POLYCLONAL ANTIBODIES

3.9.1 Immunization

Two female rabbits (New Zealand White, 2-2.5 kg) were obtained from Experimental Animal Facility, All India Institute of Medical Sciences (A.I.I.M.S.), New

Delhi. They were used for the generation of polyclonal antibodies. Animals were pre-bled one week before immunization. An emulsion was prepared by mixing 2 mL of complete Freund's adjuvant (CFA) with equal volume of PBS containing 500µg antigen protein in a hypodermic syringe. The animals were immunized with this emulsion at multiple locations intradermally. On day 14, the first booster dose was administered with the same antigen emulsified with incomplete Freund's adjuvant (IFA) in a similar fashion. The second booster dose was given on day 28. Four days later the animals were bled through the marginal ear vein. After bleeding, blood was allowed to clot for 60 min at room temperature. Serum from the blood was separated and stored in aliquots of 100 µL at –80°C.

3.9.2 Antibody Titer Determination using Enzyme Linked Immuno Sorbant Assay (ELISA)

Reagents

1. Blocking buffer: 3% BSA in PBS.
2. Pre serum: Serum collected from the rabbit before immunization.
3. Primary antibody: Anti serum collected from the rabbit after immunization.
4. Secondary antibody: Commercially available tagged with horseradish peroxidase enzyme (Goat anti rabbit IgG-HRP).
5. Phosphate citrate buffer pH 5.0.
6. 0.1 M Citric acid $C_6H_8O_7.H_2O$
7. 0.2 M Sodium dihydrogen phosphate.

8. Substrate: Orthophenylenediamine (OPD) 4 mg; Citrate buffer 0.1 M -4.8 mL, Phosphate buffer 0.2 M - 5.15 mL; Hydrogen peroxide (30%) – 4 µL. The substrate was prepared fresh and H_2O_2 was added just before the use.
9. Stopping reagent: 4N H_2SO_4, H_2SO_4- 4 mL: Distilled water 32 mL.

Procedure

The titer of the antiserum was determined in a simple ELISA protocol. Micro titer plates were coated with 50µL of immunizing antigen containing 5µg of protein in each well and incubated overnight at 4°C. Next day, the liquid content of the wells was emptied by hand jerk. All the wells were washed three times by adding 100µL washing buffer (TBS). After washing, 100µL blocking buffer (3% BSA in PBS) was added in each well and kept for 90 min at room temperature. Finally the contents were discarded followed by washing with TBS (thrice) as was done earlier.

The serial dilutions of pre-immune and anti-serum were done with 1% BSA in TBS. The wells were filled with 50µL of different serial dilutions of pre-immune serum and anti-serum in duplicate and incubated for 90 min at room temperature. After incubation, the wells were drained and washed thrice with TBS. 50µl of secondary antibody (Goat anti-rabbit IgG-HRP conjugate, 1:1000 dilution) were added to each well and incubated at 37°C for 90 min. followed by washing with TBS. Next, 10 mL of ortho phenylene diamine reagent (4 mg OPD was added in 4.8 mL of 0.1M citrate buffer and 5.15 mL of 0.2M phosphate buffer (pH 5), 4µL of H_2O_2 was added just before the use) was added and incubated at room temperature in dark for 20 min. The reaction was stopped by the addition of 50µL of 4N H_2SO_4 and the absorbance was recorded with in 10 min at 492 nm in an ELISA reader (Metertech Inc, model Σ960).

3.10 EXPRESSION OF SALT STRESS RESPONSIVE PROTEIN UNDER DIFFERENT ABIOTIC STRESS AND IN DIFFERENT CULTIVARS OF GROUNDNUT

3.10.1 Abiotic Stress Treatment

To study the effect of other abiotic stresses on NaCl induced protein, the seedlings of Kaushal cultivar of groundnut were grown hydroponically on nutrient medium as described in section 3.1 supplemented with different chemicals viz. PEG (20%), mannitol (300 mM), NaCl (150 mM), KCl (150 mM) and ABA (20 μM) separately.

To check the expression of salt stressed protein in different cultivars obtained from National Research Centre for Groundnut, Junagarh viz. TG-64, TMV-7, ICGS-37, Jawan, KRG-1, Kaushal, TG-1, Tirupati-1, Kadri-4 and Tirupati-4 were grown under 150 mM NaCl stress conditions as described in section 3.1. The expressions of two proteins 31.6 and 48 kDa were studied by Western blotting.

3.10.2 Expression of Protein by Western Blotting

Western blotting was performed as per the method of (Towbin et al., 1979) with slight modifications. It comprises a series of steps involving:

1. Resolution of a complex protein sample in a polyacrylamide gel by SDS-PAGE.
2. Transfer of the resolved proteins to a membrane called electrotransfer.
3. Identification of a specific protein on the membrane by antibody binding called immunoblotting.

3.10.2.1 Electrophoretic separation and transfer of protein bands

The protein sample was resolved on 10% SDS-PAGE as explained earlier. Unstained gel was used for electrophoretic transfer of protein bands to Polyvinylidene Difluoride (PVDF) membrane using wet blotting system in Mini Trans-Blot® Bio-Rad.

Reagents

1. Polyacrylamide gel containing the resolved proteins
2. Immobilon PVDF transfer membrane, cut to the same dimensions as the gel (including notched corner for orientation purposes)
3. Two sheets of Whatman® 3MM filter paper or equivalent, cut to the same dimensions as the gel
4. Scotch Brite® pads
5. Tank transfer system large enough to accommodate gel
6. Methanol, 100%
7. Milli-Q® water
8. Tris/glycine transfer buffer: 25 mM Tris base, 192 mM glycine, 10% (v/v) methanol, pH 8.3).
9. Powder free gloves, forceps, glass plates, glass rods etc.

Assembly of transfer cassette

1. The gel from its glass cassette was removed and stacking gel trimmed away.
2. The gel was immersed in transfer buffer for 15 to 30 min.
3. The filter papers and scotch brite pads were soaked in transfer buffer for at least 30 seconds.
4. The membrane was wet in methanol for 15 seconds.
5. Membrane should uniformly change from opaque to semi-transparent.
6. The membrane was carefully placed in Milli-Q water and soaked for 2 minutes with the help of forceps.

7. The membrane was carefully placed in transfer buffer and let equilibrate for at least 5 min.
8. The cassette holder was opened and a foam (fiber) pad placed on one side of the cassette.
9. One sheet of filter paper was placed on top of the pad.
10. On top of the filter paper the gel was placed.
11. On top of the gel the membrane was placed.
12. A second sheet of filter paper on top of the stack was placed.
13. On top of the filter paper second foam pad was placed.
14. To ensure an even transfer, any air bubbles were removed between layers by carefully rolling a pipette or a stirring rod over the surface of each layer in the stack.
15. The cassette holder was closed.

Protein Transfer

1. The cassette holder was placed in the transfer tank so that the gel side of the cassette holder is facing the cathode (–) and the membrane side is facing the anode (+).
2. Adequate amount of buffer was added to the tank to cover the cassette holder. The anode lead and cathode lead were connected to their corresponding power outputs. The system was turned on for 1 to 2 hr at 6 to 8 V/cm inter-electrode distance and the tank manufacturer's guidelines were followed (Mini Trans-Blot® Electrophoretic Transfer Cell, Bio-Rad)

3. After the transfer was complete, the cassette holder from the tank was removed and using forceps, the transfer stack was carefully disassembled.

3.10.2.2 Immunodetection

After performing electrotransfer of proteins to PVDF, the membrane was incubated with antibody specific to the protein of interest for detection of transfer.

Reagents

1. Primary antibody (specific for protein of interest).
2. Secondary antibody (specific for primary antibody), labeled with horseradish peroxidase.
3. DAB (3, 3′ diaminobenzidine).
4. 50 mM Tris base, pH 7.6.
5. 30% H_2O_2
6. Washing buffer: Phosphate buffered saline (PBS), 10 mM sodium phosphate, pH 7.2, 0.9% (w/v) NaCl.
7. Blocking solution: 3% (w/v) gelatin in PBS, 0.05% Tween-20.
8. Methanol, 100%
9. Milli-Q water.
10. Substrate: DAB/H_2O_2: (A) 5mg DAB dissolved in 10 mL of 50 mM of Tris base, pH 7.6; (B) 30mL water + 20 μL H_2O_2 (30%).

Procedure

After electro blotting, membrane was carefully placed in a blocking buffer (3% gelatin in PBS) at 37°C for 2 hr to block the non-specific binding sites. The membrane was washed thrice with washing buffer (PBS) and transferred into primary

antibody solution (1:500 dilution in 0.5% BSA in PBS) for 1 hr at 37°C. Again, the membrane was washed thoroughly with washing buffer (PBS) and incubated with goat anti-rabbit IgG-HRP conjugate (1:5000 dilution) for 1 hr at 37°C. The membrane was washed thrice with washing buffer and the blot was developed with developing solution, (A) 5 mg DAB dissolved in 10 mL of 50 mM of Tris base, pH 7.6 (B) 30 mL water + 20μl of 30% H_2O_2; Developing solution was prepared by mixing equal volume of A and B, and immunoblot was developed in dark.

3.11 IDENTIFICATION & CHARACTERIZATION OF 31.6 kDa SALT STRESS INDUCED PROTEIN

3.11.1 Protein Identification by MALDI-TOF Peptide Mass Fingerprinting

Peptide Mass Fingerprinting (PMF) is a technique for rapid identification of proteins. A pure protein (from a band in gel) is digested using a proteolytic enzyme (commonly trypsin is used) to cleave the protein into constituent peptides. The peptide masses are then measured very accurately by mass spectrometry and searched against a theoretical digest of all protein maintained in a database. Statistical scoring algorithms match the measured set of unique peptides against the theoretical set of unique peptides, and identification is achieved.

3.11.1.1 In-gel digestion of protein band

Washing and dehydration

1. Protein band of interest was excised from a stained polyacrylamide gel and cut into small particles (~1 mm^2) using a scalpel, and placed into a 0.5 mL siliconized tube (VWR *SuperSlik* microcentrifuge tubes). Also a gel piece from a protein free

region of the gel was cut out and diced, for a parallel control digestion to identify trypsin autoproteolysis products.

2. 100 μL of 25 mM ammonium bicarbonate (NH_4HCO_3; 1.98 mg/mL)/50% (v/v) acetonitrile added and vortexed for 35-40 min on a low setting (more like shaking) and pale blue solution (Coomassie staining) was removed from tube. This washing step was repeated 3-4 times.

3. Once all Coomassie has been removed, gel pieces were dehydrated with acetonitrile (100 μL). At this point the gel pieces should shrink and become an opaque-white color. If they did not, the acetonitrile was removed and replaced with fresh one.

4. Acetonitrile was removed and SpeedVac for 3-5 min.

Reduction and alkylation of cysteine residues

1. 30 μL of the 10 mM dithiothreitol (DTT, 1.54 mg/mL) in 25 mM NH_4HCO_3 solution to cover the gel pieces added, and reduced for 30-45 min at room temperature.

2. The DTT solution was replaced with 55 mM iodoacetamide (10.2 mg/mL) in 25 mM NH_4HCO_3 pH 8.0 (30 μL) and incubated for 45 min at room temperature in the dark.

3. The iodoacetamide solution was removed and gel pieces were washed with 100 μL of 25 mM NH_4HCO_3 pH 8.0, for 10 min while vortexing.

4. Washing solution was removed and dehydrated with 100 μL acetonitrile as in step 3 of washing.

5. The acetonitrile was removed and the gel pieces were dried in a vacuum centrifuge for 3-5 min.

Digestion of protein sample

1. The gel particles were rehydrated in 25 μL 0.1 mg/mL trypsin (sequence grade; Promega, Pierce) in 25 mM NH_4HCO_3 pH 8.0 solution and placed on ice for 10-15 min.
2. Excess trypsin solution was removed and overlay the rehydrated gel particles with 30 μL of 25 mM NH_4HCO_3 to keep them immersed throughout digestion and incubated for 12 to 16 hr at 37°C.

3.11.1.2 Recovery of digested peptides using a Zip-Tip

1. 5 μL of 5% aqueous TFA was added to stop the digestion.
2. The tubes containing gel pieces were shake for about 10 min and centrifuged briefly to bring the liquid to the bottom of the tube.
3. The saturated solution of HCCA (α-cyano-4-hydroxycinnamic acid, matrix) in 1:1 acetonitrile:acidified water (0.1% TFA) was prepared and this matrix solution was diluted by a factor of 2 and placed 3 μL in as many tubes as needed (one for each digestion).
4. 10 μL of 1:1 acetonitrile: 0.1% TFA through the Zip-Tip was aspirated, dispensed to waste and repeated twice with fresh solution.
5. 10 μL of 0.1% TFA was aspirated and dispensed to waste. This step was repeated twice with fresh 0.1% TFA.

6. Peptides were bind by performing 3-10 cycles of aspirating and dispensing the digest solution through the Zip-Tip. The more dilute the solution, the more cycles may be required.
7. Wash the Zip-Tip by aspirating with 10 μL of 0.1% TFA and dispensing to waste. Then it was replaced once with fresh solution.
8. 3 μL peptides were eluted using 3 cycles of aspirating and dispensing the matrix solutions prepared in step 3 through the Zip-Tip. On the fourth aspiration, 1 μL dispensed directly to the MALDI plate and allowed to dry.

3.11.1.3 Mass spectrometry and database searching

Mass fingerprints of tryptic digests were obtained by MALDI-TOF mass spectrometry on Bruker Ultraflex MALDI-TOF mass spectrometer at T.C.G.A, New Delhi. Set of peptides obtained is matched with database using search engine MASCOT and the results were anylazed.

3.12 STUDY OF SORBITOL ACCUMULATION IN SEEDLINGS GROWN UNDER SALINITY STRESS USING GAS CHROMATOGRAPHY

The accumulation of sorbitol in groundnut seedlings was studied according to the method of Tao et al. (1995) with slight modifications using gas chromatography. Tissue weighing 1 g was made to powdered in liquid N_2 and homogenized in 10 mL of 80% (w/v) methanol containing 0.5 mg mL^{-1} xylitol as an internal standard. The extract was centrifuged at 20,000xg for 10 min, and 1 mL of supernatant was collected and dried using stream of air. The resulting pellet was resuspended in 1 mL of water. Xylitol was acetylated by adding 40 μL of *N*- methylimidazole and 200 μL of acetic anhydride. After 15 min the reaction was stopped by adding 1.5 mL of water. The acetylated sample was

partitioned into 1.5 mL of dichloromethane and dried, then sample was dissolved in 100 μL of acetone and analyzed using a Perkin-Elmer 8320 gas chromatograph fitted with a DB-23 (J & W Scientific) capillary column (30 m x 0.25 mm, J & W Scientific) using H_2 as carrier gas and with the oven isothermal at 210°C. Integration of the flame-ionization detector signal was provided by Perkin-Elmer-Nelson Model 1020 data system.

3.13 EFFECT OF NaCl STRESS ON NADP-DEPENDENT SORBITOL-6-PHOSPHATE DEHYDROGENASE (S6PDH) ACTIVITY IN GROUNDNUT SEEDLINGS

3.13.1 Enzyme Extraction

To study the effect of NaCl stress on S6PDH activity, seedlings were grown under different NaCl concentrations as described previously (section 3.1). Seven days old seedlings were selected and the enzyme extraction was performed as per method described (Tao et al., 1995) with slight modifications. 5 g of tissue was homogenized in 5 mL of extraction buffer (0.1 M Tris-HCl pH 8.0, 10 mM 2-β-mercaptoethanol and 0.2 g polyvinlypolypyrollidone). The homogenate was centrifuged at 15,000xg for 20 min, the supernatant was collected and enzyme activity of crude preparation was assayed.

3.13.2 NADP-Dependent Sorbitol-6-Phosphate Dehydrogenase (S6PDH) Assay

The enzyme assay was performed as per method (Tao et al., 1995). The enzyme assay mixture contained in a total volume of 550 μL, 45 mM Tris-HCl (pH 9.3) with 0.91 mM $NADP^+$, 18 mM of sorbitol-6-phosphate and 100 μL of enzyme extract. Enzyme activity was assayed in a recording spectrophotometer by measuring increase in absorbance at 340 nm as $NADP^+$ was reduced to NADPH.

An enzyme unit of NADP-dependent sorbitol-6-phosphate dehydrogenase is defined as 1 μmol $NADP^+$ reduced per minute.

3.14 PARTIAL PURIFICATION OF S6PDH AND ANALYSIS ON NATIVE-, SDS-PAGE AND WESTERN BLOTTING

3.14.1 Partial Purification by Ammonium Sulphate Precipitation

The NADP-dependent sorbitol-6-phosphate dehydrogenase crude preparation was partially purified by Ammonium Sulphate precipitation method. Ammonium sulphate powder was gradually added to crude enzyme to achieve the final concentration of 20%, 40%, 60% and 80% saturation. Pellet obtained at each step was dialyzed against PBS (pH 7.0) at 4 °C with several changes overnight. The enzyme activity of each dialysate assayed as per method described earlier (section 3.13.2). The fractions with significant enzyme activities were stored at -80 °C for further studies.

3.14.2 Native-, SDS-PAGE and Western Blotting

The crude and partially purified enzymes samples were separated on 10% SDS and Native gels. The banding patterns were visualized by coomassie brilliant blue staining. The Western blot of Native and SDS-PAGE gels were performed using antibody raised against 31.6 kDa salt stress induced protein as per methods described earlier (section 3.10.2).

3.15 IMMUNOLOCALIZATION OF 31.6 kDa PROTEIN IN ROOT AND SHOOT SECTIONS

The localization of salt stress induced protein in the transverse sections of root and shoot of groundnut seedling was performed according to Neri et al. (1995) using Fluorescein Isothiocyanate (FITC) labeled secondary antibody. Briefly, ultra thin transverse sections of root and shoot were prepared as described previously in section 3.4. After passing through the rehydration series sections were washed with PBS several times and incubated with primary antibodies (diluted 1:100) in PBS containing 2% BSA

for 3 hr at 37 °C. Sections were washed thrice again with PBS and reacted with secondary antibody (diluted 1:50) in PBS, 2% BSA for 1 hr at 37 °C. Samples were subsequently washed 3 times with PBS and mounted in glycerol containing 1, 4-diazabicyclo-2, 2, 2-benzoctane (antifading agent) and observed under confocal microscope (Carlzeiss, U.S.A.).

3.16 STATISTICAL ANALYSIS

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

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

Where, X = individual observations

n = number of observations

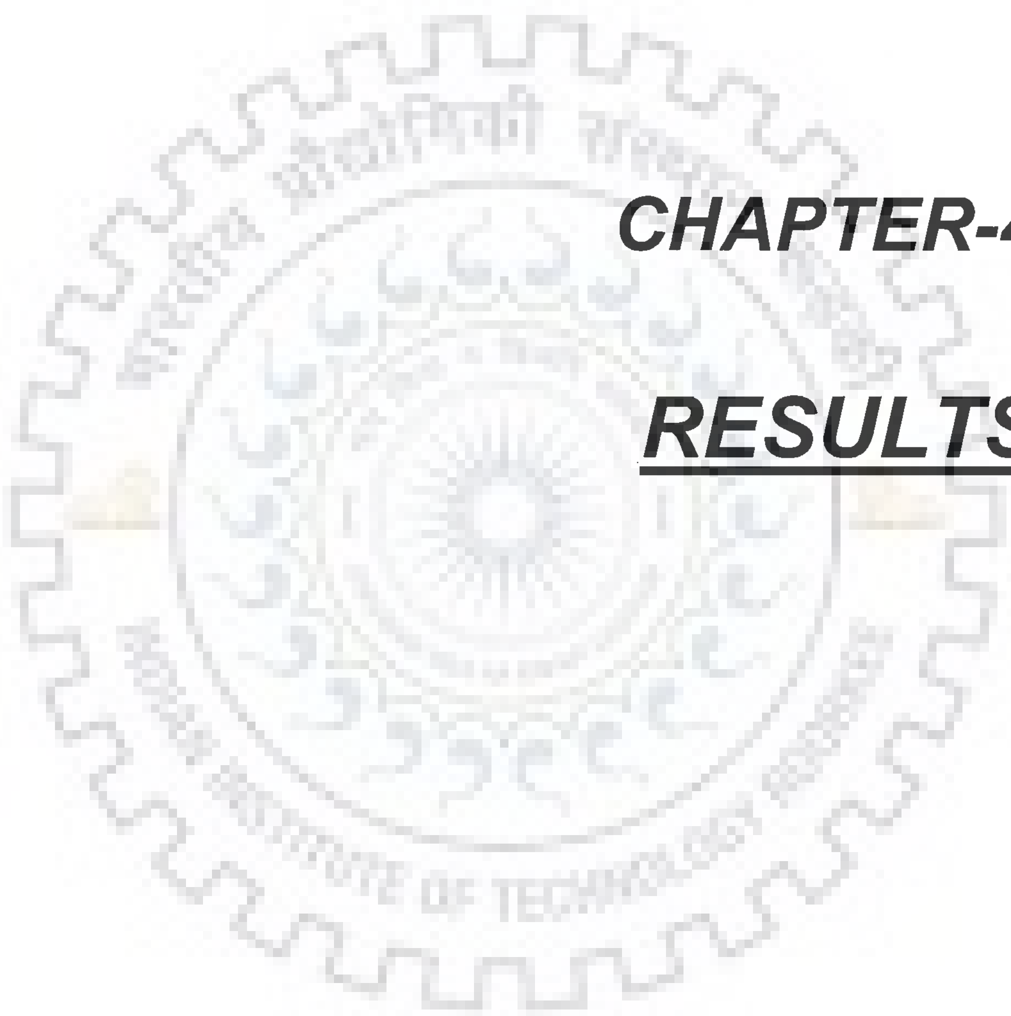
Students 't' test was used to calculate the degree of significance by the formula given below:

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

Where,

$$S = \sqrt{\frac{\Sigma X_1^2 - \frac{(\Sigma X_1)^2}{n_1} + \Sigma X_2^2 - \frac{(\Sigma X_2)^2}{n_2}}{n_1 + n_2 - 2}}$$

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



CHAPTER-4

RESULTS

CHAPTER - 4

RESULTS

4.1 EFFECTS OF SALT STRESS ON GROWTH PARAMETERS

4.1.1 Effect on Seedling, Root and Shoot Length

The salt stress found to have adverse effect on overall seedling growth. There was a reduction in the total length of groundnut seedling with increasing concentration of NaCl in growth medium from 50 to 200 mM as shown in Fig. 1A. The length of seedling grown in medium without NaCl (i.e. control seedling) was observed as 42.41 cm. The percent reduction in growth of seedling was 5.75%, 26.80%, 60.83% and 84.43% at 50, 100, 150 and 200 mM NaCl stress, respectively.

Similarly, salt stress caused gradual decrease in the length of shoot and root was as shown in Fig. 1B and 1C. The length of shoots of seedling grown with 0, 50, 100, 150 and 200 mM of NaCl were found to be 23.6, 22.0, 17.44, 7.83, and 2.15 cm stress, respectively. The length of roots of seedling was 18.81, 17.91, 13.60, 8.77 and 4.55 cm at 0, 50 mM, 100 mM, 150 mM and 200 mM of NaCl stress respectively. It has been observed that there was inhibition of shoot and root of seedlings at higher NaCl concentrations. The inhibitory effect was seen more in shoot as compared to root. The percent inhibition of length was 6.77%, 26.10%, 66.82% and 90.88% in case of shoot while 4.78%, 27.69%, 53.37% and 76.34 % in root (Fig. 2A & B). The percent increase of root length and shoot length ratio (L_r/L_s) had shown an increase inhibition of shoot length at higher NaCl concentrations. At 200 mM concentration, it was found maximum with the value of 161.08 % (Fig. 2C). This showed the inhibitory effect of NaCl stress on seedling growth.

4.1.2 Effect on Seedling Fresh Weight

The fresh weight is also an important parameter in determining the growth of a plant. Fresh weight of seedlings underwent a gradual loss under salinity. It was found that seedlings grown under controlled conditions without NaCl in growth medium had higher fresh weight. The fresh weight of shoots was found 3.79, 3.77, 2.28, 1.24 and 0.65 g per seedling grown at 0, 50, 100, 150 and 200 mM NaCl, respectively. Similar trend was found in case of roots of seedling grown at different concentrations of NaCl. It was 1.39, 1.37, 1.27, 0.69 and 0.26 g per seedling (Fig. 3A & B). It was observed that the fresh weights of shoot as well as root decreased drastically at 150 mM and 200 mM of NaCl concentration, which showed its inhibitory role on plant growth.

4.1.3 Anatomical Changes

Since salt stress has been reported to cause significant changes in anatomy of various plant organs such as root, shoot and leaves as these changes are associated to cellular response to salinity stress. Therefore, the effect of NaCl stress on root and shoot of groundnut seedling was studied. Since the growth of groundnut seedling affected most at 150 mM NaCl, therefore, the effect of NaCl stress on the anatomy of root and shoot was studied at this concentration. Some changes were found in both shoot and root transverse sections of seedlings. The size of cortical cells reduced in NaCl stress seedlings as compared to the control seedlings. The cell wall thickening, loss of intercellular spaces and change in shape of the cells were also observed at stress conditions as indicated by arrows (Fig. 4A & B; Fig. 5A & B).

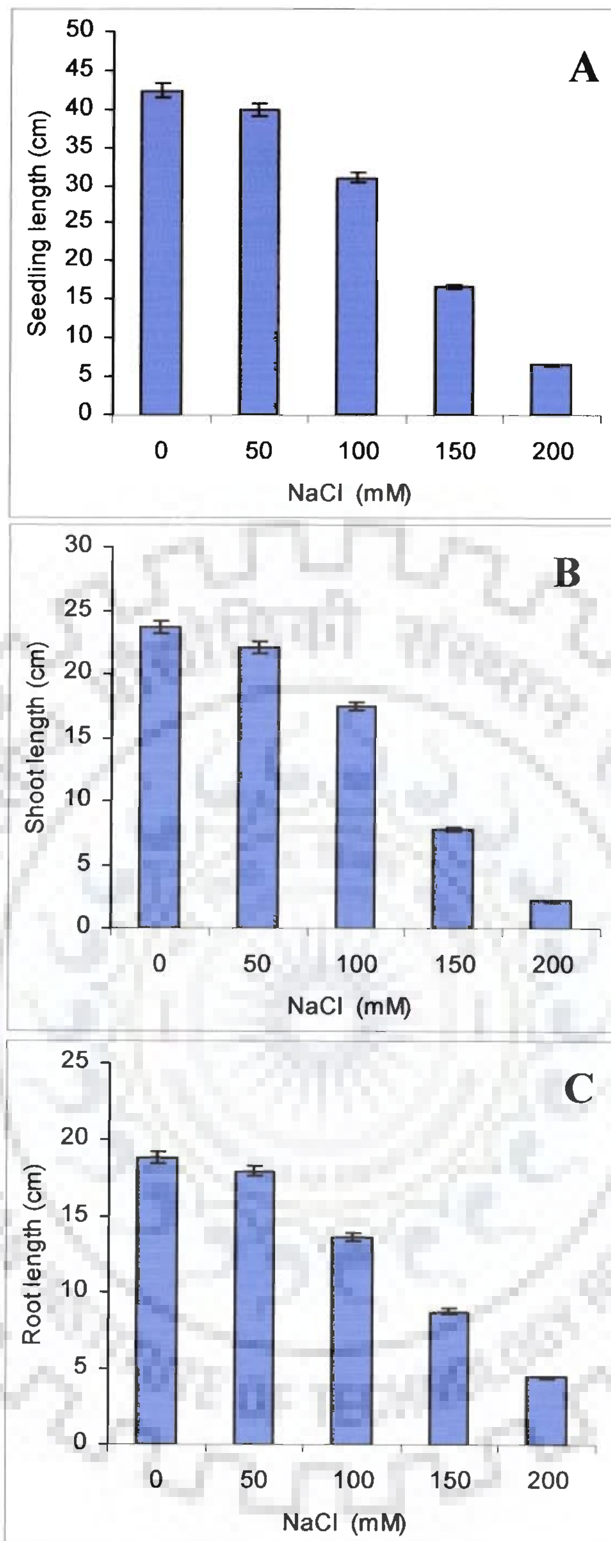


Fig. 1. Effects of salt stress on length of groundnut seedling grown at different level (0-200 mM) of NaCl concentration for seven days. (**A**) Total seedling length (**B**) Shoot length (**C**) Root length. Values are in triplicate ± S.E.

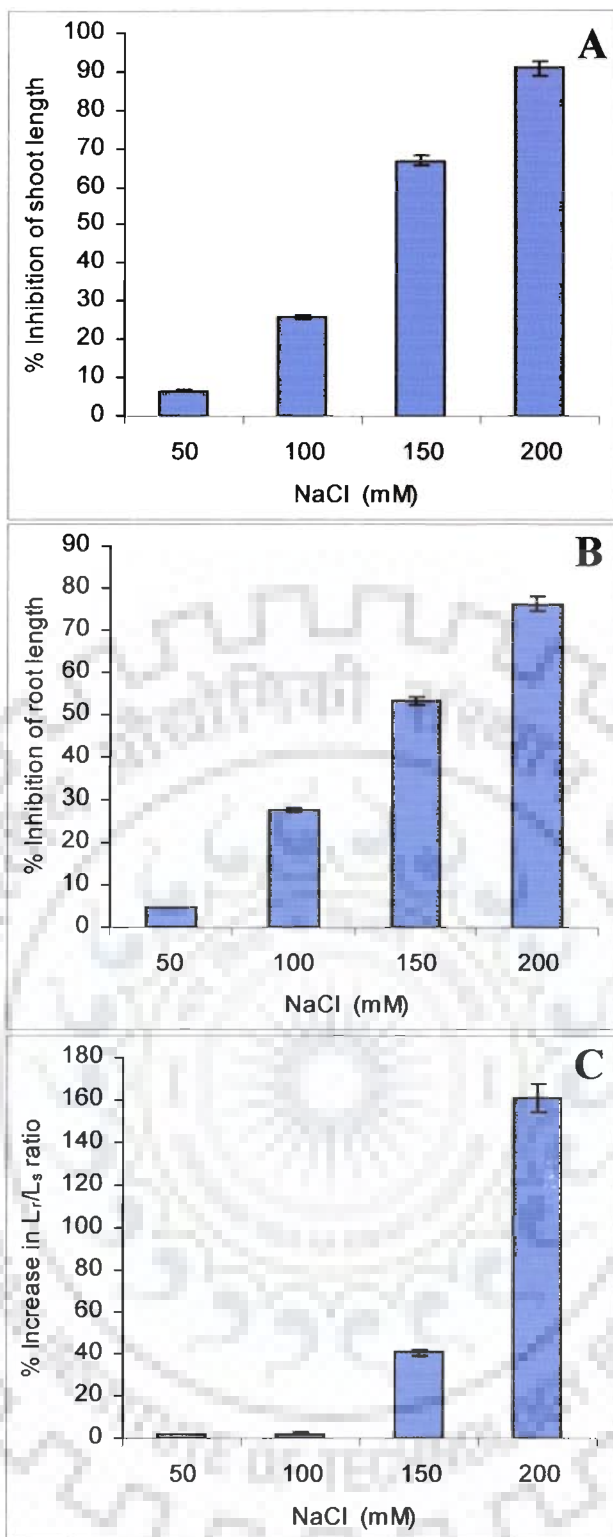


Fig. 2. Percentage inhibition of shoot and root of groundnut seedling grown under salt stress. (**A**) Shoot length (**B**) Root length (**C**) L_r/L_s ratio. Values are in triplicate ± S.E.

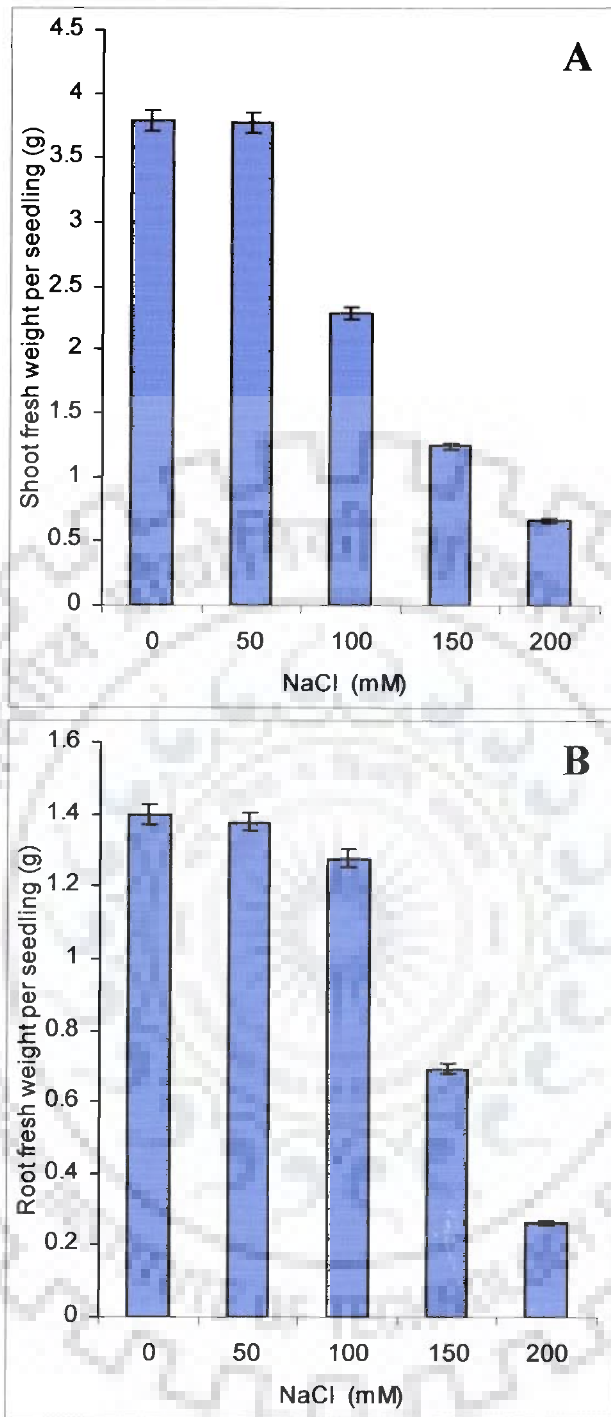


Fig. 3. Effect of salt stress on root and shoot fresh weight of groundnut seedlings grown for seven days. Values are in triplicate ± S.E.

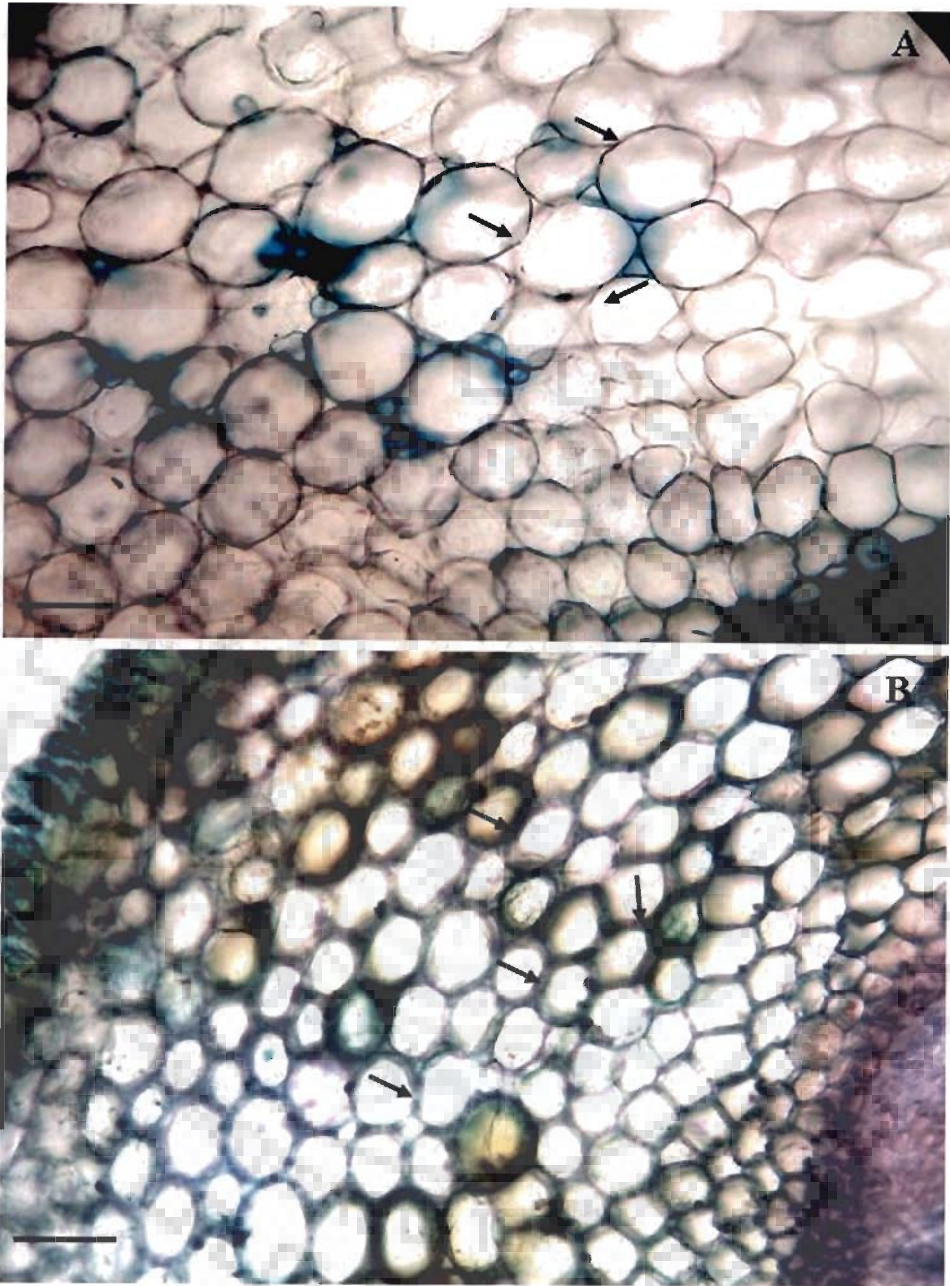


Fig. 4. Transverse section of root of groundnut seedling grown under control and salt stress condition for seven days. (**A**) Seedling grown in absence of salt (**B**) Seedling grown with 150 mM NaCl. The changes in the intracellular spaces, cell shapes and cell wall thickness are indicated with arrows. Bar (—) 50 μm

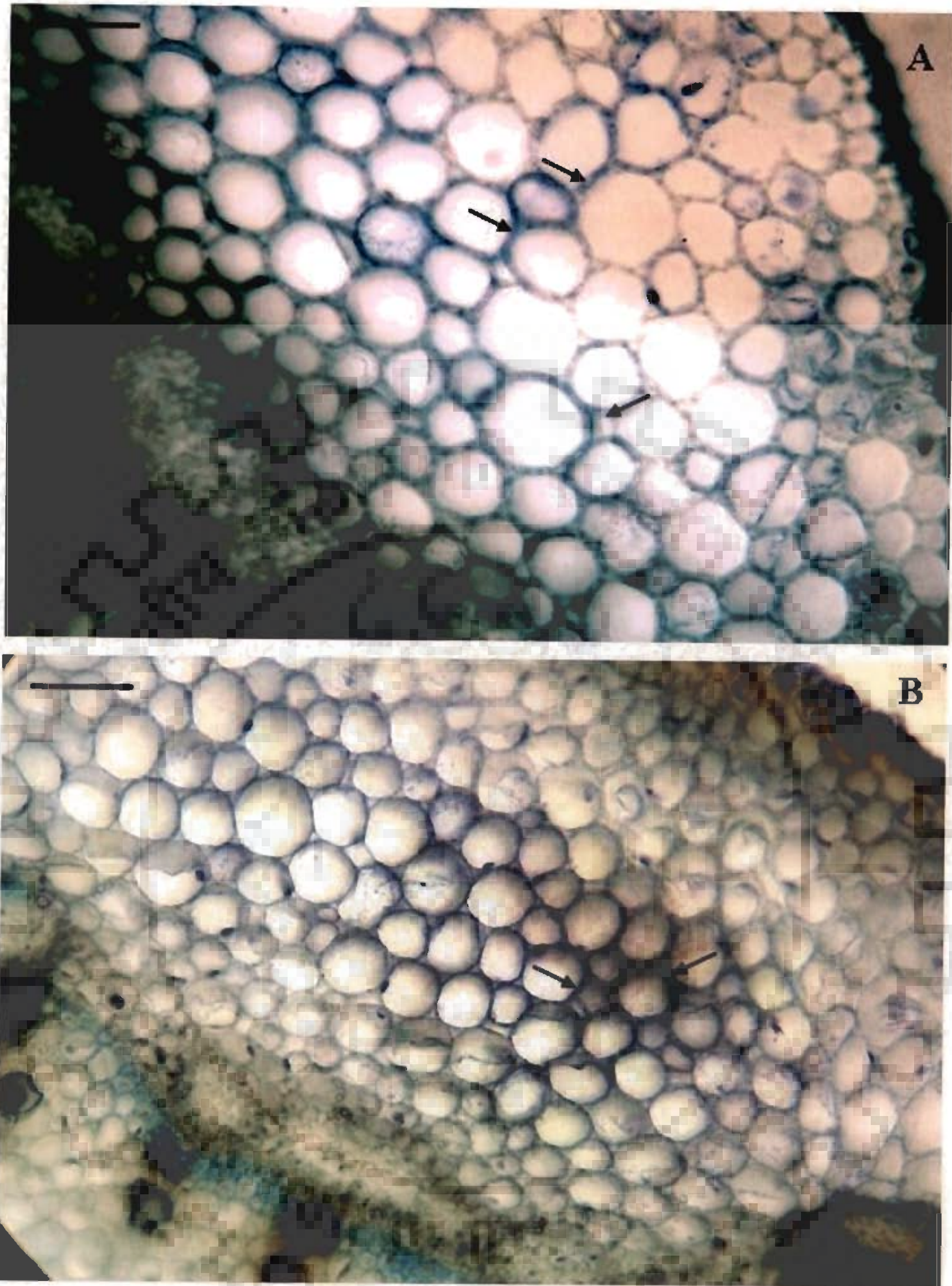


Fig. 5. Transverse section of shoot of groundnut seedling grown under control and salt stress condition for seven days. **(A)** Seedling grown in absence of salt **(B)** Seedling grown with 150 mM NaCl. The changes in the intracellular spaces, cell shapes and cell wall thickness are indicated with arrows. Bar (—) 50 μm

4.2 EFFECT OF SALT STRESS ON PROLINE ACCUMULATION

It has been generally suggested that there is positive co-relation between proline accumulation and salt tolerance. Proline accumulation in shoots and roots of groundnut seedlings grown with different salt concentrations (0-200 mM NaCl) was monitored (Fig. 6A and B). As compared to normal seedling there was an increase in proline accumulation with increase in salt stress. There was approximately eight and six fold increase in proline accumulation in shoots and roots, respectively. The increased accumulation of proline may be involved to overcome salt stress.

4.3 EFFECT OF SALT STRESS ON CELL WALL BOUND HYDROLASES (β-GALACTOSIDASE, α-GALACTOSIDASE, α-GLUCOSIDASE AND ACID PHOSPHATASE)

Cell wall bound hydrolases such as α-glucosidase, α- and β-galactosidase and acid phosphatase play a significant role in maintenance of cell wall rigidity and extensibility directly or indirectly. The effect of NaCl stress on cell wall bound preparations of these enzymes is shown in Fig. 6C. There was a decrease in the α-glucosidase, α and β-galactosidase and acid phosphatase activities under salt stress. Since the cell wall plays significant role in growth and development of cell. The observed changes in cell wall hydrolases activities may account for the change in extensibility of cell wall and ultimately growth.

4.4 EFFECT OF SALT STRESS ON PEROXIDASE ACTIVITY

It is well known that peroxidase plays a significant role in ligno-suberization of cell walls in plant under salt stress. Therefore, effect of salt stress on peroxidase activity was studied and found to be increased with increasing salt concentration in growth

medium. There was a progressive increment in peroxidase activity in seedlings grown at 50 to 150 mM NaCl concentration but at 200 mM NaCl the activity reduced down (Fig. 7A).

There found to be various peroxidase isoforms which are located in different subcellular regions. In an attempt to see which isoform is mainly affected by salt stress, peroxidase activities in various fractions such as cytoplasmic, ionically bound and covalently bound fractions of control and 150 mM NaCl grown seedlings were determined. Although, every fraction showed some increase in activity but a significant increment was observed in ionically bound peroxidase compared to other fractions. There was approximately three fold rise in the enzyme activity of ionically bound peroxidase in the seedlings grown at 150 mM NaCl concentration compared to the control seedlings (Fig. 7B).

4.4.1 Purification of Ionically Bound Peroxidase

Since the major change was observed in ionically bound peroxidase activity, it was purified by gel filtration chromatography using sephadex G-75 column and eluted fractions with peroxidase activity (fraction no. 7 to 15) were collected. The result of purification is presented in Fig. 8A. Two peaks were observed but a single peak of highest peroxidase activity was observed on elution of column with Tris-HCl buffer. Again, these fractions were pooled and subjected to further purification by step gradient DEAE-Sepharose column (Fig. 8B). A single major peak of peroxidase activity (fraction no. 5-10) was observed, which was used for further studies.

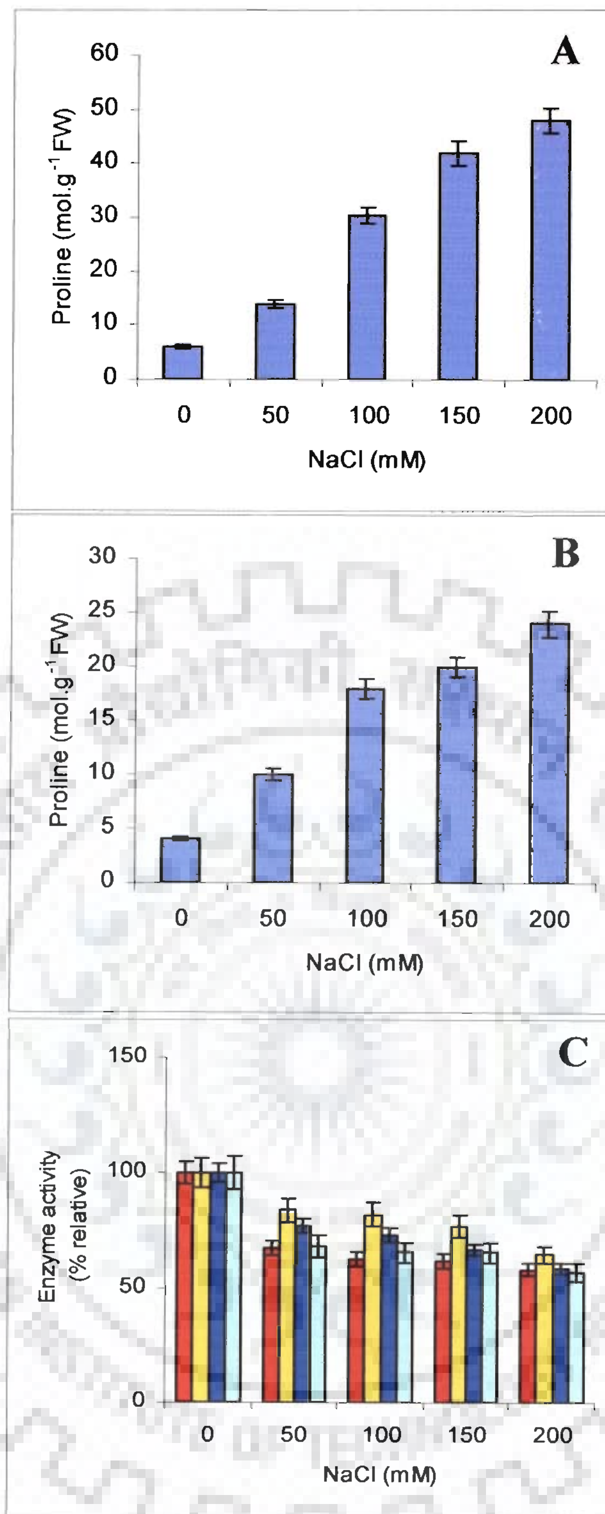
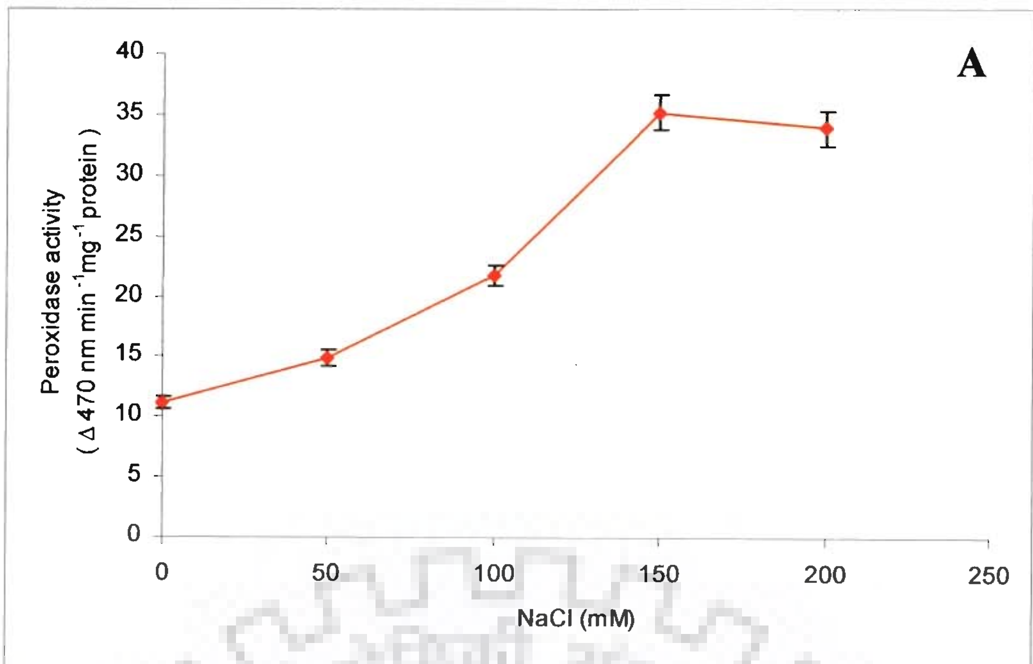


Fig. 6. The effect of salt stress on proline accumulation (**A**) in shoots (**B**) in roots. (**C**) Effect of salt stress on enzyme activities of cell wall bound hydrolases from seedlings grown at different level of salt stress.
■ α-galactosidase □ β-galactosidase ■ α-glucosidase □ acid phosphatase
Values are in triplicate ± S.E.



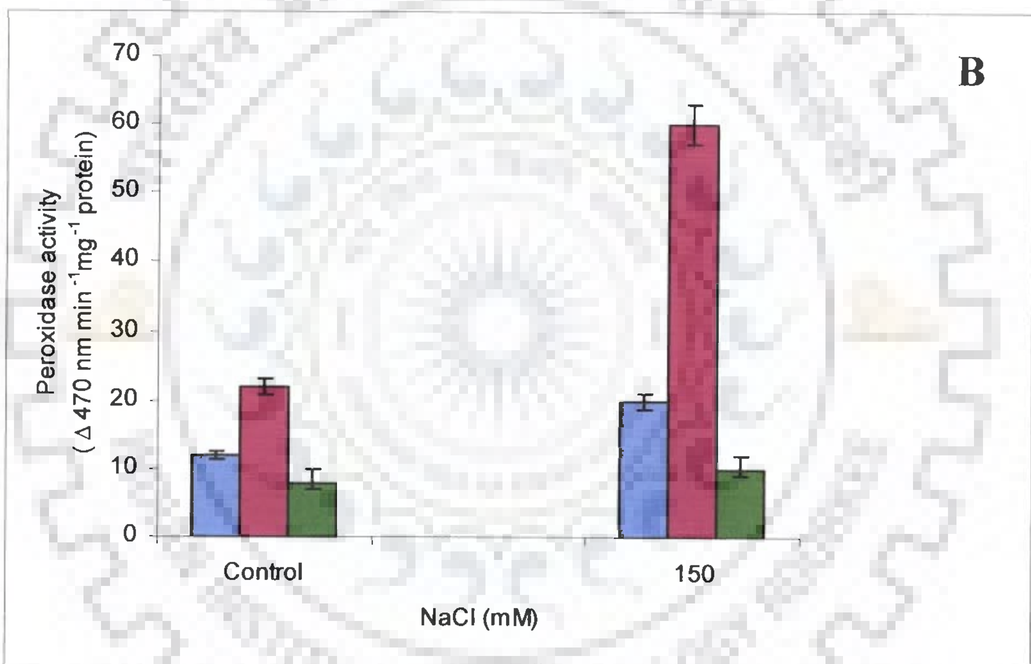
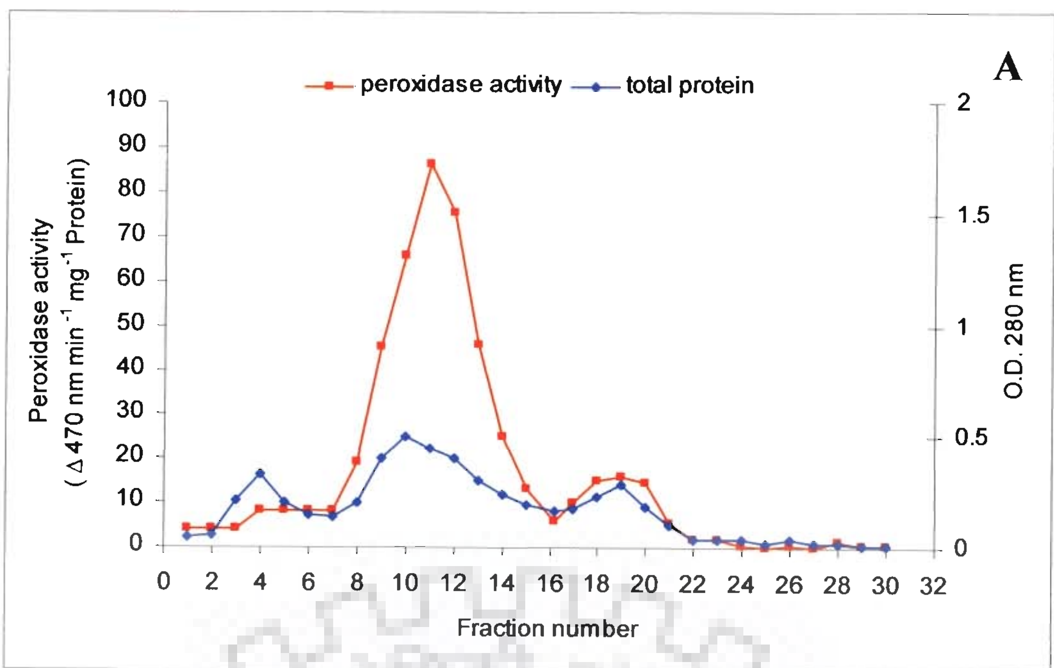


Fig. 7. Effect of salt stress on peroxidase activity (**A**) Peroxidase activity in groundnut seedlings grown at different (0-200 mM) NaCl stress concentration. (**B**) Peroxidase activity of different fractions from groundnut seedlings grown in control and 150 mM NaCl stress concentration. ▢ Cytoplasmic fraction. ■ Ionically bound fraction. ■ Covalently bound fraction Values are in triplicate ± S.E.



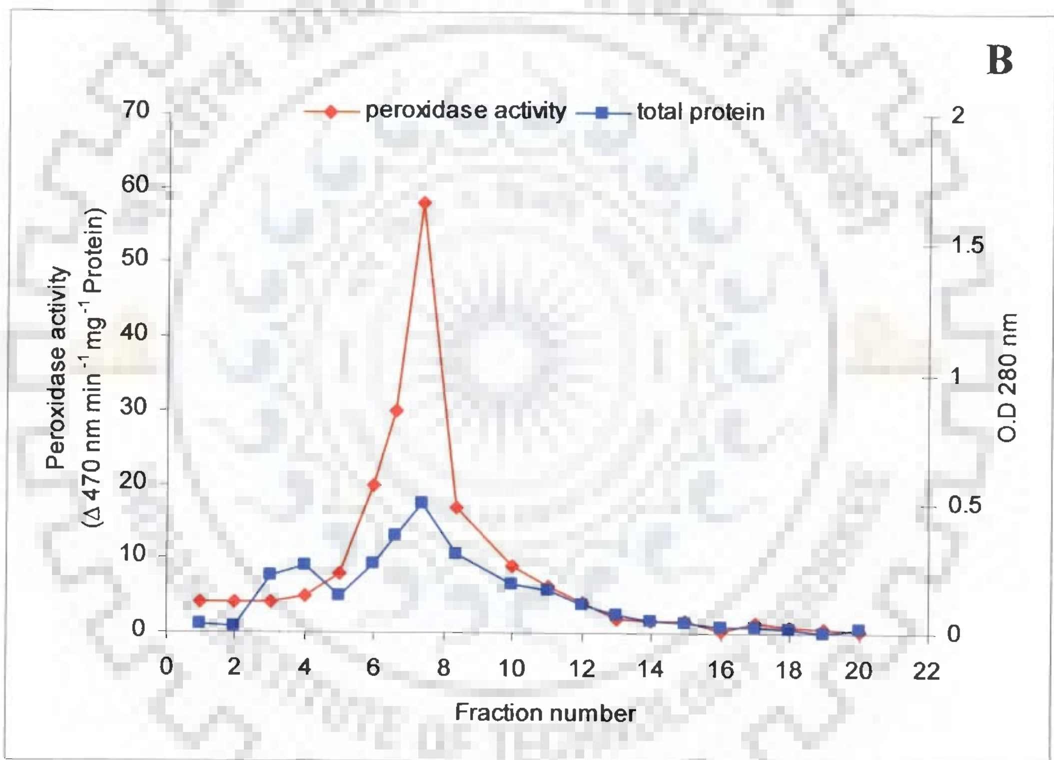


Fig. 8. Purification of ionically bound peroxidase using gel filtration & ion exchange chromatography (**A**) Elution profile of protein (monitored at 280 nm) and total peroxidase activity (monitored at Δ 470 nm) from G-75 sephadex column (**B**) Elution profile of protein (monitored at 280 nm) and total peroxidase activity (monitored at Δ 470 nm) DEAE sepharose column.

4.4.2 Determination of Molecular Weight and Isoelectric Point (pI) of Purified Ionically Bound Peroxidase

The purified ionically bound peroxidase fraction was electrophored on Native-PAGE and the gel was stained with coomassie brilliant blue (Fig. 9A) and peroxidase activity staining (Fig. 9B). A 39 kDa ionically bound peroxidase isoform was identified. The isoelectric point (pI) of peroxidase was determined using analytical IEF gel and was found to be approximately 5.5 as shown in Fig. 9C.

4.4.3 Specificity of Ionically Bound Peroxidase Isoform for Ferulic acid

Since peroxidase assay was performed using guaiacol which is a common substrate for all kinds of peroxidases. The activity of ionically bound peroxidase was assayed using ferulic acid as substrate. The ionically bound peroxidase showed specificity for ferulic acid and used it as substrate. This was clear from the absorbance of enzymic reaction at 310 nm. Analysis of the elution profile of acid insoluble product obtained from ionically bound peroxidase and ferulic acid enzymic reaction from Sephadex G25-M column using 0.1 NaOH indicated polymeric nature. This is clear from the shift in absorbance peak in comparison to free ferulic acid as absorbed at 310 nm (Fig. 10). However, we do not determine whether it is diferulate or triferulate in nature. Peroxidase mediated dimerization and trimerization of ferulic acid found to play important role in cell wall rigidity by cross linking cell wall polysaccharides and other polymeric material.

4.4.4 Effect of Exogenous Proline on Ionically Bound Purified Peroxidase Activity

Exogenous proline has been reported to influence peroxidase activity under stress. Therefore, the role of exogenous proline on the activity of ionically bound purified peroxidase was studied. Peroxidase activity of salt stressed shoots and roots were found to be significantly higher in presence of proline. In shoots grown in 200 mM NaCl with proline, the peroxidase activity was found to be 20.68% higher than in shoots of seedlings grown without proline, which was also evident from in-gel peroxidase assay shown in Fig. 11A, B & C. In roots of seedlings grown in 200 mM of NaCl stress with proline, the peroxidase activity was found to be 19.0% higher than in roots grown in absence of proline. Similar results were shown by in-gel peroxidase assay (Fig. 12A, B & C).

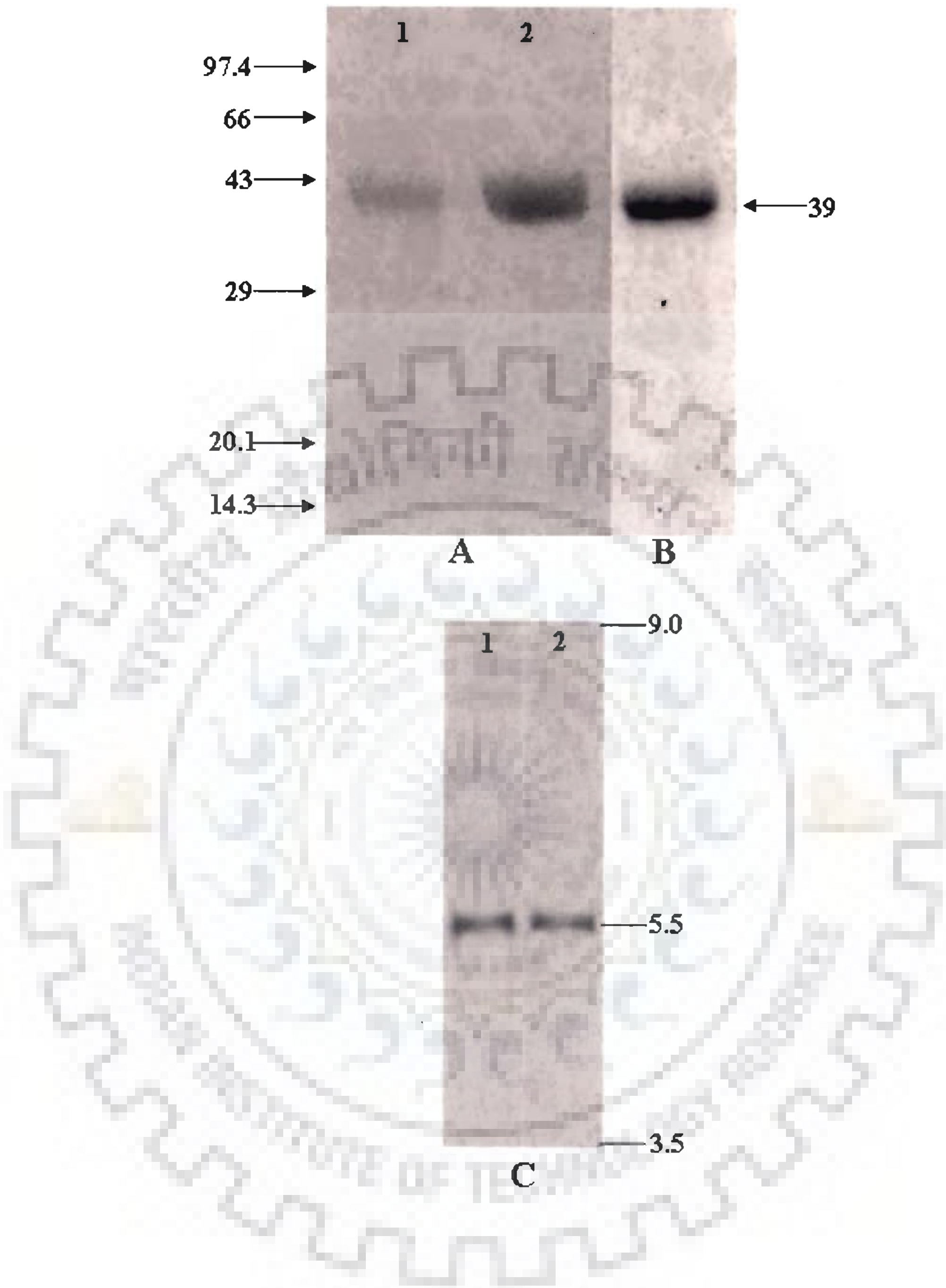


Fig. 9. (A) Coomassie blue stained Native- PAGE (10%) of purified ionically bound peroxidase fraction, lane 1 (20 μg), lane 2 (35 μg) loaded (B) In-gel peroxidase activity staining (35 μg). (C) Analysis of purified peroxidase by analytical isoelectric focusing. lane 1: coomassie stained; lane 2: activity stained analytical isoelectric focusing. The pH of anionic and cathodic ends of gel are shown in right margin.

Table 8: Purification profile of anionically bound cell wall peroxidase from 150 mM NaCl grown groundnut seedlings.

Procedure	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Purification fold
Crude	35	2940	84	1
G-75 sephadex	1.6	1251	782	9.31
DEAE-sepharose	0.25	935	3740	44.5

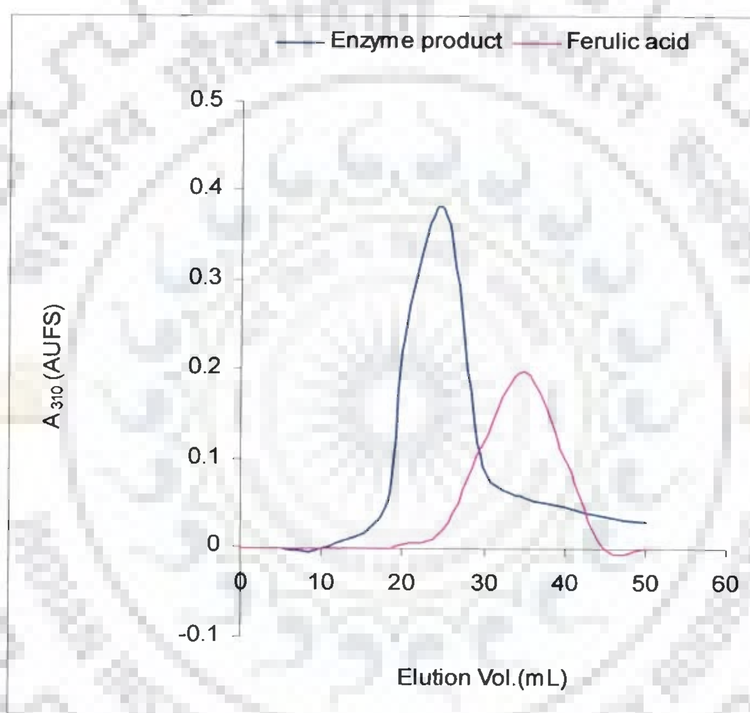


Fig. 10. Elution profile of polymeric product formed by reaction of ionically bound cell wall peroxidase with ferulic acid/H_2O_2. The acid-insoluble precipitate was dissolved in 0.1 M NaoH and loaded on sephadex G-25 column. Elution was carried out using 0.1 M NaOH and absorbance was measured at 310 nm. Free ferulic acid was used to mark the total volume of column.

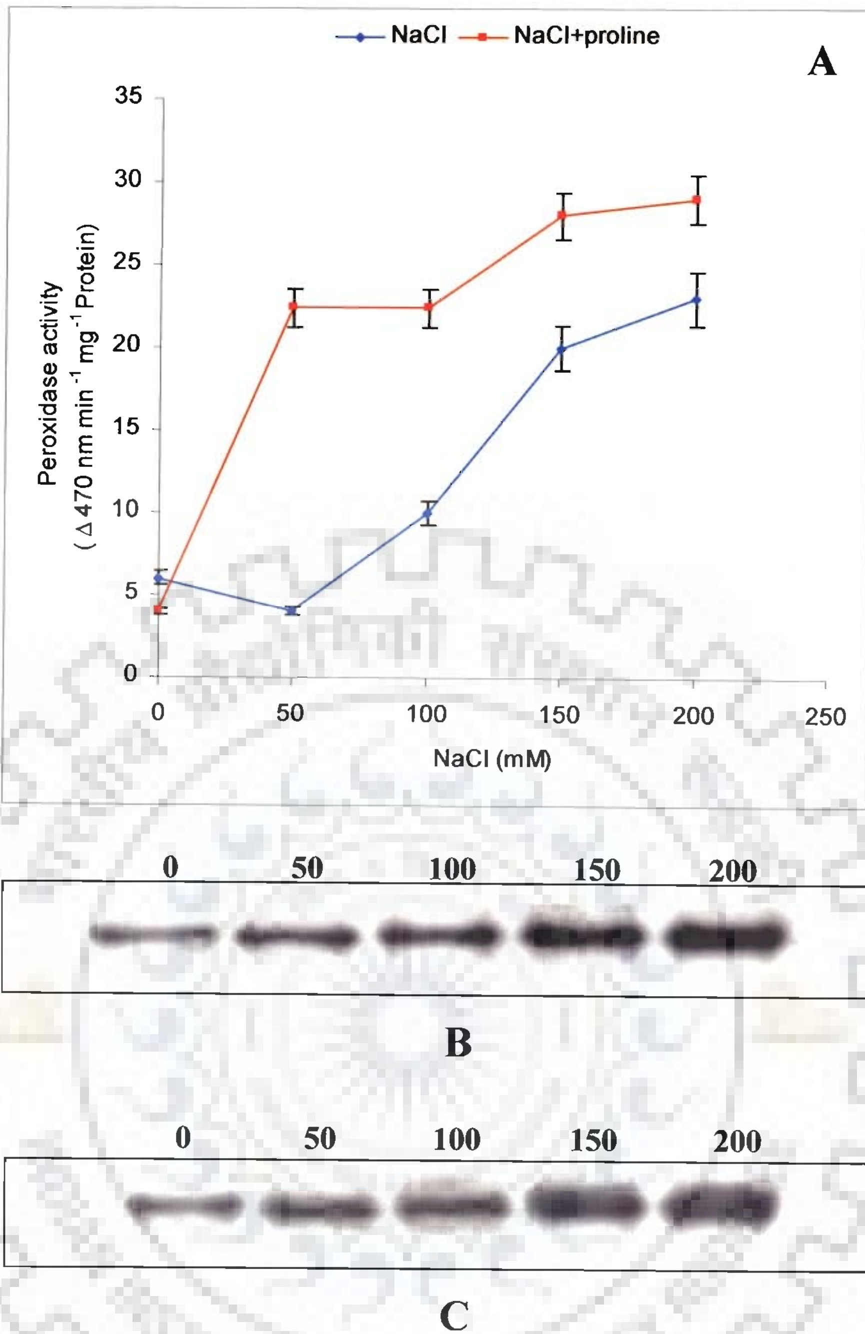


Fig. 11. (**A**) Effect of exogenous proline on ionically bound purified peroxidase activity in shoot of groundnut seedlings grown in different (0-200 mM) NaCl stress concentrations. (**B**) in-gel peroxidase activity of peroxidase purified from seedlings grown with salt stress treatment in absence of proline (**C**) in-gel peroxidase activity of peroxidase purified from seedlings grown with salt stress treatment in presence of proline. Values are in triplicate ± S.E.

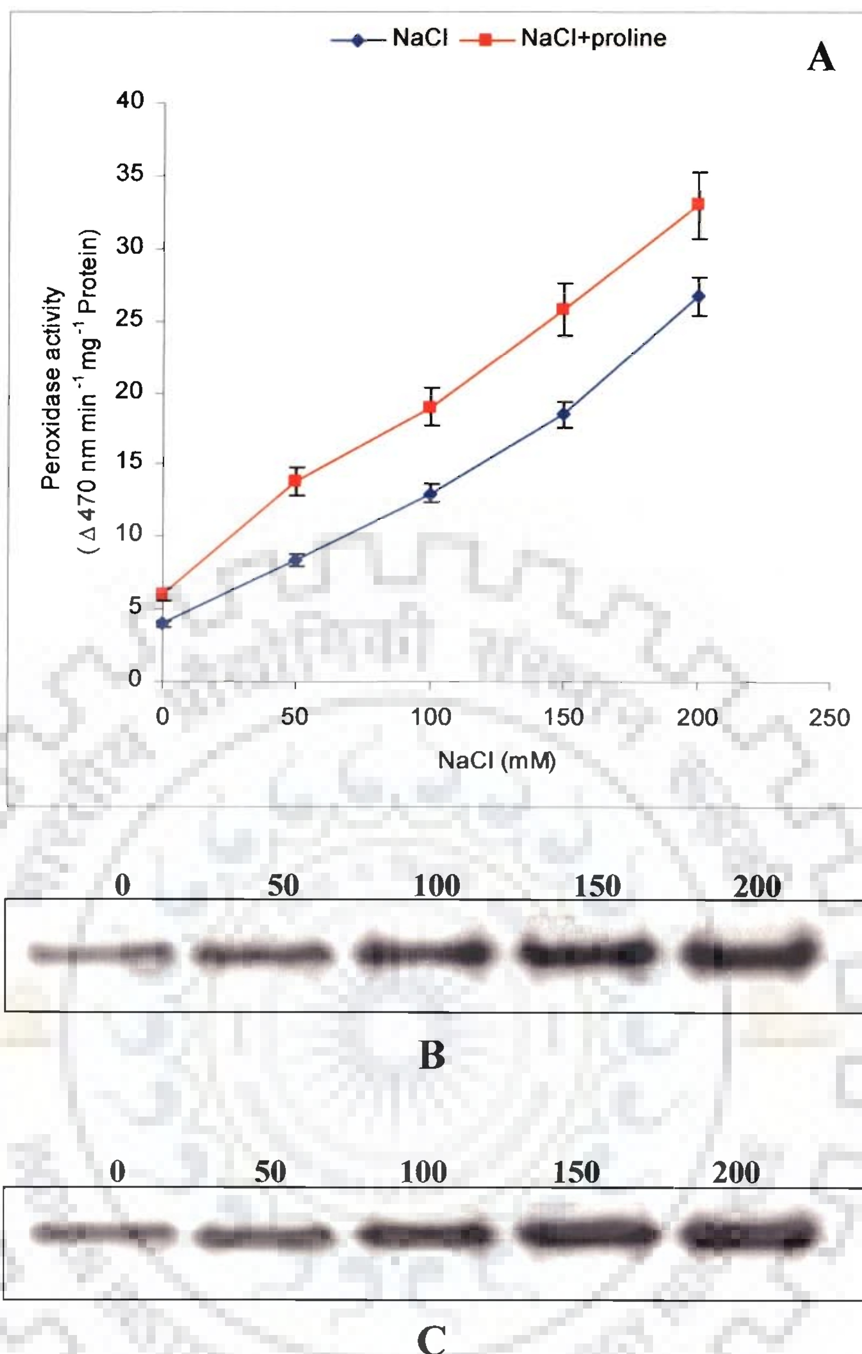


Fig. 12. (**A**) Effect of exogenous proline on ionically bound purified peroxidase activity in root of groundnut seedlings grown in different (0-200 mM) NaCl stress concentrations. (**B**) in-gel peroxidase activity of peroxidase purified from seedlings grown with salt stress treatment in absence of proline (**C**) in-gel peroxidase activity of peroxidase purified from seedlings grown with salt stress treatment in presence of proline. Values are in triplicate ± S.E.

4.5 EFFECT OF SALT STRESS ON PROTEIN PROFILE

4.5.1 SDS-PAGE Protein Profile

To examine the effect of salt treatment on protein profile, total proteins were isolated from control and salt-treated seven-day old groundnut seedlings. Analysis of proteins was done by visualizing bands in coomassie brilliant blue stained 10% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

As expected there were induction and repression of some proteins in salt stress condition compared with controls which was revealed from coomassie brilliant blue stained gel (Fig. 13A). A protein band of 31.6 kDa was found to be induced under NaCl stress and there found to be progressive increase in the expression from 50 mM NaCl (lane 2) to 200 mM NaCl (lane 5). However, the most prominent induction at NaCl concentration of 200 mM was observed. On the other hand, the expression of protein band 48 kDa was greatly repressed under salinity stress. There was steady repression of this protein with increase of NaCl stress to seedlings.

The induction of 31.6 kDa protein and repression of 48 kDa protein were also shown by quantitative densitometry analysis (Fig. 13B & C). The level of induction of 31.6 kDa protein was found to be two fold in 150 mM NaCl treated than that of control seedlings which was 26% and 13%, respectively as shown in Fig. 13B. Similarly, level of repression of 48 kDa protein was also approximately two fold, 28.5% in control and 14.8% in 150 mM NaCl treated seedlings, shown in Fig. 13C.

4.5.2 Two-Dimensional-PAGE Protein Profile

In contrast to 1-D gels, the high resolution of 2D-PAGE revealed a more complex proteins/polypeptide patterns (Fig. 14). Salt stress caused increase and decrease in syntheses of a numbers of proteins/polypeptides. The newly synthesized proteins ranged in molecular weight from 16-120 kDa and have pI from 4.5-6.5. Treatment of seedlings with NaCl did not induce any striking qualitative differences in protein profiles. Salt treatment did caused alteration in the relative amount of protein. The quantitative changes occurred in a heterogeneous group of proteins having a broad range of molecular weight and pI. A comparison of coomassie brilliant blue stained protein profiles of control and 150 mM salt treated seedlings are shown in Fig. 14A and B, respectively. The prominent among the proteins whose amount increase under salt stress were 70, 50, 35 and 23/24 kDa, pI 5.4 (spot number 2, 7, 29 and 28); 60 kDa, pI 5.6 (spot number 26) and 16 to 23 kDa pI 4.5 to 6.5 (spot number 27), respectively (Fig. 14B). Proteins which were suppressed such as 70, 64, 50 and 47 kDa, pI 5.2-6.0 (spot number 3, 4, 5, 6 and 10, Fig. 14A).

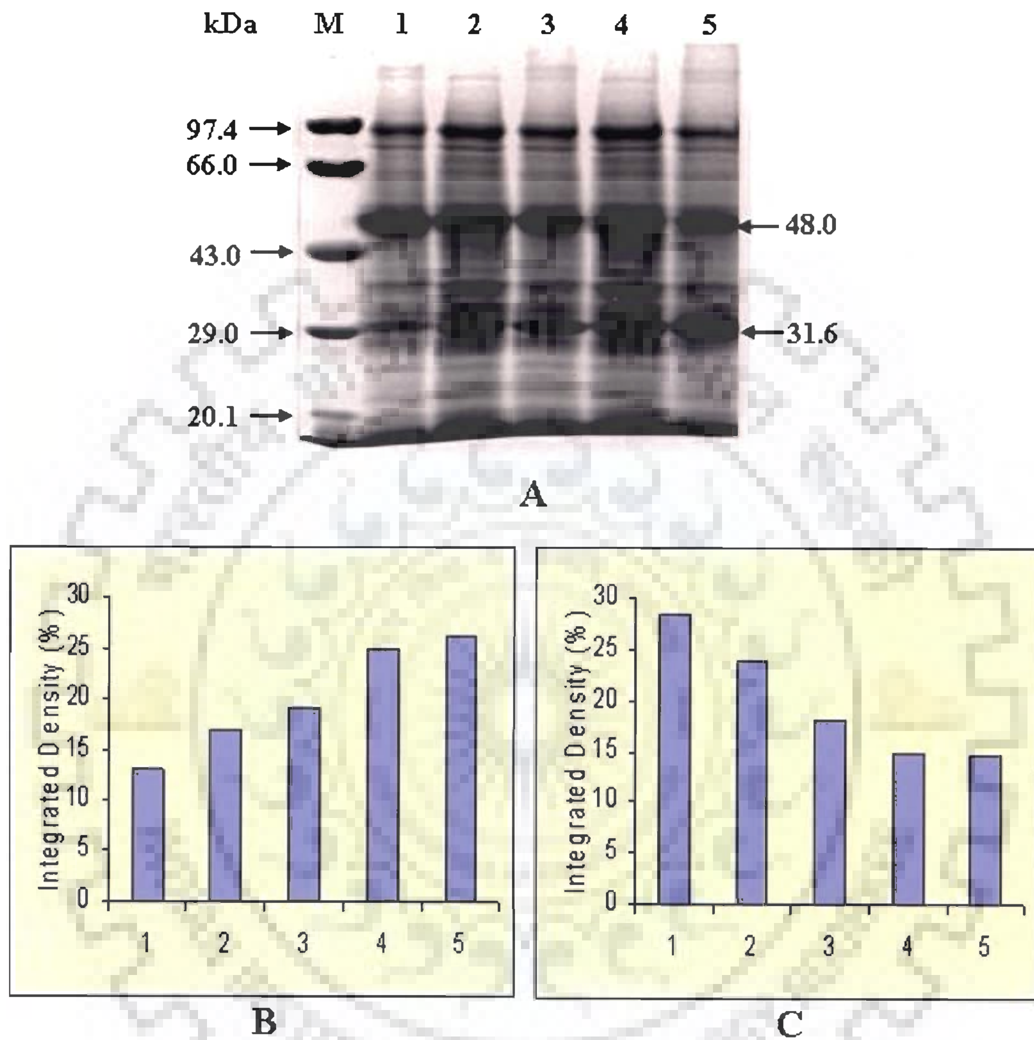


Fig.13. Protein profile of groundnut seedlings grown under different (0-200 mM) NaCl concentrations. (A) Coomassie blue stained SDS-PAGE (10%) gel. Lane 1: Control; lane 2: 50 mM; lane 3: 100 mM; lane 4: 150 mM; lane 5: 200 mM NaCl grown seedlings. (B) and (C) are the densitometry analysis of 31.6 & 48.0 kDa protein bands, respectively. Molecular markers are shown at left hand side.

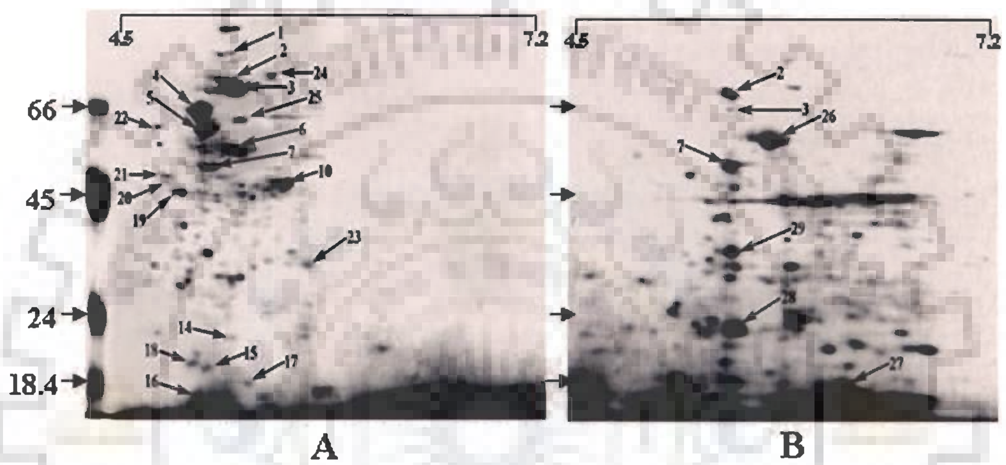


Fig. 14. Coomassie blue stained two-dimensional PAGE protein profile of groundnut seedling. (**A**) Control (**B**) Seedling grown at 150 mM NaCl stress. First dimensional IEF was performed in tube gel and the second dimension in 10 % SDS-PAGE. Salt induced proteins are indicated. Major protein spots in control and 150 mM NaCl grown seedlings are indicated by arrows.

4.6 EXPRESSION OF 31.6 kDa SALT STRESS INDUCED PROTEIN

The expression of 31.6 kDa protein was studied under salt stress, abiotic stress and in different cultivars of groundnut. The results are given in sections 4.6.1, 4.6.2 and 4.6.3.

4.6.1 Expression of 31.6 kDa Salt Stress Induced Protein under Different NaCl Concentration

The expression of 31.6 kDa protein under different NaCl concentrations was studied by Western blotting using polyclonal antibody generated against salt stressed induced 31.6 kDa protein (Fig. 15). The expression was found to be inducing gradually from control to 200 mM NaCl as shown in Fig. 15A & B.

The quantitative densitometry analysis showed a positive correlation between the levels of expression of protein and concentration of NaCl stress. It was found that the level of expression in control seedlings were 12.8 % while in seedlings grown at 150 mM of NaCl stress, it was 25.2% as shown in Fig. 15C and D. This expression pattern indicates the role of 31.6 kDa protein in salt stress tolerance of groundnut seedlings.

4.6.2 Expression of 31.6 kDa Salt Stress Induced Protein under Various Abiotic Stresses

To study the expression pattern of 31.6 kDa protein, seedlings were subjected to different abiotic stress viz. KCl, mannitol, PEG (Polyethylene glycol) and also grown in presence of ABA (Abscisic acid). There was a remarkable difference on the level of expression of the protein at different abiotic stress conditions and ABA (Fig. 16A & B). The expression of protein is almost similar in presence of NaCl and KCl as shown in Fig. 16B (lane 2 & 3), and maximum in case of PEG.

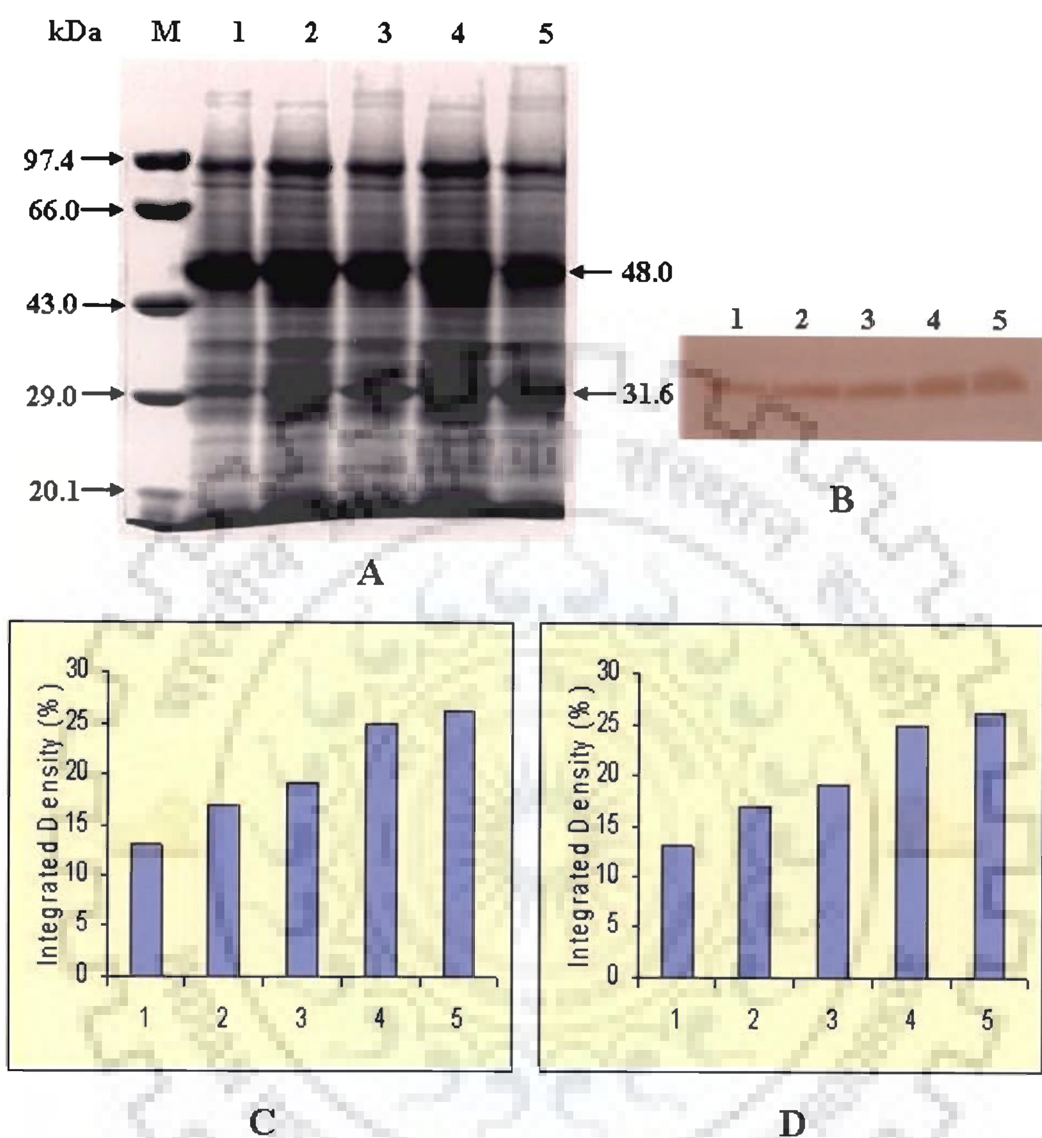
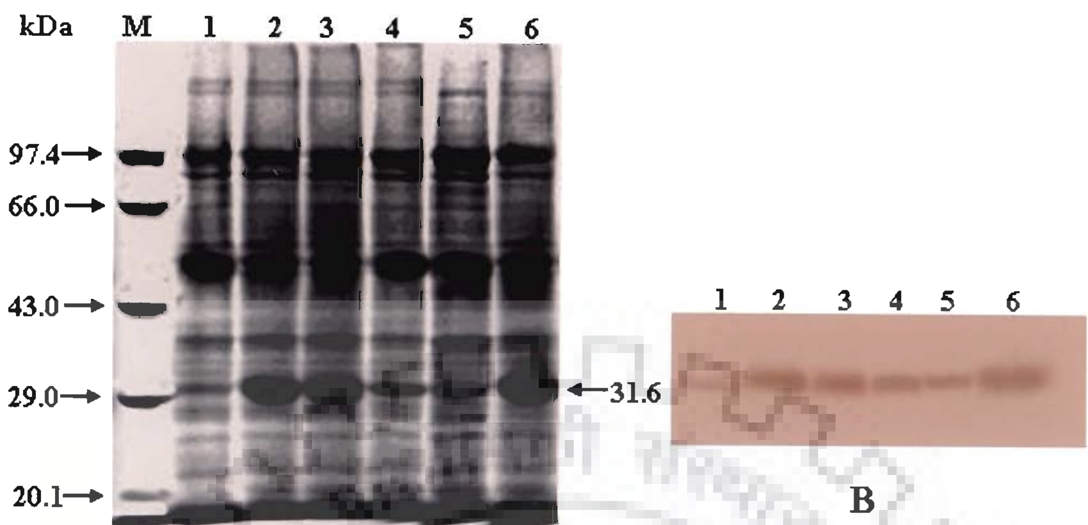
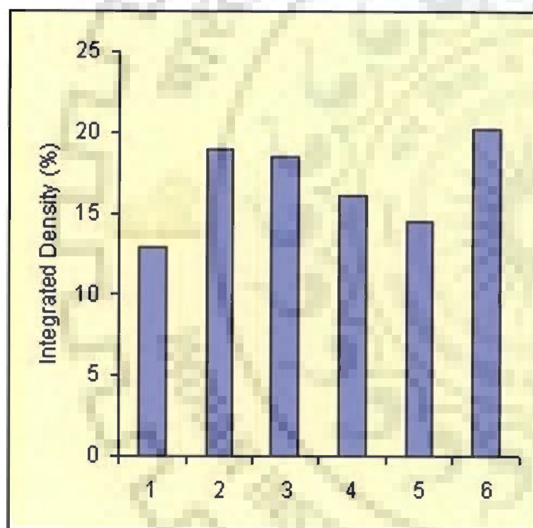


Fig. 15. Expression of 31.6 kDa salt induced protein in groundnut seedling grown in different (0-200 mM) NaCl concentrations for seven days. **(A)** Coomassie blue stained SDS-PAGE (10%) gel, **(B)** Western blot using polyclonal antibody against 31.6 kDa protein. Lane 1: Control; lane 2: 50 mM; lane 3: 100 mM; lane 4: 150 mM; lane 5: 200 mM NaCl grown seedlings. **(C)** and **(D)** are the densitometry analysis of 31.6 kDa protein band from the (A) & (B), respectively. Molecular markers are shown at left hand side.





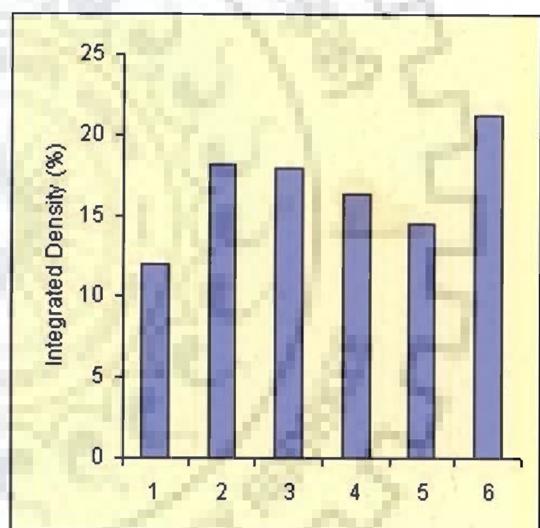
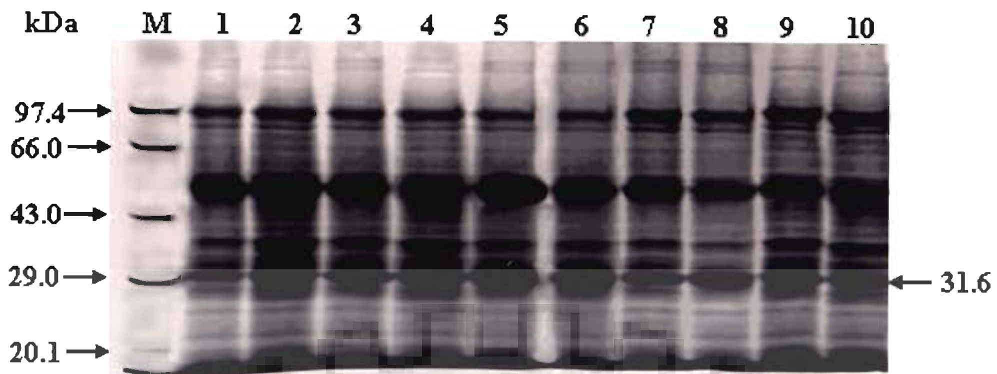
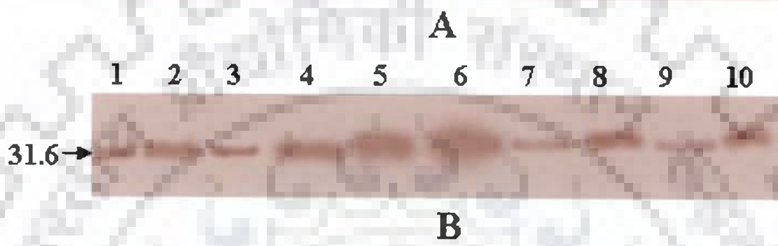


Fig. 16. Expression of 31.6 kDa protein in groundnut seedling grown under various abiotic stresses and ABA (A) Coomassie blue stained SDS-PAGE (10%) protein profile of seedling grown under different abiotic treatments (B) Western blot showing expression of the 31.6 kDa protein under different abiotic treatments. Lane 1: Control; Lane 2: 150 mM NaCl; Lane 3: 150 mM KCl; Lane 4: 300 mM Mannitol; Lane 5: 20 µM ABA; Lane 6: 20% PEG. (C) and (D) are the densitometry analysis of 31.6 kDa protein band from (A) & (B), respectively. Molecular markers are shown at left hand side.



A



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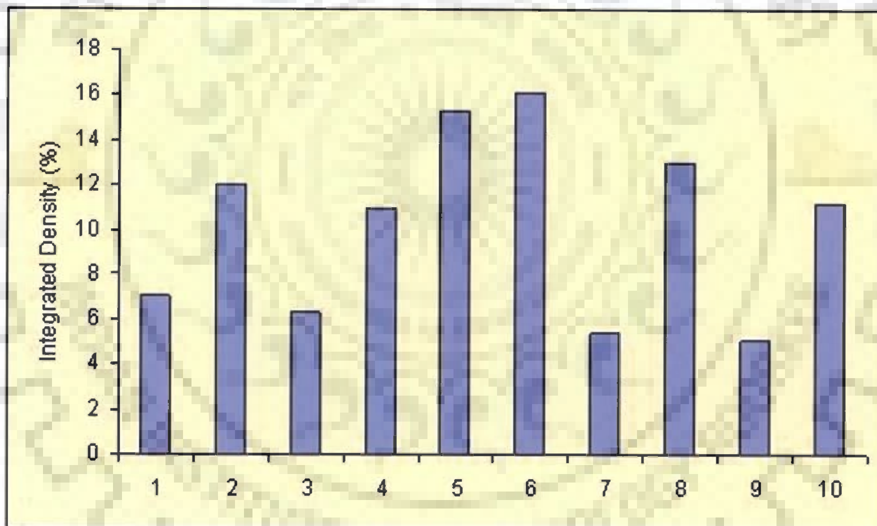
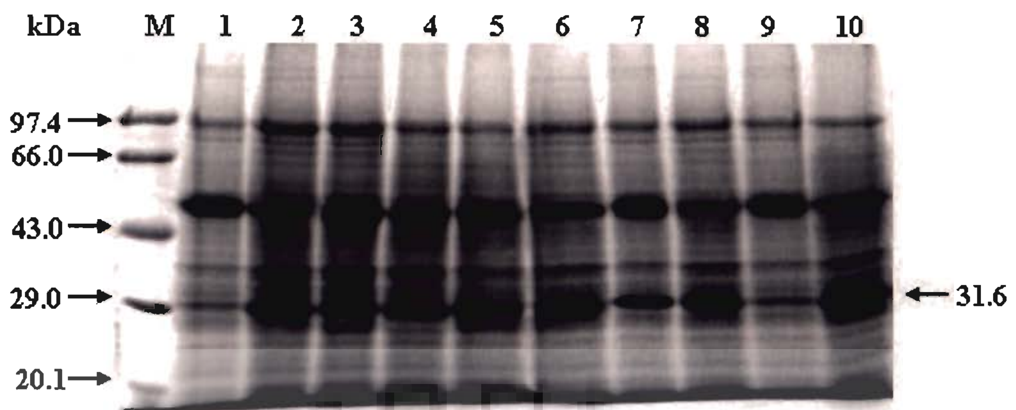


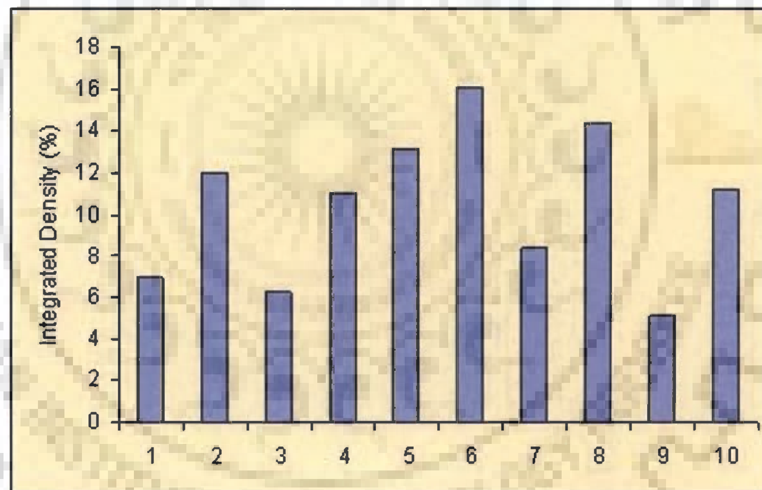
C

Fig. 17. Expression profile of 31.6 kDa protein in different cultivars of groundnut grown in control and 150 mM NaCl stress concentration. **(A)** Coomassie blue stained SDS-PAGE (10%) profile of various cultivars **(B)** Western blot showing expression pattern of the 31.6 kDa protein. Lane 1: T-64 control; lane 2: T-64 150 mM; lane 3: TMV-7 control; lane 4: TMV-7 150 mM; lane 5: ICGS-37 control; lane 6: ICGS-37 150 mM; lane 7: JAWAN control; lane 8: JAWAN 150 mM; lane 9: KRG-1 control; lane 10: KRG-1 150 mM **(C)** The densitometry analysis of 31.6 kDa protein band of the blot. Molecular markers are shown at left hand side.



A

B



C

Fig. 18. Expression profile of 31.6 kDa protein in different cultivars of groundnut grown in control and 150 mM NaCl stress concentration. (A) Coomassie blue stained SDS-PAGE (10%) profile of various cultivars (B) Western blot showing expression pattern of the 31.6 kDa protein. Lane 1: Kaushal control; lane 2: Kaushal 150 mM; lane 3: TG-1 control; lane 4: TG-1 150 mM; lane 5: Tirupati-1 control; lane 6: Tirupati-1 150 mM; lane 7: Kadri-4 control; lane 8: Kadri-4 150 mM; lane 9: Tirupati-4 control; and lane 10: Tirupati-4 150 mM (C) The densitometry analysis of 31.6 kDa protein band of the blot.

4.7 EXPRESSION OF 48.0 kDa SALT STRESS REPRESSED PROTEIN

The expression of 48 kDa protein was studied under salt stress, abiotic stress and in different cultivars of groundnut. The results are given in sections 4.7.1, 4.7.2 and 4.7.3.

4.7.1 Expression of 48 kDa Salt Stress Repressed Protein under Different NaCl Concentrations

The expression of repressing protein was studied by using polyclonal antibody raised against 48 kDa protein. The expression level of 48 kDa protein was gradually decreasing with the level of NaCl stress (lane 1-5). The expression level was highest in control seedlings and lowest in 200 mM NaCl treated seedlings as exhibited by quantitative densitometry analysis (Fig. 19B & D). There was approximately 28% of expression level in control and 13% in salt treated seedlings.

The quantitative densitometry analysis of gel as well as the blot also proved the regular decrease in the expression level of 48 kDa protein under increasing NaCl stress condition. The level of expression of protein was observed as approximately 28%, 23%, 18%, 15% and 12% in 0, 50, 100, 150 and 200 mM NaCl stress, respectively Fig. 19C & D.

The results indicated that NaCl effect the expression of protein in seedlings when grown under stress, the level of expression changes with the concentration of NaCl present in the growth medium and the repression of protein was observed.

4.7.2 Expression of 48 kDa Salt Stress Protein under various Abiotic Stresses

It was observed that the salt stress protein of 48 kDa also expressed at various abiotic stresses like KCl, Mannitol, and PEG and also in presence of ABA. The levels of expression of the protein differ greatly at different abiotic stress conditions

(Fig. 20A & B). The expression of protein was almost similar at NaCl and KCl as shown in Fig. 20B (lane 2 & 3), showed similar effect on the repression of protein under stress condition.

There was a slight decrease in the expression of protein at ABA and mannitol as compared to control (lane 4 & 1). These results were also supported by quantitative densitometry analysis which showed that level of expression approximately 22% in control, 21% in ABA stress seedlings, 19% in mannitol stress, 16-17% in NaCl & KCl stress and 5.9% in PEG stress (lane1-6, Fig. 20C & D). In any case the level of expression did not increase the level found in control seedlings and none of the used abiotic stress induced the expression of protein.

In case of PEG stress the expression level of 48 kDa protein was minimum which showed that protein is maximum repressed by PEG stress (lane 6). The level of expression of 48 kDa protein in control seedlings was found to be highest.

4.7.3 Expression of 48 kDa Salt Stress Protein in Different Cultivars of Groundnut

The effect of NaCl stress on the expression level of 48 kDa protein was also studied in ten cultivars of groundnut as described in previous section 4.6.3. Seedlings of all cultivars were grown under control and salt-stress (150 mM NaCl) conditions for seven days and isolated protein extract was used for Western blot analysis. Results showed the specificity for antibody raised against 48 kDa protein in control and salt-treated seedlings. Different level of expression patterns was observed in all cultivars (Fig. 21).

The expression level of 48 kDa protein in control and salt-treated seedlings in five cultivars viz. T-64, TMV-7, ICGS-37, JAWAN and KRG-1 was shown (Fig. 21B). The

change in level of expression was observed maximum in case control and salt-treated seedlings of ICGS-37 (lane 5 & 6) and minimum in T-64 (lane 1 & 2), which was approximately 12% and 3% respectively shown by quantitative densitometry analysis (Fig. 21C).

It was also observed that the level of protein expression in control seedlings of all cultivars was also different. It was maximum in ICGS-37 (lane 5) and minimum in JAWAN, (lane 7) approximately 17% and 10% respectively exhibited by quantitative densitometry analysis.

The level of expression of salt stress 48 kDa protein in control and salt stress seedlings of other five cultivars viz. Kaushal, TG-1, Tirupati-1, Kadri-4 and Tirupati-4 was studied and observed that there were remarkable changes in the level of expression of protein in these cultivars also (Fig. 22A & B).

It was observed that expression level of 48 kDa protein repressed maximum in salt stressed groundnut seedling of Kaushal and TG-1 cultivar (lane 2 & 4) and minimum level of repression was seen in Kadri-4 cultivar (lane 7 & 8).

The difference in the level of expression of 48 kDa protein was also observed in the control seedlings of all cultivars (lane 1, 3, 5, 7 & 9 Fig. 22B). Kaushal exhibited the highest level of expression and Kadri-4 exhibited lowest level (lane 1 & 7). Similar levels of expression were seen in Kaushal and TG-1 (lane 1 & 3) as well as in Tirupati-1 and Tirupati-4 (lane 5 & 9) as evident from densiotmetery analysis (Fig. 22C).

The repression of 48 kDa protein in salt stressed seedlings indicated the effect of NaCl stress on its expression. Different cultivars responded differently to NaCl stress on the basis of expression level of 48 kDa protein.

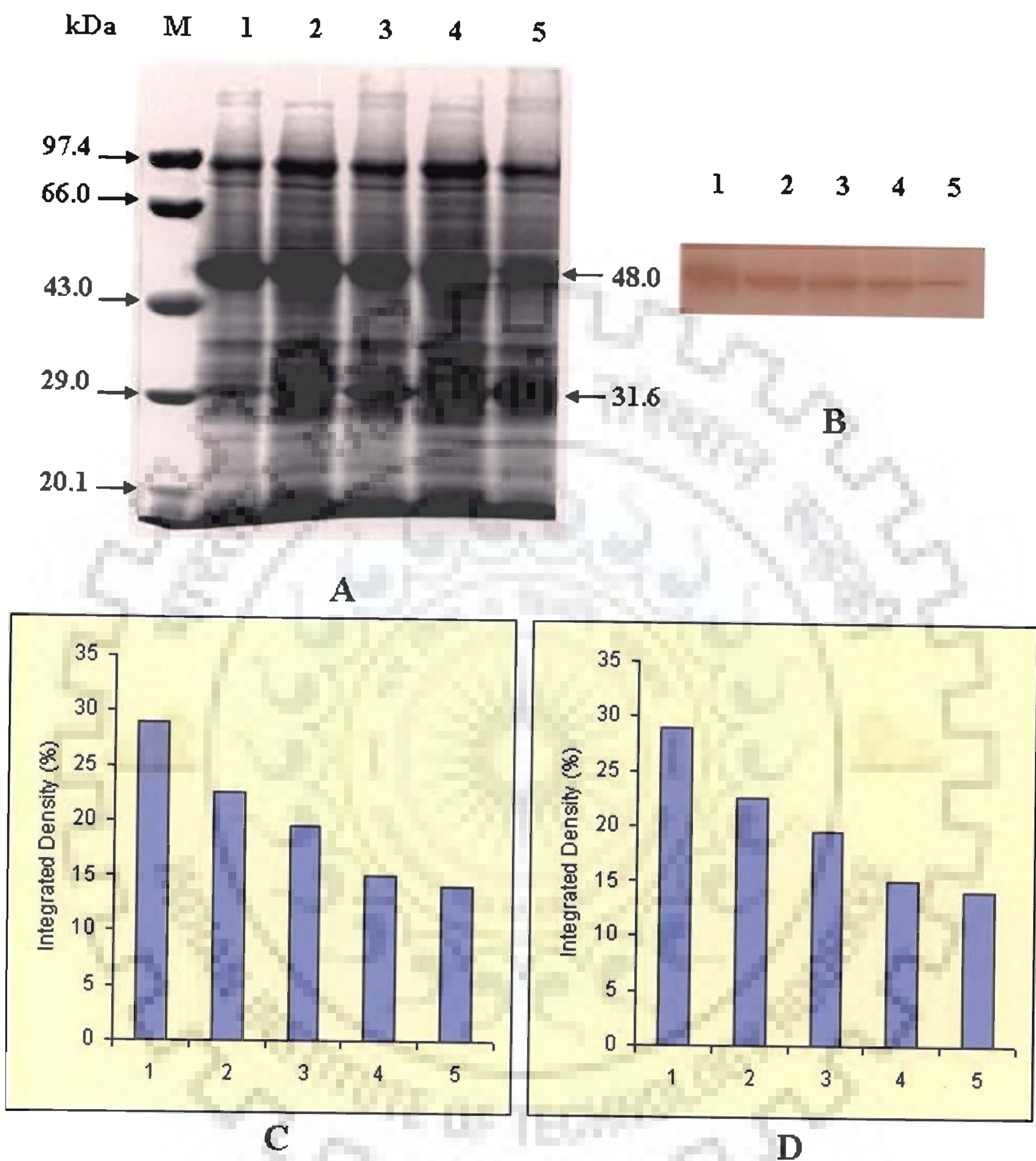


Fig. 19. Expression of 48 kDa salt stress protein from groundnut seedlings grown under different NaCl (0-200 mM) concentrations. **(A)** Coomassie blue stained SDS-PAGE (10%) gel **(B)** Western blot using polyclonal antibody against 48 kDa protein. Lane 1: Control; lane 2: 50 mM; lane 3: 100 mM; lane 4: 150 mM; lane 5: 200 mM NaCl grown seedlings. **(C)** and **(D)** are the densitometry analysis of 31.6 kDa protein band from the **(A)** & **(B)**, respectively. Molecular markers are shown at left hand side.

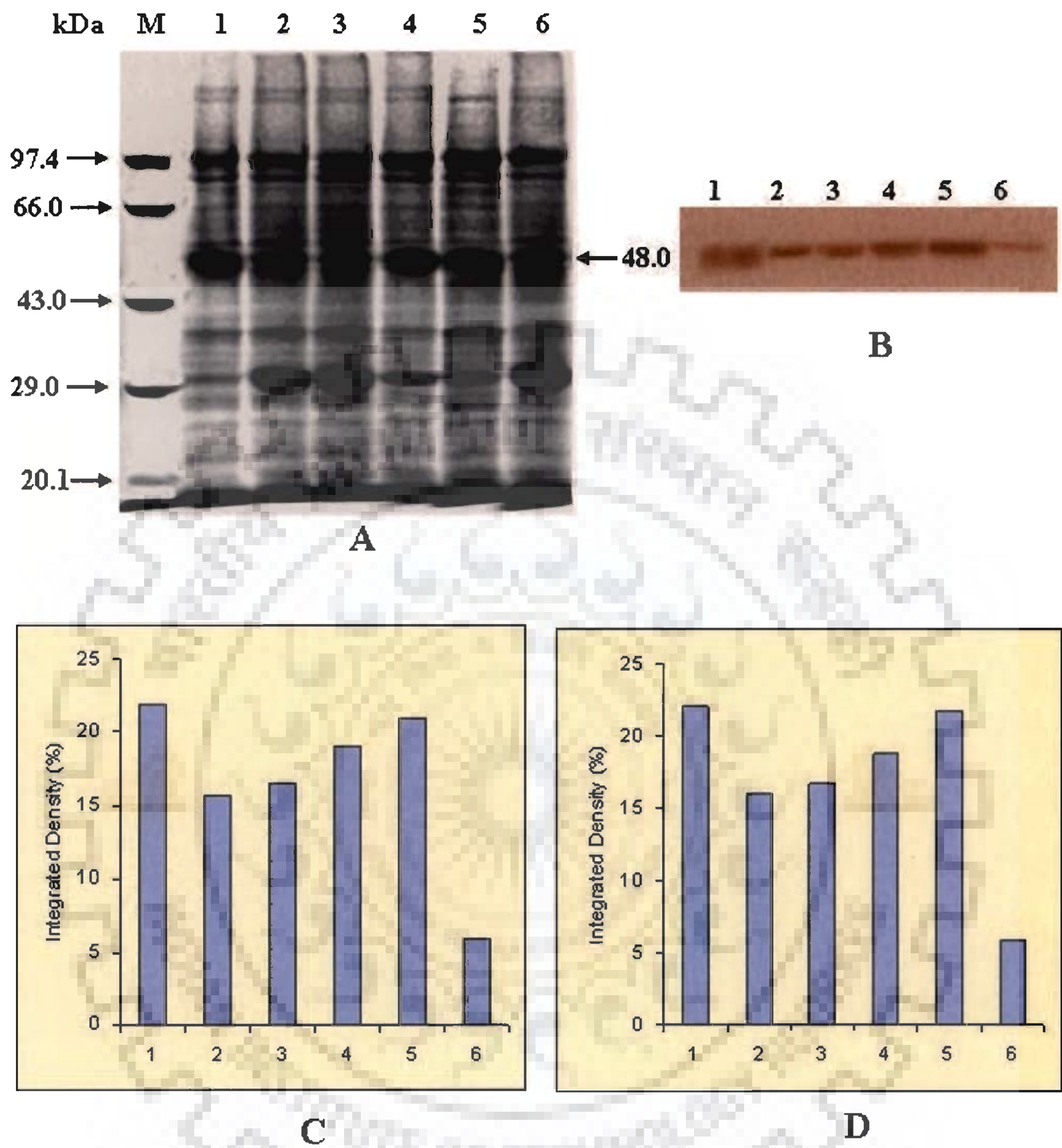
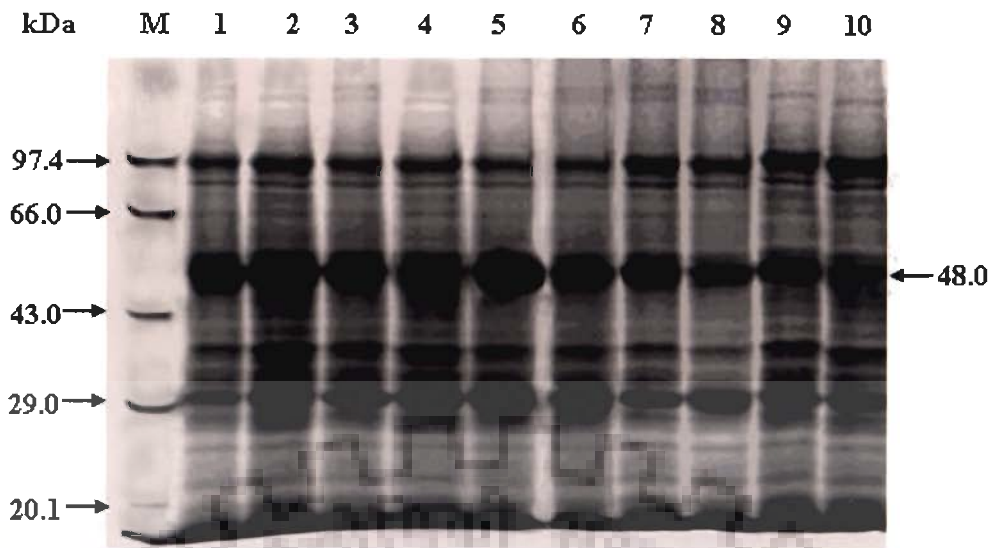
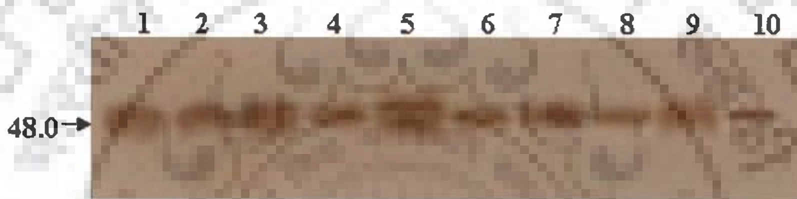


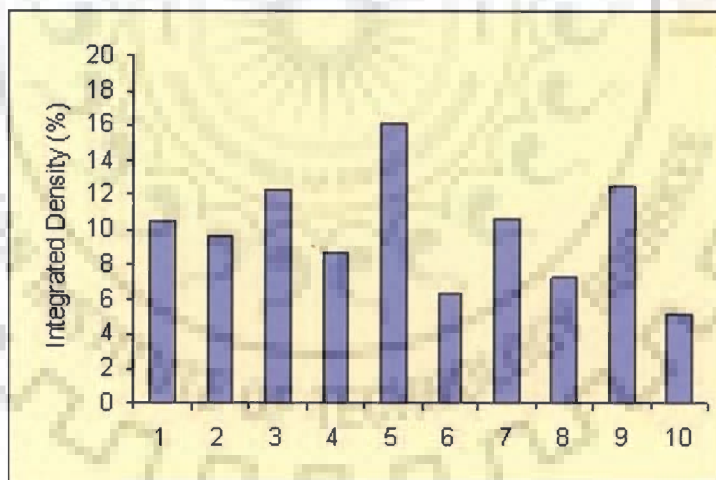
Fig. 20. Expression of 48 kDa protein in groundnut seedlings grown under various abiotic stresses and ABA (A) Coomassie blue stained SDS-PAGE protein profile of seedlings grown under different abiotic treatments (B) Western blot showing expression of the 48 kDa protein under different abiotic treatments. Lane 1: Control; Lane 2: 150 mM NaCl; Lane 3: 150 mM KCl; Lane 4: 300 mM Mannitol; Lane 5: 20 µM ABA; Lane 6: 20 % PEG; (C) and (D) are the densitometry analysis of 48 kDa protein band from the (A) & (B), respectively. Molecular markers are shown on left side.



A

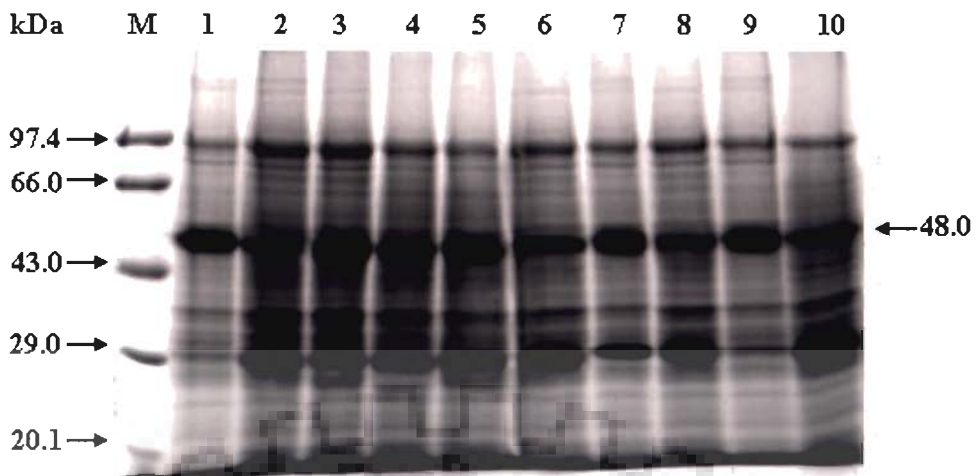


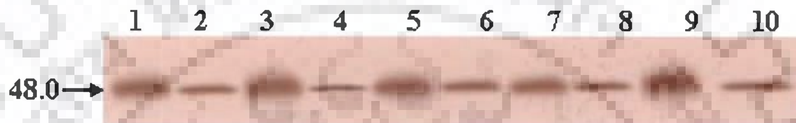
B



C

Fig. 21. Expression of 48 kDa protein in various cultivars of groundnut under NaCl stress. **(A)** Coomassie blue stained SDS-PAGE protein profile of various cultivars; **(B)** Western blot showing expression pattern of 48 kDa protein in different cultivars of groundnut. Lane 1: T-64 control; lane 2: T-64 150 mM; lane 3: TMV-7 control; lane 4: TMV-7 150 mM; lane 5: ICGS-37 control; lane 6: ICGS-37 150 mM; lane 7: JAWAN control; lane 8: JAWAN 150 mM; lane 9: KRG-1 control; lane 10: KRG-1 150 mM; **(C)** The densitometry analysis of 48 kDa protein band of the blot. Molecular markers are shown at left side





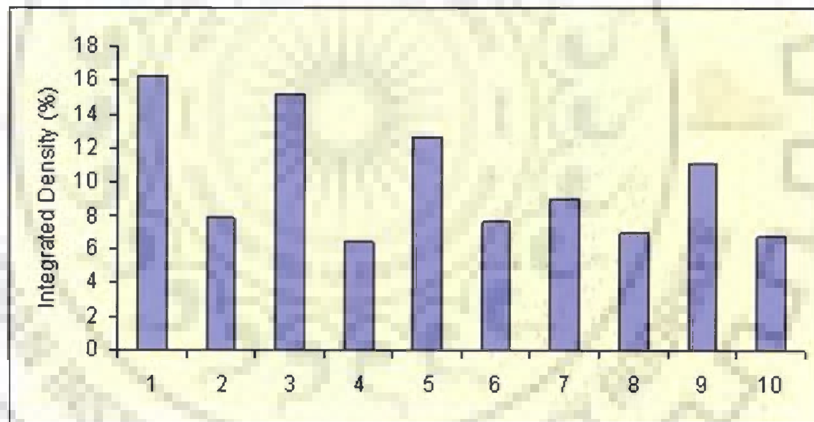


Fig. 22. Expression profile of 48 kDa protein in various cultivars of groundnut under NaCl stress (**A**) Coomassie blue stained SDS-PAGE protein profile of various cultivars, (**B**) Western blot showing expression pattern of 48 kDa protein in different cultivars of groundnut. Lane 1: Kaushal control; lane 2: Kaushal 150 mM; lane 3: TG-1 control; lane 4: TG-1 150 mM; lane 5: Tirupati-1 control; lane 6: Tirupati-1 150 mM; lane 7: Kadri-4 control; lane 8: Kadri-4 150 mM; lane 9: Tirupati-4 control; lane 10: Tirupati-4 150 mM; (**C**) The densitometry analysis of 48 kDa protein band of the blot.

4.8 EXPRESSION OF 31.6 kDa AND 48 kDa PROTEINS IN ROOT AND SHOOT TISSUE

The expression of 31.6 and 48 kDa proteins were also studied in root and shoot of seedlings using polyclonal antibody. The results for 31.6 and 48 kDa proteins are given in Fig. 23 and 24, respectively.

As observed in Fig. 23, the inducing expression of 31.6 kDa protein was seen in root and shoot of NaCl stress seedlings (lane 2 & 4). The level of expression was approximately two fold higher in shoot than root of NaCl stress seedling. It was approximately 20% in root and 38% in shoot exhibited by quantitative densitometry analysis (Fig. 23C), which indicated the role of protein at shoot tissue in salt stress conditions.

The difference in the expression level of 31.6 kDa protein in root and shoot of control seedlings was also observed (lane 1, 3 Fig. 23B) and it was approximately 25% in shoot, approximately 18% in root apparent by quantitative densitometry analysis (Fig. 23 C).

The effect of NaCl on the expression level of 48 kDa protein in root and shoot of seedlings is given in Fig. 24. The level of expression of protein repressed more in root than in shoot as shown in Fig. 24A & B. The expression of protein also showed differences in root and shoot of control seedlings. The level of expression in root was more than it was observed in shoot, approximately 32% and 27%, respectively (lane 1, 3 Fig. 24C).

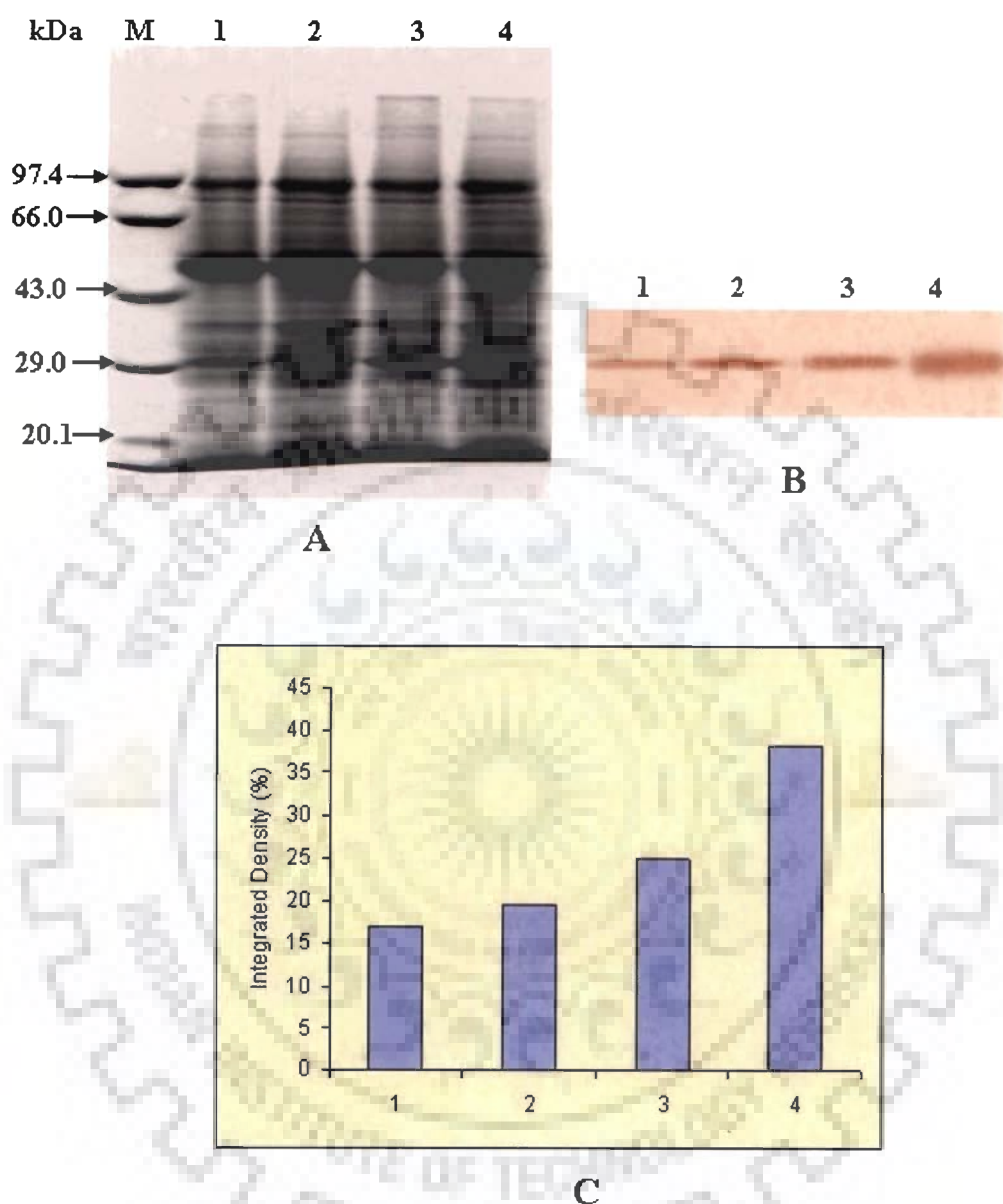


Fig. 23. Tissue specific expression of 31.6 kDa salt induced protein in shoot and root of groundnut seedling. **(A)** Coomassie blue stained SDS-PAGE protein profile, **(B)** Western blot using polyclonal antibody against 31.6 kDa protein. Lane 1: root control; lane 2: 150 mM NaCl; lane 3: shoot control; lane 4: 150 mM NaCl. **(C)** the densitometry analysis of 31.6 kDa protein band from blot. Molecular markers are shown at left hand side.

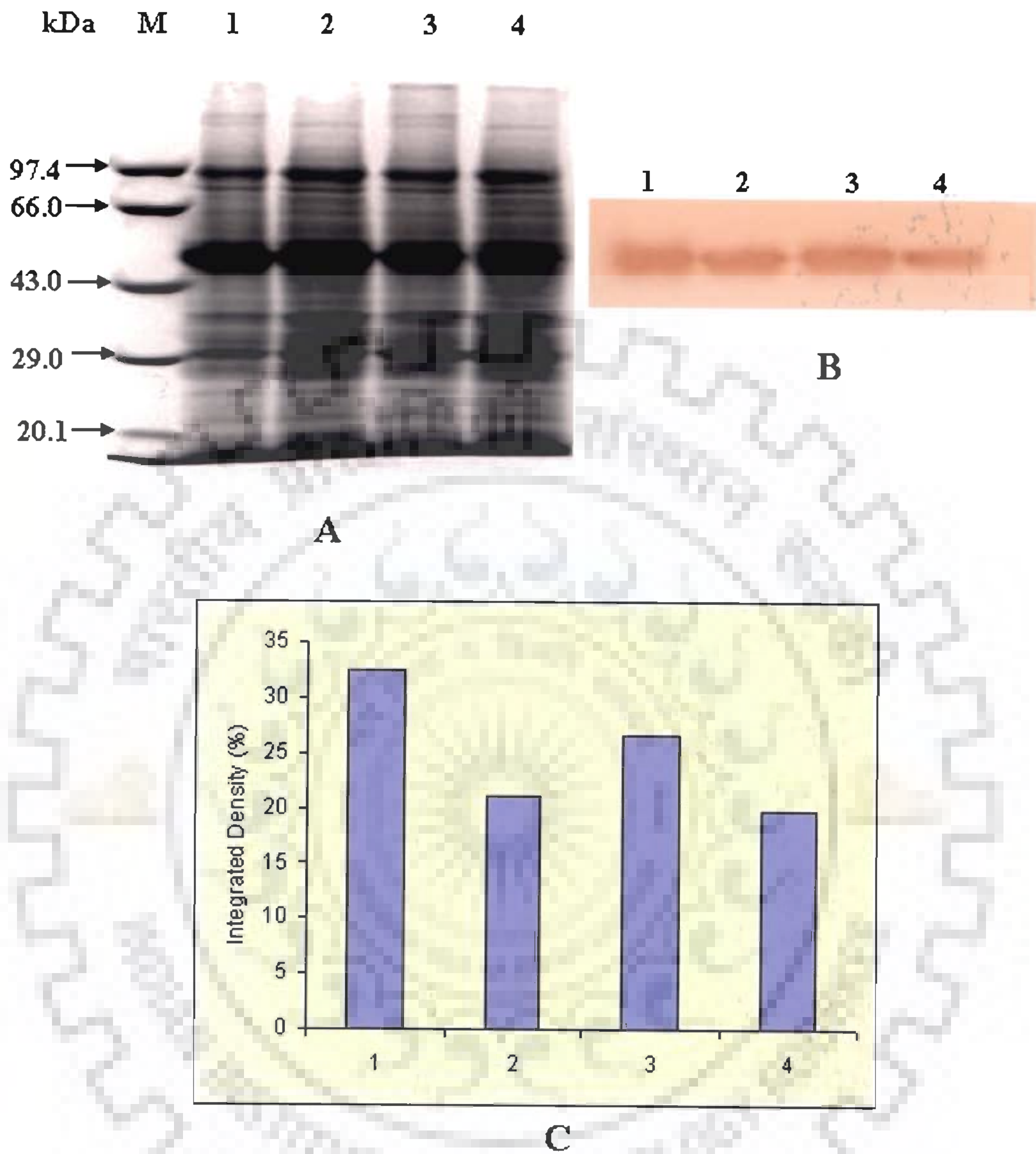


Fig. 24. Tissue specific expression of 48 kDa salt repressed protein in root and shoot of groundnut seedling. (A) Coomassie blue stained SDS-PAGE protein profile (B) Western blot using polyclonal antibody against 48 kDa protein. Lane 1: root control; lane 2: root 150 mM NaCl; lane 3: shoot control; lane 4: shoot 150 mM NaCl. (C) The densitometry analysis 48 kDa protein band from the blot. Molecular markers are shown at left hand side.

4.9 IDENTIFICATION & CHARACTERIZATION OF SALT STRESS INDUCED 31.6 kDa PROTEIN

4.9.1 Identification by MALDI-TOF Peptide Mass Fingerprinting

Attempt was made to identify the protein by peptide mass fingerprinting in which proteins was digested in-gel with trypsin and a mass spectrum of the resulting peptides (a peptide mass fingerprint) was acquired with a matrix-assisted laser-desorption/ionization time of flight (MALDI-TOF) mass spectrometer. The list of apparent peptide masses was then used to screen databases for correspondence to predict tryptic digests of known proteins.

The result obtained by MALDI-TOF peptide mass fingerprinting is given in Fig. 25, searching MSDB (Mass Spectrometry protein sequence DataBase) several proteins showed some matching with the peptides masses obtained after mass finger printing. These are- hypothetical protein T6h22.17, AE005173 NID: - *Arabidopsis thaliana*, NADP sorbitol-6-phosphate dehydrogenase-*Malus domestica* (Apple) (*Malus sylvestris*), AT3g27330/K1G2_3-*Arabidopsis thaliana* (Mouse-ear cress), Hydroxyproline-rich glycoprotein-like-*Oryza sativa* (japonica cultivar-group)., Xylanase inhibitor (Fragment)-*Secale cereale* (Rye), NBS-LRR-like protein-*Oryza sativa* (japonica cultivar-group), Pentatricopeptide (PPR) repeat-containing protein-like- *Oryza sativa* (japonica cultivar-group), Hypothetical protein.- *Oryza sativa* (Rice). Among them, NADP sorbitol-6-phosphate dehydrogenase-*Malus domestica* (Apple) (*Malus sylvestris*) was seen to have some significant role in salinity stress tolerance. Therefore, further experiments were done to study the activity of NADP sorbitol-6-phosphate dehydrogenase and sorbitol accumulation in groundnut seedlings.

4.9.2 Effect of NaCl Stress on Sorbitol Accumulation and NADP-Dependent Sorbitol-6-Phosphate Dehydrogenase activity

4.9.2.1 Detection of sorbitol in seedlings of groundnut under NaCl stress

Since, NADP-Dependent Sorbitol-6-Phosphate Dehydrogenase plays a key role in synthesis of sorbitol in plants under stress and the results obtained by peptide mass fingerprinting showed similarity of 31.6 kDa protein with NADP-Dependent Sorbitol-6-Phosphate Dehydrogenase, therefore the effect of NaCl stress on accumulation of sorbitol and NADP-Dependent Sorbitol-6-Phosphate Dehydrogenase activity was studied. The accumulation of sorbitol was determined by gas chromatography and results are given in Fig. 26 (A-F). Peaks corresponding to the retention time (7.3 min) for pure sorbitol was detected in all seedlings and area of peaks increased with the level of NaCl stress. The sorbitol concentrations in control and 150 mM NaCl stressed seedlings were 0.0342 μmol g^{-1} fresh weight and 0.0859 μmol g^{-1} fresh weight, respectively.

4.9.2.2 Assay of NADP-dependent sorbitol-6-phosphate dehydrogenase activity

NADP-Dependent Sorbitol-6-Phosphate Dehydrogenase activity increased with level of salt stress given to seedlings. The activity in control seedling was observed as 0.0021μmol min^{-1} ml^{-1} and progressively increased to 0.525 μmol min^{-1} ml^{-1} in seedlings grown in 150 mM NaCl stress (Fig. 27A). There exist a positive correlation between the NADP-Dependent Sorbitol-6-phosphate dehydrogenase (S6PDH) activity and sorbitol accumulation under NaCl stress in groundnut seedlings (Fig. 27B).

4.9.2.3 Analysis of NADP-dependent sorbitol-6-phosphate dehydrogenase by native, SDS-PAGE and Western Blotting

Since NADP-Dependent Sorbitol-6-Phosphate Dehydrogenase is reported to be dimeric in several studies, therefore the oligomeric nature of this enzyme extracted from the groundnut seedlings were determined by comparing the SDS-PAGE profile with Native-PAGE and corresponding Western blots with polyclonal antibody raised against 31.6 kDa salt stress induced protein. It was found by Native-PAGE and Western blot (Fig. 28 C & D) that the protein band had shited from its position of 31.6 kDa in SDS-PAGE to 63 kDa in Native-PAGE (Fig. 28A & B). Its migration and broad distribution indicated the homodimeric form of protein and it could be estimated from the molecular weight of the protein in Native PAGE, that it may exist in dimeric form.

4.9.2.4 Partial purification of NADP-Dependent Sorbitol-6-Phosphate dehydrogenase

Further, plant extract was precipitated by ammonium sulphate for partial purification of protein to 40%, 60% and 80% as described in materials and methods, chapter 3. The results of purification are given in Fig. 28 (E & F), where the intensity of protein band was increasing by percent of ammonium sulphate purification and an increase in enzyme activity was observed at every step.

10	20	30	40	50	60	70
MSTGTLSSGY	EMPVIGLGLW	RLEKDELKEV	ILNAIKIGYR	HFDCAAHYKS	EADVGEALAE	AFKTGLVKRE
80	90	100	110	120	130	140
ELFITTKIWN	SDHGHVVEAC	KNSLEKLQID	YLDLYLVHYP	MPTKHNAIGK	TASLLGEDKV	LDIDVTISLQ
150	160	170	180	190	200	210
QTWEGMEKTV	SLGLVRSIGL	SNYELFLTRD	CLAYSKIKPA	VSQFETHPYF	QRDSLVKFCM	KHGVLPTAHT
220	230	240	250	260	270	280
PLGGAAANKD	MFGSVSPLDD	PVLNDVAKKY	GKSVAQICLR	WGIQRKTAVI	PKSSKIQRLK	ENLEVLEFQL
290	300	310	320			
SDEDMQLIYS	IDRKYRTSLP	SKTWGLDVYA				

A

NADP sorbitol-6-phosphate dehydrogenase.- Malus domestica (Apple) (Malus sylvestris).

MSTGTLSSGYEMPVIGLGLWRLEKDELKEVILNAIKIGYRHFDCAAHYKSEADVGEALAEAFKTGLVKREELFITTKIWNS
DHGHVVEACKNSLEKLQIDYLDLYLVHYPMPTKHNAIGKTASLLGEDKVLDIDVTISLQQTWEGMEKTVSLGLVRSIGLSN
YELFLTRDCLAYSKIKPAVSQFETHPYFQRDSLVKFCMKHGVLPTAHTPLGGAAANKDMFGSVSPLDDPVLNDVAKKYGKS
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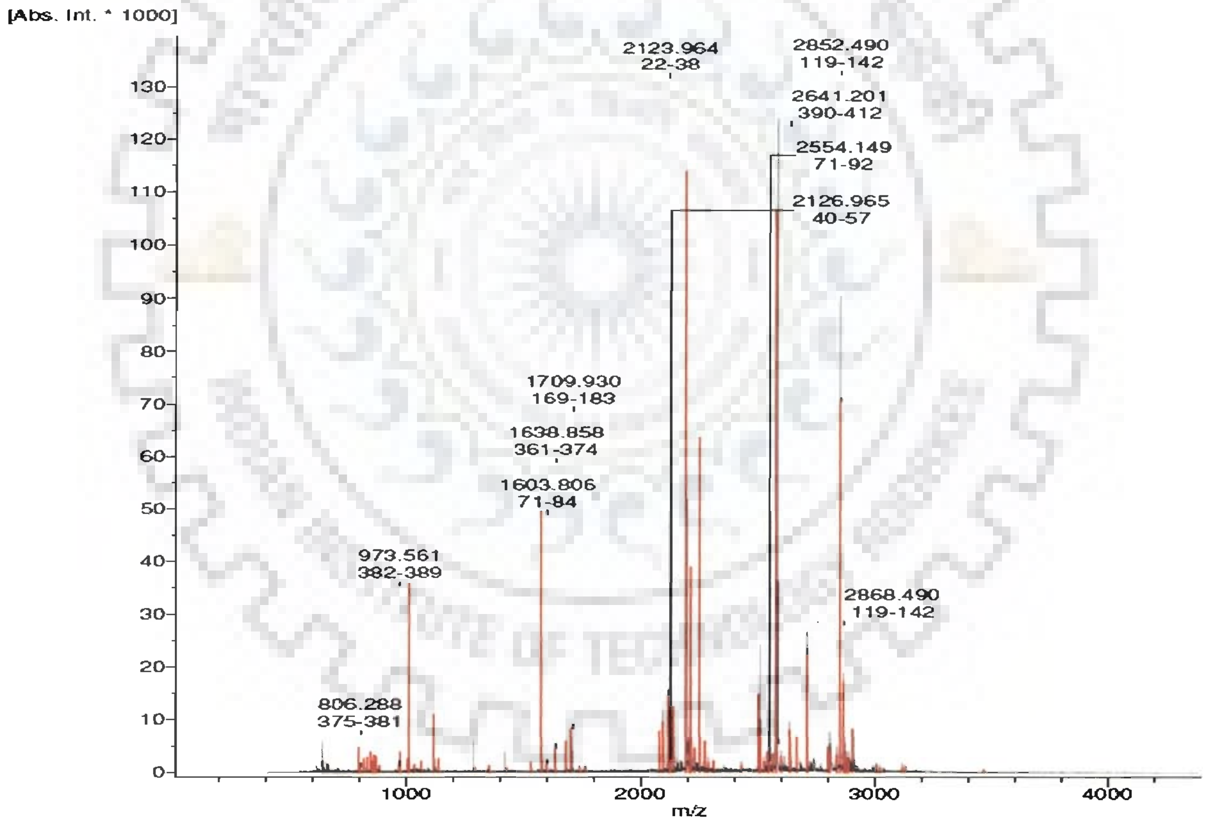
B



C

Fig. 25. Identification of 31.6 kDa salt stress induced protein using peptide mass fingerprinting. **(A)** Alignment diagram showing matching sequence 31.6 kDa protein with NADP sorbitol -6-phosphate dehydrogenase **(B)** complete sequence of NADP-dependent sorbitol-6-phophate dehydrogenase from apple (*Malus domestica*). **(C)** Mass spectra of 31.6 kDa protein.

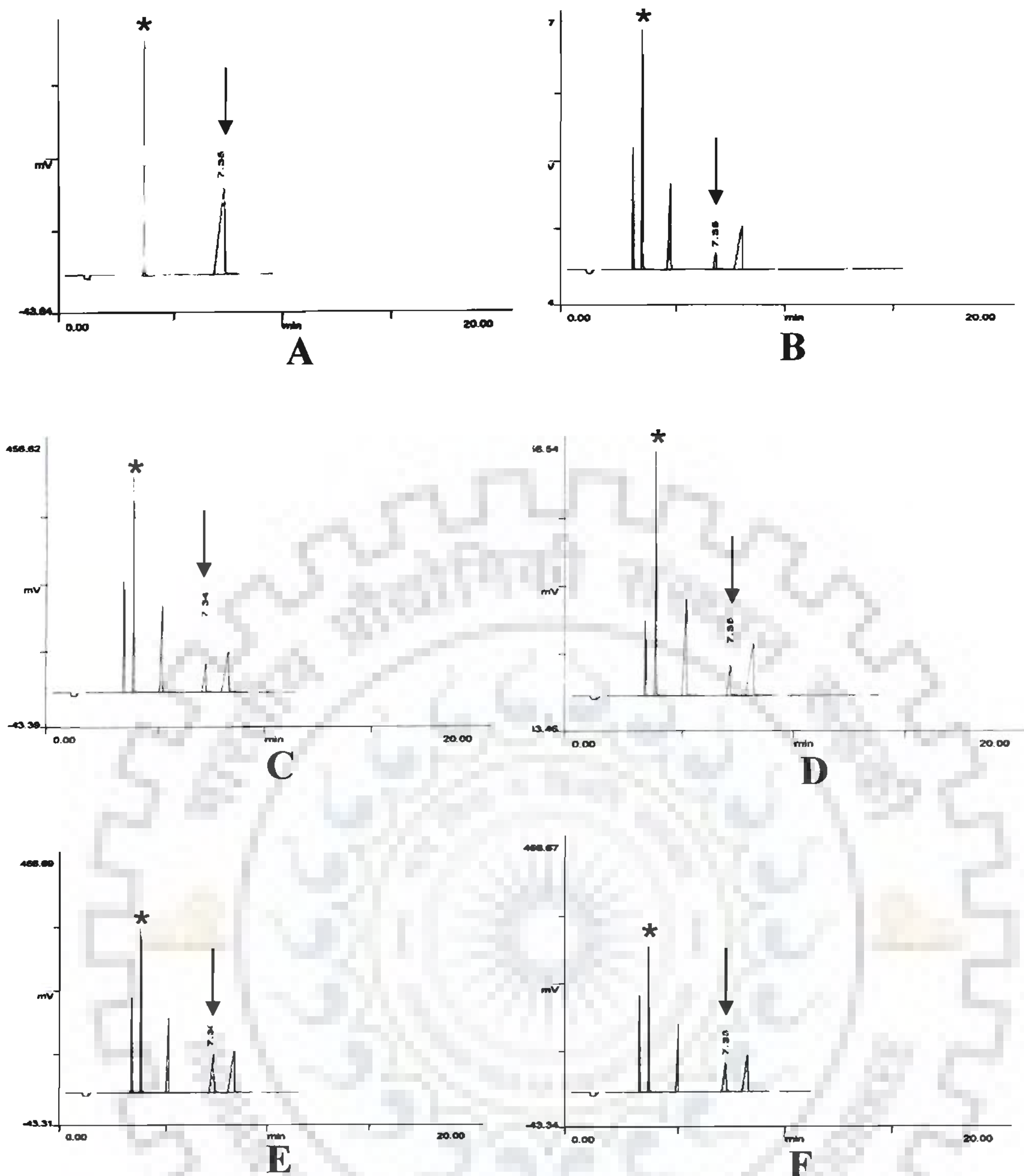
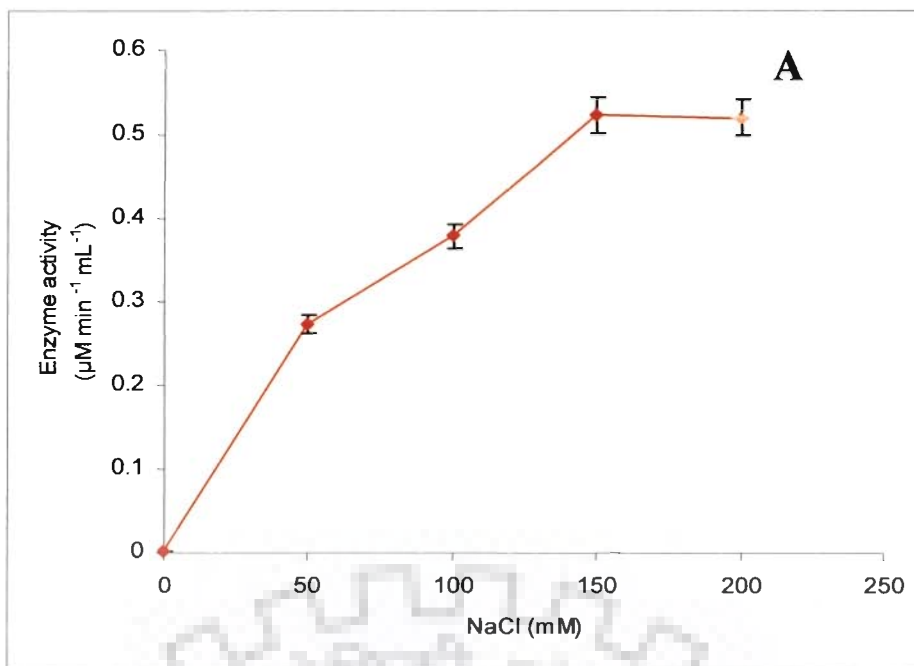


Fig. 26 Gas Chromatograph showing sorbitol accumulation in groundnut seedling grown in different (0-200 mM) NaCl concentration. (**A**) Standard sorbitol (**B**) Control (**C**) 50 mM NaCl (**D**) 100 mM NaCl (**E**) 150 mM NaCl (**F**) 200 mM NaCl . Arrow indicates the sorbitol peak. (*) indicates internal standard.



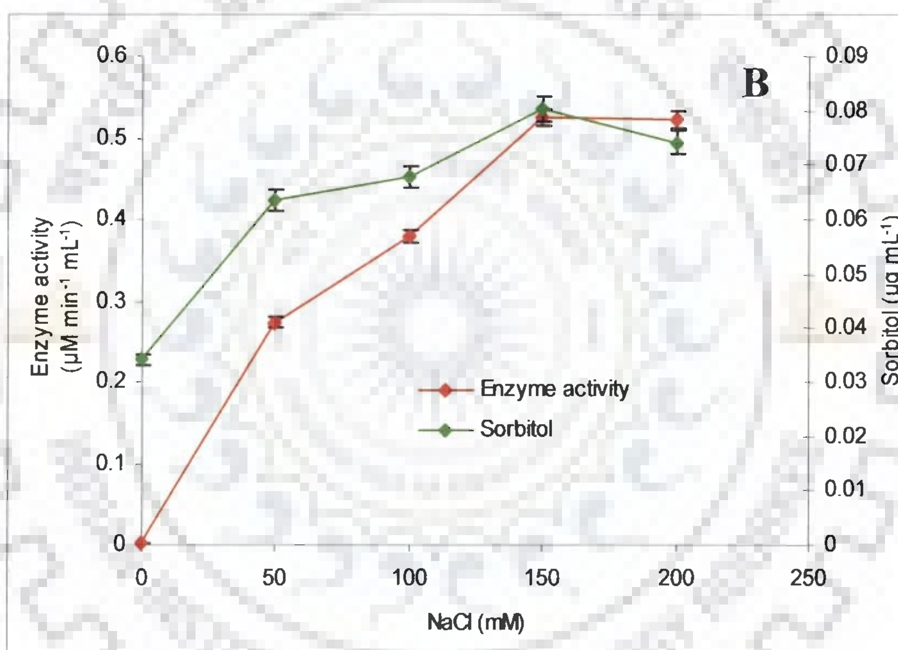


Fig. 27. Effect of salt stress on the activity of NADP-dependent sorbitol-6-phosphate dehydrogenase and sorbitol accumulation in groundnut seedling grown in different (0-200 mM) NaCl concentration for seven days. (**A**) Activity of NADP-dependent sorbitol-6-phosphate dehydrogenase. (**B**) Correlation between the of NADP sorbitol-6-phosphate dehydrogenase activity and sorbitol accumulation with increasing NaCl concentration. Values are in triplicate ± S.E.

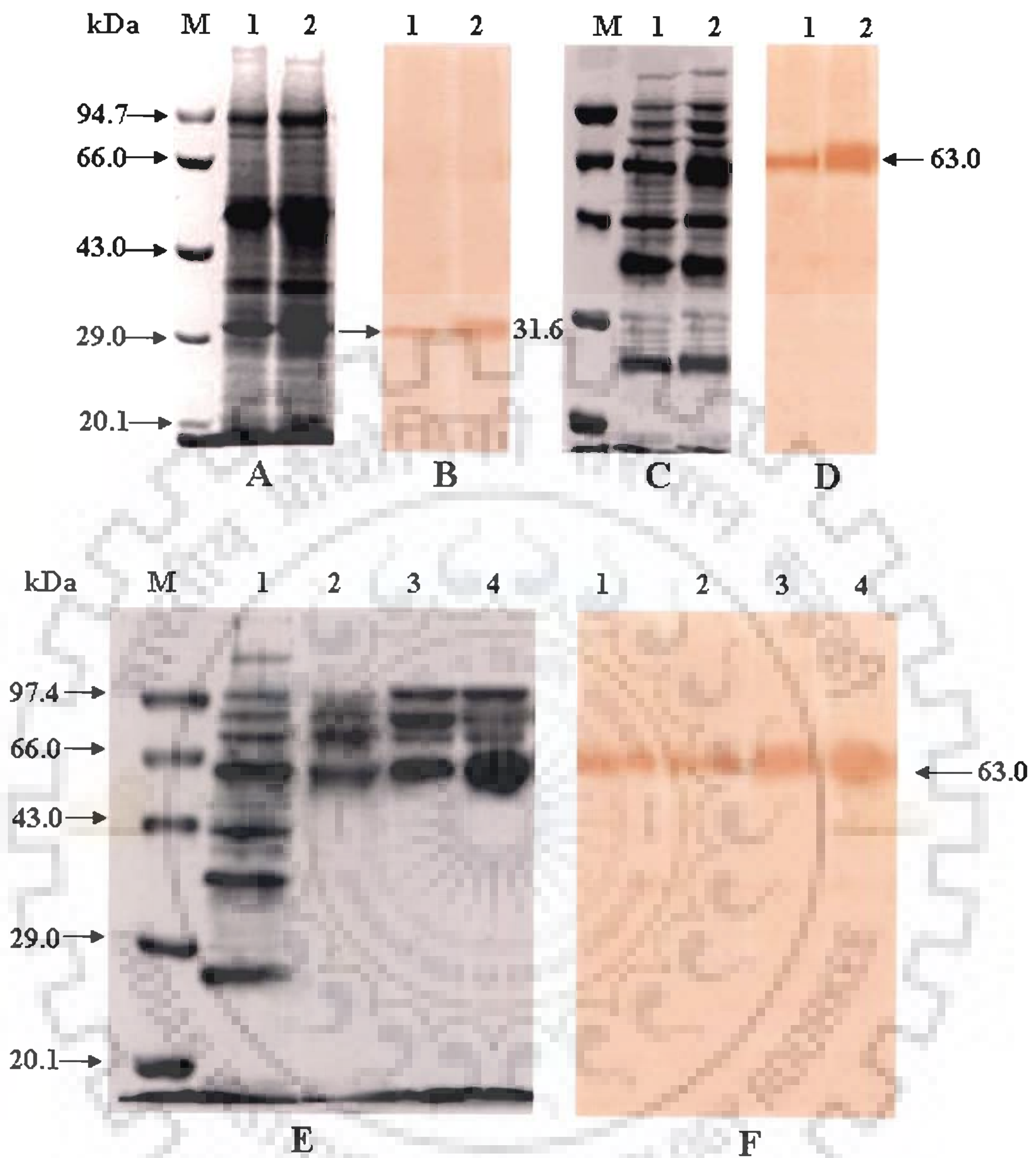


Fig. 28. Analysis of NADP-dependent sorbitol-6-phosphate dehydrogenase (A) & (C) Coomassie blue stained SDS-, and Native-PAGE of crude enzyme protein profile, respectively (B) & (D) Western blot of SDS and Native PAGE using antibody raised against NaCl stress induced 31.6 kDa protein. Lane 1: Control; lane 2: 150 mM NaCl stressed sample. (E) & (F) Coomassie blue stained Native-PAGE and Western blot of partially purified NADP-dependent sorbitol-6-phosphate dehydrogenase. Lane 1: crude; lane 2: 40% $(NH_4)_2SO_4$; lane 3: 60% $(NH_4)_2SO_4$; lane 4: 80% $(NH_4)_2SO_4$ purified enzyme (10 μg loaded). Markers are shown at left side.

4.10 IMMUNOLOCALIZATION OF 31.6 kDa PROTEIN IN GROUNDNUT ROOT & SHOOT

In an attempt to see the distribution and localization of identified 31.6 kDa protein in the seedlings of control and NaCl stress immunofluorescence study was carried out using polyclonal antibody. Goat anti-rabbit IgG- FITC were used as secondary antibody. The immunofluorescence micrographs of the observations are shown in Fig. 29 and 30. In root cell, it was observed that the level of fluorescence in control seedlings was slightly lower than 150 mM NaCl treated seedlings. The fluorescence was localized in the cytoplasmic portion of the cell indicated the presence of protein in plastid (Fig. 29B & D), Fig. 29A and C are the micrographs of the same field as immunoflourescence micrographs are shown.

In shoot cell, the level of fluorescence in 150 mM NaCl treated seedling was considerably higher than control seedling (Fig. 30B & D). Likewise to root cell, in shoot cell also the localization of fluorescence was at cytoplasmic portion and pattern of fluorescence indicated the 31.6 kDa protein may localized in plastid, Fig. 30A and C are the micrographs of the same field as immunoflourescence micrographs are shown.

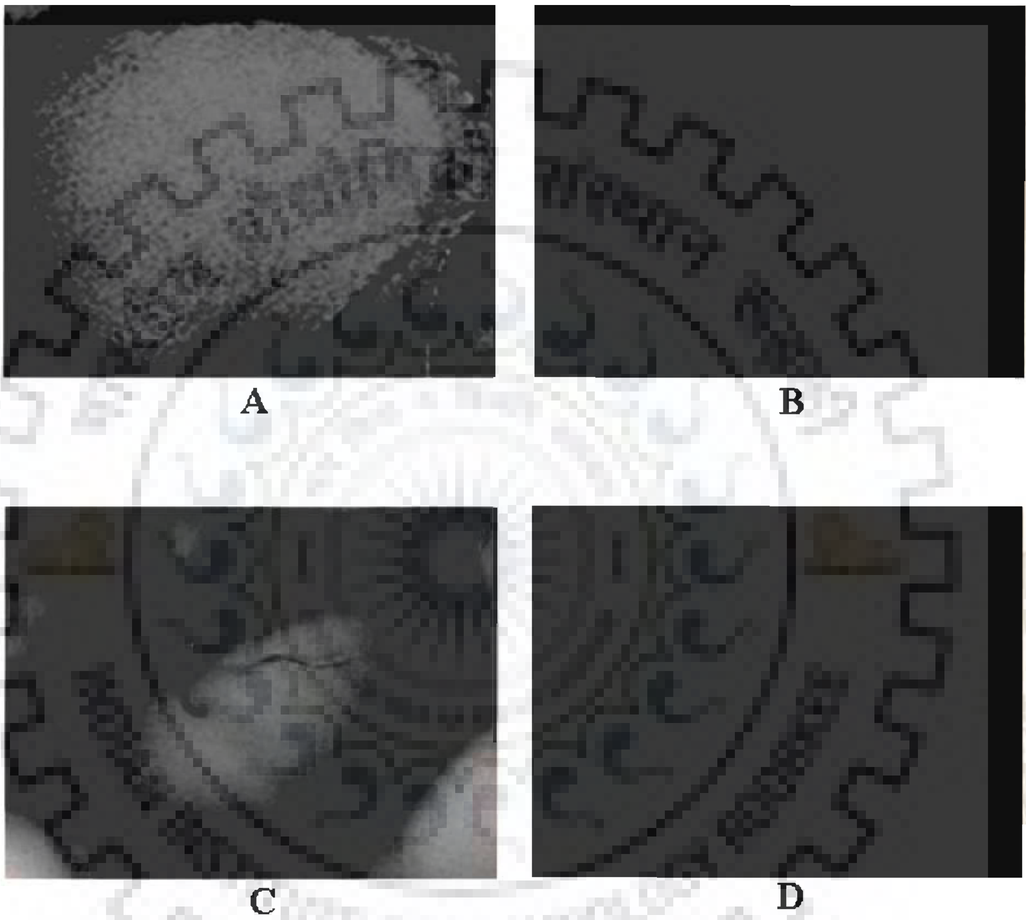


Fig. 29. Immuno-localization studies of 31.6 kDa protein in root sections of groundnut seedling grown in control and 150 mM NaCl concentration. (A) & (C) are micrographs of root cell grown in absence and 150 mM NaCl respectively. (B) & (D) are immunofluoresence images of the same area. (40 X, zoom 6)

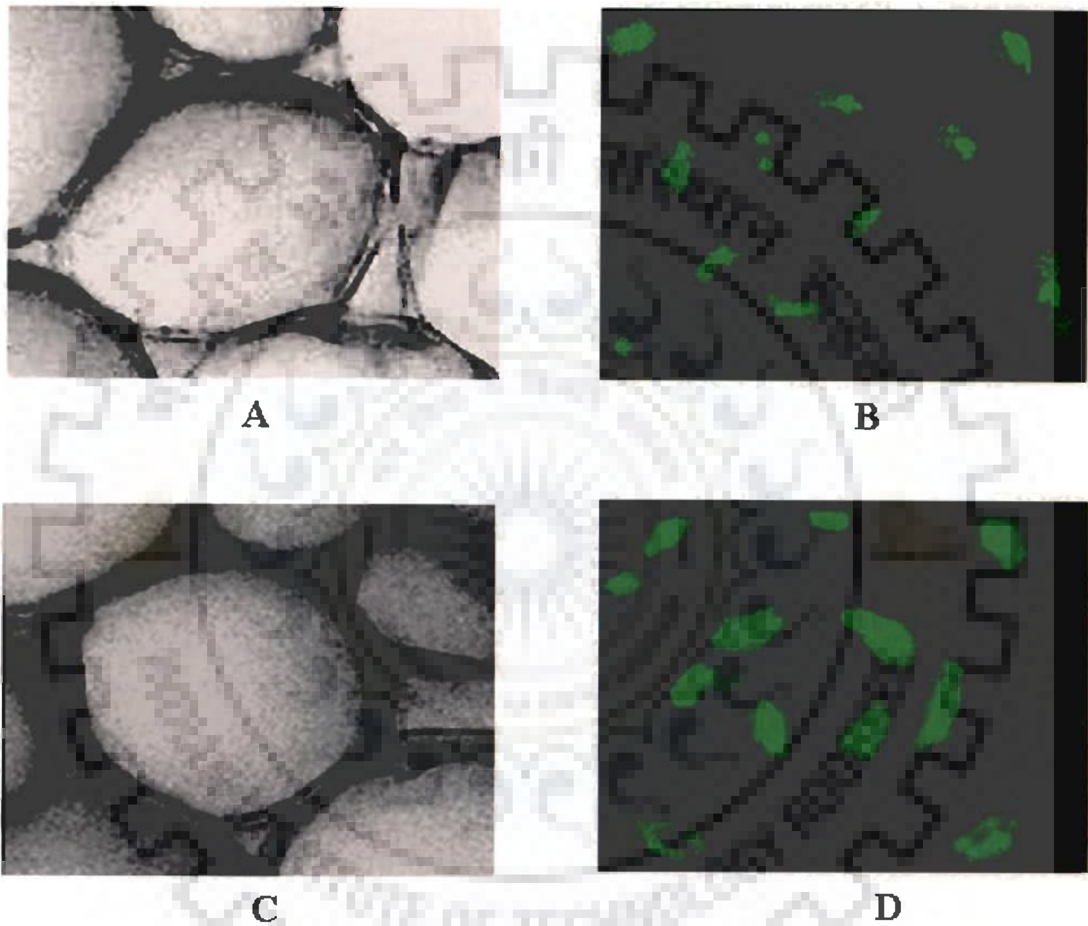


Fig. 30. Immuno-localization studies of 31.6 kDa protein in shoot sections of groundnut seedling grown in control and 150 mM NaCl concentration. (A) & (C) are micrographs of shoot cell grown in absence and 150 mM NaCl, respectively. (B) & (D) are immunofluoresence images of the same area. (40 X, zoom 6)

CHAPTER-5

DISCUSSION & CONCLUSIONS

CHAPTER -5

DISCUSSION AND CONCLUSION

Salinity of arable land is an increasing problem of many irrigated, arid and semi-arid areas of the world and it is one of the major environmental abiotic stresses affecting plant growth and productivity (Francois and Maas, 1994). Soil salinity affects almost every aspect of the physiology and biochemistry of plants which reduces yield significantly. It has direct influence on food production, therefore, it has been one of the thrust research areas globally and attempts are being made to study the effect of salt stress on various plants and to understand the basis of salinity tolerance (Sairam and Tyagi, 2004; Parida and Das; 2005; Ashraf and Harris, 2004). Salinity has been found to have adverse affects on the processes such as seed germination; seedling growth and vigour; vegetative growth; flowering and fruiting. Although all stages of plants are affected by the salt stress but the seedling and reproductive stages that are most affected and ultimately result in poor produce and low yield (Vicente et al., 2004). It has been argued that salt stress responses observed at seedling level may not be a true reflection at mature plant level. However, it has been observed in number of studies that salt tolerance shown at seedling level is also retained at mature plant level in most cases. Therefore, study of salt stress responses at the seedling level reflects the response of mature plants and understanding of the molecular mechanism at seedling level will be helpful in developing salt tolerant crops. In present work an attempt was made to study salt stress response in groundnut (*Arachis hypogaea* L.) seedlings. The effects of NaCl stress on the growth parameters like seedling root and shoot growth, fresh weight, and anatomical

changes and on various biochemical and molecular changes at the level of groundnut seedling were investigated. The findings and their possible role in salt tolerance are discussed.

5.1 MORPHOLOGICAL, ANATOMICAL AND BIOCHEMICAL CHANGES UNDER SALINITY STRESS

The effects of NaCl stress on growth parameters of groundnut seedlings were studied. The results clearly indicated that NaCl had an adverse effect on growth of groundnut seedlings and severity increases at higher salt concentration. The increase in root/shoot ratio indicated that shoot growth was more affected compared to root. The reduction in the growth of plant under salinity stress is a well known response of most of the glycophytes but there found to be great variation among species as well as cultivars and also among the different plant growth parameters recorded (Romero-Aranda et al., 2001; Meloni et al., 2001; Ghoulam et al.,2002). The extent of loss or damage by salinity stress depends on: the degree of salinity, the degree of susceptibility of plant species and varieties to salinity (Bolarin et al., 1991), environmental conditions like temperature, humidity etc (Shannon et al., 1994) and to some extent also associated with the developmental stage of plant (Vicente et al., 2004). This observed reduction in shoot and root growth of groundnut seedling in present study is in good agreement with the similar studies in other species of plant. The cane length in red raspberry (*Rubus idaeus* L. 'Autumn Bliss') reduced gradually when grown in increasing salt concentration (Neocleous and Vasilakakis, 2007). Reduction in root and shoot length of seedlings has also been reported in sunflower (*Helianthus annus* L.), cabbage (*Brassica oleracea var. capitata*), *Chrysanthemum morifolium* and, *Salvodora persica* (Hossain et al, 2004;

Ramoliya et al., 2004). The relatively higher growth inhibition of shoot compared to root of groundnut seedlings observed in present study under NaCl stress was similar to the results found in rice cultivars (Moons et al., 1995). The physiological significance of root-to-shoot ratio variations in plant stress adaptation is still an active debatable topic (Moya et al., 1999). While an increased root-to-shoot ratio is usually documented as a general response to salinity, but both theoretical (Dalton et al., 2000) and experimental (Moya et al., 1999) evidences indicate that a reduced root-to-shoot ratio may improve salinity tolerance by restricting the flux of toxic ions to the shoot and consequently by delaying the onset of the tolerance threshold (Maggio et al., 2007). The decrease in the fresh weight of root and shoot of groundnut seedlings was also observed in present study under salt stress conditions which was more pronounced with increasing NaCl concentration (50 mM to 200 mM NaCl). Decrease in fresh weight of root and shoot under salt stress is common response and there are several reports both from glycophytes (Maggio et al., 2007; Hossain et al, 2004; AliDinar et al., 1999; Moons et al., 1995;) and halophytes (Khan et al., 1999; Parida et al., 2004). The observed steady decrease in fresh weight of root and shoot in the present study is in well agreement with the earlier reports where a steady decrease in fresh weight has been reported with increasing salt concentration.

Based on the studies from various plants it has been suggested that in general, salinity can reduce the plant growth or damage the plants through: (i) osmotic effect (causing water deficit), (ii) toxic effects of ions, and (iii) imbalance of the uptake of essential nutrients. These modes of action may operate on the cellular as well as on higher organizational levels and influence all the aspects of plant metabolism (Ramoliya

et al., 2004). According to Dubey (1997) and Yeo (1998) salt causes both ionic and osmotic effects on plants and most of the known responses of plants to salinity are linked to these effects. The salt stress effects plant growth at two levels. The initial and primary effect of salinity, especially at low to moderate concentrations, is due to its osmotic effects (Munns and Termaat, 1986; Jacoby, 1994). Osmotic effects of salts on plants are a result of lowering of the soil water potential due to increasing solute concentration in the root zone. At very low soil water potentials, this condition interferes with the plant's ability to extract water from the soil and maintain turgor. Thus, in some species salt stress may resemble drought stress. However, at low or moderate salt concentrations (high soil water potentials), plants adjust osmotically (accumulate internal solutes) and maintain a potential for the influx of water (Guerrier, 1996; Ghoulam et al., 2002). Plant growth may be moderate under such conditions, but unlike drought stress, the plant is not water deficient (Shannon, 1984). At high salinity, some specific symptoms of plant damage may be recognized, such as necrosis and leaf tip burn due to Na^+ or Cl^- ions (Wahome et al., 2001). High ionic concentrations may disturb membrane integrity and function; interfere with internal solute balance and nutrient uptake, causing nutritional deficiency symptoms similar to those that occur in the absence of salinity (Grattan and Grieve, 1999).

Though we have not determined the Na^+, K^+ or other ions level in the present study, yet it is suggested that these factors are involved in the reduced growth of root and shoot of groundnut seedlings at higher NaCl concentrations (>100 mM). This is mainly because these are the common mechanism of growth reduction in most of the plants under salinity

though the salt concentrations and degree of severity may differ from plants to plant as described earlier.

A correlation was found between growth reduction and anatomical changes observed in various organs under salt stress. Anatomical changes in root and shoot tissues of groundnut seedlings grown under high NaCl (150 mM) concentration were studied. Significant anatomical changes were noticed in root and shoot of salt treated seedlings as compared to control. Reduction in cell size, cell wall thickening and disappearance of intercellular space were seen in salt grown seedling's root and shoot cells. It is likely that the stunted growth observed in groundnut seedlings in present study under salinity stress is due to these anatomical changes in root and shoot tissues. Salinity has been reported to cause anatomical changes in leaf, root and shoot in a number of plants (Longstreth and Nobel, 1979; Hilal et al., 1998; Cachorro et al., 1995; Cachorro et al., 1993; Zenoff et al., 1994; Surjus and Durand, 1996). More emphasis has been given to study the anatomical changes in leaf tissues under salinity because of it roles in photosynthesis. Studies conducted by a number of authors with different plant species showed that photosynthetic capacity was suppressed by salinity (Dubey, 1997; Kao et al., 2001; Ashraf, 2001; Romero-Aranda et al., 2001) and this is attributed to the anatomical changes in leaf tissues. A positive association between photosynthetic rate and plant growth under saline conditions has been found in different crops. Fisarakis et al. (2001) found that inhibition of vegetative growth in plants submitted to salinity was associated with a marked inhibition of photosynthesis.

Anatomical changes in root and shoot of plant grown under salinity have also been reported in earlier studies (Maggio et al., 2001; Hilal et a., 1998). In bean roots, it

was reported that excess NaCl in the growth medium induces structural changes as well as leakage of ions correlated with alterations of the cell membranes (Cachorro et al., 1995). In soybean roots an NaCl-induced acceleration of the development of secondary xylem was also observed (Hilal et al., 1998).

It is reported that tissues exposed to environments with low water availability have generally shown reduction in cell size, increase in vascular tissue and cell wall thickness (Da Silva et al, 2003). The anatomical changes like reduction cell size, cell wall thickness and inter cellular spaces in root and shoot tissues affect the transport of water and nutrient from root to leaves and metabolites from leaves to root tissues and ultimately effecting the plant growth under salt stress. Besides, it has also been observed that cell elongation is inhibited under salt stress (Nonami et al., 1995).

One of the distinctive features of most plants growing in a saline environment is accumulation of low-molecular-mass compounds, termed compatible solutes, which do not interfere with the normal biochemical reactions (Hasegawa et al., 2000; Zhifang and Loescher, 2003). These compatible solutes include mainly proline and glycine betaine (Ghoulam et al., 2002, Girija et al., 2002; Khan et al., 2000). The effect of NaCl on proline accumulation in groundnut seedlings roots and shoots was studied. A significant higher accumulation of free proline in roots (six fold) and shoots (eight fold) of groundnut seedlings grown at 150 mM NaCl or higher concentration as compared to the controls was observed. Proline has two functional roles: at high concentrations, osmotic adjustment; and at low concentrations, an unknown protective role. High level of proline enables the plant to maintain low water potential mainly by allowing additional water to be taken up by the cells and thus works as osmoticum. In addition to its role as a

cytoplasmic osmoticum, proline may function as carbon and nitrogen source for post-stress recovery and growth (Fukutaku and Yamada, 1984), a stabilizer for membranes, protein synthesis machinery (Kardpol and Rao, 1985) and enzymes (Paleg et al., 1984), scavenger of free radicals (Simrnoff and Cumbes, 1989; Saradhi et al, 1995), and as a sink for energy to reduce redox potential (Alia and Saradhi, 1993).

A positive correlation was found between proline accumulation and osmotolerance in most cases with some exceptions (Delauney and Verma, 1993; Liu and Zhu, 1997; Ashraf and Foolad, 2007). It is suggested that increased proline concentration under high salt concentration in the present study serving both functions, acting as osmoticum and also protective roles particularly as stabilizing protein structures and activities (as exogenous proline observed to increase peroxidase activity in the present study which is discussed in more detail later). This has also been proven from two earlier studies on groundnut where increase in proline under salinity stress were observed and it found to serve as osmoticum (Jain et al., 2001b; Girija et al, 2002) and also provide protection against salt stress induced lipid peroxidation groundnut cell lines (Jain et al., 2001b). Besides, there are many studies which indicate the accumulation of osmolytes particularly free proline in plants under salt stress and interaction of proline with enzymes to preserve protein structure & activities (Kavi Kishor et al., 2005; Delauney and Verma, 1993; Bohnert and Jensen, 1996; Trotel, et al., 1996; Sarvesh, et al., 1966; Schat, H., 1997; Kohl, et al., 1991) and in rice cultivars (Demiral and Turkan, 2005; Hien et al., 2003).

Salt stress has great effect on cell wall architecture. The thickening of cell wall and inhibition of cell elongation are the most common effect which are the main causes

of reduction in growth of roots and shoots under salinity. Since cell wall associated peroxidases and hydrolases (α-galactosidase, β-galactosidase, β-glucosidase and acid phosphatase) found to play important role in cell elongation and growth (Minic and Jouanin, 2006) effect of NaCl on cell wall bound peroxidases and hydrolases was studied. Salt stress found to have profound effects on various peroxidase and hydrolases (Botella et al., 1994; Lin and Kao, 1999; Quiroga et al., 2001; Roxas et al., 2000). There was decrease in hydrolases activities (α-galactosidase, β-galactosidase, β-glucosidase and acid phosphatase) under salinity stress in groundnut. The growth of plants depends on the continuous growth and development of cells which require degradation of cell wall polysaccharides, by the action of cell wall hydrolases (Minic and Jouanin, 2006). The percent relative activities of α-galactosidase, β-galactosidase, β-glucosidase and acid phosphatase decreased under salinity stress progressively with the level of stress. The decrease in α-galactosidase, β-galactosidase, and β-glucosidase 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 (Cassab and Varner 1988). Decrease in acid phosphatase activity may also imply fewer amounts of free PO_4^{3-} ions in cell wall under salinity stress.

Peroxidase has been found to play diverse role in plants and have been suggested to be involved in various metabolic steps such as auxin catabolism (Normanly et al., 1995), the formation of isodi-Tyr bridges in the cross-linking of cell wall proteins (Schnabelrauch et al., 1996), the cross-linking of pectins by diferulic bridges (Amaya et al., 1999), and the oxidation of cinnamyl alcohols prior to their polymerization during

lignin and suberin formation (Roberts et al., 1988; Whetten et al., 1998). The role of peroxidase as stress enzyme in plant has been acknowledged (Gasper et. al., 1982;, Harinasut et al., 2003). There are various isoforms of perxidases which play important role in combating the stress effects but the cell wall associated peroxidase isoforms have been of major concern due to their critical role in cell growth and development (Sanchez et al, 1996). Thus effect of salt stress on cell wall associated peroxidases and it correlation with the reduced seedling growth under salinity stress were studied. In contrast to hydrolases there was a significant increase in peroxidase activity in groundnut seedlings grown under high salt concentration (150mM or more). The most prominent increase was observed in an ionically bound anionic peroxidase isoform. NaCl induced inhibition of growth has often been related to decreases in the plastic extensibility of the growing cell walls in root, shoot and leaf (Chazen and Neuman, 1994; Neuman et al, 1994). Neuman et al, (1994) have demonstrated that root growth inhibition caused by salinity was associated with cell wall stiffening. A key role of cell wall peroxidase in the stiffening of cell wall is well established and consequently, in the growth reduction by reduction of cell elongation (Fry, 1986). A negative correlation was found between cell wall peroxidase activity and growth rate (Lin and Kao, 2001; Quiroga et al., 2000). Therefore, it is assumed that the increased ionically bound anionic peroxidase activity is associated with growth inhibition of NaCl stressed groundnut seedling by causing stiffening of cell wall. This is in good agreement with one of the earlier study where the ionically bound peroxidase activity reported to be associated with the growth inhibition of rice seedling root caused by NaCl (Lin and Kao, 1999).

Since the peroxidase activity was assayed using Guaiacol which is not a natural substrate for peroxidase in the cell-wall stiffening process and is a substrate for all kind of peroxidases. Therefore, to confirm that an increase in ionically bound cell wall peroxidase is indeed associated with NaCl induced growth reduction in groundnut, activity of ionically bound cell wall peroxidase was also assayed using ferulic acid which is a natural substrate. The ionically bound peroxidase showed specificity for ferulic acid and peroxidase mediated dimerization or trimerization of ferulic acid was observed. Thus it confirms that increase in ionically bound peroxidase is associated with NaCl induced inhibition of groundnut seedlings root and shoot. A key role in the cell wall stiffening of ferulic acid dimerization catalyzed by cell-wall peroxidase has been reported (Sanchez et al, 1996). Similar observation has been made in earlier study where the increase ionically bound peroxidase found to be associated with ferulic acid mediated NaCl induced growth inhibition of rice seedling root (Lin and Kao, 2001). There are several studies indicating an association between increase in ionically bound peroxidase activity and reduction of cell growth (Lin and Kao, 1999; Gardiner and Cleland, 1974; Goldberg et al., 1986; MacAdam et al., 1992; Rama Rao et al., 1982; Sanchez et al., 1995). It has been reported that peroxidase can catalyze phenolic crosslinks between macromolecules such as lignin, proteins, hemicellulose and ferulic acid (Gladys et al.,1988; Quiroga et al., 2000). It has been suggested that peroxidase may restrict growth by rigidifying the cell wall by catalyzing the covalent conversion of ferulyl side chain into diferulyl crosslink and by the non covalent conversion of soluble phenolics into hydrophobic quinines. Ferulyl side groups confer certain soluble polysaccharides with the property of producing gel upon oxidation with H_2O_2 and peroxidase. It has been speculated that this oxidative gelatining

of matrix polysaccharides in cell wall may effects its extensibility and thus growth rate (Gladys et al., 1988).

The salinity stress makes it difficult for plant to maintain the water content of tissues. One of the first reactions to salt stress on cellular level is hardening of the cell wall. The observed thickening and stiffening of cell wall were observed in the cells of cortex and epidermal layers salt grown seedling root and shoot of groundnut as revealed from anatomical studies. This serves to limit the cell expansion and reduced the further demand for water. This thickening of cell wall takes place due to peroxidase catalysed ferulic acid mediated suberization or lignification which contribute to control the water movement (Whetten et al, 1998). However, whether the increased ionically bound peroxidase is involved in suberization /lignification is yet to be confirmed.

It has been reported in previous studies that proline accumulation is correlated with root growth inhibition in rice (Lin and Kao, 1996), seedling induced by NaCl and that exogenous application of proline in the absence of NaCl result in reduction of root growth in rice (Chen and Kao, 1995; Lin and Kao, 1996). In present study there was found to be a correlation between proline accumulation and growth inhibition of groundnut seedling root and shoot induced by NaCl. The exogenous application of proline was also found to increase the ionically bound peroxidase activity in root and shoot of groundnut seedlings. The observations that groundnut seedlings treated with proline which resulted in an increase in ionically bound peroxidase activity in roots and shoots, reduced the growth of root and shoot in similar manner as NaCl did, further supports that the cell-wall stiffening catalyzed by ionically bound peroxidase is involved in roots and shoots growth reduction of groundnut seedlings. Similar observations have

been made in earlier study in rice where the exogenous proline increased the ionically bound peroxidase activity and involved in root growth reduction (Chen and Kao, 1995; Lin and Kao, 1996). In *Pancratium maritimum* L. roots the ionically bound peroxidase activity in presence of exogenous proline decreased at low salt level but increased at higher salt levels, while in shoots it showed increment gradually (Khedr et al., 2003). The consistent increase in peroxidase activity shows its importance and stability during salt stress condition.

5.2 MOLECULAR STUDIES IN PLANT UNDER SALINITY STRESS

As expected there were differences in protein profiles of salt treated and control groundnut seedlings. It was revealed both from SDS-PAGE and 2D-PAGE protein profiles. A number of proteins found to be induced while a few were suppressed under salinity stress. Most prominent change was seen in two proteins of 31.6 kDa & 48 kDa. There was gradual induction of 31.6 kDa protein bands and repression of 48 kDa band at higher NaCl concentrations (Fig.13). The changes in protein profiles of salt grown and control seedling better reflected in 2D-PAGE due to its better resolution compared to SDS-PAGE. Expression of a several proteins was induced while a number of them suppressed in salt treated seedling. A few newly synthesized proteins under salinity stress were also observed which were not present in control samples. The proteins ranged in molecular weight from 16-120 kDa and pI from 4.5-6.5. Salt treatment caused alteration in the relative amount of proteins. The quantitative changes occurred in a heterogeneous group of proteins having a broad range of molecular weight and pI. The prominent among the proteins whose amount increased under salt stress were 70, 50, 35 and 23/24 kDa, pI 5.4 (spot nos 2,7,29 and 28); 60 kDa, pI 5.6(spot no 26) and 16 to 23 kDa pI 4.5

to 6.5 spot no 27) (Fig 14 B) and protein which were suppressed such as 70, 64, 50 and 47 kDa, pI 5.2-6.0 (spot no 3,4,5,6 and 10) Fig 14 A, as shown in results.

Our observations are more or less similar to the earlier reports where the expression of a number of proteins induced or suppressed while a few new proteins being synthesized. A number of salt stress induced proteins have been identified from various plants using SDS-PAGE such as: a salinity induced 26 kDa protein in tobacco (Singh et al, 1985; King et al., 1986); 25, 26, 27 kDa protein in citrus and tomato (Ben-Hayyim et al., 1989); 84 kDa protein in winged bean (Esaka et al., 1992); 24 kDa glycoprotein in *Mesembryanthemum crystallinum* (Yen et al.,1994); ABA responsive 21 kDa protein in finger-millet(Aarati et al., 2003). A number of salt stress induced proteins have also been identified from various tissues and developmental stages likes 14.5 kDa, 15 kDa in rice roots (de Souza Filho et al., 2003; Salekdeh et al., 2002), 15 and 26 kDa in shoots (Shirata, et al., 1990), 26 and 27 kDa cultured cells (Shirata, et al., 1990), and 23 kDa germinating seeds (Rani & Reddy., 1994). A number of salt responsive proteins have also been identified from various crops using 2D-PAGE such as: 20-24 kDa, pI 6.3-7.2 and 26 kDa, pI 6.3 and 6.5 in barley roots (Ramagopal 1987; Hurkman and Tanaka, 1987; Hurkman, et al., 1991), 56.1-70.8 kDa, 93.8 kDa in *Brassica* (Jain et al., 1993), 22 kDa in *Raphanus sativus* (Lopez et al., 1994), 18, 19.5, 21, 26, 34, 35.5, 37, 58 kDa in cultured tobacco cells (Singh et al., 1985), and in tomato roots 21 kDa, pI 5.7; 21.5 kDa, pI 5.5; 22 kDa, pI 5.4; and 32 kDa, pI 6.4 protein (Chen and Plant, 1999).

Since salt stress response is a complex and multigenic process, it is likely that these salt induced proteins may have some role in salt tolerance or may be helpful one or other way to cope up with the salt stress related damage. Attempts have been made to

explore the possible role of some of these salt stress induced protein from various plants and role of a few proteins being established using molecular tools and transgenic plant development (Chen et al., 2007; Hmida-Sayari et al., 2005; Holmstrom et al., 2000) and while possible role of some other are being predicted based on their similarly with data bases using proteomics and genomics tools (Munns, 2005; Jain et al., 2006; Sreenivasulu et al., 2007). The salt induced proteins and their genes identified so for can be classified in various functional groups related to their physiologic or metabolic function predicted from sequence homology with known proteins viz. carbon metabolism and energy production/photosynthesis, cell wall/membrane structural components, osmoprotectants and molecular chaperons, water channel proteins, ion transport, oxidative stress defences, detoxifying enzymes, transcriptional factors. Recently a number of salt stress induced protein have been identified from salt tolerant cell line of groundnut (Jain et al., 2006) and rice using proteomics tools (Salekdeh et al., 2002). However, still a large number of salt stress proteins from various plants yet need to be identified.

Since the most remarkable change was seen in the expression levels of 31.6 kDa and 48 kDa, therefore, their expression under different abiotic stresses, tissue and cultivars specific expression were studied.

As osmotic stress is a component of salt stress, it was expected that effects caused by salt would also be induced by osmotic stress. The induction of 31.6 kDa was also seen under different abiotic stresses like KCl, Mannitol, ABA and PEG. The increased levels of expression of 31.6 kDa protein in groundnut seedlings exposed to different environmental stresses indicated us to suggest that the role of this protein is not restricted to saline stress only. The high level of expression by NaCl, KCl and PEG shows that it a

major osmotic stress protein. However, induction of this protein by ABA indicated that it is an ABA responsive protein. On the other hand the suppression of 48 kDa protein also took place in presence of KCl and PEG similar to NaCl but expression of this protein was not affected by ABA indicating it is probably an ABA-independent protein.

Salt stress induced proteins are grouped into ABA-responsive and ABA-independent based on the fact that whether their expression is effected by ABA or not. A number of salt stress induced ABA responsive (LaRosa et al., 1985; Singh et al., 1987; 1989; Claes et al., 1990; Ben-Hayyim et al., 1993; Moons et al., 1997a; 1997b; Aarati et al., 2003) and ABA independent proteins (Esaka et al., 1992; Jang et al., 1998; Chen and Plant, 1999) have been identified from several crops. Similar kind of observations have been reported in earlier studies, for example, a most acclaimed salt stress induced 26 kDa protein named osmotin from tobacco (LaRosa et al 1985), a 14.5 kDa salt stress induced protein named as SALT in rice (de Souza Filho et al., 2003; Claes et al 1990), 21 kDa from finger millet (Arati et al, 2003), LEA from rice (Moons et al., 1997a) and 23-25 kDa protein from citrus (Ben-Hayyim et al., 1993), were also induced under different abiotic stress including ABA. Several salt induced ABA non responsive protein have been reported from various plants like SAP1 a salt induced glycoprotein from winged bean (Esaka et al., 1992) and a Ca^{2+} binding salt induced protein named as AtCP1 from *Arabidopsis* (Jang et al., 1998). Although, most of the studies are on salt stress induced proteins to identify their role in salt tolerance but few are also on proteins suppressed due to stress. Zhu et al., 1993 studied the loss of arabinogalactan protein from plasma membrane of cells adapted to NaCl, which has role

in cell expansion. In present study, suppression of 48 kDa was seen under salt stress and its characterization and possible role in salt stress is yet to be studied.

The expression of 31.6 kDa and 48 kDa proteins were also studied in various groundnut cultivars. There was found to be much variation in the expression of 31.6 kDa protein in control as well as salt grown seedlings among different cultivars. Relatively high level of expression was observed in both salt grown and control seedlings of a drought adapted cultivar namely ICGS-37 compared to other cultivars. Despite the observed differences, the induced expression of 31.6 kDa protein in response to salinity was observed in all cultivars. The induced expression of 31.6 kDa protein in all cultivars under salt stress and relatively high level of expression of this protein both in control and salt stress treated seedlings of drought adapted cultivar indicates the possible role of 31.6 kDa protein in providing salt tolerance but this need to be confirmed by further investigation which are discussed later part of discussion. Variation in expression of salt stress induced protein among different cultivars has been reported in several crops (Uma et al., 1995; Morabito et al., 1996; de Souza Filho et al., 2003; Kong-ngern et al., 2005). In general there found to be a positive correlation between salt stress induced protein level and the level of salinity tolerance (Kong-ngern et al., 2005; Kumar et al., 2000). Kong-ngern et al. (2005) found a positive correlation between level of salinity tolerance and the 31 kDa protein expression levels in rice. However, there are reports which showed that there is no direct correlation between level of expression and salt tolerance level because the cultivars which showed high level of expression do not showed the greater salt tolerance (de Souza Filho et al., 2003).

Similarly the expression of 48 kDa salt repressive protein was repressed in salt treated groundnut cultivars seedlings. There were differences in expression level of this protein both in control and salt treated seedlings of different cultivars. Repression of the protein was relatively lesser in salt treated seedling of drought tolerant cultivars ICGS-37 compared to other. This indicates its possible role in tolerance; however, it is premature to state unless it is confirmed by further study.

The variation in the expression of 31.6 kDa salt induced protein was found in the roots and shoots of salt grown and control groundnut seedling. The protein expression was more in salt treated shoot compared to root. Tissue specific expression of the protein is in well agreement with earlier studies. There are a number of reports showing tissue specific expression of a number of salt induced proteins such a 31 kDa in rice protein expressed more in leaf sheath than root (Kong-ngern et al., 2005), a vascular ATPase expressed more in young leaves and root than mature leaves in ice plant (Low et al., 1996) and 26 kDa protein expressed more in root than shoot in tobacco (King et al., 1986). The tissue specific expression of these proteins is indicated to play role in salt tolerance at tissue level or at whole plant levels depending upon their expression pattern (Kong-ngern et al., 2005: King et al., 1985). Since the expression of 31.6 kDa protein was more pronounced in shoots of salt treated groundnut seedling and thus indicating its major role in shoot but the induction of proteins in roots of salt treated seedlings suggested a speculation about the involvement of proteins in whole plants.

On the other hand, 48 kDa salt repressed protein showed more repression in salt treated shoot compared to root. There are hardly any reports regarding role of salt repressed proteins in salt tolerance as expected. However, these salt repressed proteins

play indirect role in combating the salt stress conditions. Since some of these proteins have been found to play important role growth and development of plants under normal conditions, the suppression of these proteins may limit the water and nutrient demand during stress conditions.

5.3 IDENTIFICATION, CHARACTERIZATION AND IMMUNOLOCALIZATION OF 31.6 kDa SALT STRESS INDUCED PROTEIN

The major 31.6 kDa salt stress induced protein was identified by MALDI-TOF Peptide Mass Finger printing and database match search results showed the 31.6 kDa protein to be a NADP-Dependent Sorbitol-6-Phosphate Dehydrogenase (S6PDH). This opens a vista for us to find the possible role of S6PDH in salinity stress tolerance in groundnut. Since the identification 31.6 kDa protein as NADP-Dependent Sorbitol-6-Phosphate Dehydrogenase was based on the homology matched further studies were carried out to confirm that it is indeed a S6PDH. The S6PDH activity in control and seedling grown under different NaCl concentrations (50-200mM NaCl) was studied. There was increase in NADP-S6PDH activity which clearly indicates that it is a salt inducible enzyme. S6PDH nature of 31.6 kDa protein was also confirmed by the immuno-reactivity of antibody generated against 31.6 kDa protein with partially purified NADP-S6PDH preparation. The recognition of a 31.6 kDa band in SDS-PAGE and ~ 63 kDa band in native PAGE indicates that it is a homodimer protein made of two identical units of 31.6 kDa. The S6PDH from apple seedlings and loquat leaves reported to a homodimer of 36 kDa identical subunits (Kanayama and Yamaki, 1995) and homodimer of 33 kDa identical subunit (Hirai, 1981), respectively. Apple S6PDH gene has been cloned and used for generating stress tolerant transgenic plants, in tobacco (Tao et al., 1995; Sheveleva et al., 1998) and Japanese persimmon (Gao et al., 2001). S6PDH is a

key enzyme in sorbitol biosynthetic pathway and play important role in formation of sorbitol by converting glucpse-6-P to sorbitol-6-P under stress conditions (Kanayama et al., 1995, Nuccio et al., 1999). Accumulation of sorbitol under stress conditions have been reported from number of plants particulary member of rosaceace family (Gao et al., 2001). Sorbitol acts as a compatible solute like other sugar alcohols, mannitol (Tarczynski et al., 1993), D-ononitol (Sheveleva et al., 1997), pinitol (Vera-Estrella et al., 1999) etc. which accumulate during salt stress. The strong correlation between S6PDH activity and accumulation of sorbitol with increasing NaCl concentration in present study confirms the role of enzyme in sorbitol accumulation in groundnut during salt stress. The role of NADP-Dependent Sorbitol-6-Phosphate Dehydrogenase in salinity stress tolerance was also suggested by developing transgenic tobacco with apple cDNA encoding NADP-Dependent Sorbitol-6-Phosphate Dehydrogenase (Tao et al., 1995). Recently, in a study, Japanese persimmon (*Diospyros kaki* Thunb. cv Jiro) was transformed with apple (*Malus x domestica* Borkh.) cDNA encoding NADP-dependent sorbitol-6-phosphate dehydrogenase and a positive correlation between sorbitol accumulation and salt tolerance was suggested (Gao et al., 2001). The salt stress inducible S6PDH reported in the present study is 31.6 kDa protein induced presence of ABA. Our finding is in well agreement with earlier report where S6PDH from apple is reported to be an ABA inducible enzymes as it expression is enhanced in presence of ABA and it has been suggested that ABA-mediated S6PDH plays role in sorbitol biosynthesis under stress responses (Kanayama et al., 2006).

The immunolocalization study indicated that NADP-S6PDH the mainly localized in plastid and express more in shoot compared to root of groundnut seedling. NADP-

S6PDH is reported to localize in chloroplast and cytosol in apple seedling (Kanayama et al., 1992, Kanayama et al., 2006).

The accumulation of sugar alcohols in plants has been linked and correlated to environmental stress tolerance, where sorbitol may serve a multifarious roles: as a compatible solute under conditions of decreasing water potential (Hellebust 1976; Wang and Stutte, 1992), as a scavenger of free radicals under conditions of oxidative stress (Smirnoff and Cumbes, 1989), or as an anti-freeze for chill or frost (Hirai 1983).

5.4 CONCLUSION

The salt stress had adverse effect on growth parameters and there found to be changes in root and shoot histology. There found to be increase in 39 kDa ionically bound cell wall peroxidase isoform which involved in the growth retardation by reducing cell wall extensibility by thickening cell wall. The alteration was observed in protein profiles of salt treated seedling compared to control. The two 31.6 kDa and 48 kDa proteins were identified to be the most prominent salt induced and salt repressive proteins, respectively. The expression of these proteins was studied under various conditions, cultivars, and in different tissues. The 31.6 kDa protein expressed more in shoot compared to root and 48 kDa proteins repressed more in shoot under salt stress compared to root. The 31.6 kDa protein was also found to localize in plastid and identified to be NADP sorbitol-6-phosphate dehydrogenase by mass spectrometry. There was an increase in sorbitol accumulation and NADP-dependent sorbitol-6-phosphate dehydrogenase enzyme activity with increase in salt concentration. Strong correlation between sorbitol and NADP-dependent sorbitol-6-phosphate dehydrogenase and immunoreaction with anti 31.6 kDa antibodies confirmed that it indeed is NADP-

dependent sorbitol -6-phosphate dehydrogenase. The molecular weight of native enzyme molecule was found to be 63 kDa. It is suggested that sorbitol accumulation is one of mechanism of salt tolerance in groundnut and NADP-dependent sorbitol -6-phosphate dehydrogenase plays important role in it.

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