PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF WHEAT CULTIVARS TO SALT STRESS

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Veli Cengiz ÖZALP

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Prof. Dr. Alpay ANKARA

Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

Prof. Dr. Aykut KENCE

Chairman of the Depertment

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science in Biology.

Assoc. Prof. Dr. Meral YÜCEL

Supervisor

Examined Committee in Charge

Prof. Dr. Wayne E. CRISS

Prof. Dr. Ufuk GÜNDÜZ

Prof. Dr. Faruk BOZOĞLU

Assoc. Prof. Dr. Hüseyin A. ÖKTEM

Assoc. Prof. Dr. Meral YÜCEL

ABSTRACT

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ÖZALP, Veli Cengiz

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Supervisor: Assoc. Prof. Dr. Meral YÜCEL

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The responses of crop plants to salinity are important phenomena that influence their development and growth, thus the agronomic traits in arid, semi-arid and irrigated areas. Salinity increases the external water potential limitiing the availability of water for plant. Moreover, salt ions are toxic to the plant.

In the present study, the physiological and biochemical aspects of salt stress on four different wheat cultivars (Bezostaja, Gerek, Kharchia, Tosun) were examined. Salt stress was applied on six days old seedlings as different NaCl concentrations (0.2, % to 20 %) for different stress durations (1 to 5 days). The physiological responses of seedlings to salt stress were determined by Photosystem II and Membrane leakage measurements which were carried on leaf and root tissues respectively. As a result of the physiological measurements, the cultivars, Tosun and Gerek, were selected as salt tolerant and salt sensitive, respectively.

The salt stress induced changes in the in vivo protein patterns of the wheat cultivars (Tosun and Gerek) which were investigated by 35[S]-Methionine incorporation into the new synthesized proteins after 1 day treatment of 4 % NaCl. The proteins were seperated by SDS-PAGE and Two-dimensional gel electrophoresis and revealed by either flourography or silver stain. The quantitative and qualitative changes were identified in the protein patterns of both cultivars after one day of 4 % salt stress. The changes were found to be cultivar specific.

Keywords: Salt-induced Proteins, PS-II Activity, Membrane Stability, Phisiological Screening, Wheat.

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ÖZ

BUĞDAY BİTKİSİNİN TUZ STRESİNE GÖSTERDİĞİ FİZYOLOJİK VE BİYOKİMYASAL TEPKİLER

ÖZALP, Veli Cengiz

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Tuz stresi zirai bitkilerin gelişmesi ve büyümesini kurak ve sulama tarımı yapılan topraklarda etkileyen bir faktördür. Tuz stresi suyun osmotik basıncını arttırarak bitki tarafından emilmesini engeller. Bundan başka tuz iyonlari bitki için toxiktir.

Bu çalışmada dört buğday çeşidinin (Bezostaja, Gerek, Kharchia, Tosun) tuz stresi altında biyokimyasal ve fizyolojik tepkileri incelendi. Tuz stresi altı günlük fidelere değişik derişimlerde (1 %'den 20 %'e kadar) ve değişik sürelerle (1 günden 5 güne kadar) uygulandı. Fidelerin tuz stresine karsı fizyolojik tepkileri yaprak dokusunda PS-II aktivitesi ve kök dokusunda Zar geçirgenligi ölçümleri ile saptandı. Sonuç olarak Tosun tuza dayanikli ve Gerek tuza dayaniksız çesit olarak seçildi.

Tuz stresi altında protein profilleri 35[S]-Methionine'in yeni sentezlenen proteinlerde bir günlük 4 % tuz stresinden sonra kullanılması yolu ile incelendi. Proteinler SDS-PAGE ve iki-boyutlu jel electroforezi yöntemleri ile ayrıldı ve

florografi veya gümüş boyama ile görüntülendi. Her iki çeşidin protein profillerinde de nicel ve nitel farklılıklar gözlendi. Bu farklılıklar çeside özeldi.

Anahtar Kelimeler: Tuz stresi proteinleri, PS-II Aktivitesi, Zar Stabilitesi, Fizyojik seçim, Buğday.

Bilim Dalı Sayısal Kodu: 401.01.05

TABLE OF CONTENTS

CHAPTER II:MATERIALS AND METHODS

LIST OF TABLES

Page

LIST OF FIGURES

Page

NOMENCLATURE

A_x	Absorbance at x wavelenght
ABA	Abscicic Acid
BIS	N,N'-Methylene-bis-acrylamide
BME	ß-Mercaptoethanol
DCPIP	2,6-Dichlorophenolindophenol Natrium Salt
IEF	Isoelectric Focusing
kDa	KiloDalton
PPO	2,5-Diphenyloxazole
POPOP	(1,4-bis-[2-(5-Phenyloxazolyl)]benzene)
PS-II	Photosystem II Complex
SDS	Dodecylhydrogensulfate Sodium Salt
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
2D	Two-dimensional Gel Electrophoresis

CHAPTER I

INTRODUCTION

During growth and development, plants are subjected to a variety of environmental stresses such as heat, drought, cold, anaerobiosis, and salt. Salt stress is one of the major stresses affecting agricultural productivity adversely especially in arid lands. Since major crop plants are relatively low salt tolerant, it is desirable to develop salt tolerant crop plants. In contrast to halophytic plants living in sea water, crop plants are sensitive to salt. Nevertheless within salt sensitive species, there is great diversity with respect to salt tolerance (Greenway 1980).

The classical way to improve crops is through breeding which has been used for a long time. The other way is the use of recombinant gene technology. An understanding of the mechanisms that plants use to cope with those stresses is necessary to select and develop crop plants that are more tolerant to environmental stresses. One approach is to identify individual polypeptides whose levels increase in stress with the assumption that these polypeptides have a function in stress tolerance (Hurkman 1988). When the molecular mechanisms of salt tolerance are understood, then genetic engineering will have the potential to improve the salinity tolerance of crops. The methodology for the transgenic plants is readily available (Potrykus 1990).

But the limiting factor is the isolation of the genes with the capability to improve salt tolerance (halo tolerant genes) (Gaxiola 1992).

1.1 Salinity Problem In Agriculture

Salinity can be defined as the presence of excessive concentrations of soluble salts in the soil that suppress plant growth. Salinity has two components which affect plants; water stress and ion toxicity. Water stress is similar in drought stress, cold stress and salt stress because each results in the drying and desiccation in the extreme case of water stress.

Plants are subjected to salinity in two major ecosystems: i) When they are grown on inherently saline soils or ii) when they are grown on non-saline soils, but irrigated with saline water. This means mineral ions present in the soil or water constitutes salinity.

The major cations contributing to salinity are Na^{+}, Ca^{+2}, Mg^{+2}, K^{+} and anions are Cl^{-}, SO_4^{-2}, HCO_3^{-}, CO_3^{-2}, NO_3^{-2}. There exist also trace ions including B, Sr, Li, Rb, F, Mo, Ba, and Al (Tanji 1989).

Salinity is a serious problem all around the world. It is one of the major limiting factors in Agriculture. Coarse estimates show that only 10 % of the agricultural area are in non-stress conditions. Soil affected with mineral stress conditions occupies an area of 30 % of the world land area. This corresponds an area of $400x10^6$ to $950x10^6$

hectares (Eptein 1980). Of the $230x10^6$ hectares worldwide irrigated lands, one third is affected by excess salinity.

Modifying the environment (Technological approach) with irrigation, fertilisation and the use of protected environments are the central considerations in current crop management practices (Blum 1986).The use of such technologies are limited especially in developing countries because they are costly. This makes an alternative solution to the problem through modification of the physiology of plants (biological approach) attractive. On the other hand, the two approaches can complement each other.

In most agricultural ecosystems, salinity is not at a steady-state and the dynamics of the soil favour the increase in salinity with time. This is observed by the progressive salinization of irrigated lands which are referred to as Secondary salinization. This makes the problem worse since it means the loss of land once productive for agriculture . The Pencap area in India is a recent and dramatic example of the effect of secondary salinization (Flowers 1977). The salts in irrigated lands originate from a saline, high water table or salt in the applied water. The salt in water comes from the dissolution or weathering of rock sand soil. The sea water contains high amounts of salts because of its use in irrigation. These salts are carried with water to where the water goes. When the salts reach the root zone, they accumulate; and when the concentrations are high enough, the crop is no longer able to extract sufficient water from the salty soil, this results in water stress. If water uptake is appreciably reduced, the growth rate of plants is decreased and yield reduction occurs (Ayers 1985).

1.2 Responses of Plants To Salt Stress

Terrestrial plants rarely grow in a natural environment free from water stress for a period of more than a few days. Even in the humid tropics, the plant may undergo brief stress due to dynamic changes in the environment. Plants are dependent up on water for function and survival. The essential role of water in plants range from being a reactant, to serving as a medium for the ionizaton of metabolites and stabilization of biomembranes to an agent to maintain structure rigidity.

The responses of plants to a saline environment are a complex phenomenon involving both cellular and organismic levels. The root cells are the first site subjected to a salty environment. Later the ions are transported to the shoot and accumulated.

Salinity appears to affect two cellular processes. During initial exposure to salt stress, the plant experiences water stress. Water is extracted from the soil by exerting an absorptive force inside the root cells greater than that which holds the water to the soil. Drought makes the soil dry causing a decrease in availability of water to the plant. If the plant can not make sufficient internal adjustments to exert more force, it suffers water stress. This happens also when the root environment is salty. Salt decreases water availability by increasing osmotic pressure of environment. Thus, the plant must exert more force to extract water from the high osmotic pressure environment and this additional force is referred to as the osmotic effect. It is well documented that the osmotic effect causes the reduction of growth. Salinity is closely analogous to drought stress in this respect as both results in water stress and reduced growth. In addition, the plant experiences ionic stress during long term exposure to salinity. Ions leak through the plasma membrane with the water absorbed by the root tissue and accumulate in the cytoplasm where they damage metabolic processes; later

they are transported to the leaves where they accumulate during transpiration. The ions accumulate to a great extent in areas where the water loss is greatest, usually in the leaf tips and leaf edges. The accumulation to toxic levels takes time. The degree of damage depends upon the duration of exposure, concentration by the toxic ion, crop sensitivity and the volume of water transpired by plant. Although a salinity problem can be observed even when these ions are at low concentrations, toxicity is often a complicating factor accompanying water stress.

1.2.1 Physiological And Biochemical Aspects of Salt Stress

Salt affects a wide variety of physiological processes including growth, stomatal closure, osmotic adjustment and photosynthesis. These responses result in conservation of water and avoidance of ion toxicity.

Salinity induces changes in the shoot/root ratio with consequences of increasing root tissue for absorbing more water. But the overall growth is reduced to limit water loss in first place. Such water conservation responses result in a decrease in the rate of carbon uptake; and the biomass production is adversely affected. The reduced growth and a decrease in leaf conapy due to stressed induced senescence are of potential value for plant survival and adaptation.

Leaf ABA (Absiscic Acid) content increases under water stress and this increased ABA serves to close the stomata. Accumulation of ABA is an early event after the onset of stress. Thus, ABA is postulated to act as a co-ordinator of plant response to the water deficit. These responses are the closure of stomata resulting in a

limitation of transpiration, a decrease in water potential and decreased photosynthesis (Scriver 1990).

Cell membranes are the major sites for controlling the active and passive solute flux. The root plasmalemma is the first site affected by salt ions. It will response to its imbalance in ions in the root environment by changing chemical composition and structure. Cell membranes are disrupted under high salt stress. (Leopold 1981).

Plant survival under a water deficit results partly from the maintenance of full photosynthetic capacity by the leaves, thus allowing a rapid recovery after rehydration (Chaves 1991). Potential photosynthetic capacity in a salt tolerant species is only slightly impaired by salt stress. Reduced photosynthesis in stressed plants results from a combination of factors, including increased stomatal and mesophyll resistance and a decrease in total leaf area. The subcellular organelles need a constant microenvironment. Large changes in ion composition and concentrations in the cell may have pronounced effects on protein complexes that may impair the function of the organelles. The chloroplasts including the photosynthetic structures undergo severe ultrastructural changes during water stress. This process is reversible and the rehydrated plants rapidly resume the full photosynthetic capacity under water stress in tolerant species. Photosynthesis is one of the most vulnerable cell process as Heat stress is also known to affect this process very adversely. Such effects are expected under salt stress. The decrease in the photosynthetic rate under salt stress must depend upon the direct inhibition of photosynthetic processes other than the indirect effects like stomata closure, but ion concentrations of chloroplasts remain constant (Kaiser 1983).

1.2.1.1 Photosynthetic Activity Under Salt Stress

Photosynthesis is the primary path by which high-energy organic molecules are produced. Photosynthetic effieciency is the primary concern in agronomy since it is the basic processes determining the product yield.

In higher plants, the primary reactions of photosynthesis take place within specialised thylakoid membranes inside the chloroplasts. Embedded in the thylakoid membranes are various protein complexes, each of which contributes to the total photosynthetic reaction. These are PS-I, PS-II, and chloroplast ATPase.

Photosystem II (PS-II) is the protein-pigment complex that produces oxygen and traps light energy. The generation of oxygen takes place entirely within the complex of photosystem II. The essential task of PS-II is the act as a tiny capacitor , storing energy by separating and stabilising positive and negative charges on either side of the thylakoid membrane. To do this, an array of specialised pigments in PS-II absorbs a photon and efficiently converts this light energy into separation of charges.

Orchestration of the movements in the complex processes of converting light energy into separation of charge require the collaboration of many proteins in the photosystem.

The electron transfer reactions in PS-II takes place within the reaction centre. The major structural components of the reaction centre are the large polypeptides, named D1 and D2, and a smaller protein Cytochrome$_{559}$. A polypeptide with a molecular weight of 33 kDa and at least two others of different molecular weights are bound to the inner surface of the thylakoid membranes. The polypeptides serve as

stabilising matrix for the pigments and other molecules in PS-II that perform the electron-transfer and oxygen-producing reactions. Several organic ions and charged atoms (manganese, chloride, calcium, iron, and bicarbonate) are involved in catalysing electron transfer, maintaining the protein structure or regulating the photosystem's activity.

Large numbers of antenna chlorophyll molecules collect light energy and funnel it efficiently to the reaction centre. Several hundred antenna pigment molecules are associated with each reaction centre.

High temperatures cause reversible structural rearrangements suggesting that they play a physiological role in preventing overexcitation of photosystem II under high light intensities which is normally induced elevated leaf temperature (Burke 1990). Thermal adaptation involves changes in fatty acid residues of membrane lipids involved in packing the proteins such that their functional role can be preserved under prevailing thermal conditions.

1.2.1.3 Osmotic Regiulations Under Salt Stress

Osmotic adjustment is the first response to increase in osmotic potential of external environment. Osmoregulation is a specific form of solute accumulation that regulates turgor pressure during stress with positive effects on growth. This increase in the osmotic potential of the cytoplasm allows the taking up the ions like Na^+ and Cl^- from the environment and the accumulation of them inside the cell. The accumulation of ions causes ion toxicity and impairs metabolic functions. The salt

tolerant plants are thought to use this strategy up to a certain level. If the solute to be accumulated in the cytoplasm is non-toxic, higher osmotic pressures inside the cell can be achieved. These non-toxic solutes are referred to as "compatible" and they do not interfere with protein structure and function even at high concentrations (McCue 1990). Compatible osmoprotectants are organic molecules. The most common are some ammonium compounds such as glycinebetain, polyols, the amino acid proline and tertiary sulfonium compounds. Genes that encode enzymes involved in the synthesis of osmoprotectants are known to be induced under salt stress in bacteria and plants (Claes 1990).

Low molecular weight carbohydrates are well-known osmotic pressure regulators in salt tolerance and cold hardiness in many plant species. It is thought to be related to cell membrane preservation during ice-dehydration stress (Santarius 1982). Sucrose is a member of low molecular weight carbohydrate and increases in response to salt stress, desiccation stress, chilling stress, and cold hardening.

The most commonly observed osmoprotectants enhanced by drought are proline and betain. A 10 to 100 fold increase was observed in leaf tissue of many plants during water deficiency (Hanson and Hitz 1980). The increase in betain is more related to salinity. One of the enzymes in the biosynthesis of betain has been purified by Hanson (Thuan-Hua 1989). The elevated levels of proline and betain during water stress serve as osmotica to prevent further water loss from the stressed cells.

The salt response at the molecular level involves several types of ion channels in the plasmalemma and tonoplast, some osmotically active compounds called osmoprotectants and salt induced proteins. The activities of plasmalemma H^+-

translocating ATPase, K^+/Na^+ ATPase is known to increase under salt stress. But, the molecular mechanisms were not investigated.

1.3 Molecular Aspects of Salt Stress

Osmotic stresses induce a large number of different genes. Morgan suggested that osmoregulation between wheat genotypes is controlled by alternative alleles at a single locus (Morgan 1991). These genes are divided into two main groups: dehydrins and osmotins.

Several cDNAs, which code for dehydration induced proteins, have been characterised in barley and maize. An arabidopsis genomic library was screened with a maize dehydrin cDNA and a positive subclone (XERO 1) sequence contained a consensus sequence that is common to dehydrins, RAB and TAS proteins. These dehydrins are similar to each other and to basic, glycin-rich RAB (responsive to ABA) proteins that are inducible by abscisic acid and osmotic stress. Furthermore, some cotton LEA (late embryogenesis-abundant) proteins show considerable homology in their amino acid composition with the dehydrins and RAB proteins. A tomato cDNA, (TAS14) inducible by salt stress and ABA, also codes for a protein similar to LEA and the RAB proteins.

Salt induced changes in gene expression can occur at the level of transcription and post translation levels. Expression of most of the genes is not affected , but regulation of gene expression is changed to enhance and to repress the synthesis of specific genes. The total mRNA of barley roots is reduced and 21 new RNA species

are induced and several others are inhibited (Ramagopal 1987). NaCL stress invokes changes in the levels of several gene products. Adaptation of tobacco cells to salt induce the accumulation several proteins, particularly one with apparent molecular weight of 26 KD. This protein is called osmotin. The 26 KD protein was observed in the cell cultures of tomato, alfalfa, and maize under salt stress (Singh 1985). Osmotin is characterised as a cationic protein occurring in salt adapted tobacco cells in soluble (Osmotin I) and water insoluble (Osmotin II) forms. Osmotin is known to accumulate in the vacuolar inclusion bodies of cells that are adapted to NaCl (Singh 1987). It is also localized in the cytoplasm at much lower concentrations and loosely associated with isolated plasma membranes and tonoplast vesicles. The major form of osmotin synthesised by NaCl adapted tobacco cells has an apparent pI greater than 8.2. Unadapted cells synthesise mainly a cross reacting polypeptide with a lower apparent pI of 7.8 (Singh 1987). The non-adapted cells also accumulates the osmotin of both types to concentrations comparable to the adapted cells. The synthesis of osmotin in tobacco cells, millet cells, field bundweed, cotton, carrot, and potato are also induced by ABA. The biological function of osmotin is not yet determined. However, all these observations suggest that osmotin may have a role in adjusting osmotic pressure under water stress. Analysis of the amino acid sequence indicates substantial homology with thaumatin, the bifunctional maize α-amylase/proteinase inhibitor and a pathogenesis related induced proteins by tobacco mosaic virus infections. These proteins are hypothesised to be involved in defence reactions of plants to pathogens (Richordson 1987).

1..4 The Role of ABA In Salt Stress

A large number of chemically diverse growth inhibiting substances have been identified in plants. Jasmonic acid and its methyl esters are examples of such compounds that are physiologically important. However, abscisic acid is the only inhibitor that is commonly regarded as a true hormone (Moore 1989). Regulation of the growth and development of plants is dependent on the types and the amount of the various hormones. Yet the survival of plants depends also on deceleration or cessation of growth and development of tolerance to stress at critical stages of life cycles. Water stress is one of the environmentally adverse conditions imposed on plants. Coping with water stress involves a frequent and essential manifestation of the adaptation of terrestrial lands to their often changeable physical environments.

ABA is a sesquiterpen compound that is related to the monoterpenes, diterpenes, carotenoids, and triterpenes. Naturally occurring ABA is a single enantiomorph, specifically the dextrorotatory compound.

ABA is synthesised from mevalonate via farnesyl pyrophosphate. It appears that ABA biosynthesis occurs in chloroplasts. A possible alternate pathway has been described from carotenoids, specifically violaxanthin.

ABA has been implicated in the control of a diverse range of physiological processes in higher plants. Physiological responses to ABA include stomatal closure, inhibition of precocious germination of embryos and tolerance to stress. The response depends on the level of ABA and the sensitivity of competent tissue to ABA (Hethrington 1991). One of the most dramatic features about ABA metabolism is a large and rapid increase in the endogenous levels when leaves of terrestrial higher

plants are exposed to any of variety of conditions that induce stress, including mineral deficiency, flooding, injury, chilling, freezing, drought, and salt. In most of these stresses, the actual stress is deficiency of water in the protoplast. Water stress was suggested to act with turgor loss to activate genes that control ABA synthesis by effects on transcription (Salisburry 1992 and the references therein). In many cases, applied ABA can partly reduce the plant's reaction to the stress factor. For example, ABA hardens plants against frost damage (Guy 1990) and and against salt (Scriver 1990).

ABA generally exerts an inhibitory effect on the metabolism of nucleic acids and proteins. Yet, it has been shown to induce the synthesis of specific mRNAs and proteins during seed embryogenesis, dormancy, desiccation and other stresses. Experiments in tobacco cells demonstrated that ABA accelerates the rate of adaptation NaCl (LaRosa 1985).

1.5 Salt Adaptation

1.5.1 Physiological And Molecular Mechanisms of Salt Adaptation

Adaptation to salt stress is an important biological process that protects the organism against the effects of dehydration. On the other hand, the ion toxicity should be overcome during this adaptation. This type of adaptation is observed among halophytes. Mechanisms at the whole plant level can be suggested by emphasizing the ultimate dependence of salt tolerance on transport processes operating at the cellular

level, and the ways these mechanisms control solute levels passing through roots can be outlined (Gorham 1985). Plant survival in most environments requires their ability to withstand extremes of osmotic stress caused by drought, salinity and temperature. Two major mechanisms have evolved for accomplishing this, water stress avoidance and tolerance. Avoidance depends primarily on specialised adaptations in root and shoot.

Long-term changes in the photosynthetic chemistry (CAM) are also used by certain plants to reduce evaporative water loss. Water use efficiency that refers to the ratio of CO_2 fixed to water loss is an advantage if water becomes limiting for net CO_2 uptake.

Water stress tolerance, on the other hand involves, subtle changes in cellular chemistry. It appears to be the result of the accumulation of compatible solutes and of specific proteins that can be rapidly induced by osmotic stress.

Under saline conditions, a large quantity of salts, that are absorbed into the leaves and contribute to osmotic adjustment, is accumulated mainly in the vacuole when tissue concentrations exceed about 200 mol/m^3. The concentrations of inorganic ions are maintained in the range of 100 to 200 mol/m^3. The cytoplasm shows a strong selectivity for K^+ over Na^+, Mg^{+2} over Ca^{+2}, and phosphate over nitrate. Under hyperosmotic conditions, the maintenance of the osmotic equilibrium across tonoplast membranes requires the accumulation of non-toxic organic solutes which are called "compatible" solutes in the cytoplasm. The chemical nature of the compatible solutes varies from one taxonomic group to another one, but most are derivatives of polyols and nitrogen dipoles.

1.5.2 Mechanisms Operating In Roots

Solute distributions will ultimately be controlled by the active and passive transport properties of plasmalemma and tonoplast or by any pass or leak mechanism via the apoplast.

The precise sites of selective transport through the root are determined by extracellular permeability barriers. There are debates about the extent to which water and ion transport proceeds through the passage cells or plasmadesmota, and about the relative contributions of apoplastic, symplastic and transmembrane pathways of water and ion movement across roots.

In the stele, active transport processes are involved in loading solutes into the xylem stream. This offers another site for selectivity. Conversely, active transport out of the xylem transport cells may further regulate the properties and concentrations of different ions supplied to shoots. In some of the most salt sensitive crop plants, sodium may be retransported from the xylem and exported from the roots. This has been reported from Phaseolus vulgaris and squash (Gorham 1985).

The rate of water movement through roots (as determined by transpiration) has two potentially important effects on ion uptake. Local concentrations of excluded ions in the region of root surface and in the apoplast of the cortex caused by mass flow of external solution into the root effectively increases the salt stress experienced by the plant.

1.5.3 Mechanisms Operating In Leaves

The capacity of the mesophyll vacuoles to accumulate sodium and chloride may be increased by increasing the cell size and vacuole to cytoplasm ratio. This is seen successfully in the succulent Suaeda marituana. To be effective in salt including halophytes, these mechanisms may be accompanied by high growth rate. An alternative to the problem of salt accumulation in the leaves is the export of the ions outside the leaf tissue via salt exporting glands.

Mechanisms must exist for the intracellular compartmentalisation of ions that accumulates in leaves. Reduction of transpiration rates is a characteristic response to salinity. Non-stomatal water loss through the epidermis may be substantial and the cuticular thickness may be a mechanism to salt stress. In species with salt glands, cuticle function as barrier to the entering of salt, which is excluded to the surface of leaf, back into the leaf.

There are some indications about a possible association between an enhanced K^+/Na^+ discrimination character and salt tolerance. This suggest that K^+ or Na^+ accumulation in the leaves may be a mechanism for tolerance. D genome of wheat derived from the diploid grass Aegilops squarrosa contains a trait that enhances the discrimination between Na^+ and K^+ in ion transport to the shoots of wheat plants grown in the presence of salt (Shah 1987). In any given external salt concentration, the leaves of the synthetic hexaploid wheat had lower Na^+ and higher K^+ concentrations than the leaves of tetraploid wheat. Total cation transport was not affected by the trait, but the K^+/Na^+ ratios were very much higher in hexaploid wheat.

1.6 Crop Improvement For Salt Tolerance

The genetic improvement of yield is related with two major parameters. Yield potential is the maximum yield that can be achieved under optimum conditions. Yield stability refers to the complex responses and interactions with the environment.

Salt tolerance is one of the main components of yield stability in crop plants under salt stress conditions. It relates to the ability to protect the plant metabolism and cell structures when they are exposed to saline environment.

Survival is not an appropriate criterion of tolerance for improving crop plants. The crop must also produce enough of the desired product (biomass) to ensure economic viability. This crop improvement should combine high growth rates with salt tolerance since salt tolerance trait is a quantitative trait.

The main problem in improvement for salt is the determination of which of the many mechanisms of tolerance operating in the plant will produce a significant increase in tolerance without drastically reducing economic yield. It is unlikely that improvement of any single mechanism will end up with the desired result.

Genetic manipulation of crops can be accomplished by conventional breeding methods or by genetic engineering technologies. The existence of genetic variability is a prerequisite for any breeding work. Since improvements of most crop species took place under favorable conditions, their gene pools became limited. The gene pool can be enriched by the selection of mutants in tissue culture using conventional mutagenesis or by introducing genes through a wild species. There is abundant

evidence for vast genetic potential in the plant kingdom for adaptation to adverse environmental conditions (Eptein 1977, Barton 1983).

Genetic characters are defined as quantitative when they appear to be continuous, so that relative contributions of individual genes to the final phenotype are not known. But, it has been often demonstrated that polygenic traits can be actually determined by a small number of major genes or linked gene complexes that segregates as units. A prerequisite to understanding the inheritance of quantitative traits is the identification of loci that control quantitative variation.

In plant improvement, there is interest in complicated traits in relation to the biochemical and physiological processes that control yield. The increase in yield due to the breeding of crop plants was achieved by direct selection for the production of the appropriate organs under progressively improving conditions. Changes in individual physiological traits were not directly selected for. Rapid improvements in the ability of plants to stress tolerance depend upon using new selection criteria and upon understanding of the physiological processes and genetic controls that limit crop production.

Cereals contstitute a central part of human diet. A steadily growing population and limited areas available for growing crops make it necessary to secure and to extend yield potential. The estimates on the relative importance of the the major cereals, in terms of quantities grown, indicate that wheat is most important followed by rice, maize, barley, and the millet species.

1.7 Aim of The Study

In this study, the physiological and biochemical responses of wheat cultivars growing under salt stress conditions were examined. The performance of photosynthesis and the integrity of the root plasma membrane were assayed during salt stress in four wheat cultivars. The maintenance of PS-II activity and the root membrane integrity after a broad range of NaCl treatments (1-5 days) were used to rank the performance of cultivars under salt stress. Two of the cultivars that differed in tolerance were chosen for the molecular study. The polypeptide patterns of salt treated plants were analyzed first with SDS-PAGE and then with Two-Dimensional electrophoretic analysis to observe differences in accumulation of proteins under salt stress..

The characterization of the salt stress induced proteins in the salt tolerant cultivars will create a base for future studies in understanding the tolerance mechanisms which can be used in improvement of salt tolerant crop plants. Moreover, the results of this study may be used in establishment of a screening methodology for the selection of wheat cultivars for salt tolerance.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant Material

Four different wheat (Triticum aestivum) cultivars were used in experiments. Seeds of "Gerek 79" and "BezostajaI" were provided by Eskisehir Agricultural Research institute , "Tosun 144" by Ankara University, Faculty of Agriculture and seeds of "Kharchia" was a gift by National Agricultural Research Center in Islamabad-Pakistan. The origin and species names of "Gerek 79", "Tosun 144" and "Bezostaja I" were described by Kün (1988) as follows:

Gerek 79: Triticum aestivum L. ssp. vulgare Vill. v. ertyhroleucon Korn.

Tosun 144: Triticum aestivum L. ssp.vulgare Vill. v. ferrugineum Korn.

Bezostaja I: Triticum aestivum L. ssp. vulgare Vill. v. lutescens.

2.1.2 Chemicals

^{35}[S]-Methionine (2.00 mCi/ml) was obtained from Amersham International plc. (England). X ray film was Agfa Curix XP films (18x24), fixator was Agfa G350 and developer Agfa G150. PPO and POPOP were from Sigma.

The ampholines were obtained from LKB. The acrylamide and BIS were purchased from Sigma.

Commasie Blue G-250 was obtained from Serva. BSA (Bovine Serum Albumin) and Folin-Ciocalteu reagent were purchased from Sigma.

All the other chemicals were of reagent quality obtained from Fulka, Merck or Sigma. Reagents were prepared by using distilled water.

The following proteins obtained from Sigma were used as molecular weight markers in the electrophoretic analysis:

Protein	Molecular Weight (Dalton)
Phosphorylase b	94000
Bovine Serum Albumin	66000
Egg Albumin	45000
Glyceralaldehyde-3-phosphate Dehydrogenase (Rabbit muscle)	36000
Carbonic Anhydrase	29000
Tripsinogen (Bovine pancreas)	24000
Tripsin Inhibitor (Soybean)	20100
β-Lactalbumine (Bovine milk)	14400

2.2 Methods

2.2.1 Growth And Stress Conditions

Seedlings were grown from seeds that were surface sterilized with 40 % hypochlorite for 20 minutes. The seeds were left over cheesecloth suspended over 500 ml tap water in dark in a growth chamber with the temperature maintained at 25 °C ± 3. After 2 days, the chamber was illuminated by 3 fluorescent lambs (each with 40 W, 2300 lm.) which were located 30 cm above the base of the containers. The illumination was applied for a daily cycle of 8 hours dark and 16 hours light.

After 6 days of growth, the stress treatment was started by replacing the solution with. 0.2 %, 1 %, 4 %, and 20 % NaCl solutions which had been autoclaved and filtered. The stresses were given under the same condition as the growth conditions. The stress duration was 5 days for the longest treatment. Plants were harvested on the 7th, 9th and 11th days for PS-II activity and membrane leakage measurements protein analysis was on the 1st, 2nd, and 3rd days. For each harvest, photosynthetic rates and membrane leakage were measured. The proteins were extracted according to procedures given below. The root tissue, shoot tissue and hypocotyl sections were split from each other. The root tissue was washed under running tap water for 30 seconds and then twice rinsed in distilled water to remove any salt particles adhering to plant material. Washed root tissue was immediately used for the membrane leakage test or protein extraction. The shoot tissue was immediately used for the PS-II activity test or protein extraction.

2.2.2 Physiological and Biochemical techniques For Understanding The Mechanisms of Salt Stress

2.2.2.1 PS-II Activity Measurements

PS-II activity measurements were carried on the isolated thylakoid membranes. Thylakoid membranes were isolated both from control and treated plants.

2.2.2.1.1 Isolation of Thylakoid Membranes

The thylakoid membranes with photosynthetic activity were isolated from the crude extract of the shoot tissue from control and treated plants. 500 mg tissue was weighed and homogenised with 4 ml of ice-cold grinding medium by a Sorvall omni-mixer. All the steps were executed at 4 C. The homogenizer were applied in full speed for 10 second twice with 10 second intervals in between to prevent heating of the content. The grinding medium consists of 0.33 M Sorbitol, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.1 % BSA as cold protectant of proteins, and 50 mM HEPES-KOH buffer at pH:7.0. The homogenate was filtered through first 4 layers cheesecloth, and then 8 layers cheesecloth. The filtrate was centrifuged at 1.300xg for 6 minutes by a Sorvall RC-2 superspeed centrifuge. The supernatant was discarded and the pellet was soluobilized in 3 ml of suspension medium for washing. The pellet was again taken by centrifugation at 12.000xg. for 10 minutes. The pellet at the end was soulubilized in 200 µl of suspension medium (0.1 M Sorbitol, 50 mM $MgCl_2$, 50 mM HEPES-KOH buffer of pH:7.0).

2.2.2.1.2 Characterization of Isolated Thylakoid Membranes

The assay of PS-II activity was carried on an equal quantity of control and treated samples. The amount of thylakoid membranes was measured on the basis of chlorophyll amount. The chlorophyll content of samples was measured according to Arnon (1949). The chlorophyll was extracted by mixing 10 ul of sample with 1 ml of 80 % acetone. The spectrophotometer (spectronic 21) was zero-adjusted at 700 nm. against acetone and the absorbance of the sample was taken at two different wavelenghts; 645 nm. and 665 nm. The chlorophyll amount was calculated from the following formula:

$$\mu g/ml=[A_{665}(8.02)+A_{645}(20.2)]\text{xdilution factor}$$

where A_{663} and A_{645} are the absorbencies at these wavelength when the spectrophotometer were zero-adjusted at 700 nm.

2.2.2.1.3 PS-II Activity Assay

The PS-II activity of isolated thylakoid membranes was determined according to a modified method of Chetti (1988). Equal amount of thylakoid membrane based on the chlorophyll content was assayed by using DCPIP (Dichlorophenol Indophenol) as an electron acceptor of PS-II photosynthetic complex. 50 µg of chlorophyll containing thylakoid membrane was mixed in 2 ml reaction medium containing 40 mM Tricine-KOH buffer of pH=8.0, 5 mM$MgCl_2$, 2 mM K_2HPO_4, 10 mM KCl, and 0.01 % DCIP. The reaction was started by illuminating the mixture with a 40 W incandescent lamp at a 10 cm. distance. The spectrophotometric measurement at

wavelenght of 590 nm. was taken after every 20 seconds illumination for at least 3 minutes. The rate of PS-II activity was calculated as ΔA590/second.

2.2.2.2 Membrane Leakage Measurements

Ion leakage of root tissue was measured by conductivity method of Marsh (1985). The root tissue was prepared as indicated at stress treatment section. Control and treated plants were put into 50 ml distilled water with a measured electrical conductance in plastic vials. The vials were incubated at 4 °C for 24 hours and the electrical conductances (R_1) were measured with a Philips electrical conductimeter after warming the vials up to room temperature. Then, vials were left at -20 C overnight to kill the tissue completely and the second conductances (R_2) were taken to measure the total ion concentration after complete membrane disintegration. The NaCl injury to the membranes were expressed as % of control values.

$$\% \text{ Injury}=[1-(R_1/R_2)/1-(R_{c1}/R_{c2})]x100$$

where R_1, R_2 are the conductances of treated plants first time and second time respectively, R_{c1}, R_{c2} are the conductances of control plants fist time and second time respectively.

2.2.3 Protein Analysis

2.2.3.1 Protein Extraction

Proteins were extracted from root and shoot tissues according to Damerval (1986). 200 mg root or 50 mg shoot tissue were ground in liquid nitrogen until the tissue turned to white powder. The powder were solubilized in 10 % Trichloroacetic acid in acetone by further grinding and left at -20 °C for 45 minutes. The precipitated proteins were collected by centrifugation at 12000 g for 20 min., washed once with acetone by soloubilizing them in 5 ml Acetone-BME solution, incubating for 1 hour at -20 °C, and collecting the pellet by centrifuge at 12000 g for 20 minutes. The resulting pellet were dried under vacuum and solubilized in a minimum amount of SDS-PAGE sample buffer.

2.2.3.2 in vivo Protein Labelling

Proteins were in vivo labelled with ^{35}[S]-Methionine. Following NaCl treatment , 2 seedlings were placed in test tubes with the roots immersed in 2 ml of labelling medium (50 µg/ml Chloramphenicol, 25 µCi of ^{35}[S]-Methionine/ml). Following a labelling period of the last 2.5 hours of salt stress treatment, labelling was stopped by washing the tissue with cold 0.1 M methionine. Proteins were immediately extracted in the same way as explained above. Intact seedlings were labelled to avoid wound-induced changes in protein synthesis that could occur in excised root segments (Hurkman 1988).

2.2.3.3 Protein Estimation

The amount of protein was determined by Bradford protein assay (Bradford 1976) for the samples which were used for SDS-PAGE or a modified Lowry protein assay (Markwell 1981) for the samples which were used for Two-dimensional electrophoresis. The incorporation of ^{35}[S]-Methionine was determined by TCA precipitation method (Mans and Novelli 1961) and used in the quantification of radioactive samples applied to SDS and Two-dimensional electrophoresis.

2.2.3.3.1 Bradford Protein Assay

5 µl of the sample prepared for SDS-PAGE was diluted with 795 µl distilled water in a test tube. 200 µl Bradford reagent (0.01 % Serva Blau G-250, 4.7 % (w/v) ethanol, and 8.5 % (w/v) phosphoric acid) was added, the tube was vortexed, and left at room temperature for at least 10 minutes. The absorbance at 595 nm was measured against a blank consisting of 800 µl water and 200 µl Bradford reagent in a spectrophotometer. BSA was used as standard in the following quantities: 2, 5, 10, 15, and 20 mg/ml. The concentration of standard proteins were plotted against the corresponding absorbance resulting in a standard curve which was used to determine the protein in unknown samples.

2.2.3.3.2 Lowry Protein Assay

The amount of protein in Two-dimensional electrophoresis sample buffer was determined by a modified method of Lowry for SDS-containing sample buffer (Markwell 1981). 5 µl sample was diluted to 1 ml with distilled water and mixed with 3 ml of reagent C, incubated for 10 minutes. Then, 0.3 ml of 1 N Folin-Ciocalteu reagent was added and contents of each tube was vigorously vortexed immediately after addition. The mixture was incubated for 45 minutes at room temperature. The absorbances at 660 nm. were measured against a blank consisting of 1 ml water, 3 ml reagent C, and 0.3 ml of 1 N Folin-Ciocalteu reagent. BSA was used as standard in the following weights: 5, 10, 15, 20, and 30 mg. from a stock of 1 mg/ml BSA solution. The protein amount in the samples were determined from a standard curve plotted from absorbencies of BSA. The prepration of Reagent C is given in Appendix A.

2.2.3.3.3 Measurement of Radioactive Amino Acid Incorporation

The assay of Mans and Novelli (1961) was used to determine the amount of labelled amino acids incorporated during in vivo protein labelling. 2 µl of sample was added to 1 ml of ice-cold 25 % TCA and 2 % BSA solution and incubated for 30 min. to precipitate the protein products. The precipitate was collected by filtering under vacuum on Whatmann GF/C glass fibre filters and washed three times with 1 ml 5 % TCA and 1 times with 100 % acetone. The filter papers were dried at room temperature overnight. Two ml toluen based scintillation fluid was added on filter

papers, left overnight and ^{35}[S] were determined in a liquid scintillation counter (LKB 1209 Rackbeta).

2.2.3.4 SDS Polyacrylamide Gel Electrophoresis

SDS-PAGE was according to Laemmli (1970). Proteins were soloubilized by heating the pellet in boiling water for 3 min in 200-500 µL sample buffer containing 0.0625 M Tris buffer of pH=6.8, 10 % Glycerol, 2 % SDS, 0.65 M ß-Mercaptoethanol. The insoluble material were pelleted out by centrifugation at 13000 g for 20 min. Equal amounts of proteins were loaded on slab gels of 5 % stacking and 12 % separating gels. The amount of proteins were determined by Bradfod protein assay in case of non-labelled samples and by TCA precipitation method in case of labelled samples. The running buffer was consisting of 38.4 mM Glycine, 0.02 % SDS, and 1.5 mM Tris. The gels were run under constant current of 15 mA for stacking, 25 mA for separating until the Bromophenol Blue reached to the end of gel in a Bio-Rad Protean II vertical gel apparatus. After electrophoresis the gels were visualised with silver staining for non-labelled samples and with flourography for labelled samples. 2 µl of marker proteins were applied in one of the wells to determine the molecular weights of separated proteins.

2.2.3.4 Two-dimensional Gel Eectrophoresis

Two-dimensional gel electrophoresis were done according to O'Farrel (O'Farell 1975). Proteins soloubilized in SDS sample buffer were precipitated with 10 % TCA

in acetone, dried under vacuum and solublized in 100 µl IEF sample buffer containing. Equal amounts (100 µg) or equal counts (200.000 cpm) of proteins were loaded to IEF gels. The fist dimensional IEF gels contained pH=5 to 8 and pH=3 to 10 ampholines. Samples were loaded onto the acidic site of tubes of 15 cm in length and 1.5 mm in diameter. The anolyte was 0.01 M H_3PO_4, and catholyte was 0.02 M NaOH. After 14000 Vh run, the gels were excruded with the help of a syringe and placed onto a 12 % SDS slab gels for second dimension. An agar overlay (0.5 % agar) was used to fix the IEF gels in place. The second dimension was the same as SDS-PAGE procedure.

To measure the pH gradient, a gel was run without proteins, sectioned just after the IEF into 1 cm pieces. Each piece was soaked in 1.5 ml distilled water for 2 hours before measuring pH.

2.2.3.6 Gel Processing And Visualization of Seperated Proteins After Electrophoresis

After electrophoresis, gels were fixed by a fixation solution of 12 % acetic acid, 50 % methanol for at least 1 hour. The non-labelled gels were silver-stained, photographed, and dried. Labelled proteins were visualised by flourography.

2.2.3.6.1 Silver Stain

The fixed gels of SDS-PAGE or two-dimensional electrophoresis were silver stained according to Blum et. al. (Blum 1987). The gels were washed 3 times with 50 % ethanol to remove acetic acid. After pretreatment with thiosulfate, gels were rinsed with distilled water three times and incubated in silver nitrate solution with formaldehyde for 30 minutes. Rinsed gels were developed for appearance of images. The detailed procedure is given in Appendix B.

2.2.3.6.2 Fluorography

After electrophoresis, the gels were soaked in acetic acid for 5 min. and placed in 20 % (wt/v) PPO in acetic acid for 1 hour and the gels were soaked in water for 30 min, then they were dried between cellophane sheets according to Krishnan (1990). PPO treated and dried gels were exposed to X-ray films at -20 C at a close contact for 30-60 days. The films were developed and photographed.

CHAPTER III

RESULTS

3.1 Responses of Root Tissue To Salt Stress

The condition of the root plasma membrane was determined by ion leakage measurements. Ion leakage through the plasma membrane was evaluated by measuring the changes in the electrical conductance value caused by the root tissue. R_1 was the electrical conductance caused by the leakage of ions through the plasma membrane. R_2 was the electrical conductance caused by complete dissociation of the plasma membranes. The R_1/R_2 called leakage value is not a function of cell death, since it is directly proportional with the condition of plasma membrane since it corresponds to the portion of ion content leaked through plasma membrane (Blum 1985).

Table I presents the leakage values of root tissue after salt treatments of 0.2 %, 1 %, 4 %, and 20 % concentrations for 1 day, 3 days, and 5 days in four wheat cultivars. The membrane leakage of control of each cultivars was different from each other representing the genetic variability of membrane structure between cultivars. The leakage value of 7 days old seedlings was 29 for Bezostaja, 27 for Gerek, 27 for

Karchia, and 36 for Tosun. Similar variability were detected in 9 days and 11 days control leakage values

Table 1. R1/R2 of root tissue as measured by the electrical conductivity method in plants salt stressed under 0.2 %, 1 %, 4 %, and 20 % NaCl solutions and plants grown under control condition in four cultivars of wheat after 1 day, 3 days, and 5 days of treatments.

Cultivar name and NaCl Treatment	Duration of Stress 1 Day	3 Days	5 Days
Bezostaja			
Control	29	27	26
0.2 %	89	39	10
1 %	60	51	31
4 %	54	-	27
20 %	69	89	61
Gerek			
Control	27	11	51
0.2 %	35	35	57
1 %	42	48	70
4 %	55	81	92
20 %	65	85	93
Kharchia			
Control	27	39	42
0.2 %	40	55	44
1 %	60	80	57
4 %	92	100	82
20 %	93	95	100
Tosun			
Control	36	44	53
0.2 %	41	65	48
1 %	70	70	55
4 %	83	85	86
20 %	94	80	-

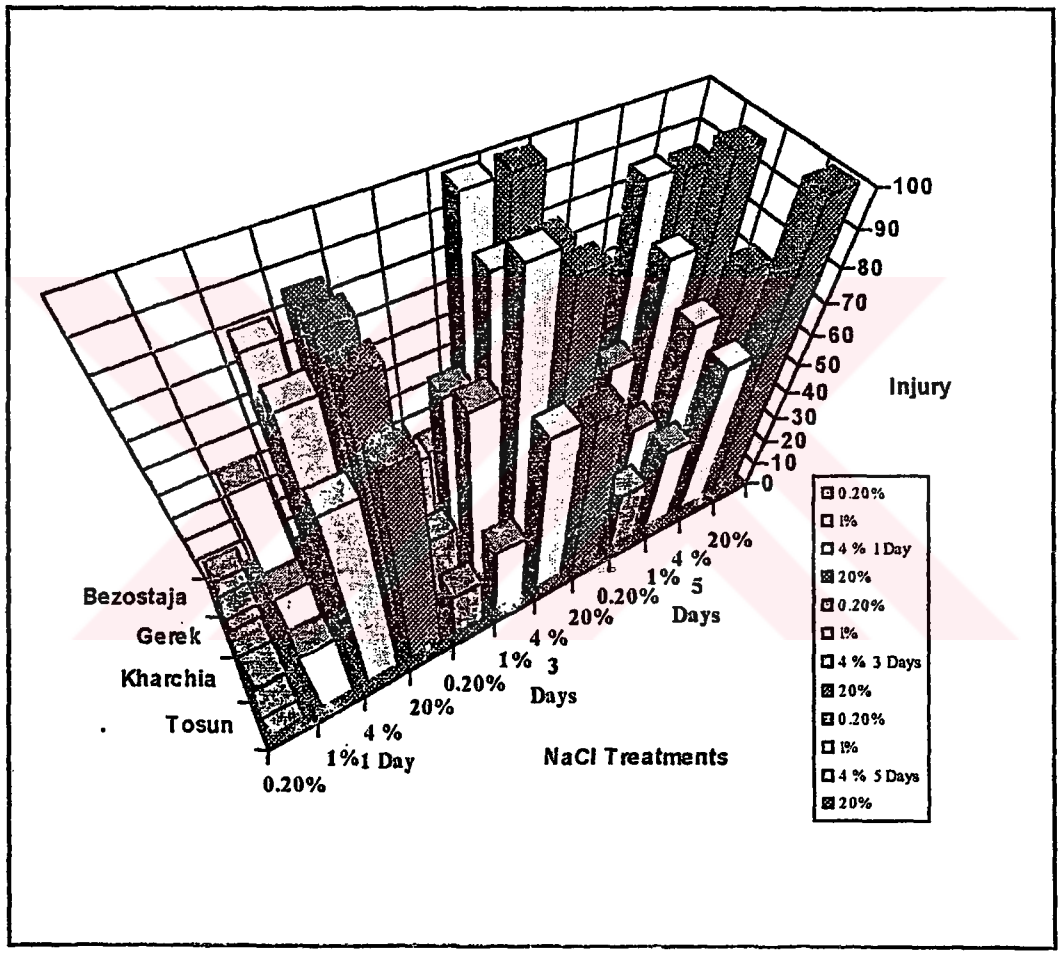


Figure1. Ion leakage of root tissue in four cultivars of Triticum aestivum under salt stress.A: injury values at 0.2 %, 1 %, 4 %, and 20 % NaCl treatments for 1 day, 3 days, and 5 days.

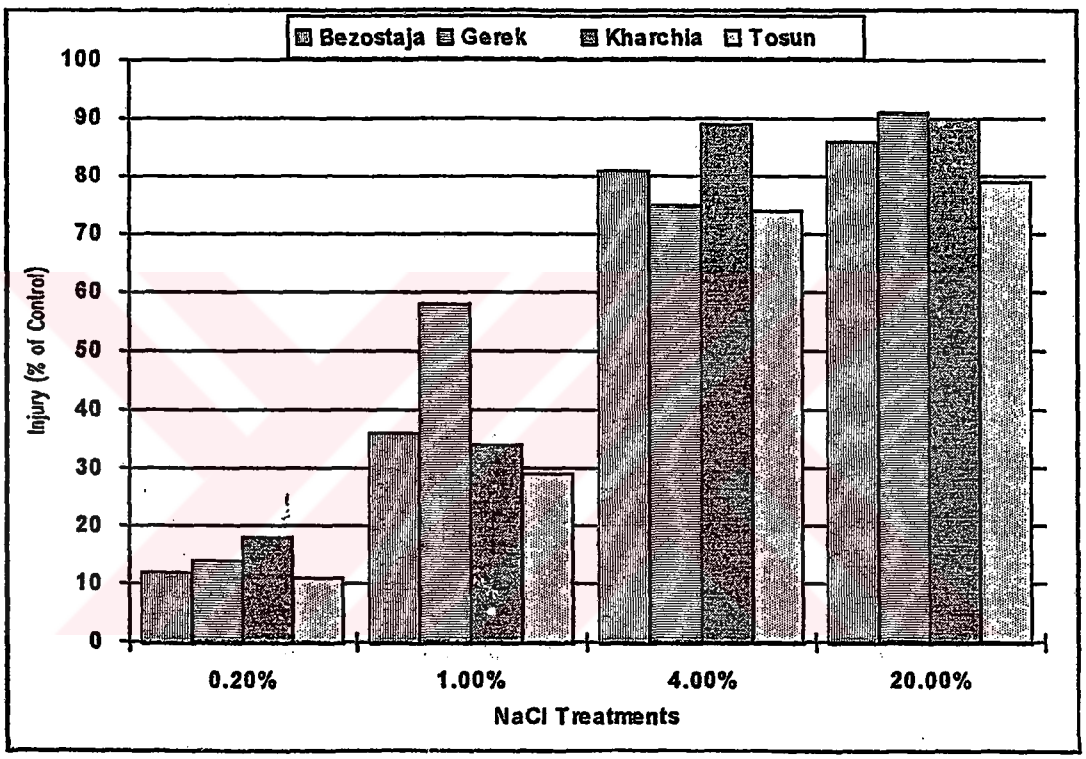


Figure 1.B Ion leakage after one day of treatment

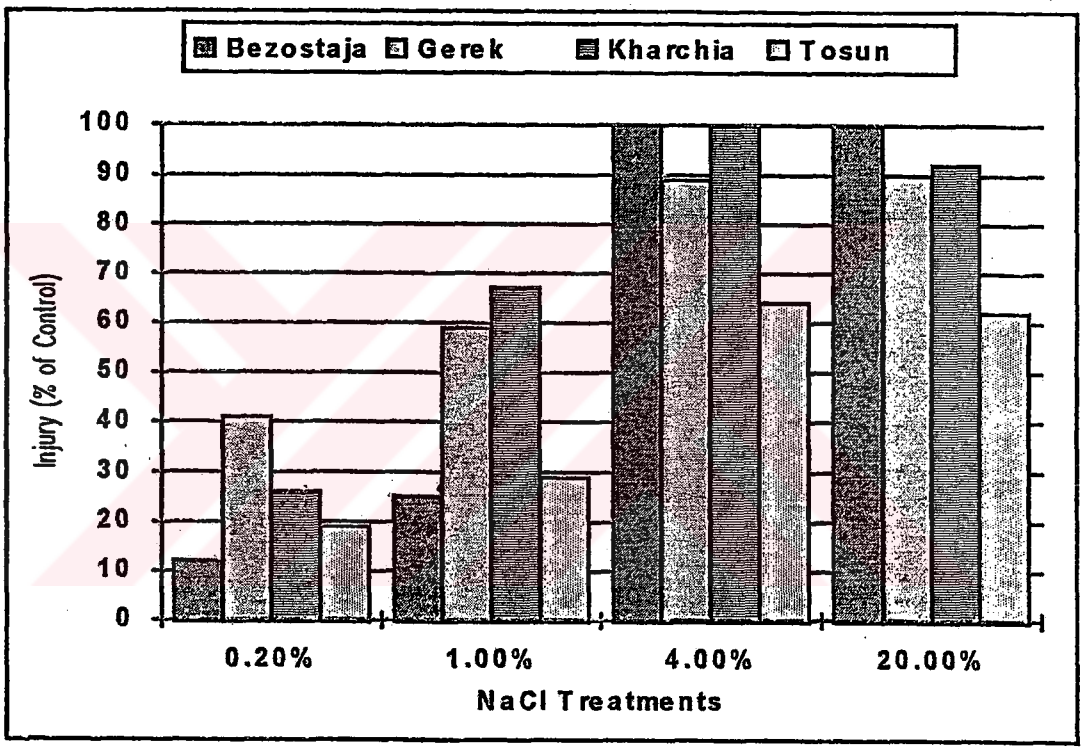


Figure 1.C Ion leakage after three days of treatment

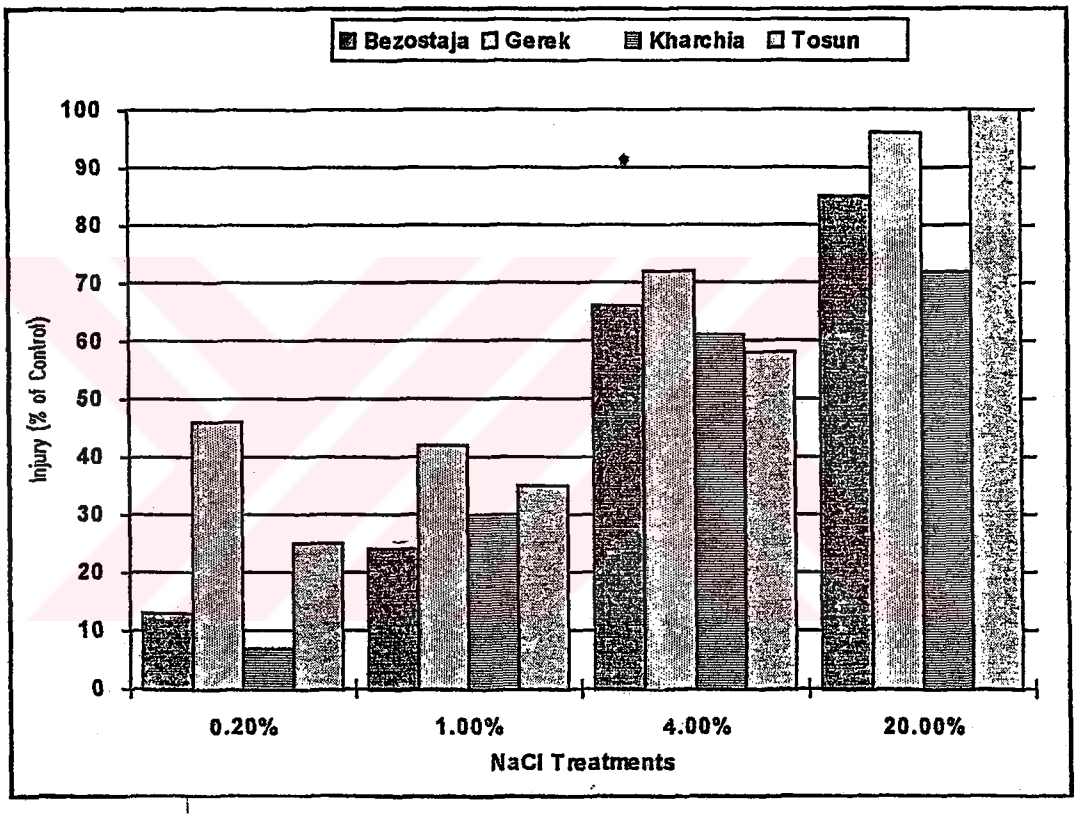
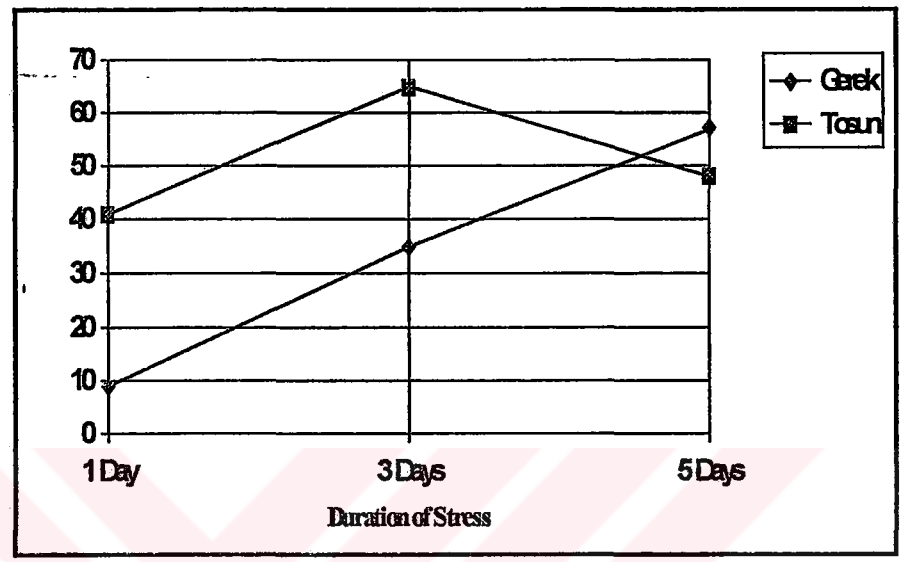


Figure 1.D Ion leakage after five days of treatment

To make a comparison of the responses of cultivars under salt stress, the condition of plasma membrane was expressed relative to the plasma membrane stability of the control plants. This relative value was "% injury" which is defined as the % leakage value of salt treated plant to leakage value of control plant. Figure 1 is a graphical presentation of % injury of four cultivars after salt stresses for the three different durations of treatments. In Figure 1., the % injury to plasma membrane is plotted against NaCl concentrations and treatment duration. A variability between cultivars were observed at 1 % and 4 % salt treatments for 1 day (Figure 1.B), but less variability were detected at very low salt concentration (0.2 %) and very high salt concentration (20 %). Salt treatments at 0.2 % and 1 % NaCl concentrations for one day did not caused lethal injury to the cells since 50 % ion leakage were thought as lethal (Chaisompongpan 1990). 4 % and 20 % salt treatments did cause lethal injury. On the other hand, the variability between cultivars was maximum at 1 % NaCl treatment with 34 % of Bezostaja, 58 % of Gerek, 32 % of Kharchia, and 29 % of Tosun for one day treatment. Therefore, 1 day treatment of 1 % NaCl put the cultivars in the following order of tolerance to salt stress: Tosun, Kharchia, Bezostaja, and Gerek. The order of tolerance in 20 % treatment was Tosun, Bezostaja, Kharchia, and Gerek. The order in 4 % was Tosun, Gerek, Bezostaja, and Kharchia. But, in these treatments, injury value was over lethal level and the observed variability in these responses was not as much as 1 % treatment (Figure 1.B). The order in 0.2 % treatment was Tosun, Bezostaja, Gerek, and Kharchia. Although this treatment caused injury below lethal level, the difference between the most tolerant and most sensitive cultivar was only 4 unit of injury. Therefore, Tosun and Kharchia were clearly ranked as relatively salt tolerant cultivars by membrane stability criteria after one day of treatment.

Injury increased in the 3 days of salt stress for all treatments compared to one day salt stress (Figure 1.C). The variability between cultivars was more in 3 days stress. Bezostaja had lowest injury value in 0.2 % and 1 % salt treatments, but high salt concentration (4 % and 20 %) caused 100 % injury. Tosun showed lowest injury comparing to other cultivars in 4 % and 20 % salt treatments with approximately 60 % injury. Kharchia and Gerek had similar values which were over 90 % injury in 4 % and 20 % salt treatments, and 60 % in 1 % salt treatment. Gerek had highest injury among cultivars in 0.2 % salt treatment. For 3 days of salt treatment, Tosun was ranked as the most tolerant.

Figure 1.D is the injury values after 5 days of salt stress. Tosun and Kharchia had lowest injury in 1 % and 4 % salt treatments. Kharchia was the cultivar with lowest injury in the other salt treatments (0.2 % and 20 %). Gerek had the highest injury in 0.2 %, 1 %, and 4 % salt treatments.



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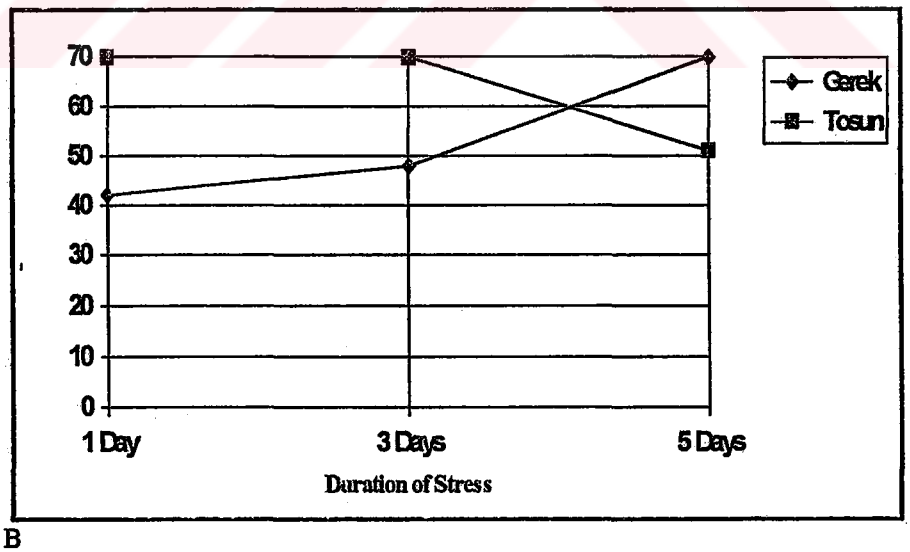


B

Figure 2. Time course analysis of injury to plasma membrane of Tosun and Gerek root tissue. A is 0.2 % NaCl treatment. B is 1 % NaCl treatment

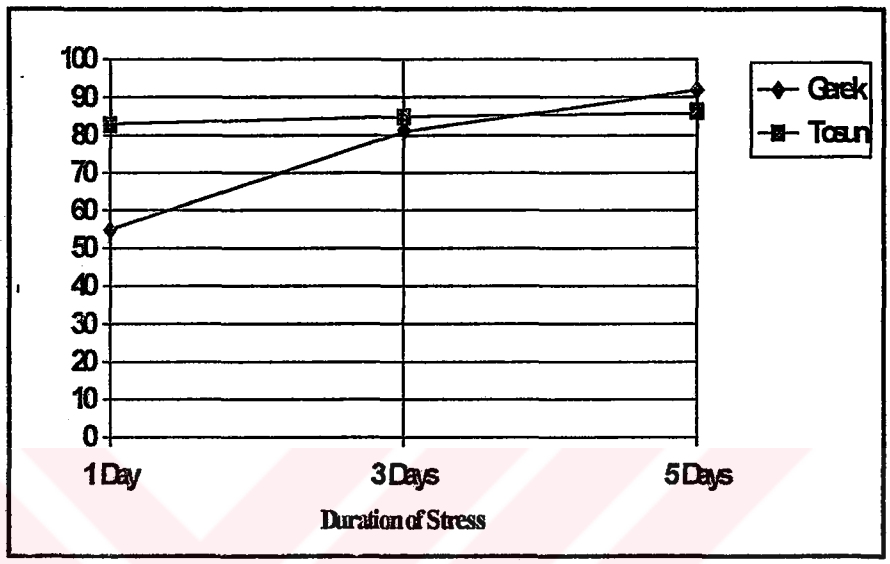


Figure 2.C Time course analysis of 4 % NaCl treatment.

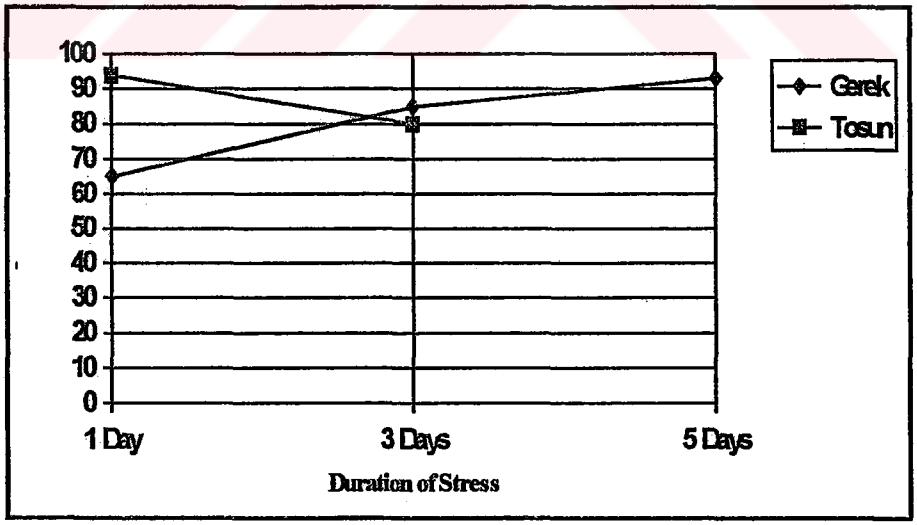


Figure 2.D Time course analysis of 20 % NaCl treatment

3.2 Response of Photosynthesis To Salt Stress

The photosynthetic activities of salt stressed and control plants were measured by the rate of electron transfer of PS-II to an artificial electron acceptor 2,4-dichlorophenol indophenol (DCPIP). The reaction was started with illumination of the assay mixture and the decrease in absorbance of DCPIP at 590 nm was recorded after each 20 seconds of illumination. The initial velocity was calculated from the slope of curves as change in absorbance per unit time. Figure 3 is the plot of absorbance versus time for Tosun after 3 days of treatment. The PS-II activity of control, 0.2 %, 1 %, 4 %, and 20 % salt treatments were calculated from the slopes of each line. The activity calculations were determined for all cultivars for 1 day, 3 days, and 5 days salt treatments separately from similar graphs.

The PS-II activities were used as an indicator of total photosynthetic activities of plants. Table II represents the activities for 1 day, 3 days, and 5 days salt treatments of 0.2 %, 1 %, 4 %, and 20 % in four cultivars. The PS-II activities of four cultivars were different from each other representing genetic variability. The activity was $902x10^{-6}$ a/sec for Bezostaja, $600x10^{-6}$ A/sec for Gerek, $700x10^{-6}$ A/sec for harchia, and $1200x10^{-6}$ for Tosun.

To make a comparison between cultivars, the PS-II activities of salt treated plants were expressed as the percentage of PS-II activities of control plants. % PS-II activity was representing the portion of control PS-II activity that is retained after salt stress.

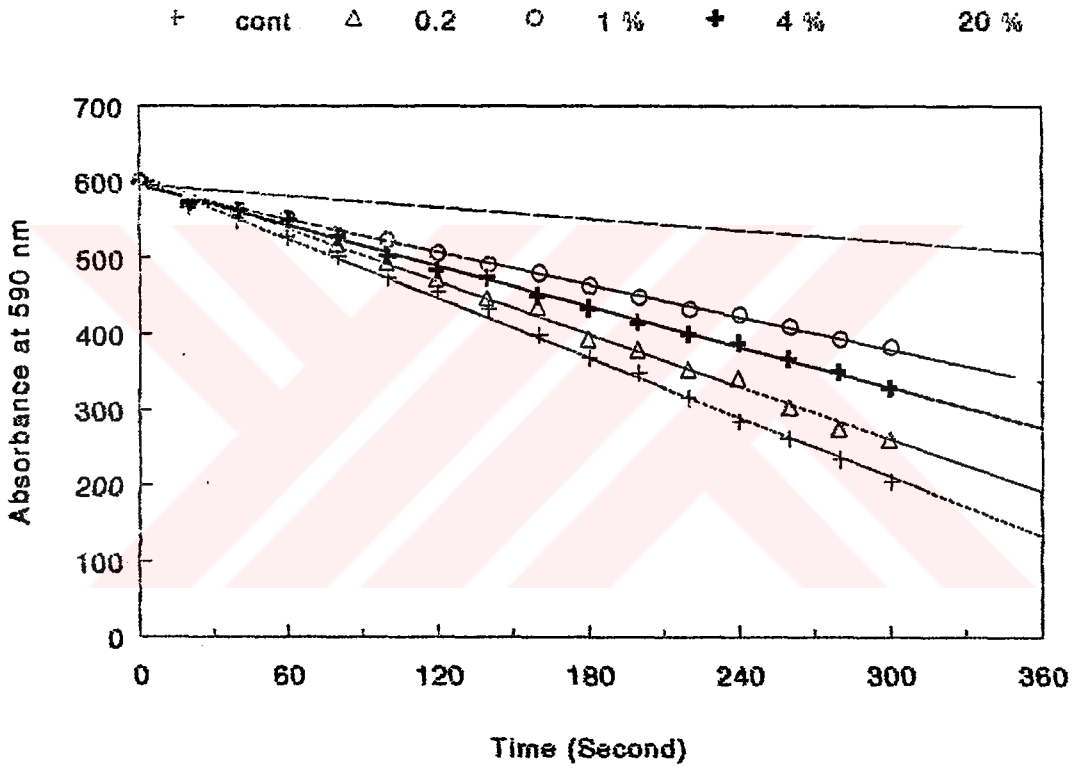


Figure 3. PS-II activity of Tosun after 5 days of stress. The slope of the lines were used as a measure of PS-II activity.

The PS-II activities of cultivars were presented in Figure 4 for comparison. In Figure 4 PS-II activities were presented against NaCl concentration and treatment duration. A variability in PS-II activity was observed after one day salt treatment (Figure 4.B). Tosun and Kharchia had the highest activity after 0.2 % salt treatment with 82 % and 100 % respectively. Bezostaja had 72 % and Gerek 60 % activity at this treatment. Tosun and Kharchia showed highest activity after 1 %, 4 % and 20 % NaCl tretaments. The cultivars can be ranked in the order of activity under salt stress as Kharchia, Tosun, Gerek, and Bezostaja after one day salt treatment for a range of salt concentrations from 0.2 % to 20 %.

The PS-II activities of Tosun and Kharchia were higher after 0.2 % salt treatment for 3 days (Figure 4.C). Tosun had highest activity in 1 % treatment, where the activities of Bezostaja and Gerek were 65 %, 46 % respectively. Kharchia had lowest activity. 4 % treatment showed similar order. The variability was very low between cultivars after 20 % salt treatment of 3 days. The order of activity after 3 days of salt stress can be stated as karchia, Tosun, Bezostaja depending on 0,2 % salt treatment. The order changes as Tosun Bezostaja, and Gerek, Kharchia when the 1 % and 4 % salt treatments were consiered.

Table 2. Effect of NaCl on PS-II activity after 0.2 %,1 %, 4 %, and 20 % treatments for a duration of 1 day, 3 days, and 5 days.The PS-II activities were calculated from the rate of reduction of Dichlorophenol Indophenol as explained in methods. The activity was A_{590}/second multiplied by 10^{-6}.

Cultiar name and NaCl treatments (%)	Stress Durations 1 day	3 Days	5 Days
Bezostaja			
Control	902	958	889
0.2 %	649	-	596
1 %	217	746	462
4 %	325	666	204
20 %	135	521	249
Gerek			
Control	667	518	326
0.2 %	410	434	506
1 %	491	350	227
4 %	514	400	460
20 %	295	204	137
Kharchia			
Control	304	600	900
0.2 %	371	480	856
1 %	265	343	60
4 %	441	175	301
20 %	259	188	74
Tosun			
Control	1055	1337	1361
0.2 %	854	975	1205
1 %	727	623	665
4 %	917	602	868
20 %	665	555	239

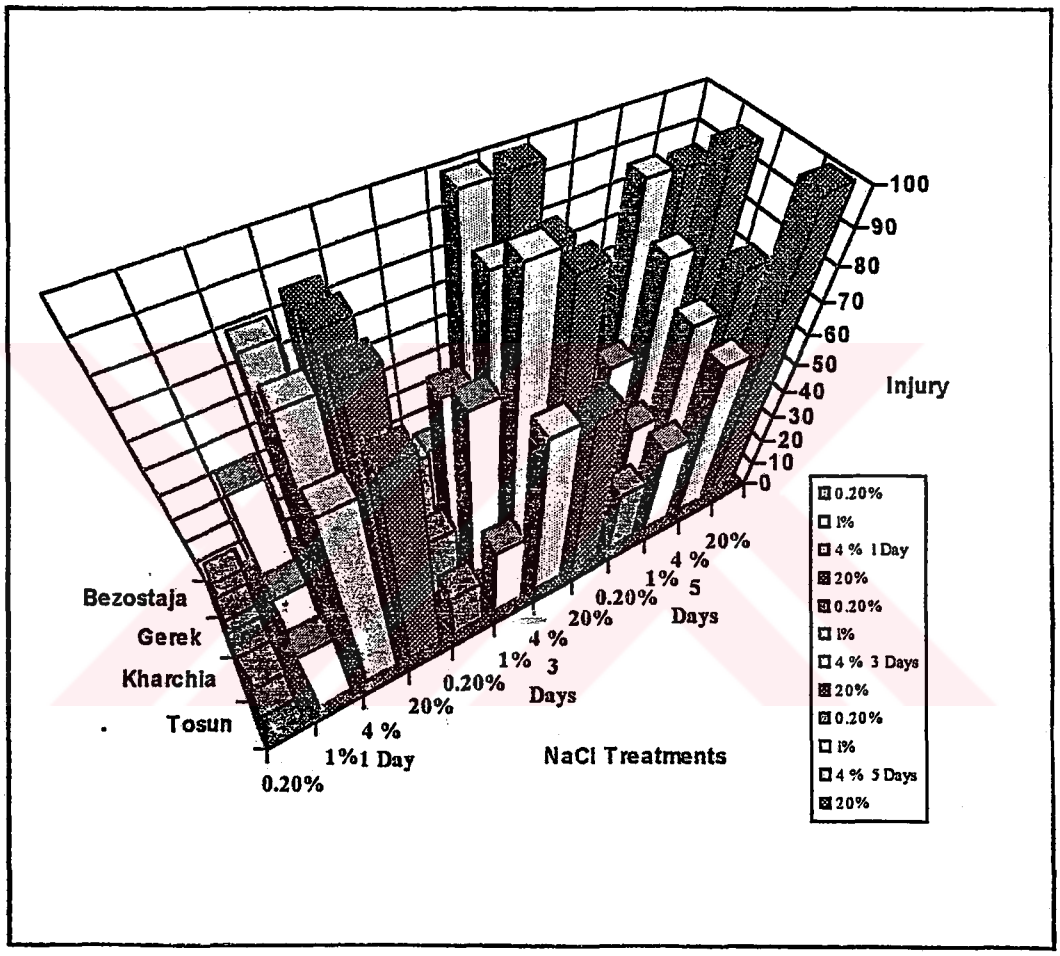


Figure 4. PS-II activities in four cultivars of Triticum aestivum under salt stress. A: PS-II activity values at 0.2 %, 1 %, 4 %, and 20 % NaCl treatments for 1 day, 3days, and 5 days.

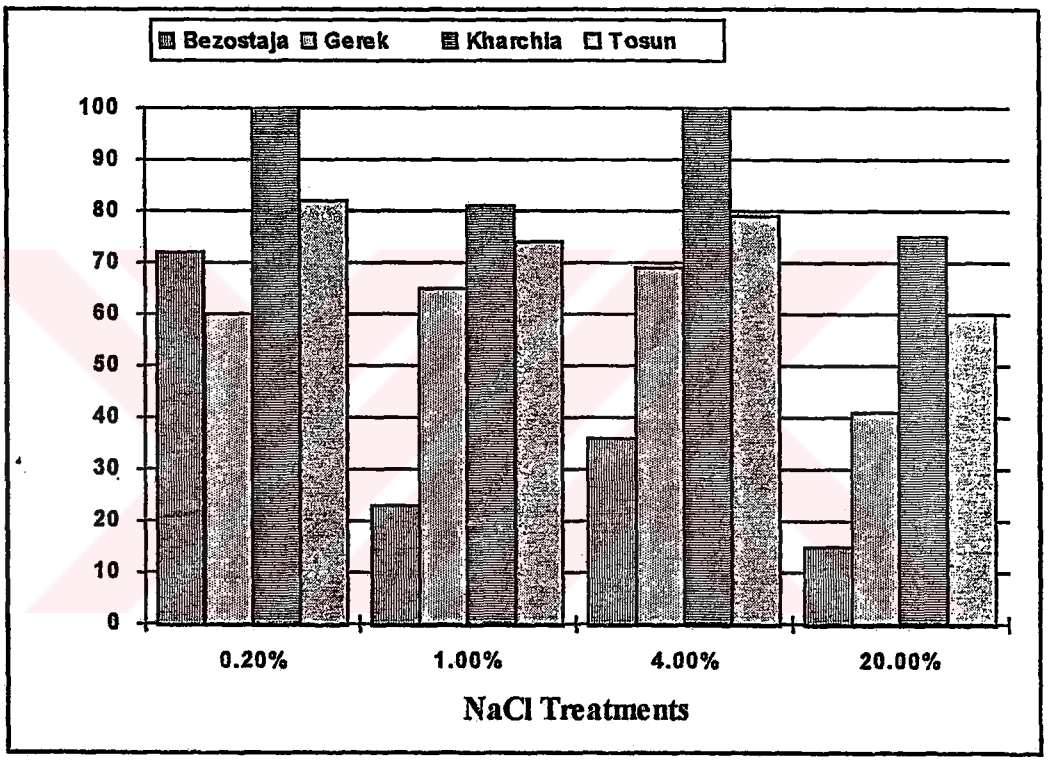


Figure 4.B PS-II activity after one day of treatment

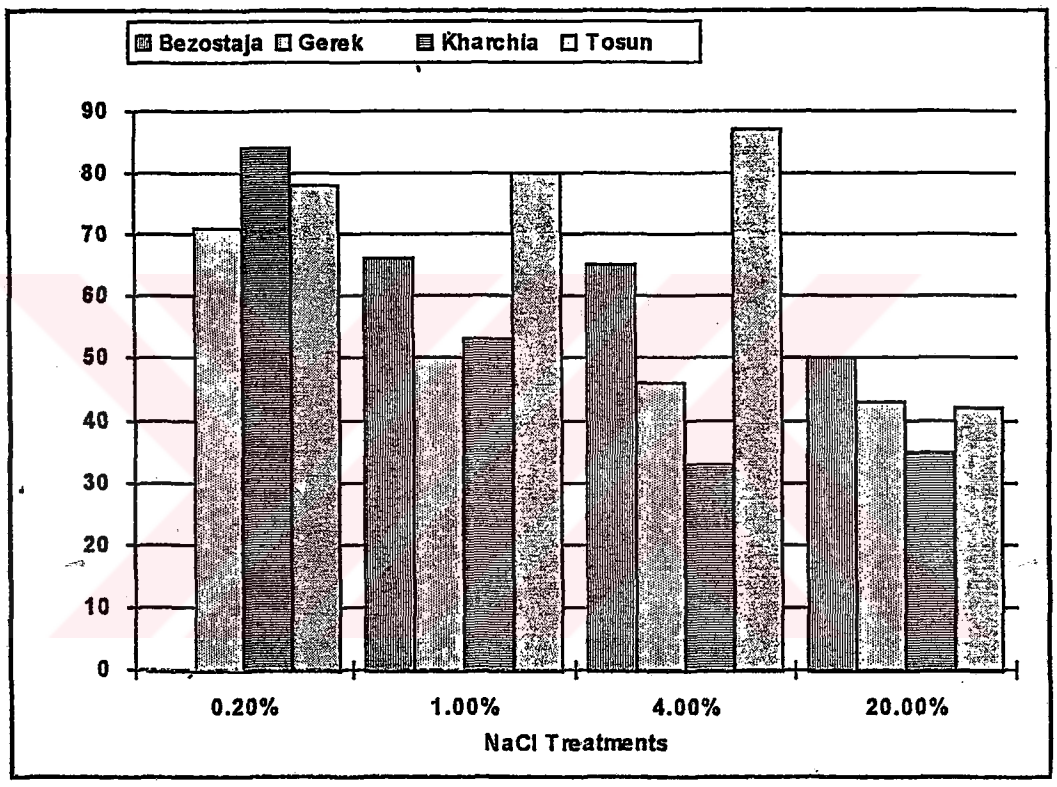


Figure 4.C PS-II activity after three days of treatment

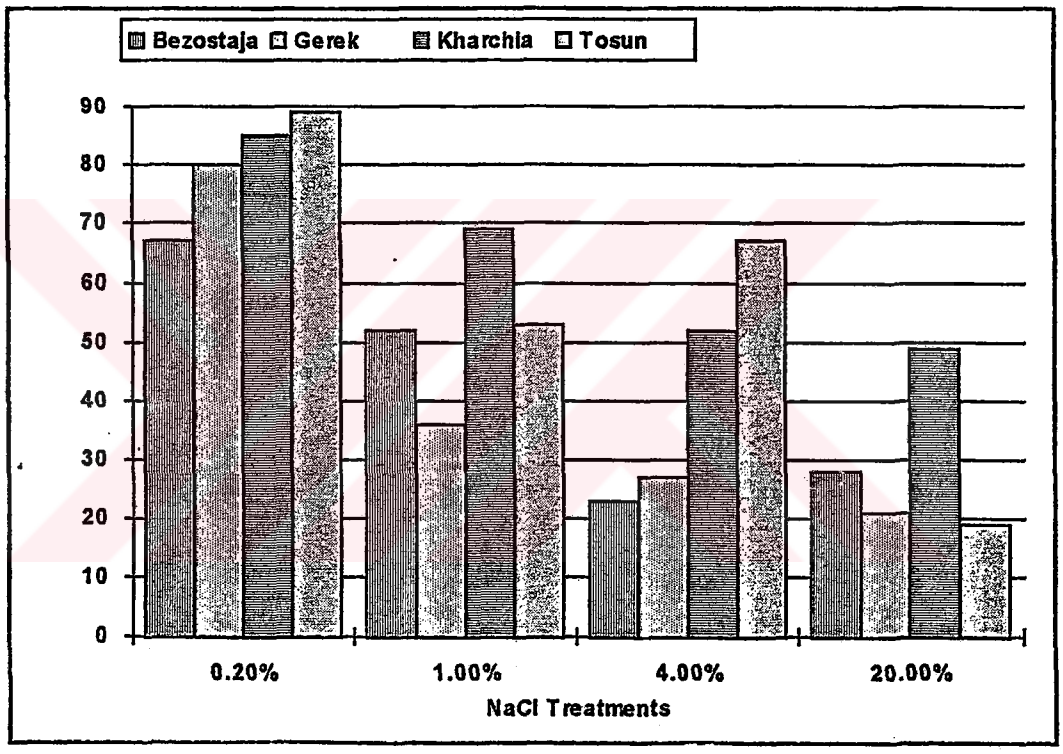
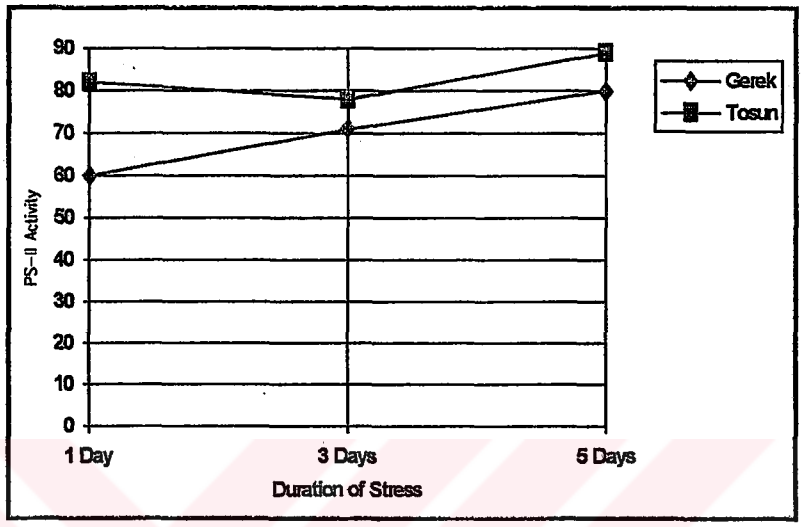


Figure 4.D PS-II activity after five days of treatment

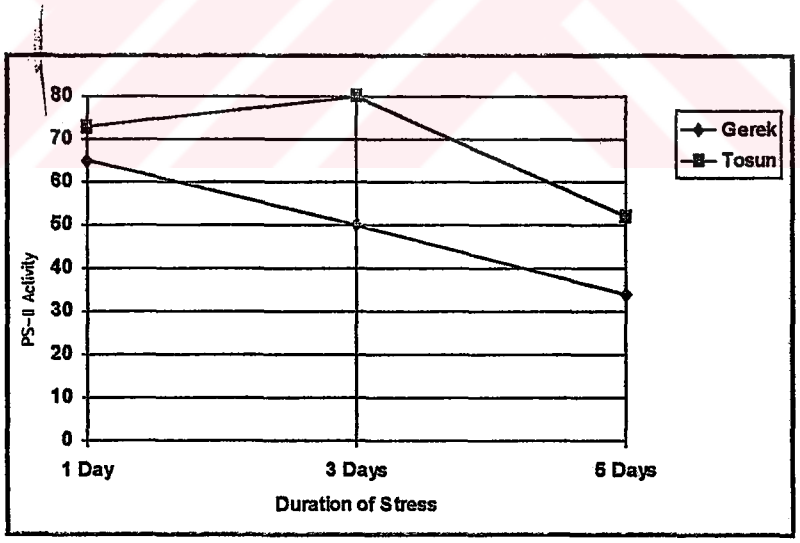
The results of 5 days of salt treatment were shown in Figure 4.D. Tosun and Kharchia had highest PS-II activity after 0.2 % salt treatment of 5 day with over 85 % of their control. Gerek had 80 %, and Bezostaja 68 % PS-II activity. In 1 % salt treatment Kharchia had 70 %, Tosun 53 % activity. At this treatment, Bezostaja had higher activity than Gerek with 51 %. 4 % treatment caused the highest variability among cultivars for 5 day of treatments. Tosun had 68 % activity, Kharchia 50 %, Gerek 28 %, and Bezostaja 22 %. at 20 % treatment, Kharchia had 50 %, Tosun and Gerek 20 %, and Bezostaja 27 % activity. Therefore , the order of photosynthetic performance of cultivars can be stated as Tosun, Kharchia, Gerek, and Bezostaja depending on the 0.2 % and 4 % salt treatments of one day.

3.3 Electrophoretic Analysis of Salt-induced Proteins

The polypeptide patterns of control and salt treated plants of two cultivars were analysed by SDS-PAGE and by Two-dimensional electrophoresis. One of the cultivars, Tosun, was identified as salt tolerant relative to the other cultivar, Gerek, according to both membrane leakage test and PS-II activity measurements. The protein analysis of root tissue were executed in both cultivars. The protein analysis of shoot tissue was executed only in Tosun. SDS-PAGE and Two dimensional analysis were carried out with both non-labelled and ^{35}[S]-Methionine labelled samples. The analysis of shoot tissue was with non-labelled samples.



A



B

Figure 5. Time course analysis of PS-II activity of Tosun and Gerek. A is 0.2 % NaCl treatment. B is 1 % NaCl treatment.

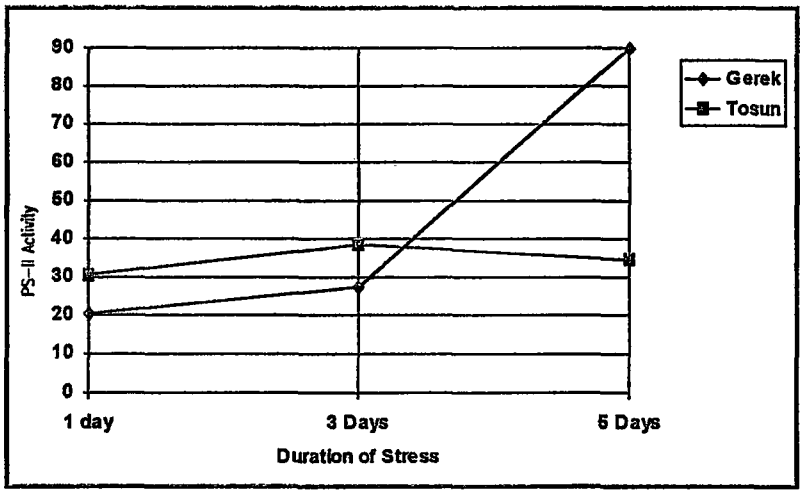


Figure 5.C 4 % NaCl treatment

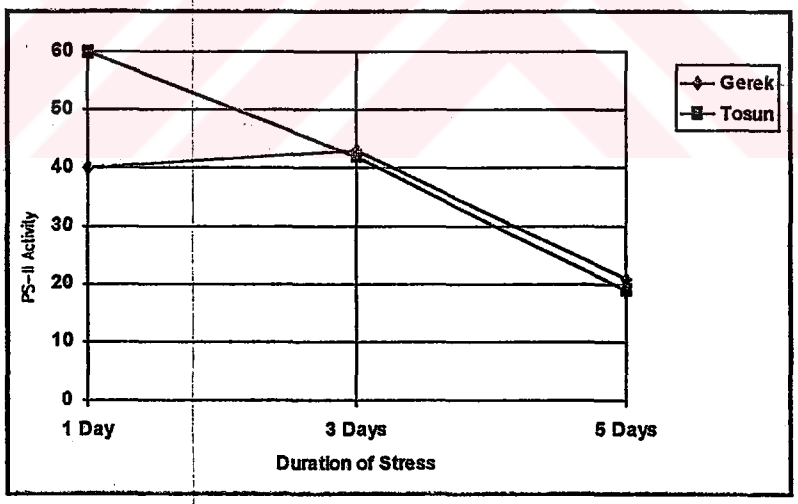


Figure 5.D 20 % NaCl treatment

3.3.1 SDS-PAGE Analysis of Root Proteins

SDS-PAGE of root tissue after one day treatment of 2 % salt caused changes in the accumulation of several proteins (Figure 6, Table III). The molecular weights of proteins which are expressed differently in salt stressed plants ranged from 17 kDa to 88 kDa. Two of these proteins (17 kDa and 22 kDa) showed the same kind of changes, increase and decrease respectively in both Tosun and Gerek. Other changes were cultivar specific. No unique protein was identified with SDS-PAGE analysis of non-labelled tissue.

SDS-PAGE analysis of labelled tissue after one day treatment of 4 % salt treatment caused changes in several proteins (Figure 7, Table IV). Some of the changes were the same as the non-labelled analysis 18 kDa and 63 kDa proteins. There were changes which was not detected in non-labelled analysis, 23.5 kDa, 24 kDa, 25 kDa, 27 kDa, 66 kDa, and 72 kDa proteins (Compare Table III and Table IV).

3.3.2 Two-dimensional Analysis of Root Tissue

Several changes in protein accumulation were identified with SDS-PAGE analysis. But, the actual changes were not clear from this analysis. The increased protein bands in SDS-PAGE could come from appearance of new proteins which were called new proteins, or simply an increase in the synthesis of continually synthesized proteins. To obtain a higher resolution in protein patterns after salt stress, the root proteins were analyzed with two dimensional gel electrophoresis.

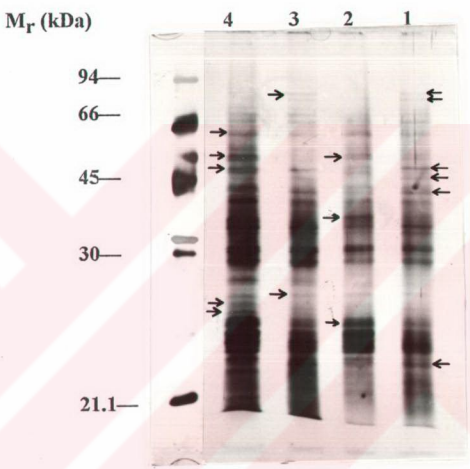


Figure 6. SDS-PAGE analysis of root tissue. The proteins were visualized by silver staining. Lane 1: Tosun seedlings grown in 2 % NaCl solution for three days. Lane 2: Tosun seedlings grown under control conditions. Lane 3: Gerek seedlings grown in 2 % NaCl solution for three days. Lane 4: Gerek seedlings grown under control conditions.

Table 3. Explanation of silver stain results given in Figure 6. (i) refers to increase in the intensity of protein bands. (d) refers to decrease in the intensity of protein bands. (N) refers to no change in the intensity of protein bands

Mr (kDa)	Tosun	Gerek
22	i	N
24	d	N
25	d	N
26	d	N
27	i	N
36	N	d
41	N	i
45	N	i
46	d	i
47	d	d
53	d	N
71	N	i
72	i	i

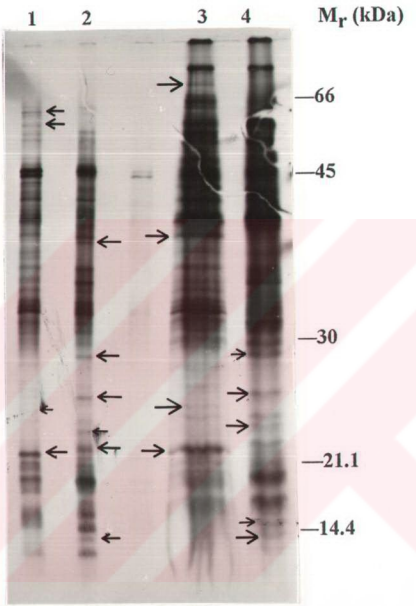


Figure 7. SDS-PAGE analysis of in vivo labelled proteins from root tissue. Lane 1 is Tosun seedlings grown under control conditions. Lane 2 is Tosun seedlings grown in 4 % NaCl solutions for one day. Lane 3 is Gerek seedlings grown under control conditions. Lane 4 is Gerek seedlings grown in 4 % NaCl solution for one day.

Table 4. Explanation of fluorography results given in Figure 7. (i) refers to increase in the intensity of protein bands. (d) refers to decrease in the intensity of protein bands. (N) refers to no change in the intensity of protein bands

Mr (kDa)_	Tosun	Gerek
17.2	i	N
17.7	i	i
18	d	N
23	i	d
23.5	i	N
24	N	d
25	i	i
27	i	i
28	i	i
32	i	i
44.3	i	N
46	N	d
64	d	N
66	d	N
72	N	d

One day treatment of 4 % salt caused changes in a large number of proteins of Tosun root tissue (Figure 8, Table V). There were 10 protein spots which increased in accumulation clearly by visual inspection compared to control tissue (Figure 8.A). There were 9 protein which decreased in accumulation under salt stress conditions (Figure 8.B). Three new proteins were identified in Tosun root tissue. The proteins changing quantitatively and qualitatively ranged in molecular weights from 22 kDa to 79 kDa and in pI value from 4.4 to 7.8 (Table V).

One day treatment of 4 % and 2 % salt caused changes in several proteins of Gerek root tissue (Figure 9, Table VI). There were 10 different proteins decreasing in accumulation under salt stress (Figure 9.A). and 3 newproteins increased under salt stress (Figure 9.B). One of the new proteins was identified under 2 % salt stress, the other two proteins appeared after 4 % treatment.No major protein spot which increases under salt stress was identified.

3.3.3 SDS-PAGE And Two-dimensional Analysis of Shoot Tissue

The protein pattern of shoot tissue under salt stress were analysed for Tosun. Three days of 4 % salt treatment resulted in changes in several proteins accumulation (Figure 10, Table VII). SDS-PAGE identified 5 proteins increasing in quantity after 2 % salt treatment. Three of these proteins were increased in quantity order 1 % salt treatment, one decreased in quantity and the other showed no difference compared to control protein pattern.

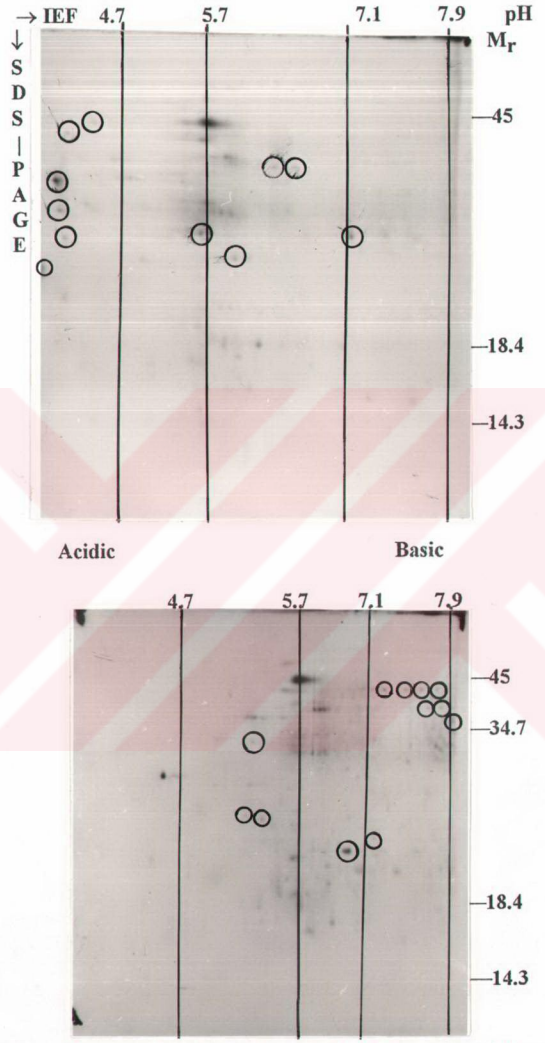


Figure 8. Two-dimensional analysis of in vivo labelled proteins of Tosun root tissue. A is seedlings grown under control conditios. B is seedlings grown in 4 % NaCl solution for one day. o indicates protein spots that changed in intensity.

Table 5. Explanation of flourography results given in Figure 8. (i) refers to increase in the intensity of protein bands. (d) refers to decrease in the intensity of protein bands.

Protein	Mr (kDa)	pI
d1	33	4.4
d2	40	6.2
d3	44	7.11
d4	45	5.7
d5	51	4.4i
d6	60	4.6
d7	66	6.7
d8	66	6.3
d9	79	4.6
d10	83	4.6
i1	22	6.9
i2	41	5.7
i3	62	7.5
i4	62	7.6
i5	69	7.8
i6	43	7.1
i7	43	7.2
i8	43	7.3
i9	43	7.6
s1	24	5.5
s2	25	5.3
s3	34	4.3

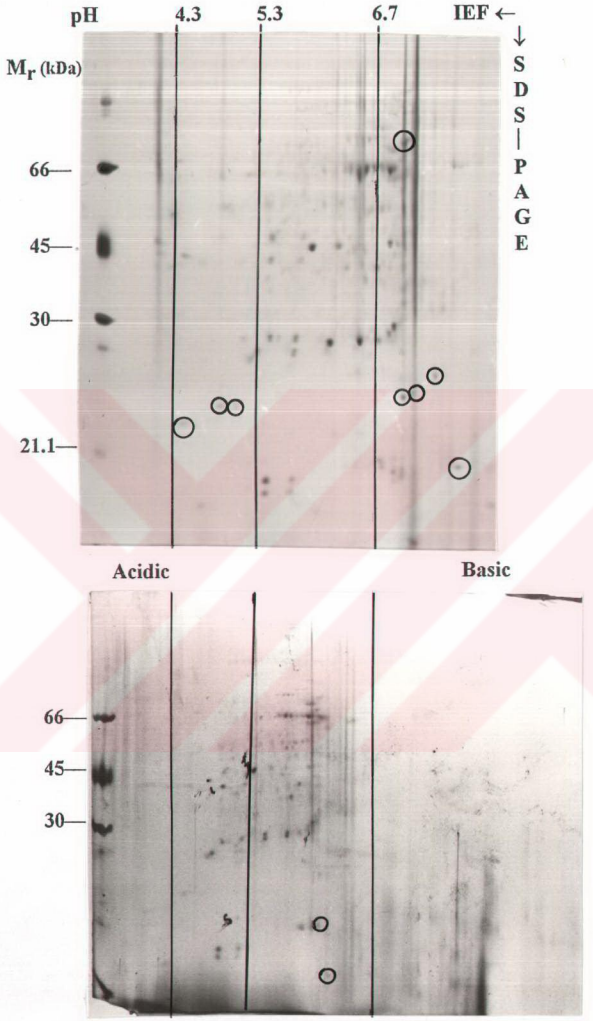


Figure 9. Two-dimensional analysis of proteins from Gerek root tissue. The proteins were visualized by silver staining. A is seedlings grown under control conditions. B is seedlings grown in 4 % NaCl for one day. C is seedlings grown in 2% NaCl for one day.

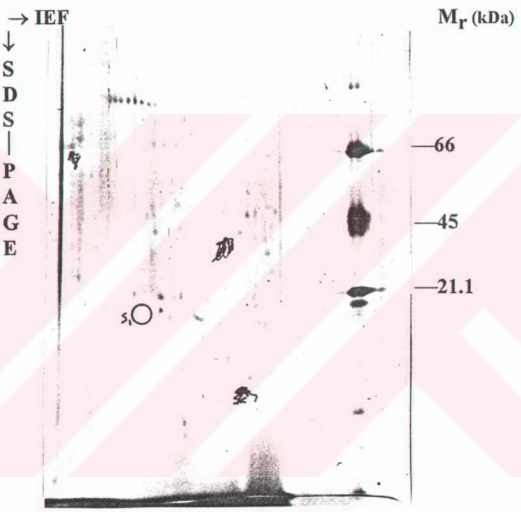


Figure 9.C Two-dimensional protein pattern of Gerek root tissue grown in 2 % NaCl for one day.

Table 6. Explanation of silver stain results given in Figure 9. (i) refers to increase in the intensity of protein bands.(Figure 9.B). (d) refers to decrease in the intensity of protein bands.

Protein	Mr (kDa)	pI
d1	16	7.8
d2	22	4.7
d3	24	5.2
d4	24	5.3
d5	26	7.2
d6	26	7.3
d7	27	7.4
d8	68	7.2
i1	28	6.5
i2	21	6.5
i3	28	6.8

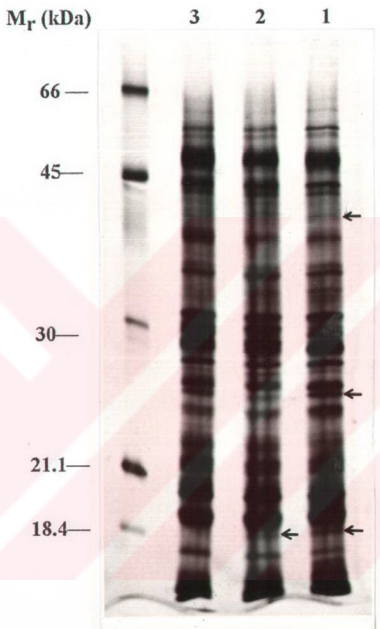


Figure 10. SDS-PAGE analisis of shoot tissue of Tosun. The proteins were visualized with silver staining . Lane 1 is the seedlings grown under control conditions. Lane 2 is seedlings grown in 1 % NaCl solution for one day. Lane 3 is seedlings grown in 2 % NaCl solution for one day. → indicates the protein bands which changed in intensity.

Table 7. Explanation of silver stain results given in Figure 10. (i) refers to increase in the intensity of protein bands. (d) refers to decrease in the intensity of protein bands. (N) refers to no change in the intensity of protein bands .

Mr (kDa)	1 %	2 %
18.5	i	i
34	N	i
39	N	i
51	N	i

To extend analysis of protein patterns , two dimensional electrophoresis was applied to shoot tissue of Tosun (Figure 11, Table VIII). There were 8 proteins decreasing in quantity (Figure 11.A) and two new proteins were identified (Figure 11.B). The proteins changing during salt stress ranged in molecular weights from 17 KD to 100 KD with pI values ranging from 4.8 to 7.0 (Table VIII).

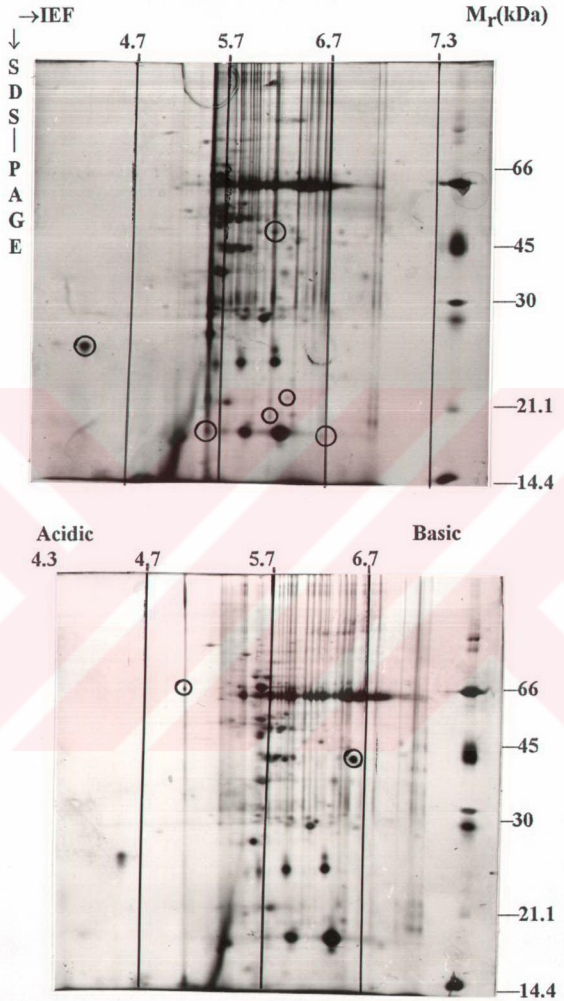


Figure 11. Two-dimensional gel electrophoretic analysis of shoot tissue of Tosun. The proteins were visualized by silver staining. A is the control, and B is the one day treatment of 4 % NaCl. "o" indicates the polypeptides that changed in intensity.

Table 8. Explanation of silver stain results given in Figure 11. (i) refers to increase in the intensity of protein bands. (d) refers to decrease in the intensity of protein bands.

Protein	Mr (kDa)	pI
d1	17	5.6
d2	19	6.0
d3	19	6.9
d4	27	4.5
d5	38	7.2
d6	51	6.5
s1	63	5.0
s2	39	6.6

CHAPTER IV

DISCUSSION

Water stress is one of the environmentally adverse conditions imposed on plants. Coping with water stress involves the adaptation of terrestrial plants to their often changeable environments. Crop plants are generally salt sensitive species. Identification of crops which give high yield under salt stress are important aims in crop improvement programs for two reasons. First, salt tolerant and high yielding crops means introducing non-arable lands to agriculture. Second, salinization of irrigated land is a serious problem. Economically feasible technological approaches can only minimize the salt amount in soil, but can not eliminate it completely. Development of salt-tolerant plants would make possible improved utilization of saline soils and water.

One strategy in the development of salt tolerant crops may be the use of recombinant gene techniques to develop a high yielding crop cultivar under salt stress by using the genetic variability within the species. This type of approach involves screening a large number of cultivars for salt tolerance, then the identifying the molecular basis of salt tolerance in the tolerant cultivars with the long-term goal of identifying and isolating the halo tolerance genes and the transfer of these genes to a high yielding cultivar. This present study contains experimental results in our our

attempt to establish consistent and accurate screening methodology and some preliminary work at the molecular level.

In the following lines, the results from physiological and biochemical studies will be discussed in attempts to characterize plant responses to salt stress, how such responses can be used in screening methodology, and how this methodology may be used in the future molecular studies.

4.1 Efects Of Different Salt Treatments

Four winter wheat cultivars were examined under salt stress for PS-II activity, membrane integrity and protein analysis.The salt stress were started by immediately placing the plant to the salty solution (salt shock) and laeving in that solution as long as stress duration when seedlings were 6 days old. The use of early seedlings is appropriate since seedling response to salt stress is highly predictive of adult plant response to salinity (Blum 1985). The age of the seedlings may be a complicating factor in the interpretation of the data for salt stress since salt treatment could alter the physiological age of cultivars and observed changes could be due, in part, to differences in developemental states. In this study, the stress duration was 5 days for the longest one. In PS-II activity and membrane leakage test, the controls were taken for a time course of 1 to 5 days and no any significant changes were observed . This can be interpreted as that the PS-II activity or membrane leakage does not show dependence on the developemental stage of seedlings for the first 6 to 11 days period of seedlings (Table I and Table II). Similarly the protein patterns from 7 and 9 days old seedlings did not give any difference by SDS-PAGE. The results on control plants

showed that the age of seedlings used in this study does not complicate the interpretation of data about salinity tolerance. Moreover, the use of aerly seedling have other advantages. A rapid evaluation of salt tolerance is possible by using early seedlings instead of wasting time in waiting growing of plant.

4.1.1 Effect of Salt Strtess On Membrane Integrity

The membrane integrity of four wheat cultivars were assesed by the electrical conductivity assay method which is the most commonly used physiological selection criteria to evaluate stress tolerance variability (Blum 1985).

Time course analysis of injury shows that the membrane stability is continuously decreasing. Figure 2 is a graphical representation of two cultivars which have a difference in tolerance as measured with ion leakage values. 0.2 % treatment did not change the injury to Gerek and Tosun in 5 days course. At the end of 5 days treatment, the injury values of both cultivars were over 80 %. 1 % Treatment caused an increase of 35 injury unit for Gerek, and 15 injury unit for Tosun. 4 % Treatment caused no variability in 5 days, but the course were different for the cultivars. The rate of injury to the plasma membrane were found to be cultivar dependent. For 1 day and 3 days , there was a difference in injury between cultivars. After 20 % Treatment, Injury to Tosun decrease in 3 days and increase up to 100 % in 5 days. Injury to Gerek is not changes too much, it is over 90 % for 1 day, 3 days, and 5 days. These results show that 1 % treatment cause a variability in injury between two cultivars. Injury to Tosun plasma membranes seemed to be lower compared to Gerek (Figure 2).

The injury to the plasma membrane of root tissue under salt stress were different , and varied among the cultivars. According to injury values, the cultivars were ranked in the order of salt tolerance as Kharchia, Tosun, Gerek, and Bezostaja (Figure 1).

4.1.2 Effect of Salt Stress On PS-II Activity

The photosynthetic activity of four wheat cultivars were assesed by the PS-II activity assay of isolated thylakoid membranes. The commonly used methods to measure the photosynthetic activity is based on the flourescence measurements on intact plants. This study takes a different approach by evaluating photosynthetic potential in vitro. In this way, the assay measures directly the genetic potential of photosynthetic machinary since the assays on intact plants are complicated by the presence of several phisiological responses to the water stress such as closure of stomata (Scriver 1990).

Time course analysis of PS-II activity shows that the activity increases with a time course of 5 days at different rates for Tosun and Gerek (Figure 5). The PS-II activity of Tosun increased from 80 % in 1 day stress to 90 % in 5 days stress for 0.2 % salt treatment (Figure 5.A). The activity decreases in 1 % treatment with time (Figure 5.B). But the decrease of Gerek is more from Tosun. The PS-II activity of Gerek maintained the same level in 5 days course for 4 % treatment, Tosun increased in activity 60 % during this time (Figure 5.C). The activity decreased to 20 % in 20 % salt treatment similarly for Gerek and Tosun. Therefore, Tosun had a higher capacity of maintenance of PS-II activity in the course of 5 days salt stress compared to Gerek.

PS-II activity ranked the cultivars according to salttolerance as Kharchia, Tosun, Gerek, and Bezostaja as shown in figure 4.

4.2 Electrophoretic Analysis

The protein patterns of 2 cultivars were analyzed with SDS-PAGE. Several appearing and disappearing bands were identified in saalt shocked plants compared to control plants. High resolution two dimensional gels confirmed and extended the SDS-PAGE results. In two dimensional gels, a lot of quantitative and qualitative differences were observed both between the treated and control plants of each cultivars and between the treated plants of two cultivars. The two cultivars used in electrophoretic studies were assayed for salt tolerance and a significant difference in salt tolerance of two cultivars were identified and thus any difference in accumulation of polypeptides specific to relatively salt tolerant cultivar may have implications for the trait of salt tolerance of wheat. The response of plants to salt is complex and involves changes in growyh, water relations, ion transport, and cell metabolism. Although the underlying mechanisms for salt tolerance remain elusive, the increases of certain polypeptides during salt stress could be important in adaptation of plants to salt stress. For example, the osmotin increases in quantity with the adaptation of the cultured tobacco cells to medium containing high levels of NaCl (Singh 1985, 1987). This correlation between tolerance to salt and increase in quantity of osmotin were taken as evidence to suggest that this protein may have a role in osmoregulation.

Quantitative results included the enhancements of some spots, appearence of new spots, decrease of spots and disappearence of spots. Two examples of subtle

adjustment of metabolic pathways is known. Many halophytes adapt to changes in soil salinity by accumulating inorganic ions in their vacoules. The osmotic potentials of cytoplasm is balanced by the synthesis and accumulation of biologically compatible solutes such as proline, betain, polyamines, sugars or sugar alcohols.(Hanson 1982). Some facultative halophytes such as Mesembryanthemum crystallinum switch to crassulacean acid metabolism (CAM) which is an alternative mode of carbon metabolism which reduces evaporative water loss(Bohnert 1988).Quantitative results may reflect adjustments in metabolic pathways in response to salt stress. Hurkman disscused the implications of two dimensional gels on the metabolic adjustments in barley roots (Hurkman 1989). Quantitative changes were polypeptides whose levels changed and most of them were specific for the cultivar, either for "Tosun" or "Gerek".Based on the differences in their genetic backgrounds, this phenomenon is probably due to variebily in the response of each of the cultivars to salt. Because seedlings of "Tosun" and "Gerek" have different tolerance to salt, the specific changes at the polypeptide levels may be related to the trait of salt tolerance.

The enhanced or suppressed polypeptides are thought as a subtle response of cells under salt stress to readjust the cell metabolism to overcome the adverse effects of salt stress.

The polypeptides which are appearing only under stress conditions and absent under non-stress conditions are called unique proteins. Unique proteins are thought to be especially important because of their association to salt condition. This may suggest a specific role under salt stress conditions which is not needed under normal growth conditions.

Visual comparison of 2 Dimensional protein patterns of "Gerek" and "Tosun" between control and salt treated plants showed that a protein with a Molecular weight 30 KD and pI of 6.8 in "Tosun" roots was unique protein. These results may show that "Tosun" may have a better performance under salt stress conditions due to the accumulation of this protein. To check this hypothesis, the gene for this protein is needed to isolated through the microsequenceing of the protein. The characterization of the gene will provide an opportunity to more accurately asses their expression during salt stress. If molecular studies confirm that the expression of this gene has a role in salt tolerance, the isolated gene can be transfered into a salt sensitive, but otherwise have desirable characters. For example, "Gerek" is the most harvested cultivar in Turkey. This performance may be taken as evidence of a general vigor combination to different type of biotic and abiotic stresses. If the field trials show that "Gerek" is not good in saline environment and "Tosun" is well, The gene for salt tolerance could be used to improve "Gerek" for saline areas by recombinant gene techniques.

Although these results are not enough to suggest a protective role for the salt-induced proteins, it is a possibility. During dehydration in C. planteriferum, a number of new transcripts were observed (Piatkowski 1990). A cDNA clone dsp-22 described in C. planterum represents a gene product that accumulates in chloroplasts upon dehydration and associates with thylakoid membranes (Bartels 1992) and possibly exerts a protective role. In addition to the tissue and subcellular localization, its sequence is similar to Elip proteins and cbr protein pointing a role related in photosynthesis. During descication, chloroplasts undergo morphological changes and lose part of their internal organization while retaining their chlorophyll. By analogy to the cbr protein, Bartels suggested that the dsp-22 protein may bind pigments or help

to maintain assembled photosynthetic structures essential for resuming active photosynthesis.

4.3 Screening of Wheat Cultivars For Salt Tolerance

Evaluation of salt tolerance of cultivars is an important objective in crop improvement The choice of identifiable stress resistance parents in breeding programs has been always the major limitation for crop improvement (Bansal 1991). There are examples of evaluation of salt resistance of wheat cultivars. The evaluation could be based on dry matter, grain yield, water status and some physiological processes. The root plasma membrane through electrical conductivity measurement is a common physiological criteria used to asses the salt and drought tolerance of plants. Photosynthesis is another important physiological processes that may be effective in stres tolerance. The growth depends exclusively on these processes. Thus, photosynthetic rate measurement under stress conditions are another commonly used parameter in tolerance assesments. Obviously salt tolerant cultivars must have stable root membranes and active photosynthetic rates under salt stress. But, it is possible that there may be cultivars which have stable root membranes and inactive photosynthetsis and vice versa resulting in a salt sensitive trait. Concequently to asses correctly the salt tolerance one should have the combination of parameters which are important in salt tolerance.

In this study, four cultivars of three varieties were evaluated for salt tolerance by two parameters. The test were used to show the variation in the genetic background of wheat cultivars in two different site which are important sites for

agronomic traits. Salt tolerance is a complex property and have effects on different sites of plants. The fist damage is to root plasm membranes. Cells retain their individual identity through the integrity of plasma membranes. Any kind of damage to the membranes will affect the performance of plant concerning the agronomic traits. We used the ion leakage assesed by electrical conductivity measurements in the same way with other studies concerning many species for evaluation of salt tolerance.

To asses the photosynthetic activity in intanct plants by fluororescence is the most common way. This type of evaluation measures whole photosynthetic machinery including physiological levels such as stomatal controls. This study takes a different aproach in this respect. The photosynthetic capability of plants were evaluated in vitro. The Photosystem II complex of phothosynthetic machinery which is thought to be most stress sensitive part, was isolated and assayed for activity.

The results of leakage test showed that cell membrane stability is higher for Tosun compared to Gerek. The results of PS-II activity shows similarly that Tosun is more active in photosynthesis under salt stress compared to Gerek. Kharchia which is a famous salt tolerant Indian variety of wheat is used as a standart to check if the tests measuring a salt tolerant variety. The performance of Kharchia was higher for the most of the experimental conditions. Tosun was second in salt tolerance. Bezostaja and Gerek were at much lower rank for both tests. Therefore, Gerek and Tosun become choice of protein studies as relative salt sensitive and relative salt tolerant cultivars respectively.

The correlation of less membrane damage in root tissue with higher PS-II activity confirms the order of salt tolerance between cultivars. Membrane stability is a parameter assesing the salt tolerance of cultivar. Photosystesis is a parameter which is

potentially important for the agronomic trait yield. Therefore, the combination of two parameters can be used to screen cultivars for salt tolerance and high yield at the same time.

CHAPTER V

CONCLUSION

In conclusion, Tosun has been determined as salt tolerant cultivar among the four cultivars by two physiological selection criteria. Tosun maintained highest PS-II activity under 4 % salt stress for 1 day, 3 days and 5 days of treatment. The most stable mebrane under salt stress was also found to be in Tosun. The biochemical analysis were conveyed thruogh SDS-PAGE and high resolution Two-dimensional gel electrophoresis. Quantitative and qualitative changes in protein patterns were revealed. This changes were cultivar specific. Tosun had at least three unique proteins with molecular weights of 24 kDa, 25 kDa and 34 kDa.

The correlation of salt tolerance of Tosun and the appearance of unique proteins only under salt stress conditions suggest that these proteins would have a role in salt tolerance trait. Further characterization of these three proteins and isolation of their genes are needed for revealing the function of these proteins.

In future studies, 2D gels may be used to isolate these polypeptides for antibody production and microsquencing. The antibody and microsquence information can be used in procedures designed to isolate the genes that corresponds to these

polyppeptides. The characterization of these genes will provide an opportunity to more accurately asses their expression during salt stress.

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APPENDICES

Appendix A

Preperation of Stock Solutions

SDS-PAGE Slab Gel Solutions

1. ACRYLAMIDE/BIS (30 % T, 2.67 % C)

30 grams of Acrylamide and 0.8 grams of N'N'-bis-methylene-acrylamide were dissolved in distilled water and diluted to 100 ml total volume, filtered through Whatman 1 filter paper and stred at 4 C in a dark bottle. The solution were used up before 30 days.

2. Seperating Gel Buffer

18.15 grams of Trizma base were dissolved in 50 ml distilled water. The pH of the solution were adjusted to 8.8 and diluted to make the total volume 100 ml (1.5 M Tris). The solution were filtered through Whatman 1 filter paper and stored at 4 C.

3. Stacking Gel Buffer

6 grams of Trizma base were dissolved in 60 ml distilled water. The pH of the solutioon were adjusted to 6.8 with concentrated HCl and diluted to 100 ml total volume. The solution were filtered thruogh Whatman 1 filter paper and stored at 4 C.

4. 10 % SDS

10 grams of SDS were dissolved in 100 ml distilled waterwith gentle stirring.

5. 10 % APS

10 milligrams of Ammonium per sulphate were dissolved in 100 µl distilled water. Always fresh solution was used.

Appendix A

6. SDS-PAGE Sample Buffer

1 ml stacking gel buffer, 0.8 ml Glycerol, 1.6 ml 10 % SDS, 0.4 ml ß-mercaptoethanol and 4.2 ml distilled water were mixed to make a 8 ml solution. Solution were prepared freshly.

7. 5x Running Buffer

60 grams of Trizma base and 360 grams of Glycine were dissolved distilled water and diluted to make the total volume 500 ml. The solution was stored at 4 C. 0.1 grams SDS was added to 100 ml 1x running buffer before use.

8. 12 % Gel Preparation

40 ml Acrylamide, 33.5 ml distilled water, 25 ml seperating gel buffer, 1 ml 10 % SDS were mixed to make 100 ml gel solution. 500 µl APS and 50 µl TEMED were added for polymerization and the solution were poured into. After 30 minutes waiting , stacking gel preparedfrom 1.3 ml Acrylamide, 6.1 ml distilled water, 2.5 ml stacking gel buffer, 100µl 10 % SDS polymerized with50 µl APS ans 10 µl TEMED were poured onto seperating gel.

Two-Dimensional Electrophoresis

1. UKS Solubilization Buffer

2.85 grams Urea, 25 mg K_2CO_3, 625 µl 10 % SDS , 25 mg Dithiothreotol, 300 µl Triton X-100, 100 µl 3-10 Ampholine and 2 ml distilled water were mixed to make 5 ml solution, filtered through 0.45 µm millipore filter and frozed at -20 C.

2. IEF Gel Solution

630 µl acrylamide solution, 160 µl 5-7 ampholine, 40 µl 3-10 ampholine, 150 µl Triton X-100, 2.75 grams of Urea, 11 mg Bis and 2 ml distilled water were mixed and frozen for storage at -20 C. The gel was poured by adding 7.5 µl APS and 5 µl TEMED for polymerization.

3. Electrolytes

2 grams of NaOH were dissoved in 500 ml distilled water and degassed under vacuum to prepare catholyte. The anolyte was 4.5 ml H_3PO_4 in 4.5 liters distilled water.

4. Agar Overlay

500 mg agar was dissolved in 100 ml distilled water with heating. 25 mg bromophenol blue was added.

Lowry Protein Assay Solutions

1. Reagent A: 2 % Na_2CO_3, 0.4 % NaOH, 0.15 % sodium tartrate, 1 % SDS,

2. Reagent B: 4 % $CuSO_4$, $5H_2O$

3.Reagent C: Mix 100 parts of Reagent A with 1 part of Reagent B.

4. Dilute Folin-Ciocalteu 2 N phenol reagent, 1:1 (v/v)

Bradford Protein Assay Solutions

1. 5X Reagent solution (prepared according to Bradford 1976)

500 mg Serva Blau were dissolved in 250 ml 95 % Ethanol. To this solution, 500 ml 85 % Phosphoric acid was added and the solution was diluted to one liter with distilled water. 1X reagent solution were filtered through Whatman 1 filter paper and left at least 24 hours at 25 C before use.

2. 1 mg BSA/1ml as standard protein solution

One mg Bovine Serum Albumine was dissolved in 1 ml distilled water by gentle mixing and stored in 0 C.

Appendix B

Procedure for Silver Staining of Proteins in Polyacrylamide gels

Steps	Solutions	Time of treatment[a)]
1. Fixation	50 %MeOH,12 % AcOH 0.5 ml 37 % HCOH/l	>1 hours
2. Wash	50 % EtOH	3 X 20 minutes
3. Pretreat	$Na_2S_2O_3.5H_2O$ (0.2 g/l)	1 minute[b)]
4. Rinse	H_2O	3 X 20 seconds[b)]
5. Impregnate	$AgNO_3$ (2 grams/l) 0.75 ml 37 % HCOH/l	20 minutes
6. Rinse	H_2O	2 X 20 minutes
7. Develope	Na_2CO_3(60 grams/l) 0.5 ml 37 % HCOH/l $Na_2S_2O_3.5H_2O$ (4 mg/l)	10 minutes[d)]
8. Wash	H_2O	2 X 2 minutes
9. Stop	50 % MeOH, 12 % AcOH	10 minutes
10. wash	50 % MeOH	>10 minutes[c)]

a)Steps 1-10 were carried outon a shaker at room temperature
b)The times indicated here should be observed exactly in order to ensure a reproducible image development
c)After step 10, transfer to 4 C for storage.
d)The time of development depends on the amount of protein in the gel