

# **STRESS RELATED RESPONSES IN SOYBEAN**

by

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## **PREFACE**

The experimental work described in this thesis was conducted in the Research Centre for Plant Growth and Development, School of Botany and Zoology, University of Natal, Pietermaritzburg, from January 1998 to June 2000 under the supervision of Professor J van Staden.

The results have not been submitted in any other form to another University and except where the work of others is acknowledged in the text, are the result of my own investigation.

**TAO LIU**  
**JUNE 2000**

I declare that the above statement is correct.

**PROF. J VAN STADEN**  
(SUPERVISOR)

## PUBLICATIONS FROM THIS THESIS

**LIU T, VAN STADEN J and CRESS WA** (2000) Salinity induced nuclear and DNA degradation in meristematic cells of soybean (*Glycine max* (L.)) roots. *Plant Growth Regulation* 30: 49-54.

**LIU T, VAN STADEN J and CRESS WA** (2000) Gene expression in soybean (*Glycine max* L.) roots exposed to low temperature: Isolation of cDNA clones. *Plant Growth Regulation* 30: 247-251.

**LIU T and VAN STADEN J** (2000) Selection and characterization of sodium chloride-tolerant callus of *Glycine max* (L.) Merr. cv. Acme. *Plant Growth Regulation* 31:195-207.

# CONFERENCE CONTRIBUTIONS FROM THIS THESIS

1. **LIU T, VAN STADEN J and CRESS WA (1998)** Characterization of the gene complex regulating the response of soybean roots to heat, cold, water and salt stress. **Twenty-fourth Annual Congress of South African Association of Botanists, University of Cape Town, Cape Town.**
2. **LIU T, VAN STADEN J and CRESS WA (1999)** Sodium chloride induced nuclear and DNA degradation in meristematic cells of soybean (*Glycine max* (L.)) roots. **Twenty-fifth Annual Congress of South African Association of Botanists, University of Umtata, Umtata.**
3. **LIU T and VAN STADEN J (1999)** Molecular basis of adaption to water and salinity stress. **First National Conference on Science and Technology of Chinese Scholars in South Africa. University of Wits, Johannesburg.**
4. **LIU T and VAN STADEN J (2000)** Effects of sodium chloride stress on callus cultures of *Glycine max* (L.) Merr. cv. Acme: Selection and characterization. **Twenty-sixth Annual Congress of South African Association of Botanists, University of Potchefstroom, Potchefstroom.**

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## ABSTRACT

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Environmental stresses such as drought, salinity and low temperature have been major selective forces throughout plant evolution and are important factors which limit crop plant distribution and agricultural productivity. An understanding of how crops adapt to adverse conditions is not only of theoretical interest, but also has considerable practical value.

Low-temperature stress subtraction libraries were constructed in a pBluescript vector with the two-step-PCR amplified cDNAs using subtractive hybridization. One insert cs18 was obtained and the sequence analysis of insert cs18 revealed that the insert cDNA had a 76% homology with the sequences of the corresponding portion of glucose dehydrogenase from *Thermoplasma acidophilum* and 62.0% homology with a genomic DNA of *Arabidopsis*. Four clones, cs18-13, cs18-14, cs18-15, and cs18-16 from low-temperature stress soybean root conventional cDNA library have been confirmed to have inserts that could hybridize to the cs18 insert. One cDNA with a *Xba* I and *Xho* I fragment of approximately 3,500 bp in length corresponded to the insert cs18, which probably encodes for glucose dehydrogenase, was obtained. Northern blot analysis indicated that cs18 mRNA was highly expressed in soybean root but moderately expressed in leaves under low temperature.

Changes in the nuclei of meristematic root cells in response to severe salinity were studied. Roots are in direct contact with the surrounding solution. Thus, they are the first to encounter the saline medium and are potentially the first site of damage or line of defence under salt stress. Nuclear deformation or degradation in the soybean root meristem with 150 mM or higher NaCl led to sequential cell degradation, cell death and cessation of plant growth. However, this study indicates that an increase in  $\text{CaCl}_2$  concentration up to 5 mM could partially prevent salt injury to the cells.

Tissue culture is an excellent tool for elucidating the correlation between plant organizational levels and salt tolerance because of the possibility it offers for studying the physiology of intact plantlets together with that of organs and single cells using homogenous plant material under uniform environmental conditions. One NaCl-tolerant cell line (R100) was isolated during this study. The R100 callus cell line was significantly more tolerant to salt than the salt-sensitive line (S100) during exposure to salt stress. Salt tolerance in this culture was characterized by an altered growth behaviour, reduced cell volume and relative water content, and accumulation of  $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{K}^+$ , proline and sugars when grown under salt stress and with its subsequent relief. The selection of this salt tolerant cell line has potential for contributing new genetic variability to plant breeders.

Sugars are not only important energy sources and structural components in plants, they are also central regulatory molecules controlling physiology, metabolism, cell cycle, development, and gene expression in plants. The



concentrations of glucose and fructose increased during salt stress and after relieving salt stress, at a rate closely corresponding to the increase in relative water content. Their accumulation was the earliest response detected during the removing of salt stress indicating that glucose and fructose may play important roles during salt-stress.

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## ABBREVIATIONS

ABA	Absciscic acid
ABRE	ABA response element
AMP	Adenosine monophosphate
BLAST	Basic local alignment search tool
bp	Base pairs
cDNA	Complementary DNA
CN	Cell nuclei
cs	Cold stress
DAPI	4'6-diamino-2-phorryliudol
DNA	Deoxyribonucleic acid
DW	Dry weight
DRE	Dehydration response element (C-repeat)
DMSO	Dimethyl sulfoxide
2,4-D	Dichlorophenoxy acetic acid
EDTA	Ethylene diamineteraacetic acid
EDX	Energy dispersive X-ray
EMBL	European Molecular Biology Laboratory
FW	Fresh weight
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
HXK	Hexokinase
IPTG	Isopropylthiogalactoside
LEA	Late-embryogenesis abundant
MB	Miller's basal medium
MOPS	3-(N-morpholino) propanesulphonic acid
mRNA	Message RNA
MS	Murashige & Skoog solution
NAA	Naphthaleneacetic acid

NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
OPPP	Oxidative pentose phosphate pathway
PCR	Polymerase chain reaction
6PG	6-phosphoglucoate
6PGDH	6-phosphoglucoate dehydrogenase
PVP	Polyvinyl-pyrrolidone
RAB	Responsive to ABA
RGR	Relative growth rate
RNA	Ribonucleic acid
RWC	Relative water content
ROS	Reactive oxygen species
$\Psi_s$	Solute or osmotic potential
SDS	Sodium dodecylsulfate
SEM	Scanning electron microscopy
SSC	Standard saline citrate buffer
TAE	Tris, acetic acid and EDTA buffer
TE	Tris and EDTA buffer
TEM	Transmission electron microscopy
TM	Melting temperature
X-Gal	5-bromo-4-chloro-3-indoyl- $\beta$ -D-galactoside

## ***Chapter One***

### **General Introduction**

Plant life is strongly dependent on the environment, and plants regulate their growth and development in response to many different environmental stimuli. Drought, salinity, temperature extremes and chemical interference are stresses often encountered. Alteration of environments and climates may also result from human activities that increase the stressful conditions under which plants must grow and survive (HALE & ORCUTT 1987). These adverse conditions may interfere with normal growth and development and in crop plants will lower food quality and yield (SACHS & HO 1986). New means of improving crop productivity must be found to increase the resources available to man. One way of doing this is to develop crops that are more tolerant to such abiotic stresses as drought, flooding, heat, radiation, salinity, chilling and freezing, so that new land can be brought under cultivation (HOLMBERG & BÜLOW 1998).

What is stress? The term stress, when used in biology, has general connotations rather than a precise definition (OSMOND *et al.* 1987). It is therefore most useful to apply the term stress in a more general sense as an 'overpowering pressure of some adverse force or influence' that tends to inhibit normal systems from functioning (JONES & JONES 1989). Plant stress is often defined as any factor that decreases plant growth and reproduction below the potential of the genotype (OSMOND *et al.* 1987). However, biotic stress remains a broadly defined and poorly understood form of

plant stress, partly because its physiological consequences are often highly variable. Abiotic factors, such as temperatures, water relations, and salinity are relatively easy to control experimentally. Stress results in an aberrant change in physiological processes brought about by one or a combination of environmental and biological factors. An inherent connotation in the term is that stress has the potential to produce injury. Injury occurs as a result of aberrant metabolism and may be expressed as a reduction in growth, yield, value, death of the plant, or a plant part (HALE & ORCUTT 1987).

Tolerance to a stress is the capacity of a plant to survive, grow and reproduce even though subjected to an unfavourable environment. The plant can sustain the effects of stress without dying or suffering irreparable injury. Whole plants can be resistant and survive stress injury, or some parts of a single plant may be resistant (seeds, buds, or dormant cells) while other parts (meristems, seedlings) are susceptible. Through the processes of evolution a plant species can become fit or adapted to an environment in which it thrives. Tolerance or resistance may change as a plant grows and develops so that at one stage of development a plant may be susceptible to stress induced injury but at another stage it may be completely resistant. In addition, a stress may change metabolism, a process called acclimation, and through such changes alter the morphology and render the plant resistant to that stress. Plants that become acclimated are hardened and can survive in the new environment (LIU 1997).

Plants respond to environmental stresses through a series of adaptive

physiological and biochemical changes that enhance their ability to survive adverse conditions. Stress relieving genes that are transcribed might encode enzymes involved in a particular metabolic pathway, regulatory proteins or proteins with specific protective properties (HOLMBERG & BÜLOW 1998). It is believed that such stress-induced proteins allow plants to make biochemical and structural adjustments that enable them to cope with the stress condition (SACHS & HO 1986).

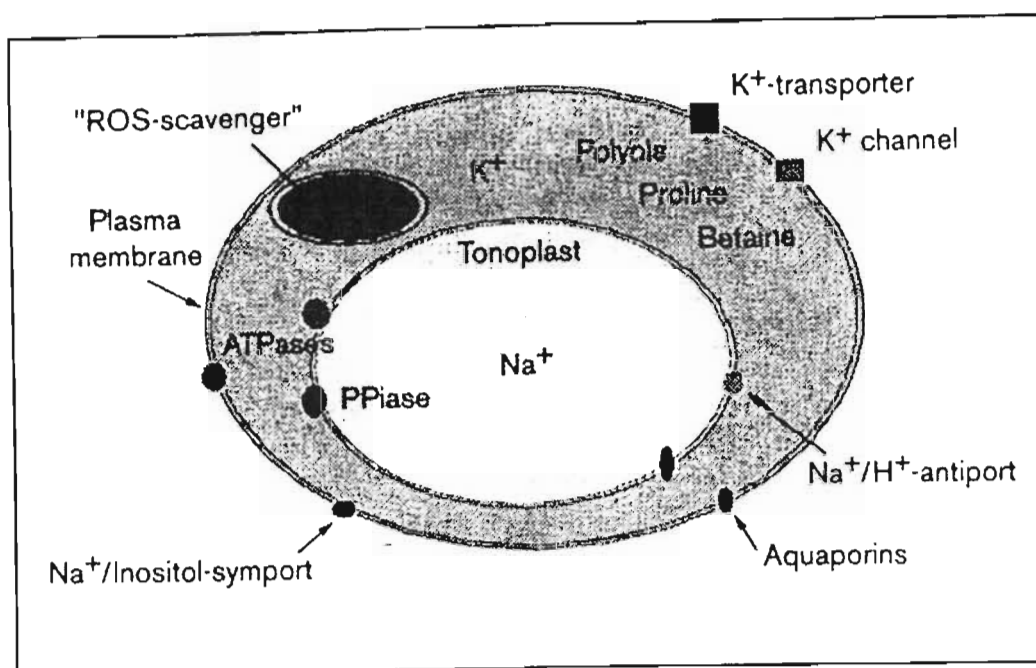
Plant growth reduction in a saline environment is commonly attributed either to ion toxicity or to water deficit (GREENWAY & MUNNS 1980). Water stress is one of the first and most evident effects of salinity. The primary effects of water deficit in plants are a decrease in the water content and a low water potential. The decrease in water potential affects water movement into growing regions and cell elongation rate (BRADFORD & HSIAO 1982; ESCOBAR-GUTIÉRREZ *et al.* 1998). Water deficit affects plant morphology and cellular metabolism. Plants have evolved mechanisms that allow them to acclimatize to a variety of environmental stresses. Low temperature is one such condition, perhaps the most dramatic manifestation of cold acclimation, or cold hardening, is the increased freezing tolerance that occurs in many plant species (LEVITT 1980). Low temperatures have a strong impact on the natural, geographical distribution of plants. This is because temperature affects the rates of biochemical processes differently and thus induces imbalances between partial processes in metabolic pathways. Adjustments to buffer low temperature effects are seen in most plant processes such as growth and development, photosynthesis, respiration, and reproduction (KÖRNER & LARCHER 1988; HÅLLGREN & ÓQUIST 1990).

A few studies of cold acclimation have begun to focus on some of the more rapid physiological and molecular responses in plants subjected to low temperatures. These studies have already revealed that plant and algal cells can rapidly alter membrane lipid composition (LYNCH & THOMPSON 1984), RNA (CATTIVELLI & BARTELS 1989) and protein content (GILMOUR *et al.* 1988 ) within hours of low temperature exposure. These findings of rapid biochemical alterations in response to low temperature are in agreement with observations of the rapid induction of freezing tolerance at inductive temperatures and at non-inductive temperatures by desiccation (SIMINOVITCH & CLOUTIER 1982).

Cellular and metabolic changes that occur during cold acclimation include increased levels of sugars, soluble protein, proline and organic acids as well as the appearance of new isoforms of proteins and altered lipid membrane composition (HUGHES & DUNN 1996). Two lines of evidence suggest a possible molecular basis for the adjustment of metabolism to low non-freezing temperature, and perhaps for freezing tolerance. The evidence consists of repeated observations that a number of enzymes show shifts in isozymic composition upon exposure to low temperature. Numerous electrophoretic studies have shown both quantitative and qualitative differences in the protein content between non-acclimated and cold-acclimated tissues (GUY 1990).

During the past 10 years, the complex interrelationship of biochemical pathways that change during stress has become appreciated, although we are far from understanding this complexity. In Figure 1.1, mechanisms for which experimental





**Figure 1.1: Enzymes, proteins, and metabolites important in plant cellular stress responses.** Stresses lead to increased production of reactive oxygen species (ROS) which are counteracted by changes in the activity and or amount of ROS scavenging systems. Stress leads to increased proton pumping across plasma membrane (P-ATPase) and tonoplast membrane (Ppi-ase, V-ATPase). Compartmentation of sodium during salt stress is accomplished by a tonoplast  $\text{Na}^+/\text{H}^+$ -antiport system and potassium levels in the cytosol are maintained to some degree. Compensating the osmotic pressure generated by vacuolar sodium, cytosolic amounts of a variety of metabolites increase (exemplified by polyol, glycinebetaine, and proline). The mechanism of entry of sodium into the cytosol is not known, potassium transporters or channels might be responsible. The stress-dependent regulation of aquaporins indicates their involvement as water channels during stress responses and some may also function in metabolite or ion transport (BOHNERT & SHEVELEVA 1998).

evidence indicates an important contribution to metabolic adjustments under stress at the cell level are illustrated with the names of proteins, enzymes and metabolites. The significance of these mechanisms is supported by gene discovery, with stress-dependent regulation of the corresponding transcripts, or by biochemical analyses (BOHNERT & SHEVELEVA 1998).

Drought, salinity and low temperature affect uptake and conductance of water. Environmental factors that affect water supply lead to changes in stomatal opening which can, if stress persists, set in motion a chain of events originating from changes in the concentration leaf-internal carbon dioxide, consecutively affecting the carbon reduction cycle, light reactions, energy change, and proton pumping (BOHNERT & JENSEN 1996a; BOHNERT & JENSEN 1996b; INGRAM & BARTELS 1996; JAIN & SELVARAJ 1997; BOHNERT & SHEVELEVA 1998). Osmotic adjustment is a fundamental adaptive response of plant cells when exposed to salinity and is necessary for survival and growth under saline conditions. Osmotic adjustment in response to salinity is a result of solute accumulation which occurs through the uptake of solute, the synthesis of organic compounds, or both (BINZEL *et al.* 1987). The solutes that accumulate during osmotic adjustment include sugars, amino acids, organic acids, proline and glycine betaine. Carbohydrates, and amino acids are major organic components affected in several plant species under salt stress, their relative contribution depending on environmental conditions (BINZEL *et al.* 1987).

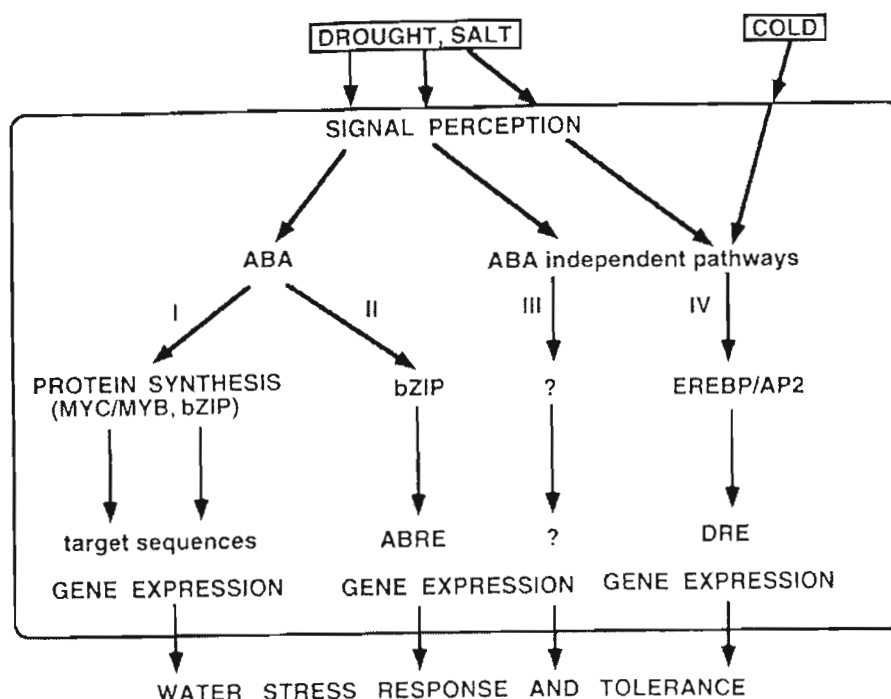
Sugars have long been known to increase in a wide range of plants grown at low moisture levels and under salinity. In higher plants, sugars are required not only to sustain heterotrophic growth but also to regulate the expression of a variety of genes. Environmental stresses, such as pathogen infection and wounding, activate a cascade of defense responses and may also affect carbohydrate metabolism (EHNESS *et al.* 1997). The rate and extent of increase in sugar content depends on the environmental conditions, species, and even on the genotype within the same species. Three sugars, glucose, fructose and sucrose accumulate in crop plants but little information is

available as to the situation in plant cells under water and salinity stresses. Amino acid accumulation does occur in a variety of monocotyledons and dicotyledons. One of the best characterized osmoregulatory responses is the accumulation of proline. In some tissues, proline levels increase as much as 100 fold in response to stress (VOETBERG & SHARP 1991).

A typical response of many plants to saline environments is to accumulate high intracellular concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  (BINZEL *et al.* 1988). In general, as the NaCl level in the plant's environment increases, both the  $\text{Na}^+$  and  $\text{Cl}^-$  levels within the plant increase. Concurrently, the levels of  $\text{Ca}^{2+}$  and  $\text{K}^+$  tend to decrease (CROUGHAN *et al.* 1978).

Plants utilize a number of protective mechanisms to maintain normal cellular metabolism and prevent damage to the structure and function of cellular components. One fundamental metabolic alteration is the accumulation of stress-induced gene products. Induction of a specific array of genes in response to water stress may represent the initial adaptation of the plant to that stress. During the last few years, a number of drought and salt responsive genes have been isolated and characterized in tomato (COHEN & BRAY 1990), maize (CLOSE *et al.* 1989), and *Arabidopsis* (GILMOUR *et al.* 1992). DNA sequence analysis of water stress-inducible cDNAs indicates that these genes encode a variety of proteins. Many of the proteins encoded by these cDNAs have been classified as LEA (Late-Embryogenesis Abundant) (BAKER *et al.* 1988), RAB (Responsive to ABA) (SKRIVER & MUNDY 1990) or dehydrin proteins (CLOSE *et al.* 1989).

Osmotic stress perception and signaling, which has become a focus of research on environmental stresses (Figure 1.2) (SHINOZAKI & YAMAGUCHI-SHINOZAKI 1997; BRAY 1997), is translated into biochemical reactions, metabolic adjustments and altered physiological state, thus re-programming the progress of development (BOHNERT & SHEVELEVA 1998). Recently studies in plants have begun to outline signal transduction mechanisms connecting osmosensation with changes in gene expression. It is thought that loss of turgor or change in cell volume resulting from different environmental stresses permits the detection of loss of water at the cellular level. One or both of these changes may activate stretch-activated channels, alter conformation or juxtaposition of critical proteins, or cause alterations in the cell wall-plasma membrane continuum (DING & PICKARD 1993), thereby triggering a signal transduction pathway(s) that induces gene expression. Relevant to the topic of stress-mediated adjustments of metabolism is the recognition that stress responses are elicited through several pathways and that these pathways are cross-linked. At least four signal transduction chains exist in plants for responding to drought, salinity and low temperature (BOHNERT & SHEVELEVA 1998). An abscisic acid (ABA)-dependent pathway responds to drought and salinity signals. Elevation in endogenous ABA content is known to induce certain water-deficit-induced genes. Therefore, ABA accumulation is a step in one of the signal transduction pathways that induces genes during water deficit (BOHNERT *et al.* 1995). A second signaling pathway, which does not depend on ABA, shows yet another bifurcation with differential responses of genes that are either affected by cold, salinity and drought, or by salinity and drought only. The receptors that sense drought or salinity are not yet identified - they may be similar to yeast osmo-sensors (POSAS *et al.* 1996). There are, however, several genes that are induced by

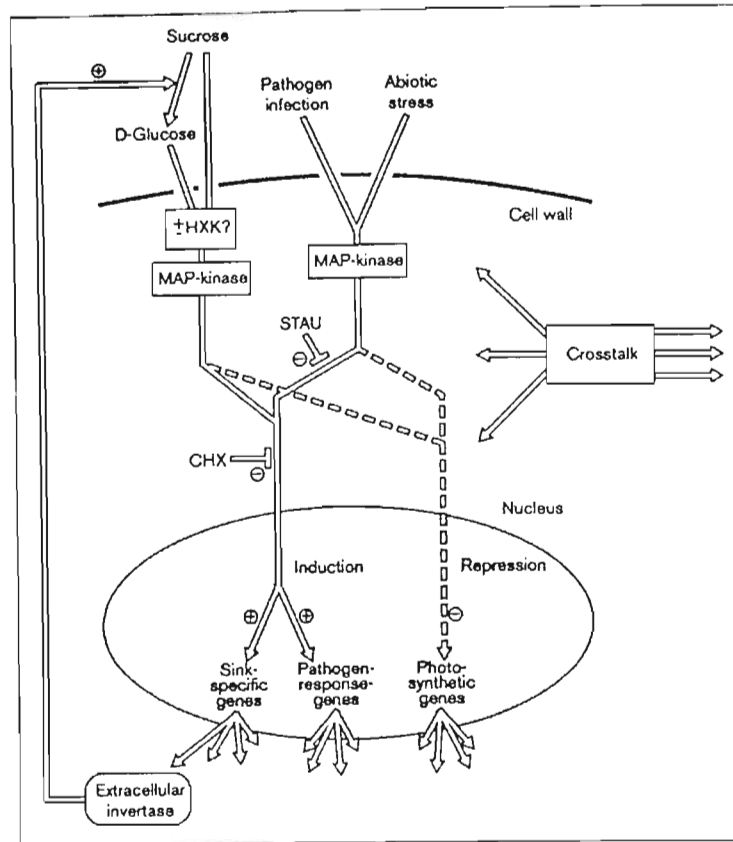


**Figure 1.2: Signal transduction pathways between initial drought-stress and/or cold-stress signals and gene expression.** At least four signal transduction pathways exist (I - IV): two are ABA-dependent (I and II), and two are ABA-independent (III and IV). Protein synthesis is required in one of the ABA-dependent pathways (I and II). There are at least four signaling pathway (I, II, III and IV) that function under drought and/or cold conditions and two pathways (III, and IV) that function under cold stress. ABRE is involved in one of the ABA-dependent pathways (II), and DRE is involved in one of the ABA-independent pathways (IV) (SHINOZAKI & YAMAGUCHI-SHINOZAKI 1997).

dehydration but not by exogenous ABA (Figure 1.2)(BRAY 1993; SHINOZAKI & YAMAGUCHI-SHINOZAKI 1996). Analysis of these genes has revealed that ABA-independent, as well as ABA-dependent, signal-transduction cascades operate between the initial signal of drought stress and the expression of specific genes. The ABA-independent expression of drought inducible genes has been analysed extensively and multiple signal-transduction cascades have been proposed between the initial

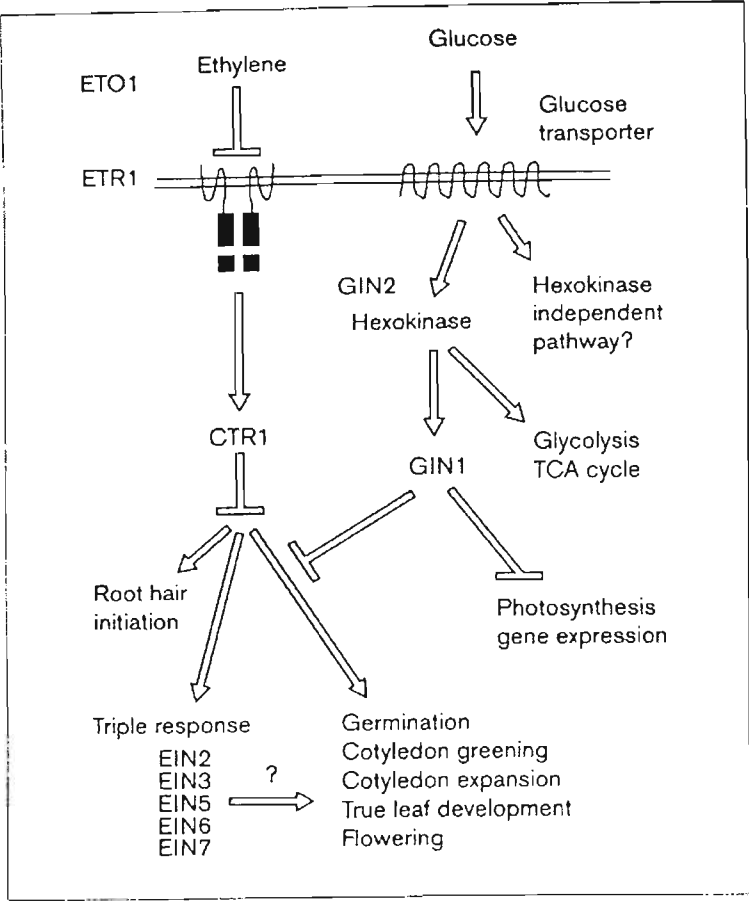
signal of drought stress and the expression of the genes (BRAY 1993; YAMAGUCHI-SHINOZAKI & SHINOZAKI 1994; SHINOZAKI & YAMAGUCHI-SHINOZAKI 1996). Other pathways are affected as a result of increased shuttling of carbon through the photorespiratory cycle (BOHNERT & JENSEN 1996a). Eventually, carbon and nitrogen allocation and storage require readjustment; reactions that lead to the consumption of reducing power to become favoured, and development and growth altered (BOHNERT & JENSEN 1996a; BOHNERT & JENSEN 1996b; INGRAM & BARTELS 1996; JAIN & SELVARAJ 1997; BOHNERT & SHEVELEVA 1998).

Sugars are not only important energy sources and structural components; they are also central regulatory molecules controlling physiology, metabolism, cell cycle, development, and gene expression in prokaryotes and eukaryotes (JANG *et al.* 1997). In higher plants, sugars affect growth and development throughout the life cycle, from germination through flowering to senescence (THOMAS & RODRIGUEZ 1994; DANGL *et al.* 1995). Recently, it has become apparent that sugars are physiological signals repressing or activating plant genes involved in many essential processes, including photosynthesis glyoxylate metabolism, respiration, starch and sucrose synthesis and degradation, nitrogen metabolism, pathogen defence, wounding response, cold stress, salt stress, cell cycle regulation, pigmentation, and senescence (CHEN *et al.* 1994). Although sugar signal transduction pathways are well characterized in prokaryotes and unicellular eukaryotes, relatively little is known about the molecular and biochemical mechanisms underlying sugar responses in multicellular eukaryotes, especially in sugar-producing higher plants. However, it is not clear whether sugars act as specific regulatory signals in plant development or merely disturb cellular metabolism and



**Figure 1.3: Model for the regulation of sink metabolism, photosynthesis and defence responses by sugars and stress-related stimuli.** Sugars and stress-related stimuli activate different signal transduction pathways that are ultimately integrated to co-ordinately regulate gene expression. Intracellular signalling may involve hexokinase (HXK) and MAP kinase and is modulated by other signal transduction pathways. Any signal that upregulates extracellular invertase will be amplified and maintained via the sugar-induced expression of this enzyme (ROITSCH 1999).

cause osmotic stress. Recent studies demonstrate that different stress related stimuli result in the same regulatory pattern of mRNAs for enzymes involved in source and sink metabolism and defence reactions. This indicates that defence responses are tightly linked to the up-regulation of sink metabolism to satisfy the energy requirements of the activation of the cascade of defence reactions (Figure 1.3)(ROITSCH 1999).



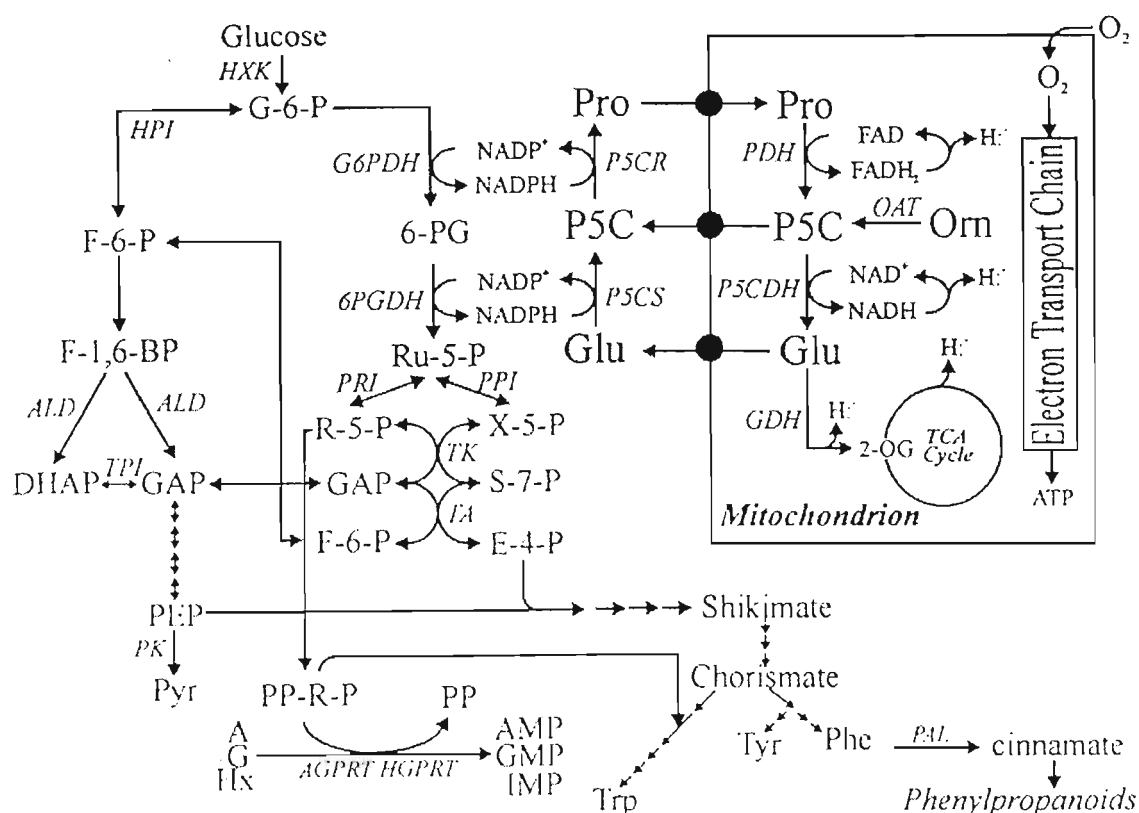
**Figure 1.4: Model for glucose and ethylene signaling.** The glucose and ethylene signaling pathways are antagonistic to each other. The balance between the glucose and ethylene signals determines plant growth and development. The convergent point between the two pathways is downstream of GIN1 and GIN2 (AtHXK1) in the glucose signaling pathway, and downstream of ETR1, CTR1, and EIN2 in the ethylene signaling pathway (SHEEN *et al.* 1999).

The specific effects of glucose on gene expression and development are characteristic of plant hormone actions. Similar to the classic plant hormones that are also plant metabolites, the synthesis, metabolism, and transport of glucose have been well studied (KOCH 1996; SHEEN1999; SHEEN *et al.* 1999). The glucose signal that triggers glucose repression remains obscure, although there is some progress on this front. The demonstration of hexokinase (HXK) as a specific glucose sensor and the



action of non-metabolizable glucose signals mediated through unknown sensors/receptors qualify glucose as a "plant hormone" with dual functions as a signalling molecule and an intermediary metabolite, but the mechanism is still elusive. The higher effective concentration of glucose relative to other hormones reflects its physiological role in allowing plants to monitor and adjust activities between sugar production and utilization. Evidence that cells can have significant concentrations of intracellular glucose raises the possibility that glucose itself is a signaling molecule (CARLSON 1998). Recently researches have uncovered intimate cross-talk between glucose and other plant hormone signaling pathways (Figure 1.4)(SHEEN *et al.* 1999).

Calcium plays an important role in the response of plants to salinity (RENGEL 1992). Elevated levels of external  $\text{Ca}^{2+}$  supply (4-10 mM) can enhance the "exclusion" of  $\text{Na}^+$  (LAHAYE & EPSTEIN 1971), prevent nuclear deformation and degradation (KATSUHARA & KAWASAKI 1996; KATSUHARA 1997), and help maintain the levels of tissue  $\text{K}^+$  (COLMER *et al.* 1994) in roots. The enhancement of net  $\text{K}^+$  to  $\text{Na}^+$  selectivity by supplemental  $\text{Ca}^{2+}$  is most pronounced in the growth zone of roots (ZHONG & LÄUCHLI 1994), and presumably result from  $\text{Ca}^{2+}$  being essential for the regulation of  $\text{K}^+$  and  $\text{Na}^+$  transport across the plasma membrane of plant cells (EPSTEIN 1961; RAINS & EPSTEIN 1967). In addition, supplemental  $\text{Ca}^{2+}$  also enhanced the accumulation of proline in salinized callus. It is not known whether external  $\text{Ca}^{2+}$  supply also modulated the levels of organic solutes in roots of intact plants exposed to high salinity.  $\text{Ca}^{2+}$  may be an important messenger in the signal transduction pathways that mediate stress-induced gene expression. Transient increases in free cytosolic  $\text{Ca}^{2+}$  have been observed in response to the imposition of several



**Figure 1.5: Possible metabolic functions of proline biosynthesis and degradation in plants during and after relief from stress.** Processes occurring in the mitochondrion are likely to be of importance only during recovery from stress (HARE 1998).

environmental stresses as well as to chemical treatments that stimulate stress-induced enhancement of oxygen activation (HARE *et al.* 1996).

During low temperature exposure increased levels of G6P have been reported in cotton (PERERA *et al.* 1995). Investigations of the flux through the oxidative pentose phosphate pathway (OPPP) under conditions of environmental stress are limited. An increase in activity of G6PDH was however, observed in water stressed barley (ARGANDONA & PAHLICH 1991) and transcripts encoding 6-phosphogluconate

dehydrogenase (6PGDH) and glucose-6-phosphate dehydrogenase (G6PDH) accumulated in alfalfa following treatment with a fungal elicitor (FAHRENDORF *et al.* 1995). The two dehydrogenases responsible for transforming glucose-6-phosphate (G6P) into ribose-5-phosphate are primarily regulated by the  $\text{NADP}^+/\text{NADPH}$  ratio, with both enzymes being strongly inhibited by NADPH. Dehydrogenase reactions that consume NADPH and produce  $\text{NADP}^+$  would positively interfere with OPPP activity. As shown in Figure 1.5, the alternating oxidation of NADPH by proline synthesis and reduction of  $\text{NADP}^+$  by the two oxidative steps of the OPPP potentially links these two pathways (HARE 1998).

Soybean (*Glycine max* (L.) Merr. cv. Acme) has been the most intensively studied of the legume crop species, mainly because of its agronomic importance. It has the highest protein content of any of the legumes (up to 50% of the seed dry weight) and produces high yields of good quality oil. The protein contains all the essential amino acids and has particularly high levels of lysine. Cultivation of soybean is an excellent candidate for providing a cheap protein source in the diet of rural populations. During 1990, human consumption of soybean products accounted for 20,000 tonnes in South Africa. This increasing importance of soybean as a crop in South Africa is illustrated by the fact that the area planted with this legume will have to increase by more than five fold over the next decade to meet the needs of the country (McKENZIE & CRESS 1992).

Although much remains to be understood concerning the response of plant genomes to stresses, research findings are already enhancing our understanding of

how plants perceive a stressful situation and respond appropriately to the challenge. The primary aim of this study was to use a combination of both molecular genetic and plant tissue physiological approaches to isolate novel genes induced by stress from *Glycine max* (L.) and to determine the effect of salinity and its subsequent relief on genomic DNA degradation, growth, organic and inorganic solute content of soybean. This information is fundamental to elucidate the impact of cultivation in saline soil on the water use efficiency of soybean. This area of research is of interest not only because it will expand our understanding of coordinate gene expression in higher organisms, but studies on the stress-induced proteins and the genes encoding them may result in the engineering of crop plants more resistant to normally encountered moderate stress conditions. Agricultural productivity is unlikely to expand until progress is made regarding soybean's tolerance to drought and temperature extremes. It would therefore be beneficial to create stress-tolerant soybean varieties. Not only would this increase productivity, but it would also be of benefit in the conservation of water, which is a seriously limiting natural resource in many parts of South Africa.

## ***Chapter Two***

### **Two-step Polymerase Chain Reaction: an efficient method for *Glycine max* (L.) cDNA library amplification**

#### **Abstract**

The Polymerase Chain Reaction (PCR), developed at Cetus Corporation, USA by Mullis, is a simple method for amplifying nucleic acids *in vitro*. Polymerase chain reaction amplification of cDNA libraries may fail due to poor denaturation, secondary structures that block elongation or non-target sequence amplification. An efficient method for the amplification of cDNA libraries from *Glycine max* roots is described. Successful amplification of a cold-stress-induced cDNA library from *Glycine max* roots was achieved in a two-step procedure using an efficient modified PCR protocol with two annealing temperatures. Non-specific PCR products were minimized by optimizing the annealing temperature. This protocol may be useful for the amplification of other cDNA libraries.

## 2.1 Introduction

The Polymerase Chain Reaction (PCR) is a very simple method for amplifying nucleic acids *in vitro* (SAIKI *et al.* 1985; MULLIS *et al.* 1986). PCR is an *in vitro* technique which allows the amplification of a specific deoxyribonucleic acid (DNA) region that lies between two regions of known DNA sequence (NEWTON & GRAHAM 1994). PCR was popularized by the introduction of automatic thermocyclers and thermostable DNA polymerase – *Taq* DNA polymerase. The PCR reaction is widely employed for the amplification of specific DNA sequences (GU 1995). The heat-resistant enzyme, *Taq* DNA polymerase can synthesize DNA at a theoretical rate of about 150 nucleotides per second per enzyme molecule at around 75 - 80°C. PCR employs oligonucleotide primers, one complementary to a sequence on the (+) strand and the other to a down stream sequence on the (-) strand. Reiterative cycles of denaturation, annealing, and extension are used to generate multiple copies of the DNA that lies between the two primers. The method uses the polymerase chain reaction to amplify even rare cDNAs, making the identification of differentially expressed genes of low abundance possible (GROENEWALD & BOTHA 1999). However, it is important to note the *Taq* DNA polymerase has a built-in error rate of about 0.1 - 0.3% (GU 1995). The primer annealing step is an important parameter in optimizing the specificity of a PCR and the calculated annealing temperature is used as a starting point for experimental work. The annealing temperature should be optimized empirically (NEWTON & GRAHAM 1994). The objective of this part of the study was to use Polymerase Chain Reaction amplified cDNA to isolate and identify the genes whose expression is up- or down-regulated in soybean roots after low temperature treatment.

## 2.2 Materials and methods

The cDNA libraries were kindly provided by Dr. S. du Plessis (Botany Department, University of Natal, Pietermaritzburg, South Africa). cDNA libraries were constructed as previously described (LIU *et al.* 2000b). In brief, seeds of soybean (*Glycine max* (L.) Merr. cv. Acme) were germinated and grown in controlled environmental chambers ( $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) under a 10 h dark (20°C)/14 h light (25°C) cycle. Two-week-old seedlings were stressed by placing them in a controlled environmental chamber (10°C) for 6 h. The roots were harvested, frozen in liquid nitrogen, and stored at -70°C. Total and poly (A<sup>+</sup>) RNA was extracted as previously described (JEPSON *et al.* 1991). Double-stranded cDNA was synthesized from poly (A<sup>+</sup>) RNA extracted from control (normal growth) or 6 h low-temperature-treated soybean roots using the method of Jepson *et al.* (1991). To prepare cDNA fragments suitable for PCR amplification, double-stranded cDNA were ligated with Uni-Amp primer (5' CCT CTG AAG GTT CCA GAA TCG ATAG-3') (Clontech, USA). The linker-ligated cDNA fragments were electrophoresed through a 1.4 % low-melting agarose gel for a short distance to remove the unligated linkers. The cDNA fragments in the size above 0.2 kb were combined.

The PCR reaction mixture contained 10  $\mu\text{l}$  1 x *Taq* buffer, 0.2 mM dNTP mix, 1.25  $\mu\text{M}$  Uni-Amp primer (5'CCTCTGAAGGTTCCAGAATCGATAG 3'), 2.5 unit of *Taq* Polymerase (Promega), 10 ng cDNA library template in a volume of 100  $\mu\text{l}$ . This was overlaid with 50  $\mu\text{l}$  mineral oil. The samples were subjected to a Hybaid Thermal Reactor (South African Scientific Products (PTY)) for 30 cycles of PCR using the

following cycling parameters: Three different programmes were used in this study. In Programme I: The DNA was denatured at 94°C for 60 sec, and the primers annealed at 60°C for 60 sec. DNA was extended at 72°C for 120 sec with a 2.4°C s<sup>-1</sup> ramp, and this cycle repeated 30 times. In Programme II: the annealing temperature was 45°C instead of 60°C. In programme III: 3 cycles of 94°C for 60 sec, 45°C for 60 sec, 72°C for 120 sec with a 2.4°C s<sup>-1</sup> ramp. Then 27 cycles of 94°C for 60 sec, 60°C for 60 sec, 72°C for 120 sec with a 2.4°C s<sup>-1</sup> ramp. At the end of the programmes, an extra extension step of 10 min at 72°C was performed to make sure that all the amplified double stranded fragments were full-length. The mineral oil was removed by extraction with chloroform. After amplification 15 µl of PCR product were electrophoresed for 2 h at 70 V in 1.1% agarose gel incorporating ethidium bromide. The DNA was visualised using a UV transilluminator. The purity of PCR products were checked using a GeneQuant DNA/RNA calculator.

### **2.3 Results**

The efficiency of the differential PCR's was tested by using three different reaction programmes. The initial amplification was carried out by using Programme I as shown in Table 2.1. This amplification programme was not efficient enough to amplify the control cDNA and the stress cDNA. cDNA products were obtained with a size between 0.5 to 1.5 Kb (Figure 2.1). The yields of amplified cDNAs were only 330 to 380 ng per 100 µl of reaction solution (Table 2.1). To obtain more cDNA for subtraction library construction, the annealing temperature was lowered to 45°C (Programme II). The cDNA PCR products obtained were between 0.5 to 4 Kb (Figure

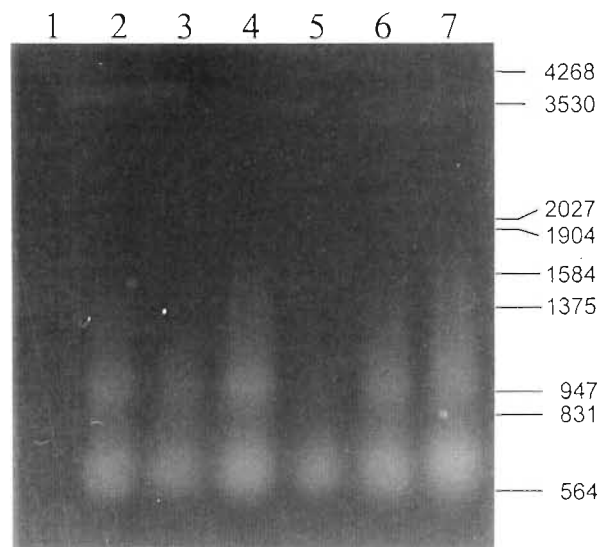


**Table 2.1: PCR amplification of the cold and control cDNAs from soybean roots.**

PCR Programme	cDNA template	Reaction conditions	Cycles	cDNA range in 1.1% agarose gel (Kb)	Yield of cDNA (ng)
I	cold-stressed or control cDNA	94°C 1 min 60°C 1 min 72°C 2 min	30	0.5-1.5	330-380
I	Control (without template)	94°C 1 min 60°C 1 min 72°C 2 min	30	N/B	0
II	cold-stressed or control cDNA	94°C 1 min 45°C 1 min 72°C 2 min	30	0.5-4.0	1100-1200
II	Control (without template)	94°C 1 min 45°C 1 min 72°C 2 min	30	0.2-0.8	50-150
III	Cold-stressed or control cDNA	94°C 1 min 45°C 1 min 72°C 2 min 94°C 1 min 60°C 1 min 72°C 2 min	3 27	0.5-4.0	920-1050
III	Control (without template)	94°C 1 min 45°C 1 min 72°C 2 min 94°C 1 min 60°C 1 min 72°C 2 min	3 27	N/B	0

N/B: Not detected by GeneQuant DNA/RNA calculator.

2.1). The yields were 1100 to 1200 ng per 100 µl of the reaction solution (Table 2.1). This higher efficiency of the PCR reaction was obtained with an annealing temperature of 45°C. It was also found that non-target PCR products, which included unexpected size and unwanted sequences, were detected by the GeneQuant DNA/RNA calculator in negative controls (without template) (Table 2.1). This suggests that the non-target DNAs may be the result of the low annealing temperature.



**Figure 2.1: Results for 30 cycles of PCR performed with different annealing temperatures.** Amplified cDNAs from soybean roots were analysed from the different reactions of negative control, control cDNA and stress cDNA. 15µl of each PCR reaction solution were subjected to 1.1% agarose gel. Negative control (without template) (Lane 1), control(Lane 3) and cold stress (Lane 5) cDNAs were amplified by PCR programme I. Control (Lane 2) and cold stress (Lane 4) cDNAs were amplified by PCR programme II. Control (Lane 6) and cold stress (Lane 7) were amplified by PCR programme III. Sizes of DNA fragments in Molecular Weight Marker are indicated in base pair.

In the present study, non-specific PCR products could be minimized by optimizing the annealing temperature. The results showed that in the control sample (without template) there was no evidence of DNA detected by the GeneQuant DNA/RNA calculator with the two-step PCR Programme III. The results in Figure 2.1 shows that all the control cDNAs and the cold stress DNAs have been amplified giving a range between 0.5 to 4 kb except for the negative control (without template).

It was hoped that there would be minimal amounts of undesired products (non-

target PCR products) when constructing the cDNA subtraction library. Reaction conditions and cycling profiles were stringently optimized for the Uni-Amp primer. The optimum conditions for the cDNA libraries PCR amplification were the following: After 3 cycles of DNA denaturation at 94°C for 1 min, primer-annealing at 45°C (1 min), elongation at 72°C (2 min), 27 cycles of denaturation at 94°C (1 min), primer-annealing at 60°C (1 min), elongation at 72°C (2 min) were carried out. The reaction was ended by a final elongation at 72°C for 10 min. With these PCR conditions, the 0.5-4.0 Kb cDNA fragments were synthesized with a yield of 800-1000 ng per 100 µl reaction.

## 2.4 Discussion

In this study, different programmes were used to amplify the soybean root stress induced cDNAs and the control cDNA (no stress treatment) by applying different annealing temperatures. Annealing temperatures for cDNA amplification were chosen according to the following formula:

$$T_m \text{ of the primers} = 81.5^{\circ}\text{C} + 16.6 (\text{Log } M) + 0.41(\text{G+C}\%)-500/n$$

where n=length of primers and M=molarity of the salt in the buffer, usually 0.047 M for DNA (BAGASRA *et al.* 1995).

Usually, primer annealing is optimal at 2°C above its melting temperature. However, this formula provides only an approximate temperature for annealing, since base-stacking, near-neighbour effects, and buffering capacity may play significant roles for a primer (BAGASRA *et al.* 1995). It was observed that the annealing temperature of 60°C, which approaches the optimum annealing temperature for the Uni-Amp primer, is not efficient in amplifying the stress-induced cDNAs or the control cDNA

(Figure 2.1). In addition, the annealing temperature of 45°C resulted in a higher yield of non-target PCR products i.e. unexpected amounts or unwanted sequences were detected by the GeneQuant DNA/RNA calculator in the PCR control (i.e. without template)(Table 1.1). This is in keeping with the findings of the randomly amplified polymorphic DNAs that result from a lower annealing temperature (JOSHI & NGUYEN 1993). During amplification, spurious products often appear in addition to those desired. Therefore, even if the reaction solutions do not contain a cDNA template homologous to the primer sequences, many artifactual bands may appear (BAGASRA *et al.* 1995). False priming could occur if the melting temperature ( $T_m$ ) between the primer and template is not accurate. Therefore, a temperature above the  $T_m$  may yield no products and a temperature too far below the  $T_m$  may give unwanted products due to false priming. Thus the determination of the optimal annealing temperature is extremely important. Many protocols have appeared in the literature to overcome false priming, including hot start (NUOVO *et al.* 1993), use of dimethyl sulfoxide (DMSO), formamide and anti-*Taq* anti-bodies (NUOVO 1994; TaqStart<sup>TM</sup>; Clontech, Palo alto, CA, USA).

According to Wang & Brown (1991), the ratio of the target PCR products and the non-specific PCR products could be changed by adjusting the annealing temperature by 2 to 10 °C. The present results showed that non-specific PCR products could be minimized by optimizing the annealing temperature in a two step PCR programme (Table 2.1). The target cDNA's and the non-specific PCR products were amplified at a low annealing temperature of 45°C. After three cycles of reaction, the annealing temperature was increased to 60°C. At this higher annealing temperature, the specificity of the target cDNA product was improved (Figure 2.1).

Amplified cDNA's of the stress and the control treatments were digested with *Eco*RI and small fragments were produced, which proved that the cDNA's of the stress and

the control plant are double stranded cDNA (LIU 1997). These results suggest that PCR could be used to amplify soybean root stress-induced cDNAs and control cDNA, and that the PCR products were suitable for further use to isolate and identify genes related to cold-stress in soybean roots.

## ***Chapter Three***

### **Gene expression in soybean (*Glycine max* L.) roots exposed to low temperature: isolation of a cDNA clone**

#### **Abstract**

A low temperature (10°C) stress, soybean (*Glycine max* L.) subtraction library, was constructed in a pBluescript vector using Polymerase Chain Reaction (PCR) amplified cDNA for subtractive hybridization. Southern blot analysis indicated that one clone cs18 from the subtraction library hybridized to the low temperature stress cDNA library. One hundred thousand plaques from the soybean low temperature stress cDNA library were screened using the insert cs18. Four clones cs18-14, cs18-15, cs18-16 and cs18-17 were confirmed to contain inserts that could hybridize to the cs18 probe. Northern blot analysis indicated that cs18 mRNA was highly expressed in roots but moderately so in leaves under low temperature. Sequence analysis of insert cs18 revealed that it had a 76% homology with the sequences of the corresponding portion of glucose dehydrogenase from *Thermoplasma acidophilum* and 62% homology with a genomic DNA of *Arabidopsis thaliana*.

### **3.1 Introduction**

Plants have evolved mechanisms that allow them to acclimatize to a variety of environmental stresses (LEVITT 1980). Low temperature is a major trigger for the acquisition of freezing tolerance in plants capable of cold acclimation. Biochemical, morphological, and physiological changes occur in plant cells during cold acclimation (GUY 1990), and direct evidence was obtained which demonstrated that low temperature regulate the accumulation of specific mRNAs during cold acclimation (THOMASHOW 1990). The appearance of novel transcripts during cold acclimation has been observed in alfalfa (MOHAPATRA *et al.* 1989), blackcurrant (KEMP *et al.* 1997), maize (KUSANO *et al.* 1995), soybean (TAKAHASHI & SHIMOSAKA 1997), and wheat (GANA *et al.* 1997). Furthermore, DNA sequences corresponding to these low temperature specific or low temperature regulated transcripts have also been isolated and characterized by differential screening of cDNA libraries constructed from these species. These mRNAs appear rapidly upon exposure of the plant to low temperatures, and the deduced amino acid sequences of the products of some of these low temperature regulated genes have been determined. The objective of this study was to investigate low temperature stress regulated gene expression in soybean roots.

### **3.2 Materials and methods**

#### **3.2.1 Plant material and cultivation**

Seeds of soybean (*Glycine max* (L.) Merr. cv. Acme) were obtained from the seed bank in Colorado, USA and germinated in the dark between water-saturated paper towels in petri dishes at 25°C for 2 days. After germination, seedlings were grown in controlled environmental chambers (150  $\mu\text{E m}^{-2} \text{s}^{-1}$ ) under a 10 h dark (20°C)/ 14 h light (25°C) cycle. Fourteen-day-old soybean seedlings were stressed by placing them in a controlled environmental chamber at 10°C for 6 h. Light conditions were the same as those used for the control.

### 3.2.2 Isolation of RNA and DNA

Total and poly(A<sup>+</sup>) RNA from 2-week-old soybean roots was extracted as previously described (JEPSON *et al.* 1991). Plasmid minipreps were performed according to the modified alkaline lysis method (FELICIELLO & CHINALI 1993). Lambda DNA minipreps were performed according to the method of Ausubel *et al.* (1989).

### 3.2.3 Construction of cDNA library

The cDNA libraries were kindly provided by Dr. S. du Plessis (Botany Department, University of Natal, Pietermaritzburg, South Africa). Double-stranded cDNA was synthesized from poly(A<sup>+</sup>) RNA extracted from control (normal growth) or 6 h low temperature-treated soybean roots using the method of Jepson *et al.* (1991). mRNA isolated from the plant material was transcribed into cDNA using a cDNA synthesis kit (Amersham). Following the ligation of an adaptor containing a suitable priming sequence (5' CCTCTGAAGGTTCCAGAATCGATAG 3' - Uni-amp adaptor/primer)



(Clontech) onto the cDNA, the cDNA was amplified via a sequence-independent PCR. Fractionated cDNA of a size range sufficient to encompass complete cDNAs were cloned into the bacteriophage vector Lambda ZAP II (Stratagene) before being packaged as phage particles, using an *in vitro* packaging reaction (Boehringer Mannheim).

#### 3.2.4 Construction of subtraction library

Subtraction library construction was performed using the modified method of Ausubel *et al.* (1989). In brief, low temperature stress cDNA and control cDNA were amplified by two step PCR, using Uni-amp primer (Chapter 2). Control cDNA was digested with *Alu* I and *Rsa* I, and low temperature stress cDNA was digested with *Eco* RI. A twenty five fold excess of control cDNA was hybridized with 1 fold of cold stress cDNA for 18 h at 37°C. The insert DNA was then ligated to pUC18, transformed into the *E. coli* host JM109 (SAMBROOK *et al.* 1989), recombinants selected in the presence of X-Gal, IPTG and ampicillin.

#### 3.2.5 Southern and Northern blot analysis

Restricted DNA was separated on a 1.1% agarose gel, transferred to a Hybond N<sup>+</sup> membrane (Amersham) using standard methods provided with the ECL<sup>TM</sup> direct nucleic acid labelling and detection system (Amersham). The cs18 insert was labelled with horseradish peroxidase according to the manufacturer's protocol (Amersham). For Northern blot analysis, equal amounts (10 µg) of total RNA from different tissue

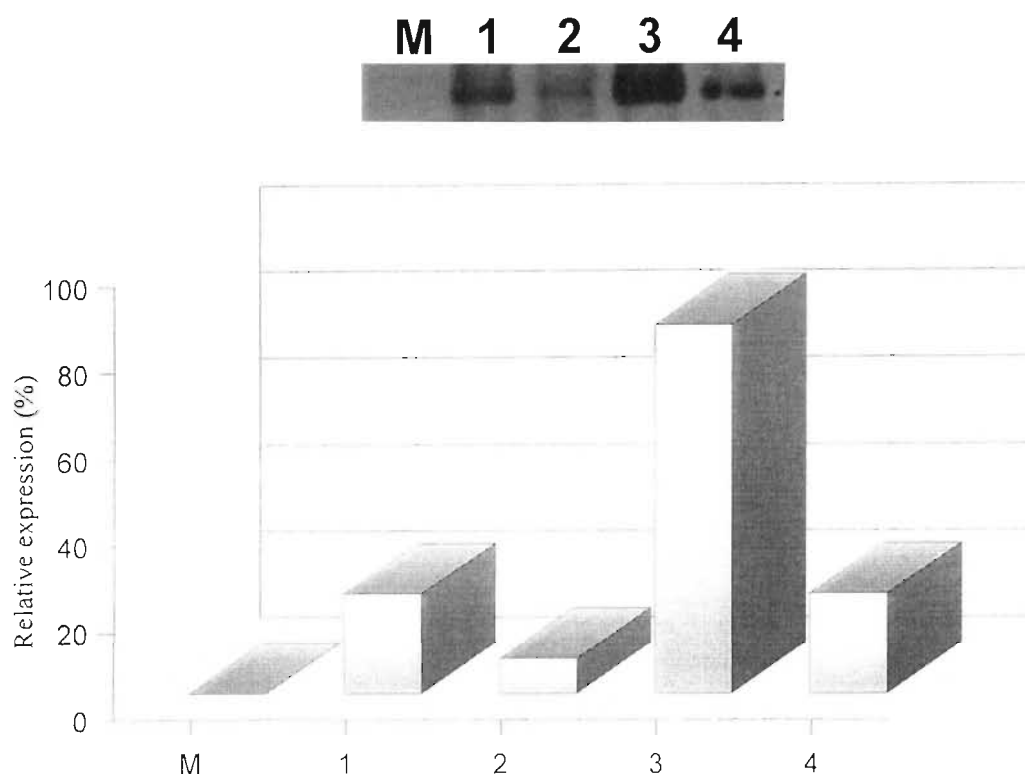
or treatments were electrophoretically separated on 1% MOPS/Formaldehyde agarose gel and transferred to Hybond N<sup>+</sup> (Amersham) according to Sambrook *et al.* (1989). After pre-hybridization of the blots, hybridization was performed for 20 h at 55°C with the <sup>35</sup>S-labeled cs18 probe in hybridization buffer (6 X SSC, 0.5% SDS, 100 µg ml<sup>-1</sup> denatured herring sperm DNA, and 5 X Denhardt's solution). Washing was carried out in 1 X SSC, 0.1% SDS at 55°C.

### 3.2.6 Screening of cDNA library, sequencing, and computer analysis

Screening of the cDNA library was done by preparing plaque lifts on Hybond N<sup>+</sup> membranes (Amersham) and hybridizing the lifts with ECL-Labeled cs18 probes from a subtractive library against poly(A<sup>+</sup>) RNA isolated from cold acclimated and non-acclimated soybean roots. About 1 X 10<sup>5</sup> plaque-forming units were screened. Plaques which showed greater hybridization with the cs18 probe representing cold acclimated plants were amplified once and rescreened with the same probe. Four plaques showing hybridization with the probe were purified and the recombinant cDNA cut from the phage and subcloned into pUC18 vector. Sequencing of cDNA cs18 was performed using the dideoxy chain-termination method (SANGER *et al.* 1977). The sequencing was conducted using the Sequenase® Version 2.0 sequencing kit (US Biochemical Corp.). Computer searches of the GeneBank, European Molecular Biology Laboratory (EMBL), were conducted using the Basic Local Alignment Search Tool (BLAST) Programs (ALTSCHUL *et al.* 1990) and accessed via the world wide web server (<http://www.ncbi.nlm.nih.gov>).

### 3.3 Results

An efficient cDNA cloning strategy is described based on the modified two step PCR technique (Chapter 2), and subtractive hybridization as outlined by Sargent and Dawin (SARGENT & DAWIN 1983). The low temperature induced subtraction library was constructed from mRNA isolated from soybean roots. Fifty two white clones were obtained from the low temperature acclimated cDNA library. The use of subtractive hybridization is to enrich the target cDNA for fragments that represent genes that are specifically expressed in the target material by removal of DNA sequences representing genes that are also expressed in the control soybean root. A 25-fold molar excess of the control cDNA, prepared from soybean roots grown under normal growth conditions, digested with two restriction enzymes that recognizes a 4 bp sequence, *Alu* I and *Rsa* I, were used, and the number of control cDNA molecules was increased. By increasing the ratio of control cDNA to stress cDNA, the hybridization could remove a sufficient amount of the control cDNA and enrich the stress cDNA library. The majority of the inserts of the subtraction cDNA library analysed were between 50 and 200 base pairs in length. This was probably due to the digestion of target cDNA with restriction endonucleases *Eco* RI. One cDNA insert *cs18* was isolated from the subtraction library of the low temperature stressed soybean roots. Since it was likely that the cDNA's represented only partial sequences in the low temperature stress induced cDNA library, the conventional cDNA library of the low temperature treatment for large cDNA inserts was screened by using *cs18* as a probe. Four inserts *cs18-14*, *cs18-15*, *cs18-16*, and *cs18-17* were found to hybridize to probe *cs18*. Digestion of *cs18-14* with *Xba* I/*Xho* I produced a



**Figure 3.1: Northern blot analysis of the expression pattern of *cs18* in soybean roots and leaves under control and low temperature conditions.** Ten µg of total RNA from each sample was separated on a denaturing agarose gel, transferred to a nylon membrane, and hybridized with a <sup>35</sup>S-labeled *cs18* probe. RNA was prepared from 14-day-old soybean seedlings as: M. RNA Molecular Weight Marker (Control); 1. Root (25°C); 2. Leaves (25°C); 3. Roots (10°C 6h); 4. Leaves (10°C 6h).

fragment of approximately 3,500 bp according to the Lambda ZAPII restriction endonuclease map. This indicated that the insert *cs18*-14 which corresponded to the *cs18* probe could be a 3.5 Kb fragment.

### 3.4 Discussion

The expression of *cs18* was analysed at the mRNA level by Northern hybridization. Northern hybridization with a <sup>35</sup>S-labeled probe showed a high expression in root and moderate expression in leaves under low temperature

cs18	49	GC	ATAATCACTT	TGCTCCGCTT	ATTATTTACT	TGCTCCAATG	90
x59788	42	GC	ATATGCACCG	TAATCAGCAT	TTGATTTACT	TGATCCATTG	83
CS18	91	GATCGATTGC	TCATGGACG	109			
X59788	84	GATCGATTGT	AAACCAACG	102			

**Figure 3.2: Nucleotide sequence similarities observed between cs18 and the glucose dehydrogenase gene of *Thermoplasma acidophilum*.**

cs18	34	AAGATGA	AAATTAGTGC	ATAATCACTT	TGCTCCGCTT	ATTATTTACT	80
AC002130	21846	AGGATAA	AAATTGTTTG	AAAGTAACTA	AGCTCCGATA	ATTATTTACT	21892
cs18	81	TGCTCCAATG	GATCGATTGC	TCATGGACGT	GTCTATTTC	119	
AC002130	21893	TTTTTAACCG	TGGAAATTGC	TTAGTCGCGC	GTCTAAATC	21931	

**Figure 3.3 Nucleotide sequence similarities observed between cs18 and genomic sequence from *Arabidopsis thaliana*.**

treatment (Figure 3.1). In contrast, the expression of cs18 in the roots was moderate and much lower in the leaves under normal temperature conditions.

In order to characterize the soybean genes which are involved in the low-temperature-response and the corresponding translation products, the insert DNA cs18 was sequenced. In total a nucleotide sequence of 196 base pairs of insert cs18 DNA was determined. Comparisons of the nucleotide sequences determined in the present study with those stored in the GeneBank database using BLAST, revealed a 60 bp region of cs18 that displayed sequence homology with portions of the

glucose dehydrogenase gene of *Thermoplasma acidophilum* (X59788) (BRIGHT *et al.* 1993) (Figure 3. 2) and a 85 bp region of cs18 that displayed sequence homology with portions of the genomic sequence for *Arabidopsis thaliana* (Figure 3.3)[DEWAR unpublished]. This revealed that the insert cDNA cs18 may be involved in a glucose dehydrogenase reaction. It is suggested that the glucose dehydrogenase could be encoded by genes regulated by different treatments. Glucose dehydrogenase expression is induced by heat shock (SODE *et al.* 1996), GA<sub>3</sub> treatment and wounding (BAILEY *et al.* 1996). These results indicate that glucose dehydrogenase may also be induced by low temperature in plants.

## ***Chapter Four***

### **Salinity induced nuclear and DNA degradation in meristematic cells of soybean (*Glycine max* (L.)) roots**

#### **Abstract**

Changes in the nuclei of meristematic root cells of soybean (*Glycine max* (L.) Merr. cv. Acme) in response to severe salinity were studied. Root growth was inhibited by 200 mM NaCl, when 1 mM  $\text{CaCl}_2$  was present in the culture media. Increasing  $\text{CaCl}_2$  up to 5 mM partially prevented this inhibition. However, inhibition also occurred with 100 mM NaCl without  $\text{CaCl}_2$ . Nuclear deformation of the cells occurred with 24 h of 150 mM or higher NaCl, and was followed by degradation of nuclei in the apical region of the root. TEM observation and agarose gel electrophoretic analysis confirmed that root tip nuclear DNA deformed or degraded with 150 mM or higher NaCl concentrations.

#### **4.1 Introduction**

Salt stress is one of the major factors limiting plant growth and productivity in arid and semi-arid areas. A high concentration of NaCl reduces growth of both the shoot and the root (GREENWAY & MUNNS 1980; CHEESEMAN 1988). Roots are in direct contact with the surrounding solution. As such, they are the first to encounter the saline medium and are potentially the first site of damage or line of defense under salt stress (KATSUHARA & KAWASAKI 1996). Root growth of many crop plants is severely inhibited by high concentrations of NaCl in the growing medium (CRAMER *et al.* 1987). Final cell size as well as the rate of cell production can be affected (KURTH *et al.* 1986). Salinization therefore results in shorter roots (AZAIZEH *et al.* 1992). Unfortunately, the cellular mechanisms of salt injury in roots are not well understood.

Ultrastructural alterations to root cells in response to salt stress have been recorded in several species. These effects of salinization include: structural damage to mitochondria in the epidermis and pericycle cells of wheat (UDOVENKO *et al.* 1970) and in *Agrostis stolonifera* (SMITH *et al.* 1982), and condensation of chromatin in the nuclei of *Hordeum vulgare* (WERKER *et al.* 1983). Kramer (1984) suggested that the appearance of various types of transfer cells in several species after exposure to salt may have a function in the adaptation of plants to salinity. Most of these observations were concerned with cells of the root elongation zone or above. There are few studies of the meristematic region. Amongst the root cells, meristematic cells are especially interesting, because growth of roots is restricted to a limited region of the root axis (BALUŠKA *et al.* 1994). Mitotic activity and most cell divisions occur in the apical-



meristem. With increased distance from the root tip, the frequency of cell division decreases and cells increase in length and age (ERICKSON & SAX 1956). Meristematic cells are also considered to be salt-sensitive (HUANG & VAN STEVENINCK 1988). These cells have been studied under exposure to moderate salinity (WERKER *et al.* 1983; HUANG & VAN STEVENINCK 1988; HUANG & VAN STEVENINCK 1990), but only few researchers focused on severe salt stress.

Calcium plays an important role in the response of plants to salinity (RENGEL - 1992; COLMER *et al.* 1996). The importance of  $\text{Ca}^{2+}$  to the function of the plasma membrane under salinity is well documented (KAFKAFI & BERNSTEIN 1996). Elevated levels of external  $\text{Ca}^{2+}$  (4 -10mM) can enhance the "exclusion" of Na (LAHAYE & EPSTEIN 1971), help maintain the levels of tissue K (COLMER *et al.* 1994), and prevent salt injury of cells (CRAMER *et al.* 1989).

The present investigation describes the changes which occur in the meristematic region of soybean root nuclei resulting from salinity treatment as observed using light and transmission electron microscopy. Biochemical changes of the nuclear DNA were also determined.

## **4.2 Materials and methods**

### **4.2.1 Plant material and culture**

Soybean seeds (*Glycine max* (L.) Merr. cv. Acme) were germinated at 28°C in the dark

in petri dishes over one-tenth MS salt solution (Appendix 2) (MURASHIGE & SKOOG 1962) at pH 5.7. After two days, seedlings were transferred to one-quarter MS salt solution (pH 5.7), and the shoots of the seedlings were exposed to sunlight ( $14 \text{ h day}^{-1}$ ) in a greenhouse with a day/night temperature of  $28.5 \pm 1 / 22.0 \pm 0.5 \text{ }^{\circ}\text{C}$ .

#### 4.2.2 $\text{Ca}^{2+}$ and NaCl treatment

For low- or high- Ca treatment, 0.1, 1.0 or 5.0 mM  $\text{CaCl}_2$  was prepared using the 1/4 MS salt solution with or without the addition of NaCl. Before addition of NaCl to 1/4 MS salt solutions, 20 seedlings (6-days-old) were sampled and fresh weights and root lengths measured. Salt shocks were carried out by adding NaCl to the nutrient solution to make concentrations of 50, 100, 150, 200, 250, 300, and 350 mM. NaCl was raised to the final concentration in one step. Values of  $\psi_s$  for the nutrient solutions were determined by thermocouple psychrometry, in order of increasing salt concentration, and ranged from -0.6 to -13 bar. One day after salt stress, the root length of 20 seedlings were measured and root growth for the one day was calculated. The growth of roots without salt stress was used as a control and allocated a 100% rate for every  $\text{CaCl}_2$  concentration tested. All measurements were recorded for 20 seedlings.

#### 4.2.3 Microscopic observation

Segments of soybean root 2 mm from the tip were fixed for 8 h in 3 % glutaraldehyde (buffered to pH 7.2 with 0.05 M sodium cacodylate), followed by two washes in the same buffer for 30 min. Post-fixation was carried out for 2 h in 2% osmium tetroxide

followed by two washes in fresh buffer. Samples were block stained in 2 % uranyl acetate for 40 min. Samples were washed in 2 x 10 min distilled water. The samples were dehydrated in a graded ethanol series, infiltrated and embedded in freshly prepared epon and polymerized at 70°C for 72 h. For light microscopy, sections 1-2 µm thick were cut by an ultramicrotome (LKB III), and stained with 0.1 % toluidine blue. For transmission electron microscopic (TEM) observation, sections were cut on a LKB III ultramicrotome and picked up on a 200 MESH copper grid. Post-staining with lead citrate for 10 min was followed by examination in a JEOL 100CX TEM operating at an accelerating voltage of 80 Kv.

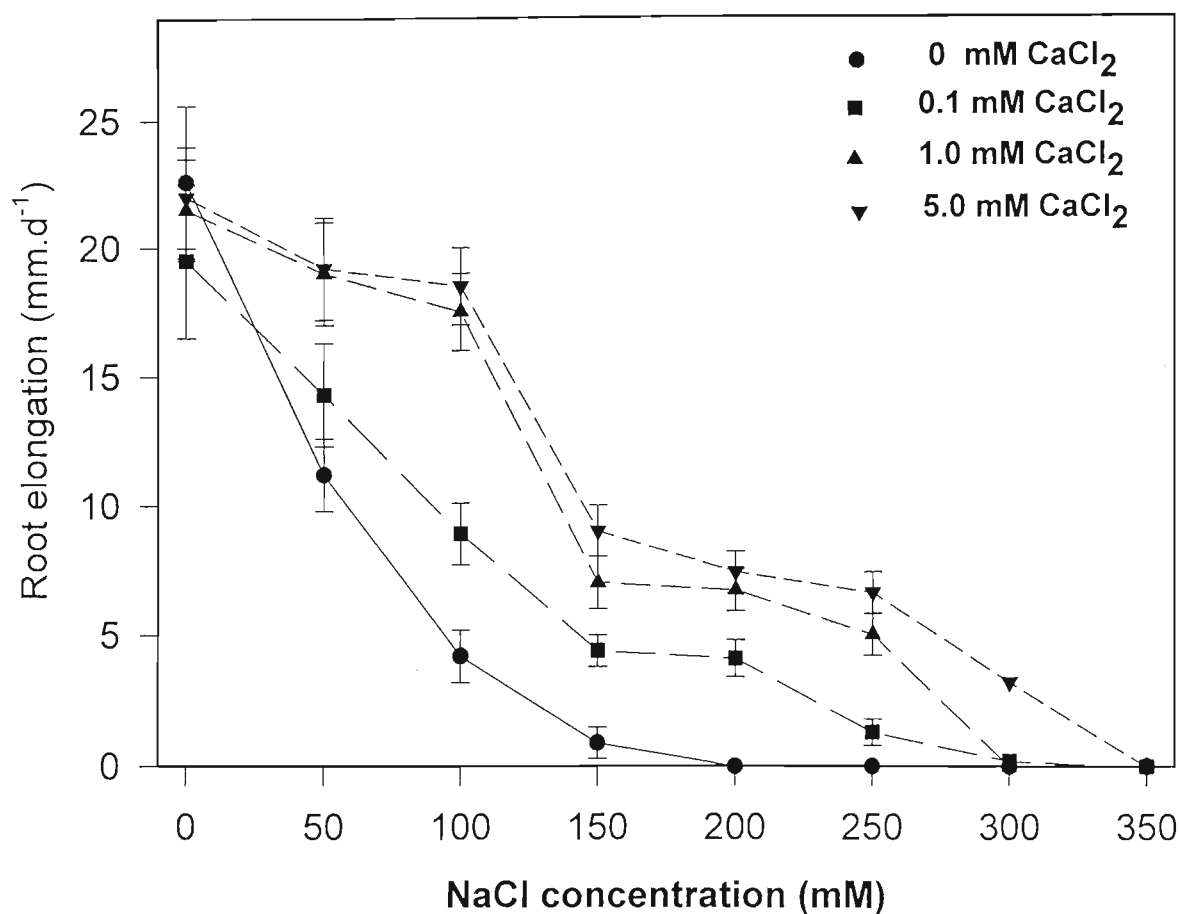
#### *4.2.4 Isolation and analysis of nuclear DNA*

Nuclear DNA was isolated according to Pich and Schubert (1993). Briefly, about 100 mg fresh root tips (10-15 mm long) including root caps were isolated from seedlings and frozen with liquid N<sub>2</sub>. Samples were placed into 600 µl DNA extraction buffer (500 mM NaCl, 50 mM Tris.HCl (pH 8.0), 50 mM EDTA, 1% 2-mercaptoethanol). The mixture was thawed and 260 µl ice cold 20% stock solution PVP was added and then 17.6 mg solid SDS was added. The mixture was incubated at 65°C for 10 min and one tenth volume of 5 M potassium acetate was added, followed by 30 min incubation on ice and centrifugation (5000 x g) for 10 min. DNA was precipitated with 0.6 volume iso-propanol. To remove RNAs, RNase A was used. Isolated DNA was kept in TE buffer. Samples (10 µl) and the marker were subjected to electrophoresis on 1.1% agarose gel in TAE buffer. DNA was stained with 0.5 µg ml<sup>-1</sup> ethidium bromide for 30 min.

### 4.3 Results

Root growth of soybean seedlings was inhibited under salinity stress. Root elongation was greatly inhibited following NaCl treatment. Figure 4.1 shows the effects of NaCl and CaCl<sub>2</sub> on root growth over 24 h. Root growth without NaCl was 22.4, 19.6, 21.3 and 22.6 mm d<sup>-1</sup> in 0, 0.1, 1, and 5 mM CaCl<sub>2</sub> respectively. There were differences with the CaCl<sub>2</sub> treatments. The inhibition of root growth was increased by increasing the concentration of NaCl in the culture solution. Root growth was inhibited when 100 mM NaCl was added to the culture media. However, when roots were treated in 100 mM NaCl with the addition of 1 mM and 5 mM CaCl<sub>2</sub>, root growth was not reduced compared with the controls (Figure 4.1). Root growth was reduced to 41% of the control when roots were treated with 100 mM NaCl combined with 0.1 mM CaCl<sub>2</sub>. Root growth was greatly inhibited when the roots were treated with 200 mM NaCl and 0.1 mM CaCl<sub>2</sub> (Figure 4.1). However, this inhibition was partly reversed (38%) when 5 mM CaCl<sub>2</sub> was applied. In treatments with 300 mM NaCl, no growth was observed at any CaCl<sub>2</sub> concentration applied.

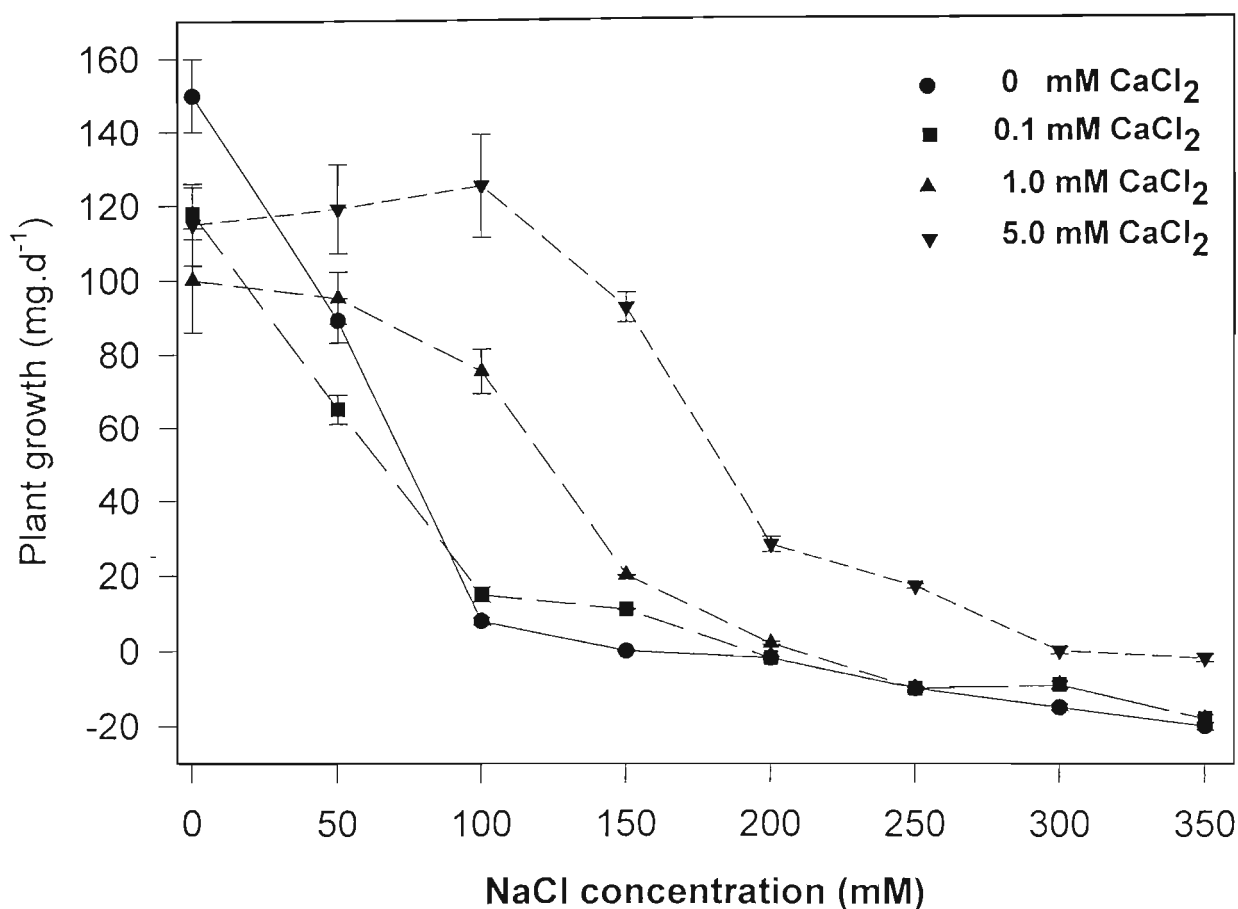
Results presented in Figure 4.2 indicate that the inhibition of plant growth resulted from NaCl- treatment and was prevented by addition of a defined concentration of CaCl<sub>2</sub>. The effects of NaCl and CaCl<sub>2</sub> on fresh weight for whole plant growth showed similar trends in that increasing NaCl concentration decreased plant fresh weight. With increasing NaCl concentrations in the culture media, the lower external osmotic potential caused the roots to lose water. The decrease was greater when the seedlings were treated with 250 mM or higher of NaCl. The “protective capacity” of CaCl<sub>2</sub> can be



**Figure 4.1:** Root relative growth (elongation) of 6-day-old soybean seedlings treated with NaCl and CaCl<sub>2</sub> for 24 h. Relative growth was calculated after normalizing the root growth without NaCl as 100%.

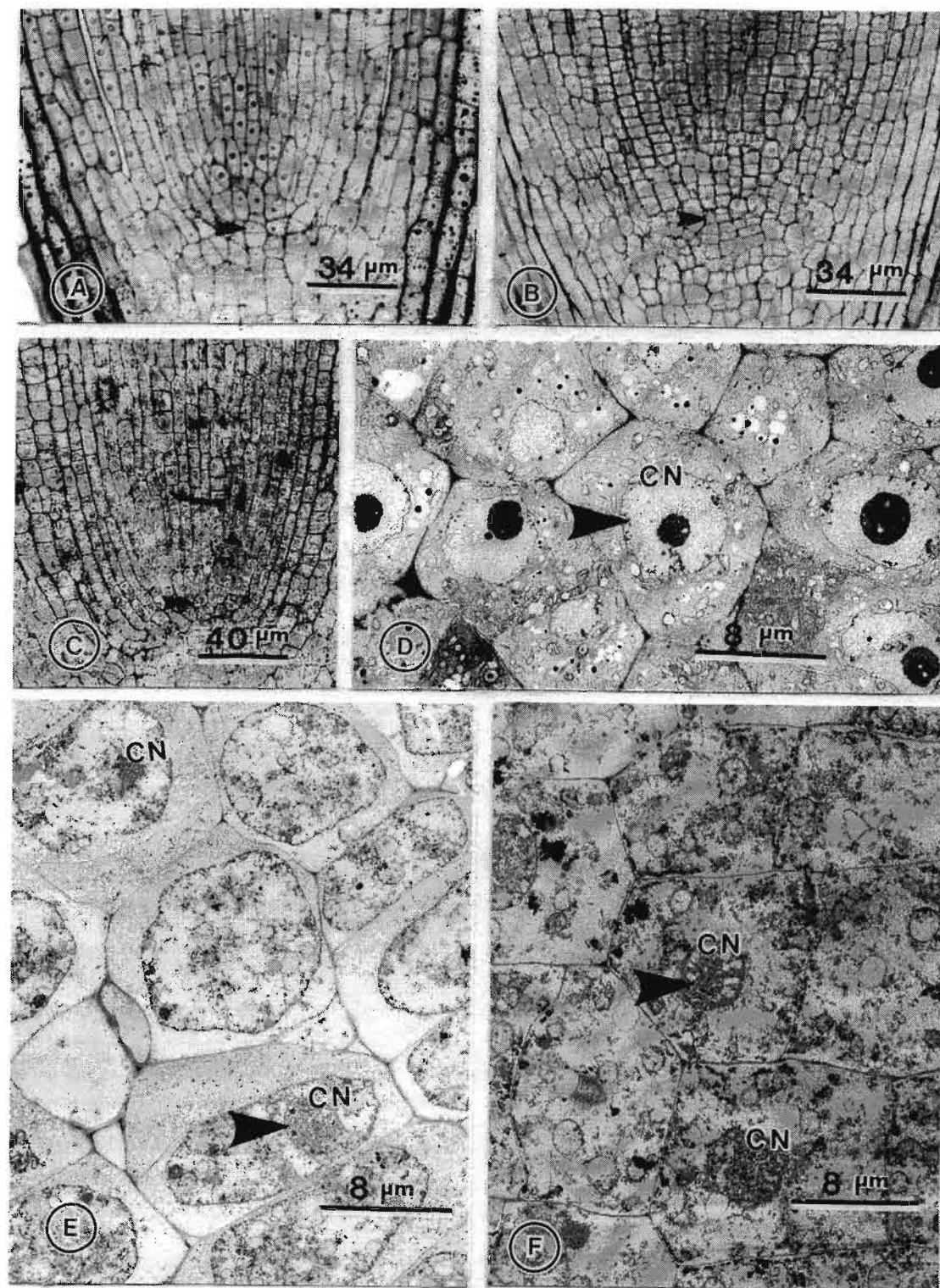
seen as the fresh weight increase was greater in 200 mM NaCl when 5 mM CaCl<sub>2</sub> was added to the test seedlings.

Light and TEM revealed changes in the nuclei in the meristematic region of soybean roots grown in 1/4 MS salt solution with different NaCl concentrations. Figures 4.3 A and 4.3 D show that cells in the meristematic region of a control soybean root grown in 1/4 MS salt solution were small with prominent nuclei with smooth and clear

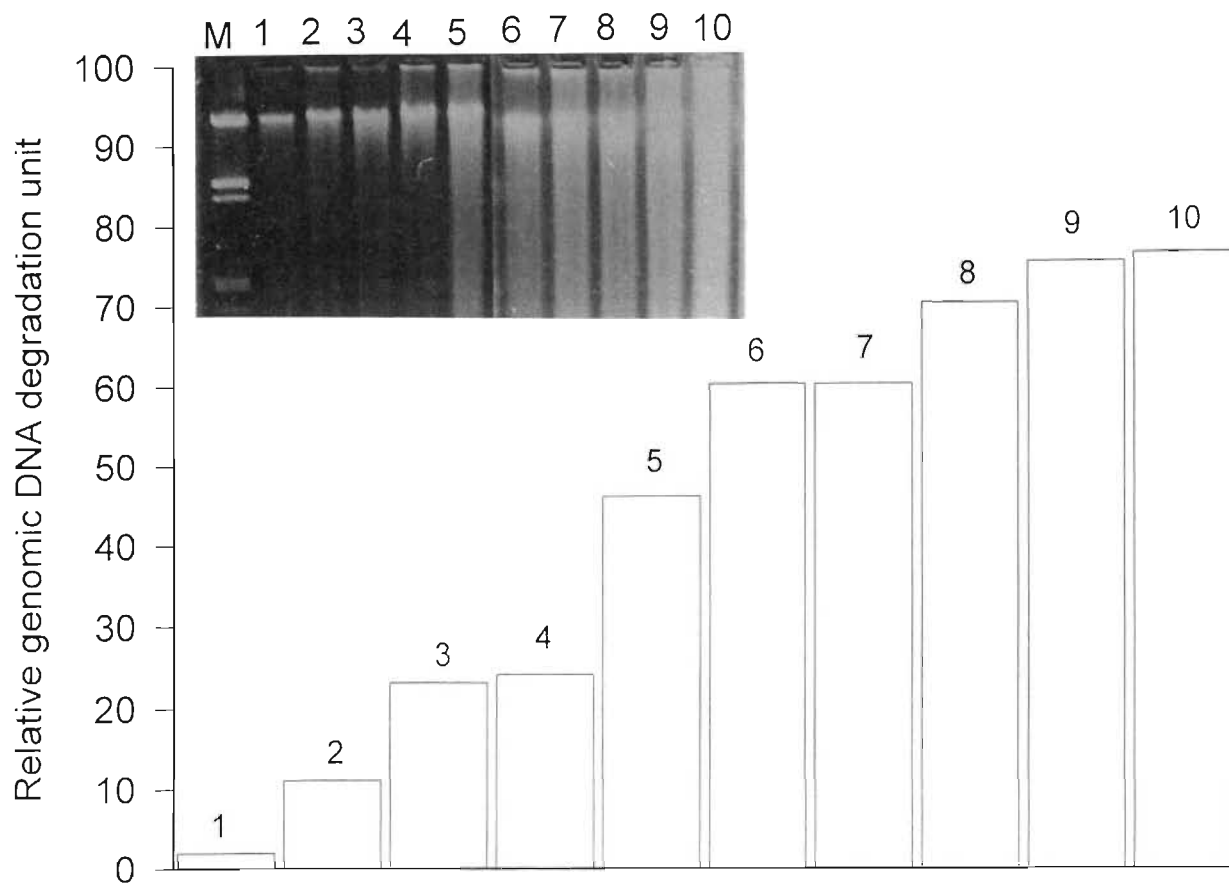


**Figure 4.2:** Plant growth (fresh weight) of 6-day-old soybean seedlings treated with NaCl and CaCl<sub>2</sub> for 24 h.

boundaries in each cell (arrows). These cells frequently showed division. In the cells of the root cap, small vacuoles and cellular organs were visible in the cytoplasm (Figure 4.3 D). After treatment with 150 mM NaCl for 24 h, the nuclei in the meristematic regions became deformed. This increased gradually until with 300 mM NaCl (Figure 4.3 C), the cell membranes separated from the cell wall, the cytoplasm became disorganized and nuclei degraded (Figure 4.3 E). Addition of 5 mM CaCl<sub>2</sub> to the test seedlings seemed to prevent nuclear deformation or degradation caused by 300 mM NaCl (Figures 4.3 B, 4.3 F and 4.6).

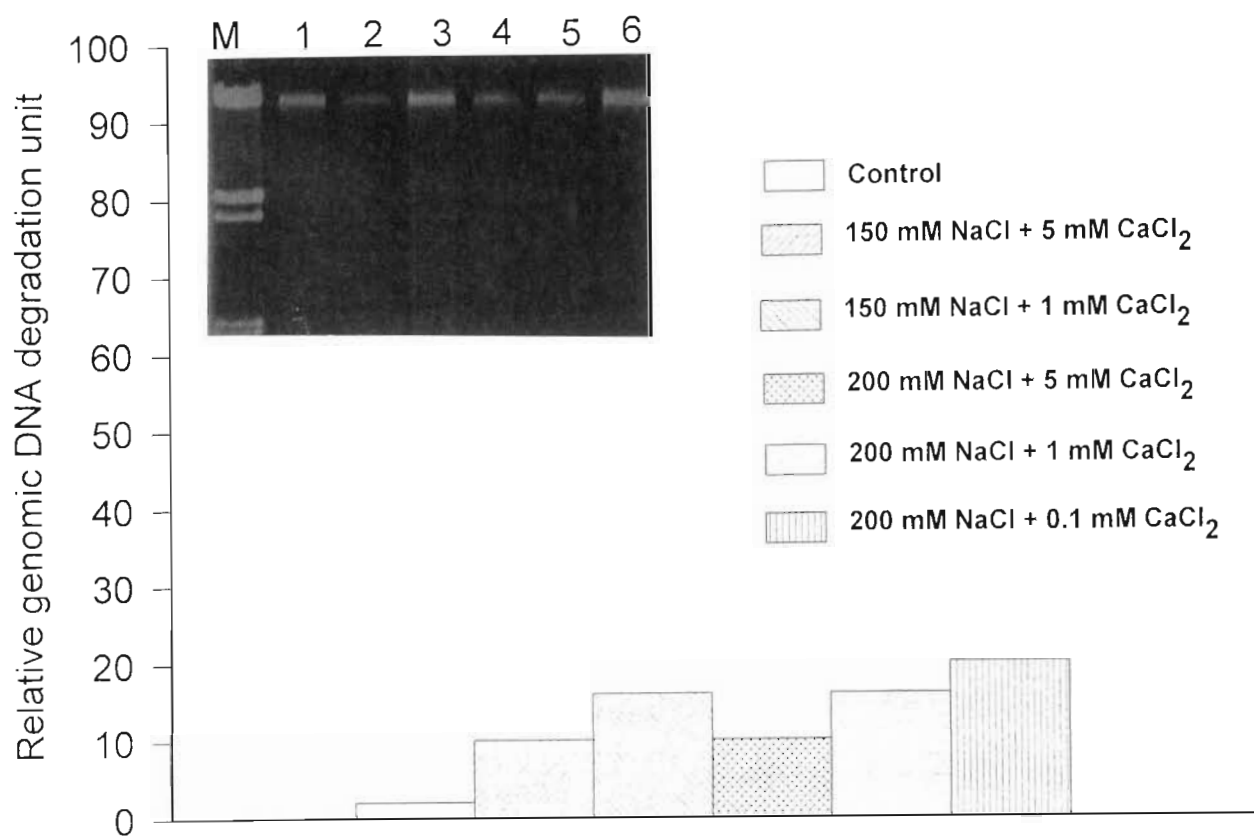


**Figure 4.3: Transmission electron micrographs of the meristemic region of the soybean root tip under salt stress and without salt stress.** (A). The appearance of cells in the meristemic region of an untreated soybean root tip. (B). The appearance of cells in the meristemic region of a soybean root tip after treatment with 300 mM NaCl and 5 mM CaCl<sub>2</sub>. Nuclei are no longer clearly visible. (C). The deformed or degraded cells in the meristemic region of a soybean root tip after treatment with 300 mM NaCl. (D). Nuclei in the meristemic cells of a control soybean root tip. CN = cell nuclei. (E). Cells in the meristemic region of a soybean root tip after treatment with 300 mM NaCl. The deformation of the nuclei and disorganized cytoplasm in the cells can be seen. CN = cell nuclei. (F). Cells in the meristemic region of a soybean root tip after treatment with 300 mM NaCl and 5 mM CaCl<sub>2</sub>. Deformation of nuclei is shown by the arrow. CN = cell nuclei.

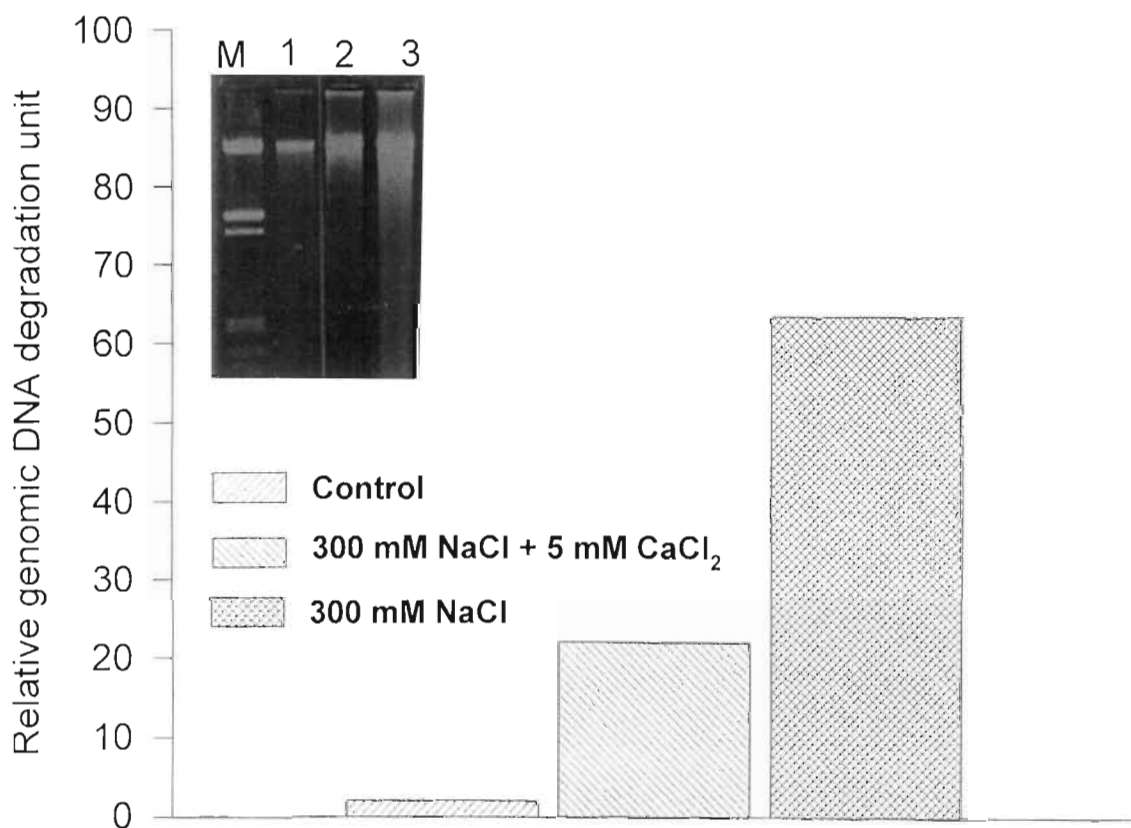


**Figure 4.4: Formation of oligonucleosomal fragments of DNA with NaCl stress.** Genomic DNA extracted from root tips of 6-day-old control soybean seedlings which had not been subjected to NaCl and from root tips of 6-day-old soybean seedlings which had been subjected to different NaCl concentrations for 24 h. Five micrograms of DNA were fractionated on a 1.1% agarose gel. The bars represent the quantification of the signal intensity by densitometric analysis. Each bar represents the quantification of the corresponding signal in the gel. The strongest signal was set at 100 and the others were quantified on the basis of this signal. (M), Molecular Weight Marker III; (1), 0 mM NaCl (control); (2), 100 mM NaCl; (3), 150 mM NaCl; (4), 200 mM NaCl; (5), 250 mM NaCl; (6), 300 mM NaCl; (7), 350 mM NaCl; (8), 400 mM NaCl; (9), 450 mM NaCl; (10), 500 mM NaCl.





**Figure 4.5: Genomic DNA from soybean root tips after treatment with different concentrations of NaCl and  $\text{CaCl}_2$  for 24 h, showing different degrees of DNA fragmentation.** Histograms represent the relative quantification of the signals as described in Figure 4.4. (M), Molecular Weight Marker III; (1), 0 mM NaCl + 0 mM  $\text{CaCl}_2$ ; (2), 150 mM NaCl + 5 mM  $\text{CaCl}_2$ ; (3), 150 mM NaCl + 1 mM  $\text{CaCl}_2$ ; (4), 200 mM NaCl + 5 mM  $\text{CaCl}_2$ ; (5), 200 mM NaCl + 1 mM  $\text{CaCl}_2$ ; (6), 200 mM NaCl + 0.1mM  $\text{CaCl}_2$ .



**Figure 4.6: DNA fragmentation with salt stress.** Five micrograms of genomic DNA from root tips of soybean were fractionated by gel electrophoresis. Histograms represent the relative quantification of the signals obtained by densitometric analysis as described in Figure 4.4. (M), Molecular Weight Marker III; (1), 0 mM NaCl; (2), 300 mM NaCl + 5 mM CaCl<sub>2</sub>; (3), 300 mM NaCl.

Biochemical analysis revealed that nuclear DNA started disintegration after salt stress for 24 h (Figure 4.4). Fragmentation of DNA was clearly detected after 150 mM NaCl stress, but not in 150 mM NaCl or 200 mM NaCl plus 5 mM  $\text{CaCl}_2$  treatment (Figure 4.5). Nuclear DNA degraded after 150 mM or higher concentrations of NaCl were applied (Figure 4.4). With the increase of NaCl concentration, more nuclear DNA degraded and the degraded genomic DNA formed a smear in the gel and the genomic DNA band became undefined. The fragments were of sizes about 180 base pairs, but not in random according to an earlier report (KATSUHARA & KAWASAKI 1996). Fragmentations were considerably suppressed in root cells treated with 300 mM NaCl plus 5 mM  $\text{CaCl}_2$  for 24 h (Figure 4.6).

#### **4.4 Discussion**

Root growth of many crop plants is severely inhibited by high concentrations of NaCl in the growing medium (CRAMER *et al.* 1987; KAFKAFI & BERNSTEIN 1996). Supplemental  $\text{Ca}^{2+}$  has a protective effect on the root growth of non-halophytes exposed to NaCl salinity (GREENWAY & MUNNS 1980; RENGEL 1992; KATSUHARA & KAWASAKI 1996). The present study confirmed these effects. Increases in the concentration of external NaCl for 24 h inhibited root growth. When external calcium increased, a higher concentration of NaCl was required to inhibit root growth.

Genomic DNA was isolated from salt-stressed roots and subjected to electrophoresis. The nuclear DNA deformed and then degraded with 150 mM or higher concentrations of NaCl. This fragmentation was suppressed when 5 mM  $\text{CaCl}_2$  was

applied together with high concentrations of NaCl. This confirmed the TEM observations that nuclei disintegrated with high NaCl treatments and that  $\text{Ca}^{2+}$  prevented such nuclear DNA degradation in root tips. Isolated DNA consisted of oligonucleosomal fragments, being in agreement with similar DNA degradation as characteristic of apoptosis in necrotic animal cells (COHEN 1993) and wheat cells (KATSUHARA & KAWASAKI 1996).

Saline solutions impose three types of stress on roots: The first is osmotic stress, resulting from lowered water potential in the root growth medium and turgor pressure for cell growth is lost; the second is ionic stress, induced by changes in concentrations of specific ions in the root growth medium and within the growing tissue; the third is cell death.

Nuclear deformation and degradation may be caused by dehydration. In the soybean root meristem, a nucleus occupies about half of a cell. Salt stress causes nuclear deformation and subsequent nuclear degradation. These deformations or degradations are assumed to be caused by cellular dehydration. The low external osmotic potential reduced the turgor pressure of root cells resulting in growth inhibition. Cells with no turgor pressure, lost most of their function, and this caused cell death. The cell death may separate the disorganised root system from other plant tissue and may prevent the influx of excess ions into the shoot. This is supported by the present findings that with 300 mM NaCl treatment, the cytoplasm became disorganized and separated from the cell wall, the nuclei were deformed or disappeared. High concentrations of  $\text{Na}^+$  inhibit  $\text{K}^+$  influx into roots (RAINS & EPSTEIN 1967; CRAMER *et*

*al.* 1987) or increase K<sup>+</sup> leakage from the cells (BEN-HAYYIM *et al.* 1987). The reduction in cell growth when Ca<sup>2+</sup> of the cell membrane was replaced by Na<sup>+</sup> was attributed to potassium leakage from the cell (LEIGH & WYN JONES 1984), the imbalances of ions may cause a loss in cell turgor pressure and lead to nuclear deformation or degradation and cell death. An increase in CaCl<sub>2</sub> concentration could prevent this from happening, because supplemented CaCl<sub>2</sub> proposedly enhance the selectivity of K<sup>+</sup> versus Na<sup>+</sup> mainly in the apical 4 - mm root region (ZHONG & LÄUCHLI 1994), reduce Na<sup>+</sup> influx into roots (RAINS & EPSTEIN 1967), and thus prevent salt injury to cells (CRAMER *et al.* 1989).

Structural changes of nuclei caused by salt stress have been reported earlier (WERKER *et al.* 1983; KATSUHARA & KAWASAKI 1996). Werker *et al.* (1983) could not explain the mechanism or physiological meaning of such developments. Katsuhara & Kawasaki (1996) studied nuclear deformation and degradation of meristematic cells in wheat roots where growth had been inhibited by salt stress using DAPI (4'6-diamino-2-phenyliudol) stain. This indicated that nuclear deformation of the cells occurred with 12 h of salt stress with 500 mM NaCl, and was followed by apoptosis-like DNA degradation. In the present study, nuclear deformation or degradation in the soybean root meristem was observed with 150 mM or higher NaCl concentration. TEM showed that the cell membrane separated from the cell wall, the cytoplasm became disorganized and nuclei degraded. This indicates that nuclear deformation or degradation in the meristematic region of the root leads to sequential cell degradation, cell death and the cessation of plant growth.

## **Chapter Five**

### **Selection and characterization of sodium chloride-tolerant callus of *Glycine max* (L.)**

#### **Abstract**

Callus cultures were initiated from soybean (*Glycine max* (L.) Merr. cv. Acme) cotyledons on Miller's basal medium supplemented with 2 mg l<sup>-1</sup> NAA and 0.5 mg l<sup>-1</sup> kinetin. Growing cells were exposed to increasing concentrations of NaCl in the medium. A concentration of 100 mM NaCl completely inhibited callus growth. After incubation for 28 days, cells which could tolerate this concentration of NaCl grew to form cell colonies. A NaCl-tolerant line of *Glycine max* (L.) Merr. cv. Acme was obtained through continuous subculturing on 100 mM NaCl. Salt tolerance in this culture was characterized by an altered growth behavior, reduced cell volume, and accumulation of Na<sup>+</sup>, Cl<sup>-</sup>, proline and sugars when grown under salt stress, as well as on normal media. These features, which proved to be stable after the culture was transferred to a salt-free medium, are characteristics commonly associated with halophytes. Presented data suggest that this salt tolerance is the result of a shift towards a halophytic behavior.

## 5.1 Introduction

Salt stress is one of the major factors limiting plant growth and productivity (LIU *et al.* 2000a). Salinity is known to affect many physiological and metabolic processes with a resulting reduction in plant growth (LÄUCHLI 1984; OLMOS *et al.* 1994). The regulation of physiological adaptation to salt stress is not clearly understood, as changes in different external factors can modify the salt response.

Plant growth reduction in a saline environment is commonly attributed either to ion toxicity or to a water deficit (GREENWAY & MUNNS 1980). Plant species adjust to high salt concentration by lowering tissue osmotic potential with the accumulation of both inorganic and organic solutes (FLOWERS *et al.* 1977). A typical response of many plants to saline environments particularly halophytes, is to accumulate high intracellular concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  (BINZEL *et al.* 1988). In general, as the NaCl level in the plant's environment increases, both the  $\text{Na}^+$  and  $\text{Cl}^-$  levels within the plant increase. Concurrently, the levels of  $\text{Ca}^{2+}$  and  $\text{K}^+$  tend to decrease (CROUGHAN *et al.* 1978). Osmotic adjustment is a fundamental adaptive response of plant cells when exposed to salinity and is necessary for survival and growth under saline conditions. Osmotic adjustment in response to salinity is a result of solute accumulation which occurs through the uptake of solute, the synthesis of organic compounds, or both (BINZEL *et al.* 1987).

Carbohydrates, and amino acids are major organic components affected in several plant species under salt stress, their relative contribution depends on

environmental conditions. Sugars have long been known to increase in a wide range of plants grown at low moisture levels and under salinity. In higher plants, sugars are required not only to sustain heterotrophic growth but also to regulate the expression of a variety of genes. Environmental stresses, such as pathogen infection and wounding, activate a cascade of defense responses and may also affect carbohydrate metabolism (EHNESS *et al.* 1997). The rate and extent of increase in sugar content depends on the environmental conditions, species, and even on the genotype within the same species. A few workers reported on the concentrations of sugars changed at different levels of water stress and recovery (ACKERSON 1981; KAMELI & LÖSEL 1993). Three sugars, glucose, fructose and sucrose accumulate in crop plants but little information is available as to plant cells under salinity stress. Free proline accumulated in a range of plants in response to a wide range of environmental stresses (HARE & CRESS 1997). It is generally accepted that under conditions of extreme salinity, proline accumulation serves as a defence against osmotic challenge by acting as a compatible solute and appears to be the preferred organic osmoticum in many plants.

Tissue culture is an excellent tool for elucidating the correlation between plant organizational level and salt tolerance because of the scope it offers for studying the physiology of intact plantlets, together with that of organs and single cells, using homogeneous plant material under uniform environmental conditions (MILLS 1989). Salt-tolerant cell lines, when compared with normal sensitive cells, can provide a useful means of measuring the capacity and range of stress tolerance and may be used in order to elucidate tolerance mechanisms at the level of the cell (KUMAR & SHARMA 1989). The selection of salt tolerant cell lines from cultured plant cells has potential for



contributing new genetic variability to plant breeders. The cell culture approach has proved effective in selecting salt tolerant cell lines of *Catharanthus roseus* (VÁZQUEZ-FLOTA & LOYOLA-VARGAS 1994), mungbean (KUMAR & SHARMA 1989), and pea (OLMOS *et al.* 1995). Vázquez-Flota and Loyola-Vargas (1994) obtained *C. roseus* cells which were capable of growth under 171 mM NaCl. Kumar and Sharma (1989) selected lines of pea cells which showed tolerance to 300 mM NaCl.

The aim of this part of the investigation was to compare the response to salinity of salt-sensitive and salt-tolerant cell lines. The different cell lines were chosen according to preliminary information on their growth under saline conditions. In this Chapter, the isolation procedure, growth characteristics,  $K^+$ ,  $Na^+$ ,  $Cl^-$ , free proline and sugar content of NaCl-sensitive and NaCl-tolerant callus cultures of soybean (*Glycine max*) exposed to salinity are outlined.

## **5.2 Materials and methods**

### **5.2.1 Establishment of tissue culture**

The original *Glycine max* (L.) Merr. cv. Acme callus culture used in this work was initiated from soybean cotyledons. The culture was maintained on Miller's basal medium (MILLER 1965) supplemented with 2 mg l<sup>-1</sup> NAA and 0.5 mg l<sup>-1</sup> kinetin (MB medium) (Appendix 2). The cultures were subcultured every 28 days and kept at 26 °C in continuous low light (0.13  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ).

### 5.2.2 Screening for a NaCl-tolerant callus line

The tolerant line was derived from callus cultures of soybean by continuous subculturing onto MB medium and supplementing it with increasing NaCl concentrations in the medium up to 150 mM NaCl. The actively-growing fresh calli were exposed to increasing concentrations of NaCl (0, 25, 50, 75, 100, 125, and 150 mM) in order to determine the concentration at which growth of normal calli was completely inhibited. The appropriate concentration, once determined, was used for selection of a callus line resistant to this concentration (100 mM) of NaCl. This NaCl-tolerant line, obtained by continuous subculturing in MB medium supplemented with 100 mM NaCl, was designated R100. Whereas callus unable to grow at the 100 mM NaCl concentration was referred to as the NaCl-sensitive line, designated S100. These lines were subcultured every 28 days.

### 5.2.3 Tolerance stability assay for soybean callus

Salt tolerance stability was assayed in cultures subjected to the continuous presence of 100 mM NaCl for 4 months. The assay was performed by exposing the R100 culture to NaCl again after it was maintained for 3 months on a NaCl-free MB medium. To establish growth rates, 1.00 g callus was inoculated into culture flasks containing 40 ml medium with 100 mM NaCl, using 27 replicates. Subsequently, at 4 days intervals, the callus from 3 culture flasks was removed and their fresh weight determined. It was then oven-dried at 80°C for 48 h prior to determination of dry weight. This procedure was repeated up to 28 days after inoculation. The salt tolerance index

was determined according to Vázquez-Flota & Loyola-Vargas (1994) as indicated in Figure 5.2.

#### 5.2.4 Scanning electron microscopy (SEM)

Samples of callus from control, R100 and S100 lines were fixed and processed for electron microscopy as described by Pan *et al.* (1997). Calli were cut into 2 x 2 mm squares and fixed in 3% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.2, for 24 h, washed twice in the 0.05 M sodium cacodylate buffer, and dehydrated through an ethanol series (30, 50, 70, 80, 90 and 100% at 30 min intervals) followed by a duplicate absolute ethanol wash for 30 min. After dehydration, the specimens were dried to critical point with liquid carbon dioxide as a transition fluid using an Hitachi HCP-2 critical point dryer, and then mounted on copper stubs. Specimens were coated with gold palladium in a polaron sputter coater and examined with a S-570 scanning electron microscope at 8.0 or 10 Kv.

#### 5.2.5 Determination of proline content

Proline was assayed as described by Bates *et al.* (1973). Two hundred milligram of callus were frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. The material was homogenised in 10 ml of 3% (w/v) sulphosalicylic acid and the homogenate centrifuged at 14,000 x *g* for 20 min at 4°C. Two ml aliquots of supernatant solution were transferred to clean tubes and reacted with 2 ml of acid ninhydrin reagent and 2 ml glacial acetic acid for 1 h at 100°C. The reaction was

terminated in an ice bath. After warming to room temperature the reaction mixture was extracted with 4 ml toluene by vigorous vortexing for 15 sec. The toluene phase containing the red chromophore was aspirated and its absorbance read at 520 nm using toluene as a blank. Proline concentration was determined using a standard curve constructed from A520 readings obtained from assaying 0.1 to 40  $\mu$ mol of L-proline.

#### *5.2.6 Determination of sugar content*

For sugar determinations, fresh callus tissue (approximately 200 mg) was ground in liquid nitrogen and extraction was conducted in 5 ml 80% ethanol for 12 h under moderate shaking. The homogenates were centrifuged at 5,000 x *g* for 10 min. Sugar was quantified using Sil-A reagent. Sugar oximes (TANOWITZ & SMITH 1984) were silylized (SWEELEY *et al.* 1963) and separated by gas chromatography on a 1.8 x 3 mm glass column packed with OV-17 on chromosorb HP 80/100 and detected by flame ionization. The column was held at 125°C for 3 min, followed by ramping at 4°C min<sup>-1</sup> to 266°C. Individual sugars were identified by co-chromatography with authentic standards, prepared as described above.

#### *5.2.7 Energy dispersive X-ray (EDX) analysis*

Samples were prepared for SEM viewing by air drying overnight and carbon coating to prevent charging as a clear picture was needed to enable selection of specific points. An Hitachi S-570 Scanning electron microscope fitted with a Link exl II EDX system was used. Specimens in the SEM were tilted to an angle of 15° towards

the X-ray detector, and the working distance in the microscope was set at 15 mm to obtain the most accurate results. An accelerating voltage of 15 Kv was used. Callus specimens were analysed at 1000 X magnification for 100 sec at at least 8 locations each. The X-ray signal intensity was quantified by counts (Y-axis label) and was indicative of the relative concentration of the elements in the samples.

### **5.3 Results**

#### *5.3.1 Callus growth*

With increasing NaCl concentration (0-150 mM), a gradual decrease in both fresh and dry weight of callus was observed (Figure 5.1 A, B). There was almost complete inhibition of callus growth at 100 mM NaCl. The NaCl-tolerant line was selected by continuous exposure to 100 mM NaCl for 4 months. As a result of this exposure, a different growth behavior was observed, characterized by a slower growth rate compared to growth on MB medium (Figure 5.4 B, D). The salt tolerance stability test consisted of maintaining the tolerant culture for 3 months in a salt-free medium. The resistant cell lines were then transferred to fresh MB medium containing 100 mM NaCl, where they grew satisfactorily. The cell line which showed resistance to NaCl was designated the 'Resistant 100' (R100) callus line, whereas callus unable to grow at the 100 mM NaCl concentration was referred to as the 'Sensitive 100' (S100) callus line.

#### *5.3.2 Growth characteristics*

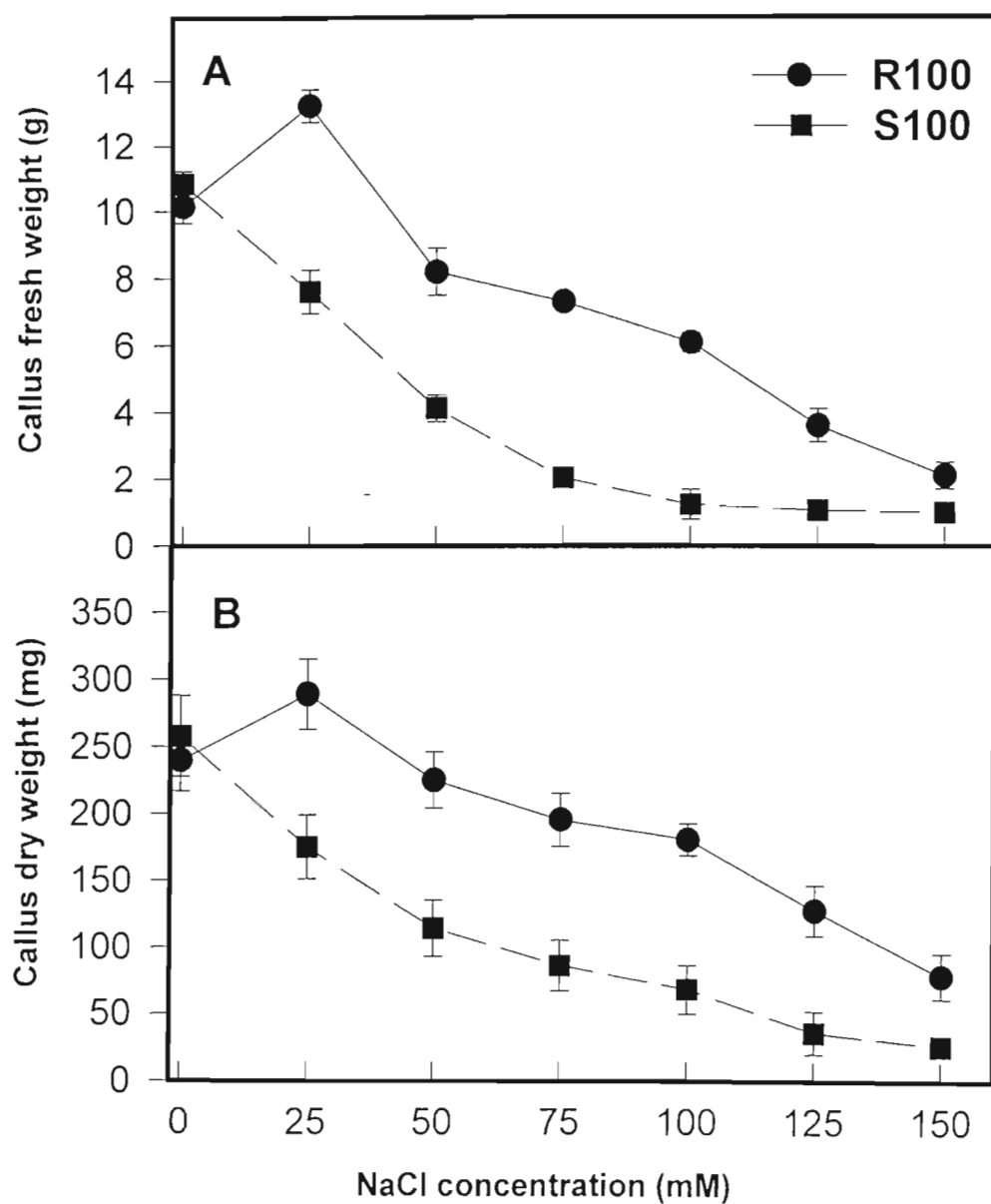
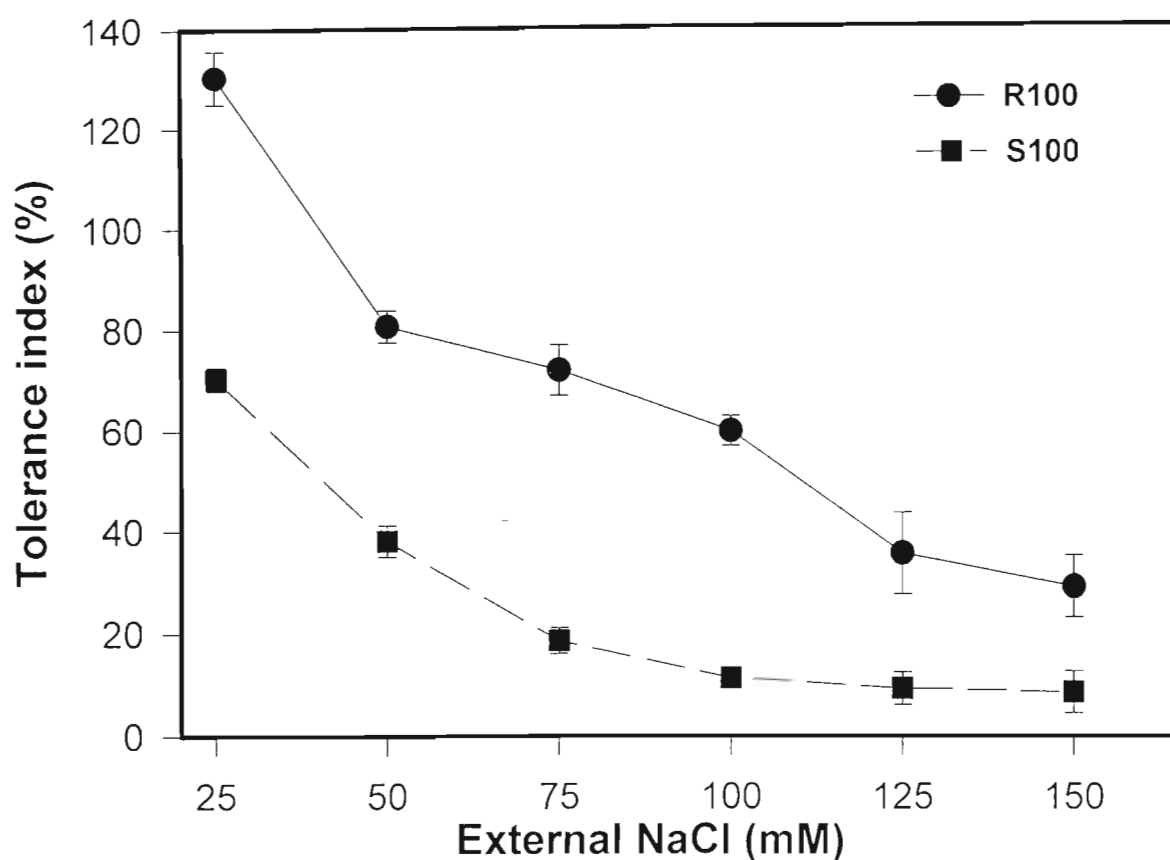
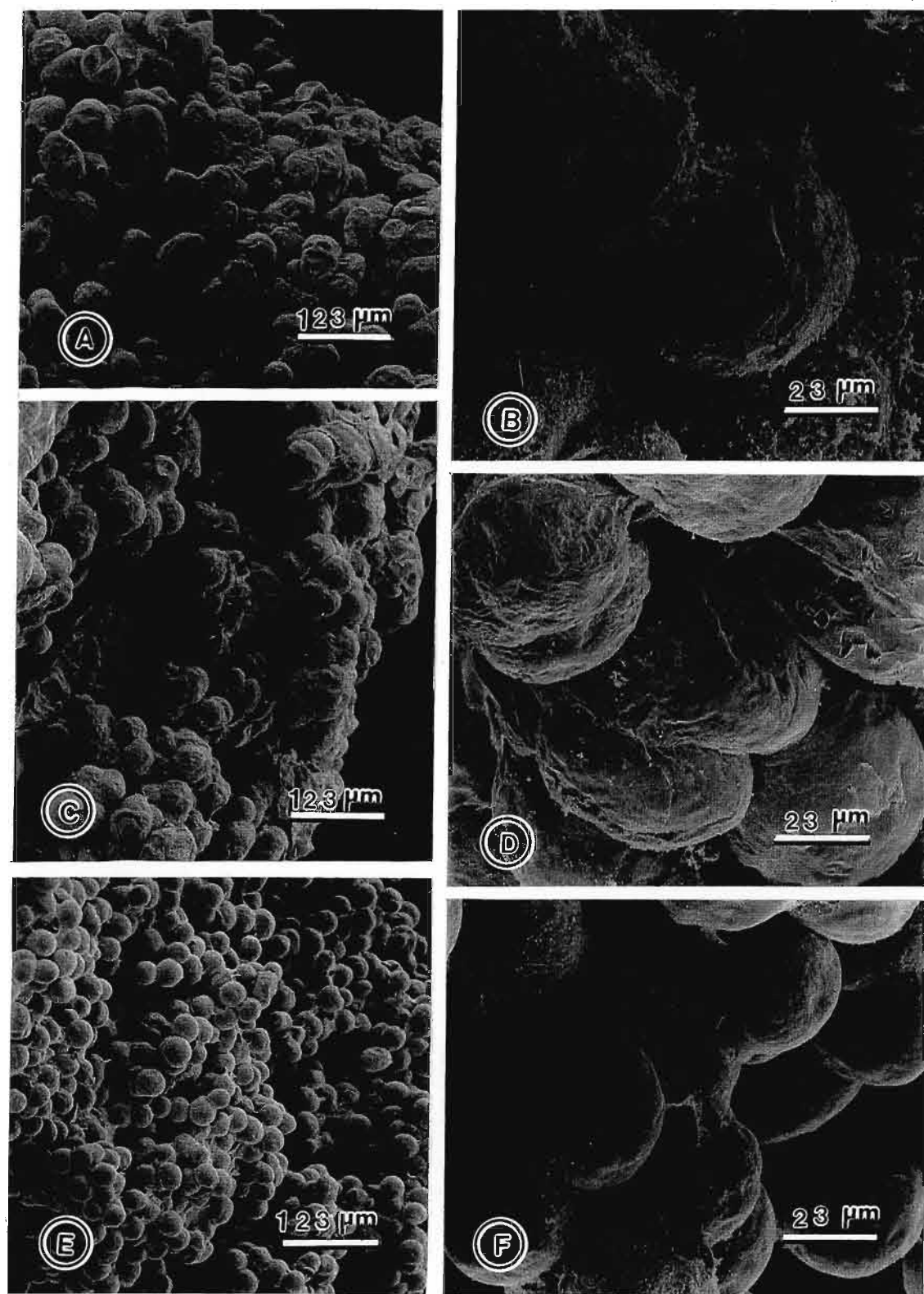


Figure 5.1: Effect of NaCl stress on the fresh (A), and dry weights (B) of NaCl-sensitive and NaCl-tolerant callus lines of *Glycine max*. Values are the average of five replications  $\pm$  SE.



**Figure 5.2: Tolerance index of the soybean NaCl-sensitive and NaCl-tolerant cell lines.** Tolerance index was calculated as a percentage of fresh weight increase without salt. Values are the average for five replications  $\pm$  SE.

With increasing NaCl concentration (0-150 mM), a gradual decrease in both fresh and dry weight of callus was observed except for NaCl-resistant R100 callus at 25 mM NaCl (Figure 5.1 A, B). Figure 5.1 displays the growth of the two cell lines as a function of increasing NaCl. When cultured on the MB medium in the absence of NaCl, the R100 line grew less than the S100 line. This was however, not statistically significant (Table 5.1). At 25 mM NaCl, the growth of R100 callus was stimulated, as evidenced by a gain in both fresh and dry weight. The final fresh weight of the R100 line was nearly double that of the S100 line. A further increase in NaCl concentration



**Figure 5.3: Scanning electron micrographs of NaCl-sensitive and NaCl-tolerant calli of *Glycine max* under salt stress and without salt stress.** Sensitive calli (A) growth on normal MB medium and (B) detail of the cells. NaCl-sensitive calli (C) and NaCl-tolerant calli (E) growth on MB medium supplemented with 100 mM NaCl. NaCl-sensitive cells (D) showing an elongated shape and NaCl -tolerant cells (F) showing spherical shape. (A, C, E) Bar = 130  $\mu\text{m}$ ; (B, D, F) Bar = 23  $\mu\text{m}$ .

**Fig 3. Liu and van Staden**



**Table 5.1: Relative growth rate, and dry weight content of salt-sensitive and salt-tolerant calli of *Glycine max* after 28 days of culture.**

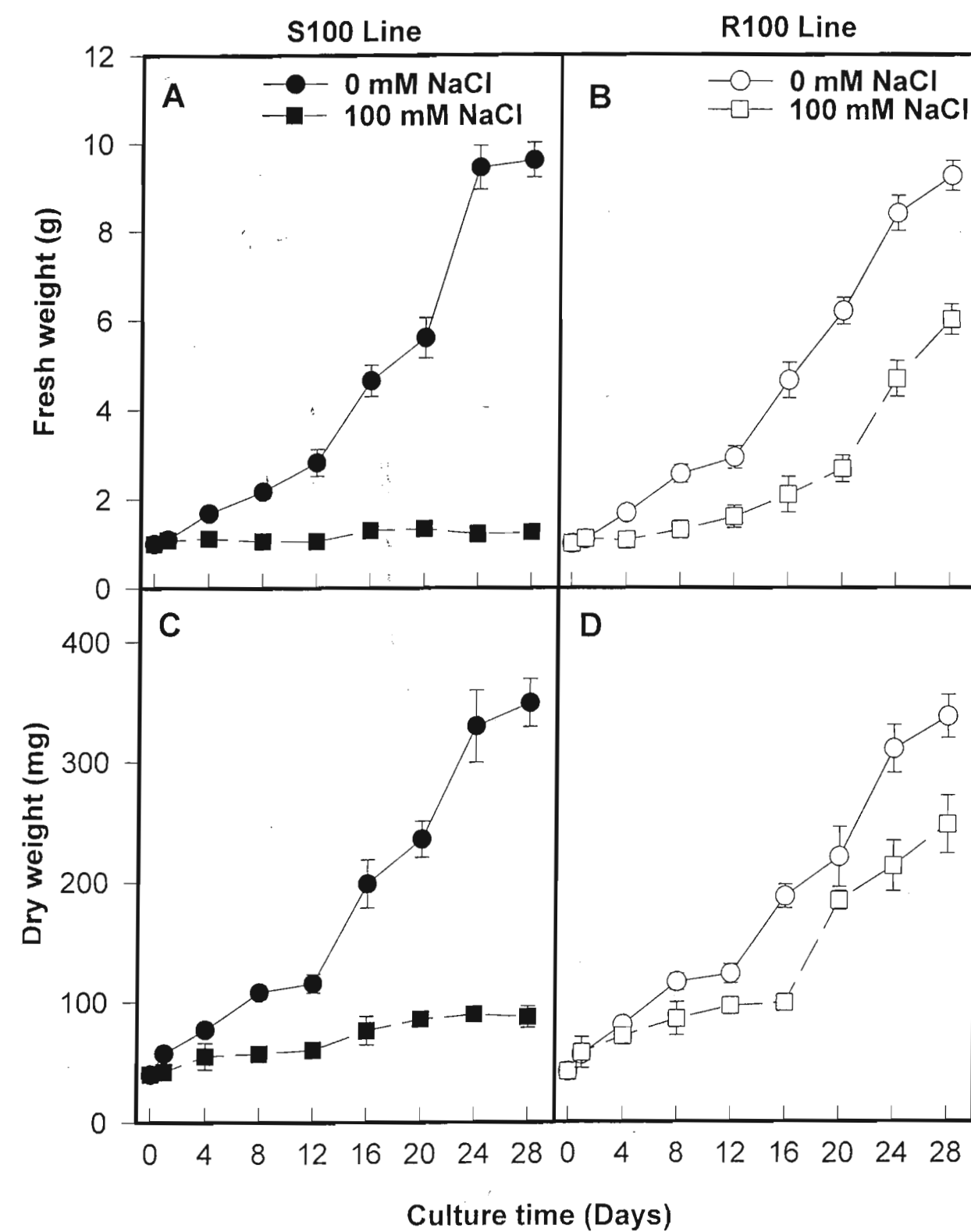
Cell Type	Treatment	Relative growth	Variation DW
		(% FW)	mg g <sup>-1</sup> FW
Sensitive	MB	985.4 ± 35.7	35.45 ± 4.3
Sensitive	MB + 100 mM NaCl	131.5 ± 16.3	55.73 ± 6.2
Tolerant	MB	957.3 ± 41.4	35.87 ± 5.2
Tolerant	MB + 100 mM NaCl	580.6 ± 45.6 *	47.4 ± 3.6 *

Each value represents the mean ± SE of ten different samples. Significant difference: (\*) p<0.001, according to Duncan's Multiple Range Test.

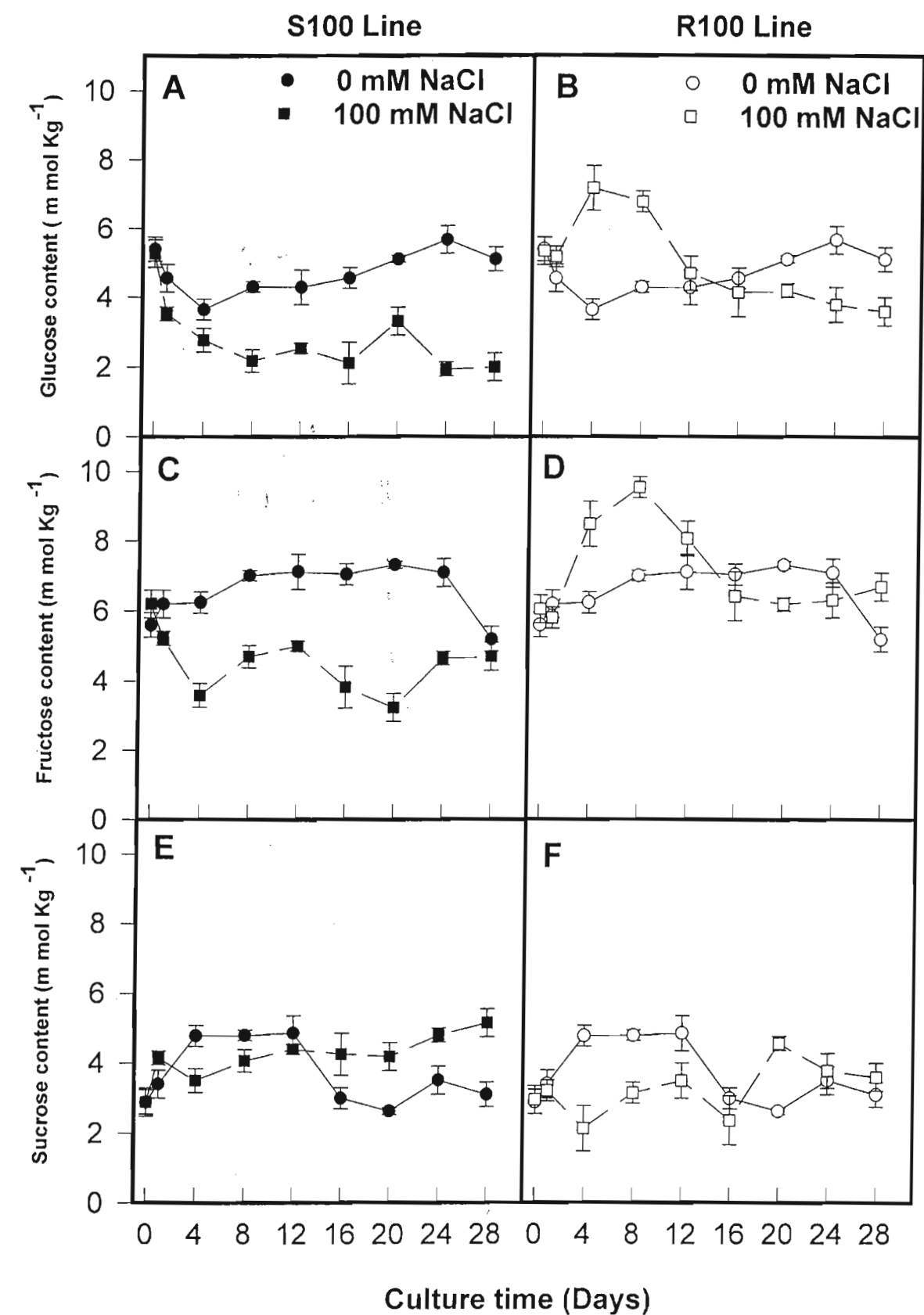
reduced growth of the S100 cell line. The R100 line was also partially inhibited at this salinity level, but its growth was substantially greater than that of the S100 line. There was almost complete inhibition of callus growth at 150 mM NaCl (Figure 5.1 A, B).

At day 28, both R100 and S100 cell lines were growing in logarithmic phase. In this phase, relative growth was significantly reduced in S100 at 100 mM of NaCl (Figure 5.1 A, B; Figure 5.4 A, C; Table 5.1). However, R100 calli showed a significant increase in Dw/Fw ratio (Figure 5.1 B; Table 5.1).

SEM observations revealed some morphological differences between the two callus lines. NaCl-sensitive cells (S100 line) were characterized by a spherical shape (Figure 5.3 A, B) under normal growing conditions but had an elongated shape with large cell volume under 100 mM NaCl-treatment (Figure 5.3 C, D). However, NaCl-



**Figure 5.4: Growth curve of NaCl-sensitive and NaCl-tolerant callus cultures of *Glycine max* at 26°C in the presence and absence of 100 mM NaCl.** Growth curves were constructed by measuring the net increase in fresh and dry weight at the indicated incubation times. S100 line grown on MB medium and NaCl (100 mM) supplemented MB medium (A, C), R100 line grown on MB medium and NaCl (100 mM) supplemented MB medium (B, D). Values are the average of five replications  $\pm$  SE.

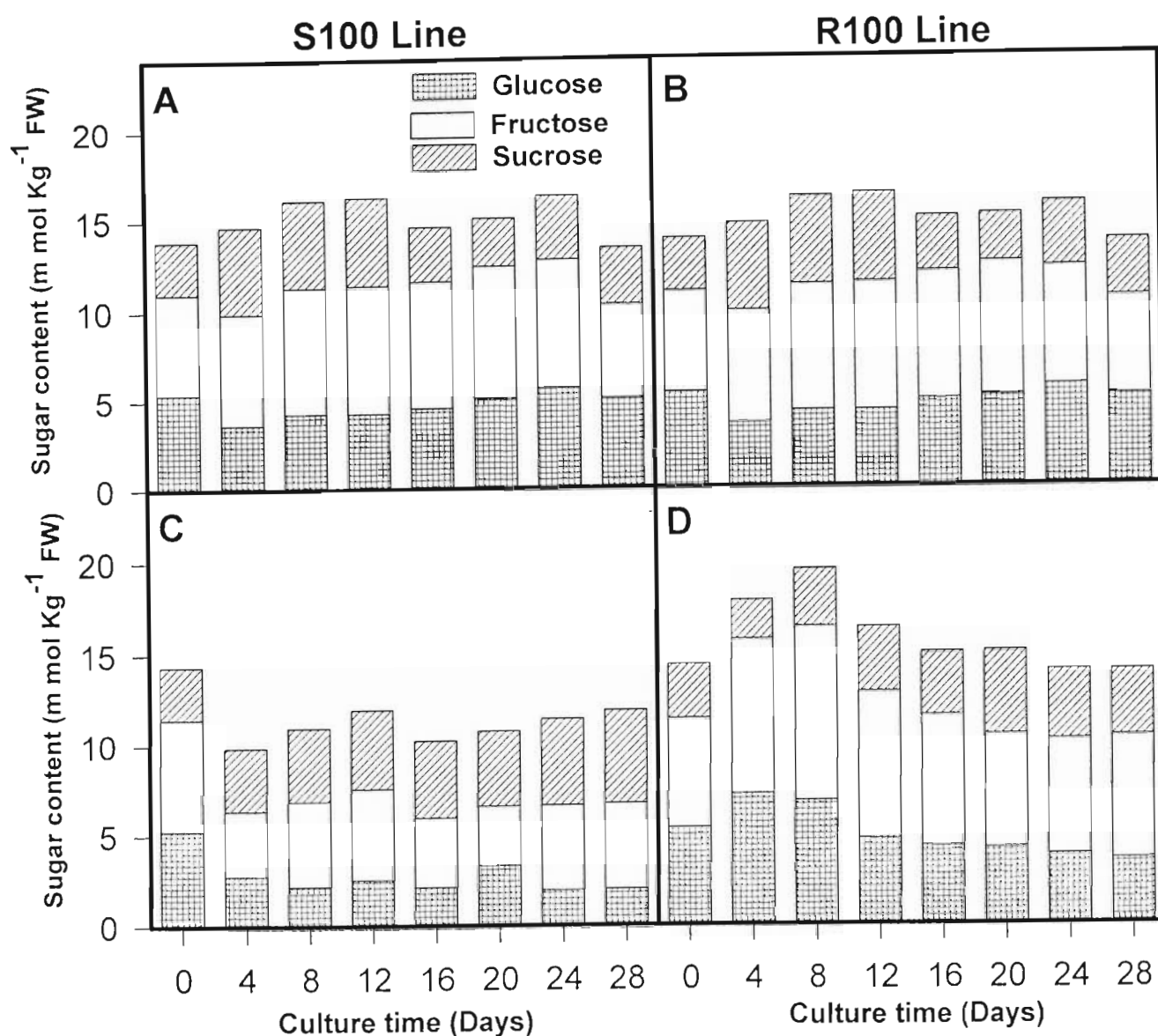


**Figure 5.5: Effects of salt stress on sugar accumulation (in mmol Kg<sup>-1</sup> FW) of calli of S100 and R100 cell lines exposed to 0 and 100 mM NaCl.** Glucose content of S100 line in MB and MB supplemented with 100 mM NaCl (A); Glucose content of R100 line in MB and MB supplemented with 100 mM NaCl (B); Fructose content of S100 line in MB and MB supplemented with 100 mM NaCl (C); Fructose content of R100 line in MB and MB supplemented with 100 mM NaCl (D); Sucrose content of S100 line in MB and MB supplemented with 100 mM NaCl (E); Sucrose content of R100 line in MB and MB supplemented with 100 mM NaCl (F). Each value is the mean of three independent samples and vertical bars represent  $\pm$  SE.

tolerant cells (R100 line) showed a more spherical shape and smaller size. Cells tolerant to 100 mM NaCl were approximately one third (volume) the size of normal cells at the growth stage used for these experiments (Figure 5.3 A, B, E, F). Both the sensitive and tolerant calli grown under 100 mM NaCl concentration were less friable than the calli growth under normal BM medium (Figure 5.3 B, D, F).

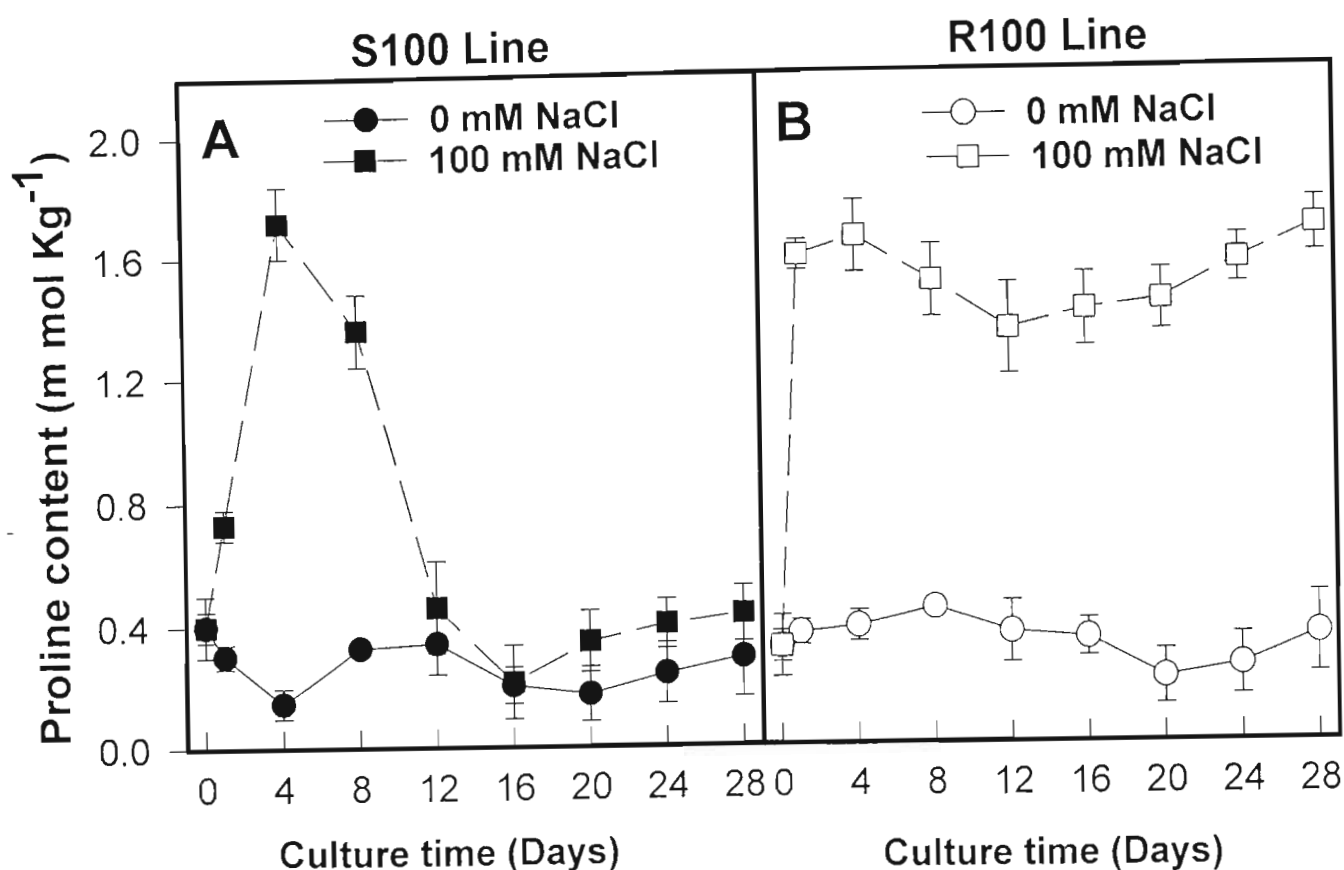
### 5.3.3 Sugar accumulation

The sensitive and tolerant cell lines exhibited differences in the time of increase of sugars, especially glucose and fructose, relative to salt stress. Under 100 mM NaCl stress, the glucose and fructose content in the R100 line increased dramatically after 24 h of stress, and reached their highest levels at days 4 and 8. The glucose content decreased rapidly after 8 days of salt stress, reaching the values of the control within 4 days, and were lower than that of the control until the end of culture (Figure 5.5 B). The fructose content in R100 callus fell rapidly after 8 days of stress, reaching the values of the control within 6 days, and remained lower than that of the control. However, at the end of the culture period (28 days), the fructose content of the R100 callus was higher than that for the unstressed R100 callus (Figure 5.5 D). The glucose and fructose content in S100 callus under salt-stress showed a different pattern. Both glucose and fructose content decreased compared to the unstressed controls. At the end of the culture period (28 days), the glucose content in the S100 callus grown under salt stress was much lower than that for the S100 callus growing under normal conditions (Figure 5.5 A, C). Sucrose, which was present in smaller amounts than the monosaccharides in both salt-sensitive and salt-tolerant stressed cells, exhibited a



**Figure 5.6:** Soluble sugar content of calli with NaCl-sensitive (A), NaCl-tolerant (B) lines grown on MB medium and NaCl-sensitive (C), NaCl-tolerant (D) lines exposure to 100 mM NaCl for 28 days.

similar response to salt stress. Both R100 and S100 calli maintained a lower sucrose content compared to the unstressed control during early culture (Figure 5.5 E, F). In the salt-sensitive cell line, callus contained significantly higher amounts of sucrose than the unstressed control after day 10. This remained at a high level until the end of the experiment (Figure 5.5 E). In R100 callus, the sucrose content fell sharply after salt



**Figure 5.7:** Proline accumulation in control and NaCl-tolerant *Glycine max* calli, respectively, as a function of NaCl concentration in the medium. S100 line in MB and MB supplemental with 100 mM NaCl (A); R100 line in MB and MB supplemental with 100 mM NaCl (B). Each value is the mean of three independent samples and vertical bars represent  $\pm$  SE.

stress and recovered to the level of the control at day 16 and increased to its maximum value by day 20 (Figure 5.5 F).

#### 5.3.4 Free proline accumulation

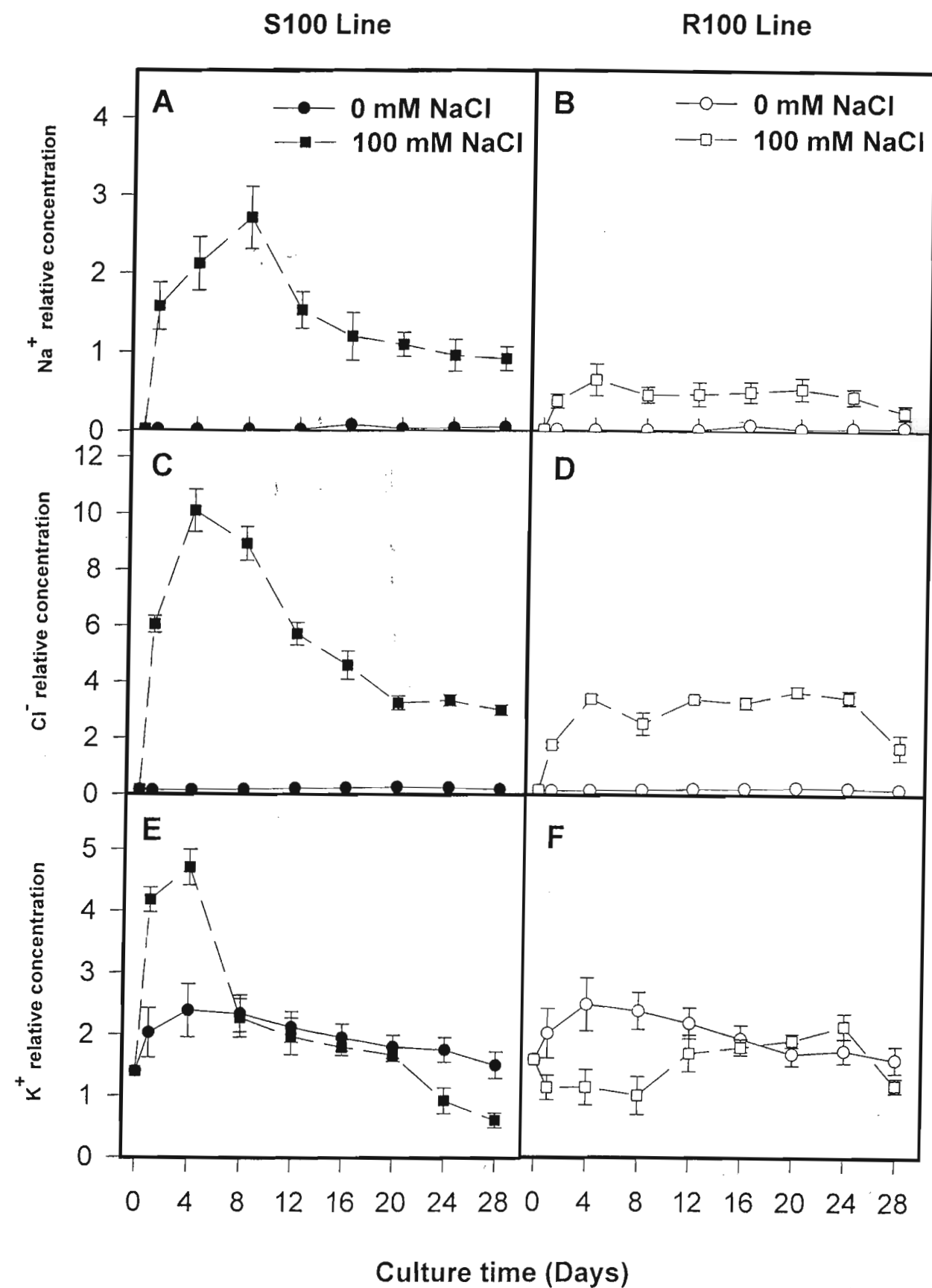
The endogenous levels of free proline were low in R100 and S100 callus grown

on normal MB medium. Proline concentrations rose sharply with salt stress in both S100 and R100 callus (Figure 5.7 A, B), reaching their maximum values by day 4. The proline content in both cell lines were more than 4-fold higher than when cell lines were grown on normal MB medium. After 8 days of salt stress, the level of proline in salt stressed S100 callus fell very rapidly and, within four days, equaled that of the controls and then decreased to levels below the control. However, under salt stress, R100 callus contained significantly higher amounts of free proline than either S100 and R100 callus lines grown on normal medium during the experiment. Similar observations have been made for several other plant species (KUMAR & SHARMA 1989; LUTTS *et al.* 1999).

#### 5.3.5 Ion accumulation

The S100 and R100 callus lines showed an increased  $\text{Na}^+$  content with 100 mM levels of NaCl in the medium, The S100 callus contained high levels of  $\text{Na}^+$  when grown under 100 mM NaCl stress, The  $\text{Na}^+$  reached its highest level at day 8 and then declined. The R100 callus maintained lower levels of  $\text{Na}^+$  than the S100 callus grown on 100 mM NaCl medium during the experiment (Figure 5.8 A, B).

S100 and R100 calli exposed to NaCl accumulated higher  $\text{Cl}^-$  levels than those maintained in normal medium (Figure 5.8 C, D). The  $\text{Cl}^-$  accumulated to a high level in the S100 line subjected to 100 mM NaCl within 24 h of culture and reached its maximum at day 4. There was a decrease after 4 days, but the  $\text{Na}^+$  levels were always higher than those in the unstressed control (Figure 5.8 C).



**Figure 5. 8: Effect of NaCl stress on  $\text{Na}^+$ ,  $\text{Cl}^-$ , and  $\text{K}^+$  content of NaCl-sensitive and NaCl-tolerant callus lines of *Glycine max*.**  $\text{Na}^+$  content of S100 line in MB and MB supplemented with 100 mM NaCl (A);  $\text{Na}^+$  content of R100 line in MB and MB supplemented with 100 mM NaCl (B);  $\text{Cl}^-$  content of S100 line in MB and MB supplemented with 100 mM NaCl (C);  $\text{Cl}^-$  content of R100 line in MB and MB supplemented with 100 mM NaCl (D);  $\text{K}^+$  content of S100 line in MB and MB supplemented with 100 mM NaCl (E);  $\text{K}^+$  content of R100 line in MB and MB supplemented with 100 mM NaCl (F). Each value is the mean of three independent samples and vertical bars represent  $\pm$  SE.



The R100 callus line maintained lower levels of  $K^+$  when grown under 100 mM NaCl stress than under normal conditions (Figure 5.8 F). When S100 and R100 callus lines were exposed to 100 mM NaCl concentrations,  $K^+$  levels in the S100 callus line increased to its highest level by day 4 and then declined steadily (Figure 5.8 E). However, the R100 callus line showed a different trend,  $K^+$  levels declined in the first 4 days and then increased continuously (Figure 5.8 F). The present findings with regard to  $K^+$  and  $Na^+$  content in the NaCl-tolerant and sensitive callus lines are in agreement with the observations of other workers ( WATAD *et al.* 1983; PANDEY & GANAPATHY 1984; BINZEL *et al.* 1987).

#### **5.4 Discussion**

Exposing cultures of *Glycine max* to an agar-solidified MB medium containing a lethal concentration of NaCl permitted selection of a cell line with increased resistance to NaCl toxicity. The NaCl-tolerant line (R100) grew well at 100 mM NaCl compared to the wild-type cells (S100), suggesting that variants could be recovered by selecting for resistance in a high salt environment. Several research groups using different plant species have reported similar growth inhibition kinetics upon exposure of cultured cells to increasing levels of NaCl (TAL 1983; KUMAR & SHARMA 1989). This R100 line displayed a number of characteristics suggesting that the tolerance was the consequence of a shift towards a halophytic nature.

The cell volume of line R100 subjected to 100 mM NaCl was lower than that in line S100 in the normal medium (Figure 5.3 A, B, E, F). the reduced cell volume of the

**Table 5.2: Relative water content (RWC) in the callus of NaCl-sensitive and NaCl-tolerant cell lines exposure for 28 days to 0 and 100 mM NaCl.** Each value is the mean of four replications  $\pm$  SE.

Days	0 mM NaCl		100 mM NaCl	
	S100	R100	S100	R100
0	95.0 $\pm$ 0.2	94.9 $\pm$ 0.15	95.0 $\pm$ 0.2	94.9 $\pm$ 0.15
1	94.9 $\pm$ 0.12	94.7 $\pm$ 0.15	94.3 $\pm$ 0.18	94.2 $\pm$ 0.13
4	94.9 $\pm$ 0.20	94.9 $\pm$ 0.16	94.3 $\pm$ 0.12	93.3 $\pm$ 0.16
8	94.9 $\pm$ 0.10	95.1 $\pm$ 0.2	94.2 $\pm$ 0.23	93.6 $\pm$ 0.21
12	95.0 $\pm$ 0.08	95.1 $\pm$ 0.12	93.7 $\pm$ 0.21	94.3 $\pm$ 0.23
16	95.6 $\pm$ 0.09	95.8 $\pm$ 0.23	93.6 $\pm$ 0.17	94.4 $\pm$ 0.25
20	96.4 $\pm$ 0.07	96.6 $\pm$ 0.3	93.4 $\pm$ 0.19	93.8 $\pm$ 0.16
24	96.3 $\pm$ 0.12	96.5 $\pm$ 0.23	92.6 $\pm$ 0.24	93.9 $\pm$ 0.18
28	95.3 $\pm$ 0.15	95.5 $\pm$ 0.21	92.6 $\pm$ 0.21	94.0 $\pm$ 0.2

R100 line appears to be caused by an increase in cell number and water loss, since the water content in this cell line was lower than that in the R100 line grown on basal medium (Table 5.2). These findings are consistent with the observations of Dix and Street (1975) and Nabors *et al.* (1975). Both groups demonstrated that the NaCl-tolerant lines grew as an increase in cell number and a change in packed cell volume. Dix and street (1975) observed that packed cell volume tended to decrease after exposure to salt despite and increase in cell number. This was attributed to a decrease in cell size. The reduction in tissue water content at 100 mM NaCl level was due to lesser absorption of water by the tissue growing at these salinities compared with that of the control tissue in which absorption of water continued up to 4 weeks (Table 5.2). A similar reduction has been observed for *Cicer arietinum* (PANDEY & GANAPATHY 1984).

Higher concentrations of soluble carbohydrates were found in R100 callus growing with 100 mM NaCl (Figure 5.6 B, D). Under 100 mM NaCl stress, the total soluble carbohydrate concentration in S100 was lower than that in the control (Figure 5.6 A, C). Glucose and fructose were the primary accumulating carbohydrates in the control. An increase of total carbohydrate content in the R100 callus was observed in 100 mM NaCl treatment. This was due to an increase in glucose and fructose concentration. Increased concentrations of glucose and fructose may result from increased sucrose hydrolysis or due to absorption from the medium. The mechanism by which soluble sugars are accumulated in salt-stressed callus is difficult to establish. Activation of the salt-stress response requires energy and thus the induction of sink metabolism. Physiological studies indicate that carbohydrate metabolism is altered in response to stress (WRIGHT *et al.* 1995; EHNESS 1997). Sucrose cleavage is the initial reaction for metabolizing sucrose in plant tissues. In plants, sugars are not only a substrate for the growth of sink tissues, which depend on the import of carbohydrates, but they regulate the expression of a variety of genes (KOCH 1996). Sugar-induced gene expression has been demonstrated for enzymes involved in pathogen and stress responses (TSUKAYA *et al.* 1991; EHNESS *et al.* 1997). It is not known whether a common mechanism is responsible for the differential metabolic regulation of these genes (EHNESS *et al.* 1997).

Free proline has been reported to accumulate in a number of plants and tissues in response to salinity stress. Proline appears to be the most abundant compatible solute accumulated during salt stress in a wide spectrum of plant species. Proline content in the R100 line, either in a NaCl-free medium, or supplemented with 100 mM,

**Table 5.3: The effect of NaCl on relative Na<sup>+</sup>/K<sup>+</sup> content of *Glycine max* callus tissue.**

Cell type	Culture time (Days)							
	0	4	8	12	16	20	24	28
S100	51	61	95	83.5	67	21	45	46
S100 + 100 mM NaCl	51	2.64	2.72	0.83	1.28	1.49	1.36	1.41
R100	56	59	78	87	74	35	39	50
R100 + 100 mM NaCl	56	2.92	1.74	2.17	3.56	3.54	3.49	4.77

(Figure 5.7 B) showed a higher level than that in the S100 line (Figure 5.7 A). These findings are consistent with the observations of Watad *et al.* (1983) and Pandey and Ganapathy (1984). These workers demonstrated that NaCl-tolerant calli maintain higher levels of proline compared to the sensitive callus at a NaCl level that completely inhibited growth of normal cells. These results suggest the possibility of an osmoregulatory role for proline. This conclusion was also reached by Kumar and Sharma (1989) and Handa *et al.* (1986).

The Na<sup>+</sup> and Cl<sup>-</sup> content of the R100 and S100 did not change during the culture cycle in the normal medium (Figure 5.8 A, C). In contrast, the Na<sup>+</sup> and Cl<sup>-</sup> contents in the S100 line was more than 3-fold higher than that of the R100 line when cultured in 100 mM NaCl medium. (Figure 5.8 A, B, C, D). In the R100 callus, the Na<sup>+</sup> level showed a similar behavior to that of Cl<sup>-</sup> (Figure 5.8 A, C). The ability for Na<sup>+</sup> and Cl<sup>-</sup> accumulation had also been observed in tobacco (BINZEL *et al.* 1988), and mung bean (KUMAR &

SHARMA 1989). It was observed that the  $K^+$  content of the R100 callus tissue decreased over the first 12 days when cultured on 100 mM NaCl medium. A similar reduction has also been obtained in tomato (TAL *et al.* 1978) and tobacco (CHEN *et al.* 1980). High concentrations of  $Na^+$  inhibit  $K^+$  influx into tissue or increase  $K^+$  leakage from the tissue and reduce cell growth. It was interesting to note that the R100 line maintained a high  $K^+$  content and higher  $K^+/Na^+$  ratio compared to S100 (Figure 5.4 A, B; Table 5.3). These data suggest that one possible mechanism of tolerance developed by the R100 line could be the uptake and compartmentation of  $K^+$  and  $Cl^-$  ions, as in *C. roseus* (VÁZQUEZ-FLOTA & LOYOLA-VARGAS 1994) and tobacco cultures (BINZEL *et al.* 1987). This indicated that  $K^+$  plays an important role in osmotic adjustment during the survival of tolerant soybean cells under NaCl stress.

## **Chapter Six**

### **Growth rate, water relations and ion accumulation of soybean callus lines differing in salinity tolerance under salinity stress and its subsequent relief**

#### **Abstract**

A selected *Glycine max* (L.) salt-tolerant callus cell line (R100) was significantly more tolerant to salt than a salt-sensitive line (S100) during exposure to salt stress. Growth (Fresh and Dry weights) of the R100 cell line declined significantly at NaCl concentrations greater than 75 mM, while growth of the S100 cell line was already impaired at 25 mM NaCl. Levels of Na<sup>+</sup> and Cl<sup>-</sup> in the callus were elevated as the salt concentration increased, while K<sup>+</sup>, Ca<sup>2+</sup> and Mg<sup>2+</sup> levels were markedly reduced. The higher  $\Psi_s$  reduction and Na<sup>+</sup> accumulation found in the S100 callus corresponded with a higher callus dehydration level during salinity. Calli grown on Miller's basal medium were supplied with 100 mM NaCl for 12 days and then transferred to medium without NaCl to relieve salinity stress. The Na<sup>+</sup> and Cl<sup>-</sup> content decreased in both R100 and S100 cell lines during the first 24 h and reached normal levels four days after transfer to the normal medium. This lower concentration was maintained until the end of the experiment. Concurrently, the K<sup>+</sup> content and K<sup>+</sup>/Na<sup>+</sup> ratio increased sharply and reached their highest levels within 24 h in both salt-sensitive and salt-tolerant cell lines. These data suggest that the inhibitory effects of salinization on growth and

accumulation of potentially toxic ions ( $\text{Na}^+$ ,  $\text{Cl}^-$ ) can be readily reversed when salinity is relieved.

## **6.1 Introduction**

Salinity is one of the most important environmental factors limiting plant growth. Salt stress can affect plant survival, biomass, plant height and plant form, where such changes in morphology affect the capacity of a plant to collect light, water and nutrients (LOCY *et al.* 1996). Salt stress is composed of two components: salt toxicity and water stress. Water stress is one of the first and most evident effects of salinity, and the determination of water relations is therefore critical for any study of plant resistance to salinity (PARDOSSI *et al.* 1998). Plants commonly react to these stresses by accumulation of solutes in cells, or osmotic adjustment, which results in improved environmental stress tolerance (GREENWAY & MUNNS 1980). The maintenance of turgor by osmotic adjustment is an important physiological adaptation for minimizing the detrimental effects of salt stress (MUNNS *et al.* 1983).

Plant species adjust to salt stress by lowering tissue osmotic potential with the accumulation of both inorganic and organic solutes (ALARCÓN *et al.* 1993). Cations [sodium ( $\text{Na}^+$ ) and potassium ( $\text{K}^+$ )] and anions [chloride ( $\text{Cl}^-$ )] are known to be major inorganic components of the osmotic potential (YAO 1983). Potassium is essential to all plant life. In most terrestrial plants,  $\text{K}^+$  is the major cationic inorganic nutrient. In contrast,  $\text{Na}^+$  is only essential for a number of  $\text{C}_4$  species where it functions as a micronutrient (MAATHUIS & AMTMANN 1999). In most other species  $\text{Na}^+$  does not act

as a nutrient in the sense that it is strictly required for growth, but its addition to the growth medium may promote growth of many plants when the  $K^+$  supply is limited (FLOWERS & LÄUCHLI 1983). That calcium plays an important role in the response of plants to salinity is well documented. The reduction in cell growth when  $Ca^{2+}$  of the cell membrane was replaced by  $Na^+$  is attributed to  $K^+$  leakage from the cell. The imbalance of ions may cause a loss in cell turgor pressure and lead to growth inhibition (LIU *et al.* 2000a).

Little is known about how these cations contribute to osmotic adjustment in soybean, or how the cations change with salinity levels. The use of *in vitro* cultures, such as callus or cell suspensions, offers a means to focus exclusively on those physiological and biochemical processes inherent to the cell which contribute to salinity tolerance (RUS *et al.* 1999). Mechanisms of salt tolerance have been examined in several crop species, but there is relatively little information on plant cell responses when salinity is relieved (ALARCÓN *et al.* 1993). In previous experiments a salt-tolerant soybean cell line was isolated and characterized. Under salt stress, differences were found in the accumulation patterns of  $Na^+$  and  $K^+$  by soybean callus cell lines differing in salt tolerance (LIU & VAN STADEN 2000). The salt-tolerant (R100) cell line maintained a high  $K^+$  content and higher  $K^+/Na^+$  ratio compared to the salt-sensitive (S100) cell line after 12 days incubation on media with 100 mM NaCl. This led to faster growth of R100 callus.

In the present study the effect of the recovery of growth upon relief of salt stress was investigated to improve our understanding of the influence of NaCl on growth and



water relations of soybean cell lines differing in salinity tolerance. The inorganic solutes in callus cultures of soybean exposed to various levels of NaCl were also investigated. Specifically, our objectives were to : determine effects of NaCl salinity on growth;  $K^+$ ,  $Na^+$ ,  $Cl^-$ ,  $Ca^{2+}$  and  $Mg^{2+}$  accumulation; the recovery of growth; and ion accumulation after relief of calli which differ in salt tolerance.

## **6. 2 Materials and methods**

### *6.2.1 Plant material, and growth conditions*

The *Glycine max* (L.) salt-tolerant (R100) and the salt-sensitive cell lines (S100) were maintained as reported earlier (Chapter 5). All cultures were kept on Miller's basal (MB)medium (Appendix 2) (MILLER 1965) supplemented with 2 mg l<sup>-1</sup> 2,4-D and 0.5 mg l<sup>-1</sup> kinetin. The cultures were subcultured every 28 days and kept at 26 °C in continuous low light (0.13  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ).

### *6.2.2 Salinity and relief treatments*

For the salinity treatment, actively-growing fresh calli were exposed to increasing concentrations of NaCl (25, 50, 75, 100, and 125 mM) in the MB media. The relief experiments consisted of exposing calli to 12 days of salinity stress and relief. Salinity treatments were conducted by adding 100 mM NaCl to the MB media for 12 days (LIU & VAN STADEN 2000). At the end of the salinity treatments, the calli were washed three times with deionized water, before the relief period was started. During relief, all

calli received MB medium only. The relief period lasted for 16 days.

### 6.2.3 *Measurement of growth*

The growth rate of the callus was measured in terms of both FW and DW. For the salt-stress treatment, a known weight of callus was inoculated into a 250 ml culture flask containing MB medium with or without different concentrations of NaCl, using 15 replicates. Subsequently at 7 day intervals, callus from three flasks was removed and FW determined. For the relief treatment, a known weight of callus was inoculated into a 250 ml flask containing MB medium with or without 100 mM NaCl, using 27 replicates. After 12 days, callus from 15 flasks was subcultured to new flasks containing MB medium without NaCl. Subsequently at 4 day intervals, callus from 3 flasks was removed and its FW determined. The calli were harvested and then air-dried for elemental analysis. Samples were oven-dried at 80°C for 48 h prior to determination of DW. This procedure was repeated up to 28 days after inoculation.

### 6.2.4 *Relative growth rate (RGR) and relative water content (RWC) measurements*

Relative growth rate was calculated from the increase in FW of callus measured at the beginning and at the end of the stress or relief period at 4 day intervals. The relative water content of calli was calculated as follows:

$$RWC (\%) = [(FW-DW)/FW] \times 100$$
, where FW is the fresh weight of callus at the time of harvest, DW was the dry weigh of the callus.

### *6.2.5 Callus sampling and elemental analyses*

Details of preparation and X-ray microanalysis have been described previously in Chapter 5. Specimens were prepared for Scanning Electron Microscopy (SEM) by air drying overnight and carbon coating to prevent charging as a clear picture was needed to enable selection of specific points. Samples were analysed by an energy-dispersive SiLi detector (Nuclear Semiconductors) combined with a Link exl II EDX system, attached to a Hitachi S-570 SEM operating at an accelerating voltage of 15 Kv. Specimens in the SEM were tilted to an angle of 15° toward the X-ray detector, and the working distance in the microscope was set at 15 mm to obtain optimum results. X-ray counts based on the peak-to-background ratios (P/B) of a specimen were compared with the standard. X-ray spectra were recorded with an electron beam of 15 Kv and spectra were collected at 1000 X magnification within 100 sec live time in a scanning mode over the area of callus for at least 8 locations each. The X-ray signal intensity was quantified by counts (Y-axis label) and was indicative of the relative concentration of the elements in the samples.

## **6.3 Results**

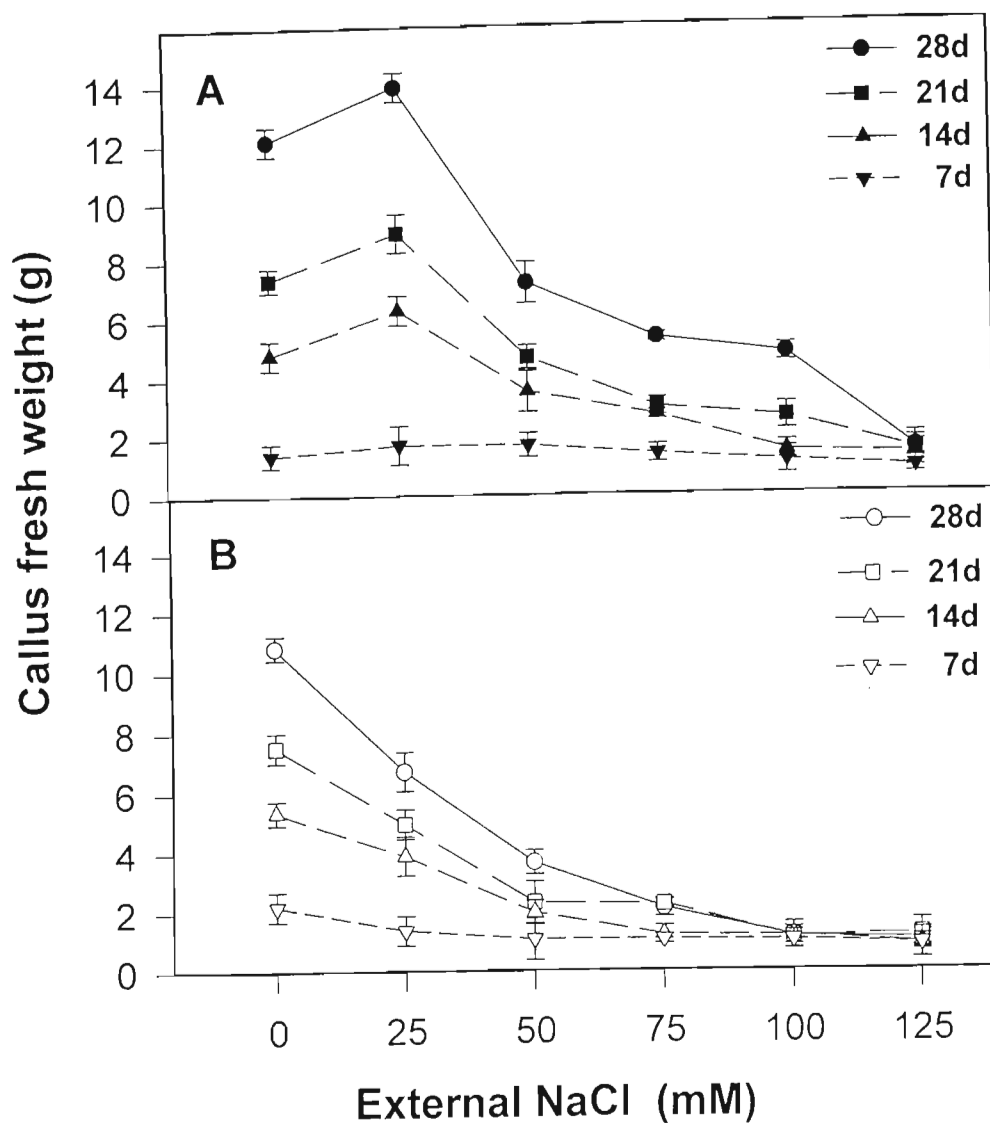
### *6.3.1 Effect of NaCl on callus growth*

Salinity markedly affected plant growth and dry matter partitioning. Sodium chloride exposure caused the callus growth (FW and DW) to significantly decrease at levels above 75 mM for the R100 cell line and above 25 mM for the S100 cell line (Figures 6.1

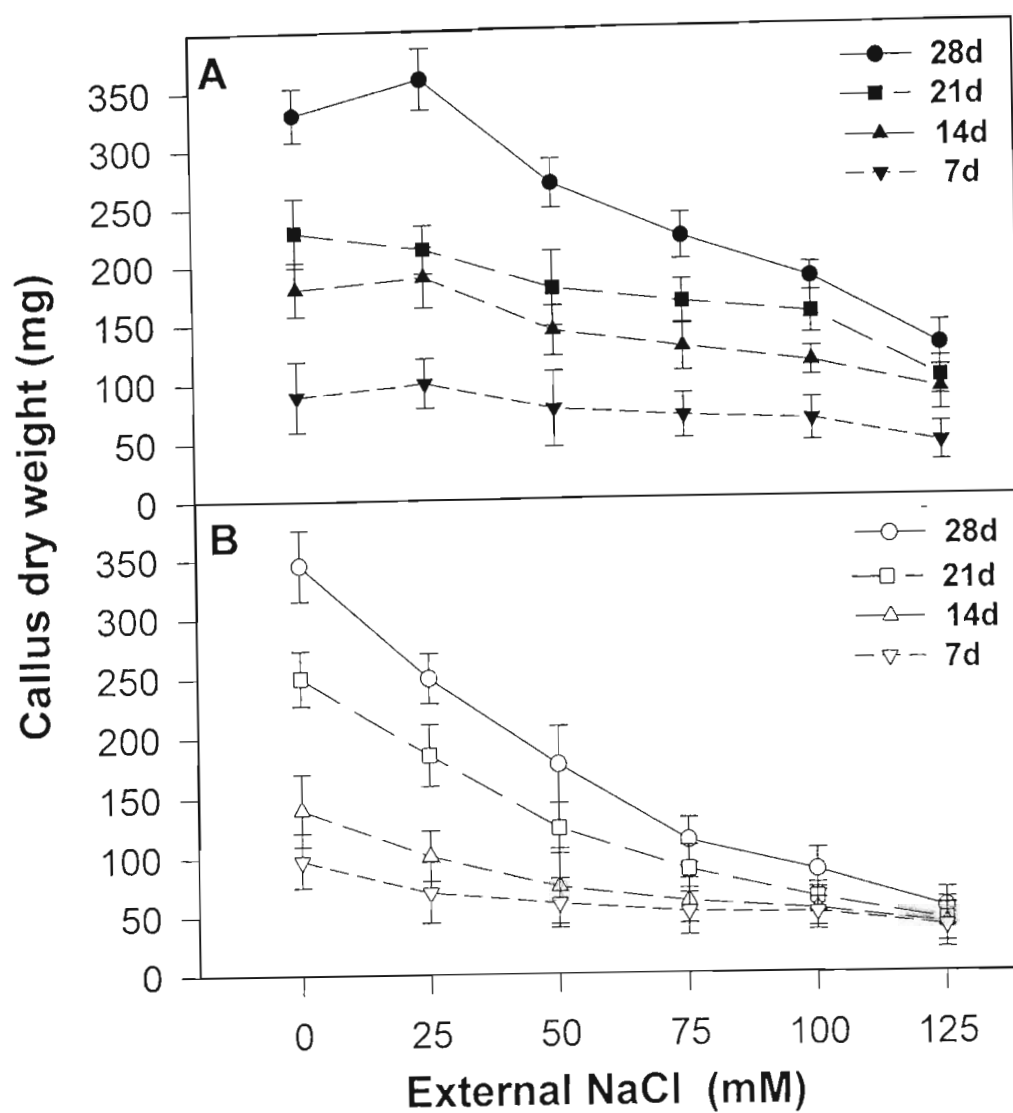
and 6.2). Salt stress reduced the FW of salt-sensitive callus (S100) by 80 % (Figure 6.1 B) and that of salt-tolerant callus (R100) by only 40% (Figure 6.1 A) respectively. The DW and FW in S100 and R100 calli under salt-stress showed a similar pattern (Figure 6.1 and 6.2). Growth resumed once salt stress was relieved. Salinized calli showed the same RGR in both cell lines during relief (Figure 6.4). By the end of the experiment (28 days), S100 callus recovered growth to nearly 60% of the control (without NaCl stress) or 450% of the 100 mM NaCl treatment (Figure 6.3 A, C) respectively. In the R100 callus line, the relief treatment was not as significant as for the S100. R100 callus growth was about 80% of the control (without NaCl stress) or 135% of the 100 mM NaCl treatment in fresh weight (Figure 6.3 B, D).

#### *6.3.2 Effect of NaCl on callus RWC*

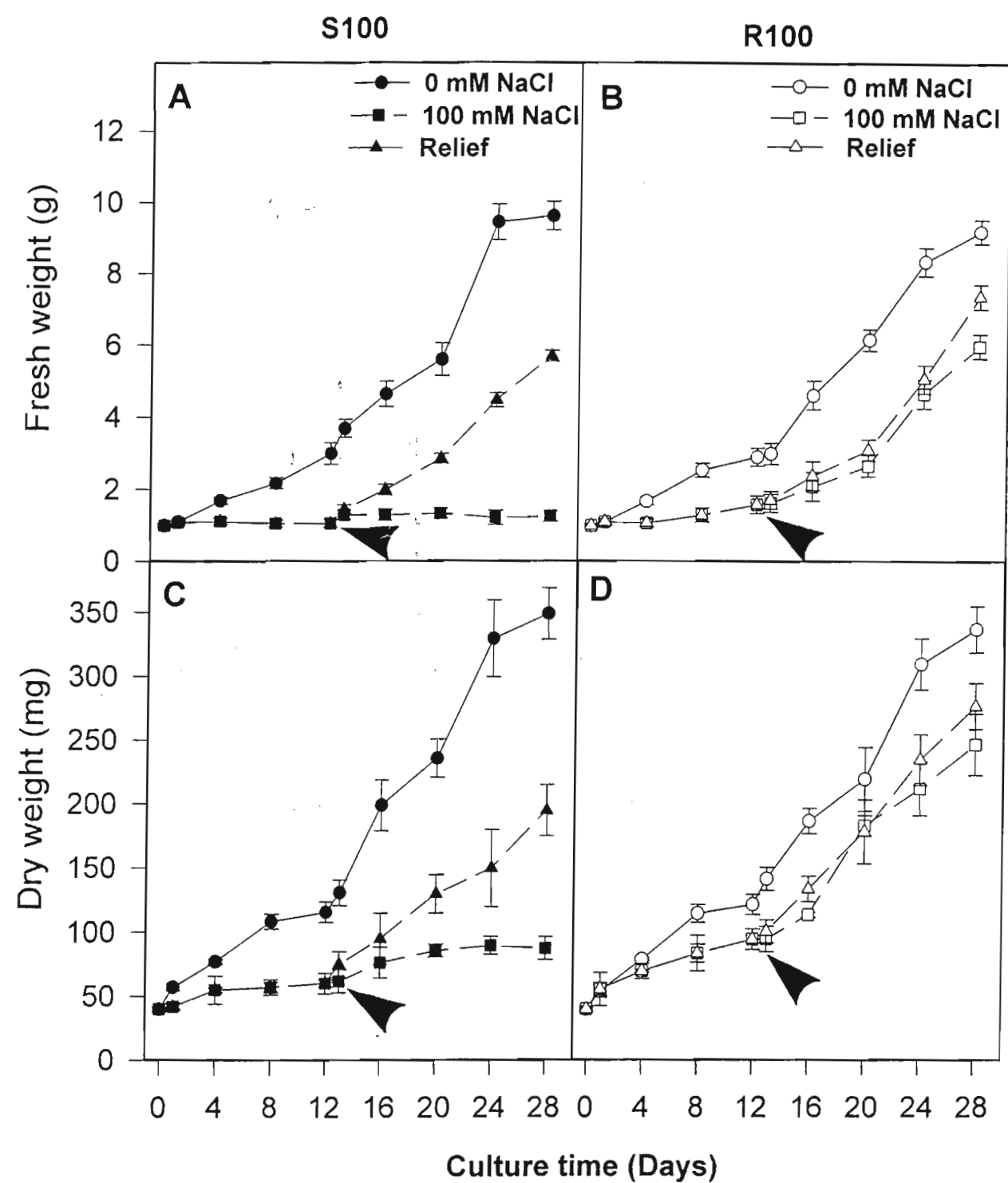
The effect of salinity on RWC varied with the salt concentration to which calli were exposed. A concentration of 125 mM NaCl caused a significant decrease in RWC during salinity treatment, but no differences emerged between RWC of control and 25 mM salt-treated calli in the two cell lines (Figure 6.5 A, B). Considering the changes throughout the growth cycle, the relative water contents of both cell lines tended to increase during the early period (days 1 to 14) in the control, whereas the opposite tendency was observed in the saline media (Figure 6.6 A, B). However, the salt-treated callus of R100 seemed to be able to increase its RWC during the second period (days 15 to 28). The salt-treated S100 callus seemed to be unable to increase RWC over the next two weeks (Figure 6.6 A, B). The different responses between cell lines seem to represent the RWC in the two time periods. Thus, S100 maintained the same tendency



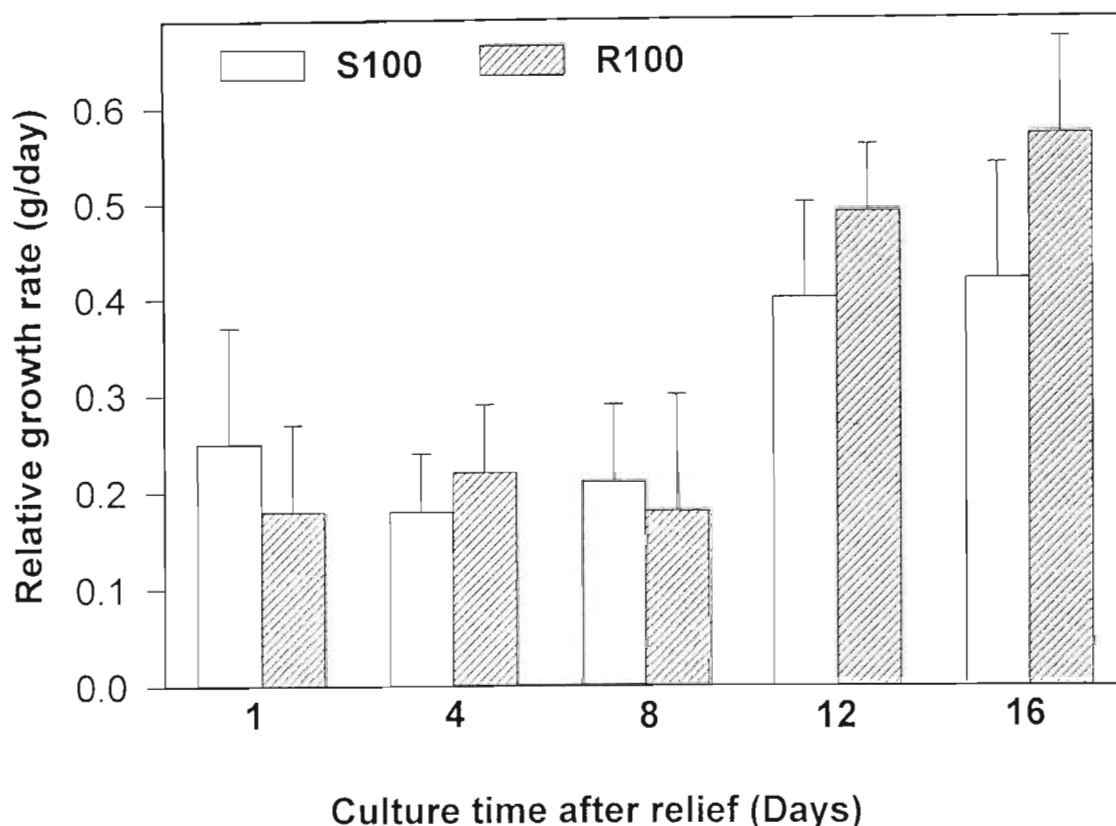
**Figure 6.1: Fresh weight changes over 28 days of exposure to various concentrations of NaCl in MB media.** Growth of *Glycine max* R100 (A) and S100 (B) callus cultures without the addition of NaCl to MB medium, and NaCl addition to MB media (between 25 and 125 mM). Each value represents the mean  $\pm$  SE of three different samples.



**Figure 6.2: Dry weight changes over 28 days of exposure to various concentrations of NaCl in MB media.** Growth of *Glycine max* R100 (A) and S100 (B) callus cultures without the addition of NaCl to MB medium, and NaCl addition to MB media (between 25 and 125 mM). Each value represents the mean  $\pm$  SE of three different samples.



**Figure 6.3: Effect of NaCl stress on the FW (A, B) and DW (C, D) of S100 and R100 callus cultures during 100 mM NaCl stress and its subsequent relief.** After 12 days of culture on the MB media with 100 mM NaCl, both the S100 and R100 callus were transferred to fresh MB media. Arrows indicate the end of stress treatment. Each value represents the mean  $\pm$  SE of three different samples.



**Figure 6.4: Relative growth rate of callus cultures of S100 and R100 cell lines after 12 days salt stress and subsequent relief from salt stress.** Each value represents the mean  $\pm$  SE of three different samples.

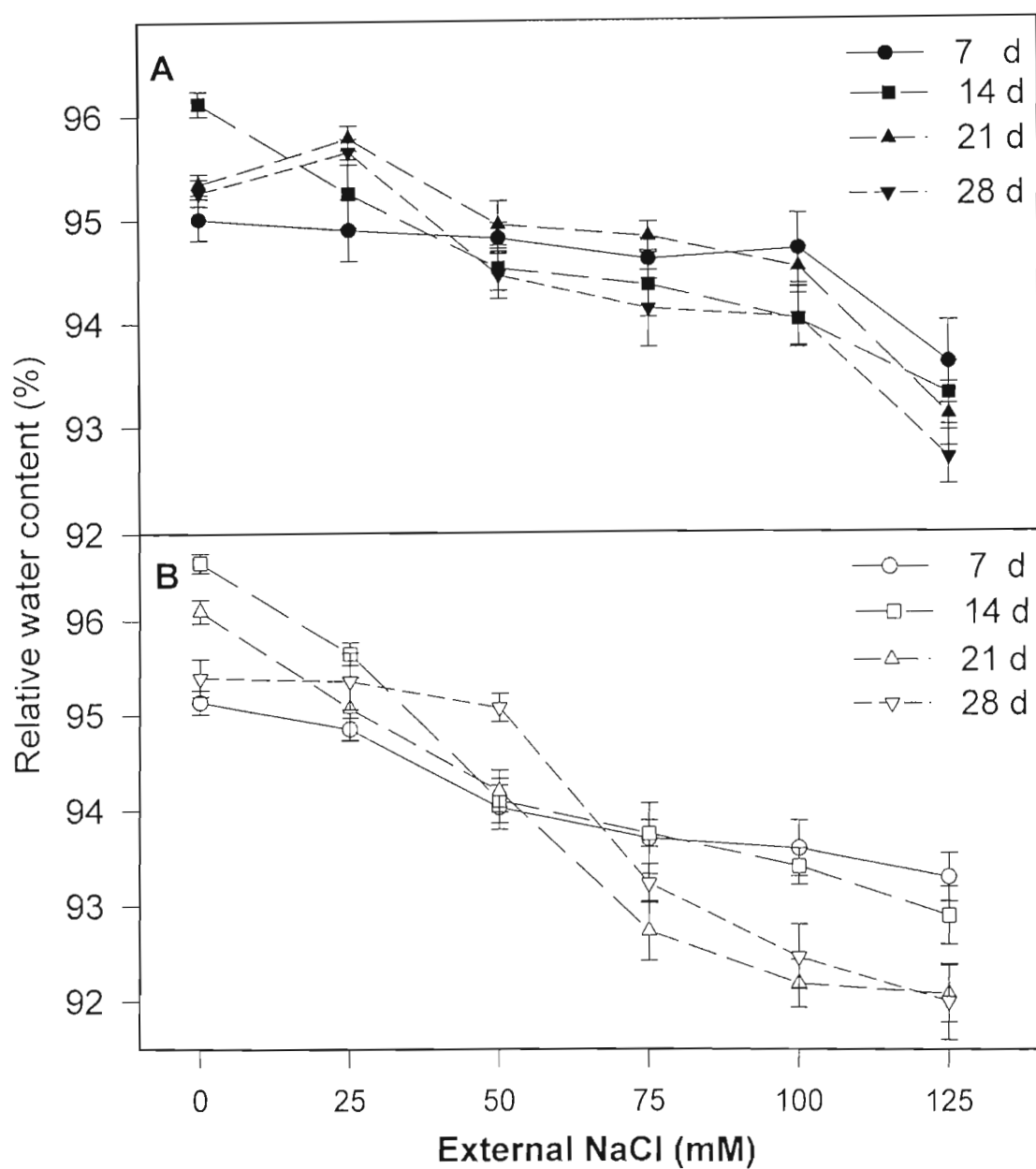
in the two periods, with the water content decreasing as the stress level increased. However, the RWC in the R100 callus was much higher for the second phase than was the case for the S100 callus. It only decreased significantly at 125 mM NaCl (Figure 6.5 A). Salt stress reduced RWC of both callus types on all dates measured (Figure 6.5 A, B). RWC was 1.5 % lower in S100 than in R100 callus by the end of the treatment. The highest RWC was observed in control callus in both cell lines (Figure 6.5 A, B). It decreased under salt-stress. With the 100 mM NaCl treatment, the RWC in S100 callus



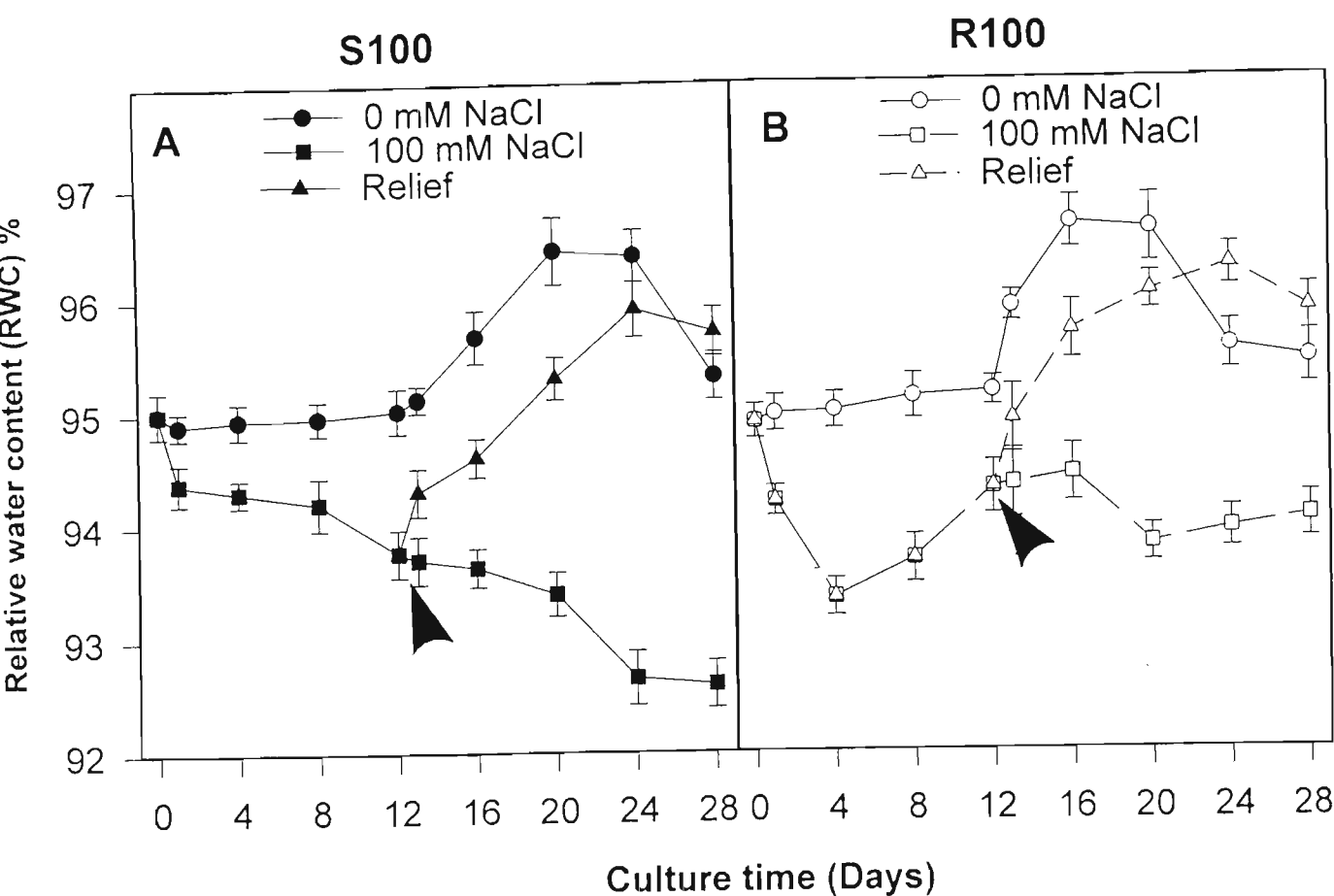
**Table 6.1: FW/DW ratio in callus cultures during the 28 days of exposure to various concentrations of salt in MB media.** R100 (A) and S100 (B) callus culture without NaCl addition to the medium (0 mM NaCl), and NaCl addition to the media (between 25 mM and 125 mM). Each value represents the mean  $\pm$  SE of three different samples.

Callus	NaCl	Fresh Weight/Dry Weight			
Line	(mM)	Culture Time (days)			
		7	14	21	28
A	0	20.8 $\pm$ 1.8	19.4 $\pm$ 1.6	34 $\pm$ 2.4	37.5 $\pm$ 3.2
	25	19.19 $\pm$ 1.3	32.1 $\pm$ 2.4	38.26 $\pm$ 3.3	38.75 $\pm$ 3.7
	50	17.4 $\pm$ 1.8	27.3 $\pm$ 2.1	26.3 $\pm$ 2.0	28.1 $\pm$ 3.1
	75	16.7 $\pm$ 1.9	23.0 $\pm$ 2.9	20.58 $\pm$ 2.8	22.08 $\pm$ 2.7
	100	16.3 $\pm$ 1.2	14.28 $\pm$ 2.1	20.01 $\pm$ 1.7	20.72 $\pm$ 2.6
	125	11.2 $\pm$ 1.6	13.7 $\pm$ 1.8	14.2 $\pm$ 1.9	14.66 $\pm$ 2.1
B	0	24.2 $\pm$ 2.3	37.85 $\pm$ 3.4	30.1 $\pm$ 2.5	36.67 $\pm$ 3.1
	25	23.30 $\pm$ 2.4	28.5 $\pm$ 2.6	27.3 $\pm$ 2.2	26.92 $\pm$ 2.5
	50	16.3 $\pm$ 1.7	20.9 $\pm$ 2.1	23.07 $\pm$ 2.8	22.2 $\pm$ 2.4
	75	15.1 $\pm$ 1.3	18.6 $\pm$ 2.1	17.9 $\pm$ 2.1	18.57 $\pm$ 1.9
	100	12.2 $\pm$ 1.4	10.3 $\pm$ 1.6	15.4 $\pm$ 1.4	12.15 $\pm$ 1.6
	125	10.4 $\pm$ 2.1	10.01 $\pm$ 1.7	10.3 $\pm$ 1.2	10.07 $\pm$ 1.5

gradually decreased over time (Figure 6.6 A). For the R100 cell line, the RWC gradually decreased over the first 4 days and recovered by day 12, with faster growth (Figure 6.6 B). Recovery of RWC in 100 mM salt-stressed calli, following salt relief, was accompanied by the same pattern. The RWC gradually increased with the faster



**Figure 6.5: Relative water content (RWC) changes over 28 days of exposure to various concentrations of NaCl in MB media.** Growth of *Glycine max* R100 (A) and S100 (B) callus without the addition of NaCl to MB medium, and NaCl addition to media (between 25 mM and 125 mM). Each value represents the mean  $\pm$  SE of three different samples.

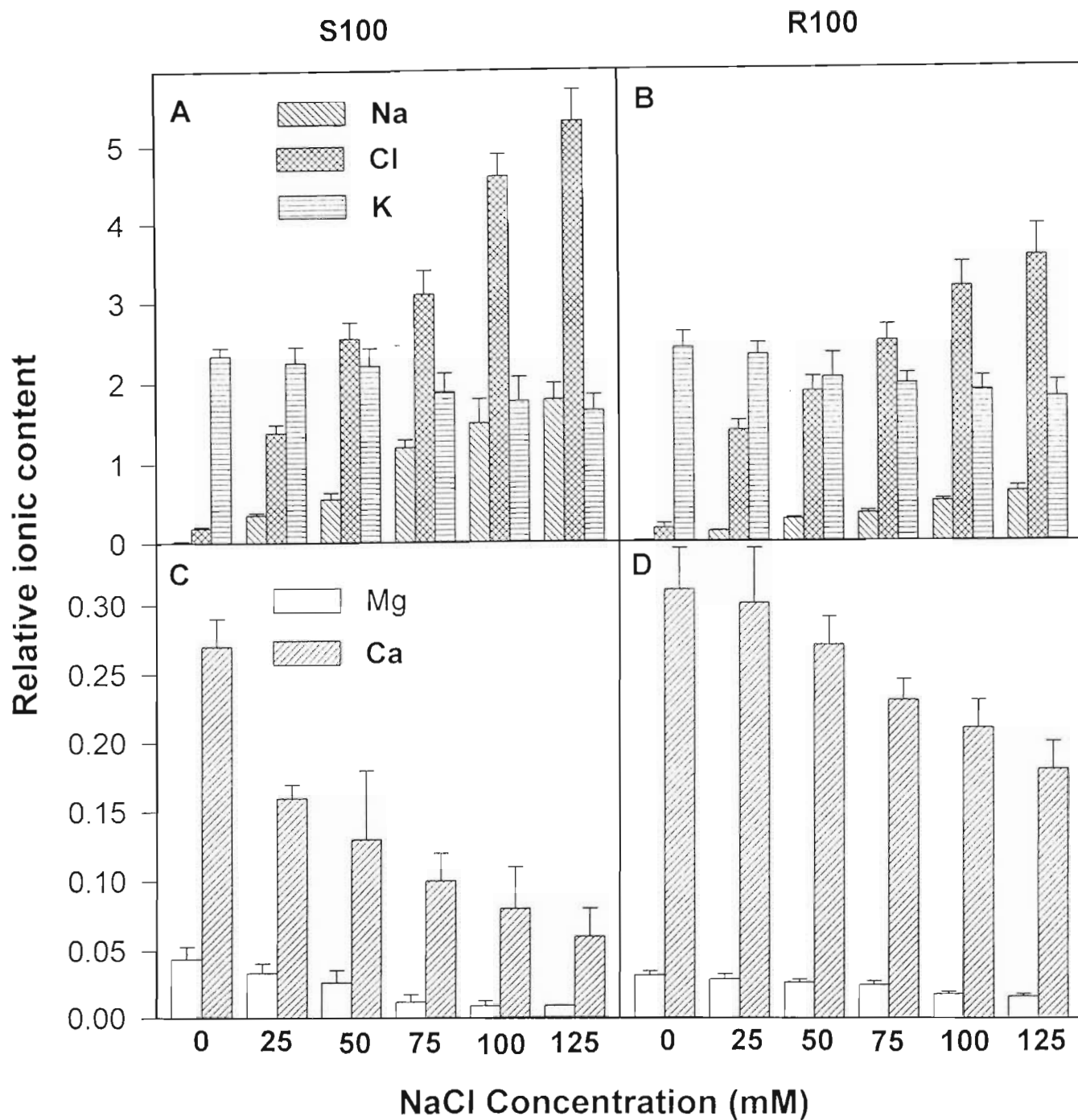


**Figure 6.6: Relative water content (RWC) changes over 28 days of exposure to 100 mM NaCl or exposure to 100 mM NaCl for 12 days and subsequently removed from salt stress. S100 (A) and R100 (B) callus culture. Arrows indicate the end of salt stress treatment. Each value represents the mean  $\pm$  SE of three different samples.**

growth in the next 12 days and then slightly decreased at the end of the experiment (Figure 6.6 A, B).

### 6.3.3 Effect of NaCl on inorganic solute accumulation

Saline treatments increased  $\text{Na}^+$  and  $\text{Cl}^-$  concentrations in both cell lines. Sodium and chloride content measured during salt stress increased linearly with an increase in the concentration of the external NaCl solution. The tolerant cell line accumulated less



**Figure 6.7: Sodium, K, Ca, Mg and Cl content changes over 28 days of exposure to various concentrations of NaCl in MB media.** Growth of S100 (A, C) and R100 (B, D) callus cultures without the addition of NaCl to MB medium and NaCl addition to media (between 25 mM and 125 mM). Each value represents the mean  $\pm$  SE of three different samples.

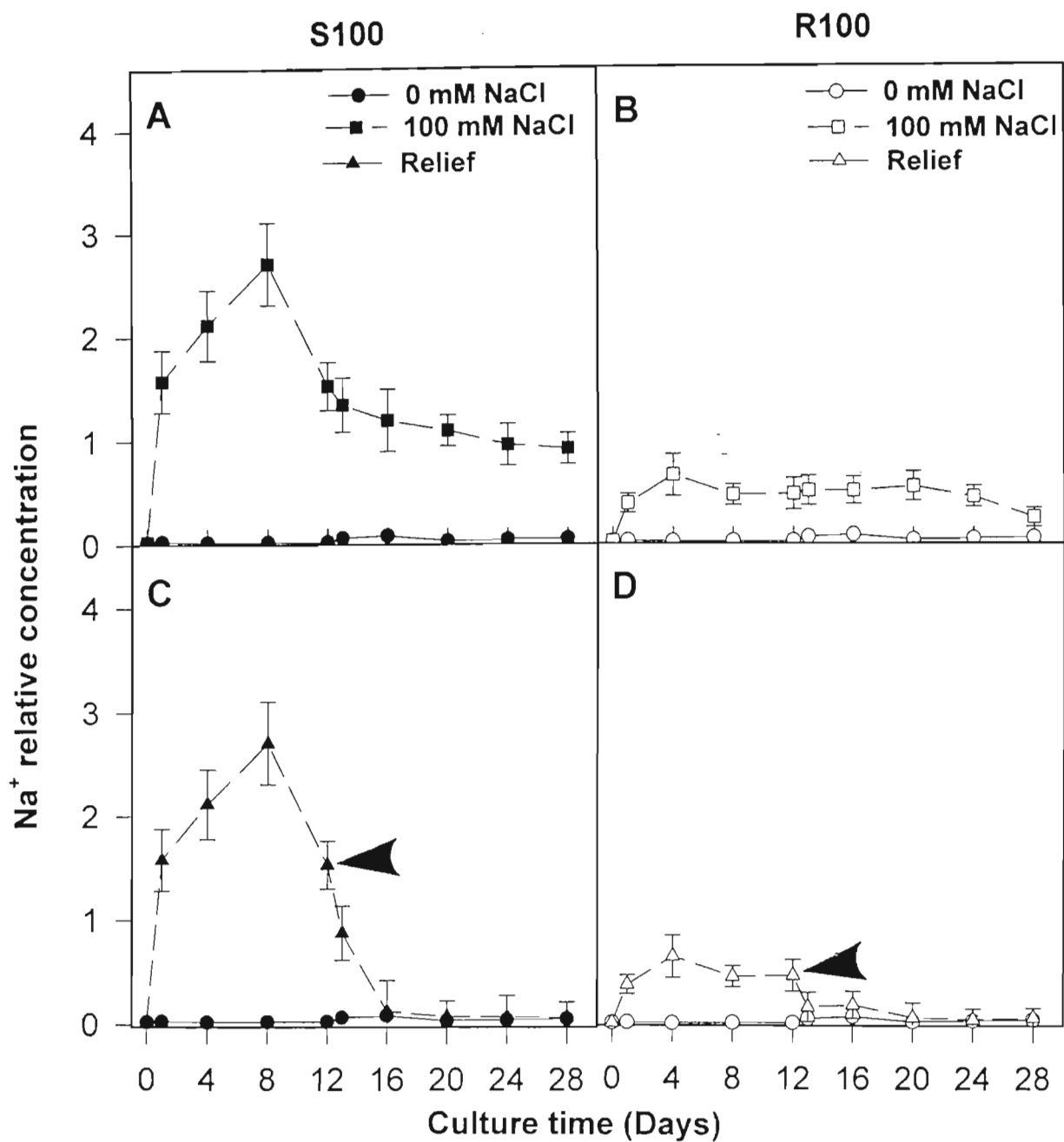
**Table 6.2: Relative K<sup>+</sup>/Na<sup>+</sup> ratio changes during 28 days of exposure to various concentrations of NaCl in MB media.** Each value represents the mean ± SE of three different samples.

Callus line	NaCl Concentration (mM)					
	0	25	50	75	100	125
R100	51	9.5	6.2	4.1	3.7	2.4
S100	49.5	6.05	3.5	1.8	1.5	1.03

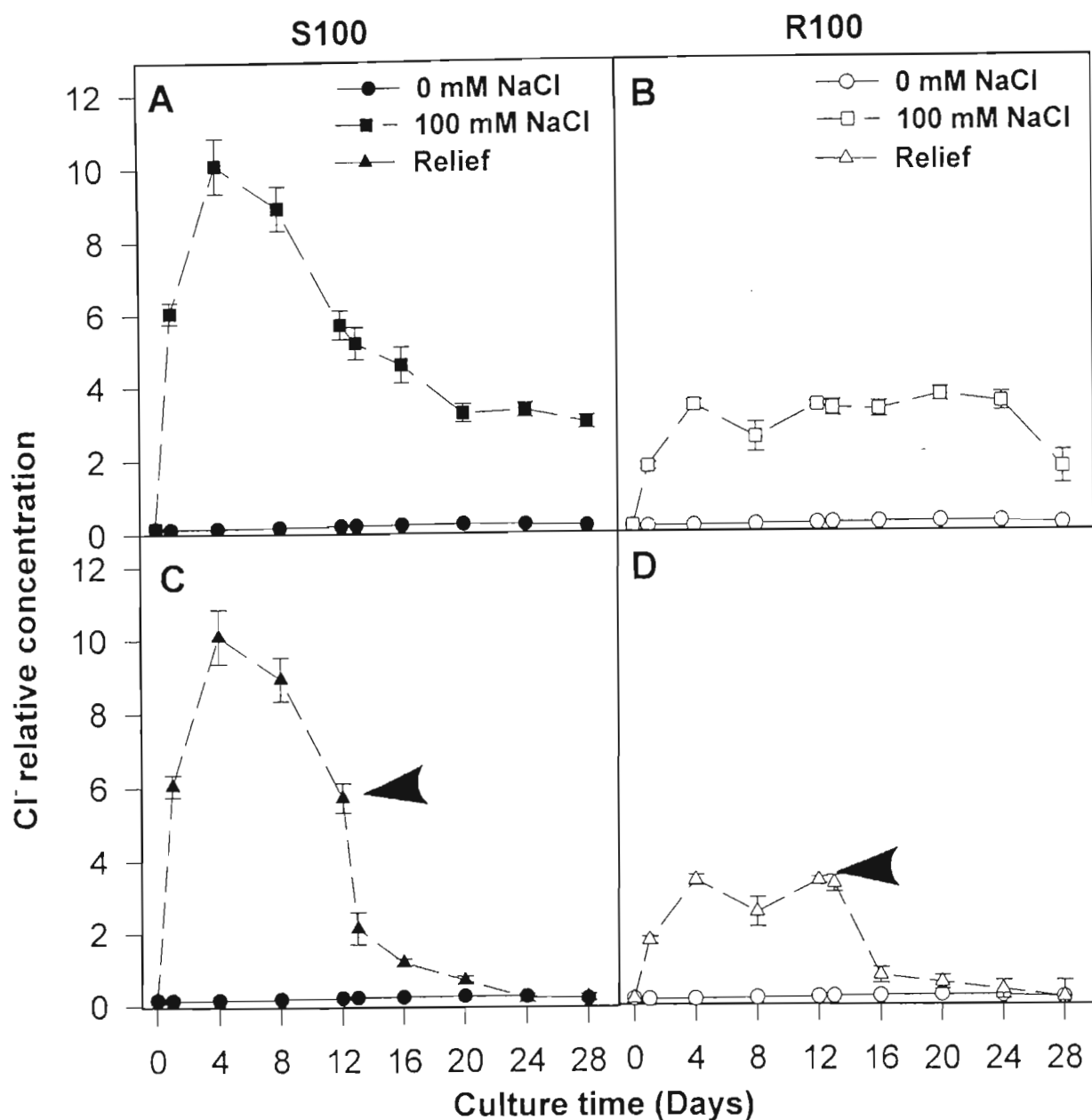
Na<sup>+</sup> and Cl<sup>-</sup> than S100 (Figure 6.7 A, B). However, S100 at elevated levels of NaCl had a significantly lower concentration of K<sup>+</sup>. Calcium and Mg<sup>2+</sup> contents followed almost the same pattern as K<sup>+</sup> in both of the callus lines (Figure 6.7 C, D). Sodium and Cl<sup>-</sup> levels were significantly elevated during the 28 days of salt exposure. As the NaCl concentration increased the levels of Na<sup>+</sup> and Cl<sup>-</sup> also increased further in both cell lines. During the same period K<sup>+</sup>, Ca<sup>2+</sup> and Mg<sup>2+</sup> concentrations in calli declined at NaCl concentrations higher than 25 mM (Figure 6.7). The K<sup>+</sup>, Ca<sup>2+</sup> and Mg<sup>2+</sup> concentrations in 100 and 125 mM salt-treated calli were significantly lower than those of the controls. The K<sup>+</sup>/Na<sup>+</sup> selectivity ratio decreased with an increasing concentration of NaCl in the external media. This decrease was much greater for the S100 than for the R100 cell line (Table 6.2).

*6.3.4 Ionic accumulations in calli during relief from salt stress*

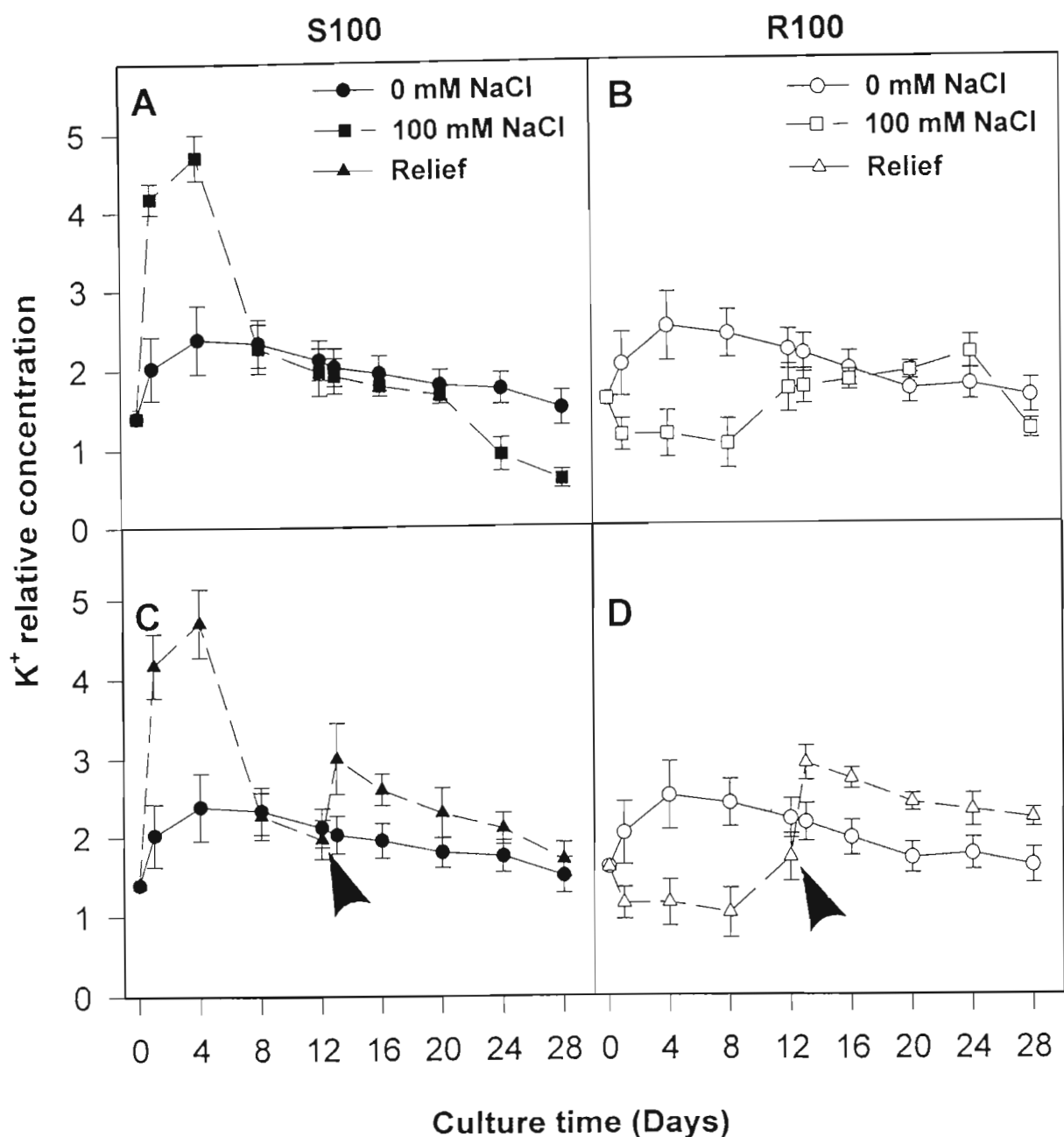
In the previous experiments in Chapter 5, the S100 and R100 callus lines showed an increased Na<sup>+</sup> content with 100 mM levels of NaCl in the medium. The S100 callus



**Figure 6.8: Sodium ion accumulation over 12 days of exposure to 100 mM NaCl and 16 days of relief.** Growth of S100 (A, C) and R100 (B, D) calli without the addition of NaCl to MB medium, and NaCl (100 mM) addition to MB medium. Growth of S100 (C) and R100 (D) calli with 100 mM NaCl addition to MB medium for 12 days and subsequent transfer to MB medium without NaCl for 16 days. The arrows indicate the end of the salt stress. Each value represents the mean  $\pm$  SE of three different samples.



**Figure 6.9: Chloride ion accumulation over 12 days of exposure to 100 mM NaCl and 16 days of relief.** Growth of S100 (A, C) and R100 (B, D) calli without the addition of NaCl to MB medium, and NaCl (100 mM) addition to MB medium. Growth of S100 (C) and R100 (D) calli with 100 mM NaCl addition to MB medium for 12 days and subsequent transfer to MB medium without NaCl for 16 days. The arrows indicate the end of the salt stress. Each value represents the mean  $\pm$  SE of three different samples.



**Figure 6.10: Potassium ion changes over 12 days of exposure to 100 mM NaCl and 16 days of relief.** Growth of S100 (A, C) and R100 (B, D) calli without the addition of NaCl to MB medium, and NaCl (100 mM) addition to MB medium. Growth of S100 (C) and R100 (D) calli with 100 mM NaCl addition to MB medium for 12 days and subsequent transfer to MB medium without NaCl for 16 days. The arrows indicate the end of the salt stress. Each value represents the mean  $\pm$  SE of three different samples.



**Table 6.3: The effect of NaCl on relative K<sup>+</sup>/Na<sup>+</sup> ratio changes during 12 days salt stress and 16 days relief from salt stress.** Growth of R100 and S100 callus with 100 mM NaCl addition to the MB medium for 12 days and subsequent transfer to MB medium without the addition of NaCl for 16 days. Values are the average of three replications ± SE.

Callus		Culture time (Days)									
line		Salt Stress				Relief					
		0	4	8	12	1	4	8	12	16	
R100		52±3.2	2.92±0.3	1.74±0.2	2.17±0.16	39±7.0	43±4.4	47±3.6	49±3.9	46±4.5	
S100		51±4.2	2.64±0.3	2.72±0.3	0.83±0.15	36±6.4	42±5.3	44±6.4	45.6±3.2	47±5.5	

contained high levels of Na<sup>+</sup> when grown under 100 mM NaCl stress, The Na<sup>+</sup> reached its highest level at day 8 and then declined. The R100 callus maintained lower levels of Na<sup>+</sup> than the S100 callus grown on 100 mM NaCl medium during the experiment (Figure 6.8 A, B). A drastic decrease in Na<sup>+</sup> content occurred within the first 24 h of both sensitive and tolerant calli but reached the normal level again at day 4 after stress relief. The Na<sup>+</sup> level remained normal until the end of the experiment (Figure 6.8 C, D).

S100 and R100 calli subjected to NaCl, accumulated higher Cl<sup>-</sup> levels than those maintained in normal medium (Figure 6.9 A, B). The Cl<sup>-</sup> accumulated to a high level in the S100 line subjected to 100 mM NaCl within 24 h of culture and reached its maximum at day 4. There was a decrease after 4 days, but the Cl<sup>-</sup> levels were always higher than in the unstressed control (Figure 6.9 A) and stressed R100 (Figure 6.9 B). The Cl<sup>-</sup> content dramatically decreased in both S100 and R100 cell lines over the first

24 h and reached the normal level 12 days after stress relief (Figure 6.9 C, D).

The R100 callus line maintained lower levels of  $K^+$  when grown under 100 mM NaCl stress than under normal conditions (Figure 6.10 B). When S100 and R100 callus lines were exposed to 100 mM NaCl concentrations,  $K^+$  levels in the S100 callus line increased to its highest level by day 4 and then declined steadily (Figure 6.10 A). However, the R100 callus line showed a different trend.  $K^+$  levels declined in the first 4 days and then increased (Figure 6.10 B). The  $K^+$  content and  $K^+/Na^+$  selectivity ratio sharply increased in both salt-tolerant and salt-sensitive calli and reached their highest levels within 24 h after relief (Figure 6.10 C, D and Table 6.3). However, the  $K^+$  content in R100 callus was higher than that in S100 callus. The content of  $K^+$  in both calli decreased gradually, but was still higher than in the control until the end of the experiment (Figure 6.10). The difference in  $K^+/Na^+$  selectivity ratios between salt stress and relief indicated a release of  $Na^+$  from the calli and the maintenance of selectivity for  $K^+$  during relief.

#### **6. 4 Discussion**

Plant cells growing in saline media must adjust osmotically, since a positive turgor is required for cell expansion and most biochemical, physiological, and developmental processes (FLOWERS *et al.* 1977; GREENWAY & MUNNS 1980). Plant cells decrease their osmotic potential by the accumulation of inorganic and organic solutes or by loss of water. In this experiment, the loss of turgor, indicated by a considerable decrease in relative water content (RWC), occurred only after increases in both FW and

DW were inhibited in salt sensitive callus after 14 days of salt stress. However, in the resistant cell line, the continuation of growth during salt stress, despite a slight decrease in water potential, is likely due to osmotic adjustment. The water relations data suggest that the salt-tolerant callus achieved osmotic adjustment by lowering the osmotic potential in response to the externally imposed stress at 100 mM NaCl. Similar effects were demonstrated in other crop plant, which enabled the plants to maintain turgor and water content (KAMEILI & LÖSEL 1993; KAMELI & LÖSEL 1995).

Growth response to salinity is often regarded as a basis of evaluation for tolerance (KUIPER *et al.* 1988). Callus, the least organized tissue, is also the most sensitive to salinity. In the present experiments, the known salt tolerant cell line, possessing inherently the highest growth rate, showed stimulation of growth at moderate salinity. A similar trend was shown by wheat salt tolerant varieties (RUS *et al.* 1999). In contrast, the salt-sensitive callus did not respond with growth stimulation at low salinity. The high FW/DW ratios (Table 6.1) in R100 indicated a relatively undisturbed water uptake, especially at high salinity, while in the rest of the callus osmotic adjustment seemed to be impaired. The range of NaCl concentrations causing 50% inhibition of growth can be estimated to vary between 75mM in R100 and 50 mM in the S100 cell line.

The RWC increase in stressed callus, immediately after relief, was due to the regaining of turgidity. Stressed callus still had lower growth rate increases than did control callus with respect to FW and DW after transfer to fresh medium. This indicates a continuing effect of NaCl on growth. Although turgor is regained slowly after relief,

it continues to limit growth. This indicates that growth can return to normal after exposure to salt stress for a short period, which suggested that processes necessary for growth were not affected. Similar conclusions have been made by other workers for re-watered maize (KAMELI & LÖSEL 1996) and tomato (GATES 1955) plants.

The general trend of solute accumulation in the present study clearly demonstrates that a NaCl-tolerant soybean cell line is able to adjust osmotically to lower the callus  $\psi$ s in response to NaCl treatment. This is apparently accomplished by the accumulation of both organic and inorganic solutes (Chapter 5; LIU & VAN STADEN 2000). However, the pattern of changes in response to salt stress is strikingly different between salt-sensitive and salt-tolerant cell lines. The high concentrations of NaCl in the tissue culture medium cause a significant increase in  $\text{Na}^+$  and  $\text{Cl}^-$  in *Glycine max* callus during exposure to NaCl, while at the same time a large efflux of  $\text{K}^+$ ,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  was evident. Accumulation of  $\text{Na}^+$  and  $\text{Cl}^-$  ions was suggested to be one of the primary causes of growth inhibition in salt-stressed plants and threshold levels of these potentially toxic ions have been calculated for various crop species (THERIOS & MISOPOLINOS 1988; WRIGHT *et al.* 1993; TATTINI 1994; TATTINI *et al.* 1995). Most studies concluded that inhibition of growth is caused by the accumulation of  $\text{Cl}^-$ . A large and permanent efflux of  $\text{K}^+$  and  $\text{Ca}^{2+}$  usually indicates damage to the membranes (KENNEDY & De FILIPPIS 1999). Under saline conditions, a constant or increased level of internal  $\text{K}^+$  is of basic importance (GALIBA & ERDEI 1986; TRIVEDI *et al.* 1991). In the present experiments, the capability of  $\text{K}^+$  maintenance and accumulation was better expressed in R100 compared with the S100 cell line. This is in agreement with earlier observations for a *Grevillea* tolerant cell line (KENNEDY & De FILIPPIS

1999).  $K^+$  efflux has been used as an indicator of cellular toxicity for a range of toxic compounds, and indeed losses of  $K^+$  and  $Ca^{2+}$  have been documented during salinity stress (GRAIFENBERG *et al.* 1995; KENNEDY & De FILIPPIS 1999). Interestingly, the relatively high salt tolerance of R100 callus as compared with S100 callus was associated with significantly high  $K^+/Na^+$  values, suggesting that the better osmotic adjustment that characterizes the R100 callus operates at the cellular level. Both the salt-sensitive and salt-tolerant calli took up a large amount of  $K^+$  from fresh external media and maintained a higher  $K^+/Na^+$  ratio, when they were transferred to fresh MB media after 12 days exposure to 100 mM NaCl. This leads to faster growth in both salt-sensitive and salt-tolerant cell lines.

## **Chapter 7**

### **Partitioning of carbohydrates and accumulation of proline in salt-sensitive and salt-tolerant soybean callus cultures under salinity stress and its subsequent relief**

#### **Abstract**

Salt tolerant (R100) and sensitive (S100) cell lines of *Glycine max* (L.), that differ in their ability to accumulate sodium (Na) and chloride (Cl) under 100 mM salt stress, were used to compare the contribution of carbohydrates and proline in osmotic adjustment. Calli were exposed to 100 mM NaCl concentrations for 12 days followed by 16 days of relief from stress to determine the effect of salinity on sugar and proline content changes of the two cell lines. The salt-tolerant and the salt-sensitive cell lines differed in the time at which, and the type of sugar, that increased during salt stress. However, recovery in sugar and proline content parameters during relief from stress were similar in the two cell lines. Proline content, G6PDH, and 6PGDH activities in the NaCl tolerant cell line decreased immediately after its relief. This indicates that proline acts as a protective compound during the period of NaCl stress. The concentration of glucose and fructose increased at a rate closely corresponding to the increase in relative water content, while the concentration of sucrose decreased to the control level, coincident with relatively rapid growth.

## 7.1 Introduction

Salinity and drought stress reduce growth and agricultural productivity more than any other environmental factors. Both salinity and drought decrease the water potential of the external medium, and both can result in growth reduction. However, the physiological mechanisms that mediate the response in each case may be different (ERDEI *et al.* 1990). Biochemical studies revealed similarities in processes induced by stress that lead to the accumulation of metabolites (McKERSIE & LESHEN 1994). Carbohydrates, amino acids and organic acids are major organic components in several plant species under water stress, with relative contributions depending on environmental conditions (MORGAN 1992).

Salt stress affects many physiological and biochemical processes in plants, resulting in the alteration of some metabolic pathways. Amongst the major effects are those involving carbohydrate metabolism, with the accumulation of sugars and a number of other organic solutes (KAMELI & LÖSEL 1993, 1995, 1996). Carbohydrates are frequently associated with active osmotic adjustment (ZHANG & ARCHBOLD 1993; WANG *et al.* 1995), and have long been known to increase in a wide range of plants grown under salinity (EATON & ERGLE 1948; ACKERSON 1981). Carbohydrate diversion plays a key role in the adaptive processes linked with NaCl-tolerance, such as  $\text{Na}^+$  and  $\text{Cl}^-$  translocation and/or compartmentation, solute synthesis for interdependent mechanisms of growth and osmotic adjustment, and protein turn-over (PEREZ-ALFOCEA *et al.* 1995). Accumulated proline has been proposed to protect enzymes (SCHOBERT & TSCHESCHE 1978; ARAKAWA & TIMASHEFF 1983, 1985),

membranes (RUDOLPH *et al.* 1986) and polyribosomes (KANDPAL & RAO 1985) during environmental perturbations.

Proline accumulation induced by stress conditions is mediated both by increased synthesis and reduced oxidation of the imino acid. Although proline may also be synthesised from ornithine (DELAUNEY *et al.* 1993; ROOSENS *et al.* 1998), metabolic labelling and molecular genetic studies indicate that most of the proline accumulated in the vegetative tissues of mature plants in response to stress and is the result of enhanced synthesis from glutamate (DELAUNEY *et al.* 1993; ROOSENS *et al.* 1998; HARE 1998). Proline accumulation in plant cells possible induce by enhanced proline synthesis on carbon flux through a redox sensitive pathway such as the oxidative pentose phosphate pathway (OPPP) (Chapter 1; HARE 1998), which is generally considered to account for at least 10-15% of the oxidation of carbohydrates in most plant tissues (AP REES 1980; HARE 1998). Although investigations of flux through OPPP under conditions of stress are limited, a four-fold increase in activity of Glucose-6-phosphate dehydrogenase (G6PDH) was demonstrated in water-stressed barley (ARGANDONA & PAHLICH 1991) and transcripts encoding 6-phosphogluconate dehydrogenase (6PDH) and G6PDH accumulated in alfalfa following treatment with a fungal elicitor (FAHRENDORF *et al.* 1995). Rapid increases in G6PDH and 6PDH activities in aluminium -resistant wheat cultivars, but not in Al-sensitive cultivars suggest a role for OPPP activity in overcoming Al toxicity (ŚLASKI *et al.* 1996).

Little is known in soybean as to how carbohydrates and proline contribute to osmotic adjustment, or how these organic solutes change after salt stress. The two cell



lines used in this study, had different capacities to accumulate sugars and proline when NaCl was applied to the media (Chapter 5). The aim of the present study was to determine the effect of salinity and subsequent relief thereof on sugar and proline accumulation of two soybean cell lines differing in salt resistance.

## **7.2 Materials and Methods**

### **7.2.1 Growth of callus**

The maintenance of *Glycine max* (L.) salt-tolerant R100 and salt-sensitive cell line S100 on Miller's basal medium (Appendix 2) (MILLER 1965) supplemented with 2 mg l<sup>-1</sup> 2,4-D and 0.5 mg l<sup>-1</sup> kinetin have been described (Chapter 5). The cultures were subcultured every 28 days and kept at 26 °C in continuous low light (0.13 μmol m<sup>-2</sup> s<sup>-1</sup>).

### **7.2.2 Salinity, relief treatments and sampling**

For the salinity experiments, the culture manipulations were similar to those outlined in Chapter 5. For relief experiments, after 12 days of incubation on the MB media with 100 mM NaCl, both the salt stressed R100 and S100 calli were transferred to fresh MB medium without NaCl. The growth rate of the callus was measured as fresh weight (FW) and dry weight (DW) changes. A known weight of callus was inoculated into a 250 ml culture flasks containing MB medium with or without 100 mM NaCl, using 27 replicates. Subsequently at 4 day intervals, callus from 3 culture flasks was removed and its fresh weight determined. The calli were harvested and frozen for subsequent

analysis.

### 7.2.3 Extraction and measurement of soluble carbohydrates

Carbohydrates were analyzed as described previously (Chapter 5). Briefly, soluble carbohydrates were extracted by grinding the tissues (ca 150 -200 mg) in liquid nitrogen. Five ml 80% (v/v) ethanol were added and the mixture was extracted overnight by shaking at 200 rpm. The homogenized samples were centrifuged at 2000 x *g* for 10 min. Four ml of the supernatant were transferred to a new vial. The extraction was repeated twice more, and the supernatant taken to dryness under nitrogen. Two hundred  $\mu$ l of 25 g l<sup>-1</sup> hydroxylamine monohydrochloride in pyridine were added and the mixture incubated at 40°C for 20 min to obtain sugar oximes. A hundred  $\mu$ l sugar oxime solution were transferred to a vial, taken to dryness under nitrogen and silylized with 100  $\mu$ l Sil-A (Sigma) at room temperature. The silylized sugars were separated by gas chromatography on a 1.8 m X 3 mm (i.d) glass column packed with OV-17 on Chromosorb HP 80/100 and detected by flame ionization. The column was held at 125°C for 3 min, followed by ramping at 4°C/min to 270°C. The final temperature was held for 5 min. Individual sugars were tentatively identified by co-chromatography with authentic standards (Merck), prepared as described above.

### 7.2.4 Determination of proline content

Free proline was extracted and quantified by the spectrophotometric method described by Bates *et al.* (1973). About 200 mg of callus tissue was used per assay. The

homogenate of plant tissue in 3% (w/v) sulphosalicylic acid was clarified by centrifugation ( $14,000 \times g$ ) for 20 min at 4 °C. The reaction mixture containing ninhydrin was extracted with toluene and the absorbance of the toluene phase containing the red chromophore was measured at 520 nm using a Beckman DU-65 spectrophotometer.

#### 7.2.5 Glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PDH) assay

G6PDH and 6PDH activities were determined based on the method described by Lozano *et al.* (1996). About 200 mg of callus tissue was homogenised in 2 ml of extraction buffer (0.05 M Tris-HCL, pH 7.9; 0.001 M Na<sub>2</sub>EDTA) in a pre-cooled mortar. The crude homogenate was transferred to microcentrifuge tubes held at 4 °C and cleared of debris by centrifugation ( $14,000 \times g$ ) for 10 min. The supernatant solutions were filtered through a 0.22 µm syringe filter (Millipore ) to remove residual insoluble material and maintained at 4°C. For both assays, 50 µl of the clarified enzyme extract was added to 950 µl of the assay buffer (0.05 M Tris-HCl, pH 7.9; 0.005 M MgCl<sub>2</sub>; 0.25 mM NADP<sup>+</sup>) in a quartz cuvette. After measuring the low level of background reduction of NADP<sup>+</sup> by the extract over 3 min at 30°C, 50 µl of 0.1 M glucose-6-phosphate or 50 µl of 6-phosphogluconate were added. The contents of the cuvette were mixed by quickly inverting the cuvette and returning it to the thermostatically regulated cuvette holder. Activity of both dehydrogenases was measured by monitoring the increase in the absorbance at 340 nm, which reflects the steady state production of NADPH. The activities were corrected for endogenous NADP<sup>+</sup>-reducing activities, and expressed as

the rate of increase in A340 per  $\mu\text{g}$  of protein.

### **7.3 Results**

#### *7.3.1 Effect of NaCl on callus growth*

Salt stress affected callus growth as described earlier in Chapter 6. Salt stress reduced the fresh weight of salt-sensitive callus (S100) by 80 % and that of salt-tolerant callus (R100) by only 40% (Table 7.1) respectively. The DW and FW in S100 and R100 calli under salt-stress showed a similar pattern (Figure 6.3). Growth resumed once salt stress was relieved and S100 callus recovered growth to nearly 60% of the control (without NaCl stress) or 450% of the 100 mM NaCl treatment (Table 7.1) respectively by the end of the experimental period (28 days). In the R100 callus line, the relief treatment was not as significant as for the S100, the R100 callus growth was about 80% of the control (without NaCl stress) or 135% of the 100 mM NaCl treatment in fresh weight (Table 7.1).

#### *7.3.2 Changes in sugar levels in NaCl-sensitive and NaCl-tolerant callus during a salt stress and following its subsequent relief*

During an earlier part of this study, under 100 mM NaCl stress, the glucose and fructose content in the R100 cell line increased dramatically after 24 h of stress (Chapter 5). The sugars reached their highest levels at day 4 and 8 (Figure 7.1 B, D). The glucose content decreased rapidly after 8 days of salt stress, reaching the values of the control

**Table 7.1: Effect of NaCl stress on the fresh weight (g) of S100 and R100 callus cultures during 28 days of salt stress or 12 days of salt stress and 16 days of relief.** After 12 days of culture on the MB media with 100 mM NaCl, both the S100 and R100 callus were transferred to fresh MB media. Each value represents the mean  $\pm$  SE of three different samples.

Callus	NaCl	Days of culture (days of relief)									
		0	1	4	8	12	13(1)	16(4)	20(8)	24(12)	28(16)
S100	0	1	1.10 $\pm$ 0.12	1.69 $\pm$ 0.15	2.18 $\pm$ 0.21	3.0 $\pm$ 0.26	3.7 $\pm$ 0.32	4.65 $\pm$ 0.37	5.61 $\pm$ 0.63	9.45 $\pm$ 1.1	9.62 $\pm$ 1.07
	100	1	1.08 $\pm$ 0.11	1.12 $\pm$ 0.11	1.06 $\pm$ 0.16	1.06 $\pm$ 0.1	1.3 $\pm$ 0.12	1.3 $\pm$ 0.2	1.34 $\pm$ 0.2	1.22 $\pm$ 0.12	1.26 $\pm$ 0.21
	Relief	1	1.08 $\pm$ 0.11	1.12 $\pm$ 0.11	1.06 $\pm$ 0.16	1.06 $\pm$ 0.1	1.45 $\pm$ 0.17	2.0 $\pm$ 0.33	2.86 $\pm$ 0.37	4.48 $\pm$ 0.6	5.7 $\pm$ 0.7
R100	0	1	1.10 $\pm$ 0.1	1.72 $\pm$ 0.2	2.56 $\pm$ 0.33	2.93 $\pm$ 0.32	3.02 $\pm$ 0.44	4.65 $\pm$ 0.54	6.2 $\pm$ 0.7	8.4 $\pm$ 1.1	9.25 $\pm$ 1.7
	100	1	1.11 $\pm$ 0.12	1.08 $\pm$ 0.13	1.31 $\pm$ 0.15	1.60 $\pm$ 0.17	1.64 $\pm$ 0.11	2.10 $\pm$ 0.22	2.68 $\pm$ 0.4	4.69 $\pm$ 0.6	6.01 $\pm$ 0.66
	Relief	1	1.11 $\pm$ 0.12	1.08 $\pm$ 0.13	1.31 $\pm$ 0.15	1.60 $\pm$ 0.17	1.72 $\pm$ 0.25	2.40 $\pm$ 0.33	3.12 $\pm$ 0.42	5.0 $\pm$ 0.71	7.40 $\pm$ 0.85

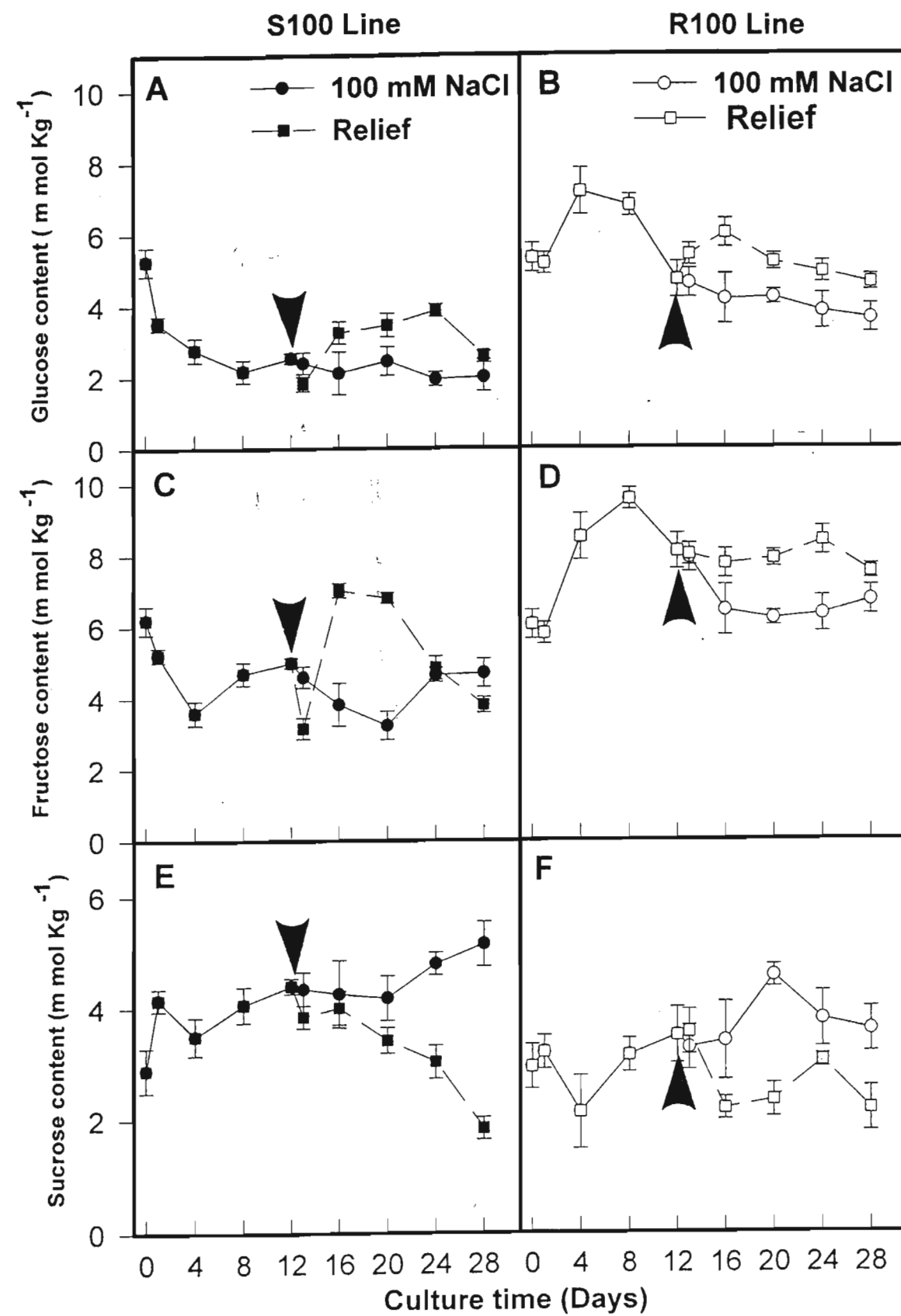


Figure 7.1: Effects of salt stress on glucose, fructose and sucrose accumulation in callus cultures of S100 and R100 cell lines growing on MB medium with 100 mM NaCl or after 12 days on MB medium with 100 mM NaCl and subsequently transferred to fresh MB medium. Arrows indicate the end of stress treatment. Each value represents the mean  $\pm$  SE of three different samples.

**Table 7.2: Changes in proline content (m mol kg<sup>-1</sup>) of different soybean cell lines in control and with 100 mM NaCl stress. Each value represents the mean ± SE of three different samples.**

Callus line	NaCl (mM)	Culture time (Days)							
		1	4	8	12	16	20	24	28
S100	0	0.42±0.07	0.38±0.04	0.44±0.05	0.43±0.04	0.45±0.06	0.42±0.09	0.43±0.03	0.44±0.04
	100	0.78±0.12	1.63±0.19	1.31±0.22	0.43±0.1	0.31±0.06	0.42± 0.07	0.44±0.03	0.44±0.04
R100	0	0.41±0.09	0.40±0.05	0.42±0.04	0.44±0.05	0.43±0.03	0.41±0.06	0.38±0.03	0.40±0.05
	100	1.61±0.47	1.66±0.38	1.56±0.23	1.45±0.18	1.50±0.33	1.52±0.4	1.54±0.32	1.56±0.55

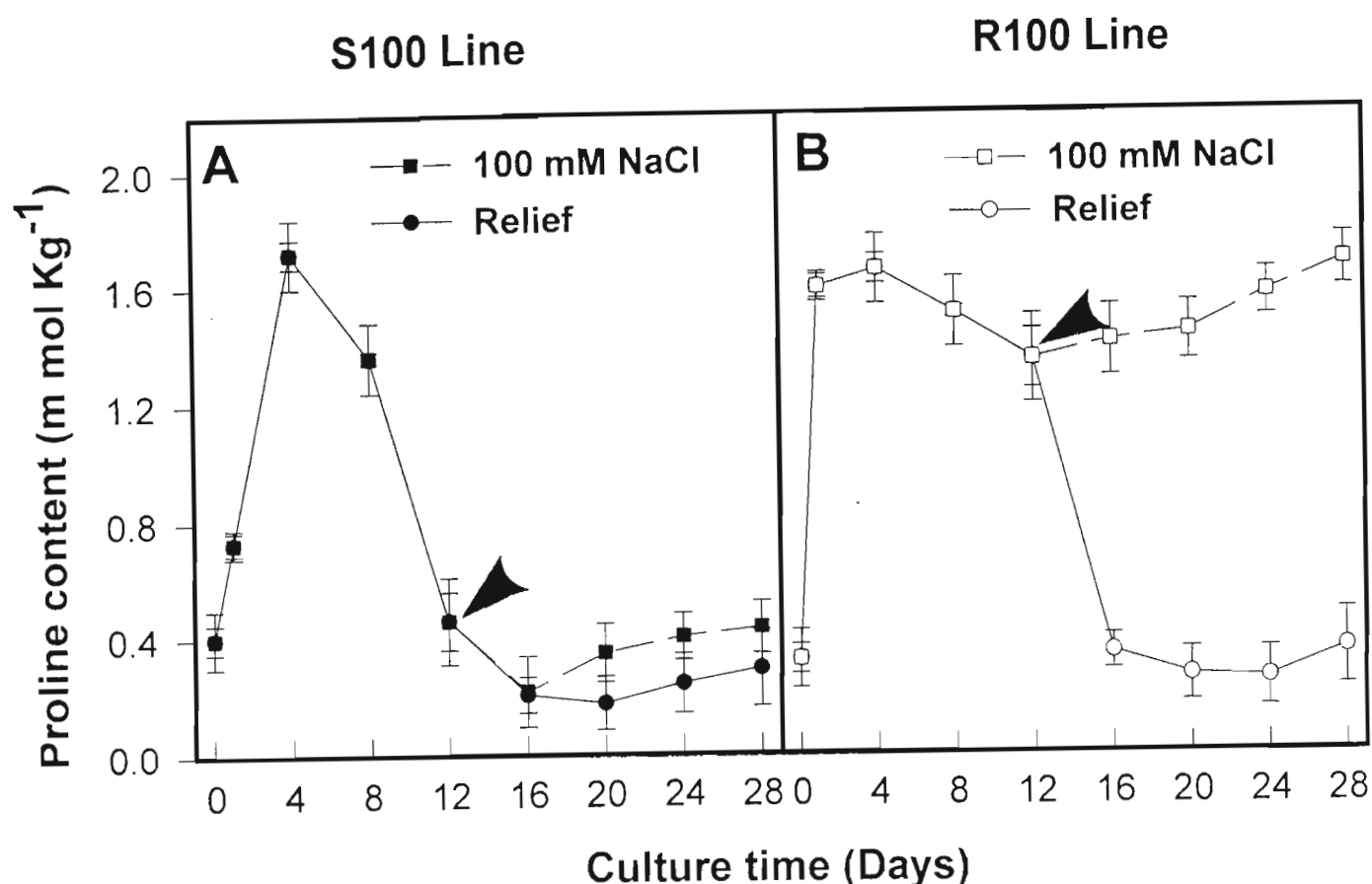
by day 12. The fructose level in R100 callus fell rapidly after 8 days of stress, reaching the values of the control within 6 days, and then remained lower than that of the control (Chapter 5).

In the relief experiment, the glucose and fructose content increased in the callus of the salt-tolerant cell line (R100) (Figure 7.1 B, D) and decreased in sensitive callus (S100) during the first 24 h (Figure 7.1 A, C). The glucose and fructose content in R100 callus again increased after relief and reached their highest levels at day 4 and day 12 after transfer to fresh MB medium. It then decreased, but the contents of glucose and fructose in relieved callus were higher than in the 100 mM salt-stressed callus (Figure 7.1 B, D). The glucose and fructose content in S100 callus under salt-stress showing a different pattern. The content of glucose and fructose declined sharply in the salt-stressed callus after relief, but dramatically increased after 24 h and reached their highest levels at day 12 and day 4 after transferred from 100 mM NaCl MB medium to fresh MB medium respectively. Sucrose, which was present in smaller amounts than the monosaccharides in both salt-sensitive and salt-tolerant cells exhibited differential responses to salt stress. A similar pattern of change with a significant decrease in both cell lines after elimination of salt stress was observed (Figure 7.1 E, F).

### *7.3.3 Proline accumulation under salt stress and its subsequent relief*

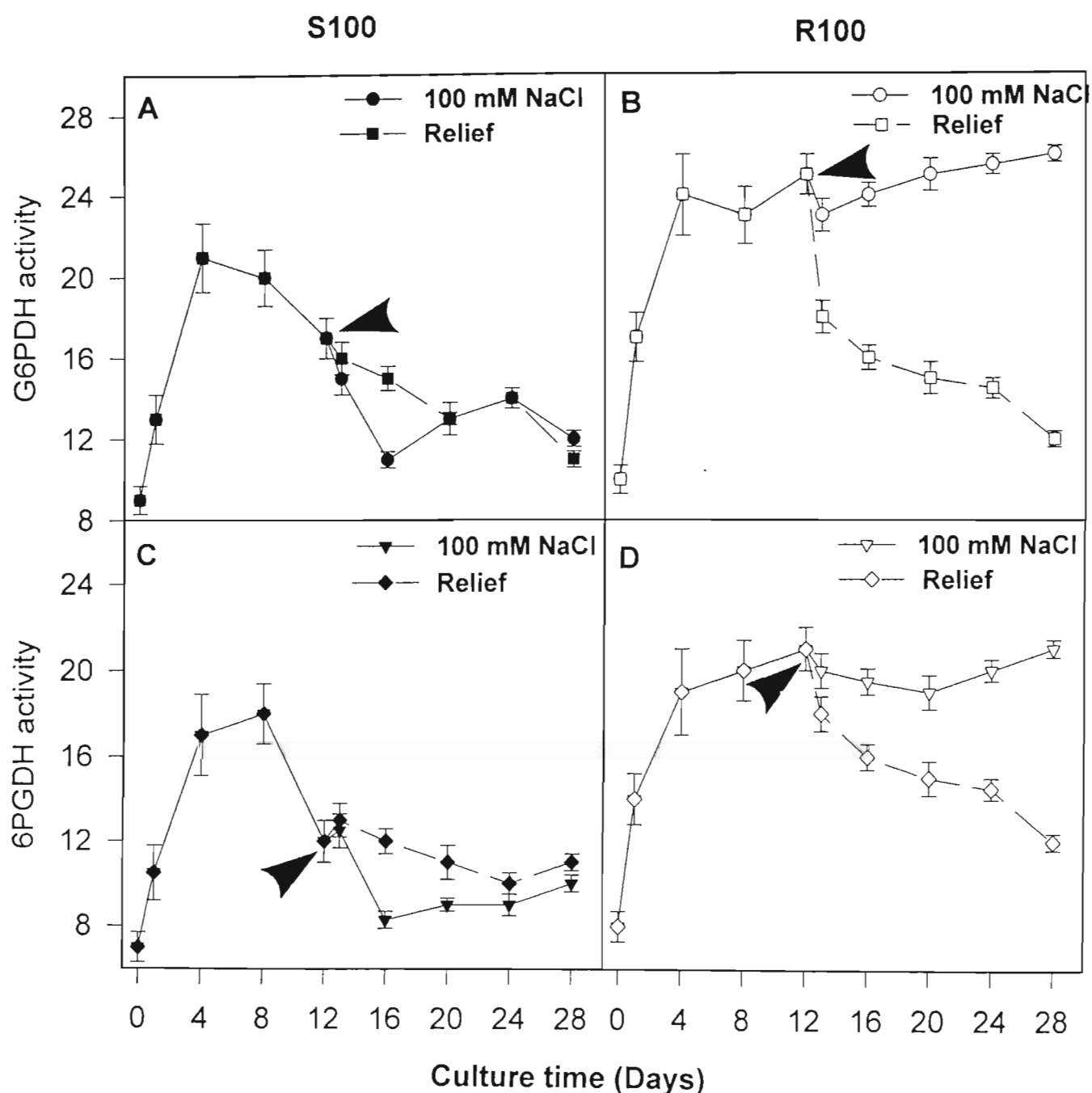
In earlier experiments (Chapter 5), the proline content rose sharply after 100 mM NaCl treatments in both S100 and R100 callus (Table 7.2), reaching maximum values by day 4. However, after 8 days of salt stress, the proline content in S100 callus fell rapidly





**Figure 7.2: Proline accumulation during 28 days of exposure to 100 mM NaCl or during 12 days of exposure to 100 mM NaCl and 16 days of relief.** Growth of S100 (A) and R100 (B) calli with 100 mM NaCl addition to the medium for 12 days and then transfer to medium without NaCl for 16 days. The arrows indicate the end of the salt stress. Each value represents the mean  $\pm$  SE of three different samples.

rapidly and decreased to very low level within 4 days. However, R100 callus contained significantly higher amount of proline during the experiment. A drastic decrease in proline content occurred in the first 24 h of R100 calli and reached the normal level at day 4 after stress relief. The proline content of S100 calli remained unchanged level during the relief experiment (Figure 7.2).



**Figure 7.3: Changes in the total extractable activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase during the course of salt stress and relief of soybean cell lines.** Changes in G6PDH specific activity ( $\Delta A_{340} \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$ ) of S100 (A) and R100 (B) exposure to 100 mM NaCl for 12 days, followed by a recovery period of 16 days. Changes in 6PGDH specific activity ( $\Delta A_{340} \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$ ) of S100 (C) and R100 (D) exposure to 100 mM NaCl for 12 days, followed by a recovery period of 16 days. The arrows indicate the end of the salt stress. Each value is the mean of three replications  $\pm$  SE.

#### *7.3.4 Glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) activity*

Specific G6PDH and 6PGDH activity could be detected in R100 and S100 callus cultures during the entire salt-stress and recovery treatment period (Figure 7.3). The mean specific activity of G6PDH was always greater than that of 6PGDH in both cell lines. A significant increase in specific G6PDH and 6PGDH activities were observed in R100 and S100 callus lines after 24 h of 100 mM NaCl treatment (Figure 7.3). Maximal G6PDH activity was observed at day 4 in both callus lines subjected to 100 mM NaCl. A drastic decrease of G6PDH and 6PGDH activity occurred 8 days after subculture of the S100 callus line on 100 mM NaCl medium. These enzymes reached normal levels at day 16 (Figure 7.3 A, C). The R100 callus line showed a different trend and the G6PDH and 6PGDH activities remained high during the NaCl-treatment (Figure 7.3 B, D). The G6PDH and 6PGDH activities decreased in R100 callus and reached its normal levels 12 days after relief (Figure 7.3 B, D).

### **7.4 Discussion**

Comparative data on the changes in sugar content in salt-tolerant soybean callus under salt stress and relief of salt stress are scarce. The present results on the effect of salinity on sugar accumulation in soybean callus demonstrate tolerance differences and provide details about the sugar components involved in salt tolerance. Sugars have long been known to increase in a wide range of plants grown under salinity (BOLARÍN *et al.* 1995). The present results confirm the fact that the soluble sugar content is a very

sensitive factor for salt tolerance improvement. The differences between the callus lines in their ability to accumulate carbohydrates were evident under stress but not under control conditions.

An increase in total carbohydrate content in the plant cells was observed in NaCl treatment. This was due to an increase in glucose and fructose concentration in the callus with high concentrations of NaCl in the medium. Salt tolerant soybean cells are characterized by a decrease in sucrose accumulation (Chapter 5). Present results agree with those of Sacher and Staples (1985), that the utilisation of carbohydrates could be the limiting factor of growth. A high sucrose content in sensitive calli, linked with their decreasing growth in saline media, could exert a feed-back inhibition on carbon metabolism. Morris and Arthur (1984) reported high acid invertase and low sucrose phosphate synthase levels with rapidly growing cells having a high demand for sucrose. The results showed a similar trend during the relief period. The sucrose concentration decreased in both sensitive and tolerant callus. Little is known about sugar changes that are induced in callus under salt stress and its subsequent relief. The results of this study showed that the relatively rapid callus growth observed in the present study, accompanied with the sharp fall in sucrose content which occurred in stressed cells during the removing of salt stress, is consistent with the view that organic solutes accumulated during salt stress are likely to be utilized in growth after stress is relieved.

Amino acid and amine accumulation may occur not only as a result of salinity *per se* but also under conditions of water stress in higher plants (FLOWERS *et al.* 1977).

Free proline, a normal constituent of the amino acid pool in many organisms, accumulate in a number of plants and tissues in response to salinity stress. Stewart and Lee (1974) indicated that proline levels up to 600 mM do not inhibit enzyme activity *in vitro*. Results in Chapter 5 showed better salt tolerance of the R100 callus line and an increased osmotolerance in proline accumulation in R100. In the present study, a decrease in proline content, G6PDH, and 6PGDH activities in the R100 line was accompanied by a relative water content increase as the callus recovered growth after stress was relieved. This supports the hypothesis that proline acts as a protective compound during periods of NaCl-stress. These results suggest the possibility that proline may function as a compatible solute in the important role of balancing cytoplasmic and vacuolar water potentials.

In the calli of salt-sensitive and salt-tolerant cell lines, the concentration of glucose increased during relief, at a rate closely corresponding to the increase in relative water content. Fructose content showed a very similar pattern, and was also well correlated with water status. The glucose and fructose content rose first and led to the growth recovery in both cell lines after the callus was moved to fresh medium. This may result from increased sucrose hydrolysis or the monosaccharides may be absorbed from the fresh medium. No-differences between the cell lines examined here in the rate and amount of glucose accumulation accompanying increasing water potential may be physiologically important in helping the cells to recover from salt stress when it is relieved. However, the glucose and fructose content of S100 callus decreased in the first 24 h of relief and then increased showing that the sensitive and tolerant calli act differently in response to the stress relief. This suggests that glucose

and fructose may play important roles during salt-stress and its relief, because they were clearly higher in stressed callus of the tolerant cell line and because their accumulation was the earliest response detected during the relief period. In plants, sugars are not only a substrate for the growth of sink tissue, which depend on the import of carbohydrates, but they regulate the expression of a variety of genes (KOCH 1996). Salt stress and water stress are the most severe environmental stresses to which plants may be subjected. Activation of the responsive machinery requires energy and thus the induction of sink metabolism. Accordingly, physiological studies indicate that both photosynthetic capacity and carbohydrate metabolism are altered in response to stresses. In most plant cells, sucrose is the main transport sugar, and cleavage of this disaccharide is the initial reaction for metabolizing sucrose in sink tissues. Invertases catalyze the irreversible hydrolysis of sucrose to glucose and fructose (AVIGADE 1982). It has been shown that genes encoding sink-specific enzymes, such as sucrose synthase (SALANOUBAT & BELLIARD 1989), granule-bound starch synthase (VISSER *et al.* 1991), and extracellular invertase (ROITSCH *et al.* 1995), are induced by glucose or sucrose. Glucose-induced gene expression has been demonstrated for enzymes involved in pathogen and stress responses (TSUKAYA *et al.* 1991; EHNESS *et al.* 1997). The specific effects of glucose on gene expression and development are characteristic of plant hormone action (SHEEN *et al.* 1999).

## ***Chapter Eight***

### **General conclusion**

Many plant species are able to increase their tolerance to adverse conditions after exposure to such environmental stresses. This adaptive process involves a number of biochemical and physiological changes. These changes include increased levels of soluble sugars, proteins, amino acids, and organic acids (THOMASHOW 1994; CAPEL *et al.* 1997). Many of these changes are regulated through changes in gene expression, and a number of genes, the expression of which is induced by environmental stress, have been isolated and characterized from a wide range of both dicotyledonous and monocotyledonous species. The precise role of such changes in gene expression, and the biological function for the majority of the environmentally induced genes remain to be determined. It is likely that use of current techniques of molecular biology may help to expand our understanding of coordinate gene expression in higher plants exposed to environmental stress. Furthermore, it is possible that studies on the stress-induced proteins and the genes encoding them may result in the engineering of crop plants more resistant to normally encountered moderate stress conditions (LIU 1997).

Plants have evolved mechanisms that allow them to acclimatize to a variety of environmental stresses. Many species of plant are capable of adapting to low temperature and/or salt stress conditions via adjustments of plant processes such as growth and development, photosynthesis, respiration, and reproduction (KORNER & LARCHER 1988). Metabolically active processes can be regulated at the gene expression level and are thus associated with the induction of specific proteins (GUY

1990; HUGHES & DUNN 1996).

Within the limits of this study, it has not been possible to provide conclusive answers to many of the questions related to abiotic stress. In attempting to present a synthesis of the most significant findings of this study, at least four significant aspects can be identified.

Firstly, an efficient cDNA cloning strategy was established based on a modified two step PCR technique. One cDNA clone, cs18-14 with a *Xba* I/*Xho* I insert of approximately 3,500 bp in length and which probably encodes glucose dehydrogenase in soybean roots subjected to low temperature, was obtained. Northern hybridization confirmed a high expression in soybean roots under low temperature treatment. Glucose dehydrogenase expression is induced by heat shock (SODE *et al.* 1996), GA<sub>3</sub> treatment (BAILEY 1996), and by phosphate starvation (GYANESHWAR *et al.* 1999). The work of Bailey (1996) showed that GA<sub>3</sub> treatment and wounding could induce glucose dehydrogenase expression. Our study indicates that glucose dehydrogenase can be induced by low temperature in plant roots.

Secondly, maintenance of root growth in saline soils is critical for plant health. Saline soils are characterized by low water potentials and high salt concentrations in the soil solution. Low water potentials have osmotic effects on plants, causing growth reduction. Salinity interferes with the uptake and transport of K<sup>+</sup>, thus disrupting K/Na-selectivity and ion homeostasis (NIU *et al.* 1995). The maintenance of adequate net uptake of K<sup>+</sup> by plants at high Na<sup>+</sup> concentrations is important. The present study



confirmed that protection against the osmotic effects on roots of salt-stressed soybean can be achieved by the presence of elevated  $\text{Ca}^{2+}$  supply where root growth was maintained. Supplemental  $\text{Ca}^{2+}$  partly mitigates the Na/K-interactions, probably through an intercellular  $\text{Ca}^{2+}$  sensor (LIU & ZHU 1998), and protects salt-sensitive plants from the loss of  $\text{K}^+/\text{Na}^+$ -selectivity at high salinity.

Thirdly, a NaCl-tolerant line of *Glycine max* (L.) Merr. cv. Acme was obtained through continuous subculturing on 100 mM NaCl. Salt tolerance in this culture was characterized by an altered growth behavior, reduced cell volume, and accumulation of  $\text{Na}^+$ ,  $\text{Cl}^-$ , proline and sugars when grown under salt stress, as well as on normal media. The pattern of salt-induced expression differed between salt-sensitive and salt-tolerant cells, but was similar upon its subsequent relief. On the basis of these results it is proposed that the salt-selected cell line R100 embodies a general shift towards a halophytic mode of salt tolerance. The culture of the salt-tolerant cell line could be used in potential regenerating programmes for increasing salt tolerance of soybean.

Fourthly, the conclusion from the data comparing the two *Glycine max* cell lines, differing in their tolerance to NaCl, appears to be that during NaCl stress glucose content, G6PDH and 6PGDH are clearly higher in the tolerant cell line, accumulation of these enzymes and sugar were the earliest response detected during the relief period, indicating that they may play important roles during salt stress. There is some evidence in this study indicating that glucose dehydrogenase is induced by low-temperature in soybean roots. Thus, glucose and relative enzymes are tightly linked to the defence responses to satisfy the energy requirements of the activation of the

cascade of defence reactions. The specific effect of glucose on gene expression and development are characteristic of plant hormone action.

The findings of this study are a small contribution to the vast and rapidly expanding body of knowledge concerning stress responses in higher plants. Nonetheless, together with much supportive evidence from the recent studies of other workers, they appear to be consistent with a long overlooked view which implicates an important regulatory role for sugar metabolism in plant growth and development.

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# Appendix One

## Media for Bacteria and Phage growth

### Luria broth (LB) per liter

NaCl	10.0 g
Bacto-tryptone	10.0 g
Yeast extract	5.0 g
Adjusted pH to	7.0

### Luria agar (LA) per liter

LB was solidified with 15 g Bacteriological agar per liter.

### NZY broth per liter

NaCl	5.0 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	2.0 g
Yeast extract	5.0 g
NZ Amine	10.0 g
Adjusted pH to	7.5

NZY medium was solidified with 15 g Bacteriological agar per liter.

### Top agar

NZY broth solidified with 0.7% Agarose (Techcomp LTD).

**Medium of bacterial cultures for titering phage per liter**

NaCl	10.0 g
Bacto-tryptone	10.0 g
Yeast extract	5.0 g
Maltose (w/v)	0.2 %
MgSO <sub>4</sub> (mM)	10.0
Adjusted pH to	7.0

- All media were autoclaved prior to use.

## Appendix Two

### Composition of media for seedling growth, tissue culture of *Glycine max* L.

#### Miller's Basal (MILLER 1965) medium (MB)

Components	Amount (mg l <sup>-1</sup> )
<b><i>Macronutrients</i></b>	
KH <sub>2</sub> PO <sub>4</sub>	300
KNO <sub>3</sub>	1000
NH <sub>4</sub> NO <sub>3</sub>	1000
Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O	500
<b><i>Micronutrients</i></b>	
MgSO <sub>4</sub> ·7H <sub>2</sub> O	71.5
KCl	65
MnSO <sub>4</sub> ·4H <sub>2</sub> O	14
NaFe.EDTA	13.2
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	3.8
H <sub>3</sub> BO <sub>3</sub>	1.6
KI	0.8
Cu(NO <sub>3</sub> ) <sub>2</sub> ·3H <sub>2</sub> O	0.35
(NH <sub>4</sub> )Mo <sub>7</sub> O <sub>24</sub> ·4H <sub>2</sub> O	0.1
Sucrose (g)	30
<b><i>Organic supplements</i></b>	
myo-inositol	100
nicotinic acid	2.0
pyridoxine HCl	0.8
thiamine HCl	0.8
NAA	2.0
Kinetin	0.5
Adjusted pH to	5.8

MB medium was solidified with 10 g Bacteriological agar pre liter.

### **Murashige and Skoog (1962) medium (MS)**

<b>Components</b>	<b>Amount (mg l<sup>-1</sup>)</b>
<b><i>Macronutrients</i></b>	
MgSO <sub>4</sub> ·7H <sub>2</sub> O	370
KH <sub>2</sub> PO <sub>4</sub>	170
KNO <sub>3</sub>	1900
NH <sub>4</sub> NO <sub>3</sub>	1650
CaCl <sub>2</sub> ·2H <sub>2</sub> O	440
<b><i>Micronutrients</i></b>	
H <sub>3</sub> BO <sub>3</sub>	6.2
MnSO <sub>4</sub> ·4H <sub>2</sub> O	22.3
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	8.6
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.25
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.025
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.025
KI	0.83
FeSO <sub>4</sub> ·7H <sub>2</sub> O	27.8
Na <sub>2</sub> EDTA·2H <sub>2</sub> O	37.3
Sucrose (g)	30
<b><i>Organic supplements</i></b>	
Thiamine HCl	0.5
Pyridoxine HCl	0.5
Nicotinic acid	0.5
Myoinositol	100
Glycine	2
Adjusted pH to	5.7

All media were autoclaved prior to use.