In the name of God

IMPROVEMENT IN SALT AND DROUGHT TOLERANCE OF ALFALFA (*MEDICAGO SATIVA* L.) USING TISSUE CULTURE AND MOLECULAR GENETIC TECHNIQUES

by
ABBAS SAFARNEJAD

Thesis submitted in accordance with the requirements
of the University of Liverpool for the degree of
Doctor in Philosophy

University of Liverpool
Department of Genetics and Microbiology

April 1996

IMPROVEMENT IN SALT AND DROUGHT TOLERANCE OF ALFALFA (*MEDICAGO SATIVA* L.) USING TISSUE CULTURE AND MOLECULAR GENETIC TECHNIQUES

ABSTRACT

Alfalfa, or Lucerne (*Medicago sativa* L.), is a valuable forage crop which is grown in areas of limited rainfall and high temperatures and where the land is often salt affected. Improved salt tolerance in this crop would be a great advantage. Attempts to increase this salt tolerance by *in vivo* screening of seeds of the commercial variety CUF 101 had produced a more tolerant progeny line, referred to as CUF101-1S. This line provided some material for further enhancement of tolerance.

The aim of this project was to produce somaclonal variation by tissue culture techniques then to screen for salt tolerance. Any changes in the genotype of salt tolerant and drought tolerant somaclones were assessed by biochemical and molecular genetic analysis.

In comparison with a non commercial line, Regen-SY, the tissue cultures of CUF101-1S showed a regeneration frequency of 6-14% while that of Regen-SY was 60-62%. The initial problem was to overcome the limited regeneration *in vitro* of the commercial variety CUF 101 and its more tolerant line CUF101-1S. This was achieved by passage of CUF101-1S through three successive cycles of tissue culture initiation, regeneration, plant growth and seed production. There was a maximum enhanced regeneration frequency of CUF101-1S in the second (70%) and third (74%) tissue culture cycle. The regenerants provided seeds for analysis of the seedling somaclones for salt and drought tolerance. The analysis was made on seeds of regenerants produced from tissue culture and also on seeds of plants derived from an *in vitro* screen. The *in vitro* screen was achieved by transferring differentiating cultures to a nutrient medium which contained 0-350 mol m^{-3} NaCl.

Seedlings from regenerant plants and from somaclones that survived an *in vitro* screen of 250 mol m^{-3} NaCl were then assessed by incubation in a concentration of 200 and 250 mol m^{-3} NaCl. The drought condition were produced by incubation in solutions of polyethylene glycol 6000 (200 and 250 g l^{-1}). The response of the seedlings was measured in the short term, 7 days, and the longer term, 14 days, as changes in growth, proline, and antioxidant enzyme activity (catalase, glutathione reductase, ascorbate peroxidase and superoxide dismutase). In comparison with the parents CUF 101 and CUF101-1S the somaclones (7R1, 6R2IV) showed a maximum increase in salt tolerance of 173% for root length and 229% for shoot length and in drought tolerance an increase of 165% for root length and 155% for shoot length. Again in comparison with the CUF 101 there was a maximum increase in the somaclones (7R1, 6R2IV) in proline accumulation in the presence of salt of 370% and drought of 152% and for the antioxidant enzymes (AP, GR) the maximum increase in the presence of salt was 147% and drought was 144%.

Anther cultures were derived from plants of a non commercial variety, Regen-SY, and also CUF101-1S. The regenerated plants from Regen-SY grew vigorously and subsequently a large number were screened for drought tolerance. There were limited survivors (6%) but no further analysis was performed on them to confirm their tolerance. Unfortunately, anther culture derived plants from CUF101-1S failed to grow.

A potentially important gene is pA9 which is known to be responsible for the production of a proline rich cell wall protein. Polymerase Chain Reaction (PCR) amplification of the pA9-like gene indicated that it was present in both tolerant and sensitive lines. Southern blotting showed that the copy number of the pA9-like was present as multiple copies in the tolerant clone (6R2IV) compared with a single copy in the parents. The DNA sequence insert in the pA9-like gene showed a 93% identity in 113 bp overlap with a proline rich protein in alfalfa.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

CHAPTER 1.

General Introduction

1. General Introduction

1.1 The response of plants to environmental stress

World food production is limited primarily by environmental stresses of which abiotic factors contribute to 70% of the reduction in yield (Boyer, 1982). In plants, stress can be defined as any factor that inhibits plant growth. Stress due to drought, waterlogging, salinity, low mineral nutrients, extremes of temperature and pH, and metals, including heavy metals and also aluminium and manganese, are common throughout the world so that it is very difficult to find 'stress free' areas where crops may approach their potential yields (Acevedo & Fereres, 1993). Arid and semi-arid regions, which are usually characterized by saline soils and water, drought, and extreme temperatures, constitute about 40% of the world's land surface (Fischer & Turner, 1978). In addition each year 22 billion tons of soil is lost through erosion and wind of droughted and salinated soil. The half-life of the world soils has been calculated to be only 100 years. This problem has become so great that the availability of arable land per person has decreased by 32% in the last 30 years (Sigurbjornsson & Maluszynski, 1995) as a result of soil loss and a world population increase of one million every 4 days. All this means that in the future the challenge is how to double food production in the next 30 years with hardly any more land available for extra food production.

Ecologists sometimes classify plants according to their response to water . (Appendices, Appendix 1.2). Crop species have also been categorized into four groups; tolerant, moderately tolerant, moderately sensitive, and sensitive on the basis of their tolerance. Barley, cotton, tall wheatgrass, sugar beet, and bermuda grass are tolerant because their threshold salinity level (maximum salinity without yield loss) ranges from 6.9 to 8.0 dS m^{-1}, whereas sorghum, wheat, perennial ryegrass, soybean, and cowpea are ranked as moderately tolerant, their threshold salinity level ranging from 4.9 to 6.8 dS m^{-1}. Moderately sensitive crops include rice, tomato, alfalfa,

potato, sugarcane, clover, etc., whereas most fruit trees and carrot, onion, and pulses are in the sensitive group, where the threshold salinity level is below 2.0 dS m^{-1} (Maas and Hoffman, 1977). Drought for these crops becomes an environmental stress when it is of sufficient duration to produce a plant water deficit or stress, which in turn causes disturbance of morphological, physiological, cellular and metabolic processes (Bray, 1994). Water stress might operate on the plant directly or indirectly. It has been suggested that it may act directly by mechanisms involving a reduction in the chemical potential of water, through a reduction in hydrostatic pressure in the cells (Shalhevet, 1993). Alternatively, water stress can act indirectly, such as through a decrease in protein content and the incorporation of amino acids into protein by increased ribonuclease activity and dissociation of polyribosomes (Galiba *et al.*, 1989).

Irrigation is the method by which crops are grown in areas of high potential water stress. The major potential sources of irrigation water in most arid areas are canals derived from rivers, tubewells, or seawater. Water from these sources contains variable amounts of salts, and their uncontrolled use has caused considerable salinization world wide (Massoud, 1974). However, inorganic ions such as NH_4^+, Ca^+, SO_4^{+2} and PO_4^{-3} must always be imported into plants in order to provide raw materials and co-factors needed to synthesize and activate the organic components of the cell. Other ions such as K^+, Na^+ and Cl^- are imported to produce the ionic balance and osmotic potential that is optimal for different physiological process. An extracellular ion excess invariably disrupts the ionic balance intracellularly, so that the influx of salt may denature or aggregate protein leading to a loss of function. Gradient-driven pumps may reverse and thus block the normal redistribution of symported molecules. Consequently the activity of some membrane components, may change, and even the entry of water may be restricted. Increasing the amount of intracellular Na^+ can have secondary effects such as a decrease in the concentration of K^+ (Ben-Hayyim, 1987; Binzel *et al.*, 1987). Salt-imposed stress has been shown to have an impact even before ions enter the cell. Extracellular Na^+ (or PEG), for

example, can leach Ca^{+2} from root cell plasmalemma, and as a result of membrane destabilization, increase K^+ efflux (Cramer *et al.*, 1985). These are only the immediate problems facing the cell. If the stress is prolonged, normal maintenance processes are impaired because both general protein synthesis (Hurkman & Tanaka, 1987) and metabolism (Criddle *et al.*, 1989) decline. Denatured proteins may form inactive complexes with otherwise functional proteins. Enzymes may be poisoned when inorganic co-factors are displaced by incoming salts (Caplan *et al.*, 1990). However, salinity can damage the plant through specific toxic effects of ions (primary salt injury); through its osmotic effect, which is equivalent to a decrease in water activity, and by disturbing the uptake of essential nutrients (secondary salt induced stress) (Shalhevet, 1993). Both of these categories exert adverse pleotropic effects on growth and development of plants, at the physiological, biochemical (Gorham *et al.*, 1985; Gossett *et al.*, 1994) and molecular levels (Hurkman *et al.*, 1989; Cushman *et al.*, 1990; Deutch & Winicov, 1994). If the plant is to adapt, each of these classes of damage must be repaired or, if possible, prevented.

The genus of *Medicago* is very extensive comprising more than 60 different species, two-thirds of which are annuals and one-third perennials (Arcioni *et al.*, 1990). The primary centre for the genus is found in the Caucasus, north western Iran and north eastern Turkey (Quiros & Bauchan, 1988). Alfalfa or lucerne (*Medicago sativa* L.) is a polymorphic species, adapted to many soils and climates. It has a chromosome complement of X=8 with both diploid and tetraploid forms and all cultivars are autotetraploids (Rumbaugh *et al.*, 1988*).* It grows from inside the Arctic circle to the Southern hemisphere and from sea level to >2500-m elevation and is cultivated without irrigation in regions where there is <200-mm annual precipitation as well as in regions that receive 2500 mm of precipitation (Rumbaugh *et al.*, 1988). Some common names include median herb, purple medic, younjeh, snail clover, burgundy hay/clover indicates its widespread nature. In spite of the various names applied to alfalfa, the crop is most commonly called alfalfa or lucerne throughout Europe (except Spain and Portugal), South Africa, Australia and New Zealand

(Michaud *et al.*, 1988). Michaud *et al.*, 1988 states that historical evidence testifies to the widespread distribution of alfalfa in Media (north western Persia) in the 1st millennium B.C. and probably the name of *Medicago* is derived from Median herb (Koocheki, A., Pers. Comm.). In the 4th century B.C., Theophrastus described how alfalfa was brought to Greece by the invading Median armies in order to feed their chariot horses and other animals (Michaud *et al.*, 1988).

Alfalfa has valuable characteristics, such as high forage quality, ability to fix atmospheric N_2 and a positive effect on soil tilth. It is now grown on over 32 million ha world-wide (Fig. 1.1) of which the most are in areas of low rainfall, high temperature and often saline soil conditions. Alfalfa productivity in these areas is reduced and in some areas growth is totally inhibited. Improved salt and drought tolerance in this crop would be a great advantage.

Fig. 1.1 World areas of cultivated alfalfa (Michaud *et al.*, 1988).

Countries	Year	Hectares
Europe	1981-1984	7, 994, 310
North America	1981-1982	13, 348, 325
South America	1981-1983	7, 770, 500
Africa	1981-1985	434, 970
Asia	1970-1984	2, 501, 800
Oceania	1981-1984	216, 700
World Total		32,266, 605

1.2 Application of tissue culture to the production of plants resistant to salt and drought stresses

Plant-breeding has had a limited success in improving the salt tolerance of crop species (Epstein *et al.*, 1980; Wenzel & Foroughi-Wehr, 1993). Although some

improved germplasm capable of germinating at high levels of salinity (Al-Khatib *et al.*, 1993) has been produced, there has been no substantial improvement in salt tolerance of crop plants (Winicov, 1991). However, the possibility of growing plants and even plant cells in the same way as micro-organisms offers a basis for new selection techniques. One advantage of this is that variation in the influence of the environment is avoided and even more importantly the selection is based on a huge single-cell population, each cell of which might be a genetically different individual (Collin & Dix, 1990). In combination with molecular biology, particularly genome and gene diagnosis coupled to the polymerase chain reaction (PCR), selection at the DNA level is already possible on small portions of the plant, and can be performed in a Petri dish (Fig.1.2). Selection of somaclonal variants takes advantage of the naturally occurring genetic variation that appears in plants regenerated from somatic cells grown in tissue culture. This variation can pre-exist in the explant tissue but more usually it arises during the tissue culture procedure itself, even in the absence of deliberate mutagens (Larkin & Scowcroft, 1981). In future the genetic engineers may provide more direct methods of transferring specific genes conferring osmotolerance or special membrane properties to control the active or passive entry of chloride ions (Ashraf, 1994). An obvious advantage of somaclonal variation is that somaclones are more simple to generate and result in a rich source of genetic variability. In addition, this source of variation has been free of the regulatory hurdles that plague products of recombinant DNA. Plants can be transferred directly to the field and evaluated as part of an ongoing breeding program.

Possible mechanisms to explain somaclonal variation include changes in gene expression as a result of chromosome structural modifications (heterochromatin and position effects), or movement of transposable elements, chromatin loss, DNA amplification, somatic crossing-over, somatic reduction, and changes in the DNA of cytoplasmic organelles (Larkin & Scowcroft, 1981; Arcioni *et al.*, 1990). Mechanisms of somaclonal variation identified in alfalfa include changes in chromosome number (chromosome doubling, aneuploidy) and structure, qualitative

genetic changes (change in gene expression, cytoplasmic changes), dominant and recessive mutations, transposable elements, and changes in chloroplasts and mitochondria (see Bingham & McCoy, 1986). It was suggested that a given variant may also carry more than one nuclear or cytoplasmic mutation.
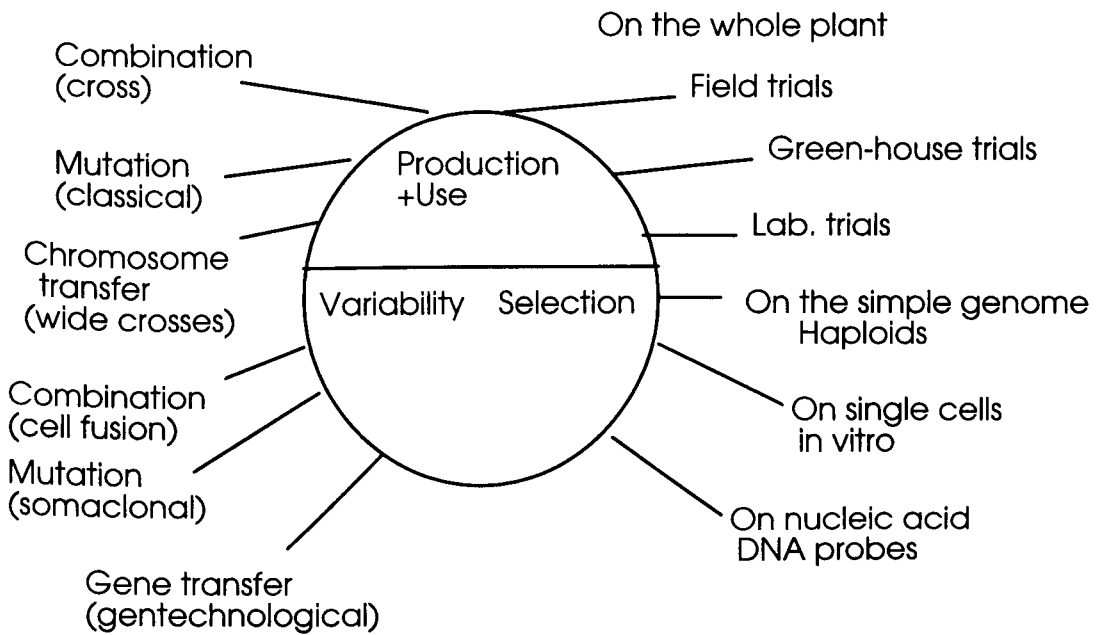
Fig. 1.2. Summary of classical (upper part) and biotechnological methods used in plant breeding (Wenzel & Foroughi-Wehr, 1993).

The importance of developing salt tolerance in plants encouraged research on selection at the cell level. Many salt tolerant somatic cell lines (reviewed by Hasegawa *et al.*, 1994) have been developed for a variety of species, including *Nicotiana sylvestris* and *Capsicum annuum* (Dix & Street 1975), alfalfa (Croughan *et al.*, 1978; McCoy 1987a), citrus (Ben-Hayyim & Kochba, 1983), *Lycopersicum peruvianum* (Hassan & Wilkins, 1988), *Nicotiana tabacum* (Nabors *et al.*, 1975) and

Pennisetum purpureum (Chandler & Vasil 1984). Somaclonal variation for salt tolerance is inherited in the progeny of tissue culture derived tobacco (Nabors *et al.*, 1980; Bressan *et al.*, 1987; Sumaryati *et al.*, 1992), sorghum (Bhaskaran *et al.*, 1986; Waskom *et al.*, 1990), flax (McHughen, 1987), rice (Vajrabhaya *et al.*, 1989), *Brassica juncea* (Jain *et al.*, 1990), alfalfa (Winicov, 1991), and sugarbeet (Freytag *et al.*, 1990) including selections that exhibit enhanced salt tolerance in the R4 generation. These results seem to indicate that intrinsically cellular mechanisms that are selected for *in vitro* will confer an enhanced salt tolerance phenotype to regenerated plants (Hasegawa *et al.*, 1994). In spite of this extensive investigation, there is limited information on the subsequent culture and stability of salt tolerance in the many of regenerated plants. Examples of somaclonal variation identified in alfalfa include changes in chromosome number and structure and changes in chloroplasts (Bingham *et al.* 1988). In studies in which alfalfa plants were regenerated from protoplasts of alfalfa with the normal tetraploid chromosome number, of five variants studied, one was hexaploid, one was near heptaploid, and three were near octoploid (Latunde-Dada & Lucas, 1983). Groose and Bingham (1984) studied aneuploidy in a tetraploid alfalfa donor that was bred to be hetrozygous for four heritable traits. The heterozygous traits were (1) flower colour (loss of a dominant alleles would change flower colour from purple to white); (2) multifoliolate leaves (loss of dominant alleles would shift the plant from trifoliolate to multifoliolate leaves); (3) ability to regenerate (loss of a dominant allele would reduce or prevent regeneration); and (4) nuclear restoration of cytoplasmic male sterility (loss of dominant alleles would shift plant from male fertile to sterile. Johnson *et al.* (1984a) assessed 11 protoclones with chromosome structural changes among about 100 protoclones. A change in chlorophyll pigmentation arose in the tissue culture of a progeny of WFM reported by Bingham and McCoy (1986). A variant with chlorophyll deficient sectors (CDS) regenerated along with 25 normal green plants. The white-flowered donor did not yield regenerates that were revertant for flower colour; flower colour was stable white (Bingham & McCoy, 1986). The

application of tissue culture techniques to a crop for the production of somaclones requires that cultures can be initiated readily and then subsequently regenerated into multiple regenerants, which in turn can be screened for variation *in vitro* or *in vivo*. Direct *in vitro* selection for salt tolerant genotypes of alfalfa (*Medicago sativa*) has involved the addition of up to 171 mol m^{-3} NaCl to callus media (Croughan *et al.*, 1978; McCoy, 1987; Smith & McComb, 1983; Winicov, 1991). To identify mutations, selection pressure is applied *in vitro* to a large population of somatic cells with the aim of uncovering the desired phenotype. In this way the number of plants that eventually require field evaluation can be reduced thus representing a considerable saving in time and space (Collin & Dix, 1990). Selection can be carried out *in vitro*, by culturing either callus, cell suspensions, protoplasts, plantlets, embryos, or microspores in the presence of a screening agent (Collin & Dix, 1990).

Most of the past attempts at the isolation of salt tolerance by cell culture have been inconclusive. Selected salt tolerant cell lines of tobacco have only given rise to one NaCl tolerant plant, and there were preliminary reports of regenerated salt tolerant plants obtained from oats and rice (Nabors *et al.*, 1980, Nabors & Dykes, 1985) but no subsequent commercial cultivars. Watad *et al.* (1991) indicated that resistance to salt in salt tolerant somaclones of tobacco was effective and stable at the cellular level and after plant regeneration. Heritable somaclonal variation has been observed in wheat (Larkin *et al.*, 1984) with both homozygous and heterozygous mutations obtained in the primary regenerated plants. Nevertheless in several examples using alfalfa, salt tolerance in cell lines have not always been transmitted to the whole plant of alfalfa (Bingham & McCoy, 1986; McCoy, 1987a). Another problem was that although salt tolerant cell lines have been selected in a variety of alfalfa, only a limited number of plants could be regenerated from these lines, and their growth was reduced (Stavarek & Rains, 1984), or the regenerated plants were not salt tolerant (Smith & McComb, 1983). Selection of alfalfa callus produced cell lines able to grow in the presence of 171 mol m^{-3} NaCl added to the medium (Croughan *et al.*, 1978; Smith & McComb, 1983). However there was a poor correlation between

growth under NaCl conditions in culture, and the response of the whole plant (Smith & McComb, 1983). In most of their research, salt-tolerant cell lines could not be regenerated, or if plants were established, they did not show the salt tolerance of the cell lines from which they were derived. However, one report on alfalfa (Winicov, 1991) indicated that NaCl tolerance was expressed in plants regenerated from NaCl-tolerant cell lines of the non-commercial variety Regen-S. In some cases, regenerated alfalfa showed abnormal growth, sterility, non salt tolerance, or even increased salt-sensitivity (Smith & McComb, 1981; Stavarek & Rains, 1984; McCoy, 1987). The limits of detection of somaclonal variation are being extended by biochemical analysis (e.g. gel electrophoresis); advanced cytogenetic techniques (e.g. *in situ* hybridization and improved banding techniques); and additional use of molecular tools (e.g. molecular probes). The combination of improved detection methods and proper genetic strategies to separate desired from undesired variation might enhance the usefulness of somaclonal variants as research tools and as gene donors for crop improvement (Bingham & McCoy, 1986).

1.3 Proline accumulation

Plants are exposed to many environmental stresses including extremes of temperature, flood-induced anaerobiosis, high irradiance and hyperosmotic stress. Among these, hyperosmotic stress caused by drought and salinity are the most important factors limiting plant growth and crop productivity (Boyer, 1982). Since the water potential of a saline growth medium or soil is lowered by the osmotic potential of the dissolved solutes, salt and water stresses produce similar plant responses (Ben-Hayyim, 1987). To counteract the effects of osmotic stress, plants have evolved adaptive mechanisms which may be classified into four categories (McCue & Hanson, 1990). Three of these adaptations, developmental traits (e.g. time of flowering), structural traits (e.g. leaf waxiness), and physiological mechanisms (e.g. the ability to exclude salt while maintaining the absorption of

water and the ability to compartmentalize ions within the vacuole), involve complex interactions. Hence the gene products controlling these traits remain largely uncharacterized. The fourth category involves metabolic responses, such as alteration in photosynthetic metabolism (Cushman *et al.*, 1992) and the accumulation of compatible osmolytes. The latter could be due to a small number of gene products some of which may be identified by investigation of osmotically induced changes in protein and mRNA populations (Singh *et al.*, 1985; Winicov *et al.*, 1989). The best characterized biochemical response of plant cells to osmotic stress is the accumulation of organic osmolytes, most commonly proline and betaines (McCue & Hanson, 1990). A large body of data indicate a positive correlation between proline accumulation and adaptation to salt or drought stress (Delauney & Verma, 1993). There is also evidence that proline accumulation is an integral component of an adaptation by the cell to hyper osmotic stress and this comes from studies of osmoregulation in bacteria. Proline has been shown to be an efficient osmoprotectant in bacteria, and proline-over-producing mutants of *Escherichia coli* do exhibit increased osmotolerance (Csonka, 1989; Hanson *et al.*, 1994). Exogenous applied proline protected differentiated (seedlings) but not undifferentiated (callus) *N. sylvestris* against salt stress (Dix *et al.*, 1984). Santos-Dias and Ochoa-Alejo (1994) observed a positive correlation between the proline levels in the culture cells of *Capsicum annuum* L. and *Larrea tridentata* and the degree of resistance to PEG. The accumulation of this amino acid may be part of a general adaptation to adverse environmental conditions, having been documented in response to several stresses (Delauney & Verma, 1993). Proline appears to be the most widely distributed osmolyte accumulated under stress conditions not only in plant but also in eubacteria, protozoa, marine invertebrates and algae (see McCue & Hanson, 1990). Several possible physiological functions have been suggested which induce proline accumulation as a consequence of water shortage, such as osmoregulation, a sink for energy and nitrogen, a signal of senescence, and an indicator of drought resistance (Aspinall & Paleg, 1981; Heuer, 1994). Proline may

affect the solubility of various proteins (Paleg *et al.*, 1984), thus protecting them against denaturation under water-stressed conditions, or it may do so by controlling the cell pH (Venekamp, 1989; Irigoyen *et al.*, 1992). There is evidence that proline improves the stability of some cytoplasmic and mitochondrial enzymes (Nash *et al.*, 1982) and enhances the desiccation tolerance of somatic embryos (Kim & Janick, 1991). Proline was the most actively accumulated amino acid, both in terms of the total amount of proline accumulated and the percentage increase during drought stress. The increase resulted from an increased flux of glutamate to pyrroline-5-carboxylate and proline in the proline biosynthetic pathway (Rhodes *et al.*, 1986, Goods & Zaplachinski, 1994). The biosynthetic pathway leading to proline synthesis comprises four steps under the control of only two enzymes (Appendix, 3.1). A bi-functional enzyme, 1-proline-carboxylate synthetase (P5CS) initiates first the phosphorylation of glutamate as a result of its γ-glutamylkinase activity, followed by the reduction of γ-glutamyl phosphate to glutamic-γ-semialdehyde (GSA) by GSA dehydrogenase. The genes encoding P5C synthase and P5C reductase were both cloned from plant c-DNA banks by complementation of the corresponding bacterial auxotrophic mutants (Delaunay & Verma, 1990a; Hu *et al.*, 1992). The availability of a cloned P5C synthase gene has already shown that the plant enzyme is feedback inhibited by proline and that the level of P5CS transcripts can be enhanced under salt stress (Delaunay & Verma, 1993). The possibility of proline synthesis via ornithine has also been suggested by Delaunay and Verma, (1993). In alfalfa growth reduction due to drought stress was accompanied by increased amino acid levels in shoots, roots and nodules (Schubert *et al.*, 1995). Withholding irrigation stimulated proline accumulation in leaves and nodules (Irigoyen *et al.*, 1992) and proline levels were shown to be always higher in drought-stressed plants (Antolin & Sanchez-Diaz, 1992).

This effect was not corroborated by studies on *Lycopersicum esculentum* and *L. pennellii* (Pérez-Alfocea *et al.*, 1994). In addition, salt-induced proline synthesis was not always rapid, beginning only when cell injury was evident (Moftah & Michel,

1987). Furthermore, elevated levels of proline were maintained for as long as a month after the stressed cells were returned to normal osmotic conditions (Chandler & Thorpe, 1987).

The absence of a positive correlation between proline accumulation and osmotolerance in some species does not negate an adaptive role for proline; rather, it may reflect the predominance in these species of osmoregulatory mechanisms other than osmotic adjustment, e.g. morphological or physiological mechanisms. Moreover, these results can be rationalized if the osmoprotective function of proline is assumed to involve minimizing the effects of a particular form of cell damage and not simply adjusting the intracellular osmotic potential (Caplan et al., 1990). This is consistent with indications from bacterial studies that proline (and glycine betaine) enhance the stability of proteins and membranes in environments of low water activity or high temperatures (Csonka, 1989).

1.4 Active oxygen species and antioxidative systems

Water and salt stresses have been proposed as oxidative stresses which can damage cell structure and metabolism. Plant cells are normally protected against such effects by the action of enzymatic and non-enzymatic defence systems. When molecular oxygen is incorporated into organic molecules, relative intermediates such as superoxide anion (O_2^-), hydroxyl radical (OH^-), single oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2) are produced (Badiani et al., 1990; Scandalios, 1990). The complete four-electron reduction of oxygen to water proceeds most readily via a stepwise pathway which can also generate partially reduced intermediates, Fig. 1.3 (Scandalios, 1990).

However, physiological events (Asada, 1993, Gossett et al., 1994) and biotic or abiotic pathogenesis (Doke et al., 1993, Foyer et al., 1994) can enhance the formation of toxic oxygen species and damage can occur (Badiani et al., 1990).

Active oxygen species are produced, directly or indirectly, by many diverse cellular reactions and most intracellular compartments have been implicated in their generation. The chloroplasts for instance are potentially the most powerful source of oxidants in plant tissue (Foyer & Harbinson, 1993).

$$^1O_2$$

$$O_2 \xrightarrow[H^+]{+e^-} O_2^{-} \xrightarrow[H^+]{+e^-} H_2O_2 \xrightarrow[H^+]{+e^-} OH^- \xrightarrow[H^+]{+e^-} H_2O$$

(with hv and $+e^-$, H^+ pathway to $^1O_2$)

(Overall reaction $O_2 + 4e^- + 4H^+ \longrightarrow 2H_2O$)
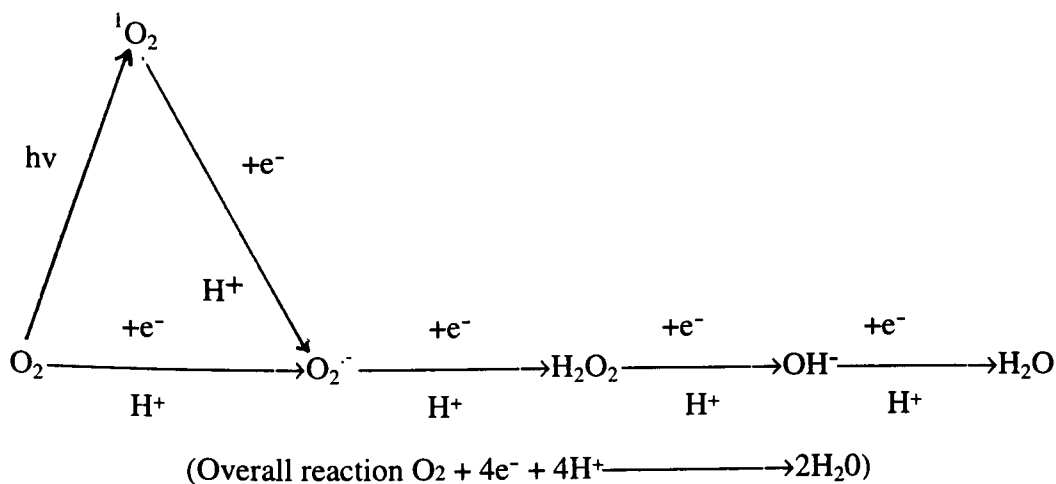
Fig. 1.3 Pathways of oxygen reduction to water leading to the formation of various intermediate reactive oxygen species.

Activated oxygen species can damage cell structure and metabolism either through their oxidizing activity or by inducing the formation of organic free radicals. The effect of oxidative stress may range from simple inhibition of enzyme function to the production of random lesions in proteins and nucleic acids (Scandalios, 1990). The main cellular components susceptible to damage by free radicals of oxygen are lipids (peroxidation of unsaturated fatty acids in membranes), proteins (denaturation of enzymes), carbohydrates (cleavage of polysaccharides), and nucleic acids (Monk & Davies, 1989). These highly reactive oxygen species can react with unsaturated fatty acids causing peroxidation of essential membrane lipids in the plasmalemma or in intracellular organelles (Fridovich, 1976). Peroxidation damage to the plasmalemma leads to the leakage of cellular contents, rapid desiccation and cell death. Several

Calvin-cycle enzymes within plant chloroplasts are very sensitive to hydrogen peroxide, and high levels of H_2O_2 can directly inhibit CO_2 fixation (Robinson et al., 1980; Charles & Halliwell, 1981). Most importantly, the hydroxyl radical can attack lipids and proteins indiscriminately and causes lesions in the DNA (Lesko et al., 1980). Some sources and effects of activated oxygen species are shown in Table 1.3.

Injury induced by drought stress on the photosynthetic apparatus, for instance chlorophyll destruction, may be due to these toxic oxygen forms. The available evidence clearly suggests that the production of active oxygen species is a general alarm signal which alerts metabolism and gene expression for possible modifications. It is the first defence against invading stress, and if unchecked, leads to disruption of cell function and death (Scandalios, 1990). Active oxygen species are highly destructive; their prolonged presence is not compatible with cell function or structure. The capacity of the antioxidative system thus determines the fate of the cell; whether the cell continues to function or suffers photo-oxidation, destruction of tissue and death (Foyer et al., 1994). Resistance to stress may therefore involve the antioxidant enzyme activities.

To counteract the toxicity of active oxygen species, a highly efficient antioxidative defence system, composed of both non-enzymatic and enzymatic constituents, is present in all plant cells (Halliwell & Gutteridge, 1985). The non-enzymatic antioxidants are generally small molecules and low-molecular-weight non-protein sulphyryls, such as glutathione, cysteines and cysteinylglycine (Foyer et al., 1994). The enzymatic constituents include enzymes capable of removing, neutralizing or scavenging free radicals and oxyintermediates, such as catalase, superoxide dismutases, ascorbate peroxidase and other peroxidases (Scandalios, 1990).

Table 1. 3. Some sources and effects of activated oxygen species[a]

Oxygen species	Sources	Biological effects
Molecular oxygen (O_2)	Atmospheric oxygen, photosystem II ($2H_2O \rightarrow 4H^+ + 4e^-O$, and various enzymes (catalase and superoxide dismutase)	Inhibition of photosynthesis (competes with CO_2 for RUBP carboxylase) and random production of free radicals
Superoxide radical (O_2^-)	Illuminated chloroplasts, herbicides (paraquat and nitrogen), and enzymatic reaction (uricase, aldehyde oxidase, xanthine oxidase, flavin dehydrogenases, and oxidation of ferredoxin$_{red}$)	Membrane lipid peroxidation, enzyme inactivation, depolymerization of polysaccharides, reaction with H_2O_2 to form $OH_.$, aging, and autoimmune diseases
Hydrogen peroxide (H_2O_2)	Glycolate oxidase in glyoxysomes, illuminated chloroplasts, and enzymatic reactions (superoxide dismutase, urate oxidase, and various peroxisomal enzymes)	Inhibition of CO_2 fixation, marking of some proteins (e.g., glutamine synthetase) for proteolytic degradation, oxidation of flavonols and sulfhydryls, mutagenesis, and inactivation of Calvin cycle enzymes
Hydroxyl radical ($OH^.$)	Haber-Weiss reaction $H_2O_2 + O_2^{-.} \rightarrow OH^- + OH^. + O_2$ Fenton reaction $H_2O_2 + Fe^{2+} \rightarrow OH^- + OH^. + Fe^{3+}$	Most potent oxidant known! Causes DNA lesions, protein degradation, peroxidation of membrane lipids, ethylene production, and is implicated in rheumatoid arthritis
Singlet oxygen ($^1O_2$)	Photoexcited chlorophyll molecules in triplet state, air pollutants (NO_2, O_3, etc.), and pathogenic fungi (cercosporin toxin)	Mutagenesis, membrane lipid peroxidation, and photooxidation of amino acid

a From Scandalios (1990).

Catalases (EC 1.11.1.6; H_2O_2: H_2O_2 oxidoreductase; CAT) and peroxidase (EC 1.11.1.7) are classes of enzymes that remove H_2O_2 very efficiently, as depicted below:

$$H_2O_2 + H_2O_2 \xrightarrow{\text{CAT}} 2H_2O + O_2 \quad (K1 = 1.7 \times 10^7 \ M^{-1} \ Sec^{-1})$$

$$H_2O_2 + R(OH)_2 \xrightarrow{\text{POD}} 2H_2O + R(O)_2 \quad (K4 = 0.2\text{-}1.10^3 \ M^{-1} \ Sec^{-1}$$
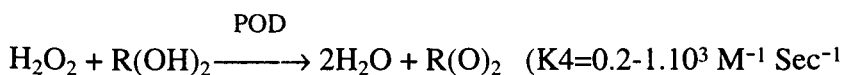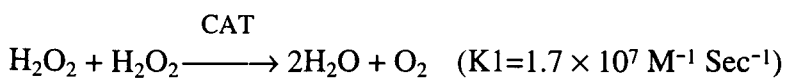
Superoxide dismutase (EC 1.15.1.1; $O_2{}^-$: $O_2{}^-$ oxioreductase: SOD) is a class of metal-combining proteins that are highly efficient at scavenging the superoxide radical ($O_2{}^{\cdot-}$). The enzyme catalyses a disproportionation reaction at a rate very near that of diffusion.

$$O_2 + e^- \longrightarrow O_2{}^{\cdot-}$$

$$O_2{}^{\cdot-} + O_2{}^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

$$H_2O_2 + H_2O_2 \xrightarrow{\text{CAT or GR}} 2H_2O + O_2$$

$$H_2O_2 + R(OH)_2 \xrightarrow{\text{AP}} 2H_2O + R(O)_2$$
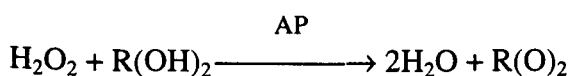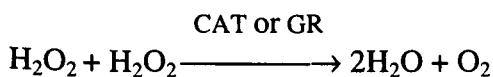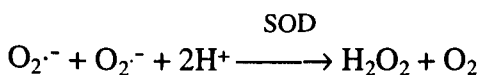
It is perhaps this series of co-operative interactions that play a more crucial role in the detoxification of reactive oxygen species rather than the elimination of $O_2{}^{\cdot-}$ or H_2O_2. This process operates in the cytoplasm and chloroplast (Scandalios, 1990; Polle & Rennenberg, 1993). Although activated oxygen is produced as a by-product of normal cell metabolism, its levels are enhanced by exposure to chemical and environmental stress. Plants with high levels of antioxidants, either constitutive or induced, have been reported as having greater resistance to this oxidative damage (Gossett *et al.*, 1993; 1994; Foyer *et al.*, 1994). In addition, prior exposure of cells to

agents that induce superoxide dismutase (SOD) and catalase (CAT) activity caused a partial protection against oxidative stress (Gregory & Fridovich, 1973). Catalase prevents oxidative damage to DNA during aerobic growth and protects cells from oxidative damage by converting this active-oxygen species into oxygen and water (Halliwell, 1982). Ascorbate is present in chloroplasts, cytosol, vacuole and apoplastic space of leaf cells at high concentrations (Polle et al., 1990; Foyer et al., 1991a). Ascorbate peroxidase (AP) is perhaps the most important antioxidant in plants with a fundamental role in the removal of hydrogen peroxide (Foyer et al. 1994). Irigoyen et al. (1992) found SOD activity in alfalfa leaves was not affected by drought stress. In contrast, CAT and peroxidase were significantly inhibited by slight drought stress. Glutathione has a major role in sulphur transport and is involved in a number of cellular processes in plants. It acts as a protein disulphide reductant which detoxifies herbicides by conjugation, either spontaneously or by the activity of one of a number of glutathione-S-transferases, and regulates gene expression in response to environmental stress and pathogen attack (Dron et al. 1988; Wingate et al., 1988). There was a progressive increase in glutathione reductase (GR) activity and in the cellular antioxidant system in the leaves of water stressed field-grown cotton (*Gossypium hirsutum* L.) plants compared to the irrigated controls (Burke et al., 1985).

1.5 The effect of environmental stress on the antioxidant system

Plants are exposed in their environment to a wide range of different stresses, which can originate from human activities or may have natural causes. As plants have only limited mechanisms for stress avoidance, they require flexible means for adaptation to changing environmental conditions (Polle & Bennenberg, 1993). Activate oxygen species such as superoxide, hydrogen peroxide, and hydroxyl radical are associated with a number of physiological disorders in plants. Increased production of activated oxygen species has been associated with the development of injury symptoms

resulting from diverse environmental stresses, including drought (Price & Hendry, 1991) and salinity (Gossett et al., 1994). Reactive oxygen species are also generated in plant cells during normal metabolic functions, especially in chloroplasts during photosynthesis (Salin, 1988).

Tolerance to these environmental stresses correlates with an increased capacity to scavenge or detoxify activated oxygen species (Kendall & Mckersie, 1989; Malan et al., 1990). There was an increase in glutathione reductase activity and in the cellular antioxidant system in the leaves of water stressed field-grown cotton (Gossypium hirsutum L.) plants compared to the irrigated controls (Burke et al., 1985). Irigoyen et al. (1992) found catalases were significantly stimulated by moderate drought stress. Safarnejad (1992), found that with increasing salt and mannitol, the activity of catalase, superoxide dismutase, and glutathione reductase increased in the callus of tolerant and moderately tolerant wheat varieties, while is remained constant or decreased in the sensitive ones. Gossett et al. (1993) examined the relationship between antioxidant enzymes and salt tolerance in eight-week-old cotton leaves of two salt-tolerant and two salt-sensitive cultivars. The more salt-tolerant cultivars contained significantly higher constitutive levels of catalase (CAT) and induced levels of peroxidase and glutathione reductase (GR). They also found a positive correlation between increased antioxidant activity and salt tolerance in both the callus tissue and whole plant studies. Cross breeding of two maize cultivars, which resulted in increased activity of two antioxidant enzymes, superoxide dismutase (SOD) and GR, improved protection from oxidative stress (Malan et al., 1990). During the initial period of salt stress, mesophyll cells of barley were able to maintain higher SOD activity and with increasing intensity of salt stress, SOD activity decreased (Ming et al., 1989). The activity of SOD, which is responsible for dismutating superoxide ($O_2^{\cdot-}$) to H_2O_2, showed a marked decline in Vigna catjang and Oryza sativa under salinity stress (Singha & Choudhuri, 1990). Although the reduced activity of SOD minimized the generation of H_2O_2 from $O_2^{\cdot-}$, the simultaneous decrease in CAT activity and the increase in glycolate oxidase activity

can explain the accumulation of H_2O_2 in the tissues of *Vigna catjang* and *Oryza sativa* under salinity stress. This H_2O_2 may thus be responsible for the degrative reactions of plant tissues subjected to salinity stress (Singha & Choudhuri, 1990). Deleterious effects also occurred in transgenic plants in which manganese superoxide dismutase was slightly overproduced (Bowler *et al.*, 1991). It was suggested that over production of SOD caused an increased H_2O_2 production which overwhelmed the capacity for H_2O_2 decomposition and, thereby, shifted O_2^- and H_2O_2 levels towards a ratio favourable for $OH\cdot$ production (Bowler *et al.*, 1991). In experiments conducted with leaf peroxisomes purified from two cultivars of *Pisum sativum L.* with different sensitivity to NaCl, salt stress decreased catalase activity (Olmos *et al.*, 1994). However, despite this research on the effect of salt-stress in activating oxygen related enzymes further work needs to be carried out to examine this role in the overall response of plants to salt stress.

1.6 Molecular markers for salt and drought resistance

For thousands of years man has been breeding plants by selecting individuals that are higher yielding, better tasting, or otherwise more attractive (Tanksley *et al.*, 1989). Crop varieties first selected by early man and later improved by modern plant breeders form the basis of the sophisticated and highly productive agricultural economy (Tanksley *et al.*, 1989). Biochemical and molecular genetic markers are now finding many applications in plant breeding programs and genetic studies. Biochemical methods, such as isozyme analysis, have been used to distinguish between homozygous and heterozygous individuals and to determine the degree of genetic variability in plant populations (Melchinger *et al.*, 1991). However, isozyme analyses are limited by the small number of marker loci that are available, a general lack of polymorphism for these loci in elite breeding materials, and the variability in banding patterns due to plant development (Tanksley & Orton, 1983; Tanksley *et al.*, 1989). Molecular analysis of the genome at the DNA level can provide a greater

advantage because DNA sequences are the same in all of the living cells of a plant, regardless of physiological or developmental states of the tissue. Direct DNA diagnosis would allow the immediate detection of specificity or alteration of the plant genome. Molecular genetic markers are increasingly finding applications in plant breeding programs and genetic studies. These markers have been used to develop genetic linkage maps for many agriculturally important species, including alfalfa (Echt *et al.*, 1993). Gene analysis or genome analysis, using DNA hybridization techniques, have become a useful tool in the selection of certain character to improve crops. Saiki *et al.* (1985) developed a polymerase chain reaction (PCR) protocol to study very small amount of plant material (1 mg fresh weight). Thus, the presence of a defined nucleotide sequence can be determined at a very early stage of development. In combination with PCR, the whole spectrum of techniques making use of molecular markers can now be applied to *in vitro* selection (Wenzel & Foroughi-Wehr, 1993).

Screening for polymorphisms at the population level requires that a large number of individuals are compared. This is achieved by amplifying specific regions of DNA by PCR followed by direct sequencing, then analysing the pattern of change across the sequence. Random amplified polymorphic DNA (RAPD) is one of PCR techniques that is relevant to the study of population-level variation (Williams *et al.*, 1990). Bagheri *et al.* (1995) showed RAPDs to be useful for clarifying phylogenic relationships within a species, and also to provide useful genetic markers for varietal identification in peas (*Pisum sativum* L.). RAPD patterns have been used to determine genetic distances among heterogenous population and cultivars which correspond to their known relatedness (Yu & Pauls, 1993). RAPD analysis of sugarcane plants regenerated from embryogenic callus revealed very few polymorphisms (Taylor *et al.*, 1995). Whereas RAPDs detected gross genetic change in protoplast derived sugarcane callus (Taylor *et al.*, 1995). Increases and decreases in copy number of repeated sequences were observed in alfalfa plants from successive regeneration cycles (Kidwell & Osborn, 1993), indicating that specific

repeated nuclear DNA sequences have changed copy number in plants regenerated from tissue culture.

The list of genes whose transcription is up-regulated in response to stress is rapidly increasing. These polypeptides are believed to have a role in stress physiology (Bartels & Nelson, 1994). Drought induced plant responses are largely the result of changes in gene expression. Major changes in gene expression also occur in response to NaCl, which results in altered patterns of mRNA or protein accumulation (Hurkman & Tanaka, 1987; Hurkman *et al.*, 1991; Winicov *et al.*, 1989; Winicov & Deutch, 1994). However, it has often been difficult to distinguish between the primary and secondary effects of salt stress and to distinguish between those responses which are functionally adaptive, and those which are simply indicative of salt toxicity (Winicov & Deutch, 1994). Some responses may be correlated to cell damage while others may be a result of an adaptive response (Bray, 1994). During the last few years a number of drought-induced genes have been identified in a wide range of plant species. These include RAB17 which is a maize protein known to accumulate in desiccating embryos and to be induced by ABA and water stress in seedlings (Vilardell *et al.*, 1990). Ferritin is an iron storage protein which is induced by ABA and water stress in maize plantlets (Zivy *et al.*, 1995). The 26-kDa protein osmotin is involved in the adaptation of cultured tobacco cells in response to NaCl and water stress (Singh *et al.*, 1985). The gene for this protein has been cloned (Singh *et al.*, 1989) and in tomato a similar gene (*Np24*) has been identified (King *et al.*, 1988). However osmotin also occurs in the basal leaves and floral organs of tobacco (Neale *et al.*, 1990). The *salT* gene is expressed in the roots and sheaths of mature plants and seedlings of rice in response to salt, abscisic acid (ABA), or dehydration (Claes *et al.*, 1990). Some other gene products have been found to accumulate in barley roots during salt stress, such as two germin-like proteins (GS1 and GS2) which had high sequence similarity to germin (Hurkman *et al.*, 1991). Transcripts were also found encoding the enzymes involved in proline biosynthesis in mothbeans and peas (Hu *et al.*, 1992; Williamson & Slocum, 1992).

One of the methods used is the polymerase chain reaction (PCR) which is a basic tool in molecular biology and is employed in a variety of applications including forensic and clinical diagnostic procedures (Saiki *et al.*, 1985; Kazzazian, 1989). A particular advantage of PCR is that it requires little biological material as a starting template and offers a rapid method for the analysis of DNA samples (Panaccio & Lew, 1991). The PCR is an *in vitro* method for the enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. A repetitive series of cycles involving template denaturation, primer annealing, and the extension of the annealed primers by DNA polymerase results in the exponential accumulation of a specific fragment whose termini are defined by the 5' ends of the primers. Because the primer extension products synthesized in one cycle can serve as a template in the next, the number of target DNA copies approximately doubles at every cycle. Thus, 20 cycles of PCR yields about a million-fold (2^{20}) amplification. This method was invented by Kary Mullis (Mullis & Faloona, 1987).

Three other PCR techniques that are relevant to the study of population variation are RAPD (random amplified polymorphic DNA) (Williams *et al.*, 1990) PCR, the analysis of variation in microsatellite DNA (Tautz, 1989), and the use of GC-clamps on denaturing gradient gels (Myers *et al.*, 1989).

In recent years, attention has focused on alterations in gene expression. Drought induced plant responses are largely the result of changes in gene expression. Major changes also in gene expression, occur in response to NaCl (Winicov & Deutch, 1994). For example, the 26-kDa protein osmotin is involved in the adaptation of cultured tobacco cells in response to NaCl and water stress (Singh *et al.* 1985) a gene for this protein has been cloned (Singh *et al.* 1989). The *salT* gene is expressed in the roots and sheaths of mature plants and seedlings of rice in response to salt, ABA, or dehydration (Claes *et al.*, 1990). In alfalfa, Winicov *et al.* (1989) have shown that the acquisition of salt-tolerance by callus cells is accompanied by

increased expression of a subset of genes. These genes may form the basis of a marker system for salt and drought tolerance.

1.7 Anther culture of alfalfa

The approach that could offer a significant contribution to the improvement of alfalfa is the induction of haploid plants through microspore, anther, or ovule culture. The haploids are of fundamental importance for the study of the genetic structure, and represent the material for the constitution of tetraallelic lines, avoiding the use of lines with high production of unreduced gametes; in addition they could be used for obtaining somatic hybrids between sexually incompatible species without increasing the DNA content (Arcioni *et al.* 1990). The most appropriate starting material for the production of haploids are the gametes. Consequently, two principal methods exist for the induction of plants with a simpler genome, either starting from the female (parthenogenesis) or male gametophyte (androgenesis), which means the *in vitro* regeneration of pollen or the induction of haploid tissue from the egg cell (Wenzel *et al.*, 1995). The principal attraction of anther culture is that (1) a huge population of single haploid cells is available; (2) selection can be carried out at the earliest breeding stage; (3) the genotypes derived from chromosome doubling will be homozygous for selected individuals and recessive traits as a consequence of chromosome doubling (Wenzel & Foroughi-Wehr, 1993).

The production of embryos from developing pollen cells in *Datura* (Guha & Maheshwari, 1964) has led to considerable interest in the use of anther-derived haploids in breeding and genetic studies. Anther culture is well developed in species such as barley and tobacco and a considerable body of knowledge has been generated on the biology of androgenesis, both *in vitro* and *in vivo* (Foroughi-Wehr & Wenzel, 1993). Although there have been isolated cases of haploid plant regeneration from cultured legume anthers, there has been no systematic study of anther culture with these species. The production of haploid callus from cultured

anthers has been reported in a number of legumes including *Cicer arietinum, Vigna mungo, Pisum sativum* (Gosal & Bajaj, 1988). The pasture legumes have not been studied as frequently. Attempts to culture anther tissue from the legumes, such as alfalfa (Bingham, 1971; Saunders & Bingham, 1972), produced callus only from the maternal anther-wall tissue. There are only isolated reports of haploid plant regeneration from *Lotus corniculatus* (Tomes & Pterson, 1981), *Trifolium pratense* (Bhojwani *et al.* 1984), *T. alexandrium* (Mokhtarzadeh & Constantin, 1978) and *M. sativa* (Zagorska *et al.*, 1984; Zagorska & Dimitrov, 1995). In the past no data was provided for the frequency of alfalfa haploid plant regeneration from cultured anthers. Anther culture of *M. sativa* lines, selected for high regeneration ability through somatic embryogenesis, was carried out by Piccirilli *et al.* (1989) and Tanner *et al.* (1990), but all the regenerated plants showed the normal somatic chromosome number (2n=4x=32).

1.8 Research programme

The aim of this Ph.D was to investigate the application of *in vitro* techniques and molecular biology to improve salt and drought tolerance of alfalfa. The variety of alfalfa cv. CUF 101 is grown extensively and does show some salt tolerance. Attempts to increase this salt tolerance by *in vivo* screening using seeds have produced a more tolerant line, CUF101-1S (Al-Khatib *et al.*, 1993). Our attention has been focused on the production of further variation by tissue culture from this line, the selection for more NaCl tolerant lines and their analysis by molecular genetic techniques. The objectives were as follows:

1. Initiation of tissue culture and regeneration of the commercial variety CUF 101 and the line CUF101-1S.

2. Maximizing the regeneration frequency in tissue culture of CUF 101 and CUF101-1S.

3. Initiation of an *in vitro* screen for NaCl tolerance using regenerating cultures derived from CUF 101 and CUF101-1S.

4. Regeneration of CUF101-1S from anther culture.

5. Determination of the whole plant response of CUF 101, CUF101-1S and progeny of regenerants of CUF101-1S to salinity and osmotic stress.

6. Characterization of progeny of somaclones based on biochemical and molecular techniques.

CHAPTER 2.

Regeneration of Callus Cultures

2. Regeneration of Callus Cultures

2.1 INTRODUCTION

Plant regeneration from tissue culture is fundamental to the application of biotechnology for crop improvement. Many species of plants can now be regenerated from tissue culture and particularly species such as *Nicotiana, Petunia,* and *Lycopersicum,* within the family *Solanacae.* Major crops such as maize (*Zea mays* L.), wheat (*Triticum aestivum* L.) and many forage legumes, however, are more difficult to regenerate from callus culture (Quesenberry & Smith, 1993). Embryo induction and plant regeneration from tissue cultures are determined by the genotype and physiological status of the donor plant, the plant organ used as source of explants, the culture medium, and the interaction between them (Mathias & Simpson, 1986c; Bregitzer, 1992). The ability of callus tissue to regenerate plants also appears to be under the genetic control of major genes in two systems, one located in the nucleus and the other in the cytoplasm (Nesticky *et al.,* 1983; Wan *et al.,* 1988). The genes that control somatic embryogenesis, as reviewed by Henry *et al.* (1994), seem to be independent of those that control microspore embryogenesis in wheat (Agache *et al.,* 1988), rye (Lazar *et al.,* 1987) and maize (Armstrong *et al.,* 1992). Taylor and Veilleux (1992) also found in *Solanum phureja* no significant correlation between anther culture competence, protoplast culture ability and leaf disc regeneration, implying three separate genetic mechanisms.

The induction of embryogenesis in alfalfa (*Medicago sativa* L.) showed wide variation between genotypes (Bingham *et al.,* 1975). Callus growth and embryogenesis showed variation both between cultivars and within many of the 76 cultivars of alfalfa (*M. sativa* L., *M. falcata* L., *M. varia* Martyn) tested by Brown & Atanassov (1985). Mitten *et al.* (1983) found that of 35 tetraploid alfalfa cultivars

---

only a few genotypes could be regenerated from hypocotyl derived tissue cultures. Even though the frequency of regeneration within a cultivar or genotype may be high there was much variation in the efficiency of regeneration (Mitten *et al.*, 1984). Any variation was attributed to the intervarietal and intravarietal heterogeneity in alfalfa, which is derived from the fact that it is an open pollinated species (Wan *et al.*, 1988). *In vitro* embryogenic responses of nine varieties of alfalfa grown in Mexico were examined by Fuentes *et al.* (1993). The response of each variety was low when tested using four tissue culture protocols. Percentage of regeneration in the non-commercial variety, Regen-S increased from 12% initially to 67% after two cycles of recurrent selection for regenerated callus (Bingham *et al.*, 1975) which suggested that *in vitro* regeneration is a highly heritable character and that regeneration potential can be increased through one or a few cycles of selfing or backcrossing.

The range of germplasm of alfalfa from which plant regeneration has been reported is very narrow. From a total of 443 genotypes, only 27 were capable of regenerating plants from hypocotyl callus (Matheson *et al.*, 1990). They represented an overall regeneration frequency of 6.1% that was the highest in Regen-Y and Sc-3702, which both contain *M. falcata* germplasm. In general the number of regenerative genotypes from which plant populations have been established and evaluated is even more limited. Most studies of this kind were limited to a non-commercial variety, Regen-S, which was developed specifically for its ability to regenerate reliably from callus (Bingham *et al.*, 1975).

The aim of the work described in this chapter was to initiate regeneration and to select for increased capacity for plant regeneration from callus tissue cultures of a commercial alfalfa variety, CUF 101.

2.2 MATERIALS AND METHODS

2.2.1 Plant materials

Six progeny lines of CUF 101 (second generation seeds, S2) showing moderate salt tolerance, were produced at the University of Liverpool (Al-Khatib *et al.*, 1993). The lines were designated CUF101-1S, -6N, -3F, -4A, -2A and -5R. Seeds of parent CUF 101 were supplied by Cleanseed Pty Ltd (POBox 31, Bongendore NSW, Australia 2621). Regen-SY seeds were supplied by Prof. E.T. Bingham (University of Wisconsin, Madison, W153706).

2.2.2 Initiation and regeneration of tissue culture

Seeds were sterilised by 0.1% w/v mercuric chloride and 0.1% w/v sodium dodecyl sulphate for 10 min., followed by 15% v/v 'Domestos' bleach for 20 min., then washed in five changes of sterile distilled water. Surface sterile seeds were placed singly on the surface of 10 ml aliquots of a germination media containing either Murashige and Skoog (1962) (MS), or half strength Schenk & Hildebrandt (1972) (SH) media, with 1% w/v sucrose, no growth regulators and 0.8% Difco Bacto Agar. The media was contained in Universal vials which were maintained at 25°C under fluorescent light giving a 12h photoperiod.

After 8 days, cotyledon tissue and 1 cm length of hypocotyl were placed separately on the surface of 10 ml BII medium (Saunders & Bingham, 1975) and modified SH medium (Schenk and Hildebradt, 1972) in Universal vials (Table 2.1). After two weeks, each complete explant with all its attached callus was transferred to regeneration media (Table 2.1), Boi2y (Saunders & Bingham, 1975), modified SH medium (Walker *et al.*, 1979) (DS), modified SH medium (Stavarek *et al.*, 1980) (KS), and SHAP medium (Wan *et al.*, 1988), and incubated as before. After two to five weeks large numbers of buds were visible on the callus (Fig. 2.1) in Boi2y

medium but not in the others. When larger these shoots (Fig. 2.2a,b) were individually transferred to a modified Schenk and Hildebrandt medium (1972) (GS) containing 1% w/v sucrose, no growth regulators and 0.8% Difco Bacto Agar. After two weeks, well rooted plantlets (Fig. 2.3a) were transferred to Jiffy-7 peat moss containers (Jiffy products Ltd., Norway) in a glasshouse (Fig.2.3b). After 10 days plants were transplanted into a soil-sand-peat moss mixture (3:1:1) and grown in a glasshouse to flowering. *Medicago sativa* is normally considered to be self-incompatible (Gartner, 1965). However, Ockendon and Currah (1971) has shown that self pollination in *Brassica oleracea* can take place by pollination between flowers in inflorescences on different branches of the same plant. Inflorescences were self pollinated by hand and covered immediately with non moisture-proof glassine bags for a week (Sayers & Murphy, 1966). The bags were then removed to allow seeds to mature. The seeds from regenerants were then subjected to two further cycles of tissue culture initiation and regeneration, to allow assessment of regeneration frequency. The plants derived from the second and third tissue culture cycles of cotyledon and hypocotyl material were grown to flowering and cross pollinated within plants derived from the same clone and allowed to set seed (Fig. 2.4).

2.3 RESULTS

2.3.1 Regeneration of tissue culture

Two weeks after explants of CUF 101, the 6 progeny lines and Regen-SY were transferred to BII and SH media, callus was initiated from all genotypes. At this stage, the explants with attached callus were transferred to Boi2y medium. Two weeks after transfer to Boi2y, occasional buds were visible on some of the explants of CUF 101, and three progeny lines and most of the Regen-SY seedling explants (Fig. 2.5 and 2.6a,b). Three samples of callus showing multiple green areas on the

surfaces were selected from each genotype. During 2 to 5 weeks after transfer of callus to Boi2y medium, many buds and shoots became visible on explants of Regen-SY, CUF 101, and the progeny CUF101-1S,-3F,-6N. The data in Table 2.2 show the regeneration responses of each genotype tested to the media. The number of buds and plantlets were counted on each of 3 calli selected after the stage showing formation of organized green areas. Genotypes with the highest frequency of regeneration were Regen-SY, CUF 101 and CUF101-1S (Table 2.2). Lines CUF101-3F and CUF101-6N also gave a small number of regenerated plantlets. Two genotypes (CUF101-5R, CUF101-2A) did produce buds, but these showed no continued development and no plantlets were produced. One genotype (CUF101-4A) produced no buds. The CUF101-1S line showed the greatest regeneration capacity after Regen-SY, although the frequency of regeneration was still low when compared to Regen-SY. No significant difference was observed between callus initiated from shoot or root explants on SH medium and BII medium ($P>0.05$) (Fig. 2.7). The data showed that both frequency of regeneration and mean number of buds per callus in hypocotyl tissue was in general greater than that from cotyledon explants (Fig. 2.8 and 2.9). On the regeneration medium callus initiated on SH medium was marginally more productive than that initiated on the BII medium (Fig. 2.8 and 2.9).

For example on the callus initiated on the BII medium, the frequency of the regeneration in cotyledon explants was 6% for the parent, CUF101-1S, and the highest somaclone 8R1 was 42% while for hypocotyl explants the regeneration frequency was 3% for CUF101-1S and 8R1 was 57%. The mean number of buds per callus from the cotyledons was 0.39 (CUF101-1S) compared with the highest, 3.11 (9R1) and for the hypocotyl it was 0.07 (CUF101-1S) and 12.00 (8R1) (Fig. 2.8). Of the cotyledon callus initiated on SH medium, the frequency of regeneration was 14% for CUF101-1S and 50% in 7R1 and in hypocotyl derived callus it was 0% for CUF101-1S and 70% in 6R1. The mean number of buds per cotyledon callus was

0.29 (CUF101-1S) and 6.5 for 7R1 and for hypocotyl callus it was 0 for CUF101-1S and 10 for 6R1 (Fig. 2.9).

2.3.2 Improvement in regeneration

Regeneration frequencies were found to be different between explants and genotypes. Most notable was that the percentage regeneration increased significantly in the 2nd cycle of tissue culture for cultures derived from both explant (cotyledon and hypocotyl) and in all genotypes ($P<0.05$) (Fig. 2.10 and 2.11).

In cotyledon explants, the frequency of regeneration showed a 8, 5, 3 and 6 fold increase in 6R, 8R, 7R and 9R, compared with their control. The regeneration frequency increased significantly, in the 3rd cycle of tissue culture in 7R and 9R ($P<0.05$) but there were no significant difference between the 2nd and 3rd tissue culture cycle for 6R and 8R ($P>0.05$) (Fig. 2.10a). There was in general a significant increase in mean number of buds per callus in the 2nd and 3rd tissue culture cycle in all genotypes and a further increase was observed in the 3rd tissue culture cycle of 7R ($P<0.05$) (Fig. 2.10b).

In tissue of hypocotyl origin, the frequencies of regeneration in the 2nd cycle of tissue culture showed a 6, 4, 4 and 5 fold increase in 6R, 8R, 7R and 9R compared with their control. However, there was no significant difference between the 2nd and 3rd cycle of tissue culture of any genotype ($P>0.05$) (Fig. 2.11a). The mean number of buds per callus in the 2nd tissue culture cycle showed a 11, 48, 22 and 28 fold increase in 6R, 8R, 7R and 9R respectively compared with their control (Fig. 2.11b). A further increase was observed for the mean number of buds in the 3rd tissue culture cycle of 72 and 74 fold in 6R and 7R compared with their control ($P<0.001$) but there was no significant difference between the 2nd and 3rd tissue culture cycle of 8R and 9R ($P>0.05$) (Fig. 2.11b).

2.4 DISCUSSION

Although it has been possible to regenerate alfalfa from tissue cultures for almost 20 years, there is no common protocol for the *in vitro* regeneration of alfalfa varieties. The majority of research into regeneration of alfalfa has involved the use of Regen-S and Regen-SY, both lines developed specifically for their ability to regenerate from callus (Bingham *et al.* 1975; Bingham, 1991). In the results reported here, the parent CUF 101 showed very limited regeneration to the plantlet stage compared to Regen-SY, but some independent plants of CUF 101 did survive. By comparison, one of the progeny lines, CUF101-1S, was relatively productive. The data showed not all the CUF 101 progeny were as productive, since some did not show any plantlet formation despite early evidence of bud formation. Although plantlets were produced from CUF101-3F these did not survive to the flowering stages. The frequency of regeneration and bud formation was in general greater from hypocotyl than cotyledon tissues. Fuentes *et al.* (1993) found a genotype dependent response for Mexican alfalfa varieties. Although the response in each variety was low, plants from two varieties (Moapa 69, Sintetico) were regenerated. Skotnicki (1986) found that the callus showed limited green and early bud formation on leaf and stem callus of CUF 101 and plantlet formation on leaf callus. The formation of small green areas on alfalfa callus of recalcitrant varieties has been regarded as an indicator of potential bud formation (Skotnicki, 1986; Fuentes *et al.*, 1993), but then further development into plantlets was not always successful.

The procedure for enhancing regeneration by passage of a genotype through one or more cycles of tissue culture and regeneration was used to produce the Regen-S variety (Bingham *et al.*, 1975). It was shown that a similar approach was successful with genotypes of alfalfa which show a low frequency of regeneration such as CUF 101 and a range of Iranian varieties such as Yazdi, Ghara, Cimerjencaya and Hamedani younjeh and also *Medicago polymorpha* (Safarnejad *et al.*, in press). The results demonstrated that recurrent selection can be applied to increase the frequency

of plant regeneration from callus tissue culture and increase the frequency of plants per explant. The frequency of regeneration of CUF101-1S in the 2nd and 3rd cycles of tissue culture increased up to 70% and 74%. Bingham *et al.* (1975) were also able to increase regeneration of the non-commercial line, Regen-S to 67% in two cycles of tissue culture. Quesenberry and Smith (1993) made five cycles of recurrent phenotypic selection for increased plant regeneration via somatic embryogenesis in Red clover (*Trifolum pratense* L.). The percentage regeneration in the 5 generations (referred to as RPS1, RPS2, RPS3, RPS4, and RPS5) increased from 4, 9, 16, 20, 41, to 72%. Matheson *et al.* (1990) found of 443 alfalfa genotypes screened only 27 regenerated plants. Wan *et al.*, (1988) found differences in the efficiency of callus regeneration among tissue culture derived regenerants of alfalfa. They suggested three possible factors which may have affected regeneration from these parent plants. In addition to the major genes for ability to regenerate, there may be modifiers influencing the efficiency, or a separate genetic system controlling efficiency, or a gene dosage effect. Hendriks and de Vries (1995) identified three secreted proteins present in carrot suspension cultures that play a role in somatic embryogenesis. Extracellular proteins released from tissue cultures of *Picea abies* have a putative regulatory effect on embryo development; these include, arabinogalactan proteins, zeamatin-like proteins, chitinase and peroxidases (von Arnold *et al.* 1995). Mordhorst *et al.* (1995) identified a cellular 85 kDa polypeptide in barley suspension cultures which accumulated during the loss of regenerative ability. Following the pattern of proteins secreted to the medium, polypeptides were characterized and correlated with the embryogenic capacity (46 kDa), or the loss of regenerative potential (17.4 and 40.5 kDa), respectively.

In further studies it would be informative to establish the basis for the improved regeneration ability in the somaclones by identifing the proteins and gene(s) that are correlated with this change.

Table 2.1 Media used for callus initiation and regeneration of *M. sativa* cv. CUF 101, CUF101-1S and somaclones derived from tissue culture.

	Callus Induction Medium		Regeneration Medium				Root and Shoot Medium	
	SH	BII	Boi2y	KS	DS	SHAP	MS	GS
SH	*			*	*	*		*
MS							*	
Blaydes		*	*					
2,4-D	2 mg/l	2 mg/l		1 mg/l	50 µM/l			
Kinetin	2 mg/l	2 mg/l		21.5 mg/l	5 µM/l			
NAA	2 mg/l	2 mg/l						
Sucrose (w/v)	3%	3%	3%	3%	3%	3%	1%	1%
Inositol			100 mg/l					
Yeast extract			2 g/l					
L-alanine						30 µM/l		
L-proline						50 µM/l		
Agar (w/v)	1%	1%	1%	0.6%	1%	1%	0.8%	0.8%
pH	5.6-5.8	5.9-6	5.9-6	5.9-6	5.6-5.8	5.9-6	5.9-6	5.9-6

Medium abbreviations:

MS   Murashige and Skoog (1962)

SH   Schenk and Hildebrandt (1972)

BII   Blaydes (1966) basal medium , modified by Saunders & Bingham (1975)

Boi2y   Blaydes (1966) basal medium, modified by Saunders & Bingham (1975)

SHAP   Modified SH medium (Wan *et al.*, 1988)

KS   Modified SH medium (Stavarek *et al.*, 1980)

DS   Modified SH medium (Walker *et al.*, 1979)

2, 4-D   2, 4-dichlorophenoxyacetic acid

NAA   Naphthaleneacetic acid

Table 2.2. Bud and plantlet production from explants of *M. sativa* isolated from cotyledon (C), hypocotyl (H) and root (R) of seedlings placed on a callus initiation medium, BII, for 14 days then on a regeneration medium, Bio2y, for 28 days. In each case the record regeneration from the best callus.

Varieties or genotypes	Explant Source	No. regenerants callus^{-1}	
		buds	plantlets
Regen-SY	C	97	88
	H	189	171
	R	64	62
CUF 101	C	8	2
	H	2	1
	R	0	0
CUF101-1S	C	9	7
	H	16	11
	R	0	0
CUF101-3F	C	7	3
	H	3	0
	R	1	0
CUF101-6N	C	2	0
	H	1	0
	R	1	1
CUF101-5R	C	2	0
	H	0	0
	R	0	0
CUF101-2A	C	1	0
	H	0	0
	R	0	0
CUF101-4A	C	0	0
	H	0	0
	R	0	0

Fig. 2.1 Callus of *M. sativa*, CUF101-1S on regeneration medium showing multiple buds after 28 days. Scale represent 1 mm.

Fig. 2.2a,b Callus of *M. sativa*, CUF101-1S on regeneration medium showing shoots at different stage of regeneration after 28 days. Scale represent 1mm.

a

b
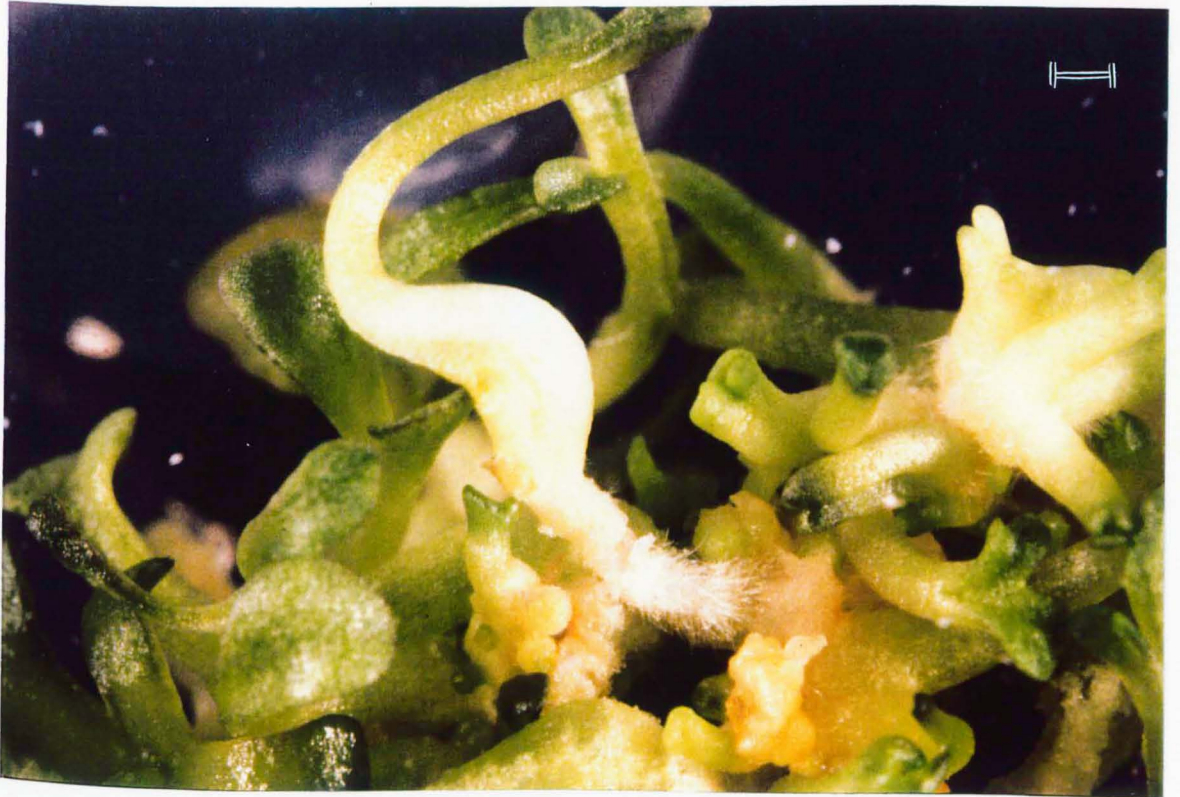
Fig. 2.3 a) Tissue culture derived regenerated plantlets of CUF101-1S at different stages of development on a root induction medium.

b) Tissue culture derived plantlet of CUF101-1S transferred to a Jiffy-7 peat moss containers and maintained under high humidity.

Scale represent 1 cm.

a

b

Fig. 2.4 Protocol of selection for improvement of regeneration of *Medicago. sativa*, cv. CUF 101.

CUF 101
(parent variety)

↓ *In vivo* selection

CUF101-1S
(Partial salt tolerant line)

↓

Tissue culture
(Initiation of callus)

↓

Regeneration
(Shoot and root formation)

↓ Plant growth *in vivo*

Flowering

↓ Self pollination

Seed progeny (1st cycle)

↓

Tissue culture

↓

Regeneration

↓

Seed progeny (2nd cycle)

↓

Tissue culture

↓

Regeneration

↓

Seed progeny (3nd cycle)
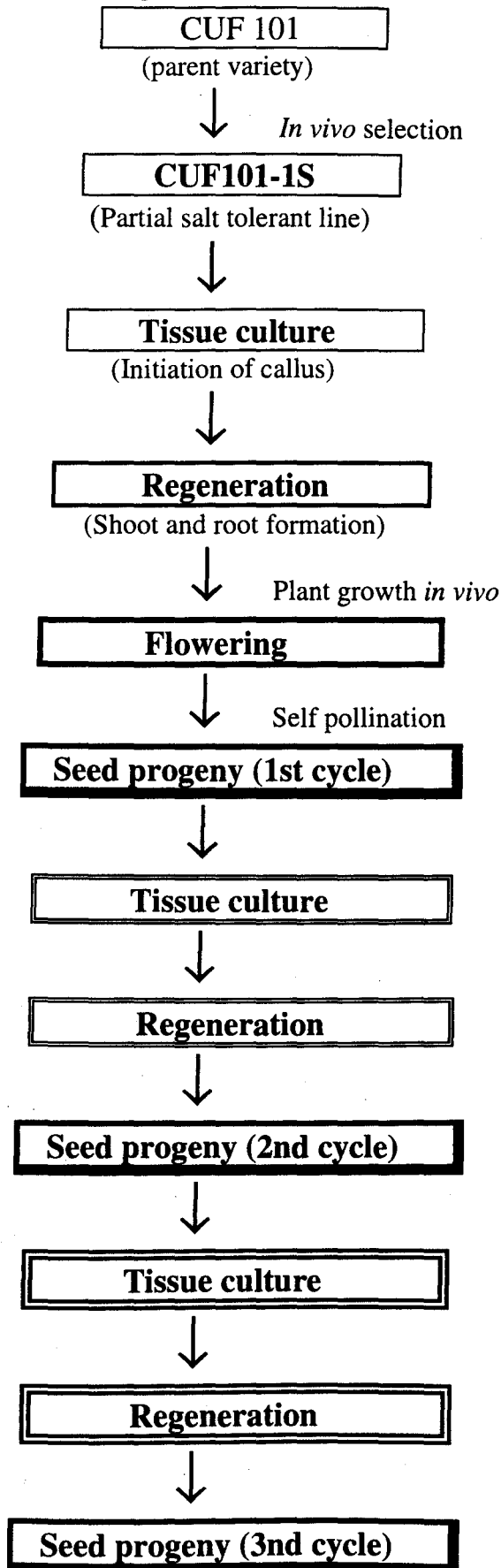
Fig. 2.5 Regeneration frequency of alfalfa callus at 28 days derived from

a) CUF101-1S.

b) Regen-SY.

Scale represent 1 cm.

a
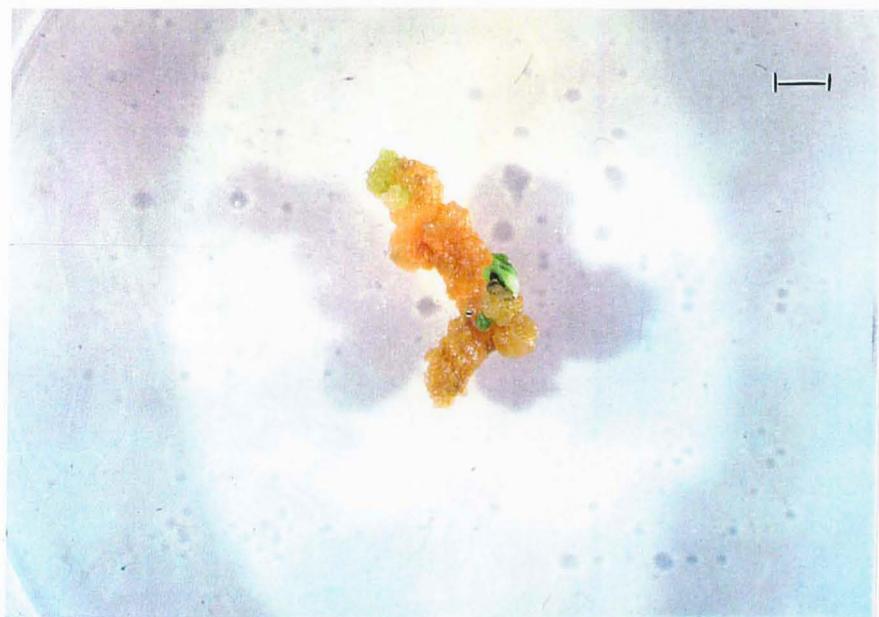
b
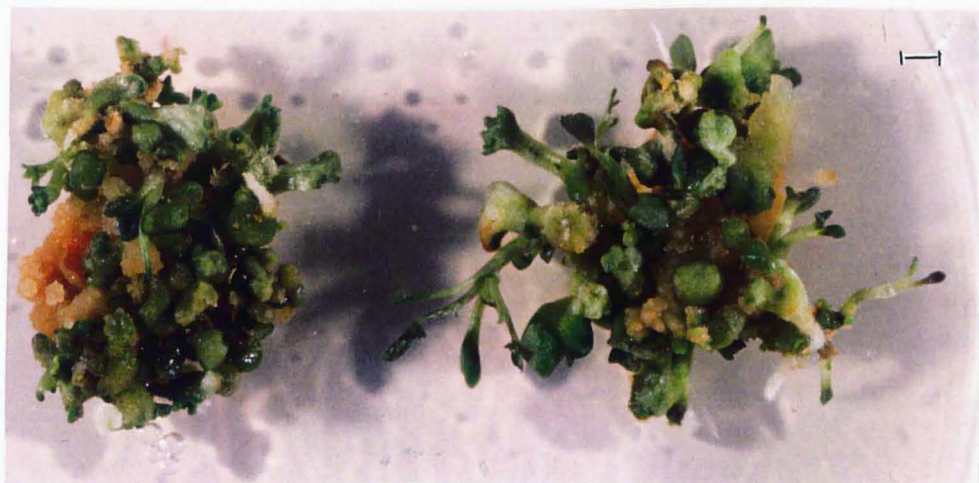
Fig.2.6 a) Comparison of regeneration frequency in callus of 1) CUF 101, 2) CUF101-1S, 3) and 4) progeny of regenerants of CUF101-1S after 28 days.

b) Sequence of regeneration from callus to plantlet.

Fig.2.7 Initiation of callus of *M. sativa,* CUF101-1S on different media.

a) Callus derived from cotyledon explant.

b) Callus derived from root explant.

Callus fresh weight   □

Callus dry weight   ■

a)

Callus growth (g)

2.0

1.5

1.0

0.5

0.0

BII   SH

b)

Callus growth (g)

1.8

1.5

1.2

0.9

0.6

0.3

0.0

BII   SH
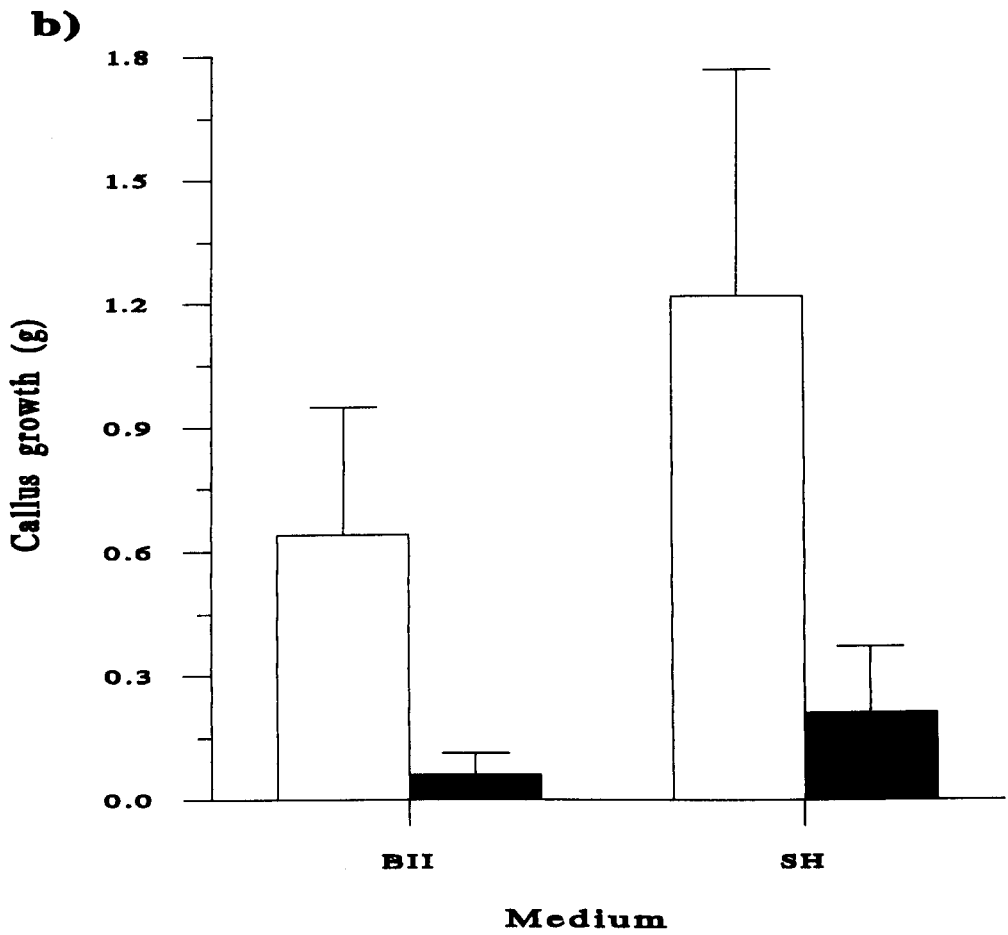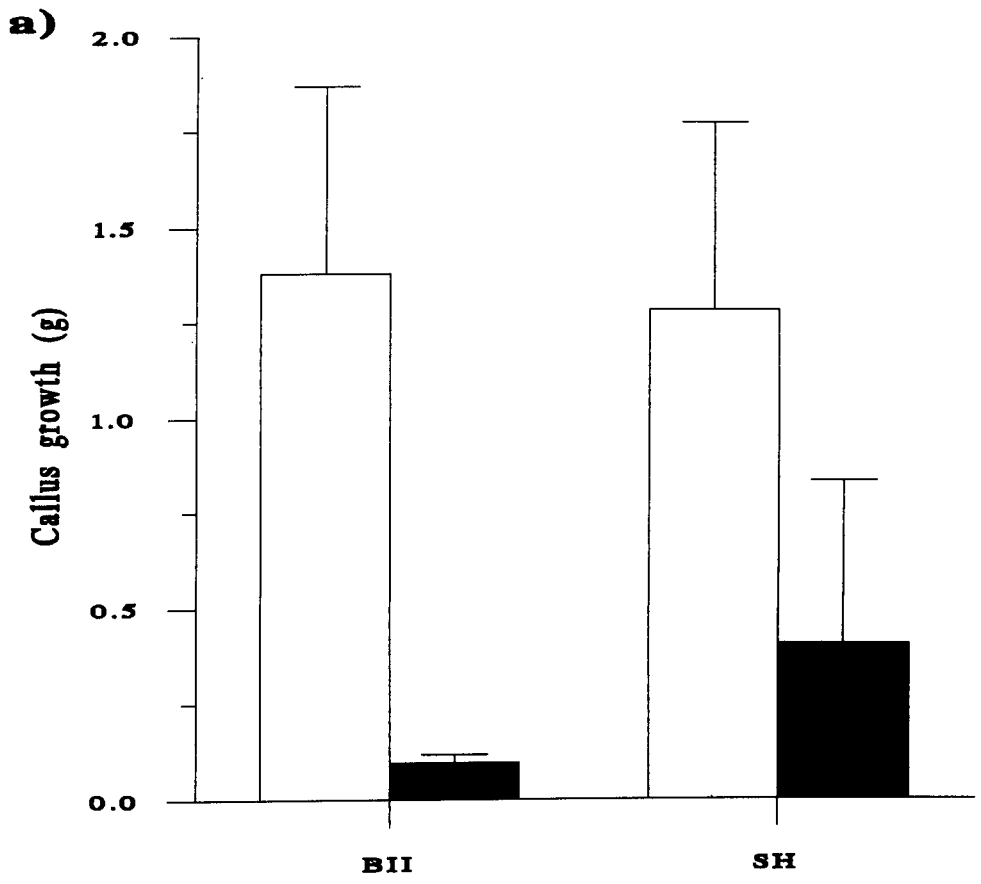
Medium

Fig. 2.8 Regeneration of *M. sativa*, CUF101-1S and somaclones (6R1, 7R1, 8R1, 9R1). Callus initiated on the BII medium.

a) Regenerated callus as a percentage of total callus (%).

b) Mean number of buds per callus ± SE.

Cotyledon ☐
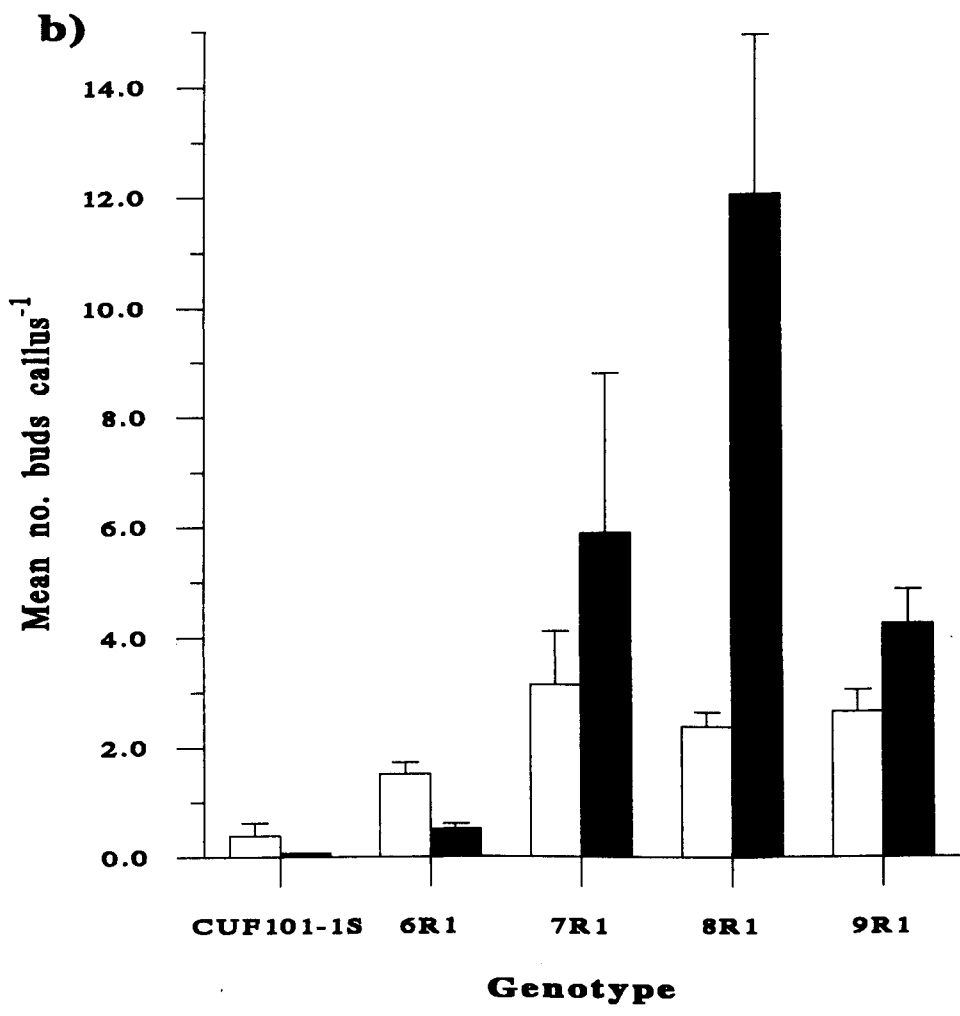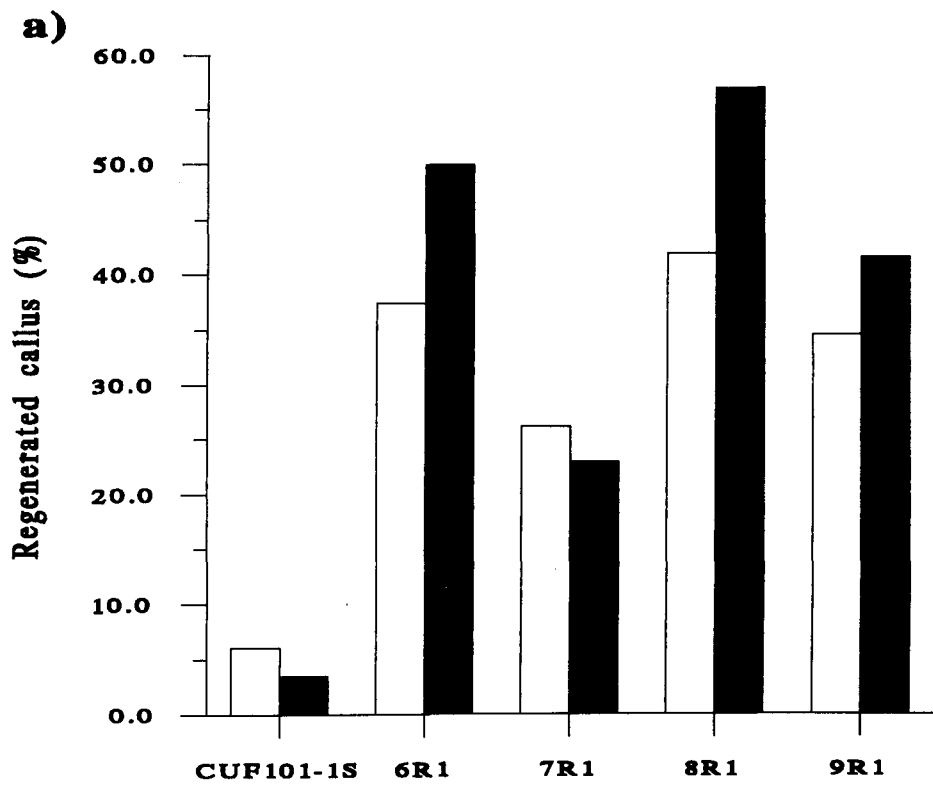
Hypocotyl ■

Fig. 2.9 Regeneration of *M. sativa*, CUF101-1S and somaclones (6R1, 7R1, 8R1, 9R1). Callus initiated on the SH medium.

a) Regenerated callus as a percentage of total callus (%).

b) Mean number of buds per callus ± SE.

Cotyledon   □

Hypocotyl   ■

No survivors   ✳

a)
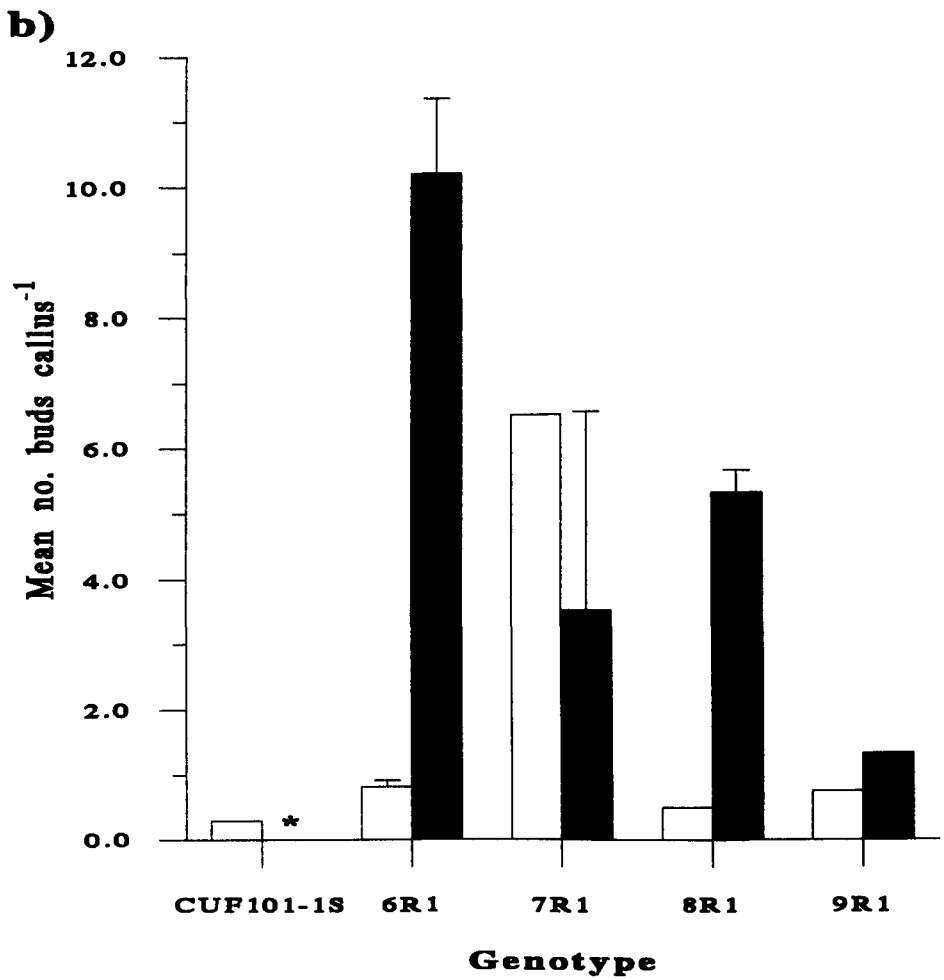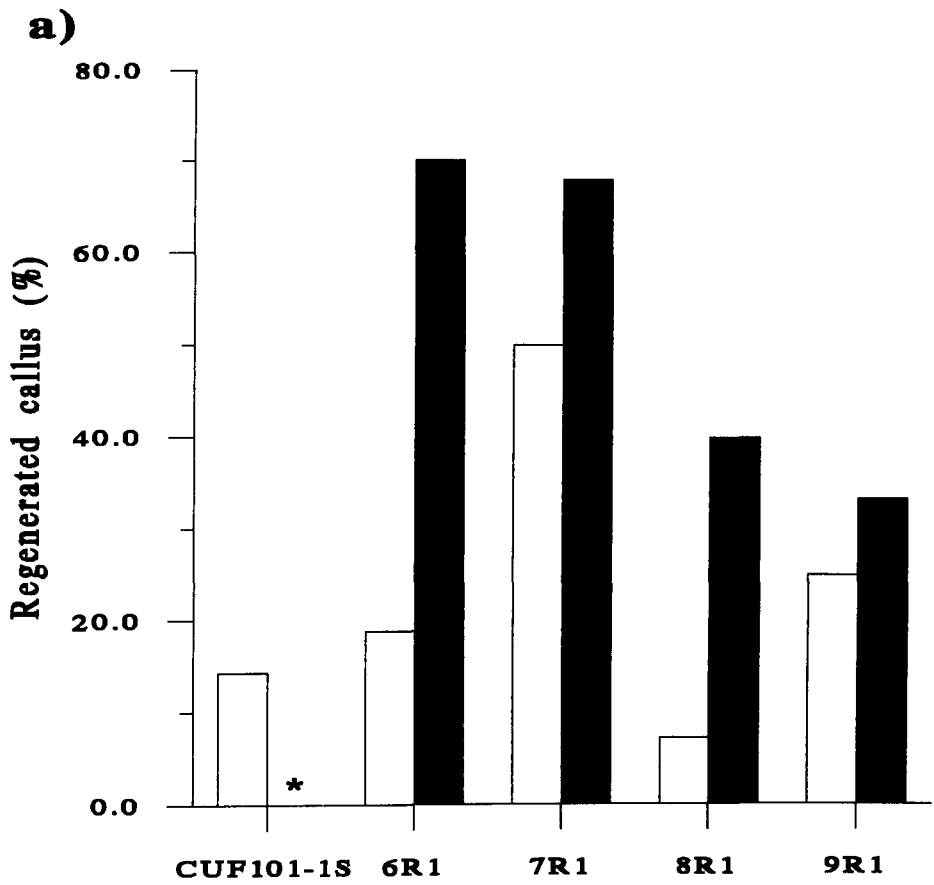
Fig. 2.10 Selection for improved regeneration *in vitro* in repeated cycles of tissue culture of *M. sativa* somaclones (6R, 8R, 7R, 9R). Callus derived from cotyledon explant.

a) Regenerated callus as a percentage of total callus (%).

b) Mean number of buds per callus ± SE.

1st cycle   □

2nd cycle   ▨

3rd cycle   ■

Not measured ✶

a)

Regenerated callus (%)

70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0

Regen-SY   6R   8R   7R   9R

**

b)

Mean no. buds callus^{-1}

10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0

Regen-SY   6R   8R   7R   9R

**

Genotype
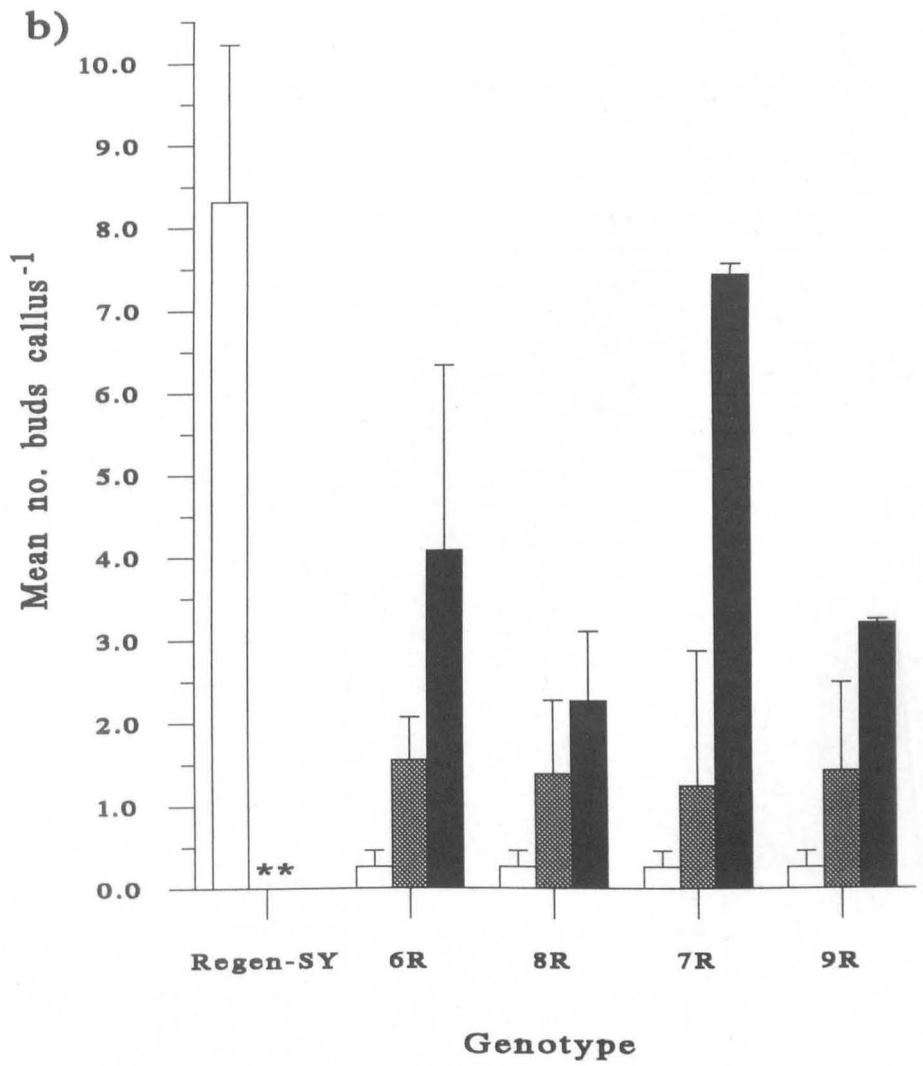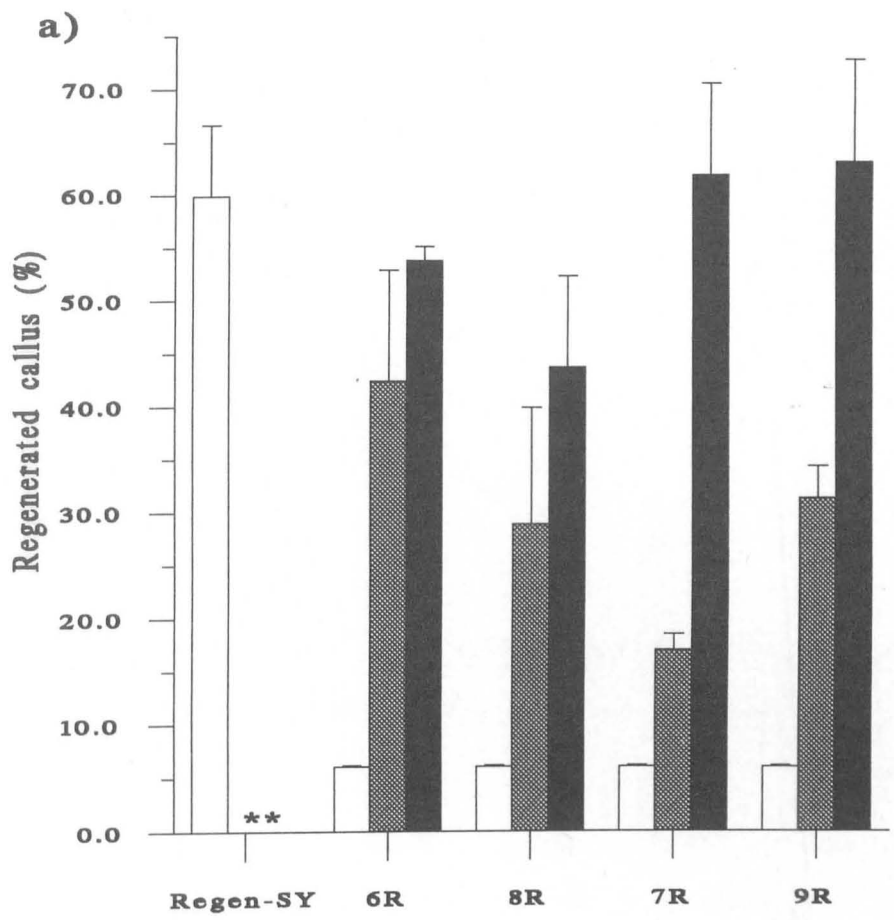
Fig. 2.11 Selection for improved regeneration *in vitro* in repeated cycles of tissue culture of *M. sativa* somaclones (6R, 8R, 7R, 9R). Callus derived from hypocotyl explants.

a) Regenerated callus as a percentage of total callus (%).

b) Mean number of buds per callus ± SE.

1st cycle   ☐

2nd cycle   ▨

3rd cycle   ■

Not measured   ✶
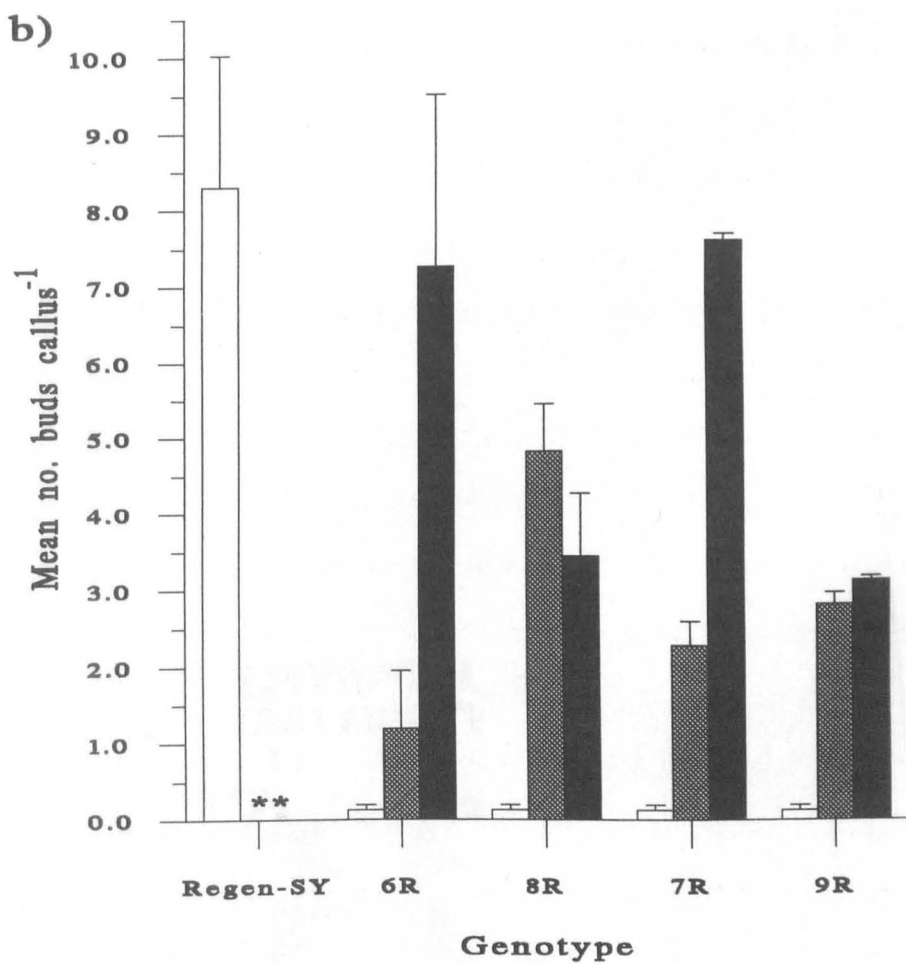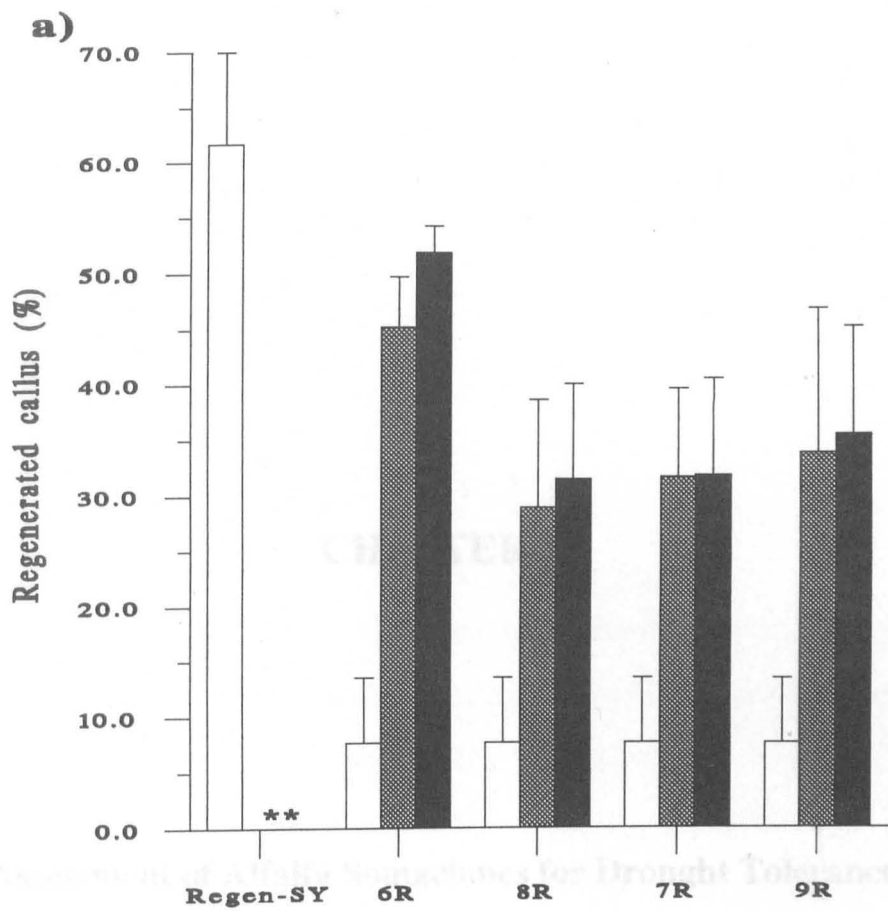
CHAPTER 3.

Assessment of Alfalfa Somaclones for Drought Tolerance

3. Assessment of Alfalfa Somaclones for Drought Tolerance

3.1 INTRODUCTION

3.1.1 Growth response to drought stress

Plant species sown for pasture and range improvement in semi-arid conditions must be able to germinate, grow and flower under marked drought stress (Rumbaugh & Johnson, 1980). Such plants survive water stress through modifications to their morphology and metabolic processes. These adaptative mechanisms include traits promoting the maintenance of high tissue water content, as well as those promoting tolerance to low water availability. Although there are plants which tolerate extreme cellular dehydration such as *Larrea divaricata*, a desert shrub which is able to reduce its fresh weight to 30% of its original fresh weight before its leaves being to die, in most plants, levels of 50 to 75% weight reduction are lethal. In plant species such as alfalfa which are grown in arid environments, tolerance to water stress usually involves the development of low osmotic potentials (Santos-Diaz & Ochoa-Alejo, 1994).

Analysis of the growth has shown that alfalfa responds to any onset of drought by a reduction in shoot and root elongation (Carter & Sheafer, 1983a,b; Hall, 1993), and in the number of basal buds and shoots (Brown & Tanner, 1983; Perry & Larson, 1974). Brown and Tanner (1983) also found the development of water stress reduced leaf size, internode length, and dry matter production of alfalfa during the first 14 days after the stress was initiated. Schubert *et al.* (1995) induced drought stress in symbiotically nitrogen-fixing alfalfa and whilst root dry weight was not affected by drought stress, shoot and nodule dry weight production were inhibited. Interestingly, Antolin *et al.* (1995) found that nitrogen-fixing alfalfa plants were more drought

tolerant than nitrate-fed ones which may have been a consequence of an increased root/shoot ratio in the nitrogen-fixing plants absorbing from soil.

3.1.2 Proline accumulation in response to drought stress

Water deficits have been shown to induce a lowering of the osmotic potential in crops as a means of maintaining their turgor (Neumann, 1995a). This decline in the osmotic potential as a response to water deficits can be achieved by solute accumulation within plant cells (Delauney & Verma, 1993; Santos-Dias & Ochoa-Alejo, 1994), or by decrease in cell volume. An increase in concentration of osmotic solutes occurs as water leaves the vacuole, thus preventing damage from cellular dehydration by balancing the osmotic strength of the cytoplasm with that of the environment (Santos-Dias & Ochoa-Alejo, 1994). Osmotic adjustment is an important mechanism in drought tolerance because it enables a continuation of cell expansion (Wyn Jones & Gorham, 1983), stomatal and photosynthetic adjustments, and better plant growth and yield production (Heuer, 1994). The compounds involved in osmotic adjustment are mainly soluble sugars, potassium, organic acids, chloride, and free amino acids (Delauney & Verma, 1993). The degree of osmoregulatory processes is affected by the rate of stress, stress pre-conditioning, organ type and age, and genetic difference between and within species (Morgan,1984). The most frequent and extensive response of crops to water stress or increasing osmotic pressure is the accumulation of proline on dehydration (Delauney & Verma, 1993; Good & Zaplachinski, 1994).

Proline was also shown to occupy a key role in alfalfa grown under stress. The response of alfalfa to drought stress was that growth reduction was accompanied by increased proline concentration in shoots, roots, and nodules (Schubert *et al.* 1995). Similarly withholding irrigation stimulated proline accumulation in leaves and nodules (Irigoyen *et al.*, 1992). In seven annual *Medicago spp.* and *M. sativa* grown

under drought stress, Naidu *et al.* (1992) found proline, and proline concentrations were always higher in drought-stressed plants (Antolin & Sanchez-Diaz, 1992).

3.1.3. Antioxidant enzyme activity in response to drought stress

When molecular oxygen is incorporated into organic molecules, relative intermediates such as superoxide anion ($O_2 \cdot^-$), hydroxyl radical (OH^-), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2) are produced (Badiani *et al.*, 1990; Scandalios, 1990). Physiological events (Asada, 1993, Gossett *et al.* 1994) and biotic or abiotic pathogenesis such as salinity, drought, high and low temperature, flood, high light intensities, freezing, exposure to UV irradiation, herbicide treatment and mineral deficiency (Doke *et al.*, 1993, Foyer *et al.*, 1994) can enhance the formation of toxic oxygen species and damage can occur (Badiani *et al.*, 1990). Plants possess very efficient scavenging systems for active oxygen species that protect them from destructive oxidative reactions, and often result in increases in antioxidant defence such as the activity of the antioxidant enzymes (Scandalios, 1990; Polle *et al.*, 1992; Gossett *et al.*, 1994; Foyer *et al.*, 1994). A balanced increase in activities of the antioxidants is required to obtain increased stress resistance.

3.1.4 Production of somaclones resistance to drought

One possible approach to improving the ability of a crop to tolerate drought is to make use of the variation provided by tissue culture derived somaclonal variation. Selection for drought tolerance is most frequently carried out by including molecules like PEG 4000 or PEG 6000 in the culture medium (Nabors, 1990) which have the effect of reducing extracellular free water availability (Caplan *et al.*, 1990) and simulating drought stress. However there are problems with this approach. Handa *et al.*, (1983) and Bressan *et al.* (1981) found that cultured tomato cells rapidly adapted to PEG stress but did not retain resistance in the absence of stress. They concluded

that phenotypic adaptation rather than genotypic selection was involved. A PEG selection was not necessary and they were also able to obtain many stable PEG-tolerant lines from somaclonal variants that were isolated in the absence of stress. In another approach Sumaryati *et al.* (1992) produced a regenerant mutant from protoplast cultures of *Nicotiana plumbaginifolia* maintained in 25% PEG then found that the resistant line produced 10-25 times more proline than the wild type when grown on a non-selective medium. In wheat, improved tolerance to drought was found in plants regenerated in the presence of 25% PEG (Bajji *et al.* 1995). In alfalfa selection for drought tolerance was attempted during the callus phase using a short term exposure to 10% PEG, after which the callus was transferred to a PEG free medium, and regeneration was initiated via embryogenesis (Dragiiska *et al.* 1995). Although details of the methods used for selection by Bajji *et al.* (1995) and Dragiiska *et al.* (1995) were limited, there was evidence that drought tolerant somaclones had been produced based upon selection in the presence of PEG but little evidence for this drought tolerance progeny of the somaclones.

The objectives of the present study was to assess progeny of tissue culture derived regenerants of alfalfa for drought tolerance and to test the selected material for stability of any tolerance characteristics. The progeny were also to be examined for the effects of drought stress on proline levels and activity of the antioxidant enzymes, GR, SOD, CAT and AP.

3.2 MATERIAL AND METHODS

3.2.1. Choice of PEG treatment

A seedling screen was used to provide a rapid method of assay for the level of drought tolerance in the somaclones. It was necessary to establish the maximum concentration of PEG that could be used in the seedling screen without inhibiting germination of the alfalfa (LD90). Uniform sized seeds of parent CUF 101 were

surface sterilised in 2% v/v sodium hypochlorite for 2 minutes, rinsed in sterile water and dried at room temperature. For each replicate, 20 seeds were evenly spaced over a raft of alkathene beads in plastic pots containing 300 ml of nutrient solution (Fig. 3.1). The solution used was a modified half strength Rorison solution (see Appendix 3.2; Hewitt, 1966) plus 4 mol m^{-3} $CaCl_2$. The pots contained 0, 50, 100, 150, 200, 250 g l^{-1} concentration of PEG 6000 with three replicates for each treatment. The pots were arranged randomly in a shallow tray filled with water and placed under a clear plastic cover in a growth room at 25-27 °C. After two weeks, shoot lengths were measured from the base of the hypocotyl to the base of the youngest trifoliate leaf and root lengths from the tip to the top of root.

3.2.2. Seedling response to PEG in the long term

Seeds of CUF 101 and the somaclones 7R1, 4R1, 3R1 derived from tissue culture (Fig. 3.2) and 6R2IV (see Chapter 2, Section 2.2.2 and Chapter 5, Section 5.2.2) were germinated and grown in 0, 200 and 250 g l^{-1} PEG using the procedure described in Section 3.2.1. Three replicate pots were used for each treatment. These concentrations were chosen because, although 250 g l^{-1} PEG severely restricted the growth of cv. CUF 101, it did allow of germination and growth. After two weeks shoot and root length were measured and the seedlings grown in the 200 g l^{-1} PEG treatment were frozen in liquid nitrogen for subsequent biochemical analysis.

Analysis of variance was carried out for each character by SAS packages (1985). Bonferroni's method (Maxwell & Delaney, 1990) was used to compare means (Appendix 3.3).

3.2.3 Seedling response to PEG in the short term

Seedlings of CUF 101 and somaclones (7R1, 4R1, 3R1, 6R2IV) (see Chapter 2, Section 2.2.2 and Chapter 5, Section 5.2.2) were grown for 7 days in Rorison

solution without PEG, supported individually in a polythene frame on the top of a 2.5 litre volume black polythene container. They were then transferred to the former solution containing 0 and 200 g l^{-1} PEG, and left in these solution for 12h, and 1, 2, 4, and 7 days. After these treatments whole plants were analysed for proline concentration and antioxidant enzyme activity.

3.2.4 Preparation of extracts for proline estimation

Seedling shoot tissue were frozen in liquid nitrogen then when required were homogenised in methanol: chloroform: water (MCW 12:5:1 /V) at room temperature, using 0.2 g tissue in 2 cm^{-3} of MCW and the proline concentrations estimated by the method described by Singh *et al.* (1973) with the following modification. The homogenate was centrifuged at 5 × 1000 rpm for 5 minutes in a Gallenkamp bench top centrifuge and the supernatant retained. The pellet was re-extracted in the same volume of MCW for 5 minutes. The two supernatants were pooled and separated into a lower chloroform and upper methanol water layer by adding 1.5 cm^3 water and 1 cm^3 chloroform to every 4 cm^3 of extract.

The upper layer was used for proline estimation. Samples of 1.0 cm^3 were transferred to boiling tubes to which were added 1.25 cm^3 glacial acetic acid and 1.25 cm^3 of acid ninhydrin [1.25 g ninhydrin was warmed in 30 cm^3 glacial acetic acid and 20 cm^3 orthophosphoric acid (85%)]. The boiling tubes were heated in a boiling water bath for 65 minutes, and after cooling 5 cm^3 toluene were added. The tubes were shaken on a whirlimixer and left for about 20 minutes for separation of the two layers (lower and upper layer). The absorbance of the upper (toluene) layer was measured at 515 nm against a toluene blank. The assay was calibrated with standard solution of l-proline (Appendix 3.4). Analyses of variance were carried out for three measurements on extracts from 3 independent experiments for each

treatment. Bonferroni's method (Maxwell & Delaney, 1990) was used to compare means.

3.2.5 Preparation of extracts for enzyme assays

Frozen seedlings shoot tissue (400 mg) were homogenized in 400 µl 0.1 M K_2HPO_4 (PH=7.0) with a mortar and pestle. The homogenized samples were washed with 2×200 µl 0.1 M K_2HPO_4 into Eppendorf tubes and centrifuged for 10 minutes (10000 rpm) in an Eppendorf microfuge. The pellet was resuspended in 800 µl 0.1 M K_2HPO_4 and centrifuged, and the combined supernatant was used for the determination of activities of glutathione reductase (Halliwell & Foyer, 1978), ascorbate peroxidase (Nakano & Asada, 1981), superoxide dismutase (Misra & Fridovich, 1972), and catalase (Beers & Sizer, 1952) (Appendix, 3.5).

Three parallel activity measurements were carried out from 3 independent samples for each extract. Bonferroni's method (Maxwell & Delaney, 1990) was used to compare means.

3.3 RESULTS

3.3.1 Bioassay response to PEG

Shoot and root lengths of 14-day-old seedlings were reduced with an increase in PEG concentration. Shoots showed a nearly linear decrease in length in the presence of PEG, but in contrast, there was no significant reduction in root length after 50 g l^{-1} PEG (P>0.05). The effect of PEG on root length was significantly different only in the presence of 50 and 250 g l^{-1} PEG (P<0.05) (Fig. 3.3). Root/shoot ratios increased with increasing PEG concentration. In 250 g l^{-1} PEG the ratio was 4 compared with 2.1 at zero PEG (Table 3.1).

3.3.2. The response of alfalfa somaclones to different concentrations of PEG

Root length decreased in all genotypes in the presence of PEG. Differences between the parent plant and the somaclones were most obvious at the highest concentration, 250 g l^{-1}, where root length of the CUF 101 was reduced to 30% of the control in zero PEG compared with 60% for 7R1 and 54% for 6R2IV. These differences were significant at $P<0.05$ (Fig. 3.4a). The shoot length showed a similar pattern but there were greater reductions growth in 250 g l^{-1}. Thus the parent plants, CUF 101, were reduced to 19% of the control and 7R1 to 33% and 6R2IV to 32%. These differences were significant at $P<0.05$ (Fig. 3.4b).

There was no significant difference between CUF 101, 7R1, 4R1 and 6R2IV for germination in the presence of 200 g l^{-1} PEG. A reduction in seed germination was shown by CUF 101 and 3R1 (Fig. 3.4c). Root/shoot ratios increased in all five genotypes with increase of PEG concentration (Table 3.2).

3.3.3 Estimation of proline in seedlings

Proline accumulation in 14-day-old seedlings of CUF 101 and somaclones (7R1, 4R1, 6R2IV) exposed to 200 g l^{-1} PEG 6000 are shown in Fig. 3.5. Some difference in proline accumulation was detected in genotypes in the absence of PEG. Proline accumulation was higher in CUF101-1S, 7R1, 6R2IV at the 0 g l^{-1} PEG than CUF 101. At 150 g l^{-1} PEG, proline increased in all genotypes and a significant increase (×14) was observed in 7R1 at 150 g l^{-1} PEG compared with 0 g l^{-1} PEG ($P<0.001$). At 200 g l^{-1} PEG, proline accumulation increased in all genotypes with a very much larger increase in 4R1, 6R2IV, CUF 101 and 7R1. Proline accumulation in 4R1, 6R2IV, CUF 101 and 7R1 at 200 g l^{-1} PEG increased 42, 31, 30 and 23 fold compared with 0 g l^{-1} PEG levels and higher levels were in 7R1 and 6R2IV than that measured in CUF 101 ($P<0.001$).

In the short term experiment, proline levels of 7-day-old seedlings at a concentration of 200 g l^{-1} PEG 6000 was detected after 1, 2, 4 and 7 days in both CUF 101 and 7R1 with an earlier increase in 7R1 (Fig. 3.6). Proline level increased after 2 days in CUF 101 (Fig. 3.6a), and 1 day in 7R1 (Fig.3.6b), but proline levels in both genotypes decreased thereafter (Fig. 3.6).

3.3.4 Antioxidant enzyme assays

The activity of SOD, AP, GR and CAT antioxidant enzymes in 14-day-old seedlings of parent CUF 101 and somaclones 7R1, 4R1 and 6R2IV exposed to 200 g l^{-1} PEG 6000 was shown in Fig. 3.7 to 8. There was no significant difference in GR activity among genotypes CUF 101, 7R1 and 4R1 in the absence or in the presence of PEG (P>0.05) (Fig 3.7a). A reduction in GR activity was observed in 6R2IV in the presence of 200 g l^{-1} PEG compared with 0 g l^{-1} PEG. SOD activity in 200 g l^{-1} PEG increased 125% ± 8% in CUF 101 and 160% ± 5% in 4R1 whereas it decreased 89% ± 6% in 7R1 compared with their 0 g l^{-1} PEG levels (Fig. 3.7b).

There was a significant difference (P<0.05) in CAT activity between the control and those treatments containing PEG in 7R1, CUF 101 and 4R1 respectively (Fig. 3.8a). No difference in CAT activity was detected in 6R2IV in the absence and presence of PEG (P>0.05) (Fig.3.8a). At 200 g l^{-1} PEG , small increases in AP activity in CUF101-1S, 7R1 and 6R2IV and further increases were observed in 7R1. The activity of AP was reduced in CUF 101 in the presence of PEG. No difference in AP activity was measured in the absence and in the presence of PEG in 4R1 (P>0.05) (Fig. 3.8b).

In the short term experiment, CAT activity decreased in the presence, and the absence of PEG in CUF 101, and after two days the reduction was less in the presence of 200 g l^{-1} PEG than 0 g l^{-1} PEG treatment (Fig. 3.9a). Addition of 200 g l^{-1} PEG caused a reduction in CAT activity in 7R1 after 2 days growth and then increased. After 4 days, activity decreased in the control (Fig. 3.9b). AP activity in

CUF 101 increased after 1 day and was unchanged after 2 days in the presence of 200 g l^{-1} PEG while it also increased in the control 0 g l^{-1} PEG treatment (Fig. 3.10a). The activity of AP in 7R1 increased at 0 g l^{-1} PEG until 4 days, and it was unchanged thereafter. In 200 g l^{-1} PEG, by contrast, AP activity was unchanged until day 4, and then increased (Fig. 3.10b).

3.4 DISCUSSION

This work was undertaken to detect any increase in osmotic tolerance in alfalfa somaclones in response to simulated drought. A decrease in water potential induced by the addition of different amounts of PEG 6000 to the nutrient solution caused a gradual increase in drought stress, which reduced root and shoot growth in alfalfa seedlings. The reduction of shoot and root length by drought can be explained in terms of turgor limitation or cell wall hardening (Matsuda & Riazi, 1981; Chazen & Neumann, 1994, Neumann, 1995c). The onset of significant wall hardening in growing leaf tissue may be advantageous for the survival of crops grown under severe water deficit conditions leading to terminal drought. Since turgor pressure inside the cell is the response to the pressure exerted on the protoplast by the cell walls (Cosgrove, 1993) hardening the cell walls should increase turgor pressure. The ability to harden expanding cell walls in response to severe water deficits could therefore delay the stress induced loss of turgor pressure, and consequently irreversible wilting in developing leaf tissues. Moreover, wall hardening is associated with production of smaller mature cells which are known to have an increased capacity to adjust osmotically and maintain turgor pressure (Neumann, 1995a). Finally, wall hardening also leads, in the longer term, to production of smaller leaves and plants. This would in turn reduce plant transpiration rates (Neumann, 1995c).

The experiments reported here showed that the R/S ratio of alfalfa seedlings increased with increasing PEG concentration. This response would agree with the

fact that shoot growth is generally more affected by water stress than root growth, not only because the transpiring parts of the plant usually develop greater and longer water deficits (Nielsen & Halvorson, 1991), but because the shoot itself is usually more sensitive to water deficits (Sharp *et al.*, 1988). Another explanation is that the root has more capacity for osmotic adjustment than the shoot (Munns & Weir, 1981).

Selection for drought tolerance is most frequently carried out by including molecules like PEG 4000 or PEG 6000 in the growth medium (Nabors, 1990) which reduces the extracellular free water concentration (Caplan *et al.*, 1990). There has been limited reports of stable, heritable tolerance to PEG in plant species (Sumaryati *et al.* 1992; Bajji *et al.*, 1995). Except for the limited finding of Dragiiska *et al.* (1995), no previous work has been reported on selection for drought tolerance in alfalfa using somaclonal variation. Dragiiska *et al.* (1995) exposed callus to 10% PEG, after which the callus was transferred to a PEG free medium, then regeneration was initiated via embryogenesis. Unfortunately there was no progeny test to show whether the tolerance was stable. A progeny analysis is essential if somaclonal variation is to be regarded as a useful source of variation.

The data here showed, that one of the somaclones (7R1) was significantly more tolerant at 250 g l^{-1} PEG 6000 level than CUF 101. The basis for the tolerance may be proline production or change in activities of the antioxidant enzymes. The PEG-induced increase in proline in CUF 101 and in the somaclones 7R1, 4R1 and 6R2IV indicated a positive correlation between proline accumulation and response to osmotic stress. The drought-tolerant genotype (7R1) showed a larger and earlier response in proline level than the more drought-sensitive cultivar (CUF 101) in the presence of PEG. The accumulation of this amino acid may be part of general adaptation to adverse environmental condition (Delauney & Verma, 1993). A large body of data has indicated that there is a positive correlation between proline accumulation and adaptation to drought stress (review by Delauney & Verma, 1993). Corcuera *et al.* (1989), in potato cells exposed to 10% PEG, observed a 9 fold

increase in proline compared with their control. In tomato cells exposed to 25% PEG, there was a 319 fold increase in proline content compared with control cells (Handa *et al.* 1983; Rhodes *et al.*, 1986). A positive correlation between the proline levels in cultured cells of drought sensitive chili pepper *(Capsicum annuum L.)* and *Larrea tridentata*, a plant species highly tolerant to drought, and the degree of resistance to PEG was found by Santos-Diaz & Ochoa-Alejo (1994). However, other studies have shown no such correlation (Bhaskaran *et al.*, 1985). This is due to the fact that a proline increase seems to depends on the length of time and concentration of osmotic or salinity stress (Bray *et al.*, 1991), the type of tissue, and the age of the plant, and its genotype. Thus proline accumulation differs between cultivars adapted to certain growth conditions or regions, as well as differing within species which are tolerant to drought or salinity (Heuer, 1994). In the experiments reported here proline level increased up to 42 fold over control levels in the alfalfa somaclones, and levels in 7R1 and 6R2IV were higher than that measured in CUF 101. In the tolerant somaclone (7R1), at 150 g l^{-1} PEG, proline content was 21 times higher than that measured in the sensitive cultivar CUF 101. In seedlings of three annual *Medicago* species, the most osmotically tolerant species exhibited the highest potential for proline accumulation in response to osmotic and salinity stress (Refoufi & Larher, 1989) suggesting that it was involved in the osmotic adjustment of alfalfa plants during stress. Water stress imposed on alfalfa plants by withholding irrigation, also stimulated proline accumulation in leaves and nodules (Irigoyen *et al.*, 1992). They observed that the free proline content of the most severely stressed nodule and leaf tissues were 9 and 17 times the content of the well watered nodule and leaf controls respectively. The threshold water potential triggering such an accumulation was higher in nodules tissue, suggested that under severe water stress, nodule metabolic enzymes and structural proteins may be protected by this process.

This study did not show any correlation between increase of GR and SOD activities and osmotic tolerance in seedlings of alfalfa but did show a positive correlation between increase of CAT and AP activities and osmotic tolerance. The PEG-induced

increase in AP and larger increase in CAT activities in 7R1 suggest that this more drought-tolerant somaclone has a higher antioxidant scavenging system than the more salt-sensitive CUF 101. Resistance to environmentally induced oxidative stress has been shown in several instances to be associated with high levels of both CAT and SOD (Harper & Harvey, 1978; Hassan & Fridovich, 1979). In previous studies by Safarnejad (1992), the results suggested that changes in the activity of the antioxidant enzymes examined are probably genotype-dependent, and that they are correlated with the stress tolerance of wheat varieties. With increasing salt and mannitol concentrations, the activities of CAT, SOD, and GR increased in the callus of tolerant and moderately tolerant wheat varieties, while remained constant or decreased in the sensitive ones. The AP activity increased in the sensitive variety, Cappelle Desprez, and the moderately tolerant variety, Chinese Spring, while its level remained unchanged in the tolerant variety, Kharchia. The most pronounced changes in the activity of the antioxidant enzymes were detectable after two days. Some cultivars of *Conyza bonariensis, Lolium perenne,* and *Nicotiana tabacum,* in which several components of the antioxidative systems were concurrently increased, were more resistant to environmental stresses than cultivars with lower antioxidative capacity (Shaaltiel & Gressel, 1986; Shaaltiel *et al.,* 1988). The activity of SOD, showed marked decline in *Vigna catjang* and *Oryza sativa* under salinity stress (Singha & Choudhuri, 1990). In cotton, analysis of GR, an enzyme of the cellular antioxidant system, showed that the decline in enzyme activity associated with plant development under irrigated conditions was inhibited by water stress (Burke *et al.,* 1985). The data presented here shows that the activity of SOD was constant in the tolerant somaclone (7R1), whilst it was increased in the sensitive somaclone 4R1 and the sensitive cultivar CUF 101. AP activity increased significantly at 200 g l^{-1} PEG in 7R1 whereas it decreased in CUF 101 compared with 0 g l^{-1} PEG. At 200 g l^{-1} PEG, CAT activity was 1.4 fold higher in 7R1 than that measured in CUF 101. GR activity was unchanged during drought stress. SOD activity in the alfalfa cultivar Argon, remained unchanged during drought stress treatment, suggested that the

alfalfa leaves kept their ability to dismutase superoxidase radicals. In contrast, CAT and peroxidase were significantly inhibited by slight drought stress (Irigoyen *et al.*, 1992). However, catalase and peroxidase responded differently to the more severe stresses. While peroxidase underwent additional inhibition, CAT activity was highly stimulated at moderate drought stress (-2.0 MPa) (Irigoyen *et al.*, 1992).

In alfalfa parents and somaclones there was a positive correlation between proline accumulation, the activity of the antioxidant enzymes, CAT, AP, and seedling growth response to osmotic stress. This correlation requires further examination to establish whether the response of proline synthesis and antioxidative enzyme activity is an inductive one.

Fig. 3.1 Seedlings of *M. sativa* were grown on a raft of alkathene beads in plastic pots containing 300 ml of Rorison solution (Hewitt, 1966).

Fig. 3.2 Assessment of *M. sativa* cv. CUF 101, somaclones derived from tissue culture and parent CUF101-1S for drought tolerance

```
            ┌─────────────────┐
            │    CUF 101      │
            └─────────────────┘
             (parent variety)
                    │
                    ▼
            ┌─────────────────┐
            │   CUF101-1S     │
            └─────────────────┘
          (partial salt tolerant line)

                    │
                    ▼
          ┌───────────────────┐
          │  Tissue culture   │
          └───────────────────┘
            (Initiation of callus)

                    │
                    ▼
        ╔═══════════════════════╗
        ║    Regeneration       ║
        ╚═══════════════════════╝
          (Shoot and root formation)

                    │
                    ▼         Plant growth *in vivo*

          ┌─────────────────────┐
          │    Flowering        │
          └─────────────────────┘

                    │
                    ▼         Cross pollination within plants derived
                                  from the same clone

        ┌───────────────────────────┐
        │  Seed progeny (1st cycle) │
        └───────────────────────────┘
        (1st cycle of tissue culture derived progeny)

                    │
                    ▼

      ╔═══════════════════════════════╗
      ║  Assess in 0-250 g l⁻¹ PEG    ║
      ╚═══════════════════════════════╝
          (Seedlings assessed *in vivo*)
```

Assess in 0-250 g l^{-1} PEG

Fig. 3.3 Growth of 14-day-old seedlings of *M. sativa* cv. CUF 101 in response to osmotic stress (PEG).

a) Mean root length ± SE.

b) Mean shoot length ± SE.

Concentrations of PEG were: 0- ▢, 50- ▥ , 100- ▤ , 150- ▨ ,
200- ▦ , and 250 g l^{-1} - ■.

a)

b)

Conc. PEG (g l^{-1})
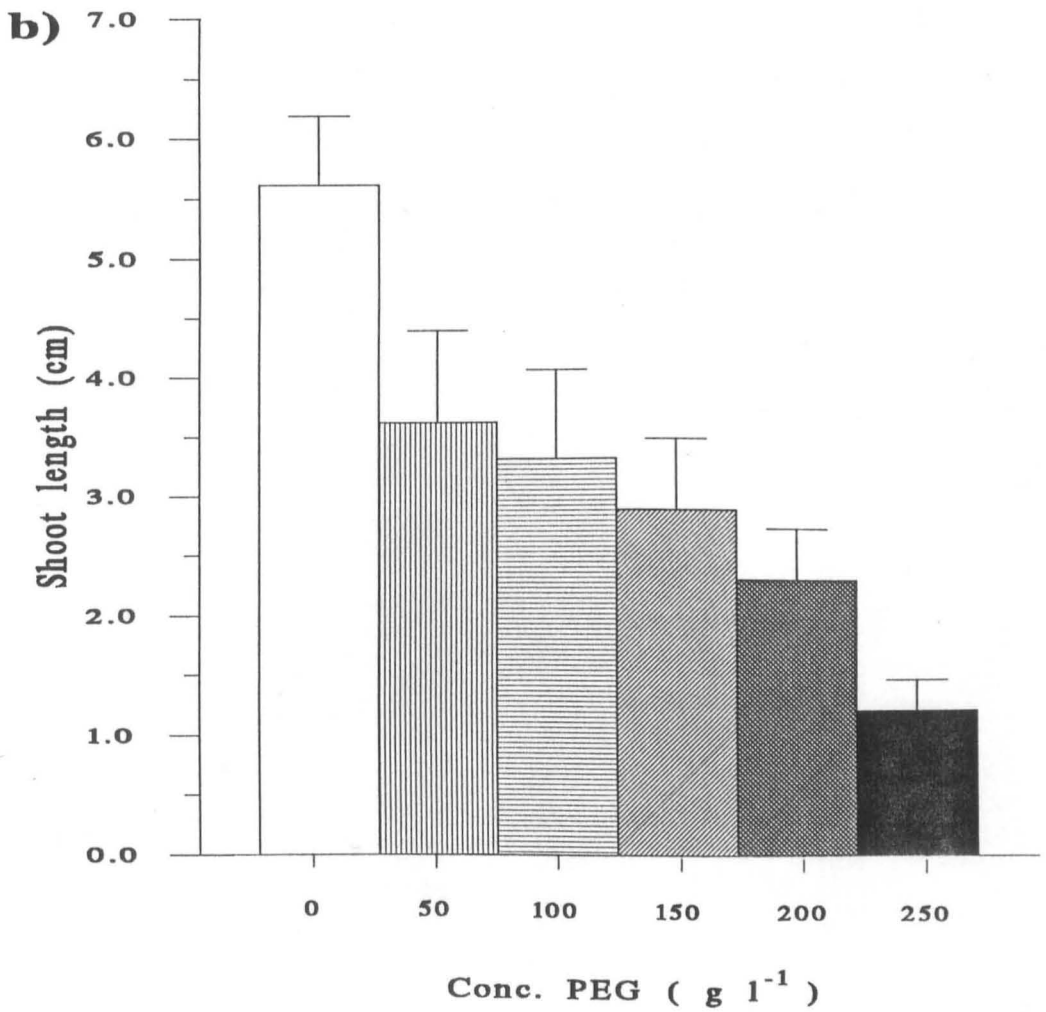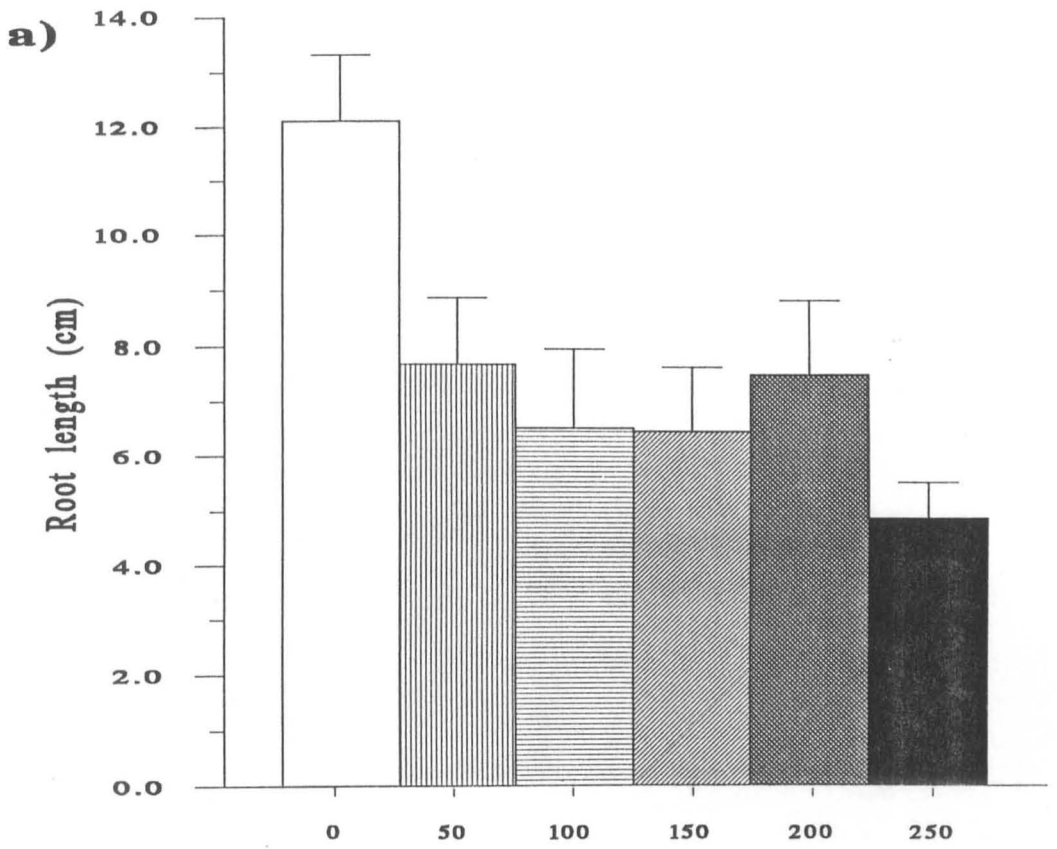
Fig. 3.4 Growth of 14-day-old seedlings of *M. sativa* cv. CUF 101 and somaclones (7R1, 4R1, 3R1, 6R2IV) in response to osmotic stress (PEG).

a) Mean root length ± SE.

b) Mean shoot length ± SE.

c) Percentage germination ± SE.

Concentrations of PEG were: 0-□, 200- ▨ , 250 g l^{-1} -■, and no survivors ✶ .

Fig. ... Root length (a), shoot length (b) and germination (c) of seedlings of five alfalfa genotypes CUF 101, 7R1, 4R1, 3R1 and 6R2IV ... under osmotic stress (PEG). Means ... were significantly different at ... Data are means ± SE.
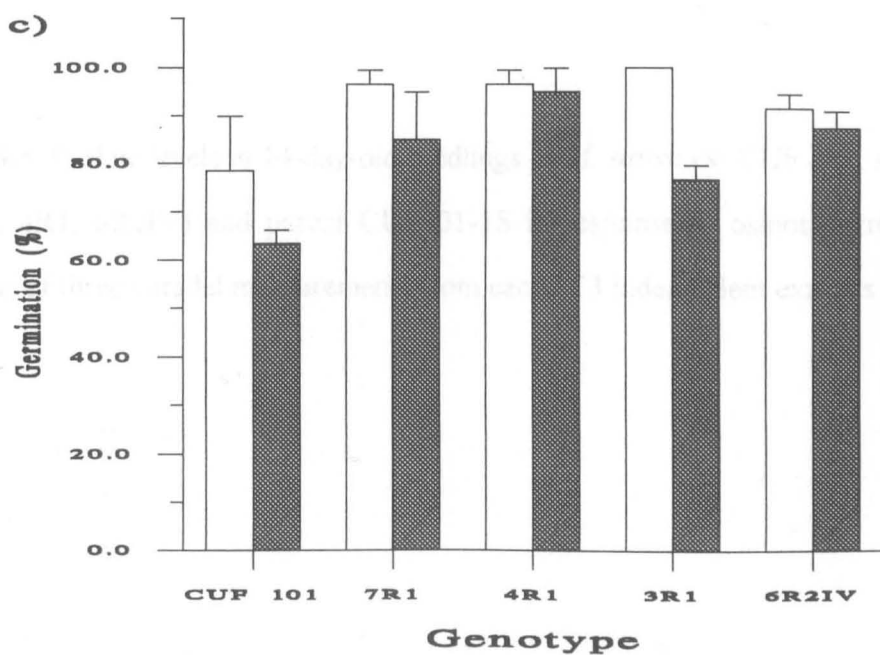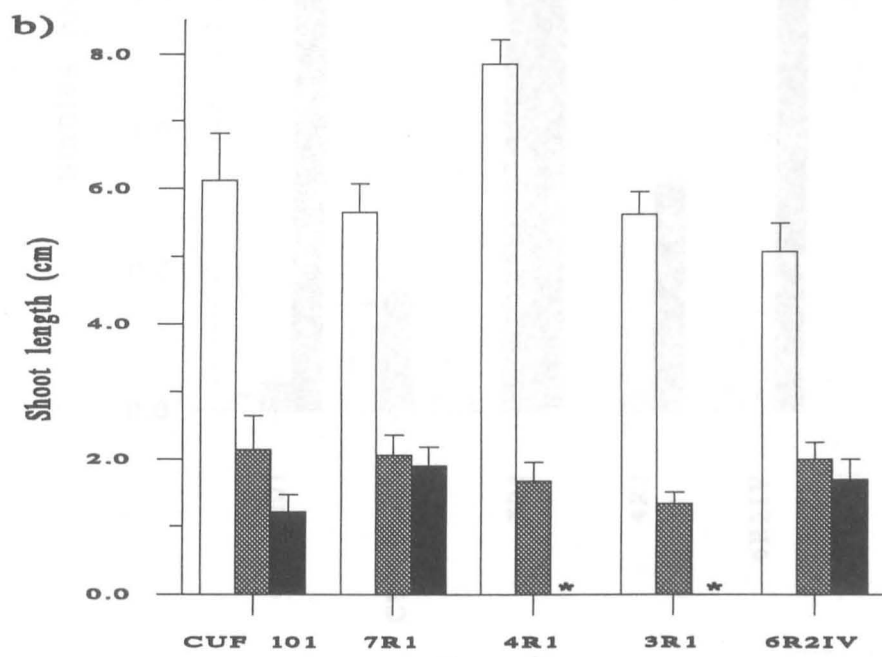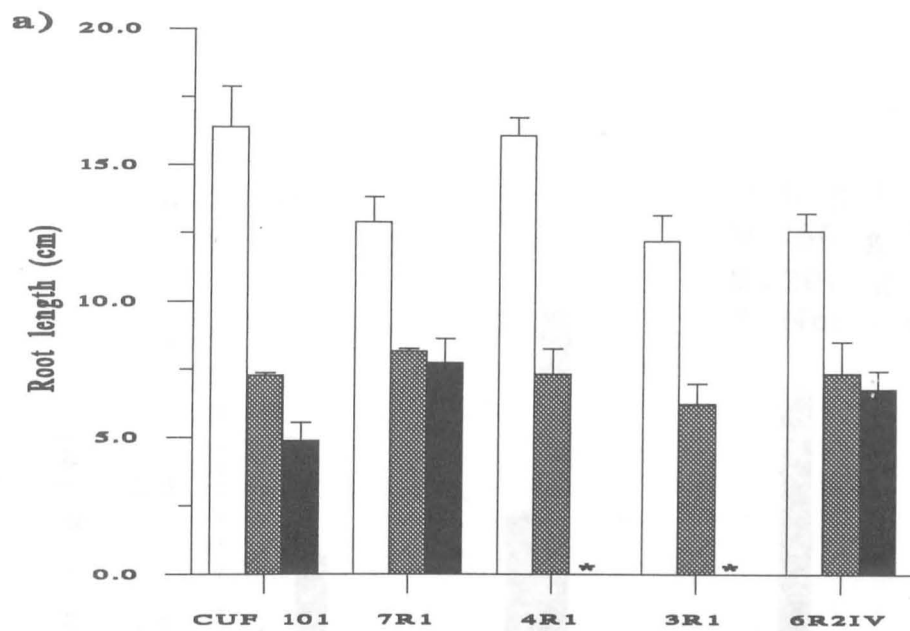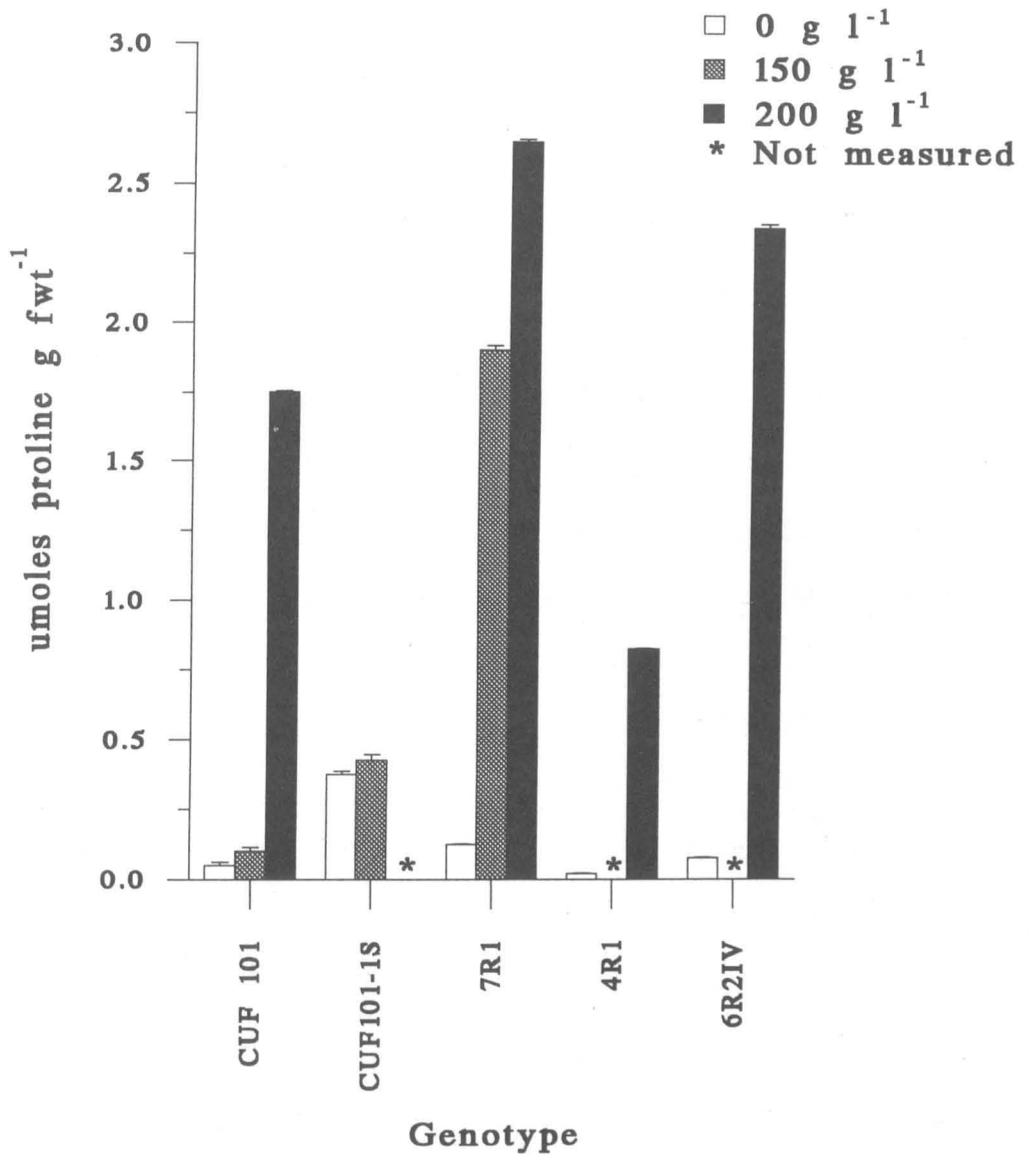
Fig. 3.5 Proline levels in 14-day-old seedlings of *M. sativa* cv. CUF 101, somaclones (7R1, 4R1, 6R2IV) and parent CUF101-1S in response to osmotic stress (PEG). Means of three parallel measurements from each of 3 independent extracts ± SE.

Fig. 3.6 Proline levels of 7-day-old seedlings of *M. sativa* cv. CUF 101, somaclone (7R1) in response to osmotic stress (PEG) after 1, 2, 4, and 7 days. Means of three parallel measurements from each of 3 independent extracts ± SE.

a) Cultivar CUF 101.

b) Somaclone 7R1.

Concentrations of PEG were: 0-□ and 200 g l^{-1}-■.

a)

b)

Time of incubation (days)
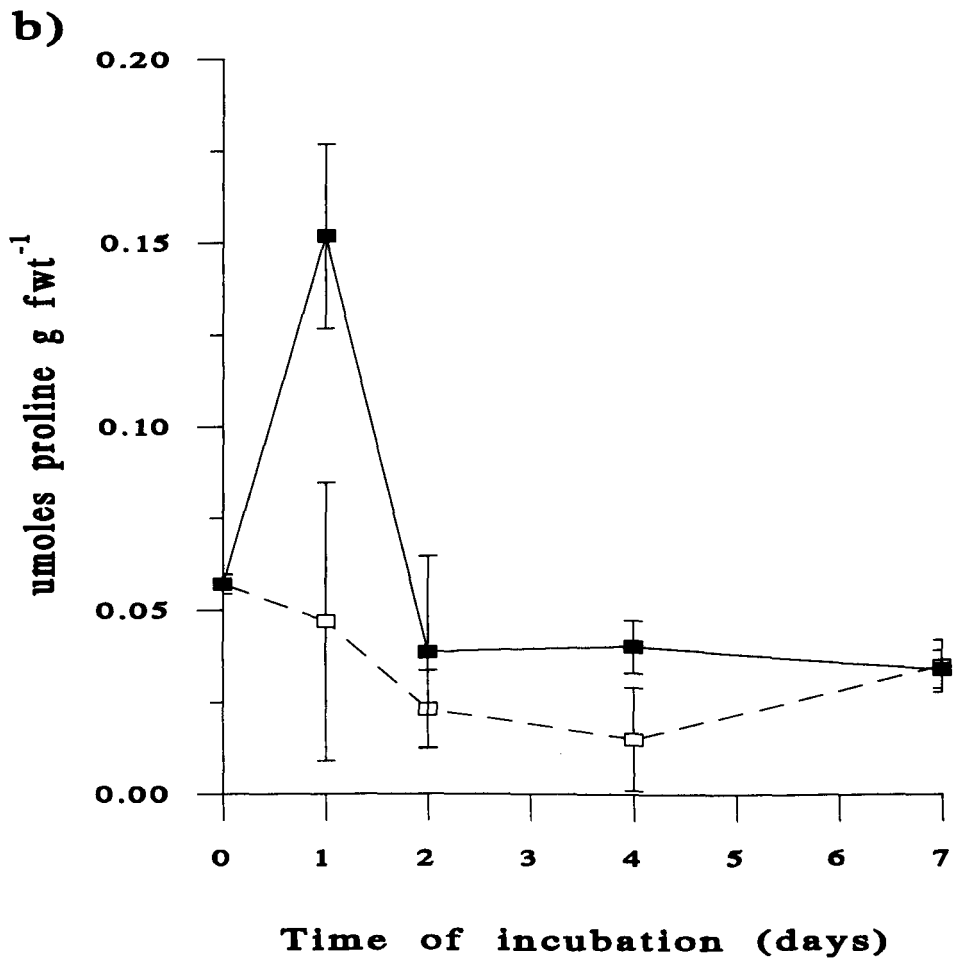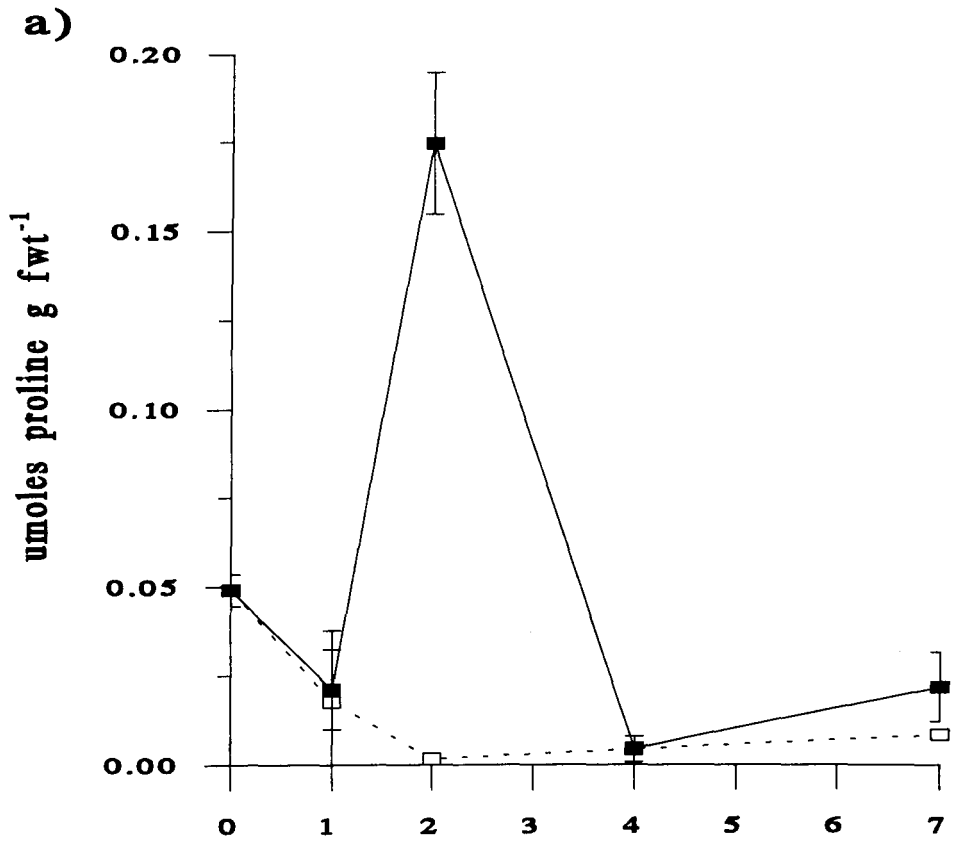
Fig. 3.7 Activity of antioxidant enzymes in 14-day-old seedlings of *M. sativa* cv. CUF 101 and somaclones (7R1, 4R1, 6R2IV) in response to osmotic stress (PEG). Means of three parallel measurements from each of 3 independent extracts ± SE.

a) Glutathione reductase.

b) Superoxide dismutase (somaclone 6R2IV not measured).

Concentrations of PEG were: 0-□ and 200 g l^{-1}-■.

a)

b)

Genotype
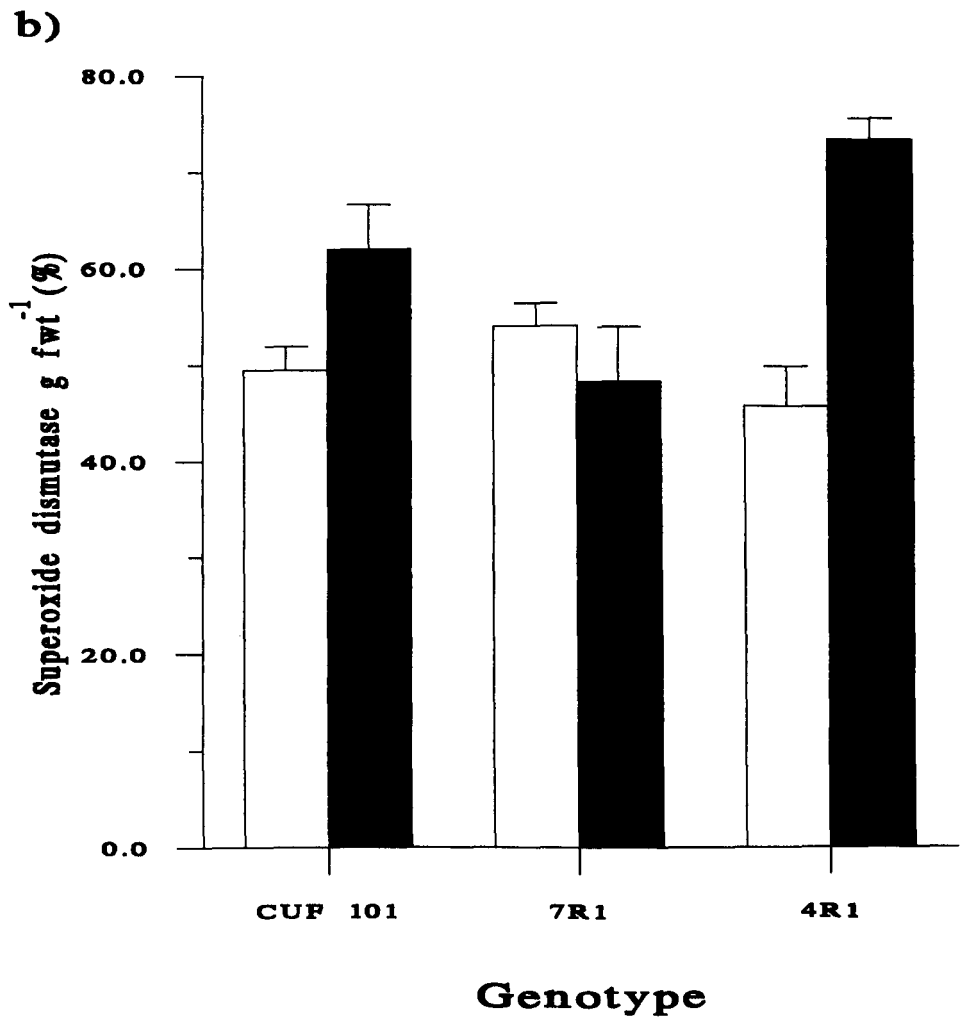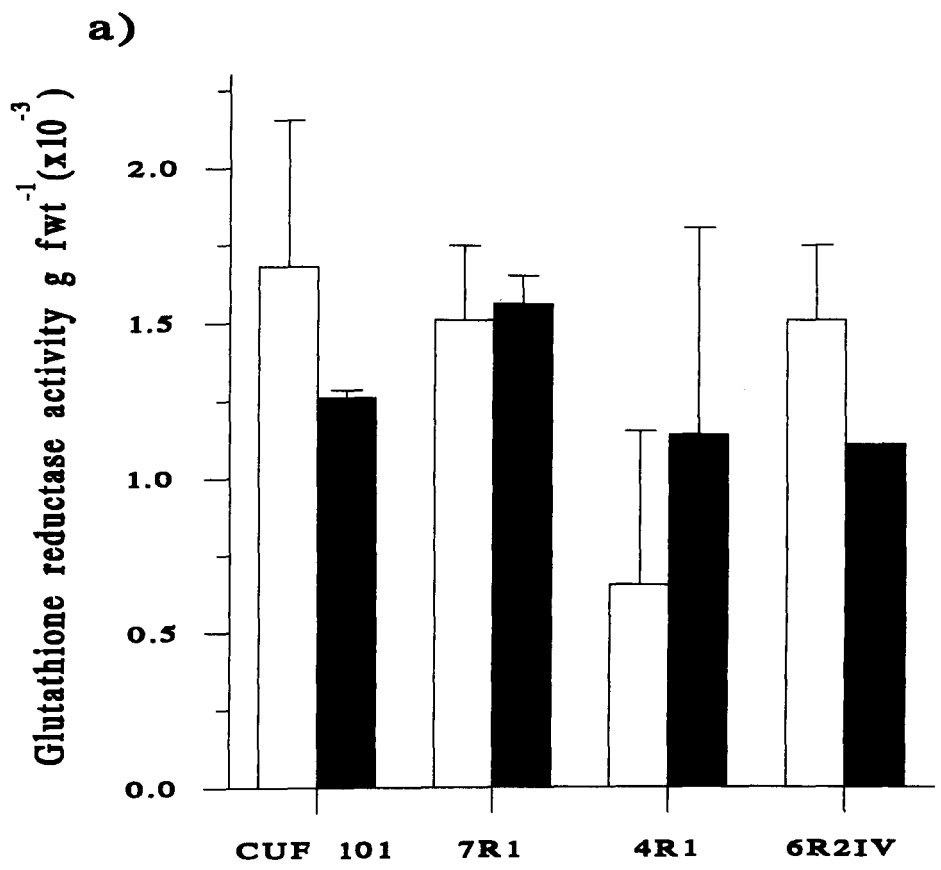
Fig. 3.8 Activity of antioxidant enzymes in 14-day-old seedlings of *M. sativa* cv. CUF 101, somaclones (7R1, 4R1, 6R2IV) and parent CUF101-1S in response to osmotic stress (PEG). Means of three parallel measurements from each of 3 independent extracts ± SE.

a) Catalase (CUF101-1S not measured).

b) Ascorbate peroxidase.

Concentrations of PEG were: 0-□ and 200 g l^{-1}-■.

b)

Ascorbate peroxidase activity g fwt^{-1} (x10^{-3})

Genotype

a)

Catalase activity g fwt^{-1} (x10^{-3})
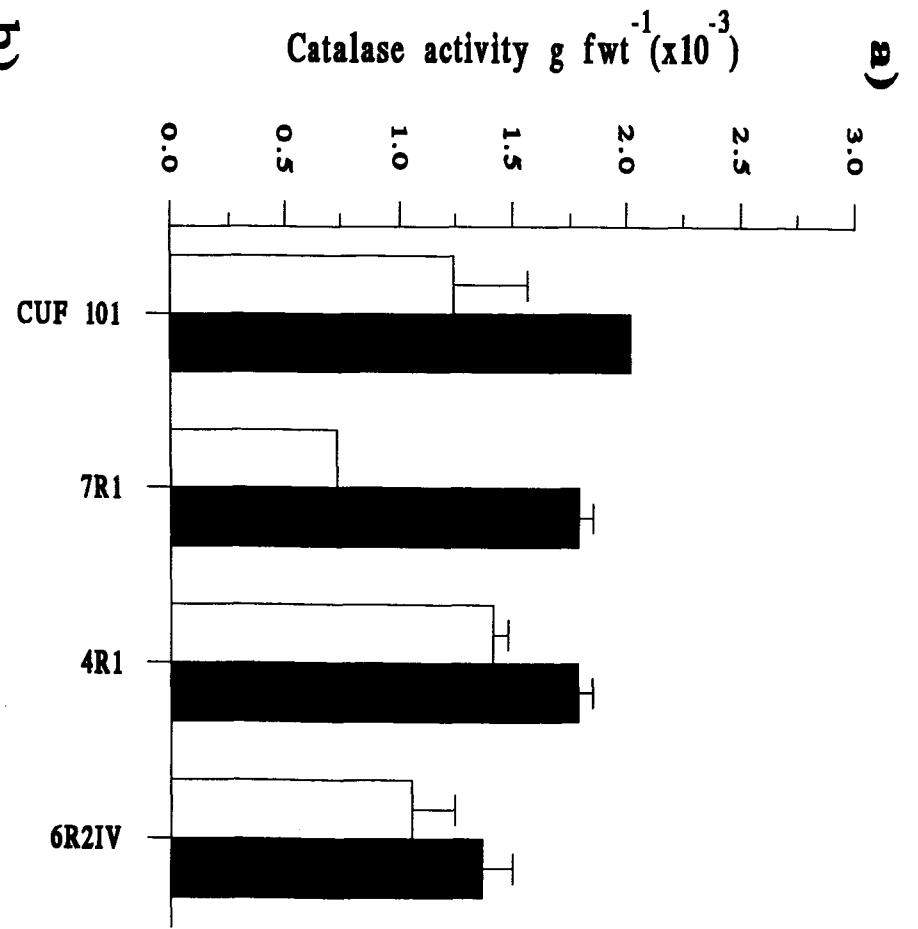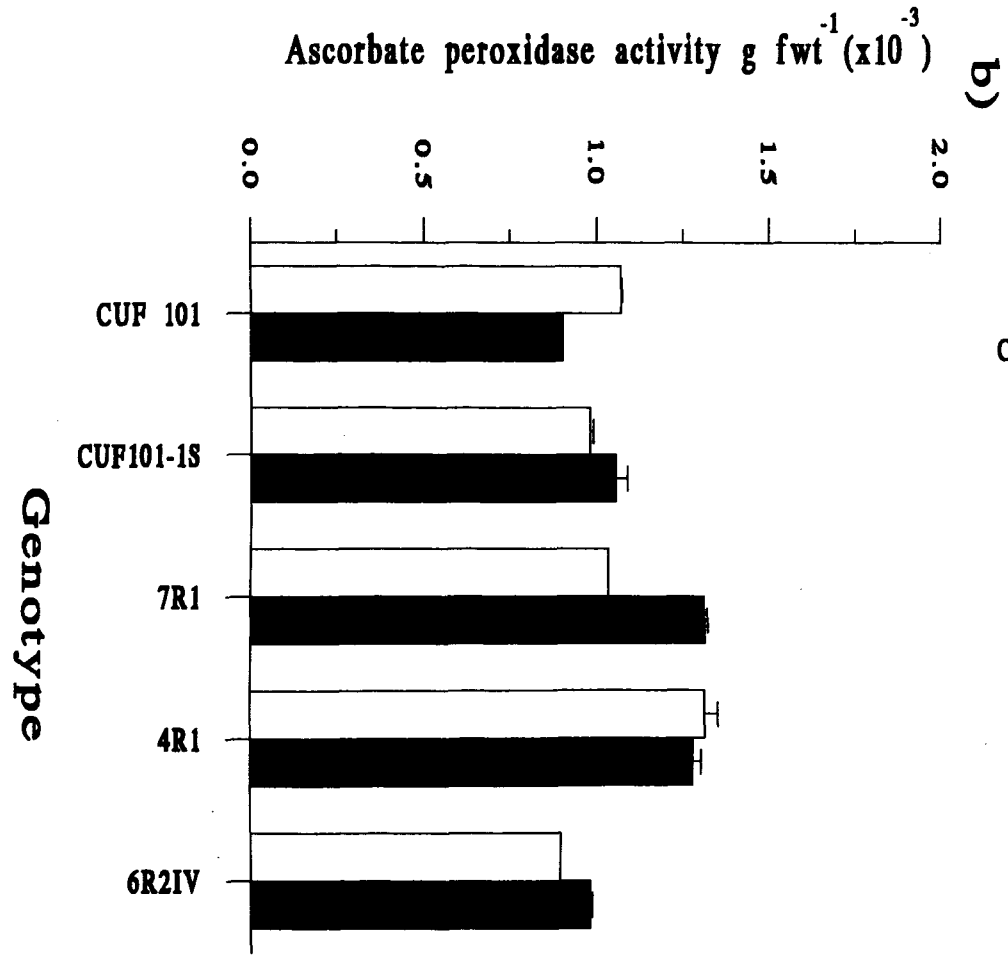
Fig. 3.9 Activity of antioxidant enzyme, catalase, in 7-day-old seedlings of *M. sativa* cv. CUF 101 and somaclone (7R1) in response to osmotic stress (PEG) after 1, 2, 4, and 7 days. Means of three parallel measurements from each of 3 independent extracts ± SE.

a) Cultivar CUF 101.

b) Somaclone 7R1.

Concentrations of PEG were: 0-□ and 200 g l^{-1}-■.

a)

Catalase activity g fwt^{-1} ($\times 10^{-3}$)

b)

Catalase activity g fwt^{-1} ($\times 10^{-3}$)

Time of incubation (days)
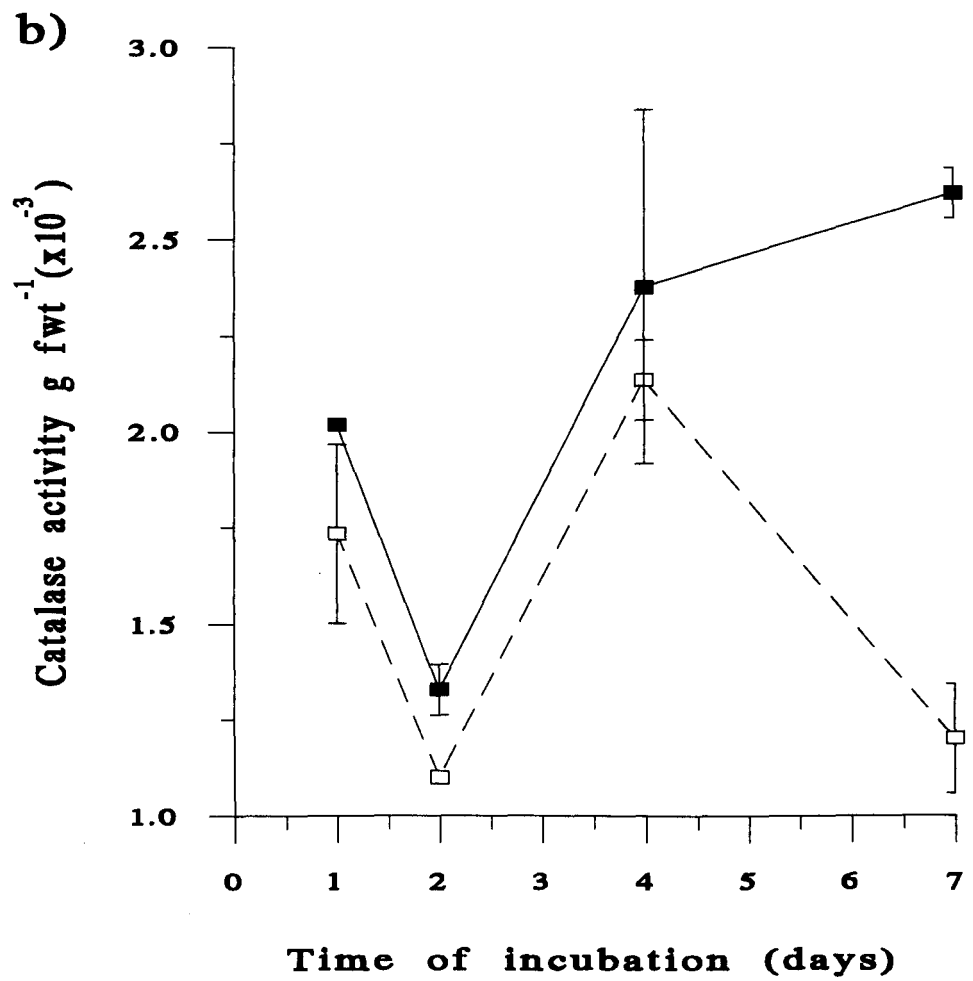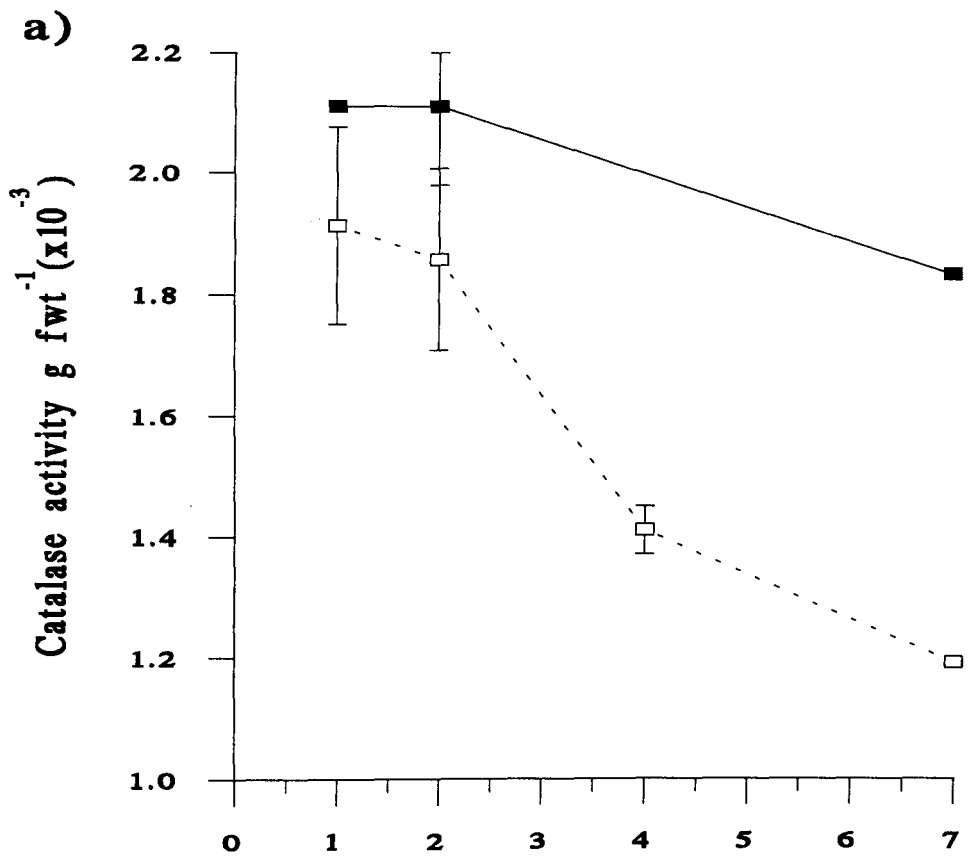
Fig. 3.10 Activity of antioxidant enzyme, ascorbate peroxidase, in 7-day-old seedlings of *M. sativa* cv. CUF 101 and somaclone (7R1) in response to osmotic stress (PEG) after 1, 2, 4, and 7 days. Means of three parallel measurements from each of 3 independent extracts ± SE.

a) Cultivar CUF 101.

b) Somaclone 7R1.

Concentrations of PEG were: 0-□ and 200 g l^{-1}-■.
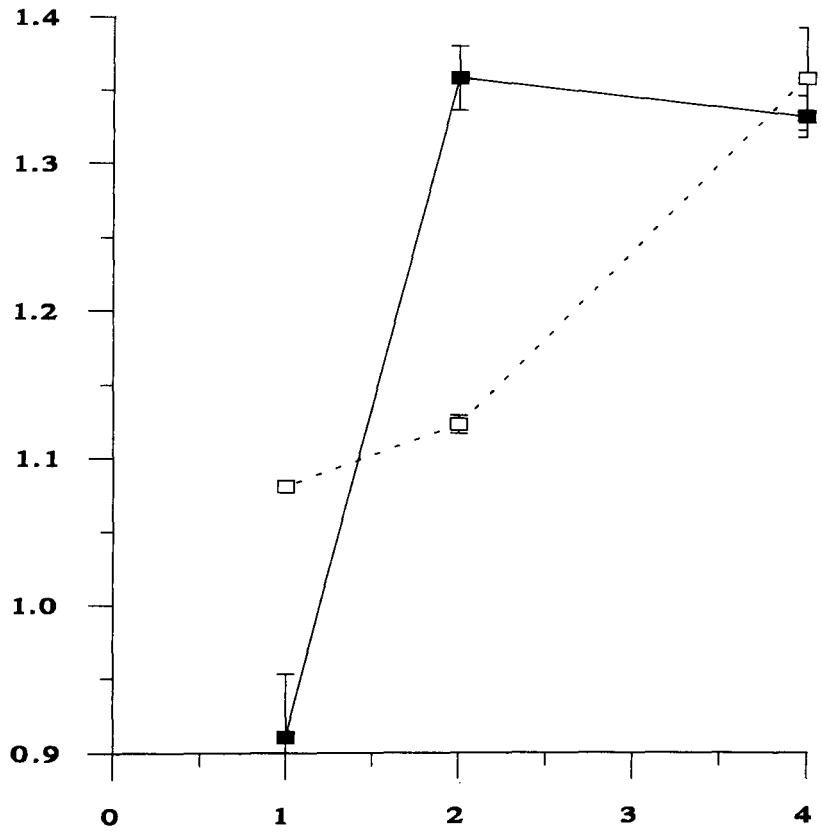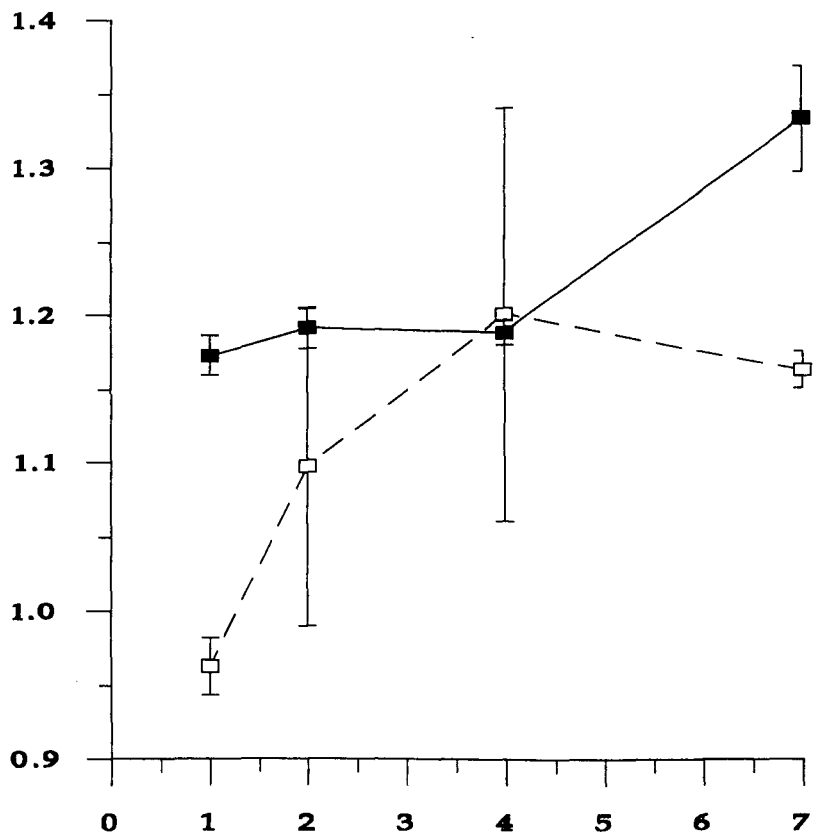
Table 3.1 Summary of root and shoot length means of 14-day-old seedlings of *M. sativa* cv. CUF 101 in the presence of 0-250 g l^{-1} PEG.

Genotype	NaCl (mM)	Mean Root L.	SE	Mean Shoot L.	SE	R/S
CUF 101	0	12.11	1.22	5.612	0.577	2.117
CUF 101	50	7.68	1.65	3.62	0.777	2.122
CUF 101	100	6.51	1.45	3.312	0.75	1.961
CUF 101	150	6.45	1.18	2.88	0.593	2.233
CUF 101	200	7.59	1.32	2.3	0.43	3.3
CUF 101	250	4.89	0.64	1.21	0.26	4.041

Table 3.2 Summary of root and shoot length means of 14-day-old seedlings of *M. sativa* cv. CUF 101 and somaclones (7R1, 4R1, 3R1, 6R2IV) in the presence of 0, 200 and 250 g l^{-1} PEG.

Genotype	PEG (g l^{-1})	Mean Root L.	SE	Mean Shoot L.	SE	R/S
CUF 101	0	16.41	1.464	6.114	0.692	2.68
CUF 101	200	7.28	0.078	2.138	0.51	3.41
CUF 101	250	4.89	0.64	1.21	0.26	4.04
7R1	0	2.89	0.932	5.64	0.419	2.29
7R1	200	8.19	0.092	2.128	0.303	3.85
7R1	250	7.75	0.89	1.89	0.29	4.1
4R1	0	16.122	0.66	7.835	0.364	2.06
4R1	200	7.370	0.93	1.66	0.292	4.44
4R1	250	0	0	0	0	0
3R1	0	12.21	0.969	5.603	0.327	2.18
3R1	200	6.264	0.759	1.331	0.169	4.71
3R1	250	0	0	0	0	0
6R2IV	0	12.571	0.66	5.051	0.255	2.49
6R2IV	200	7.372	1.159	1.981	0.250	3.72
6R2IV	250	6.78	0.67	1.67	0.31	4.06

CHAPTER 4

Assessment for Salt Tolerance of Somaclones Derived from Callus of Alfalfa

4. Assessment for Salt Tolerance of Somaclones Derived from Callus of Alfalfa

4.1 INTRODUCTION

In classical plant breeding programmes, selection is carried out on large populations normally in the field and some have produced an improved germplasm (Al-Khatib *et al.*, 1993). Field selections are influenced by environmental conditions, and can be uncertain and lengthy, especially in the case of breeding for quantitative characteristics with a polygenic background. Such traits may in some cases show slight changes per selection cycle, thus a long time is required to improve a desired agronomic trait (Wenzel & Foroughi-Wehr, 1993). Plant biotechnology techniques will complement these traditional methods and should be of most use where no natural resistance is found within a species, or where conventional breeding practices are difficult (Collin & Dix, 1990).

Plant tissue culture techniques are of interest to the plant breeder, since they provide a method for the production of somaclonal and gametoclonal variation that might enhance salt tolerance (Nabors *et al.*, 1980; Bhaskarans *et al.*, 1986; McHughen, 1987; Watad *et al.*, 1991; Winicov, 1991). Somaclonal variation technology takes advantage of the naturally occurring genetic variation that appears in plants regenerated from somatic cells grown in tissue culture. This variation can pre-exist in the explant tissue but more usually it arises during the tissue culture procedure itself, even in the absence of mutagens (Larkin & Scowcroft, 1981). The selection of genotypes expressing cellular NaCl tolerance *in vitro* has many apparent advantages for crop improvement, but the poor relationship between tolerance *in vitro* and *in vivo* and extreme somaclonal variation resulting from extended culture periods in saline media have reduced the usefulness of such procedures in alfalfa breeding according to McCoy (1987). In fact standard cell culture protocols may

involve significant osmotic stress. Unintentional selection for plants that perform well in non-saline tissue culture may therefore result in plants capable of improved growth at higher levels of NaCl. For example, plants regenerated from callus cultures of Regen-S alfalfa (line W75RS) (Bingham *et al.* 1988) showed apparent tolerance to NaCl (Smith & McComb, 1981; Johnson & Smith, 1992) indicating that salt tolerance in callus cultures may arise at random in the absence of NaCl in the culture media. This means that a short tissue culture stage can be used as a source of somaclones and not necessarily for *in vitro* screening.

The aim of the work described in this chapter was to determine whether heritable changes in salt tolerance were present in progeny of regenerants derived directly from cell cultures not exposed to NaCl.

4.2 MATERIAL AND METHODS

4.2.1. Seedling screen for NaCl tolerance

A seedlings screen was used to assess the level of NaCl tolerance in alfalfa, cv. CUF 101, CUF101-1S and somaclones derived from the first and second tissue culture cycles. To develop an effective screening procedure it was necessary to use different calcium chloride and sodium chloride concentrations. Three calcium chloride treatments (0, 1.5, 4 mol m^{-3}) and four NaCl treatments (0, 50, 100, 150 mol m^{-3}) were used to examine this interaction.

Seeds of cv. CUF 101 were surface sterilised in 2% v/v sodium hypochlorite for 2 minutes, rinsed in sterile water and dried at room temperature. Within each of 3 replicates, 20 seeds were evenly spaced on a raft of alkathene beads in pots containing 300 ml of nutrient solution. The solution used was modified half strength Rorison solution (Hewitt, 1966) (see Appandix 3.2) with 3 concentration of CaCl$_2$ (0, 1.5, 4 mol m^{-3}) for each NaCl concentration (0, 50, 100, 150 mol m^{-3}). Pots were arranged randomly in a shallow tray filled with water and placed under a clear plastic

in a growth room at 25-27°C. After two weeks shoot length and root length were measured from the base of the hypocotyl to the base of the youngest trifoliate leaf or the cotyledon and from the tip to the top of root, after which the seedlings were separated into shoot and roots which were dried in an oven at 80°C for 48 hours.

The same seedling screen was used with nutrient medium which contained concentrations of 0, 200 and 250 mol m^{-3} NaCl and 4 mol m^{-3} CaCl$_2$ to assess the tolerance of seeds of cv. CUF 101, CUF101-1S and the seeds of somaclones 1R, 2R, 3R, 4R, 5R, 6R, 7R, 8R, 9R derived from the first and second tissue culture cycles (see Chapter 2, Section 2.2.2) (see Fig. 4.1). A concentration of 4 mol m^{-3} CaCl$_2$ allowed a significant level of germination and growth in the highest concentration of NaCl. There were three replicates for each treatment. After two weeks in the growth chamber, shoot and root length were measured, and seedlings from the 200 mol m^{-3} NaCl treatment were frozen in liquid nitrogen for subsequent biochemical analysis.

Analysis of variance was carried out for each character by SAS packages (1985). Bonferroni's method (Maxwell & Delaney, 1990) was used to compare between the means (Appendix 4.2, 4.5 and 4.7).

4.3 RESULTS

4.3.1 The effect of CaCl$_2$ on the response of NaCl

The data showed that root length was reduced with an increase in NaCl concentration. The inhibition by NaCl was in general greater at 0 mol m^{-3} CaCl$_2$ and was less with each increase in CaCl$_2$ (Fig. 4.2 and 4.3). Root length of CUF 101 seedlings at 150 mol m^{-3} NaCl was 23% ± 8% and 67% ± 18% of that in 0 mol m^{-3} NaCl contains 1.5 and 4 mol m^{-3} CaCl$_2$ respectively (Fig. 4.2a). Shoot length showed the same pattern with 23% ± 8% and 48% ± 14% in 1.5 and 4 mol m^{-3} CaCl$_2$ compared with their zero control (Fig. 4.2b). There was no germination in the highest NaCl concentration without added CaCl$_2$. There was a significant effect of

adding CaCl₂ on root length, but there was no significant effect of 1.5 and 4 mol m⁻³ CaCl₂ on shoot length ($P>0.05$) (Fig. 4.2).

There was no significant difference between root and shoot dry weight of CUF 101 seedlings at 150 mol m⁻³ NaCl containing 1.5 and 4 mol m⁻³ CaCl₂ ($P>0.05$) (Fig. 4.3). There was also no significant effect of NaCl concentration in each level of CaCl₂ on root and shoot dry weight, but there was a significant difference between 100 and 150 mol m⁻³ NaCl containing 4 mol m⁻³ CaCl₂ on root dry weight ($P<0.05$) (Fig. 4.3a).

4.3.2 Seedling response to NaCl

Regenerated plants that appear to be variants of the donor plant are shown in Fig. 4.4. In this study, most of the 687 regenerated plants were normal, 3 plants were dwarf, 2 sterile and some showed morphological abnormalities.

Root and shoot lengths were reduced in all the material grown at 200 mol m⁻³ NaCl. The reduction in root length was less in the somaclonal derived lines 7R1, 6R1, 8R1, 3R1, 9R1 and 4R1, compared with their parental CUF 101 and CUF101-1S (Fig. 4.5a). Shoot length showed the same pattern with the least reduction in 7R1, 4R1, 6R1, 8R1, 3R1 and 9R1, compared with parental CUF 101 and CUF101-1S (Fig. 4.5b). As a result of the significant difference in NaCl tolerance of 7R1 in comparison with CUF 101 and CUF101-1S, all further investigation concentrated on the response of 7R1 ($P<0.05$).

In the presence of 200 mol m⁻³ NaCl, root length of seedlings of CUF101-1S was reduced to 9% of the zero control and 7R1 to 19% of the control (Fig 4.6a), while shoot length was 9% of the control for CUF101-1S and 20% for 7R1 (Fig. 4.6b). These differences were significant at $P<0.05$.

There was a significant difference between root and shoot length means of 7R1 at 250 mol m⁻³ NaCl compared with 0 mol m⁻³ NaCl ($P<0.05$). A 1.9 fold increase in root length (Fig. 4.6a) and 2.4 fold increase in shoot length were observed in 250

mol m^{-3} compared with CUF101-1S at 250 mol m^{-3} NaCl (Fig. 4.6b). Shoot and root length in the first (7R1, 8R1, 9R1) and second (7R2, 8R2, 9R2) tissue culture cycles were measured at 200 mol m^{-3} NaCl. All genotypes showed a reduction of 28-31% in the first tissue culture cycle for shoot and root length and a reduction of 7-13% in the second tissue culture cycle compared with their 0 mol m^{-3} NaCl controls (Fig. 4.7).

4.4 DISCUSSION

The effect of adding CaCl$_2$ to the media containing NaCl was investigated. In whole plant studies, Kurth *et al.* (1986) suggested that Ca^{2+} ameliorates salt toxicity by mitigating the toxic effects of Na$^+$ ions rather than the osmotic effects associated with salt stress (Bliss *et al.*, 1986). In salt stressed *M. sativa* cell cultures, increased Ca^{2+} resulted in increased proline accumulation and increased growth (Shah *et al.*, 1990) suggesting that Ca^{2+} also has a role in ameliorating the osmotic component of salt toxicity.

The data from alfalfa somaclones showed that there was a significant increase of ($p<0.05$) root and shoot length of 7R1 at 250 mol m^{-3} NaCl compared with CUF101-1S (Fig. 4.5). Regenerants grown on medium without added NaCl could still enhance the performance of tissue culture derived plants when placed under NaCl stress. This is consistent with the increase in salt tolerance shown by regenerated plants of Regen-S (Smith & McComb, 1981; Johnson & Smith, 1992). Smith and McComb (1981) reported improved salt tolerance in alfalfa plants derived from a population selected for improved capacity for regeneration in culture (Regen-S). They showed that selection for improved callus growth and regeneration without being exposed intentionally to any osmotic stress also affected the response of plants of Regen-S to osmotic stress. Johnson and Smith (1992) also showed that regenerated plants of Regen-S had a higher salt tolerance than the parent plants, and

they concluded that the salt tolerance must have been a consequence of passage through tissue culture.

There were some somaclones which showed a greater inhibition of growth (2R1, 5R1) in the presence of NaCl compared with their parent CUF101-1S (Fig. 4.5). In an assessment of somaclones derived from callus of potato, over a period of five years in field conditions 13 economically important characters such as growth of stem and leaves, maturity, tuber number, tuber weight, time of blooming and others showed significantly positive and negative differences in comparison with the control population (Thieme *et al.*, 1995). The data for the assessment of progeny of the second cycle of tissue culture showed that the passage through a 2nd cycle had caused a decrease in the salt tolerance shown by somaclones (Fig. 4.7). A given variant may carry more than one nuclear or cytoplasmic mutation (Bingham & McCoy, 1986). Genetic phenomena identified in alfalfa include the direct expression of recessive mutations in the diploid genome and an unstable genetic system for anthocyanin in a tetraploid line (Bingham & McCoy, 1986) suggested that in the latter case, there was a functional as a result of culture then it reverted to full function in the next cycle of culture. The mechanism leading to this loss of salt tolerance in the 2nd cycle of tissue culture is unknown.

Fig. 4.1 Method of assessment of *M. sativa* cv. CUF 101, CUF101-1S and somaclones for salt tolerance

```
                    ┌─────────────────┐
                    │    CUF 101      │
                    └─────────────────┘
                     (Parent variety)
                            │
                            ▼        In vivo selection
                    ┌─────────────────┐
                    │   CUF101-1S     │
                    └─────────────────┘
                  (Partial salt tolerant line)
                            │
                            ▼
                    ┌─────────────────┐
                    │ Tissue culture  │
                    └─────────────────┘
                   (Initiation of callus)
                            │
                            ▼
                    ┌─────────────────┐
                    │  Regeneration   │
                    └─────────────────┘
                 (Shoot and root formation)
                            │
                            ▼        Plant growth in vivo
                    ┌─────────────────┐
                    │   Flowering     │
                    └─────────────────┘
                            │
                            ▼      Cross pollination within plants derived
                                        from the same clone
                    ┌──────────────────────┐
                    │ Seed progeny (1st cycle) │
                    └──────────────────────┘
                            │
                            ▼
                    ┌──────────────────────┐
                    │ Assess in 0-250 mM NaCL │
                    └──────────────────────┘
                            │
                            ▼
                    ┌──────────────────────┐
                    │   Tissue culture     │
                    └──────────────────────┘
                            │
                            ▼
                    ┌──────────────────────┐
                    │   Regeneration       │
                    └──────────────────────┘
                            │
                            ▼
                    ┌──────────────────────┐
                    │ Seed progeny (2nd cycle) │
                    └──────────────────────┘
                            │
                            ▼
                    ┌──────────────────────┐
                    │ Assess in 0-250 mM NaCl │
                    └──────────────────────┘
                   (Seedlings assessed in vivo)
```

Fig. 4.2 Growth of 14-day-old seedlings of *M. sativa* cv. CUF 101 in NaCl (0,

50, 100, 150 mol m^{-3}) in the presence of CaCl$_2$ (0, 1.5, 4 mol m^{-3}).

a) Mean root length ± SE

b) Mean shoot length ± SE

Concentrations of NaCl were: 0- □, 50- ▤ , 100- ▦ , 150 mol m^{-3} - ■,

and no survivors ✱ .

a)

Root length (cm)

20.0

15.0

10.0

5.0

0.0

*

0   1.5   4

b)

Shoot length (cm)

8.0

6.0

4.0

2.0

0.0

*

0   1.5   4

Conc. CaCl$_2$ (mM)
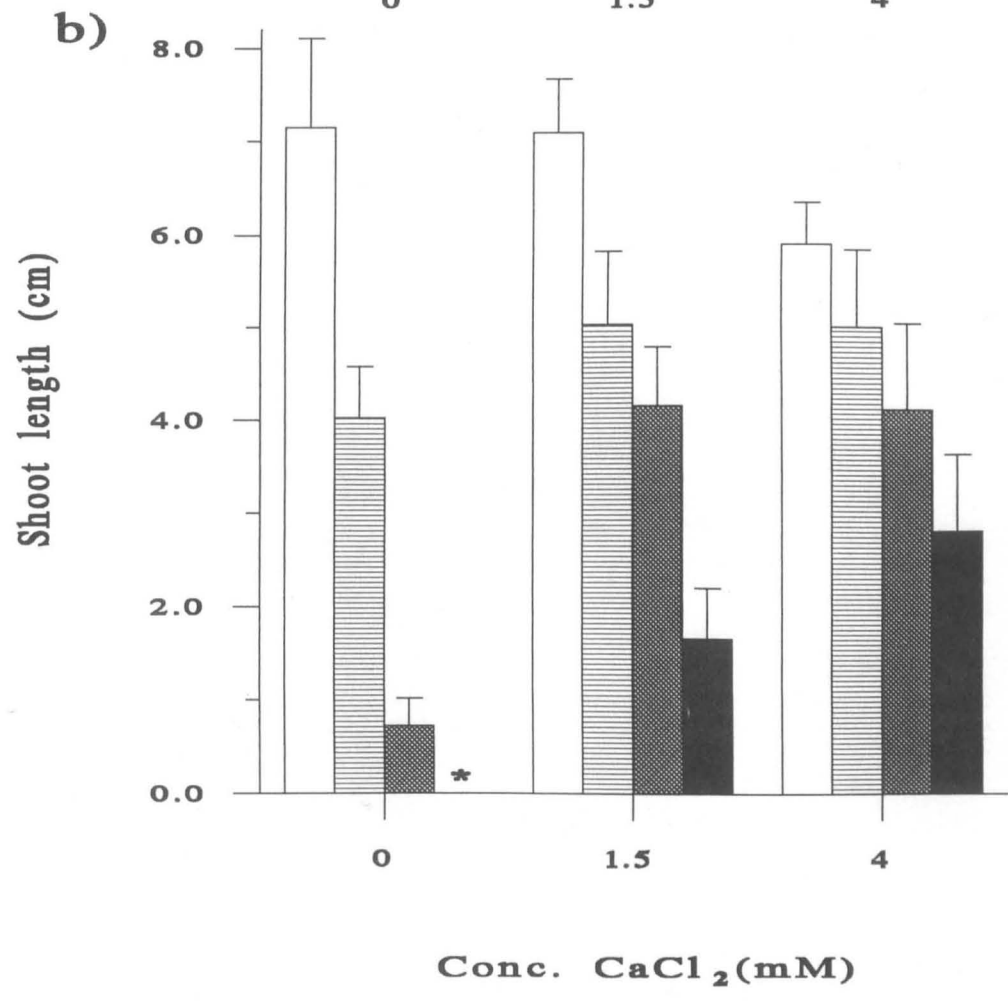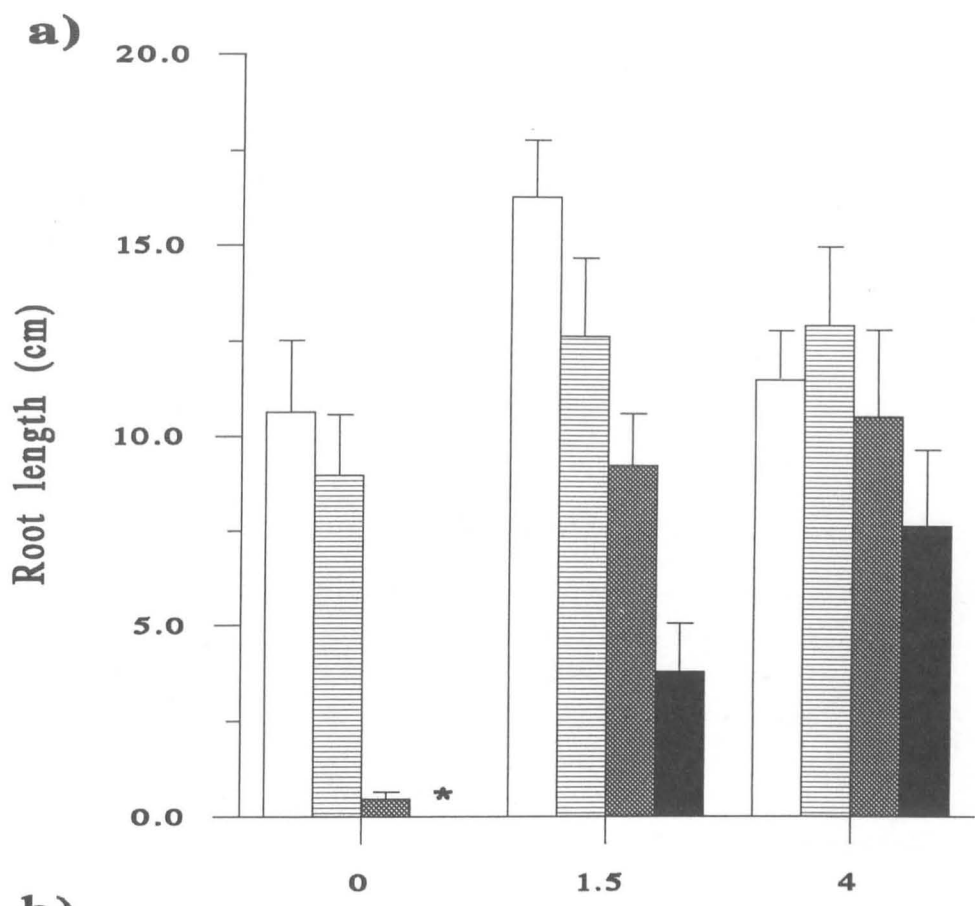
Fig. 4.3 Growth of 14-day-old seedlings of *M. sativa* cv. CUF 101 in NaCl (0, 50, 100 and 150 mol m^{-3}) in the presence of $CaCl_2$ (0, 1.5 and 4 mol m^{-3}).

a) Mean root dry weight ± SE

b) Mean shoot dry weight ± SE

Concentrations of NaCl were: 0- □, 50- ▤ , 100- ▨ , 150 mol m^{-3} - ■, and no survivors ✱ .

a)

b)

Conc. CaCl$_2$ (mM)
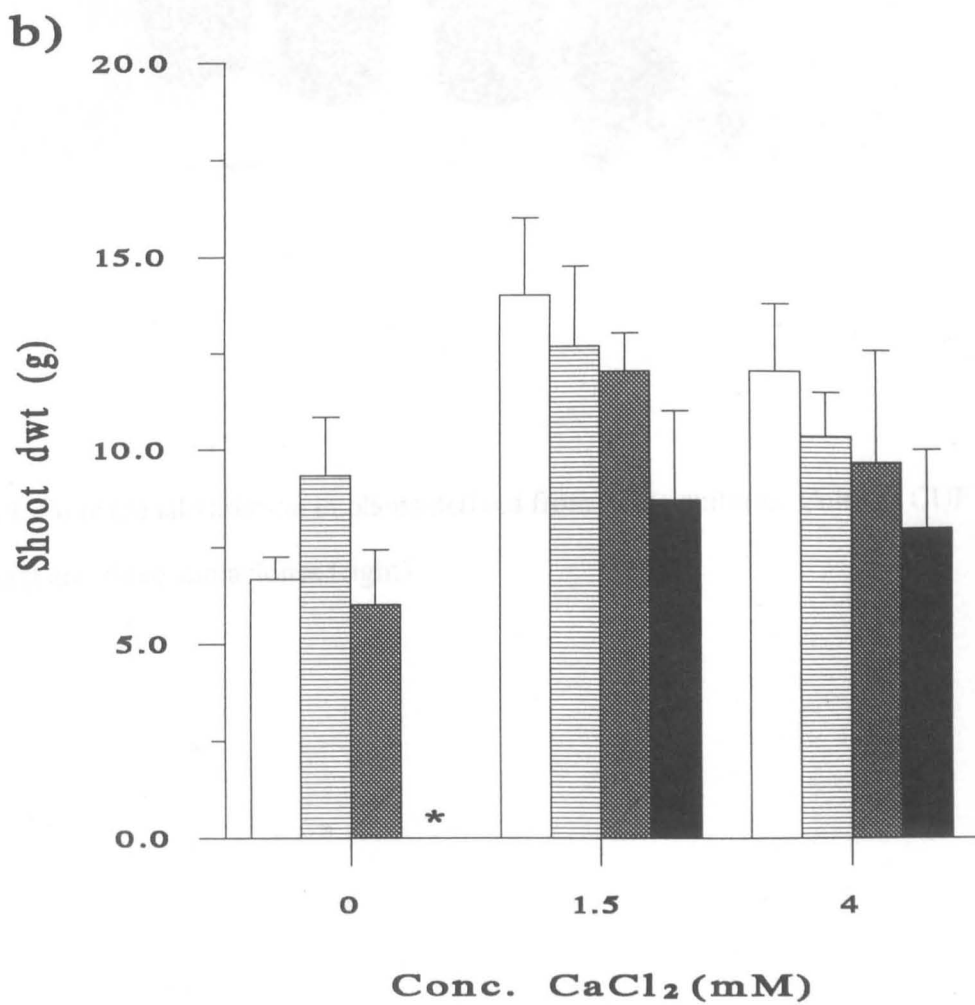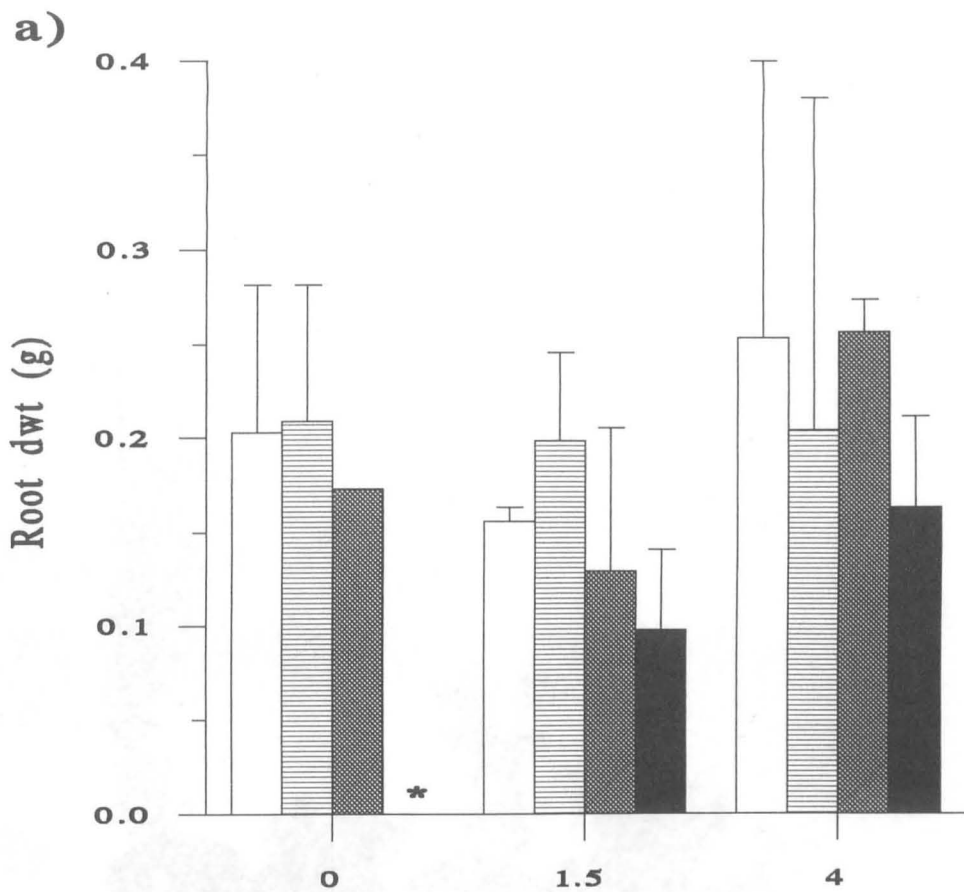
Fig. 4.4 Somaclonal variation in plants derived from tissue cultures. Cultivar CUF 101 (left) and three somaclones (right).

Fig. 4.5 Growth of 14-day-old seedlings of *M. sativa* cv. CUF 101, nine

somaclones (1R1, 2R1, 3R1, 4R1, 5R1, 6R1, 7R1, 8R1, 9R1) and parent

CUF101-1S in response to salinity stress (NaCl).

a) Mean root length ± SE.

b) Mean shoot length ± SE.

Concentrations of NaCl were: 0- $\square$ and 200 mol m^{-3} - $\blacksquare$.
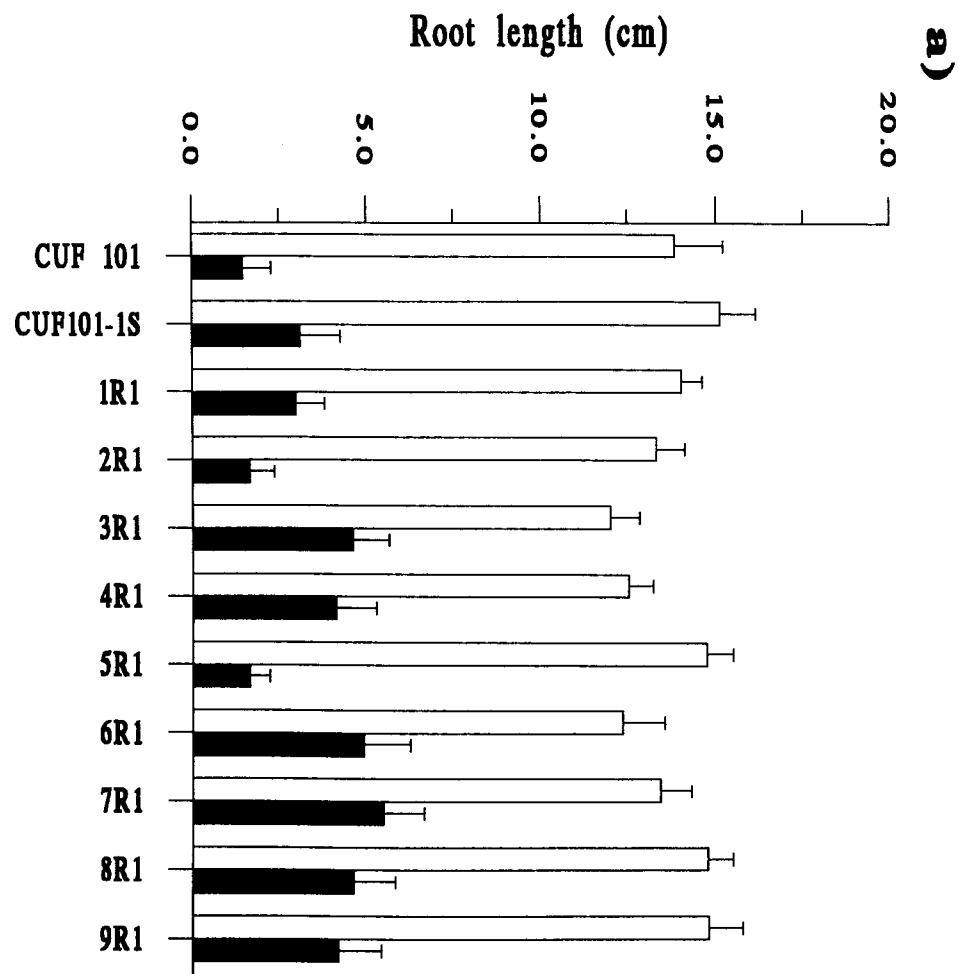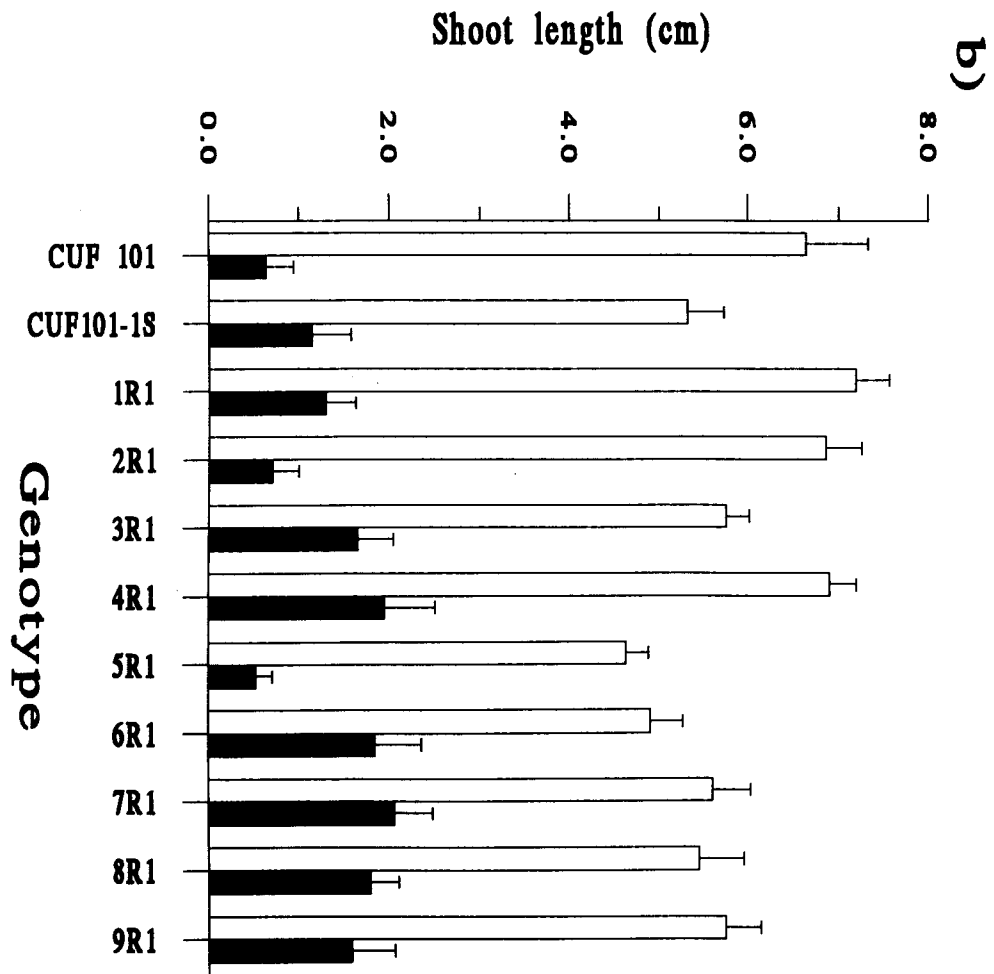
Fig. 4.6 Growth of 14-day-old seedlings of *M. sativa* cv. CUF 101, somaclone

(7R1) and parent CUF101-1S in response to salinity stress (NaCl).

a) Mean root length ± SE

b) Mean shoot length ± SE

Concentrations of NaCl were: 0-□, 200- ▓ and 250 mol m^{-3} -■.

a)
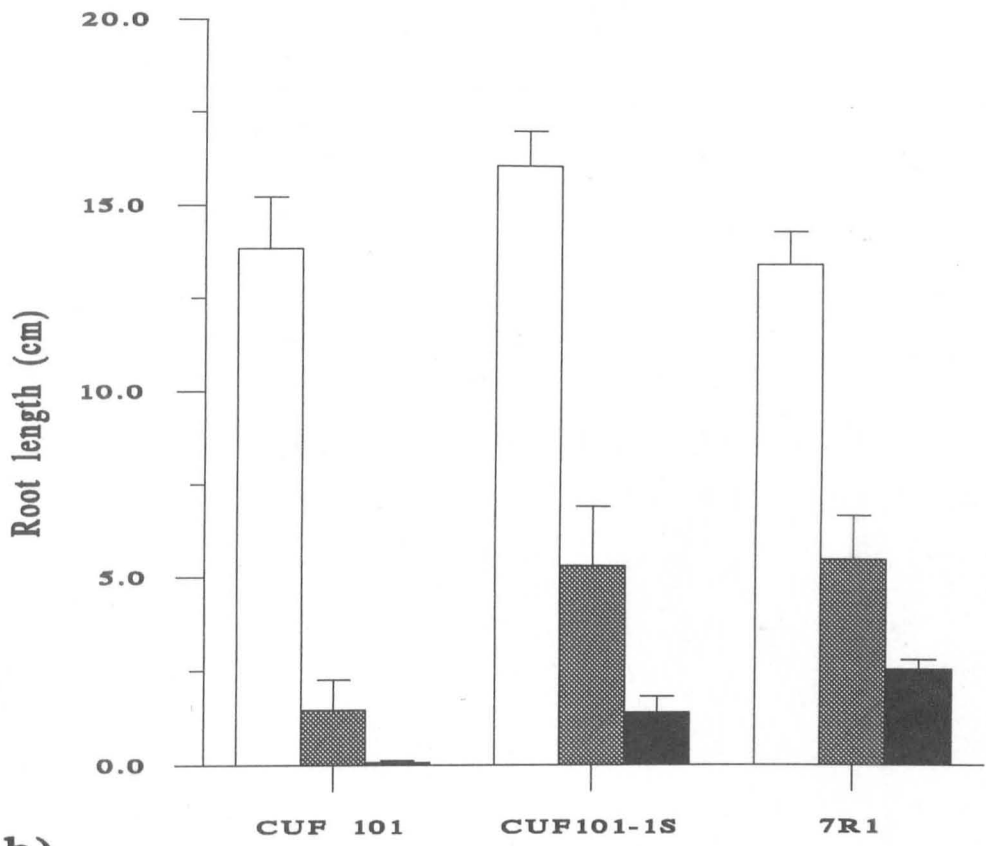
b)
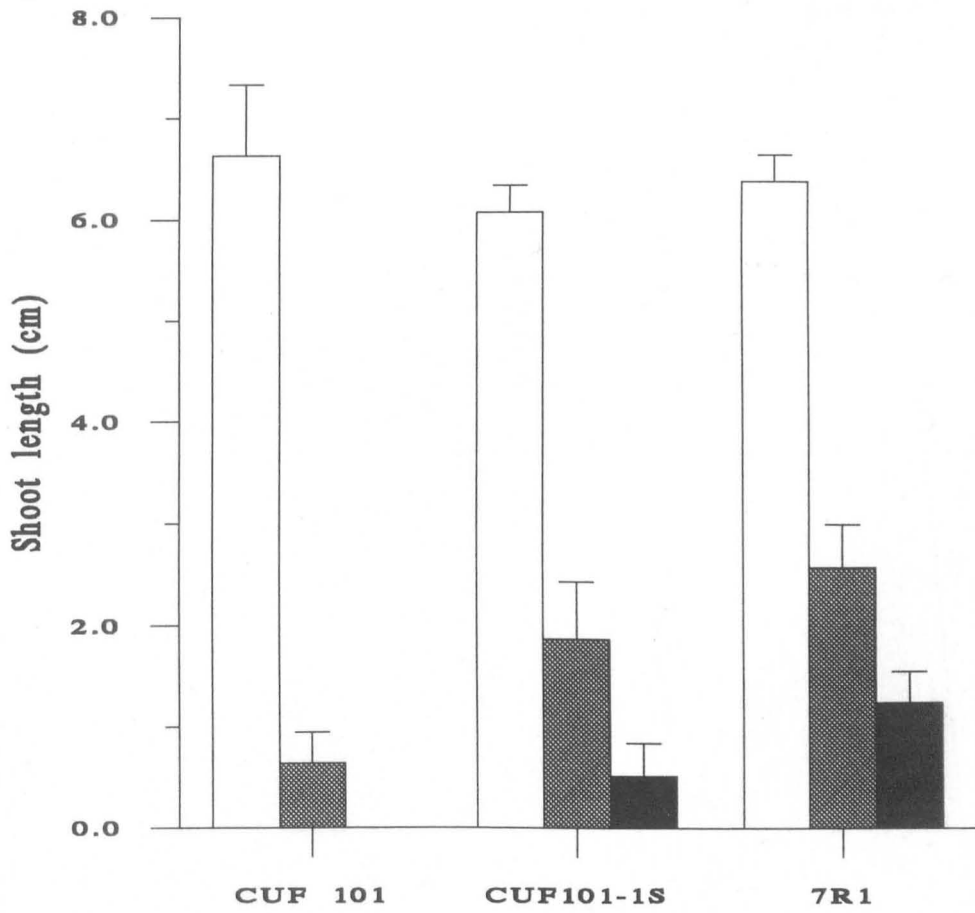
Genotype

Fig. 4.7 Growth of 14-day-old seedlings of *M. sativa* cv. CUF 101, from progeny of 1st (7R1, 8R1, 9R1) and 2nd (7R2, 8R2, 9R2) tissue culture cycles in response to salinity stress (NaCl).

a) Mean root length ± SE.

b) Mean shoot length ± SE.

Concentrations of NaCl were: 0- □ and 200 mol m^{-3} - ■.

a)

Root length (cm)

Genotype: CUF 101, CUF101-1S, 7R1, 7R2, 8R1, 8R2, 9R1, 9R2

b)

Shoot length (cm)

Genotype: CUF 101, CUF101-1S, 7R1, 7R2, 8R1, 8R2, 9R1, 9R2
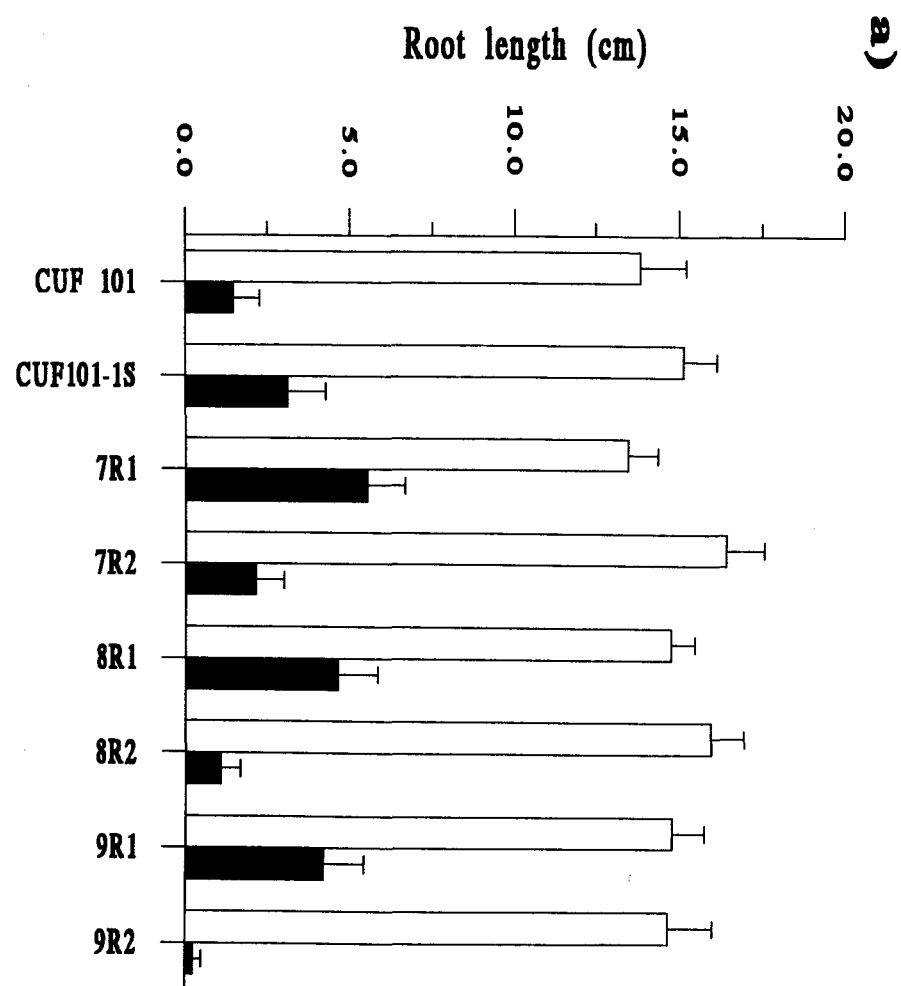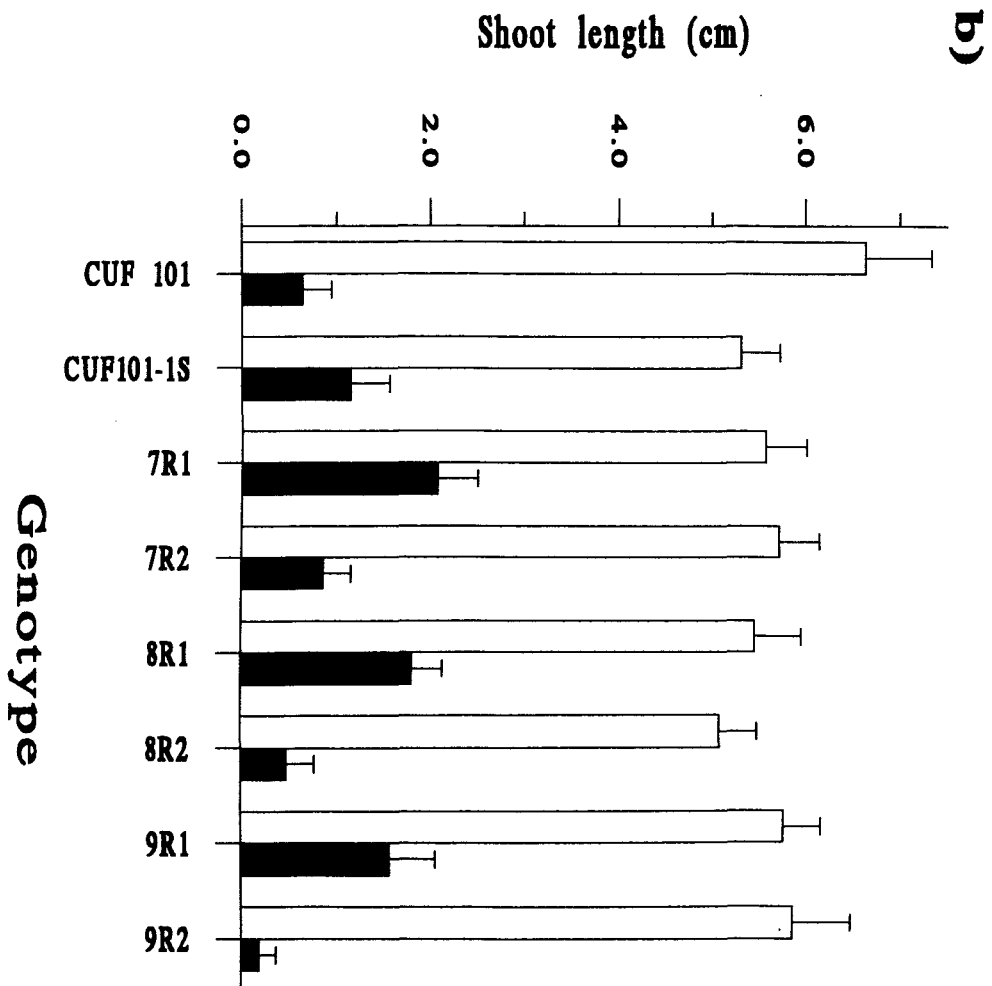
CHAPTER 5

In vitro Selection for Salt Tolerance

5. *In vitro* Selection for Salt Tolerance

5.1 INTRODUCTION

Selection through tissue culture technology offers an additional route for improvement to breeding in alfalfa (Arcioni *et al.*, 1990).

The difficulties associated with the regeneration of the cell lines and loss of salt tolerance in the regenerants may be overcome by the use of an *in vitro* screen where the callus is placed on a regenerating medium containing NaCl at high concentration. Shoot and root formation would then occur in the presence of NaCl thus imposing selection at the differentiation stage so that it is more likely that selected material will have a response to NaCl closer to whole plants. Further, there is no need for a separate cell selection then a regeneration stage. Although, from the literature the success rate in selection for salt tolerance from somaclones was quite low, it was thought that an *in vitro* screen via differentiating culture would overcome some of the problems.

The aim of this work was to screen for increased salt tolerance in the commercial variety of alfalfa, CUF 101, a selected more tolerant line CUF101-1S, and their regenerants using an *in vitro* screen. The *in vitro* selection was based on the use of differentiating cultures so that selection for salt tolerance would occur during the regeneration process.

5.2 MATERIAL AND METHODS

5.2.1 Selection of NaCl treatment for callus

Before using *in vitro* selection it was necessary to establish first the concentration of NaCl in the nutrient media which reduced the callus growth. Explants from cotyledons of CUF 101 were transferred to medium (BII). The callus was divided into equal sized portions of approximately 0.15 g and a portion placed in separate Universal vials on to media containing 0, 50, 100, 150, 200 and 250 mol m^{-3} NaCl. There were six replicates for each treatment, arranged in a randomised block design in a growth room at 25°C. After 28 days fresh weights were taken, the callus wrapped in foil and placed in an oven at 80°C for 48h then the dry weights measured. The results showed that a range of 0-350 mol m^{-3} NaCl was suitable for *in vitro* selection.

5.2.2 *In vitro* selection

Explants from cotyledon and hypocotyl of seedlings of CUF101-1S and four regenerant lines (6R1, 7R1, 8R1, 9R1) were transferred to BII medium to initiate callus (see Chapter 2, Section 2.2.2). After 2 weeks, callus having been initiated, the explants (cotyledon, hypocotyl) with attached callus were transferred to the regeneration medium (Boi2y) containing concentrations of 0-350 mol m^{-3} NaCl (Fig. 5.1). Shoots formed on these treatments were transferred to GS medium for root induction and shoot elongation. The resulting plantlets were potted into a soil-sand-peat moss mixture (3:1:1) and placed in a glasshouse. The plants were allowed to flower, and were cross pollinated within plants derived from the same callus and allowed to set seed. This seed was derived from plants that had been through two tissue culture cycles.

5.3 RESULTS

5.3.1 Response of callus to NaCl

Explants of CUF 101 were placed on the callus initiation medium and the growth assessed (Appendix 5.2). Growth of callus was reduced by NaCl but even at the highest concentration, 250 mol m^{-3} NaCl, there was some growth of callus from cotyledon explants (Fig. 5.2). Both fresh and dry weights decreased with increasing external NaCl. Concentration in excess of 150 mol m^{-3} NaCl caused brown coloration and necrosis in most of the cultures.

5.3.2 Regeneration in the presence of NaCl

Regeneration frequencies in callus and mean numbers of bud per culture from CUF 101, CUF101-1S line and 4 somaclones (6R2IV, 8R2IV, 7R2IV, 9R2IV) (Fig. 5.1) in the presence of 0-350 mol m^{-3} NaCl are shown in Fig. 5.3 to 6 (Appendix 5.3 and 5.4).

There was limited regeneration of CUF 101 and CUF101-1S in the first tissue culture cycle whereas regeneration in the 2nd tissue culture cycle was enhanced 15 fold (Fig. 5.3 to 6). The percentage of regenerated callus and mean number of buds per callus decreased with increase of NaCl in the medium. No regeneration of callus derived from cotyledons occurred in the presence of 300 and 350 mol m^{-3} NaCl (Fig. 5.3), whereas there was regeneration in callus derived from hypocotyls for 6R2IV at those NaCl concentration and for 8R2IV at 300 mol m^{-3} NaCl. However, there were no survivors from these treatments (Fig. 5.4). This investigation concentrated therefore on the response to the highest level of NaCl from which surviving plants could be obtained (i.e. 200, 250 mol m^{-3} NaCl) (Fig. 5.5 to 6). Callus derived from the cotyledon of all somaclones and parent CUF101-1S showed regeneration in the presence of 200 mol m^{-3} NaCl (Fig. 5.5a). At 250 mol m^{-3} NaCl, callus from

CUF101-1S, 6R2IV and 9R2IV were regenerated. There were no surviving regenerants of CUF 101 at 200 and 250 mol m^{-3} NaCl and no survivors at 250 mol m^{-3} NaCl for the regenerants 8R2IV and 7R2IV somaclones. One of the somaclones (6R2IV) showed enhanced regeneration at 200 and 250 mol m^{-3} NaCl ($P<0.05$) (Fig. 5.5a). The mean number of buds per callus derived from cotyledon explants was a significantly greater in 6R2IV than in CUF101-1S at 200 and 250 mol m^{-3} NaCl ($P<0.05$). At 250 mol m^{-3} NaCl a 5.2 fold increase was observed in mean number bud per callus in 6R2IV compared with CUF101-1S (Fig. 5.5b).

Callus derived from the hypocotyl of all somaclones showed regeneration in the presence of 200 and 250 mol m^{-3} NaCl (Fig. 5.6). There were no surviving regenerants of CUF 101 at any NaCl concentration and no survivors at 200 and 250 mol m^{-3} NaCl for the parent CUF 101-1S (Fig. 5.6). Two somaclones (6R2IV, 9R2IV) showed enhanced regeneration at 250 mol m^{-3} NaCl ($P<0.05$) (Fig. 5.6a). The mean number of buds per callus derived from hypocotyl explants were significantly greater in 9R2IV, 6R2IV and 8R2IV than in 7R2IV at 250 mol m^{-3} NaCl ($P<0.05$) (Fig. 5.6b).

5.4 DISCUSSION

The aim of the work described in this chapter was to select for increased salt tolerance in alfalfa by screening differentiating cultures in the presence of high concentrations of NaCl. The advantage of this method is that shoot and root induction and growth occur in the presence of salt so that selection is based more on a whole plant response to NaCl. This also avoids having a separate selection stage via callus then a regeneration stage. An additional advantage of this method of selection is that regeneration can be initiated rapidly, thus avoiding any losses in regeneration capacity (Collin & Dix, 1990). The only problem with this approach in alfalfa was that the capacity of the parental sources to regenerate was very limited initially. This was overcome by passage of the material through one cycle of tissue

culture. Large numbers of regenerants were then produced in the second cycle which provided the stage for the *in vitro* selection. In the past, intensive efforts have been made to isolate variants with enhanced salt tolerance and a number of selection methods have been used to produce salt tolerant lines. Most of the selections have been based on either a direct or a stepwise approach (Dix, 1986; Collin & Dix, 1990). Direct selection is when callus pieces are transferred onto callus maintenance media containing high concentrations of NaCl. The surviving cells are then isolated, and bulked (Kavi-Kishor, 1988). In a stepwise (step up) selection, callus cultures are exposed to a gradual increase in NaCl levels (McCoy, 1987a,b). A direct approach was employed in the *in vitro* selection here.

Long term stability in selected cell lines and regenerated plants is a serious problem. Selection may produce physiologically adapted cells, but this can be lost over a period of time in the absence of the selection pressure. Nam and Heszky (1986) considered long term exposure to salt as a way of increasing the frequency of variants in plant cell populations. However, long term exposure to a selection pressure such as NaCl may also inhibit plant regeneration (Croughan *et al.* 1978) and, if plants are produced, the plants may show reduced growth (McCoy, 1987b; Stawarek & Rains, 1984). This could be a result of ploidy levels being altered during the prolonged tissue culture phase. For example, in 16-month-old colonies of *M. sativa* derived from cell suspension protoplasts, 50% of the calli were tetraploid, 40% octoploid and 10% decaploid (Arcioni *et al.* 1990). Chromosome loss was also observed at each level of ploidy (Atanassov & Brown, 1984). In the report by McCoy (1987b), all the plants which regenerated from NaCl tolerant cell lines were abnormal and many (44.7%) were extreme dwarfs. A further problem was that most of them had unbalanced polyploid chromosome sets with the most-extreme cytogenetic variant having 106 chromosomes. The increase in ploidy level was related to the rapid loss of regeneration capacity, and the additional chromosomal and/or genetic alteration was probably a consequence of the extended culture periods (Arcioni *et al.*, 1990; Bingham & McCoy, 1986). Of seven plants regenerated by

McCoy (1987b), only two expressed salt tolerance, and only one of these plants flowered and it was both male and female sterile. In an earlier experiment, Smith and McComb (1983) found that regenerated plants were as salt sensitive as the initial plants but this may have been due to cells within the callus not being uniformly exposed to the selective agent (Collin & Dix, 1990) and some cells may escape the stress (Bingham & McCoy, 1986). Many selection procedures do not result in immediate killing of sensitive cells and in some cases the selective agents may not be lethal, but merely growth inhibitory (Dix, 1986). At least seven major tolerance mechanisms for salt tolerance have been proposed and they are discussed by Levitt, (1980). Some of these may operate only at the cellular level and thus be expressed *in vitro*, while some mechanisms may operate at the organismal level (Bingham & McCoy, 1986). There are therefore serious problems associated with selection for salt tolerance *in vitro* using undifferentiated cells.

The procedure followed in the experiment described in this chapter has largely overcome the limitation of *in vitro* cell selection by regeneration of alfalfa in high concentration of NaCl. It has been shown that some somaclones (e.g. 6R2IV) were more tolerant than others (7R2IV, 8R2IV, 9R2IV). In a similar approach Nam and Heszky (1986) measured frequency of regeneration of rice tissue culture in 0, 1 and 2% NaCl but they found no significant difference between selected callus and non selected at 1-2% NaCl and between ploidy levels (n, 2n) in the capacity to regenerate in the presence of NaCl. They concluded the application of selection pressure during the regeneration was essential for increasing of the expression of salt tolerance in a regenerated plant. They also found different levels of NaCl tolerance in regenerated lines. Ibrahim (1990) suggested that there were three types of cells in culture; (1) those with a reduced growth rate and which may die during the selection process, these representing the majority of cells in tissue culture exposed to NaCl, (2) cells that exhibit a gradual resistance to salt and become salt adapted cells and (3) cells that are salt tolerant which can survive and grow at sub-inhibitory concentration of salt.

The use of a high level of NaCl in a selection programme may allow only the third group to grow. These are the resistant cells and these will also differentiate in the presence of NaCl. The method used here was able to select for increased salt tolerance in alfalfa by regeneration in the presence of 250 mol m^{-3} NaCl. Since this NaCl tolerance was also stable and expressed in the progeny of the somaclones and the method of screening was successful where many other methods of selecting for NaCl tolerance *in vitro* had failed in the past.

Fig. 5.1 *In vitro* selection of *M. sativa* cv. CUF 101, somaclones derived from the 1st cycle of tissue culture and the parent CUF101-1S for salt tolerance.

```
            ┌─────────────────────┐
            │      CUF 101        │
            └─────────────────────┘
               (parent variety)
                      │
                      ↓      In vivo selection
            ┌─────────────────────┐
            │     CUF101-1S       │
            └─────────────────────┘
            (Partial salt tolerant line)
                      │
                      ↓
            ┌─────────────────────┐
            │   Tissue culture    │
            └─────────────────────┘
             (Initiation of callus)
                      │
                      ↓
            ┌─────────────────────┐
            │    Regeneration     │
            └─────────────────────┘
            (Shoot and root formation)
                      │
                      ↓     Plant growth in vivo
            ┌─────────────────────┐
            │     Flowering       │
            └─────────────────────┘
                      │
                      ↓    Cross pollination within plants derived
                                  from the same clone
            ┌─────────────────────┐
            │ Seed progeny (1st cycle) │
            └─────────────────────┘
                      │
                      ↓
            ┌─────────────────────┐
            │   Tissue culture    │
            └─────────────────────┘
                      │
                      ↓
            ┌─────────────────────┐
            │ Regeneration in 0-350 mM NaCl │
            │    (in vitro selection)       │
            └─────────────────────┘
                      │
                      ↓
            ┌─────────────────────┐
            │ Seed progeny (2nd cycle) │
            └─────────────────────┘
```
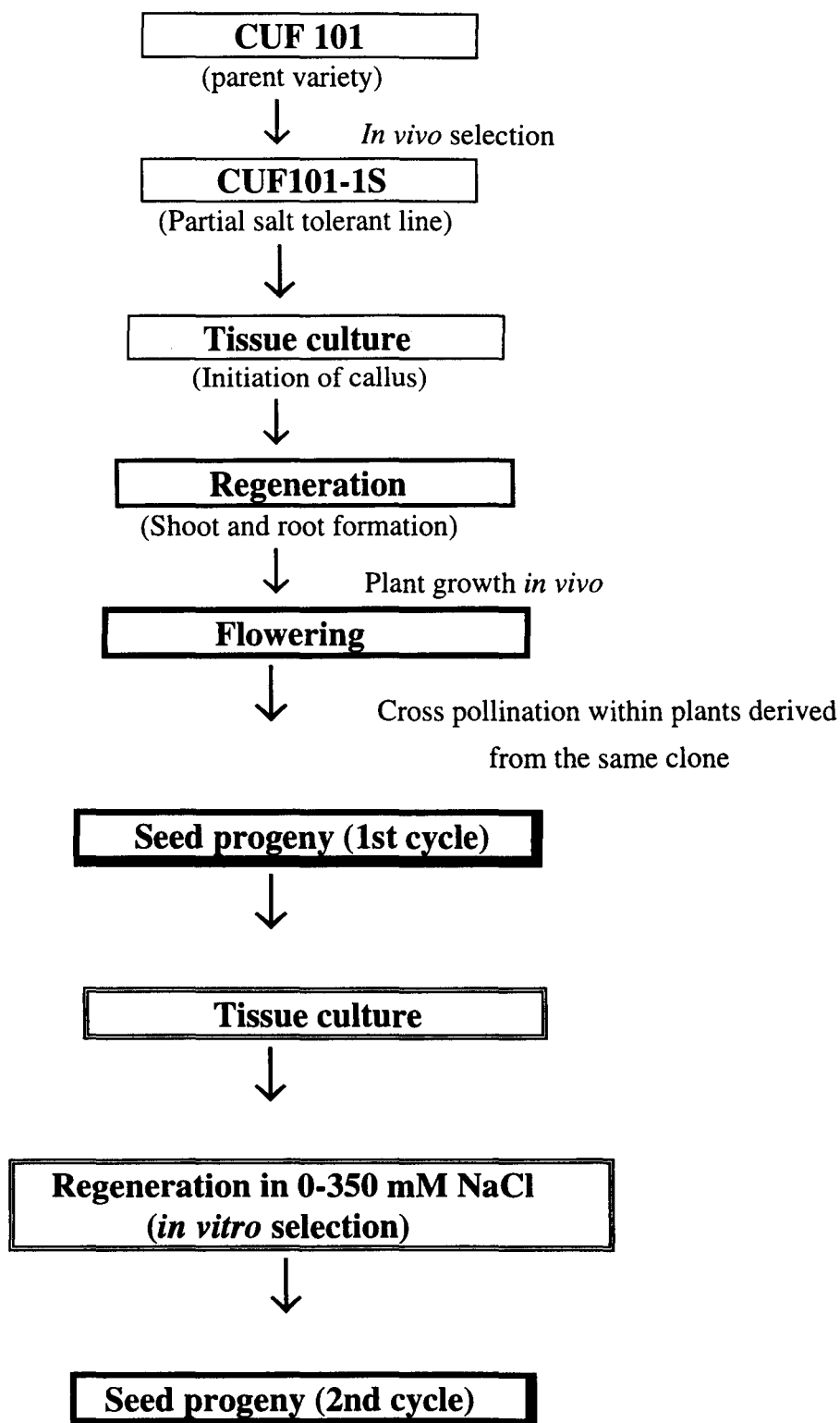
Fig. 5.2 Callus growth of *M. sativa* cv. CUF 101 at different concentrations of NaCl after 28 days. Callus derived from cotyledon explants.

   a) Mean callus fresh weight ± SE.

   b) Mean callus dry weight ± SE.

a)

b)

Conc. of NaCl (mM)
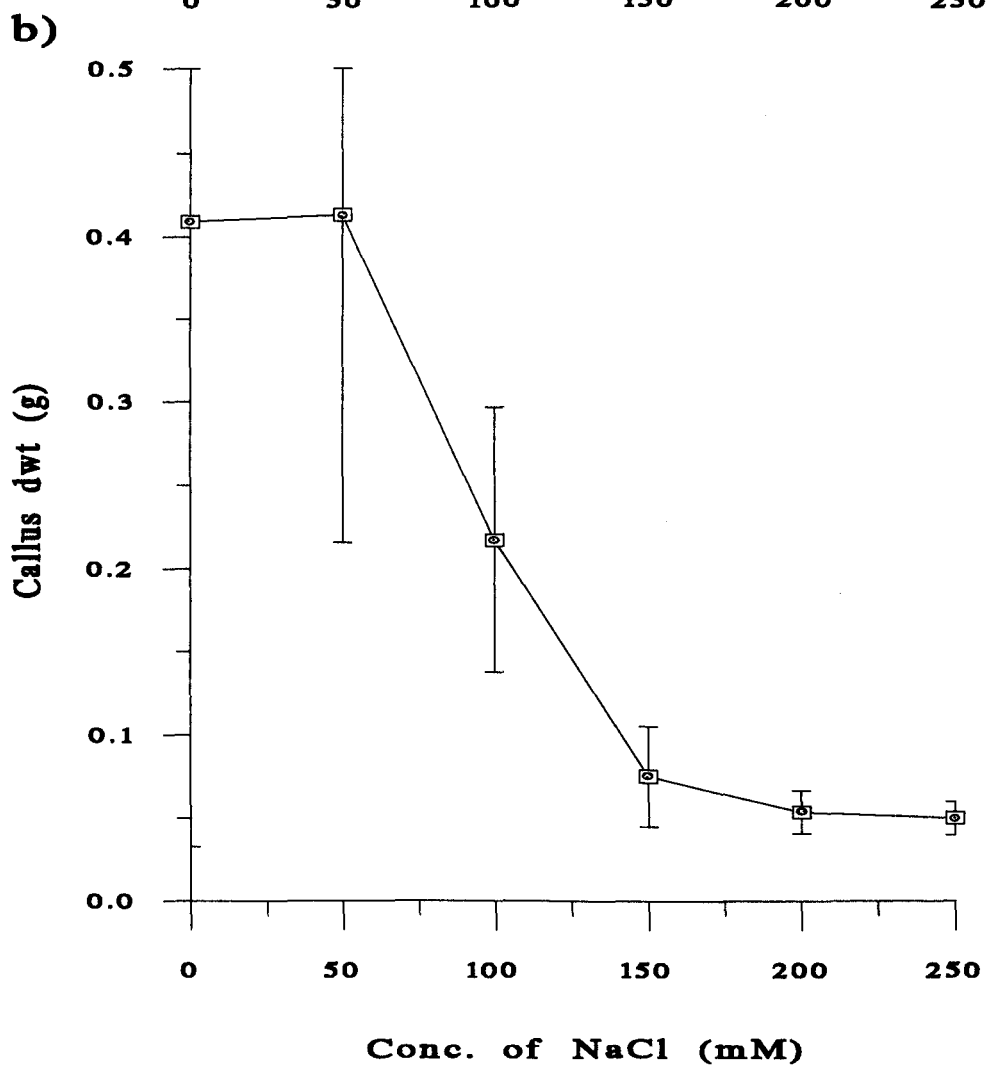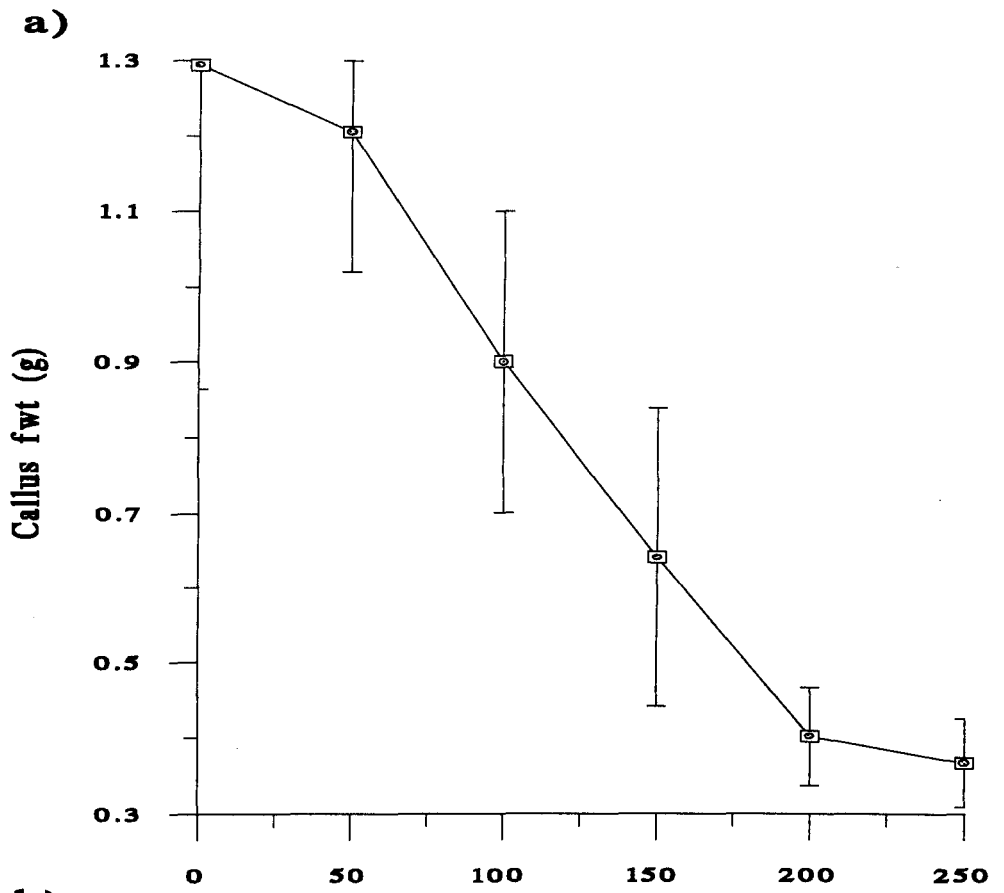
Fig. 5.3 Regeneration in callus during *in vitro* selection of *M. sativa* cv. CUF 101, somaclones (6R2IV, 8R2IV, 7R2IV, 9R2IV) and parent CUF101-1S in the presence of 0-350 mol m^{-3} NaCl. Callus derived from cotyledon explants.

a) Regenerated callus as a percentage of total callus (%).

b) Mean number of buds per callus $\pm$ SE.

Concentrations of NaCl were: 0- ☐, 50- ▤ , 100- ▥ , 150- ▨ , 200- ▦ , 250- ■, 300- ▦ , 350 mol m^{-3} - ▧ , and no survivors- ✶.

a)

Regenerated callus (%)

b)

Mean no buds callus^{-1}

Genotype

CUF 101

CUF101-1S

6R2IV
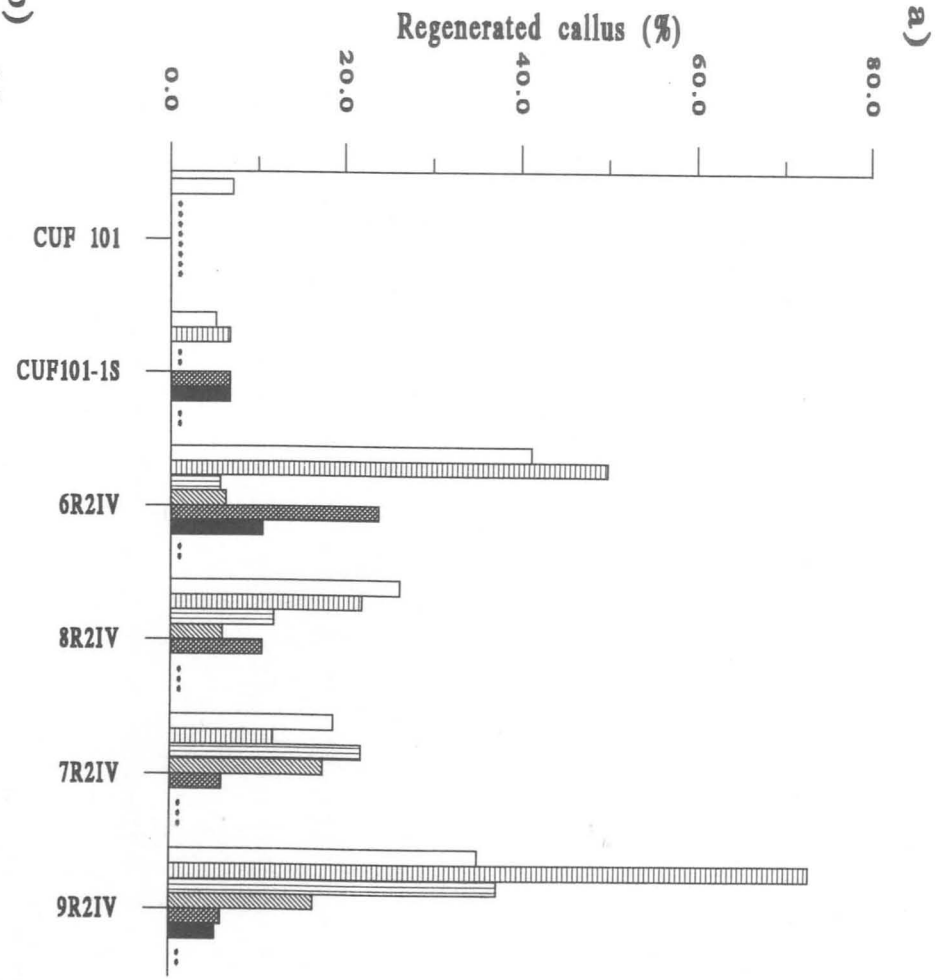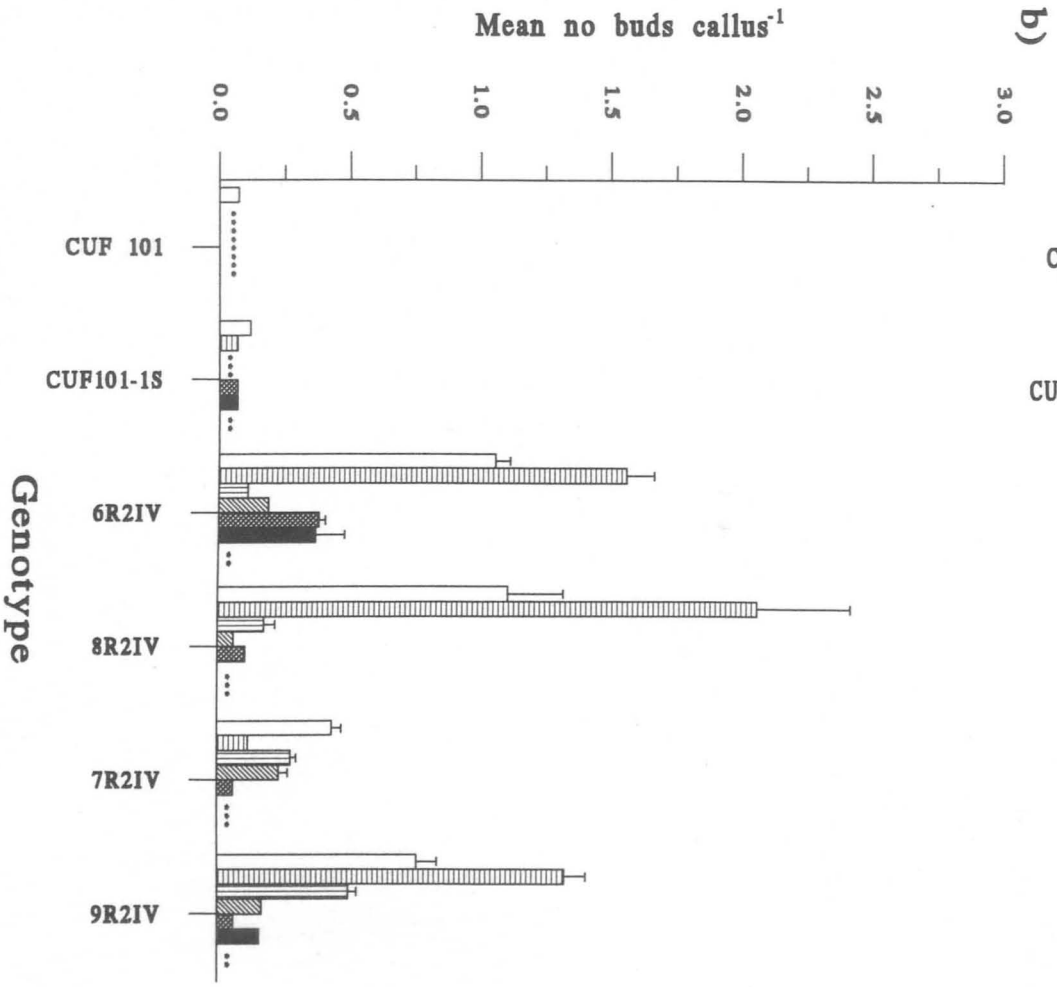
8R2IV

7R2IV

9R2IV

Fig. 5.4 Regeneration in callus during *in vitro* selection of *M. sativa* cv. CUF 101, somaclones (6R2IV, 8R2IV, 7R2IV, 9R2IV) and parent CUF101-1S in the presence 0f 0-350 mol m^{-3} NaCl. Callus derived from hypocotyl explants.

a) Regenerated callus as a percentage of total callus (%).

b) Mean number of buds per callus ± SE.

Concentrations of NaCl were: 0- □, 50- ▤ , 100- ▥ , 150- ▨ , 200- ▩ , 250- ■, 300- ▦ , 350 mol m^{-3} - ▧ , and no survivors- ✳ .

Fig. 5.5 Regeneration in callus during *in vitro* selection of *M. sativa* cv. CUF 101, somaclones (6R2IV, 8R2IV, 7R2IV, 9R2IV) and parent CUF101-1S in the presence of 0, 200 and 250 mol m^{-3} NaCl. Callus derived from cotyledon explants.

a) Regenerated callus as a percentage of total callus (%).

b) Mean number of buds per callus ± SE.

Concentrations of NaCl were: 0- □, 200- ▓ , 250 mol m^{-3} - ■, and no survivors- ✱ .

Fig. 5.6 Regeneration in callus during *in vitro* selection of *M. sativa* cv. CUF 101, somaclones (6R2IV, 8R2IV, 7R2IV, 9R2IV) and parent CUF101-1S in the presence of 0, 200 and 250 mol m^{-3} NaCl. Callus derived from hypocotyl explants.

a) Regenerated callus as a percentage of total callus (%).

b) Mean number of buds per callus ± SE.

Concentrations of NaCl were: 0- □, 200- ▒ , 250 mol m^{-3} - ■, and no survivors- ✶ .

a)

Regenerated callus (%)

b)

Mean no. buds callus^{-1}

Genotype

CUF 101

CUF101-1S

6R2IV

8R2IV

7R2IV

9R2IV
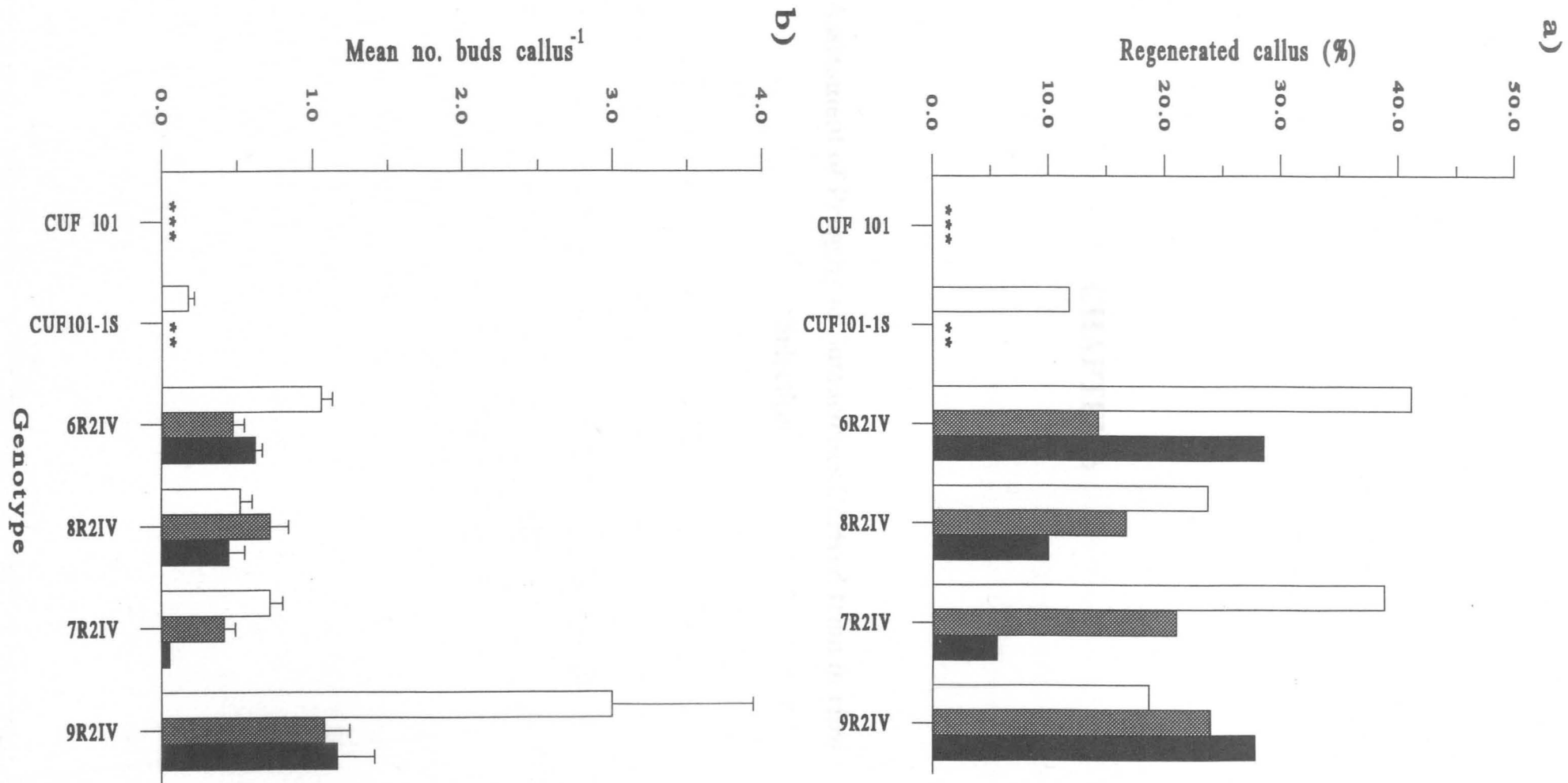
CHAPTER 6

Assessment of Progeny of Somaclones Derived from *in vitro* Selection

6. Assessment of Progeny of Somaclones Derived from *in vitro* Selection

6.1 INTRODUCTION

Crop plants exhibit salt tolerance at germination and at later stages of growth (Maas, 1986). Some of the most salt tolerant agricultural crops (e.g., sugarbeet, barley and cotton) are more sensitive during germination or early seedling growth than they are at later growth stages. In contrast, corn, pea, and beans are more sensitive during later stages of development (Subbarao & Johansen, 1994). Information on the response to salinity of alfalfa at each growth stage is important in adopting suitable genetic and management strategies for saline soils (Subbarag & Johansen, 1994). It has been found that alfalfa is more sensitive to salinity stress at germination than at later growth stages (Johnson *et al.*, 1992). Assessment of the response at germination and early seedling growth would be easier, provided there was a clear relationship to the whole plant response. Use of simple measures of growth-root length or shoot length have revealed genetically based variation in response to salinity (McNeilly, 1990). Ashraf *et al.* (1987) and Al-Khatib *et al.*, (1993) selected individuals for increased alfalfa shoot length after 14 days of growth of seedlings in saline solution cultures (-0.82 MPa NaCl). The selected populations at 4 weeks produced about 30% more dry weight than unselected plants when grown at -0.58 MPa (Ashraf *et al.*, 1986), establishing that a seedling screen was a valid method for obtains salt tolerant adult plants. This conclusion was confirmed by Rumbaugh and Pendery (1990) who measured the resistance of 761 alfalfa (*Medicago sativa* L. Emend) plant introduction accessions to NaCl during germination. They found germplasm from the Indian and African centres showed a high level of NaCl resistance during germination.

The aim of this study was to assess the progeny of plants derived from the *in vitro* and plantlet screening and to test the heritability of the resistance in the progeny of selected plants using a seedling test.

6.2 MATERIAL AND METHODS

6.2.1 Seedling salt tolerance

Seed from CUF 101, CUF101-1S and from somaclones 6R2IV(250 mol m^{-3} NaCl), 7R2IV(100, 150, 200 mol m^{-3} NaCl), 9R2IV(100, 200, 250 mol m^{-3} NaCl) that survived these concentration of NaCl in an *in vitro* screen (see Chapter 5, Section 5.2.2) were used to test the heritability of salt tolerance (Fig. 6.1). Seeds were obtained by cross pollination within regenerants derived from the same callus. These seeds were grown in 0 and 200 mol m^{-3} NaCl for 14 days (see Chapter 4, Section 4.2.1) then germinated seeds were counted and shoot and root lengths were measured, frozen in liquid nitrogen and stored for subsequent biochemical analyses.

6.2.2 Growth at the mature plant stage

Growth of the somaclones was assessed as mature plants over three harvests. Seeds derived from adult plants of the two somaclones 7R1 and 6R2IV, and from the parent CUF 101 were germinated on Whatman No. 2 filter paper in 10 cm Petri dishes. Uniform and well rooted 5-day-old seedlings were potted into a soil-sand-peat moss mixture (3:1:1) and grown. Each genotype was replicated eight times. After 45 days, when 10-15% of the plants had flowered, plant height was measured and number of branches per plant were counted. Shoot fresh weights were measured, and the shoot tissue wrapped in foil and placed in an oven at 80°C for 96h, and dry weights measured. Plants were allowed to grow, and again once 10-15% flowering

was observed, a second harvest was taken as in the 1st harvest and a further third harvest was made following the same protocol as before.

6.2.3 Plantlet testing

To test the response to NaCl of plantlets derived from the somaclones 6R2IV, 7R2IV, 8R2IV, 9R2IV and of the parent CUF101-1S, 40 well rooted plantlets of each genotype from nonsaline controls (see Chapter 5, Section 5.2.2) were transferred from GS medium (see Chapter 2, Section 2.2.2) to continuously aerated tap water. Plantlets were grown, supported individually in a polythene frame on the top of a 2.5 litre. volume black polythene container, for 3 days to wash the agar from the roots. They were then transferred to half strength Rorison solution (Appendix 3.1) containing 4 mol m^{-3} CaCl$_2$ and 0 or 200 mol m^{-3} NaCl. After 7 days, surviving plantlets were potted into a soil-sand-peat moss mixture (3:1:1) in a glasshouse.

6.3 RESULT

6.3.1 Growth measurement in NaCl

Germination was markedly reduced at 200 mol m^{-3} NaCl, and there was significant differences ($P<0.05$) between somaclones and parents in the presence of NaCl compared with 0 mol m^{-3} NaCl (Fig. 6.2). Both percentage and rate of seed germination declined after exposure to 200 mol m^{-3} NaCl with the least reduction in 6R2IV derived from differentiating cultures in the presence of 250 mol m^{-3} NaCl (Fig. 6.2). As salt levels increased in the regeneration medium, there was no indication that the plants regenerated from the highest salt levels were more tolerant than those from the lower salt levels. There were no significant differences in response to 200 mol m^{-3} NaCl ($P>0.05$) (Fig. 6.3) in shoot and root lengths of 9R2IV derived from differentiating cultures in the presence of 100, 200 and 250 mol m^{-3}

NaCl. There was no significant difference (P<0.05) between shoot and root lengths of somaclone 7R2IV derived from differentiating cultures in the presence of 100, 150 and 200 mol m^{-3} NaCl, except shoot length in response to 200 mol m^{-3} NaCl (P<0.05) (Fig. 6.3; Appendix 6.3). Shoot and root lengths of somaclone 6R2IV(250 mol m^{-3} NaCl) was significantly greater than all the other somaclones (7R2IV100, 150, 200 mol m^{-3} NaCl) and parental CUF 101 and CUF101-1S (P<0.05) (Fig. 6.3). As a result, further investigations concentrated on the somaclones 6R2IV(250 mol m^{-3} NaCl) and 9R2IV(250 mol m^{-3} NaCl). Root length was reduced in all genotypes in the presence of 200 mol m^{-3} NaCl. The reduction was 50% of the 0 mol m^{-3} NaCl for 6R2IV and 20% of the 0 NaCl in CUF101-1S (Fig. 6.4a). The reduction in shoot length showed the same pattern with less reduction (40%) in 6R2IV and CUF101-1S (20%) of their 0 mol m^{-3} NaCl (Fig. 6.4b). However, at 200 mol m^{-3} NaCl, there was a difference between genotypes and further significant increases was observed in 6R2IV compared with CUF 101, CUF101-1S and 9R2IV (Fig. 6.4). At 200 mol m^{-3} NaCl, a 2 fold increase was observed in root and shoot length means of 6R2IV compared with CUF101-1S (P<0.05) (Fig. 6.4). Compared with nonsaline controls, the 200 mol m^{-3} NaCl treatment increased the R/S ratio of 6R2IV whereas it was decreased in parent CUF 101 (Table 6.1). The data indicated that survival and growth rates differed among the salt tolerant plants since some regenerants showed greater survival and growth rate whereas other somaclones showed a survival and growth rate lower than that of the CUF101-1S control selected line (Fig. 6.4). Although NaCl reduced shoot and root growth of all genotypes, the magnitude of the reduction varied amongst them.

6.3.2 Growth measurements of mature plants

Regenerant progenies that had been adapted to NaCl continued to exhibit slower growth than CUF 101 when grown in the absence of salt in the 1st harvest (Fig. 6.5 and 6.6), whereas no significant differences were detected between selected

somaclones and parent CUF 101 of the 2nd and 3rd harvest for shoot fresh weight (P>0.05) (Fig. 6.6a). No difference was observed in shoot fresh weight between the 1st, 2nd and 3rd harvest for CUF 101, whereas, significant differences were detected between the 1st and 2nd harvest but not between the 2nd and 3rd harvest for 7R1 and 6R2IV (P<0.05) (Fig. 6.6a).

Shoot dry weight showed the same pattern with a greater reduction in 7R1 and 6R2IV compared with CUF 101 in the 1st harvest but no significant difference between somaclones (7R1, 6R2IV) and parent CUF 101 in the 2nd and 3rd harvest (P>0.05) (Fig. 6.6b). Also there was no significant difference between the 1st, 2nd and 3rd harvest for shoot dry weight in CUF 101 and 7R1 (P>0.05). In somaclone 6R2IV there was a significant difference between the 1st and 2nd harvest but no significant difference between the 2nd and 3rd harvest (P<0.05) (Fig. 6.6b). No significant difference in branch number (Fig. 6.7a) and the height (Fig. 6.7b) occurred when the somaclones (7R1, 6R2IV) and parent CUF 101 were grown in the absence of salt and harvested at the three harvest time (P>0.05). Analysis of variance (ANOVA) showed in Appendix 6.6 and 6.7.

6.3.3 The response of plantlets to NaCl

Plantlets derived from the somaclones (6R2IV, 7R2IV, 8R2IV, 9R2IV) and the parent CUF101-1S were tested in the presence of 200 mol m^{-3} NaCl to assess the response of the plant. Only two plantlets derived from 7R2IV survived. These surviving plantlets (7R2VO) were transferred to soil in a glasshouse and flowered but they did not set seed. DNA was extracted from their leaves for molecular analyses.

6.4 DISCUSSION

Salt tolerance of regenerated plants was examined by growth of the F_1 generation of somaclones, derived from *in vitro* selection, in comparison with parental CUF 101 and CUF101-1S for 14 days in the solution culture containing 200 mol m^{-3} NaCl. This comparison showed that somaclonal variation could arise in a differentiating culture and contribute to the modified genotype of the selected shoots. The evidence from the *in vivo* assessment confirms that variation for salt tolerance does exist within seed populations of the original variety (McNeilly, 1990), but this variation, as would be expected, is exceeded when the material is passed through a tissue culture stage. The additional variation resulting from the *in vitro* selection could be due to mutation during tissue culture. These results demonstrate that the salt tolerance characteristics expressed in cell culture can contribute to increased salt tolerance of the whole plant. The results also strongly suggest that salt tolerance is stable in the regenerated plant and its progeny. Almost all of the regenerated plants were self fertile; thus both male and female fertility was retained in these plants.

Selection at 250 mol m^{-3} NaCl *in vitro* produced somaclones with increased germination performance in saline environments. Rumbaugh and Pendery (1990) in a study of 761 accessions of alfalfa from throughout the range of the species, found a 50% reduction in germination after 7 days with each 42.8 mol m^{-3} increase in NaCl concentration. Accessions that originated in more arid regions exhibited a generally higher germination percentage at any given NaCl concentration. Salt tolerance in these natural populations was expressed at germination in the some way as the somaclones. There are different genetic mechanisms involved in salt resistance during germination, seedling emergence and development (Assadian & Miyamoto, 1987). Salinity may greatly reduce germination, and therefore seedling emergence, if seeds are sown less than 10 mm from the surface of soil, where moisture may be low and salts accumulate. Shalhevet and Bernstein (1968) suggested that alfalfa can tolerate high salinity in the lower portions of the root zone (at 1.8 m) by increasing

water uptake from higher root zone regions that are lower in salinity. Hanks *et al.*, (1977) reported that reduced salinity in the lower levels of the root zone (because of a high water table) were associated with absence of reductions in yield with the application of saline irrigation water.

Although shoot and root lengths of all genotypes decreased significantly in the presence of NaCl, the degree of reduction varied between salt tolerant somaclones and parental material. The results were consistent with the results of Kapulnik *et al.* (1989) and Noble *et al.*, (1984) where stem production in alfalfa decreased as salinity increased, and that salt-tolerant plants had more stems than salt-sensitive plants as salinity increased.

The changes in R/S ratios in response to NaCl recorded in this study were correlated with salt tolerance during germination. For example, compared with control, the 200 mol m^{-3} NaCl treatment increased the R/S ratio in 6R2IV, the most salt-tolerant somaclone during seed germination, while it decreased the R/S ratio of CUF 101. The somaclone 6R2IV that showed high seed germination and shoot and root length in the presence of NaCl, provided the most promising material for increasing salinity tolerance in alfalfa (Safarnejad *et al.*, 1996). The positive correlation between high germination and shoot and root length in the presence of NaCl may be a chance presence of these two normally independent character (Johnson *et al.*, 1992) or possibly in this material, the two character may be linked. Further work would readily identify this situation.

Even in the absence of NaCl, plants derived from *in vitro* selection at the 250 mol m^{-3} NaCl (6R2IV) and salt tolerant somaclone (7R1) showed highly reduced growth rates compared with the parent CUF 101 in the 1st harvest (Fig. 6.6) but the final yield in the 2nd and 3rd harvest was similar. Results from the 1st harvest agreed with the observation of Bressan *et al.* (1987) and Watad *et al.* (1991) that cells and plants regenerated from a salt tolerant cell lines exhibit slow growth in the absence of NaCl. The reduced growth in the 1st harvest may have been due to the tissue

culture conditions expressed by the regenerant. These epigenetic effects were not however expressed in any later harvest.

These results suggest that assessment of individual variants at the seed stage is an essential step in deriving vigorous salt tolerant plants and a stable salt tolerance at the whole plant level. The fact that the tolerance was shown in seeds produced by regenerant plants, suggested that the selection process selects potentially mutant plants. The regenerated plants described here may therefore offer a valuable resource of genotypes in plant breeding for salt tolerance, and as experimental plants for isolation, identification, and manipulation of genes contributing to salt tolerance in alfalfa.

Fig. 6.1 Assessment of progeny following *in vitro* selection of *M. sativa* cv. CUF 101, somaclones derived from the 1st cycle of tissue culture and the parent CUF101-1S for salt tolerance


```
                    ┌──────────────────┐
                    │     CUF 101      │
                    └──────────────────┘
                      (parent variety)
                            │
                            ↓    In vivo selection
                    ┌──────────────────┐
                    │    CUF101-1S     │
                    └──────────────────┘
                  (Partial salt tolerant line)
                            │
                            ↓
                    ┌──────────────────┐
                    │  Tissue culture  │
                    └──────────────────┘
                   (Initiation of callus)
                            │
                            ↓
                    ┌──────────────────┐
                    │   Regeneration   │
                    └──────────────────┘
                 (Shoot and root formation)
                            │
                            ↓    Plant growth in vivo
                    ┌──────────────────┐
                    │    Flowering     │
                    └──────────────────┘
                            │
                            ↓   Cross pollination within plants derived
                                      from the same clone
                    ┌──────────────────┐
                    │ Seed progeny (1st cycle) │
                    └──────────────────┘
                            │
                            ↓
                    ┌──────────────────┐
                    │  Tissue culture  │
                    └──────────────────┘
                            │
                            ↓
                    ┌──────────────────────────┐
                    │ Regeneration in 0-350 mM NaCl │
                    │      (in vitro selection)     │
                    └──────────────────────────┘
                            │        ↘
                            │   ┌──────────────────────────────┐
                            │   │ Assess of plantlets in 200 mM NaCl │
                            │   └──────────────────────────────┘
                            ↓
                    ┌──────────────────────────┐
                    │   Seed progeny (2nd cycle)    │
                    └──────────────────────────┘
                            │
                            ↓
                    ┌──────────────────────────┐
                    │   Assess in 0-250 mM NaCl     │
                    └──────────────────────────┘
                 (Seedlings assessed in vivo)
```
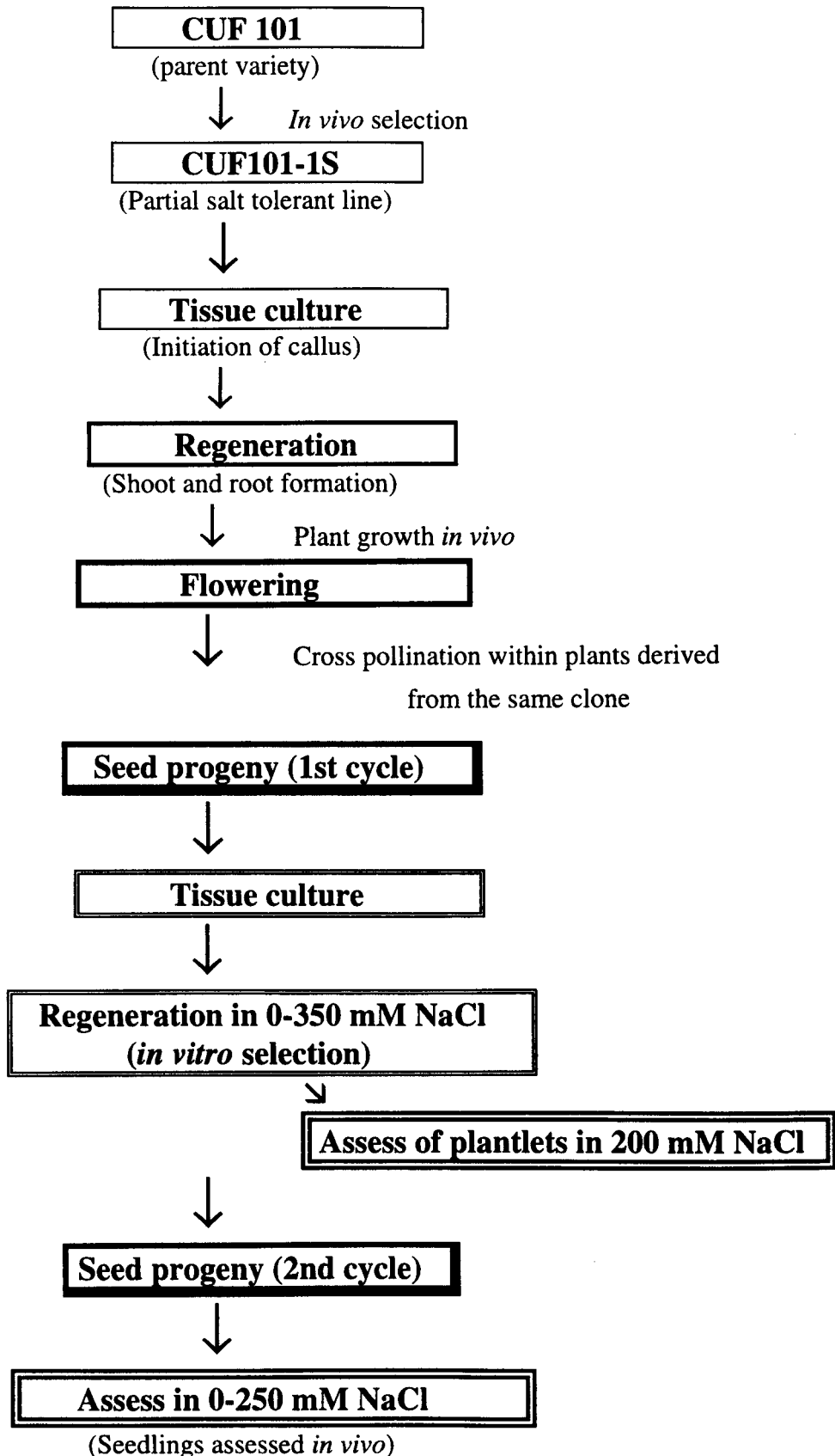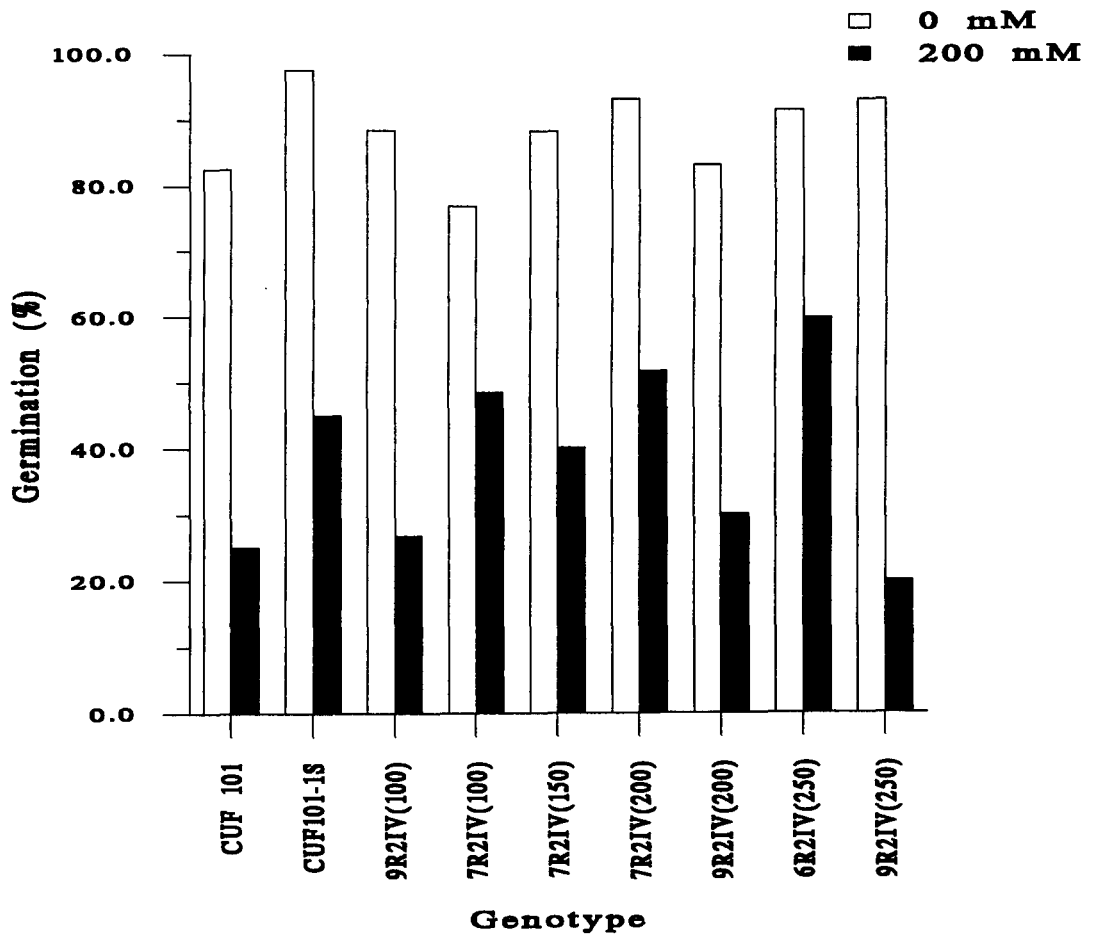
Fig. 6.2 Germination in 200 mol m^{-3} NaCl of seed progeny of parental and somaclonal genotypes of *M. sativa*. Somaclones were derived from an *in vitro* screen at 100-250 mol m^{-3} NaCl.

Fig. 6.3 Growth in 200 mol m^{-3} NaCl of 14-day-old seedlings of parental and somaclonal genotypes of *M. sativa*. Somaclones were derived from an *in vitro* screen at 100-250 mol m^{-3} NaCl.

a) Mean root length ± SE.

b) Mean shoot length ± SE.

Concentrations of NaCl were: 0- □ and 200 mol m^{-3} - ■ .

a)
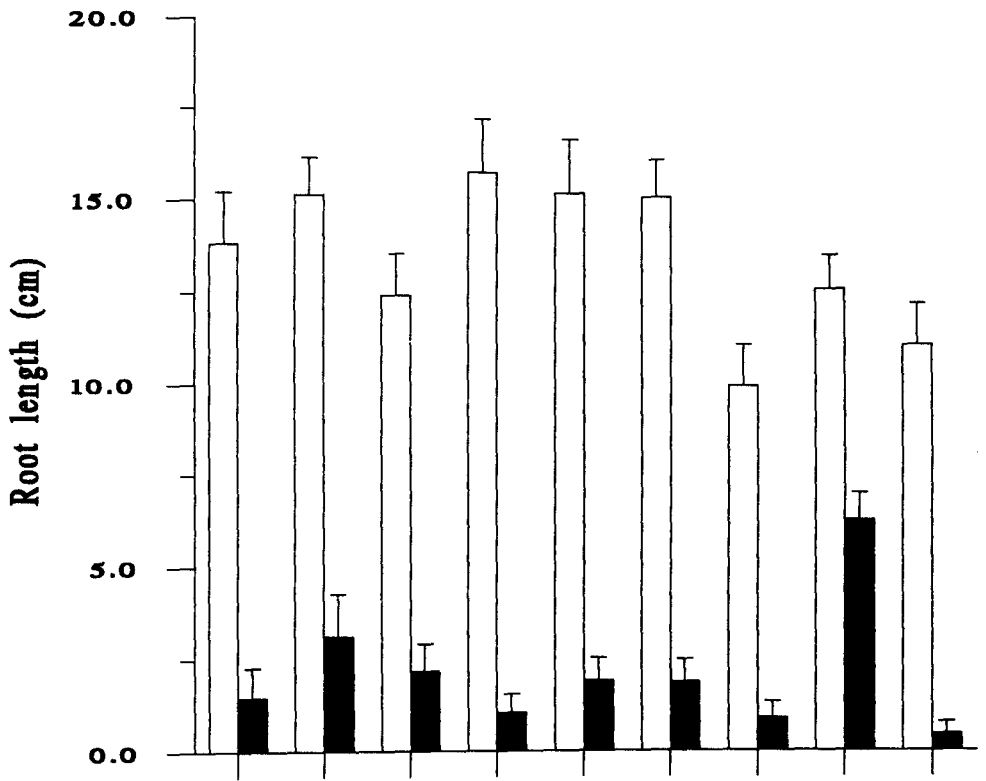
b)
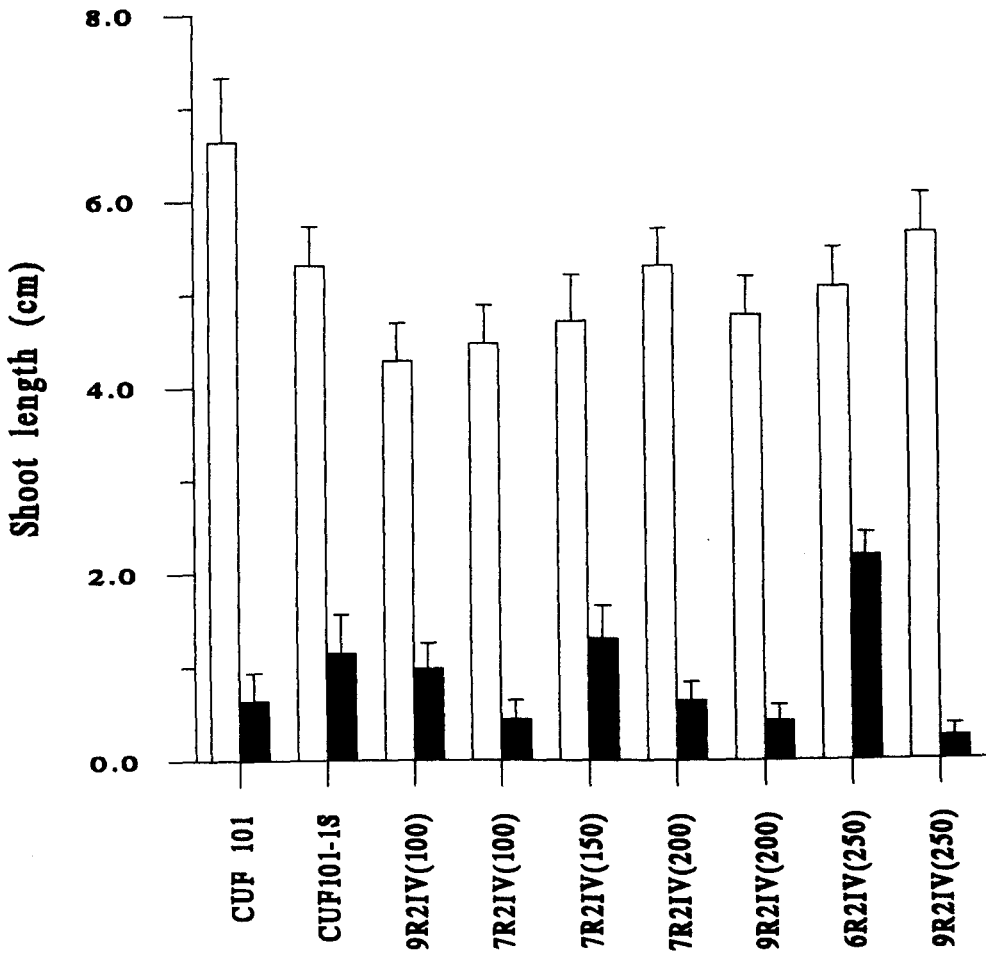
Genotype

Fig 6.4 Growth in 200 mol m^{-3} NaCl of 14-day-old seedlings of parental and somaclonal genotypes of *M. sativa*. Somaclones were derived from an *in vitro* screen at 250 mol m^{-3} NaCl.

a) Mean root length ± SE.

b) Mean shoot length ± SE.

Concentrations of NaCl were: 0- □ and 200 mol m^{-3} - ■.

a)

b)
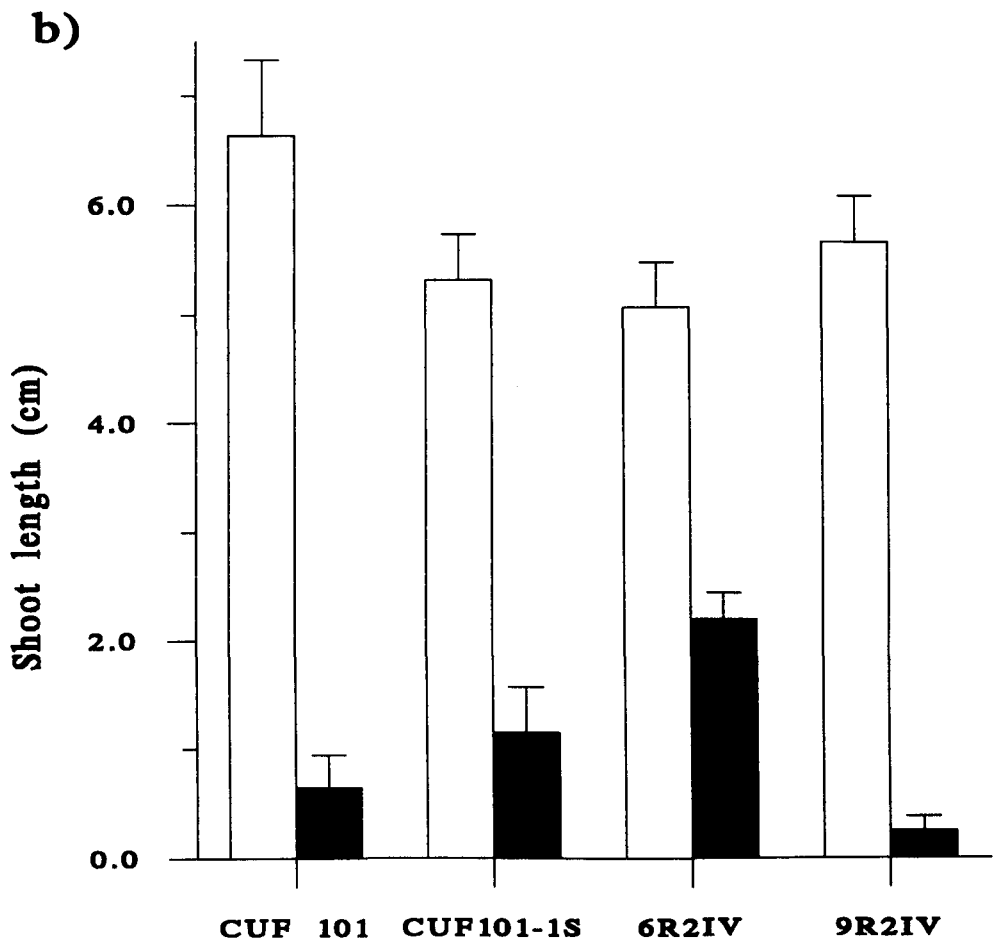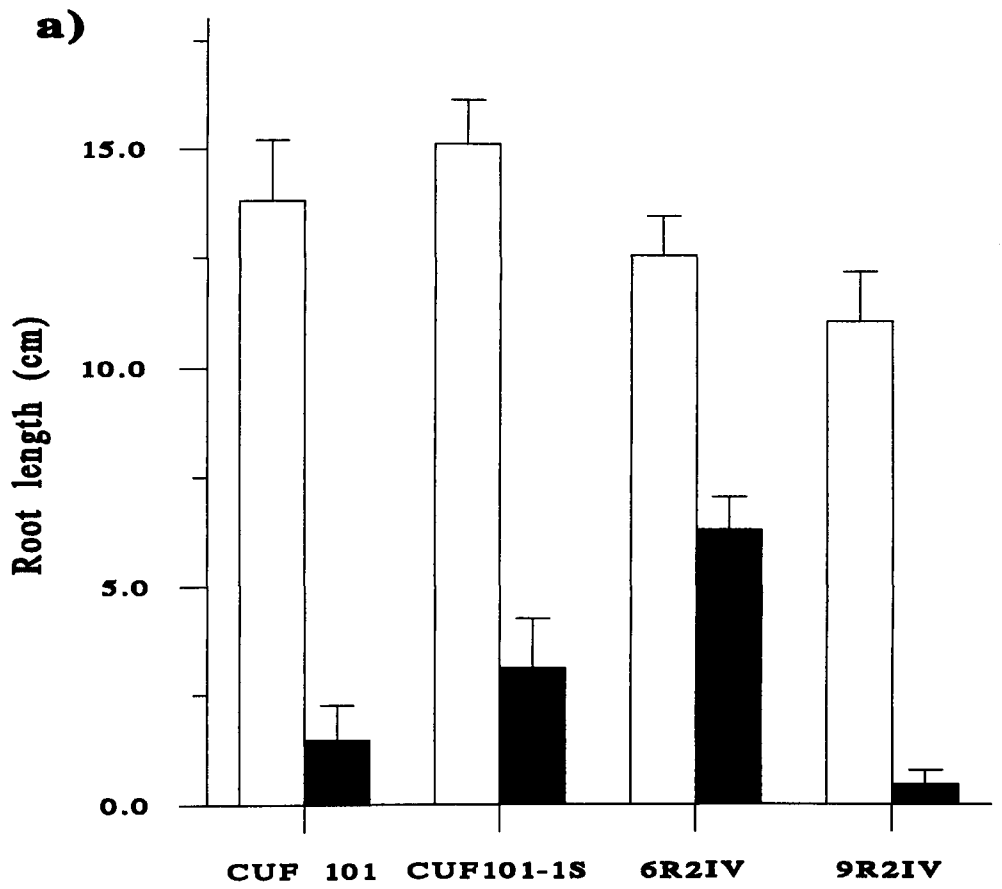
Fig. 6.5 Comparison of mature plants of *M. sativa* cv. CUF 101 and somaclones (7R1, 6R2IV).

  a) Cultivar CUF 101 (left), 7R1 (right).

  b) Cultivar CUF 101 (left), 6R2IV (right).

Fig. 6.6 Comparison of mature plants of *M. sativa* cv. CUF 101 and somaclones (7R1, 6R2IV) in three different harvests.

a) Mean shoot fresh weight ± SE.

b) Mean shoot dry weight ± SE.

1st harvest - □

2nd harvest - ▨

3rd harvest - ■

a)
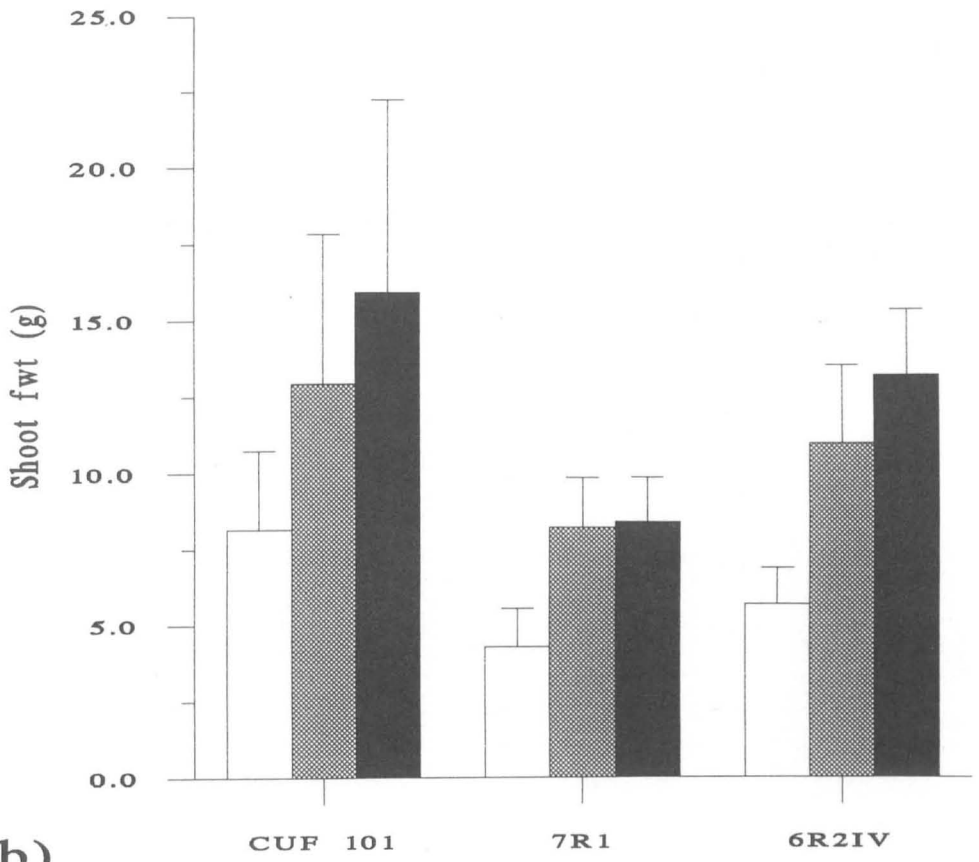
b)
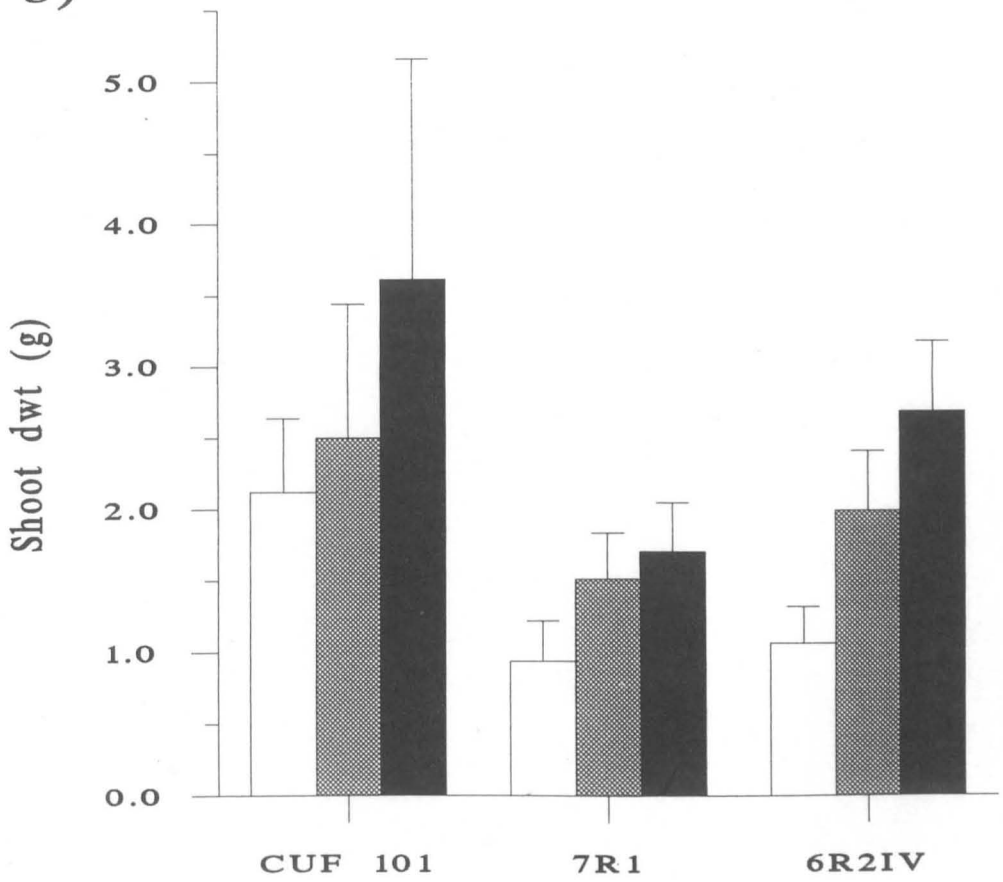
Genotype

Fig. 6.7 Comparison of mature plants of *M. sativa* cv. CUF 101 and somaclones (7R1, 6R2IV) in three different harvests.

a) Mean number of branches ± SE.

b) Mean height ± SE.

1st harvest - □
2nd harvest - ▩
3rd harvest - ■

a)

b)
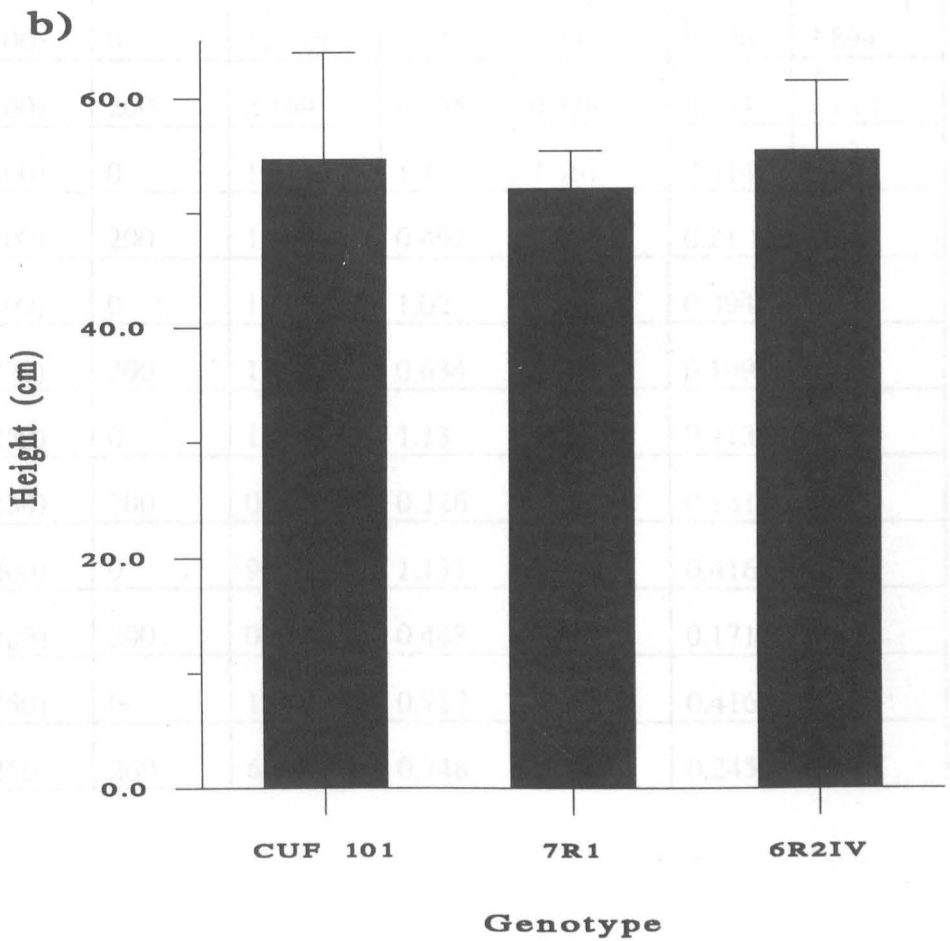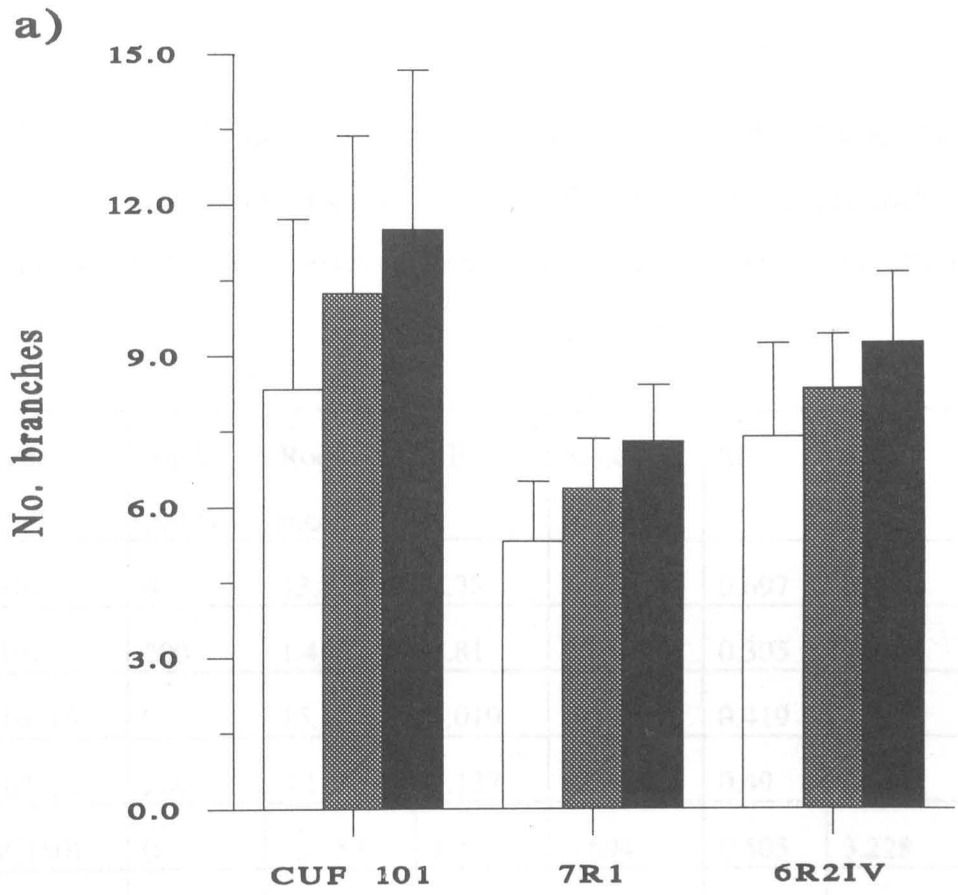
Genotype

Table 6.1 Summary of root and shoot length means of 14-day-old seedlings of *M. sativa* cv. CUF 101, seven somaclones and parent CUF101-1S in the presence of 0 and 200 mol m^{-3} NaCl. Somaclones derived from an *in vitro* selection at 0-250 mol m^{-3} NaCl.

Genotype	NaCl (mM)	Root L. mean	SE	Shoot L. mean	SE	R/S
CUF 101	0	13.826	1.38	6.634	0.697	2.684
CUF 101	200	1.456	0.81	0.638	0.305	2.282
CUF101-1S	0	15.12	1.019	5.313	0.419	2.846
CUF101-1S	200	3.11	1.137	1.147	0.49	2.711
7R2IV(150)	0	15.153	1.47	4.694	0.505	3.228
7R2IV(150)	200	1.898	0.619	1.298	0.35	1.462
9R2IV(100)	0	12.418	1.13	4.291	0.406	2.894
9R2IV(100)	200	2.169	0.735	0.978	0.274	2.218
7R2IV(100)	0	15.733	1.44	4.467	0.414	3.522
7R2IV(100)	200	1.039	0.497	0.439	0.21	2.367
7R2IV(200)	0	15.05	1.02	5.28	0.394	2.85
7R2IV(200)	200	1.863	0.634	0.64	0.199	2.911
9R2IV(250)	0	11.058	1.13	5.639	0.413	1.961
9R2IV(250)	200	0.448	0.326	0.248	0.131	1.806
9R2IV(200)	0	9.953	1.131	4.741	0.416	2.099
9R2IV(200)	200	0.89	0.448	0.425	0.171	2.094
6R2IV(250)	0	12.573	0.917	5.051	0.416	2.489
6R2IV(250)	200	6.31	0.748	2.197	0.245	2.872

CHAPTER 7.

Biochemical Analyses of Somaclones for Salt Tolerance

7. Biochemical Analyses of Somaclones for Salt Tolerance

7.1 INTRODUCTION

7.1.1 The physiological role of proline accumulation under salt stress

In monocotyledonous and dicotyledonous plants, adaptation to drought and salt stresses are accompanied by the accumulation of low-molecular weight compounds termed compatible solutes (Csonka, 1989). In suspension culture cells of both glycophytes and halophytes, these compounds accumulate after salt stress, as do presumably, osmoprotectant proteins after salt stress (Ben-Hayyim & Kochba, 1983). One of the best studied responses of this type involves the accumulation of proline under water or salt stress (Bartels & Nelson, 1994). The accumulation of this amino acid results from an increased flux of glutamate to pyrroline-5-carboxylate and proline in the proline biosynthetic pathway, as well as decreased rates of proline catabolism (Delauney & Verma, 1993; Heuer, 1994). Elevated levels of proline synthesis in both salt sensitive and salt tolerant cell cultures of *Nicotiana sylvestris* in response to salt stress was reported by Dix and Pearce (1981). In some tissues, proline levels may increase as much as 100-fold in response to stress (Voetberg & Sharp, 1991). Exogenously supplied proline can greatly facilitate cellular osmotic adjustment, cellular repair (Poljakoff-Mayber, 1982; Munns & Termaat, 1986), and growth in salt-containing medium (Hasegawa *et al.*, 1986), and these putative osmoprotectants may provide relief from the osmotic and/or salt stress (Flower *et al.*, 1977; Greenway & Munns, 1980). Exogenous proline applied to non-adapted cultured rice callus significantly increased growth in the presence of NaCl (Kavi-Kishor, 1989). A clear protective effect of exogenously applied proline in the presence of salt has been demonstrated, suggesting elevated levels of endogenous proline synthesis as a basis for improved salt tolerance (Dix *et al.*, 1984). In the same way, tolerant callus cultures of *Brassica napus* contain 5-6 times more proline than

unselected callus cultures (Chandler & Thorpe, 1987). Stable NaCl-selected *Nicotiana* cells differ from wild type cells by their ability to accumulate markedly more proline under NaCl treatment (Abd-Elrahem *et al.*, 1983). Marked increases in free proline has been reported in NaCl stress conditions in many plants such as barley, sorghum, rice, pearl millet, soybean, pea, tomato, and pistachio (reviewed by Heuer, 1994). In alfalfa, Fougère *et al.* (1991) reported that proline increased 11.3, 12.8, and 8.0 fold in roots, cytosol and bacteriods respectively, after 150 mol m^{-3} NaCl treatment.

7.1.2 The effect of salt stress on the antioxidant enzyme

The production of active oxygen species is, perhaps, a general alarm signal which serves to alert or notify metabolism and gene expression for possible modifications. It is the first defence against invading stresses, and if unchecked, leads to disruption of cell function and death (Foyer *et al.*, 1994). To prevent damage, plants posses an antioxidative defence system composed of low molecular weight antioxidants and protective enzymes (Asada & Takahashi, 1987; Scandalios, 1990). The activity of the antioxidative defence system must be equal to the task of destruction of radicals and active oxygen species in normal metabolism, and at times when the plant suffers stress. Evidently the antioxidative defence system of plants is quite limited in its capacity to respond to stress; the activities of component enzymes, or the antioxidant levels, usually only double in response to many stress situations (Foyer *et al.*, 1994). Biochemical studies have suggested that there is a positive correlation between the increase in antioxidant enzyme activities and salt tolerance in alfalfa plants, suggesting that increased activity of antioxidative enzymes may provide plants with better protection against salt stress (Safarnejad *et al.*, 1996). The hypothesis that stress tolerance may be improved by increasing the endogenous antioxidant capacity of plants, has aroused considerable interest in recent years. It is based on the observation that the production of toxic oxygen derivatives is increased as a result of

all types of environmental or man-made stress. The suggested sequence of changes is as follows (1) the occurrence of oxidative damage to membranes and other cellular constituents during or following stress; (2) increases in maximum extractable levels of active oxygen species and free radicals in stress situations; (3) marked increases in the enzyme activity of the antioxidative system and in the levels of ascorbate and glutathione in response to the imposition of certain stress conditions. (4) Cross-tolerance to several forms of stress occurs once the antioxidative defences have been increased (Foyer *et al.*, 1994).

The aim of the work here, was to examine effects of salt stress (NaCl) on proline accumulation, and antioxidant enzyme activity (GR, SOD, CAT and AP) using salt-tolerant and salt-sensitive alfalfa genotypes as a comparison.

7.2 MATERIAL AND METHODS

7.2.1 Exposure of seedlings to NaCl

Seedlings of 14-day-old of CUF 101, CUF101-1S, and somaclones 7R1 and 6R2IV were grown in nutrient solutions containing 0-200 mol m^{-3} NaCl as described in Chapter 4, Section 4.2.1 and Chapter 6, Section 6.2.1 then frozen at -70 °C for biochemical analyses.

In a further experiment 7-day-old seedlings of CUF 101, CUF101-1S and somaclones, 7R1, 4R1, and 6R2IV from seeds germinated in a salt free Rorison solution (Appendix 3.2) were transferred to the nutrient solution containing 4 mol m^{-3} CaCl$_2$ and 0, and 200 mol m^{-3} NaCl. Seedlings were grown on a polythene frame at the top of a 2.5 volume black polythene container (Fig. 7.1), removed and frozen after 12h, and 1, 2, 4, and 7 days for biochemical analyses.

Proline and antioxidative enzymes GR, SOD, CAT, and AP were extracted from the seedlings and estimated as described in Chapter 3, Section 3.2.4. Analysis of variance was carried out on three parallel measurements from each of the 3

independent extracts. Bonferroni's method (Maxwell & Delaney, 1990) was used to compare means.

7.3 RESULTS

7.3.1 Proline estimation

Proline accumulation in shoot tissue of the 14-day-old seedlings of CUF 101, CUF101-1S and somaclones 7R1 and 6R2IV exposed to 200 mol m^{-3} NaCl is shown in Fig. 7.2a. Proline levels were significantly higher ($P<0.05$) in 7R1 and 6R2IV than in CUF 101 and CUF101-1S in the absence of NaCl. Proline increased markedly in the salt-tolerant somaclones (7R1, 6R2IV) in the presence of salt, while only a small increase was recorded in the parental non-tolerant CUF 101, and the partial salt tolerant CUF101-1S (Fig. 7.2a). At 150 mol m^{-3} NaCl, proline accumulation increased in CUF 101, CUF101-1S and 7R1, with the largest increase in 7R1. No measure was made at 150 mol m^{-3} NaCl for 6R2IV. At 200 mol m^{-3} NaCl, proline accumulation increased in all five genotypes with the larger increases in 7R1 and 6R2IV ($P<0.001$). This increase was 12.4 fold in 7R1, and 16.7 fold in 6R2IV compared with the values at 0 mol m^{-3} NaCl (Fig. 7.2a). There were 3.4 fold increases in proline in 7R1, and 3.8 fold increase in 6R2IV, compared with that in CUF 101 and CUF101-1S ($P<0.001$) at 200 mol m^{-3} NaCl.

In root tissue, in the absence of NaCl, proline levels were higher in CUF101-1S and 7R1 than that in CUF 101 (Fig. 7.2b). No significant differences were observed between CUF 101, CUF101-1S and 7R1 in the presence of 150 mol m^{-3} NaCl ($P>0.05$).

Where seedlings were transferred to a NaCl solution and the changes examined in the short term, the level of proline increased 10 fold by day 4 in CUF 101, then declined (Fig. 7.3a). In somaclone 7R1 it increased 5 fold at day 1 and remained constant until day 2, then the levels declined until day 4 (Fig. 7.3b). Although CUF

101 achieved a higher level of proline this did not occur until much later than in 7R1.

7.3.2 Antioxidant enzyme assays

Data for the four antioxidant enzyme activities in 14-day-old seedlings are shown in Fig. 7.4 to 5. There was no significant difference ($P>0.05$) in GR activity between different genotypes maintained in 0 mol m^{-3} NaCl. However in the 200 mol m^{-3} NaCl treatment GR activity increased 234% $\pm$ 15% in 7R1, and 246% $\pm$ 32% in 6R2IV compared with their control values. The GR activity in the 200 mol m^{-3} was increased 148% $\pm$ 14% for CUF 101 and 227% for CUF101-1S compared with their control values (Fig. 7.4a). At 200 mol m^{-3} NaCl, GR activity was 27% higher in 7R1 and 32% in 6R2IV than that in CUF 101 and CUF101-1S.

SOD activity was more varied. It decreased in CUF101-1S and 6R2IV ($P<0.05$) in the presence of 200 mol m^{-3} NaCl, increased in 7R1 ($P<0.05$) and showed no significant difference in CUF 101 and 4R1 ($P>0.05$) (Fig. 7.4b).

When 7R1 was grown in media with 200 mol m^{-3} NaCl, CAT activity increased significantly ($P<0.05$) by 151% compared with the control without NaCl, while under the same conditions no significant differences ($P>0.05$) were observed in CUF 101, CUF101-1S and 6R2IV (Fig. 7.5a).

At 200 mol m^{-3} NaCl, AP activity increased in all genotypes with the larger increase over control in 7R1 at 131% $\pm$ 1% (Fig. 7.5b). In the somaclone 7R1 CAT, AP, SOD and GR activities increased significantly when seedlings were grown at 200 mol m^{-3} NaCl. On the other hand, the activity of SOD and CAT decreased or was unchanged when CUF 101 seedlings were grown in 200 mol m^{-3} NaCl, while AP and GR activities were increased slightly.

The data for GR and AP activities in 7-day-old seedlings which were transferred to 200 mol m^{-3} NaCl is shown in Fig. 7.6 and 7.7. GR activity in the presence of 200 mol m^{-3} NaCl decreased in CUF 101 (Fig. 7.6a), whereas in 7R1 with the same

treatment GR activity increased after 2 days and then decreased (Fig. 7.6b). The only obvious trend was shown by GR where the activity increased after day 2 then declined.

7.4 DISCUSSION

The study sought to examine a correlation between proline accumulation and the ability of alfalfa seedlings to grow in nutrient solutions containing NaCl (Chapter 4 and 6). In fact the NaCl induced increases in proline in CUF 101 (×7), CUF101-1S (×7) and in somaclones 7R1 (×12) and 6R2IV (×17) indicated a positive correlation between proline accumulation and adaptation to salt or osmotic stress. The accumulation of this amino acid may be part of a general adaptation to adverse environmental condition (Delauney & Verma, 1993). That there is no correlation in some studies (see Delauney & Verma, 1993) may be because proline increases may depend on the length of time and concentration of the salt stress and plant genotype (Bray *et al.*, 1991, Safarnejad *et al.*, 1996). However, there is good evidence of enhanced proline production with increased NaCl stress. Proline levels differ between cultivars and species adapted to stress conditions (Heuer, 1994). Exposure of pea plants to 120 mol m^{-3} NaCl in nutrient solution resulted in increased free proline, from approximately to 1 to 3% of the total free amino acid content in roots (Bar-Nun & Poljakoff-Mayber, 1977). Free proline was found to increase in rice cultivars grown in sand culture and exposed to salt treatments and salt-tolerant cultivars had higher protein and amino acid concentrations (Dubey & Rani, 1989). The levels of proline in the halophyte *Distichlis spicata* L. increased rapidly following initiation of 200 mol m^{-3} NaCl-induced stress and stabilised at an elevated level within 48h of exposure to NaCl (Ketchum *et al.*, 1991) indicating a rapid response to stress.

Salt stress induced a large increase in amino acids in alfalfa cv. Europe (Fougère *et al.*, 1991) and of the amino acids, proline showed the largest increase, 11.3-, 12.8-,

and 8.0-fold in roots, cytosol and bacteroids, respectively in response to 150 mol m^{-3} NaCl. In the work reported here, proline level increased up to 17 fold in 6R2IV in response to 200 mol m^{-3} NaCl and significantly increased in response to PEG stress (Chapter 3). It may be that the increased content of proline under conditions of high NaCl may be a response to increased water deficit. High levels of proline in the tolerant somaclones compared with levels in the sensitive cultivar (CUF 101) in response to NaCl and PEG may be due to an osmoprotective effect in alfalfa. The different levels of proline in roots and shoots in long and short term exposure of seedlings to stress showed that the level of proline depends upon the type of tissue, the age of the plant, and genotype.

This study show a positive correlation between increases in antioxidant enzymes GR, SOD, CAT, and AP activities and salt tolerance in seedlings of the somaclone 7R1. It is unknown whether these increases were due to an up regulation of the genes controlling the synthesis of these enzymes, or an increased activation of constitutive enzyme pools (Gossett *et al.*, 1994). It has been reported that in *Vigna catjang* and *Oryza sativa* leaves, O_2^- radical and H_2O_2 could play an important role in the mechanism of salt injury (Singha & Choudhuri, 1990). A possible competitive inhibition of SOD activity in salt-treated cowpea plants was also suggested (Hernandez *et al.*, 1993). In experiments in two cultivars of *Pisum sativum L.* with different sensitivity to NaCl, salt stress decreased CAT activity (Olmos *et al.*, 1994). The NaCl-induced increase in SOD activity in 7R1 suggests that this salt-tolerant somaclone may, as a consequence, have higher O_2^- scavenging and dismutating capacity than the more salt-sensitive CUF 101. GR activity also increased significantly in 7R1 and 6R2IV when the seedlings were grown in 200 mol m^{-3} NaCl, but the activity of this enzyme in CUF 101 was significantly less than that observed in 7R1 and 6R2IV. This suggests that the 7R1 and 6R2IV somaclones have a more active ascorbate-glutathione cycle in the presence of salt. The NaCl-induced enhancement of these enzymes in 7R1 indicates that the more salt-tolerant genotype has a higher capacity for decomposition of the H_2O_2 generated by SOD. Ming *et al.*

(1989) found high SOD activities during the initial period of salt stress in wheat at the whole plant level. Gossett *et al.* (1994) reported, that at 150 mol m^{-3} NaCl, callus of the salt-tolerant cultivar of cotton showed significant increases in SOD, CAT, AP and GR activities compared to callus tissue grown without NaCl. In contrast, callus tissue of the salt-sensitive cultivar, at the presence 0, 75 and 150 mol m^{-3} NaCl showed no difference in the activities of enzymes. In a study of the NaCl response of whole cotton plants, the more salt-tolerant cultivars contained significantly higher levels of CAT activity and induced levels of peroxidase and GR activities (Gossett *et al.* 1993).

The results obtained from the work reported in this Chapter are in agreement with results from the work assessing response to PEG (Chapter 3), or those observed in callus tissue of wheat in the presence of salt and mannitol (Safarnejad, 1992). Salt and mannitol stresses induced higher activities of CAT, SOD and GR in the callus of the NaCl tolerant wheat varieties "Chinese Spring" and "Kharchia". In the sensitive variety (Cappelle Desprez), the activity of these enzymes remained unchanged or decreased after the treatments. These results suggest that tolerant genotypes would appear to have more effective enzyme defence mechanisms than the sensitive ones, and that different stress conditions may induce a common stress response that might be related to osmotic stress.

Fig. 7.1 Growth of 7-day-old seedlings of *M. sativa* after 7 days in Rorison solution containing 0 and 200 mol m^{-3} NaCl on a polythene frame at the top of a 2.5 litre volume black polythene container. Scale represent 1 cm.

a) Control: CUF 101 (left) and 7R1 (right).

b) 200 mol m^{-3} NaCl: 7R1 (left) and CUF 101 (right).
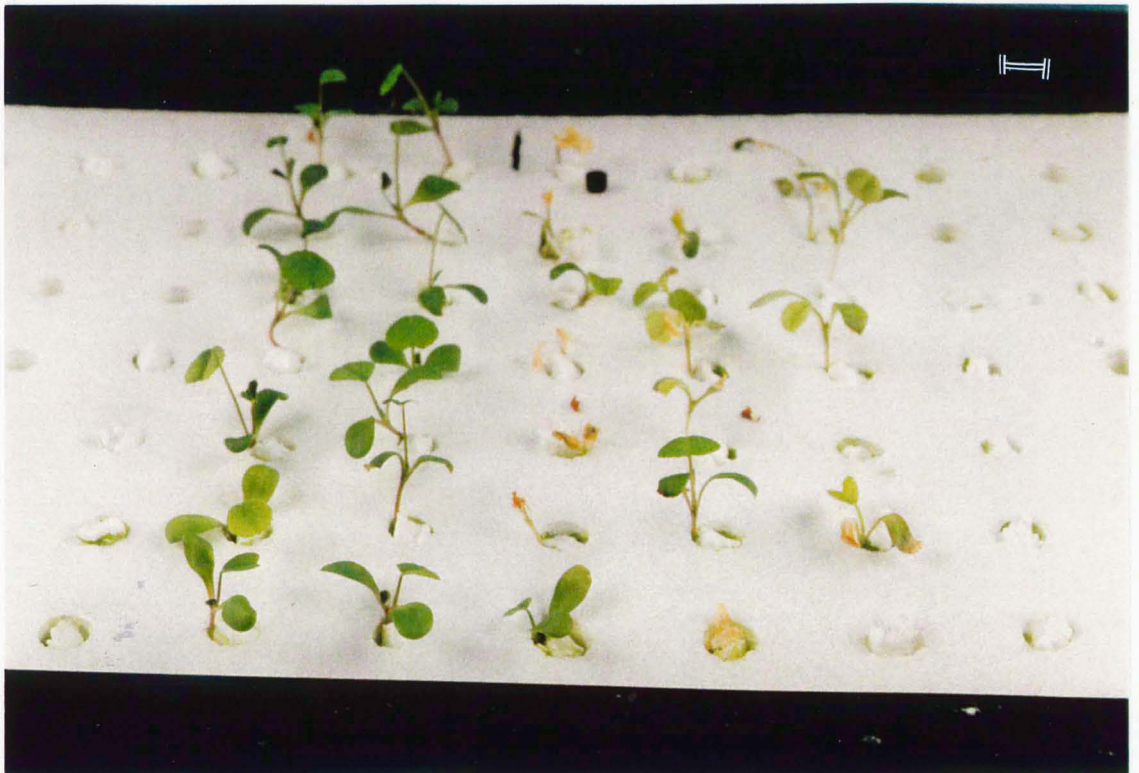
Fig. 7.2 Proline accumulation in 14-day-old seedlings of *M. sativa* cv. CUF 101 (NaCl sensitive), NaCl tolerant somaclones (7R1, 6R2IV) and partially tolerant parent CUF101-1S in response to salt stress (NaCl). Means of three parallel measurements from each of 3 independent extracts ± SE.

a) Shoot tissue

b) Root tissue

Concentrations of NaCl were: 0- □ , 150- ▓ , 200 mol m^{-3} - ■, and not measured- ✶ .

a)

umoles proline g fwt $^{-1}$

CUF 101 CUF101-1S 7R1 6R2IV

b)

umoles proline g fwt $^{-1}$

CUF 101 CUF101-1S 7R1

Genotype
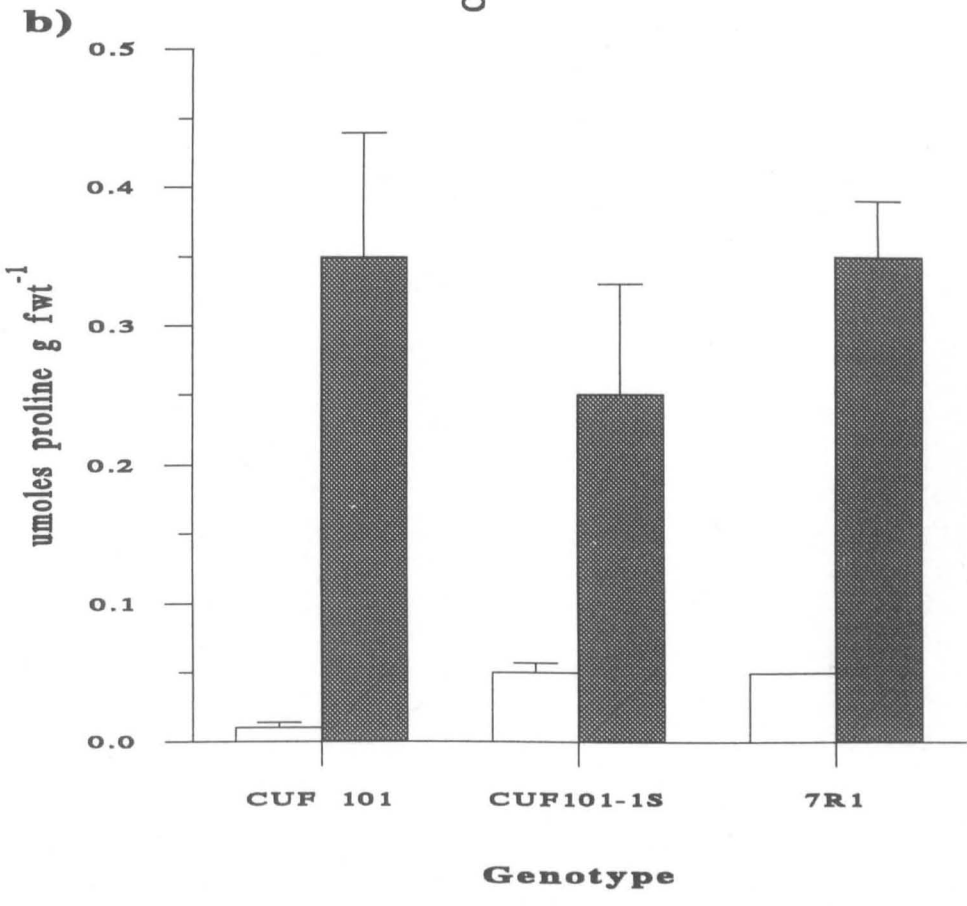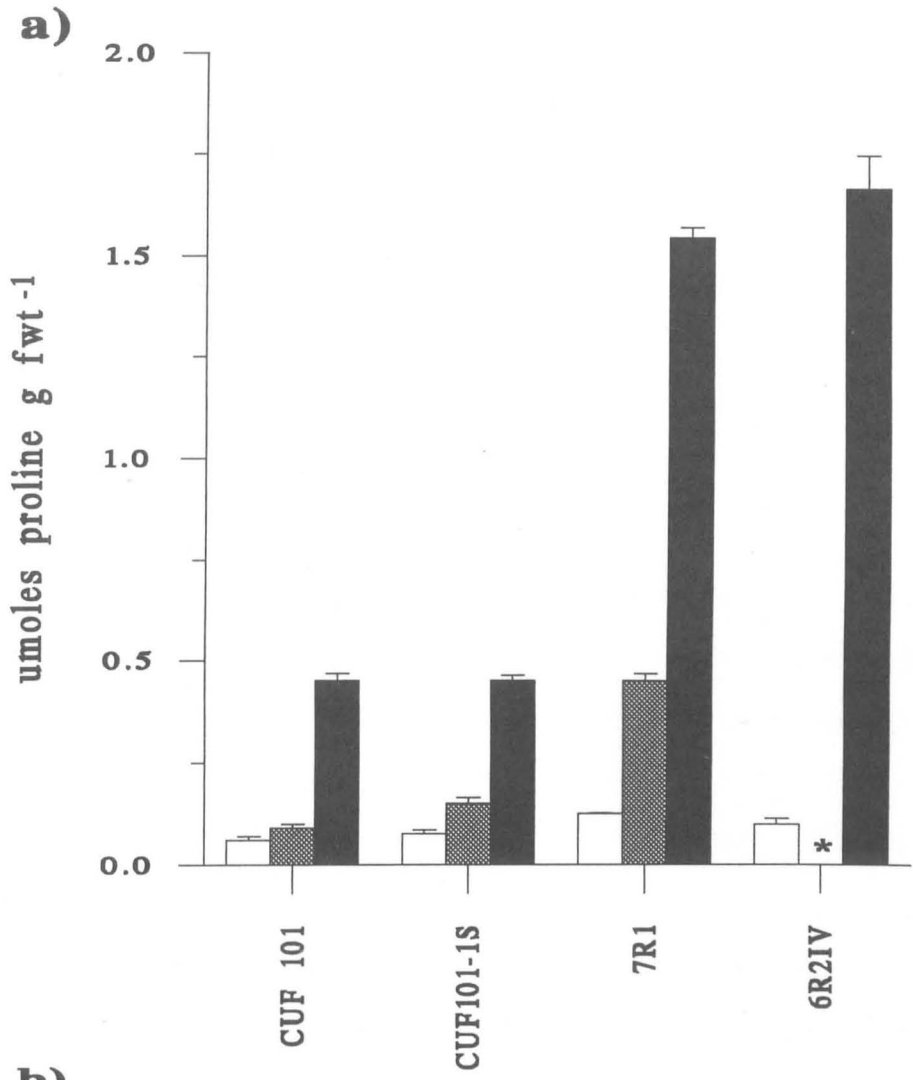
Fig. 7.3 Proline accumulation in 7-day-old seedlings of *M. sativa* cv. CUF 101 and somaclone (7R1) in response to salt stress (NaCl). Means of three parallel measurements from each of 3 independent extracts ± SE.

a) Cultivar CUF 101

b) Somaclone 7R1

Concentrations of NaCl were: 0- □ and 200 mol m^{-3} - ■.
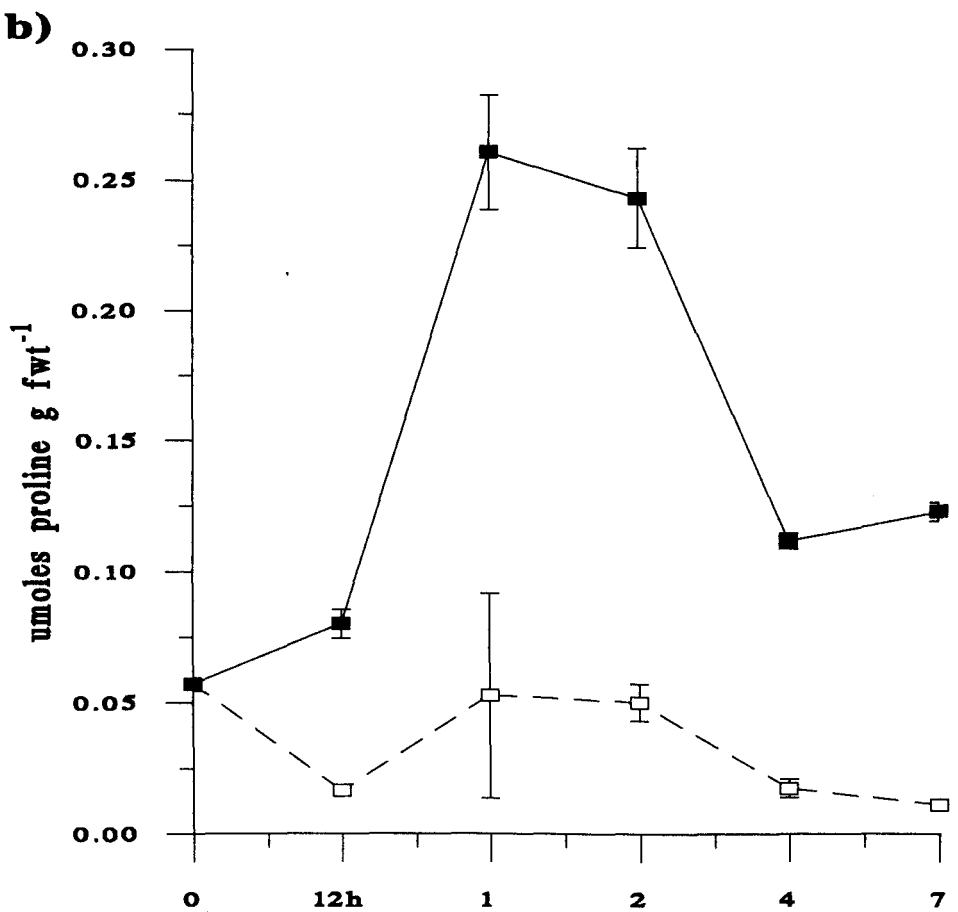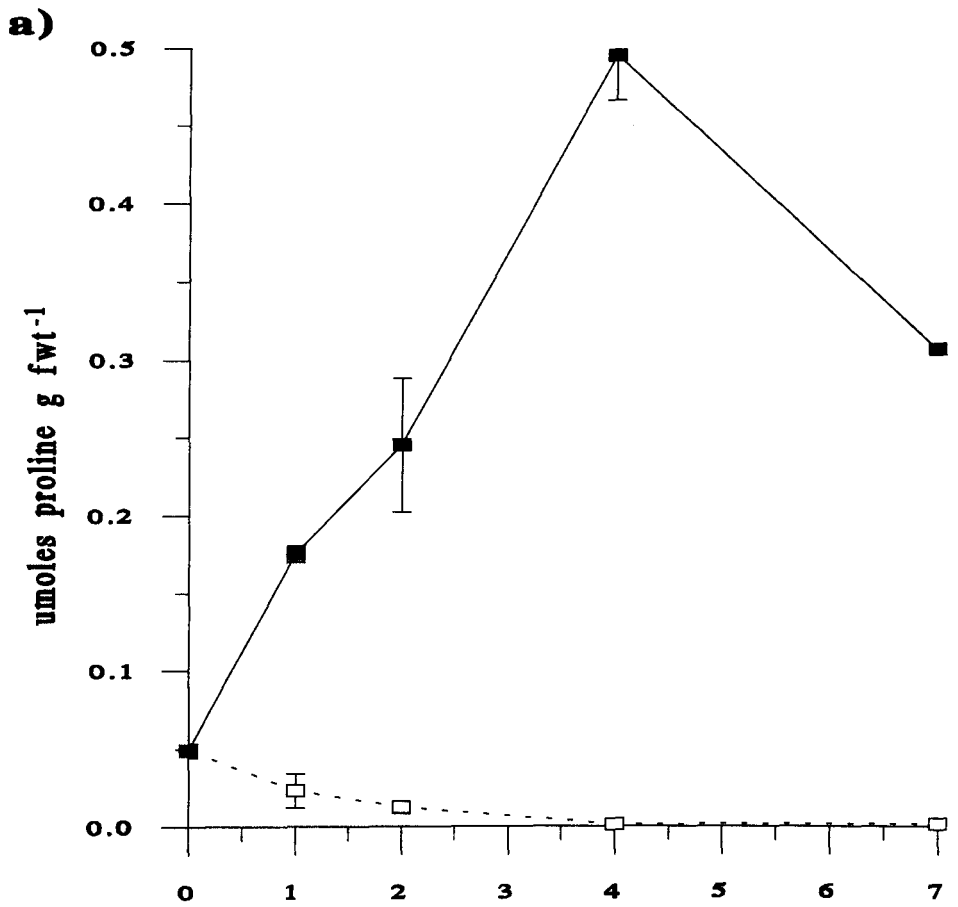
Fig. 7.4 Activity of antioxidant enzymes in 14-day-old seedlings of *M. sativa* cv. CUF 101, somaclones (7R1, 6R2IV) and parent CUF101-1S in response to salt stress (NaCl). Means of three parallel measurements from each of 3 independent extracts ± SE.

a) Glutathione reductase

b) Superoxide dismutase

Concentrations of NaCl were: 0- □ and 200 mol m^{-3} - ■.

b) Superoxide dismutase g fwt^{-1} (%)

a) Glutathione reductase activity g fwt^{-1} (x10^{-4})

Genotype

CUF 101
CUF101-1S
7R1
4R1
6R2IV
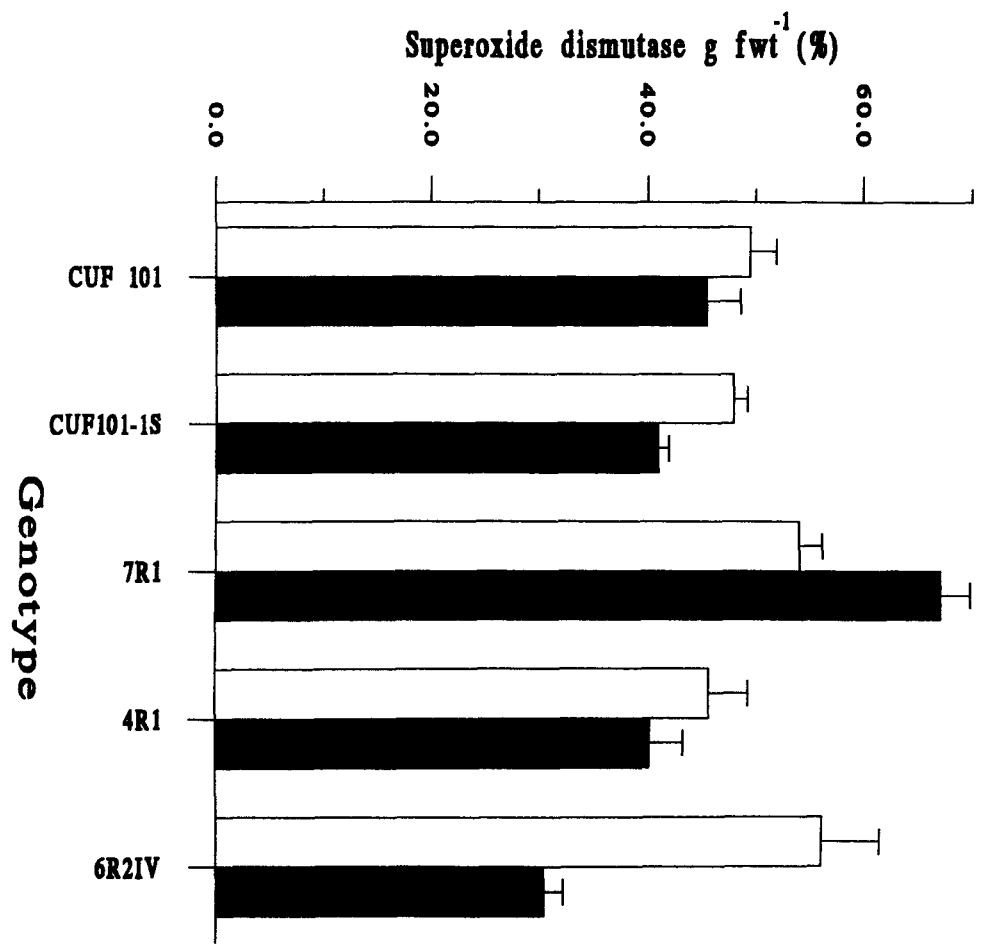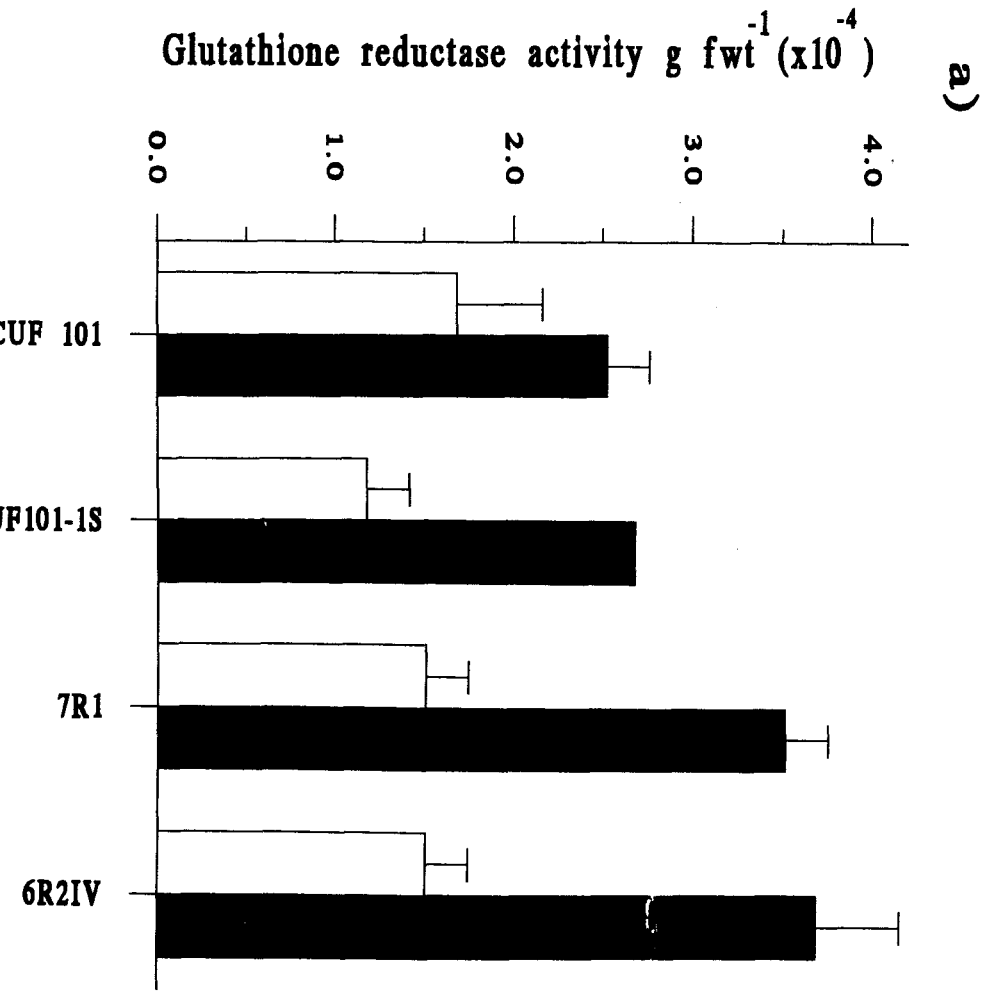
Fig. 7.5 Activity of antioxidant enzymes in 14-day-old seedlings of *M. sativa* cv. CUF 101, somaclones (7R1, 6R2IV) and parent CUF101-1S in response to salt stress (NaCl). Means of three parallel measurements from each of 3 independent extracts ± SE.

a) Catalase

b) Ascorbate peroxidase

Concentrations of NaCl were: 0- □ and 200 mol m^{-3} - ■ .

a)

Catalase activity g fwt^{-1} (x10^{-3})

b)

Ascorbate peroxidase activity g fwt^{-1} (x10^{-3})

Genotype

CUF 101

CUF101-1S

7R1

6R2IV

0.0   0.5   1.0   1.5   2.0
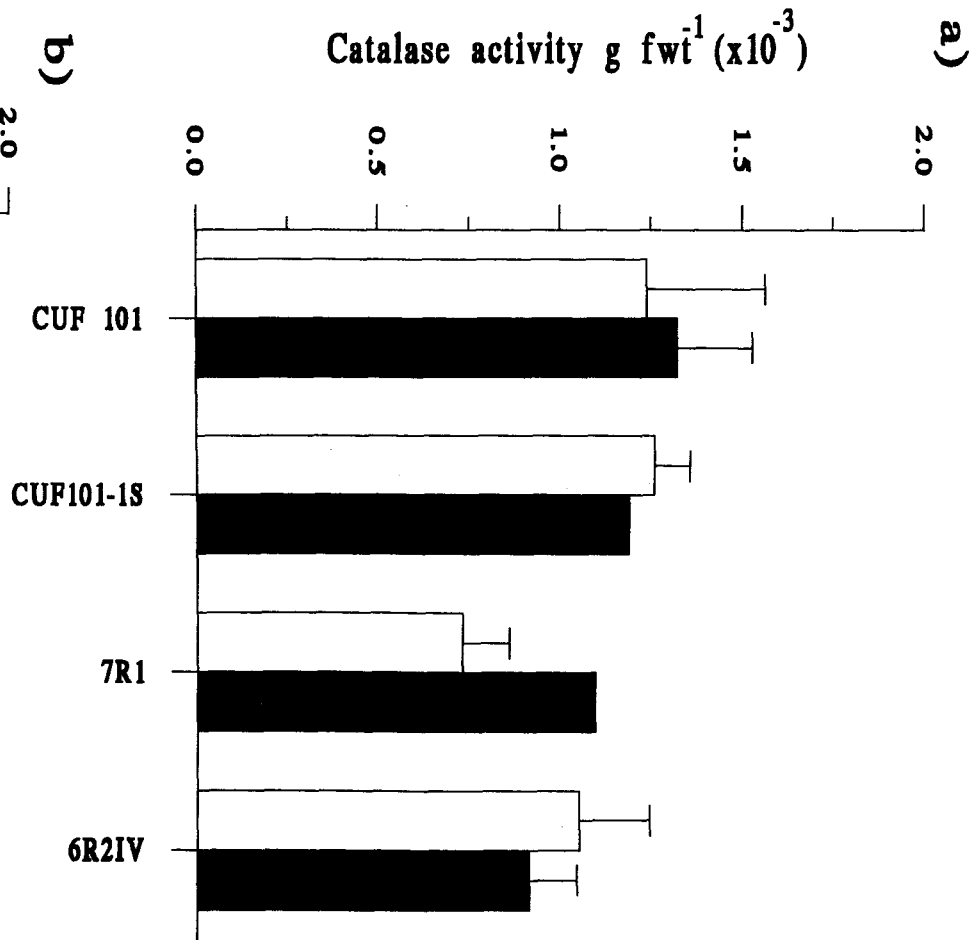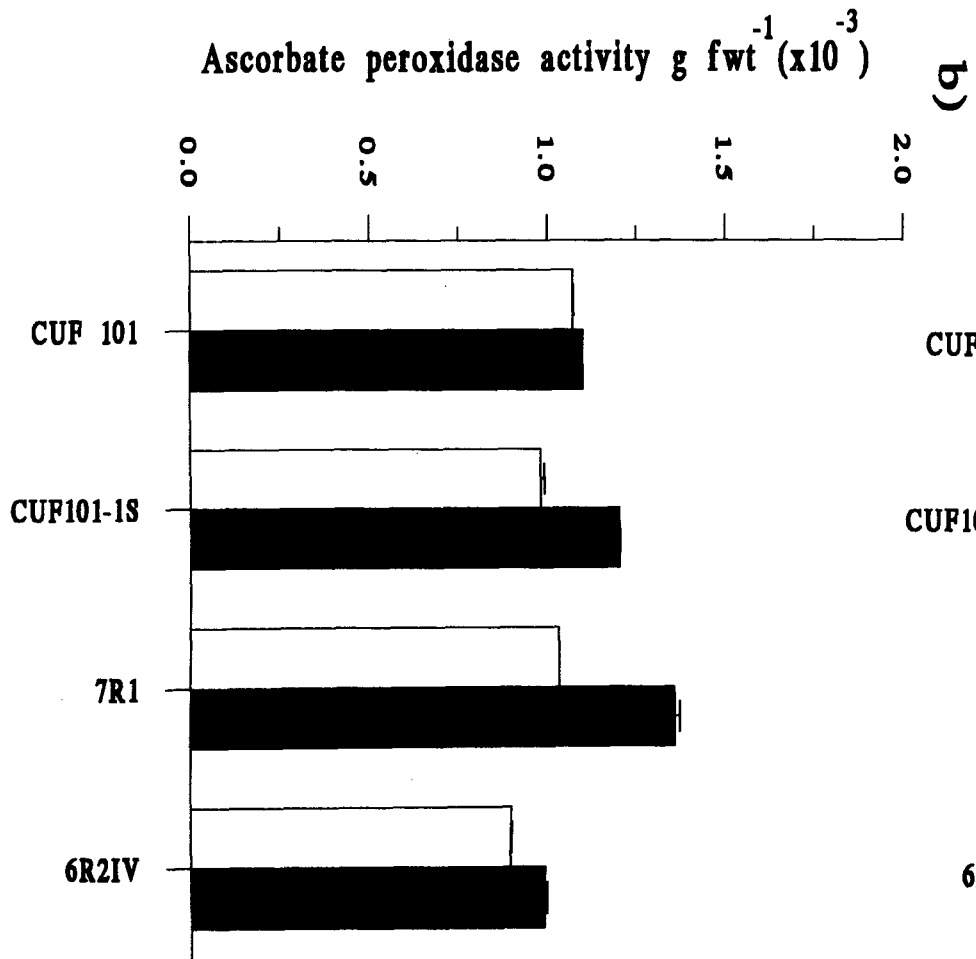
Fig. 7.6 Activity of glutathione reductase in 7-day-old seedlings of *M. sativa* cv. CUF 101 and somaclone (7R1) in response to short term of salt stress (NaCl). Means of three parallel measurements from each of 2 independent extracts ± SE.

a) Cultivar CUF 101

b) Somaclone 7R1

Concentrations of NaCl were: 0- ☐ and 200 mol m^{-3} - ■.

a)

b)

Time of incubation (days)
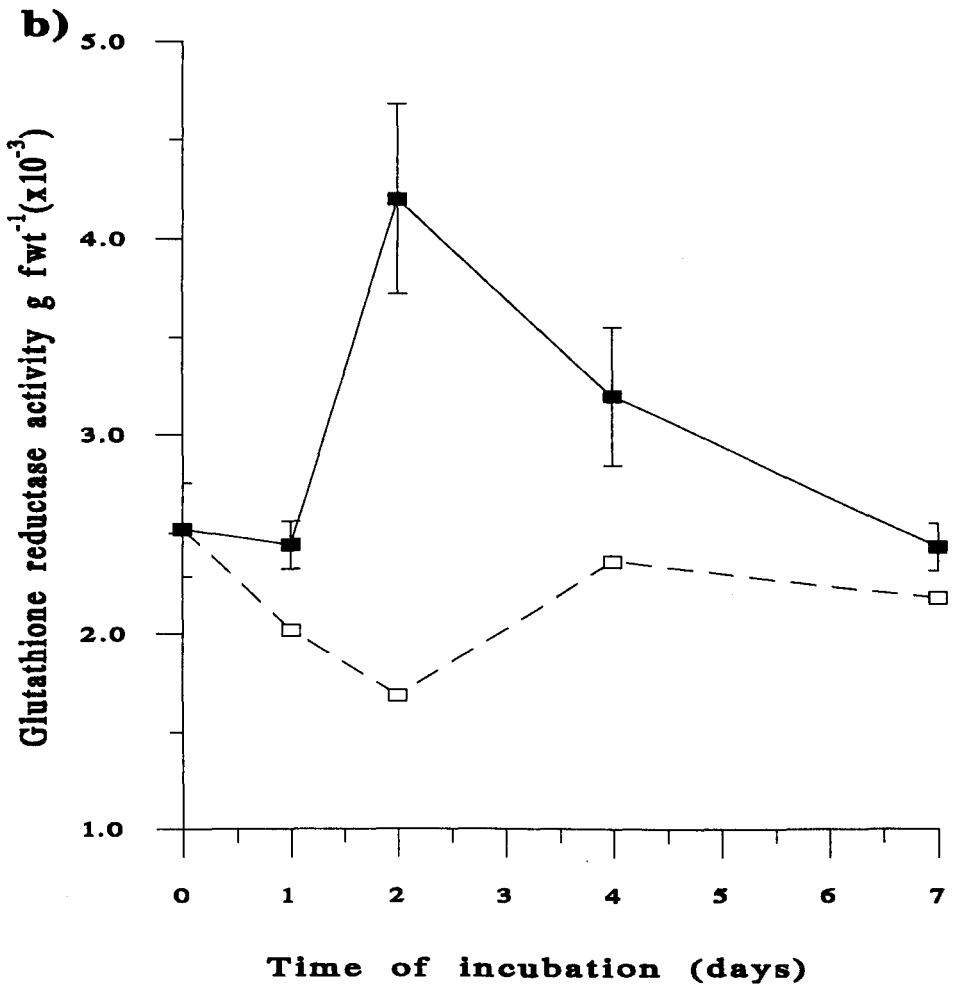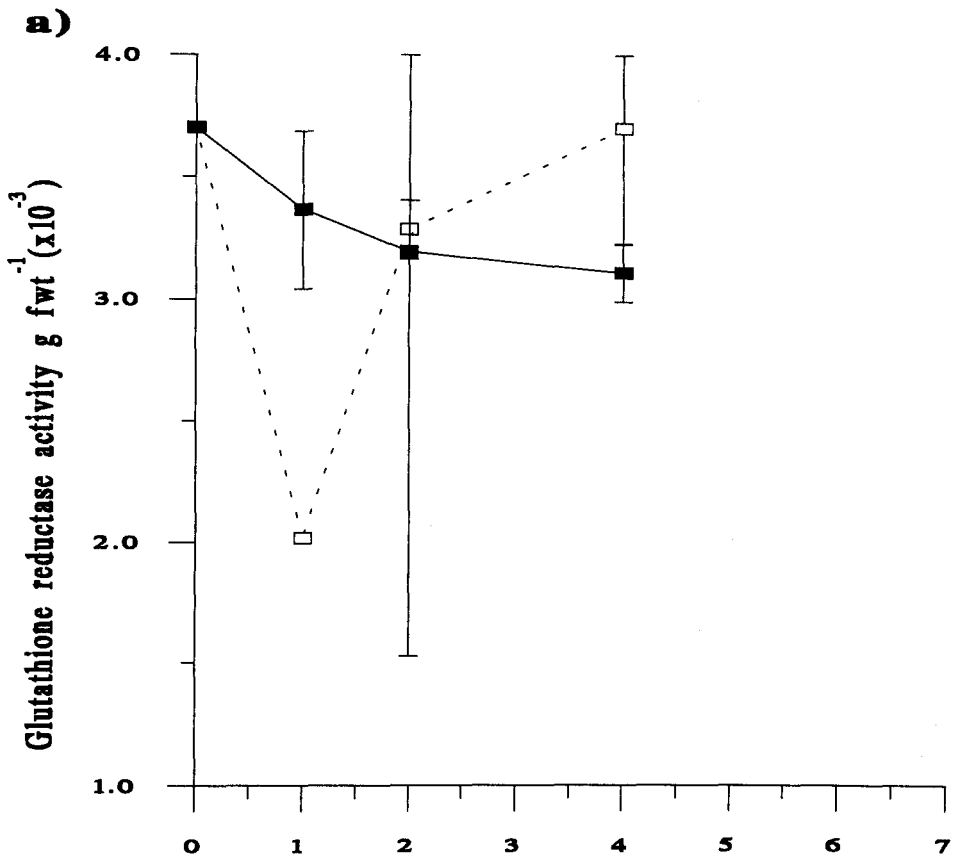
Fig. 7.7 Activity of ascorbate peroxidase in 7-day-old seedlings of *M. sativa* cv. CUF 101 and somaclone (7R1) in response to short term of salt stress (NaCl). Means of three parallel measurements from each of 2 independent extracts ± SE.

a) Cultivar CUF 101

b) Somaclone 7R1

Concentrations of NaCl were: 0- □ and 200 mol m^{-3} - ■ .

a)

b)
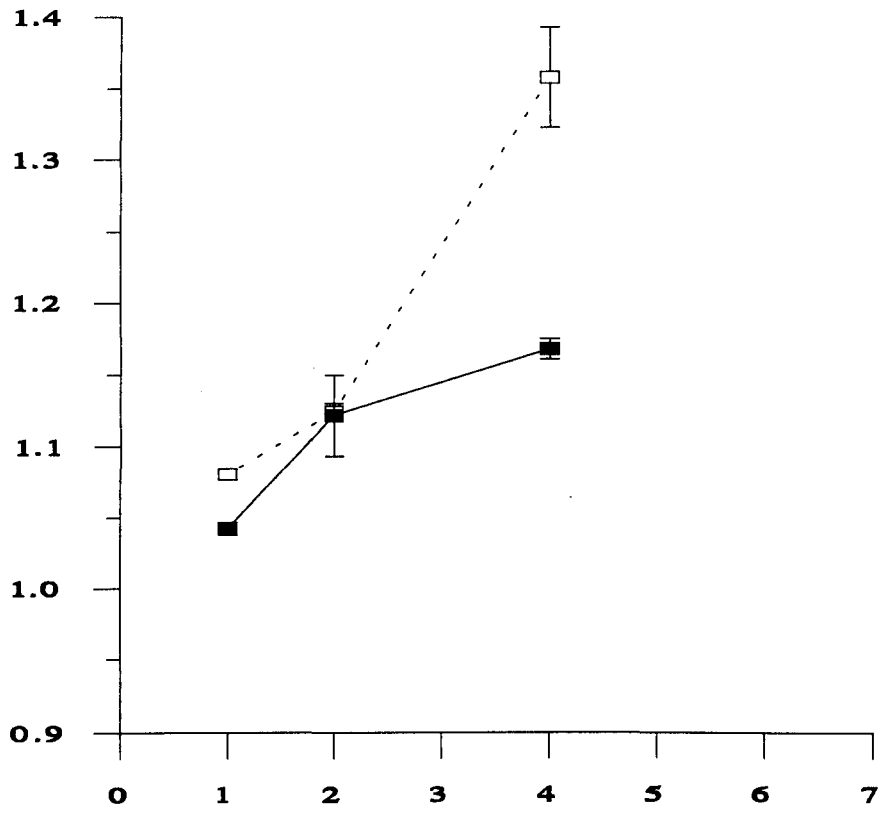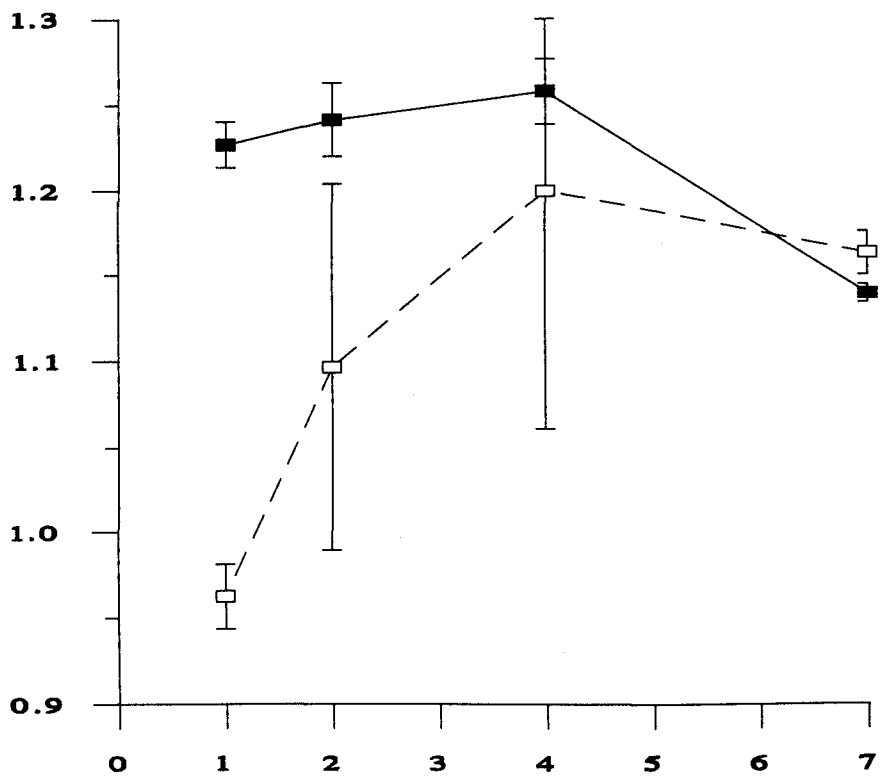
Time of incubation (days)

CHAPTER 8

Molecular Characterization of Drought and Salt Stress Responses in Somaclones of Alfalfa

8. Molecular Characterization of Drought and Salt Stress Responses in Somaclones of Alfalfa

8.1 INTRODUCTION

Traditionally a combination of morphological and agronomic traits have been used to measure genetic diversity. However the phenotype of a plant is determined not only by its genetic composition, but also by the environment. The phenotype provides therefore, an imperfect measure of the genetic potential of plants (Tanksley *et al.*, 1989). To overcome this problem biochemical and molecular techniques are being used to monitor genetic variability in plant responses (Bagheri *et al.*, 1995).

Winicov *et al.* (1989) have shown that the acquisition of salt-tolerance by callus cells of alfalfa is accompanied by increased expression of a subset of genes. Some of these genes are expressed constitutively, but other are induced by the presence of NaCl including several nuclear and chloroplast genes encoding photosynthetic functions (Winicov & Seemann, 1990; Winicov & Button, 1991). Winicov & Deutch, (1994) have used the salt-tolerant plants to characterise a cDNA clone that identifies transcripts accumulated specifically in roots and which could not be found in leaves in response to NaCl. They isolated a cDNA clone by differential screening of a cDNA library from cells of salt-tolerant alfalfa using cDNA probes derived from poly-A+ RNAs from salt-tolerant and salt-sensitive callus which was designated pA9 and which contained a 600 bp insert that hybridizes to a 1.4 kb RNA transcript. The sequence of the pA9, suggested that it represents a gene encoding a protein related to proline-rich cell wall proteins previously found in tomato, maize and carrot (Winicov & Deutch, 1994). The evidence for Alfin-1 (Winicov, 1993) and pA9 (Winicov & Deutch, 1994) indicated that accumulation of mRNAs from these genes were NaCl inducible in regenerated salt tolerant plants derived from the non-commercial variety Regen-S, but there was no data on the extent to which these observations could be extended to other variants or plant species.

The aim of this study was to determine the presence of pA9-like sequences in tolerant and sensitive lines of alfalfa to characterize the gene then establish whether any changes in the copy number of this specific gene and the DNA sequence of the alfalfa insert in the region of clone pA9 had occurred.

8.2 MATERIAL AND METHODS

8.2.1 Plant materials

DNA was extracted from young leaves from glasshouse grown plants of CUF 101, CUF101-1S (see Chapter 2, Section 2.2.1), somaclones derived from different tissue culture cycles 7R1, 7R2 (see Chapter 4, Section 4.2.1), 7R2VO (see Chapter 6, Section 6.3.3), and somaclones derived from *in vitro* selection 6R2IV, 9R2IV (see Chapter 5, Section 5.3.2).

8.2.2 DNA preparation

Genomic DNA was extracted from an alfalfa leave material which had been excised using a sterile scalpel then the tissue frozen in liquid nitrogen (Yu & Pauls, 1993). The frozen leaf tissue was ground to a powder in a mortar and pestle and homogenized in 400 µl of extraction buffer (10 ml TRIS-HCl pH 7.4, 2.5 ml 5 M NaCl, 2.5 ml 0.5 M EDTA pH 8.0, 2.5 ml 10% SDS and 32.5 ml double-distilled water) in a sterile Eppendorf tube at approximately 500 rpm for 30 second. The homogenate was centrifuged for 1 min in a microfuge (13000 rpm) then DNA was precipitated from 300 µl of the supernatant, in a new Eppendorf tube, by adding 300 µl of isopropanol. After 5 min on ice, the DNA was pelleted by centrifugation for 10 min, the supernatant was removed and after air drying the DNA pellet was dissolved in distilled water to give a concentration of 1 mg/ml then stored at -20°C for PCR.

For Southern blot analysis, DNA was extracted following Doyle *et al.* (1990) with the following modification:

1. The buffer (10 ml) was preheated in a centrifuge tube to 60°C in a water bath (Yu & Pauls, 1993).

2. The frozen fresh leaf tissue (0.5 to 1.0 g) was ground to a fine powder in liquid nitrogen using chilled mortar and pestle.

3. The powder was scraped directly into preheated buffer and swirled gently to mix.

4. The sample was incubated at 60°C for 30 min. with occasional gentle swirling.

5. The sample was extracted once with 1 volume of chloroform-isoamyl alchol (24:1, v:v), mixing gently but thoroughly and centrifuged at 3000 rpm for 10 min. at room temperature. The aqueous phase was removed with a wide-bore pipette and transferred to a clean, centrifuge tube.

7. Two-third volumes of cold isopropanol and 20 µl of 10 M NaCl were added then the tube inverted gently and left at -20 °C for 30 min. to precipitate the nucleic acid by one of the following options:

-If strands of DNA were visible, the nucleic acids were spooled with a glass hook and transfered to 10 to 20 ml of wash buffer [70% (v/v) ethanol].

-If the DNA appeared flocculent, the solution was centrifuged at (3000 rpm) for 10 min. as much of the supernatant as possible was gently poured off, leaving the precipitate as a diffuse and very loose pellet.

8. A volume of 10 ml 70% ethanol was added then centrifuged at 3000 rpm for 10 minutes (for three time).

10. The nucleic acids were allowed to dry.

11. The pellet was dissolved in distilled water and stored at -20 °C.

8.2.3 PCR amplification

Genomic DNA was used for PCR. Primers (OMID 1 and OMID 2; Table 8.1) were added to 50 mol m^{-3} dNTP mixture (dATP, dCTP, dGTP and dTTP), together with

target DNA, 2.5 U of Taq DNA polymerase (Boehringer, UK) and 10 µl of 10 × Taq DNA polymerase reaction buffer. Reaction volumes were made to 100 µl with sterile distilled water and overlaid with 120 µl mineral oil. Amplification conditions were one cycle of 94 °C for 4 min., followed by 30 cycles (94 °C 1 min., 55 °C 1 min. and 72 °C 2 min) and one cycle of 72 °C for 10 min using a Perkin Elmer Cetus 480 thermocycler.

Table 8.1 Primers for analysis of putative osmotic somaclones of alfalfa (*M. sativa* L.)

OMID 1

5' CAA GGA CTT GTT GAT TTG GAT G 3'

OMID2

5' TAA GCT GGG CAC TTT AAA CCC T 3'

8.2.4 Preparation of DNA probe

The probe was prepared as follows. A gel (0.35 g Agarose, 0.84 µl buffer (50 × TAE) and 42 ml dH$_2$O) was heated to dissolve the Agarose and allowed to cool to 50 °C. Then 1µl ethidium bromide (10 mg ml^{-1}) was added and mixed. PCR products were loaded and electrophoresed for an hour with the band were cut and put in a tube. Three × dH$_2$O was added. The DNA probe was radiactively labelled with ^{32}P dCTP following the protocol of the random labelling kit (Boehringer Mannhelm). The probe was purified using a Sephadex G-50 column. The labeled probe fraction were pooled and denatured for use in Southern blotting.

8.2.5 Southern blotting

To estimate the number of genes corresponding to the pA9 sequence within the alfalfa genome and to evaluate the somaclones derived from each of two successive tissue culture cycles, genomic DNA from tetraploid plants *M. sativa,* CUF 101, CUF101-1S, somaclones 7R1, 7R2, 7R2VO and somaclones derived from *in vitro* selection with 250 mol m^{-3} NaCl 9R2IV, 6R2IV were investigated. Genomic DNA extracted from these genotypes were quantified and digested using the restriction endonuclease *Eco* RI (10 µg/lane) then subjected to electrophoresis. The gel was transferred to a nylon membrane, and hybridized to radioactive probe inserts derived from clone pA9. Hybridisation protocols used were as recommended in the Gene Screen ^{TM} Plus instruction manual (DuPont), with the exception that 65°C washes were used in place of 60 °C in order to detect sequence similarity of greater than 70 %. Membranes were exposed to the Fuji RX film at -70 °C.

8.2.6 DNA sequencing

PCR product were prepared for sequencing using Mllrdspin 400 (Pharmcia) columns as recommended by the Manufacturer.

The alfalfa insert in the pA9-like gene was sequenced by the dye-labled cabellen terminator method (Perkin-Elmer, 1995) using sequencing kits containing AmpliTaq® DNA Polymerase FS and the ABI 373 DNA Sequencer.

The DNA sequence was analysed using the FASTA programme within the GCG suite at the Daresbury laboratories.

8.3. RESULTS

8.3.1 PCR amplification

To distinguish between salt tolerant and sensitive lines of alfalfa, initial experiments using the polymerase chain reaction (PCR) amplification of a region of the pA9 gene indicated that it was present in the salt tolerant lines, 6R2IV (Fig. 8.1, no.6), 9R2IV (Fig. 8.1, no. 2), the partially salt tolerant line, CUF101-1S (Fig. 8.1, no.4) and also in the sensitive cultivar, CUF 101 (Fig. 8.1, no. 3). Thus, the response is not qualitative and therefore quantitative evaluation was required.

8.3.2 Southern blot analysis

Southern blot analysis, in which PCR products were used as a probe, showed that a single band was observed in CUF101-1S (Fig.8.2, no. 4), two bands in somaclone 6R2IV (Fig. 8.2, no. 6), two bands in somaclone 7R1 (Fig. 8.2, no. 7), three bands in somaclone 9R2IV (Fig. 8.2, no.2). However, no bands were observed in CUF 101 and none were observed in somaclones 7R2VO and 7R2 (Fig. 8.2, no. 3, 5 and 8, respectively). This indicated that pA9-like was present as multiple copies in the tolerant clone 6R2IV and 7R1, compared with a single copy in the parent CUF101-1S and no band in the sensitive somaclone 7R2 and CUF 101. There was no seed for specific genotype 9R2IV and 7R2VO to analysis the level of their tolerance.

8.3.3 DNA sequencing

The DNA sequence of the pA9-like gene was determined (Fig. 8.3). It showed sequence similarity with the DNA sequences summarized in Fig. 8.4, with certain regions showing similarities of some proteins ranging up to 93%. The pA9-like sequence showed 93% identity in 113 bp overlap to the corresponding portion of the

pA9 sequence found in alfalfa, derived from Regen-S (Winicov & Deutch, 1994), 75.3% identity in 97 bp overlap to the TPRP-F1 sequence in young tomato fruits (Salts *et al.* 1991) 70.4% identity in 98 bp overlap to HyPRP gene found in *Cuscuta reflexa* (Subramaniam *et al.* 1994), 66.3% identity in 101 bp overlap to *corC* gene found in *Medicago sativa* (Castonguay *et al.*, 1994).

Translation of the DNA sequence from the 5' end revealed one sequence of 36 amino acids (Fig. 8.3) which exhibited a strong sequence similarity to some proline-rich protein predicted from cDNA clones (Fig. 8.5). The pA9-like sequence showed 86% amino-acid identity with the corresponding region of the pA9 found in alfalfa (Winicov & Detuch, 1994), 78% identity to the TPRP-F1 sequence in tomato fruits (Salts *et al.* 1991), 61% identity to the HyPRP sequence in maize (Jose-Estanyol *et al.*, 1992), 58% identity to the HyPRP sequence in *Cuscuta reflexa* (Subramanian *et al.* 1994), 56% identity to the ADR11.2 sequence in soybean (Datta *et al.*, 1993), 53% identity to the ZRP3 sequence in maize (John *et al.*, 1992), 50% identity to the Dc2.15 sequence in carrots (Aleith & Richter, 1990) and 47% identity to the SAC51 sequence in rape (Coupe *et al.*, 1993).

8.4 DISCUSSION

Biochemical and molecular studies have demonstrated that a complex strategy is employed by plants to adapt to drought and salinity stress. In such a multifactorial response, the basis of tolerance could be related to biosynthesis of intracellular compatible solutes (Chapter 3, 7), the level of antioxidant enzymes (Chapter 3, 7), ionic flux (Niu *et al.* 1993) and increased synthesis of certain proteins in some plants (Singh *et al.* 1985; Hurkman *et al.*, 1987, 1991). Experiments using polymerase chain reaction (PCR) amplification of a region of the pA9 gene indicated that it was present in tolerant and sensitive lines. The results from this study showed an increase in copy number of pA9-like gene between salt sensitive and salt tolerant lines, and

have revealed changed copy number in plants regenerated from different tissue culture cycles.

The intensities of some of the polypeptide bands increased with increasing levels of NaCl adaptation, while the intensities of other polypeptide bands are reduced (Singh *et al.*, 1985; Hurkman *et al.*, 1987). They found that enhanced levels of 43- and 26-kilodalton (kDa) polypeptides were present in both NaCl and PEG-induced water stress adapted cells but were not detectable in unadapted cells. They suggested an involvement of the 26-kDa polypeptide in the adaptation of cultured tobacco cells to NaCl and water stress. The protein patterns for control and 200 mol m^{-3} NaCl-stressed plants of barley were qualitatively similar, but the net synthesis of a number of proteins was qualitatively changed (Hurkman *et al.*, 1987). The most change was a significant increase in two proteins. Each pair consisted of protein of approximately 26 and 27 kDa. Both GS1 and GS2 (26-kDa polypeptides) increased in barley roots during salt stress and had a high sequence similarity to germin, a protein that increases significantly in germinating wheat seeds (Hurkman *et al.* 1991). Holland *et al.* (1993) reported a gene 'csa' encoding for a citrus salt-stress-associated protein (cit-SAP) was cloned from *Citrus sinensis* salt treated cells. It was a plant GP-like protein which was induced as a result of exposing the plant to salt stress. Holland *et al.* (1993) and Dhindsa (1991) also reported that glutathione peroxidase activities were increased as a result of drought.

Variation in copy number of pA9-like was consistent with a previous report of changes in repeated DNA sequence among alfalfa somaclones (Kidwell & Osborn, 1993). They showed that specific nuclear repeated DNA sequences, including a ribosomal DNA sequence, changed copy number in plants regenerated from tissue culture. Both increases and decreases in copy number of repeated sequences were observed in alfalfa plants regenerated from successive cycles of tissue culture (Kidwell & Osborn, 1993). When DNA extracted from potato regenerants, was probed by Southern hybridization with cloned potato DNA fragments (Landsmann & Uhrig, 1985), a de-amplification (e.g. reduction) in genes coding for ribosomal RNA

was found, although this alteration was not associated with a change in plant morphology. Some variation in copy number is due to asymetrical mitotic exchanges which results in the exchange of segments, loss of homologues owing to somatic reduction-duplication and loss of chromosome segments to chromosome breakage (Kidwell & Osborn, 1993). They considered that change of copy number among the alfalfa somaclones may be located in or close to regions of heterochromatic DNA that were susceptible to chromosome breakage during tissue culture. RAPDs revealed gross polymorphisms in protoplast-derived callus which had been in tissue culture for over two years and had lost the ability to differentiate shoots and regenerate plants (Taylor *et al.*, 1995). The sensitivity of RAPDs was not sufficient to detect genetic changes in tissue cultured plants of sugarcane that result from somaclonal variation (Taylor *et al.*, 1995). Chowdhury and Vasil (1993) also found no DNA variation in sugarcane plants regenerated from *in vitro* cultures, using RFLP analysis. They suggested that those were most likely due to the use of embryogenic cultures or apical buds, both of which are less prone to genetic changes because of stringent selection in favour of normal cells during somatic embryo formation, or they may have been due to the use of unsuitable probes.

Winicov and Deutch (1994) demonstrated that the clone pA9 responded to salt in both whole plants and callus derived from Regen-S, and suggested its role in cellular salt tolerance was tissue-specific, but there was no data to suggest that their observations could be applied to other variants or plant species. However, linking the expression of a gene to a higher degree of tolerance within a genotype might provide arguments for its role in adaptation (Moons *et al.*, 1995). Southern blot analysis showed changes in copy number during tissue culture. The pA9-like gene was present as multiple copies in the tolerant clone compared with a single copy in the parents which provided support for salt inducible gene expression in alfalfa (Winicov & Deutch, 1994). A cell line from diploid alfalfa which was tolerant to 171 mol m^{-3} NaCl in the medium and noticeably more green than the parent line was assessed to see if increased chlorophyll accumulation was related to chloroplast

development, a greater photosynthetic activity and increases in chloroplast-gene expression in this cell line (Winicov & Button, 1991). They found that the chloroplast-DNA copy number in the salt tolerant line increased by 50% from that of the salt-sensitive parent line, suggesting that activation of the physiological systems in the chloroplasts was contributing to the ability of the cells to withstand prolonged salt stress. Winicov and Seemann (1990) demonstrated that the chlorophyll accumulation and response to salt was associated with large increases in the two photosynthesis related mRNAs on the *rbcL* and the *rbcS* gene and a substantial increase in the activity of the holoenzyme. They suggested that the salt-induced increase in mRNA and protein accumulation involved in photosynthesis may play a significant role in the salt tolerant capability of salt tolerant alfalfa cells.

The DNA sequence insert in the region of pA9-like showed a 93% identity in 113 bp overlap with DNA sequence of the alfalfa in clone pA9 (Winicov & Deutch, 1994). The pA9-like gene was present as multiple copies in the tolerant somaclones 6R2IV and 7R1 which showed significantly less reduction in root and shoot lengths, in the presence of NaCl and PEG, compared with that measured in CUF101-1S and CUF 101 and none were observed in the sensitive somaclone 7R2. Multiple copies were in those clone (6R2IV, 7R1) where the proline level increased 16.7 and 12.4 fold at 200 mol m^{-3} NaCl compared with their control values and showed a 3.8 and 3.4 fold increase compared with that measured in CUF101-1S. Proline level in 6R2IV and 7R1 at 200 g l^{-1} PEG increased 31 and 23 fold compared with 0 g l^{-1} PEG and levels were higher in 6R2IV and 7R1 than that measured in CUF 101. Different stress conditions may induce a common stress response that might be due to osmotic stress. Le Rudulier *et al.* (1982) and Jakowec *et al.* (1985) reported a recombinant plasmid carrying a gene which governs proline overproduction and which confers an increased osmotolerance when transferred to *E. coli* and *Klebsiella pneumoniae*. Many of the proline-rich proteins that have been characterized were isolated from tissue capable of growth and cell expansion, and they may have a role in cell wall formation and structure (Cassab & Varner, 1988). The size of proline-rich proteins

varies from approximately 14 kDa for maize and carrot proteins, to about 36 kDa for the tomato protein. Because pA9 hybridizes to a 1.6 kb mRNA, it is likely that full-length transcripts encode a protein with 30-40 kDa (Winicov & Deutch, 1994). Although, the DNA sequence of pA9-like was not particularly proline-rich, it did exhibit a 93% identity in 113 bp overlap with a proline-rich protein (Deutch & Winicov, 1994), 75.3% identity in 97 bp over lap with proline rich protein (TPRP-F1 gene) in tomato (Salts et al., 1991). Interestingly, the tolerant somaclone (6R2IV, 7R1) revealed elevated levels of the antioxidant enzymes (see Chapter 3, Section 3.3.4 and Chapter 7, Section 7.3.2). An example of any induced protein in the susceptible and resistance alfalfa may reveal a relationship between the protein and the antioxidant enzymes.

PCR PRODUCTS
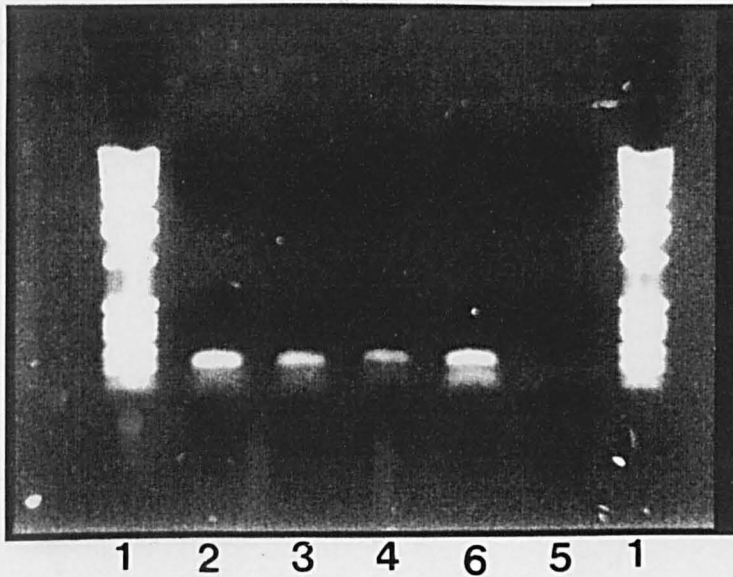
Fig. 8.1 PCR products of *M. sativa* cv. CUF 101 (3), somaclones [9R2IV (2), 6R2IV (6) and parent CUF101-1S (4)] and sterile distilled water (5).
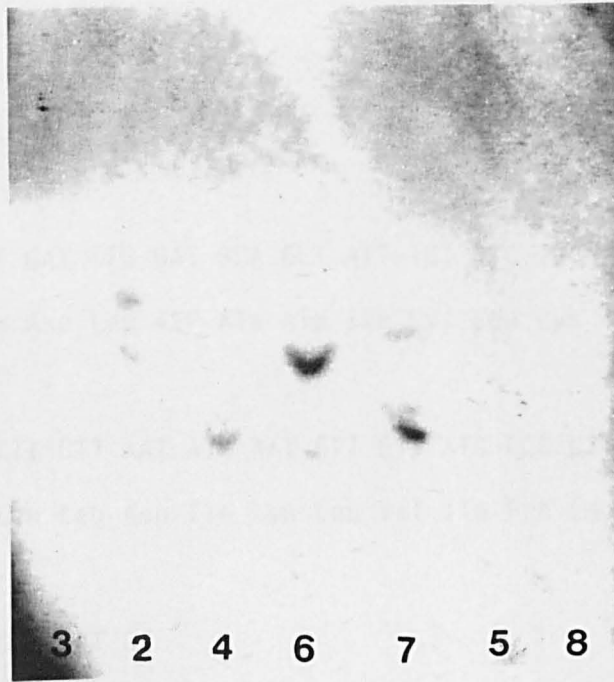
Fig. 8.2 Southern blot analysis of alfalfa genomic DNA. Total genomic DNA, isolated from alfalfa leaves, was digested with the restriction endonuclease *Eco*R1. The resulting digested (10 µg/lane) were separated by electrophoresis, transferred to a nylon membrane and probed with ^{32}P-labelled derived from clone pA9. Hybridization of PCR products of *M. sativa* cv. CUF 101 (3), somaclones [9R2IV (2), 6R2IV (6), 7R1 (7), 7R2VO (5), 7R2 (8) and parent CUF101-1S (4).

```
G CGA GGA CTT GAT TTG GAT GCA GCT ATT TGT CTC TGC ACC ACT ATT
  Arg Gly Leu Asp Leu ASP Ala Ala Ile Cys Leu Cys Thr Thr Ile


AGA CNT AAA CTT CTT AAT ATC AAT CTT GTT ATC CCC CTT GCT CTT NAG
Arg  N  Lys Leu Leu Asn Ile Asn Leu Val Ile Pro Leu Ala Leu  N


G-T CTC NGT GNG TGT GG
 N  Leu  N   N  Cys
```

Fig. 8.3 Nucleotide sequence of the cDNA insertion of clone pA9-like and derived amino-acid sequence.

```
pA9-like   1GCGAGGACTT  10GTTGATTTGG  20ATGCAGCTAT  30ATGTCTCTGC

pA9            T.A.......  ..........  ..........  ..........

TPRP-F1        AG......A   ..A..C....  .......C..  T.....T...

HyPRP          AG.......   .CA.GG....  .C..C.GG..  ...C..A...

(Cuscuta reflexa)

ALFCORC        T.A...TT.G  .C........  .......AG.  T.....T..T

ZRP3           .GAG..T..G  ..G..CC.C   .C..C..AT.  G..C......

DC2.15         .AAG.TCT.G  TCAACC.C.A  GGC.GCGGTA  TGCCT.TGCA

HyPRP          ...GT.CGGA  CG..CCCGAT  CGA.ACGCTG  .A.CTGAACG

(Zea mays)

ADR11-2        ...CA.G..A  CG..CCCCAT  TGA..CGCTC  .AACTTGGTG

SAC51          A.AGCC---.  AA..CCC.AG  AGA.GCTCT.  .AACTACTAG



pA9-like   40ACCACTATTA  50GACNTAAACT  60TCTTAATATC  70AATCTTGTTA

pA9            ..........  ...T......  ..........  ..........

TPRP-F1        ..A.......  ...TC..G..  CT.A..C..A  ..CA.CA..C

HyPRP          .....C....  A.GCA..G..  .T.G..C..A  ..CA.AA..C

(Cuscuta reflexa)

ALFCORC        ...G......  AGGC...TA.  ....GG....  ..CT.GAA.G

ZRP3           ...G.C..C.  AGGCC..CG.  C..CGGC...  C.C.....CG

DC2.15         CTGC.AT.A.  AG.CA.C.TA  CTGGG.A.GC  .TCTCAA.CC

HyPRP          CGTG.GTGGA  CGTGCTG.GC  GGCCTGATC.  ACCTGGTGAT

(Zea mays)

ADR11-2        C.TGTGTGGA  TCTTCTTGGA  GGG.TGGT..  .CAT.G...T

SAC51          TCTG.GCCAA  CGTGCTC.GC  GGTCT.CTCA  .CATCACCCT
```

```
pA9-like    80TCCCCCTTGC   90TCTTNAGGTC   100TCNGTGNGTG   110TGG

pA9         ..........     ....C.....     ...AT..A..      ...

TPRP-F1     .T...A....     ...AC....T     CTTA.TGA..      ATT

HyPRP       .A...A....     ....C.A...     CTCA.CGAC.      AC.

(Cuscuta reflexa)

ALFCORC     .T..TA.CA.     A...A--...     ..CTACTACT      .A.

ZRP3        .G......AG     C..CA.CTC.     ATCCCTCAAC      AAC

DC2.15      ..TTAT.GC.     CTCAGCTTGG     .TCTAAACAC      CT.

HyPRP       CGGGCAGGAG     G.CAGGTCCA     AGTGCTGCCC      GCT

(Zea mays)

ADR11-2     GGGTGACCCT     GG.GCGAAC.     AGT.CTGCCC      C.T

SAC51       .GGGAAGCCA     C..GTGAAG.     CAT..TGCAC      CCT
```

Fig. 8.4 Nucleotide sequence of the cDNA insertion of clone pA9-like and the homologous of some proline-rich proteins. The pA9-like sequence were aligned with the corresponding gene product sequence: alfalfa derived from Regen-S, pA9 (Winicov & Deutch, 1994); tomato, TPRP-F1 (Salts *et al.* 1992); maize, HyPRP (Jose-Estanyol *et al.*, 1992); *Cuscuta reflexa*, HyPRP (Subramaniam *et al.*, 1994); soybean, ADR11.2 (Datta *et al.*, 1993); maize, ZRP3 (John *et al.*, 1992); carrot, DC2.15 (Aleith & Richter, 1990) and rape, SAC51 (Coupe *et al.*, 1993). Nucleotide identity to the pA9-like sequence is shown by a dot (.).

```
pA9-like    RGLVDLDAAI   CLCTTIRNKL   LNINLVIPLA   LNNLNN

pA9         Q.........   .......L..   ..........   ..Q.LI

TPTP-F1     G.........   .......L..   .....IIL..   ..Q.LI

HyPRP       Q.VA.....L   .......AR.   ....I.L.I.   .NLLIT

(Zea mays)

HyPRP       G..AG...G.   ......KA.,   ....IIL.I.   .QVLID

(cuscuta reflexa)

ADR11.2     Q...EVE..V   .....LKL..   ..L.IWV...   .QLL.T

ZRP3        E........L   ....A.KANV   .G.H.NV..S   .NFILN

DC 2.15     E...N.E..V   ....A.KANI   .GK..NL.I.   .SL.LN

SAC51       K..A..E..A   ....ALKANI   .G...N..IS   .SLLLN
```

Fig. 8.5 Alignment of the predicted carboxyl-terminal pA9-like and the homologous of some proline-rich proteins. The pA9-like sequence were aligned with the corresponding gene product sequence: alfalfa derived from Regen-S, pA9 (Winicov & Deutch, 1994); tomato, TPRP-F1 (Salts *et al.* 1992); maize, HyPRP (Jose-Estanyol *et al.*, 1992); *Cuscuta reflexa*, HyPRP (Subramaniam *et al* , 1994); soybean, ADR11.2 (Datta *et al.*, 1993); maize, ZRP3 (John *et al.*, 1992); carrot, DC2.15 (Aleith & Richter, 1990) and rape, SAC51 (Coupe *et al.*, 1993). Amino acid identity to the pA9-like sequence is shown by a dot (.).

CHAPTER 9.

The Response to Anther Culture of Alfalfa

9. The Response to Anther Culture of Alfalfa

9.1 INTRODUCTION

9.1.1 Haploids in plant breeding

Since the discovery of anther culture as a method to produce large numbers of haploids (Guha & Maheshwari, 1964), interest has been generated in the breeding applications of microspore-derived haploids and doubled haploids. In a breeding program, both genetic and time-saving benefits can result from utilizing haploids and the fertile doubled haploids plants derived from them (Takashima et al., 1995). Their potential use in research would be similar to that of potato haploids and the elaboration of efficient methods of inducing haploids in alfalfa would be of enormous significance to further work on the implementation of the genetic selection program involving this species (Arcioni et al., 1990).

Plant breeders have been interested in haploid techniques which allow segregation materials to be rapidly advanced to complete homozygosity in a single generation (Schnell et al., 1980). Haploids are produced either by androgenesis, in which the male gamete develops into the haploid or doubled haploid plantlet, or by parthenogenesis, when the female gives rise to the haploid but normally parthenogenesis is more laborious than androgenesis since it demands both intensive greenhouse work and in vitro equipment (Wenzel et al., 1995). It has been shown that regeneration from microspores can be achieved both via embryogenesis (Chu & Hill, 1988) and organogenesis, but the embryogenic system was found to be more efficient (Heszky, 1992). Haploid plants derived from anther or microspore culture have potential uses in mutation induction studies because recessive genes are not masked. The advantages of using microspores for selection purposes are: (1) a huge population of single haploid cells is available; (2) selection can be carried out at the

earliest breeding stage; (3) the genotypes derived from chromosome doubling will be homozygous for selected individuals and recessive traits as a consequence of chromosome doubling (Wenzel & Foroughi-Wehr, 1993).

9.1.2 Application of anther culture in alfalfa improvement

Successful anther culture is an important goal in breeding commercial tetraploids such as alfalfa because it would allow the production of homozygous lines which can not be obtained from continued self-fertilisation owing to limited self-fertility, and the loss of vigour with increasing homozygosity (Zagorska *et al.*, 1984). Haploids from several monocotyledon and dicotyledon species have been successfully induced by *in vitro* culture (Foroughi-Weher & Wenzel, 1993). The anther culture line IR51500-AC11-1 had very good salinity tolerance at maturity, and had a comparatively higher seasonal average grain yield than other lines and varieties of rice tested (Alejar *et al.*, 1995). Attempts have been made to culture anthers from legumes, and there are reports of successful haploid plant regeneration from *Pisum sativum* (Gosal & Bajaj, 1988), *Lotus corniculatus* (Tomes & Peterson, 1981), *Trifolium pratense* (Bhojwani *et al.*, 1984) and *Trifolium alexandrium* (Mokhtarzadeh & Constantin, 1978).

In *Medicago sativa* reports of attempts to induce haploid plant production are limited. The first report was by Bingham (1971) who isolated several haploids from crosses between tetraploid alfalfa and diploid *M. sativa* and *M. falcata*. The 4x × 2x cross method proved to be effective, and had the advantage of obtaining haploids from specific parents. Nevertheless, no more than one or two small pods per raceme were obtained, and each pod often contained only one, not necessarily haploid, seed. Because this method of emasculating the mother plants, crossing the parental and identifying the haploids is laborious, an *in vitro* approach was attempted.

The aim of the work in this chapter was to initiate anther cultures of CUF 101 and Regen-SY so as to produce homozygous lines and to generate gametoclonal variation.

9.2 MATERIAL AND METHODS

9.2.1 Anther culture

Immature flower buds of healthy plants of Regen-SY and CUF101-1S grown in natural conditions were excised from 7.30 to 9.30 a.m., since mitoses in microspores within these anthers are more or less synchronised and cell division occurs more regularly, when the flower buds are 2-4 mm long, with pollen at the uninucleate stage (Appendix 9.1). Buds were surface sterilized by 10% v/v "Domestos" bleach plus 0.1% w/v sodium dodecyl sulphate for 15 min. then washed in five changes of sterile distilled water. Anthers were placed on a modified SH medium (Schenk and Hildebrandt, 1972) containing 100 mg l^{-1} serine, 10% w/v sucrose in solid medium (10 g l^{-1} Agar), semi-solid medium (3 g l^{-1} Agar) and filter-sterilised liquid medium, incubated at high and low temperature pre-treatments.

For high temperature pre-treatment, inflorescences were excised and the stem portion was placed in water prior to maintenance in darkness at 25°C and 40°C for 4 hours. Following the pre-treatment buds were surface-sterilized, the anthers were excised and cultured on the medium in Petri dishes and incubated for 1 day at 35°C, followed by 1 day at 30°C, prior to transfer to 25°C.

For low temperature pre-treatment, anthers were placed directly on to the medium in Petri dishes (at least 200 anthers per dish). The dishes were sealed and incubated at 4°C for 3 days, then maintained at 25°C under fluorescent light with a 16h photoperiod. After 3 weeks any embryo or callus that was produced was transferred to regeneration media, SHAP medium (Wan et al., 1988), Boi2y (Saunders and Bingham, 1975) (see Chapter 2, Section 2.2.2) and a modified Blayeds (1966)

medium supplemented with 1 mg l^{-1} 2IP [N^6-(2-isopentyl) adenine] (Zagorska *et al.*, 1984), and incubated as before. From 3-5 weeks after transfer of callus to regeneration media, large numbers of buds and shoots became visible on callus of Regen-SY and a low ratio in CUF101-1S in modified Blayeds (1966) medium supplemented by 1 mg l^{-1} 2IP but not on the others. When larger, these shoots were transferred individually to modified Schenk and Hildebrandt medium (1972) (GS) containing 1% w/v sucrose, no growth regulators, and 0.8% Difco Bacto Agar. After 2 weeks growth, well rooted plantlets were transferred to Jiffy-7 peat moss containers (Jiffy Products Ltd., Norway) in a glasshouse mist bed. After 10 days, plants were transplanted into a soil-sand-peat moss mixture (3:1:1) and grown in a glasshouse to flowering. Inflorescences were self pollinated by hand and cross pollinated between plants derived from the same clone. Inflorescences were covered immediately with non moisture-proof glassine bags for 7 days (Sayers & Murphy, 1966) then the bags were removed to allow seeds to mature.

9.2.2 Assessment of anther derived plant for drought tolerance

Fifty anther derived plants were multiplied vegetatively and grown for 90 days for two harvests. In June, 10 days before the third harvest, 50 pots containing uniform plants were arranged in a randomized complete-block design with 3 replicates and this was repeated for the donor plant. The plants were placed outside the greenhouse for 30 days during the summer of 1995, without any irrigation, when there was no precipitation.

9.3 RESULTS

9.3.1 Anther response and callus induction

Anther cultures were initiated and regenerated from Regen-SY (Fig. 9.1a). Regenerated shoots were subsequently isolated and grown into flowering plants (Fig. 9.1b). There were some morphological differences between the anther derived plants and the donor plant. Most of the plants were fertile, some were fertilised by hand and a few plant were self-incompatible.

Regeneration of CUF101-1S was less productive but regenerating callus was obtained from anther culture (Fig. 9.2a) and shoots were isolated and grown into plantlets (Fig. 9.2b)

The more productive medium for callus induction and development was semi-solid medium (3 g l^{-1} agar) kept at a temperature of 4°C for 3 days. For regeneration, the most productive medium was modified Blayeds (1966) medium supplemented by 1 mg l^{-1} 2IP (see Section 9.2.1).

9.3.2 Drought tolerance of anther derived plants

After a 30 day period of drought all donor plants and most of the anther derived plants died (Fig.9.3). Three anther derived plants survived (Fig. 9.4).

9.4 DISCUSSION

Anther culture was used more successfully for Regen-SY than CUF101-1S. Several factors may be involved in determining a successful anther culture: the physiological condition of the donor plants (Sunderland, 1971), the genotype of the donor, the developmental stage of the pollen plant (Foroughi-Wehr & Wenzel, 1993), the composition of the culture medium (Ouyang et al., 1986) and the culture

conditions such as pre-treatment of anthers (Huang, 1990). The data collected here indicated that a semi-solid medium (3 g l^{-1} agar) with low temperature pre-treatment at 4°C for 3 days was more effective for callus induction than other media and treatments. Modified Blayeds (1966) medium was the most productive for regeneration of the anther cultures. Chu and Hill (1988) showed that a liquid medium is better than a solid one for anther culture of wheat. Alfalfa microspores are very sensitive to oxygen and at low O_2 concentration, they die. It is very difficult to maintain anthers on the surface of liquid medium, and when they sink to the bottom they are exposed to low concentration of O_2 and die. Use of solid media could eliminate this problem. However on solid media moisture condenses in drops around cultured anthers and can interfere with their oxygen uptake. Use of small amounts semi-solid medium that does not aggregate in droplets but remains in a thin layer so that anthers are constantly in contact with both the medium and oxygen was effective. Genotype, induction medium and cold-pre-treatment was found to be important for AC plants production. This work also indicated the importance of sucrose and serine in the induction medium.

High frequencies of AC plant were obtained in cold-pre-treatment at 4°C for 3 days. Cold pre-treatments are usually used to inhibit mitosis and accumulation of cells before the first mitosis or at the G1 boundary of the cell. A period of cold pre-treatment of alfalfa increased the proportion of pollen undergoing division in subsequent culture periods (Tanner *et al.*, 1990). They found when buds underwent an additional cold pre-treatment at 4°C for one week prior to culture, the proportion of viable pollen after culture for one week, was increased to 30%. Cold pre-treatment also plays an important role in sunflower (*Helianthus annus* L.) and the effect was more evident when cold-pre-treatment was coupled with a condition of 12% sucrose and exclusion of exogenous hormone (Yang *et al.*, 1990). Chu and Hill (1988) also showed that sucrose (9%) was required for wheat anther culture. Both pollen embryoid frequency and plant regeneration in anther cultures of wheat (Chu & Hill, 1988) benefited from utilizing amino acid such as serine.

Variation in the chromosome number were shown in anther cultures of species such as rice (Chen, 1986). Most chicory regenerated plants had become polyploid spontaneously *in vitro* leading to 1n + 2n, 2n, 2n + 4n and other mixoploid plants (Theiler-Hedtrich & Hunter, 1995). Zagorska *et al.* (1984) and recently Zagorska & Dimitrov (1995) obtained regenerants from anther culture of alfalfa with a reduced chromosome number and regenerants with a high degree of variability. Some of the plants were mixoploid, while others were normally diploid. Anther culture of lines of *M. sativa*, selected for high regenerative ability through somatic embryogenesis, has been carried out by Piccirilli *et al.* (1989) and Tanner *et al.* (1990), but all the regenerated plants showed the normal somatic chromosome number ($2n=4x=32$).

Some anther culture derived lines showed good drought tolerance as mature plants. Further research is needed to identify the conditions required for successful anther culture of CUF 101 and CUF101-1S since these are commercial varieties.

Fig. 9.1 Regeneration from anther derived callus of Regen-SY showing

a) Shoot buds.

b) Anther derived plant.

Scale represent 1 mm.

a
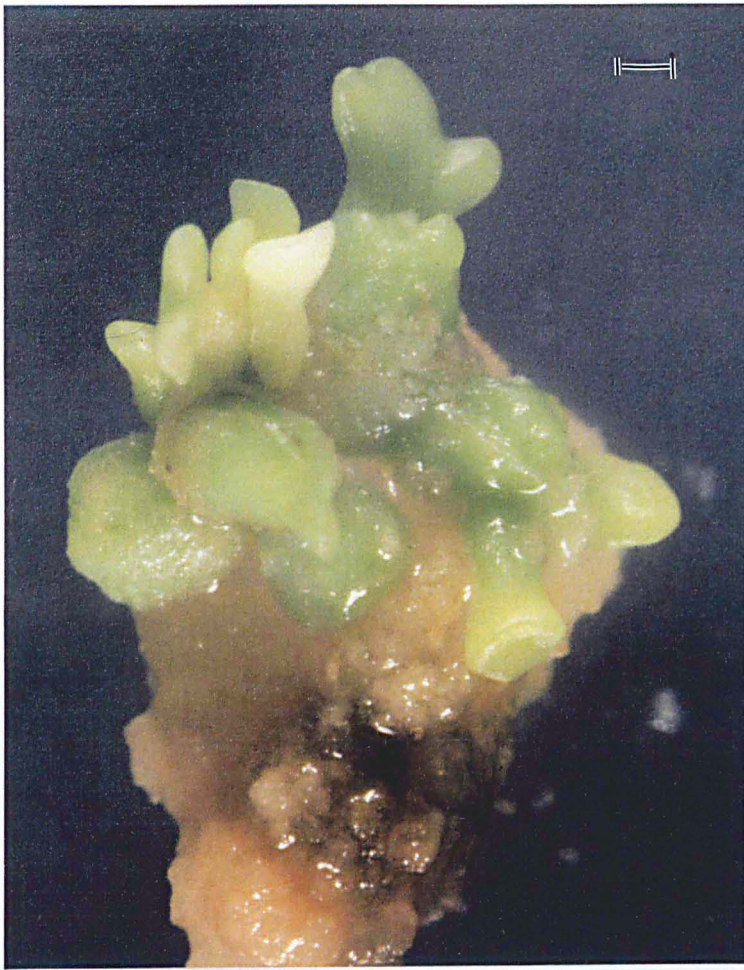
b

Fig. 9.2 Regeneration from anther derived callus of CUF101-1S showing

a) Shoot buds.

b) Anther derived plantlet.

Scale represent 1 mm.

a

b

Fig. 9.3 Anther derived plants after a period of 30 days drought during the summer of 1995. The plants were placed outside without any irrigation, over a period when there was no precipitation.

Fig. 9.4 Anther derived plants after a period of 30 days drought in summer without any irrigation.

CHAPTER 10.

General Discussion

10. General Discussion

Soil salinity is an increasing problem in arid and semi-arid areas of the world. One approach to overcoming soil salinity is to breed for increased salt tolerance in crops. There have been numerous reviews during the past decade related to breeding for salt tolerance (Epstein & Rains, 1987; Shannon & Noble, 1990; Miles, 1991; Shalhevet, 1993). However, there is only a limited number of commercially available varieties specifically selected for salt tolerance (Shannon & Noble, 1990). These tolerant varieties as cited in Shalhevet (1993), are in barley (Epstein *et al.* 1980), wheat (Kingsbury *et al.*, 1984), lettuce (Shannon, 1984), melon (Pasternak & de Malach, 1987) and rice (Yeo & Flowers, 1982). The traditional breeding approach is still of most interest to breeders because of the limited facilities required and the lack of regulations. However, this approach has generally failed to identify a clear genetic basis for a trait that is influenced by environmental conditions and which is responsive to selection. In the immediate future, the challenge is how to double the world food production in the next 30 years with hardly any more land available for extra food production. Biotechnology (molecular biology, molecular genetics, tissue culture), is likely to play a significant role in overcoming this problem. The application of biotechnology to breeding programmes will be in the development of improved selection for both qualitative and quantitative traits.

10. 1 Improvement of regeneration

Plant tissue culture has an important role in the genetic manipulation of plants and in the contribution to crop improvement (Henry *et al.*, 1994). However, the major crops, i.e. the cereals and many forage legumes are more difficult to regenerate and show wide differences in genotype responses (Quesenberry & Smith, 1993). The process of somatic embryogenesis in all these species can be divided into (1) initiation of somatic embryos from the primary explant, (2) proliferation of embryogenic

cultures (3) maturation of somatic embryos and (4) plant regeneration (von Arnold *et al.* 1995) of which stage 1 is often the most difficult. The results from this thesis showed that positive selection for embryogenic tissue culture can increase the frequency of regeneration in callus cultures and thus increase the frequency of producing regenerant plants. The frequency of regeneration in the 2nd and 3rd cycle of tissue culture (Chapter 2) increased to 70% and 74%, suggesting that regeneration is a heritable character that is susceptible to selection. The somaclones with improved regeneration also showed enhanced osmotic tolerance (Chapter 3 and 4) and multiple copies of the pA9-like gene (Chapter 8). It is possible that the gene involved in osmotic tolerance may have a role in regeneration ability. It is more likely that this correlation was coincidental since there are now considered to be embryogenic proteins whose function is to initiate and regenerate embryogenesis (Hendriks & De Vries, 1995). When progeny from the 2nd and 3rd cycle of regeneration were used as sources of explant, the regeneration frequency was compariable to the 1st cycle of regeneration in Regen-SY (Bingham, 1991). The basis for the initial differences in regeneration frequency between Regen-SY and CUF 101 is not known. Hendriks and De Vries (1995) identified three secreted proteins present in carrot suspension cultures that may play a role in somatic embryogenesis. Hendriks and De Vries (1995) suggested that some of the proteins were representative of a class of proteins that has been implicated in plant defence against biotic and abiotic stresses. Studies of the extracellular chitinase, zeamatin-like proteins as well as extracellular peroxidase, revealed a close correlation between the presence of specific chitinases in *Picea abies* and embryo morphology and development (von Arnold *et al.*, 1995). The proteins may determine the level of regeneration frequency and hence genotype differences.

The factors influencing regeneration are still poorly understood and need to be investigated in more detail.

10.2 Somaclonal variation

Plant breeders are always searching for plant material that represents an improvement on existing cultivars. Spontaneous mutation occurs at a rate, on average, of 1 in 10^6. It is generally too low for practical exploitation, and mutation breeding is difficult to control and frequently yields mosaics and deleterious changes (Lindsey & Jones, 1989). In the last two decades, the advances in molecular biology, molecular genetics, and tissue culture have proceeded at an astonishing rate, allowing breeders to alter plants by directly modifying their genetic make-up more quickly and more precisely than ever before. One approach has been to use the variation produced by somaclonal variation. This source of variation can produce variants in 15% or more of the progeny. Regenerant plants are usually not mosaics and the process of regeneration removes genotypes with the most deleterious changes (Lindsey & Jones, 1989).

Genetic analysis of the alfalfa somaclones described in Chapter 8 show that copy number of the pA9-like gene had changed in somaclones regenerated from tissue culture. Many of the genetic changes that have been detected either in spontaneously generated mutants, or as a result of induced mutations, also arise via somaclonal variation, e.g. cytoplasmic gene changes, chromosome rearrangements, mitotic crossing over, gene expression and copy number changes (Evans, 1989). Somaclonal variation has been screened for salt tolerance, and the indications from tobacco, sorghum, flax, rice, *Brassica juncea*, alfalfa, and sugarbeet, are that enhanced salt tolerance is present in the R4 generations showing it to be a stable change (reviewed by Hasegawa *et al.*, 1994).

In vitro selection by screening differentiating cultures was used in this present study to increase salt tolerance in alfalfa. The results (Chapter 6) suggested that salt tolerance was selected and was stable in the regenerated plant and progeny. The use of an *in vitro* screen consisting of differentiating cultures does attempt to overcome the problem of loss of totipotency associated with cell selections. The *in vitro* screen

although useful did not select genotypes with any greater tolerance than an *in vitro* screen of the tissue culture regenerants. It may be that the tissue is capable of only so much variation and it will be detected by whatever screening method. This presence of limited capacity for variation is confirmed by the absence of significant variation induced by the 2nd and 3rd tissue culture cycle.

10.3 Correlation between salt tolerance and PEG tolerance

Drought and high salinity are important environmental factors that cause osmotic stress and as a result reduce plant growth and crop productivity. The deleterious effect of salt on plant cells has two components, namely osmotic stress and ion toxicity. Osmotic stress also results from desiccation and therefore it is a common component of drought and salt stress (Serrano and Gaxiola, 1994). It seems likely that some of the mechanisms involved in drought tolerance would also contribute to salinity tolerance (Ben-Hayyim, 1987; Yeo, 1994). In the same way that salinity tolerance mechanisms need to be chosen to target the environment in which an improvement is sought, so it is the case for drought. For drought tolerance a combination of a deep tap root for absorbing water, some mechanism for keeping leaf turgor at least above zero, and in many cases drought tolerance, drought avoidance or drought escape, or tolerance of high leaf temperatures is necessary (Yeo, 1994). Maiti *et al.* (1994) identified several sorghum lines which showed resistance to drought, salinity and high temperature. These lines had longer roots and a higher root mass in drought conditions compared to plants under control treatment but, under salinity, root length and dry mass were not affected. This suggests that the mechanism of adaptation to salt stress may be at a biochemical level involving a specific compound able to act as an osmotic regulator, while it is a morphological mechanism for adaptation to water stress (Maiti *et al.*, 1994). Kawasaki *et al.* (1983) found no differences in dry matter yield of beans, sorghum and corn between iso-osmotic concentrations of sodium chloride and PEG. Potassium, calcium and magnesium contents in the tops and roots were severely

depressed by salt but not by PEG. In a study of sour orange citrus seedlings (*Citrus aurantium*) growing under different salt and PEG concentrations, Zekri and Parsons (1990) found both root and shoot growth to be less adversely affected by salt (39 and 47% yield reduction, respectively) than by PEG (47 and 56% reduction, respectively). Heyser and Nabors (1981) reported that NaCl-adapted tobacco cells could also grow for longer periods on media containing iso-osmotic concentrations of dextran than non-adapted cells. Harms and Oertli (1985) showed that carrot cell lines adapted to mannitol, were more resistant to salt-stress than non-resistant cells. Ben-Hayyim (1987) reported that salt tolerant cell lines of *Citrus sinensis* were more tolerant to PEG stress also. In contrast *C. aurantinum*, salt tolerant selected lines had no advantage over non-selected lines when exposed to PEG stress, from which it was concluded that adaptation to salt in *C. sinensis* (a salt excluder) is probably osmotic whereas in *C. aurantinum* (a salt accumulator) it is not so (Chowdhury & Vasil, 1993). Jensen (1982) did not find any differences in dry matter yield or wilting percentages in response to water stress between barley plants (*Hordeum distichum*) pre-treated with saline water and not pre-treated. Clearly, there are some mechanism that are specific for each stress while some are common mechanism for both stress adjustment. Alternatively, under saline stress, osmotic adjustment may be achieved by uptake of inorganic ions from the soil solution (Prat & Fathi-Ettai 1990), whereas under water stress, the plants may only adjust osmotically by synthesizing organic compounds (Delauney & Verma, 1993). Thus, the interrelation between the two stress factors is of particular significance for irrigated crops (Shalhevet, 1993). The reduction of water uptake by roots is the major cause of damage due to salinity and drought, which are essentially similar in their effect on growth in that both cause tissue desiccation, although salt effects in addition a specific ion toxicity (Shalhevet, 1993).

In the experiments described in chapter 4 and 6, somaclones 7R1 and 6R2IV showed significant increase in NaCl tolerance. The response to NaCl was a 3-4 fold increase in proline levels and a significant increase in some of the antioxidant

enzymes. Under osmotic stress one of the somaclones (7R1) was significantly more tolerant at 250 g l^{-1} PEG 6000 level than CUF 101, and a positive correlation between proline accumulation, antioxidant enzymes (CAT, AP) and response to osmotic stress was recorded in the somaclones. Increase in CAT and AP activities and proline level was greater in the osmotic-tolerant somaclone 7R1, but GR and SOD was unchanged or decreased (Chapter 3).

10.4 Characterization of somaclones using biochemical and molecular analysis

The genetic improvement of stress tolerance is an urgent need for the future of agriculture and it is an important challenge to biotechnology (Bartels & Nelson, 1994). Molecular genetic markers are finding many applications in plant breeding programs and genetic studies (Chunwongse et al., 1993). The data indicated a positive correlation between proline accumulation (Chapter 7), increase in selected antioxidant enzyme activities in the highest tolerant somaclone 7R1 (Chapter 7) and an ability of alfalfa seedlings to grow on nutrient solution containing NaCl (Chapter 4 and 6). Assessment of PEG tolerance also indicated a positive correlation between proline level and response to osmotic stress in the osmotic-tolerant genotype (7R1) which showed a larger and early response in proline level than the osmotic-sensitive CUF 101 (Chapter 3). There is evidence that proline accumulation does appear to differ between cultivars adapted to certain growth conditions or regions, as well as differing within species which are tolerant to drought or salinity (Heuer, 1994). Assessment of response to PEG stress did not show any correlation between increased GR and SOD activities and osmotic tolerance in seedlings of alfalfa, but it did show a positive correlation between increased CAT and AP activities and osmotic tolerance (Chapter 3).

The tolerant genotypes appeared to have a more effective enzyme defence mechanism than the sensitive genotype. However, different stress conditions may induce a common stress response that might be related to osmotic stress since, under osmotic

stress, it is appears that newly synthesized proteins, such as pA9-like gene, together with amino acids and antioxidative enzymes, act as components of an osmotic (salinity and drought) mechanism.

Previous evidence has shown that stress tolerance is dependent upon the genetic and biochemical characteristic of plants at the level of species down to genotype, and different levels of soluble proteins and proteolytic enzymes exist in the two sets of genotypes differing in stress tolerance (Dubey & Rani, 1990). Stress situations affect protein metabolism in plants and in a range of different types of environmental stresses, such as salinity and drought, new stress-specific proteins are synthesized (Dubey, 1994). The levels of total and soluble proteins differ in stress-tolerant and stress-sensitive plant genotypes in both non-stressed and stressed conditions (Mittal & Dubey, 1991). Drought and salinity also alters gene expression in plants, leading to synthesis of specific gene products and an increased abundance of their translatable mRNAs and proteins. As a result these stress-specific proteins possibly endow the plants with the capacity to adapt to a stressful environment by physiological and biochemical adjustments (Dubey, 1994).

Clone pA9 identified a potentially important gene that was regulated in a tissue-specific manner by NaCl (Winicov & Deutch, 1994). Although, the DNA sequence of pA9-like was not particularly proline-rich, it did exhibit a 93% identity in 113 bp overlap with a proline-rich protein (Deutch & Winicov, 1995), and a 75.3% identity in 97 bp over lap with proline-rich protein (TPRP-F1 gene) in tomato (Salts et al., 1991).

10.5 Conclusions

The following was achieved:

1. Limited regeneration of commercial variety CUF101-1S from callus.
2. Enhanced regeneration of CUF101-1S in a second and third tissue culture cycle.

3. Initiation of an *in vitro* screen for NaCl tolerance using regenerating cultures derived from CUF101-1S.

4. An increase in NaCl and PEG tolerance detected in progeny derived from *in vitro* selection.

5. Enhanced tolerance to salt and PEG stress in the progeny plants derived from 1st tissue culture stage.

6. A positive correlation identified between shooting and rooting ability, proline level and activities of selected antioxidant enzymes in alfalfa in response to salt and drought stress.

7. PCR amplification of a region of the pA9 gene indicated that pA9-like gene was present in both the tolerant and sensitive cultivar.

8. The pA9-like gene was presented as multiple copies in the tolerant somaclones compared with a single copy in the parent.

9. The DNA sequence of the alfalfa with 113 bp insert in the pA9-like gene exhibited up to 93% identity with a proline rich cell wall protein.

10. Plants of Regen-SY and CUF101-1S were regenerated from anther culture.

10.6 Future work

The project on alfalfa could be developed as follows:

1. Biochemical and molecular analysis of somaclones of alfalfa to identify the proteins and genes that are correlated with the increase in regenerative ability in alfalfa.

2. Examination of inheritance of osmotic tolerance in seed progeny of 2nd and 3rd generation in plants derived from the *in vitro* selection method.

3. Determine the role of antioxidant enzymes in the overall response of plants to salt and drought stresses.

4. Examination of pA9-like gene in other plant species using PCR.

5. Multiplication of pA9-like gene then transfer to alfalfa to see if tolerance enhanced.

6. Identification of osmotic-specific proteins using 2 dimension gel electrophoresis.

7. Production of haploid plants from dihaploid plants.

8. Selection of anther derived plants for osmotic tolerance using morphological, biochemical and molecular studies.

REFERENCES

REFERENCES

Abd-Elrahem, A.W., L. Reinhold, & H.R. Lerner, 1983. Comparison between stable NaCl-selected *Nicotiana* cell line and the wild type. Plant Physiology, 73: 624-629.

Acevedo, E. & E. Fereres, 1993. Resistance to abiotic stresses. In: M.D. Hayward, N.O. Bosemark & I. Romagosa (eds.), Plant Breeding: Principles and Prospects, Chapman & Hall, London, pp. 406-421.

Agache, S., J. De Buyser, Y. Henry & J.W. Snape, 1988. Studies on the genetic relationship between anther culture and somatic tissue culture abilities in wheat. Plant Breeding, 100: 26-33.

Al-Khatib, M., T. McNeilly & J.C. Collins, 1993. The potential of selection and breeding for improved salt tolerance in Lucerne (*Medicago sativa* L.). Euphytica, 65: 43-51.

Aleith, F. & G. Richter, 1990. Gene expression during induction of somatic embryogenesis in carrot cell suspensions. Planta, 183: 17-24.

Alejar, M.S., F.J. Zapata, D. Senadhira, G.S. Khush & S.K. Datta, 1995. Utilization of anther culture as a breeding tool in rice improvement. In: M. Terzi, R. Cella & A. Falavigna (eds.), Current Issues in Plant Molecular and Cellular Biology, Kluwer Academic Publishers, Netherlands, pp. 137-142.

Antolin, M.C. & M. Sanchez-Diaz, 1992. Photosynthetic nutrient use efficiency, nodule activity and solute accumulation in drought stressed alfalfa plants. Photosynthetica, 27: 595-604.

Antolin, M.C., J. Yoller & M. Sanchez-Diaz, 1995. Effect of temporary drought on nitrate-fed and nitrogen-fixing alfalfa plants. Plant Science, 107: 159-165.

Arcioni, S., F. Damiani, M. Pezzotti & E. Lupotto, 1990. Alfalfa, Lucerne (*Medicago spp.*). In: Y.P.S. Bajaj (ed.), Biotechnology in Agriculture and Forestry. Vol. 10, Legumes and Oilseed Crops I, Springer-Verlag Berlin, pp. 197-241.

Armstrong, C.L., J. Romero-Severson & T.K. Hodges 1992. Improved tissue culture response of an elite maize inbred through backcross breeding, and identification of

chromosomal regions important for regeneration by RFLP analysis. Theoretical and Applied Genetics, 84: 755-762.

Asada, K. & M. Takahashi, 1987. Production and scavenging of active oxygen in photosynthesis. In: D. Kyle, C. Osmond & C. Arntzen (eds.), Photoinhibition, Elsevier, Amsterdam, pp. 227-287.

Asada, K., 1993. Production and action of active oxygen species in photosynthetic tissue. In: C.H. Foyer & P.M. Mullineaux (eds.), Causes of Photo-Oxidative Stress and Amelioration of Defense System in Plants, CRC Press, Boca Raton, FL, pp 77-104.

Ashraf, M., 1994. Breeding for salinity tolerance in plants. Critical Reviews in Plant Sciences, 13: 17-42.

Ashraf, M., T. McNeilly & A.D. Bradshaw, 1986. The response to NaCl and ionic content of selected salt-tolerant and normal lines of three legume forage species in sand culture. New Phytologist, 104: 463-471.

Ashraf, M., T. McNeilly & A.D. Bradshaw, 1987. Selection and heritability of tolerance to sodium chloride in four forage species. Crop Science, 227: 232-234.

Assadian, N.W. & S. Miyamoto, 1987. Salt effects on alfalfa seedling emergence. Agronomy Journal, 79: 710-714.

Atanassov, A. & D.C.W. Brown, 1984. Plant regeneration from suspension culture and mesophyll protoplasts of *Medicago sativa* L., Plant Cell Tissue and Organ Culture, 3: 149-162.

Badiani, M., M.G. De Biasi, M. Colognola & F. Artemi, 1990. Catalase, peroxidase and superoxide dismutase activities in seedlings submitted to increasing water deficit. Agrochimica, 34: 90-102.

Bagheri, A., J.G. Paull, P. Langridge & A.J. Rathjen, 1995. Genetic distance detected with RAPD markers among selected Australian commercial varieties and boron-tolerant exotic germplasm of pea (*Pisum sativum* L.). Molecular Breeding, 1: 193-197.

Bajji, M., J.M. Kinet & J. Bouharmont, 1995. Characterization of progenies issued from drought tolerant plants of durum wheat selected *in vitro*. Proceedings of Interdrought, 30th August-2th September, Montpellier-France, Abstract VII 2.

Bar-Nun, N. & A. Poljakoff-Mayber, 1977. Salinity stress and the content of proline in roots of *Pisum sativum* and *Tamarix tetragyna*, Annals of Botany, 41: 173-179.

Bartels, D. & D. Nelson, 1994. Appoaches to improve stress tolerance using molecular genetics. Plant, Cell and Environment, 17: 659-667.

Beers, F.R. & I.W. Sizer, 1952. A spectrophotometric method for measuring the break down of hydrogen peroxide by catalase. Journal of Biological Chemistry, 195: 133-140.

Ben-Hayyim, G. & J. Kochba, 1983. Aspects of salt tolerance in a NaCl-selected stable cell line of *Citrus sinensis*. Plant Physiology, 72: 685-690.

Ben-Hayyim, G., 1987. Relationship between salt tolerance and resistance to polyethylene glycol-induced water stress in cultured citrus cells. Plant Physiology, 85: 430-433.

Bhaskaran, S., R.H. Smith & K.F. Schertz, 1986. Progeny screening of sorghum plants regenerated from sodium chloride selected callus for salt tolerance. Journal of Plant Physiology, 112: 205-210.

Bhaskaran, S., R.H. Smith & R.J. Newton, 1985. Physiological changes in cultured sorghum cells in response to induced water stress. Plant Physiology, 79: 266-269.

Bhojwani, S.S., K. Mullins & D. Cohen, 1984. Intra-varietal variation for *in vitro* plant regeneration in the genus *Trifolium*. Euphytica, 33: 915-921.

Bingham, E.T. & T.J. McCoy, 1986. Somaclonal variation in alfalfa. In: J. Janick (ed.), Plant Breeding Reviews, Vol. 4. AVI, Westport, Connecticut, pp. 123-152.

Bingham, E.T., 1971. Isolation of haploids of tetraploid alfalfa. Crop Science, 11: 433-435.

Bingham, E.T., 1991. Registration of alfalfa hybrid Regen-SY germplasm for tissue culture and transformation research. Crop Science, 31: 1098.

Bingham, E.T., L.V. Hurley, D.W. Kaatz & J.W. Saunders, 1975. Breeding alfalfa which regenerates from callus tissue in culture. Crop Science, 15: 719-721.

Bingham, E.T., T.J. McCoy & K.A. Walker, 1988. Alfalfa tissue culture. In: Alfalfa and Alfalfa Improvement, Agronomy Monograph no. 29, ASA-CSSA-SSSA, USA, pp 903-929.

Binzel, M.L., P.M. Hasegawa, D. Rhodes, S. Handa, A.K. Handa & R.A. Bressan, 1987. Solute accumulation in tobacco cells adapted to NaCl. Plant Physiology, 84: 1408-1415.

Blaydes, D.F., 1966. Interaction of kinetin and various inhibitors in the growth of soybean tissue. Physiologia Plantarum, 19: 748-753.

Bliss, R.D., K.A. Platt-Aloia & W.W. Thomson, 1986. Osmotic sensitivity in relation to salt sensitivity in germinating barley seeds. Plant, Cell and Environment, 9: 721-725.

Blum, A., 1988. Plant breeding for stress environments. CRC Press Inc., Boca Raton, Florida, USA, pp 163-178.

Bowler, C., L. Slooten, S. Vandenbranden, R. De Rycke, J. Botterman, C. Sybesma, M. Van Montagu & D. Inzé, 1991. Manganese superoxide dismutase can reduce cellular damage mediated by oxygen radicals in transgenic plants. EMBO Journal, 10: 1723-1732.

Boyer, J.S., 1982. Plant productivity and environment. Science, 218: 443-448.

Bray, A., 1994. Alterations in gene expression in response to water deficit. In: A.S. Basra (ed.), Stress-Induced Gene Expression in Plants. Harwood Academic Publishers, London, pp. 1-23.

Bray, L., D. Chriqui, K. Gloux, D. Le Rudulier, M. Meyer & J. Peduzzi, 1991. Betaines and free amino acids in salt stressed vitroplants and winter resting buds of *Populus trichocarpa × deltoides*. Physiolgia Plantarum, 83: 136-143.

Bregitzer, P., 1992. Plant regeneration and callus type in barley: Effects of genotype and culture medium. Crop Science, 32: 1108-1112.

Bressan, R.A., N.K. Singh, A.K. Handa, R. Mount, J. Clithero & P.M. Hasegawa, 1987. Stability of altered genetic expression in cultured plant cells adapted to salt. In: L. Monti & E. Proceddu (eds.), Drought Resistance in Plants, Physiological and Genetic Aspects, Commission of the European Communities, Brusselles, pp 41-57.

Bressan, R.A., P.M. Hasegawa & A.K. Handa, 1981. Resistance of cultured higher plant cells to polyethylene glycol-induced water stress. Plant Science Letters, 21: 23-30.

Brown, D.C.W. & A. Atanassov, 1985. Role of genetic back ground in somatic embryogenesis in *Medicago*. Plant Cell Tissue and Organ Culture, 4: 111-122.

Brown, P.W. & C.B. Tanner, 1983. Alfalfa stem and leaf growth during water stress. Agronomy Journal, 75: 799-805.

Burke, J.J., P.E. Gamble, J.L. Hatfield & J.E. Quinesberry, 1985. Plant morphological and biochemical responses to field water deficit. I. Response of glutathione reductase activity and paraquat sensitivity. Plant Physiology, 79: 415-419.

Caplan, A., B. Claes, R. Dekeyser & M. Van Montagu, 1990. Salinity and drought stress in rice. In: R.S. Sangwan & B.S. Sangwan-Norreel (eds.), The Impact of Biotechnology in Agriculture, Kluwer Acadamic Publishers, Dordrecht, pp. 391-402.

Carter, P.R. & C.C. Sheaffer, 1983a. Response of alfalfa to soil water deficits. I. Growth, forage quality, yield, water use, and water use efficiency. Crop Science, 23: 669-675.

Carter, P.R. & C.C. Sheaffer, 1983b. Alfalfa response to soil water deficits. II. Plant water potential, leaf conductance, and canopy temperature relationships. Crop Science, 23: 676-680.

Cassab, G.I. & J.E. Varner, 1988. Cell wall proteins. Annual Reveiw of Plant Physiology and Plant Molecular Biology, 39: 321-353.

Castonguay, Y., S. Laberge, P. Nadeau & L.P. Vézina, 1994. A cold-induced gene from *Medicago sativa* encodes a bimodular protein similar to developmentally regulated proteins. Plant Molecular Biology, 24: 799-804.

Chandler, S.F. & I.K. Vasil, 1984. Selection and characterization of NaCl tolerant cells from embryogenic cultures of *Pennisetum purpureum* Schum. (Napier grass). Plant Science Letters, 37: 157-164.

Chandler, S.F. & T.A. Thorpe, 1987. Proline accumulation and sodium sulfate tolerance in callus cultures of *Brassica napus* L. cv. Westar. Plant Cell Reports, 6: 176-179.

Charles, S. & B. Halliwell, 1981. Light activation of fructose biphosphatase in isolated spinach chloroplasts and deactivation by hydrogen peroxide- a physiological role for the thioredoix system. Planta, 151: 242-246.

Chazen, O. & P.M. Neumann, 1994. Hydroulic signals from the roots and rapid cell wall hardening in growing maize leaves are primary responses to PEG induced water deficits. Plant Physiology, 104: 1385-1392.

Chen, Y. 1986. Anther and pollen culture of rice. In: H. Hu & H. Yang (eds.), Haploids of Higher Plants *In vitro*. Springer-Verlag Berlin, pp. 3-25.

Chowdhury, M.K.U. & I.K. Vasil, 1993. Molecular analysis of plants regenerated from embryogenic cultures of hybrid sugarcane cultivars (*Saccharum spp*), Theoretical and Applied Genetics, 86: 181-188.

Chu, C.C. & R.D. Hill, 1988. An improved anther culture method for obtaining higher frequency of pollen embryoids in *Triticum aestivum* L., Plant Science, 55: 175-181.

Chunwongse, J., G.B. Martin & S.D. Tanksley, 1993. Pre-germination genotypic screening using PCR amplification of half-seeds. Theoretical and Applied Genetics, 86: 694-698.

Claes, B., R. Dekeyser, L.R. Viliarroel, M.van Den Buicke, G. Bauw, M. van Montagu & A. Caplan, 1990. Characterization of a rice gene showing organ-specific expression in response to salt stress and drought. The Plant Cell, 2: 19-27.

Collin, H.A. & P.J. Dix, 1990. Culture systems and selection procedures. In: P.J. Dix (ed.), Plant Cell Line Selection, Procedures and Application, VCH Publishers, Weinheim, pp. 3-18.

Corcuera, L.J., M. Hintz & E. Pahlich, 1989. Proline metabolism in *Solanum tuberosum* cell suspension cultures under water stress. Journal of Plant Physiology, 134: 290-293.

Cosgrove, D.J. 1993. Wall extensibility: its nature, measurement and relationship to plant cell growth. Tansley review 46. New Phytologist, 124: 1-23.

Coupe, S.A., J.E. Taylor, P.J. Issac & J.A. Roberts, 1993. Identification and characterization of a proline-rich mRNA that accumulates during pod development in oilseed rape (*Brassica napus* L.). Plant Molecular Biology, 23: 1223-1232.

Cramer, G.R., A. Läuchi & V.S. Polito, 1985. Displacement of root cell. Plant Physiology, 79: 207-211.

Criddle, R.S., L.D. Hansen, R.W. Breidenbach, M.R. Ward & R.C. Huffaker, 1989. Effects of NaCl on metabolic heat evolution rates by barley roots. Plant Physiology, 9: 53-58.

Croughan, T.P., S.J. Stavarek & D.W. Rains, 1978. Selection of a NaCl-tolerant line of cultured alfalfa cells. Crop Science, 18: 959-963.

Csonka L.N., 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiol Reveiw, 53: 121-147.

Cushman, J.C., D.M. Vernon, & H.J. Bohnert, 1992. ABA and the transcriptinal control of CAM induction during salt stress in the common ice plant. In: D.P.S. Verma (ed.), Control of Plant Gene Expression, CRC Press, Boca Raton. FL, pp. 287-300.

Cushman, J.C., E.J. DeRocher, & H.J. Bohnert, 1990. Gene expression during adaptation to salt stress. In: F. Katterman (ed.), Environmental Injury to Plants, CA: Academic Press, San Diego, pp. 173-203.

Datta, N., P.R. LaFayette, P.A. Kroner, R.T. Nagao & J.L. Key, 1993. Isolation and characterization of three families of auxin down-regulated cDNA clones. Plant Molecular Biology, 21: 859-869.

Delauney, A.J. & D.P.S. Verma, 1990a. A soybean gene encoding Δ^1-pyrroline-5-carboxylate reductase was isolated by functional complementation in *Escherichia coli*. and is found to be osmoregulated. Molecular General Genetics, 221: 299-305.

Delauney, A.J. & D.P.S. Verma, 1993. Proline biosynthesis and osmoregulation in plants. The Plant Journal, 4: 215-223.

Deutch, C.E. & I. Winicov, 1995. Post-transcriptional regulation of a salt-inducible alfalfa gene encoding a putative chimeric proline-rich cell wall protein. Plant Molecular Biology, 27: 411-418.

Dhindsa, R.S., 1991. Drought stress, enzymes of glutathione metabolism, oxidation injury, and protein synthesis in *Tortula ruralis*. Plant Physiology, 95: 648-651.

Dix, P.J. & H.E. Street, 1975. Sodium chloride-resistant cultured cell lines from *Nicotiana sylvestris* and *Capsicum annuum*. Plant Science Letters, 5: 231-237.

Dix, P.J. & R.S. Pearce, 1981. Proline accumulation in NaCl-resistant and sensitive cell lines of *Nicotiana sylvestris*. Zeitschrift fuer Pflanzenphysiologie, 102: 243-248.

Dix, P.J., 1986. Cell line selection. In: M.M. Yeoman (ed.), In Plant Cell Culture Technology, Botanical Monographs, Vol. 23, Blackwell Scientific Publications, pp 143-201.

Dix, P.J., U.A. McLysaght & R.S. Pearce, 1984. The potential of cell cultures for the production of salt tolerant cultivars. In: W. Lange, A.C. Zeven & N.G. Hogenboom (eds.), Efficiency in Plant Breeding, Pudoc, Wageningen, Netherlands, pp. 219-223.

Dobrenz, A.K., M.H. Schonhorst, J.E. Stone, R.K. Thompson, S. Allen & D. Smith, 1983. Registration of AZ-GERM SALT-I nondormant alfalfa germplasm. Crop Science, 23: 807.

Doke, N., Y. Miura, L. Sanchez & K. Kawakita, 1993. Involvement of superoxide in signal transduction; response to attack by pathogens, physical and chemical shocks and UV irradiation. In: C.H. Foyer & P.M. Mullineaux (eds.), Causes of Photo-Oxidative Stress and Amelioration of Defense System in Plants, CRC Press, Boca Raton, FL, pp 177-198.

Doyle, J.J., J.L. Doyle & L.H. Bailey Hortorim, 1990. Isolation of plant DNA from tissue. Focus, 12: 13-14.

Dragiiska, R., V. Redeva, P. Denchev, A. Atanassov & D. Djilianov, 1995. *In vitro* selection for drought tolerance in alfalfa. Proceedings of Interdrought, 30th August-2th September, Montpellier-France, Abstract VIII 37.

Dron, M., S.D. Clouse, R.A. Dixon, M.A. Lawton & C.J. Lamb, 1988. Glutathione and fungal elicitor regulation of a plant defense gene promoter in electroporated protoplasts. Proceedings of the National Academy of Science, USA, 85: 6738-6742.

Dubey, R.S. & M. Rani, 1989. Influence of NaCl salinity on growth and metabolic status of proteins and amino acids in rice seedlings. Journal of Agronomy Crop Science, 162: 97-106.

Dubey, R.S. & M. Rani, 1990. Influence of NaCl salinity on the behavior of protease, aminopeptidase and carboxy-peptidase in rice seedlings in relation to salt tolerance, Australian Journal of Plant Physiology, 17: 215-222.

Dubey, R.S., 1994. Protein synthesis by plants under stressful conditions. In: M. Pessarakli, (ed.), Handbook of Plant and Crop Stress, Marcel Dekker, Inc., New York, pp. 277-299.

Echt, C.S., K.K. Kidwell, S.J. Knapp, T.C. Osborn & T.J. McCoy, 1993. Linkage mapping in diploid alfalfa (*Medicago sativa*). Genome, 37: 61-71.

Epstein, E. & D.W. Rains, 1987. Advances in salt tolerance. Plant and Soil, 99: 17-29.

Epstein, E., 1985. Salt tolerant crops: origin, development and prospects of the concept. Plant and Soil, 89: 187-98.

Epstein, E., J.J. Norlyn, G.W. Rush, R.W. Kingsbury, D.W. Kelly, G.A. Cunningham & A.F. Wrona, 1980. Saline culture of crops: a genetic approach. Science, 210: 399-404.

Evans, D.A., 1989. Somaclonal variation genetic basis and breeding applications. Trends in Genetics, 5: 46-50.

Fischer, R.A. & N.C. Turner, 1978. Plant productivity in the arid and semi-arid zones. Annual Review of Plant Physiology, 29: 277-317.

Flowers, T.J., P.F. Troke, & A.R. Yeo, 1977. The mechanism of salt tolerance in halophytes. Annual Review of Plant Physiology, 28: 89-121.

Foroughi-Weher, B. & G. Wenzel, 1993. Andro- and parthenogenesis. In: M.D. Hayward, N.O. Bosemark & I. Romagosa (eds.), Plant Breeding: Principles and prospects, Chapman & Hall, London, pp. 261-277.

Fougère, F., D.L. Rudulier & J.G. Streeter, 1991. Effects of salt stress on amino acid, organic acid and carbohydrate composition of roots, bacteroids and cytosol of alfalfa (*Medicago sativa* L.). Plant Physiology, 96: 1228-1236.

Foyer, C.H. & J. Harbinson, 1993. Oxygen metabolism and the regulation of photosynthetic electron transport. In: C.H. Foyer & P. Mullineaux (eds.), Causes of Photooxidative Stresses and Amelioration of Defense in Plants. CRC Press, Boca Raton, FL, pp. 1-42.

Foyer, C.H., M. Lelandais, E.A. Edwards & P.M. Mullineaux, 1991a. The role of ascorbate in plants, interactions with photosynthesis and regulatory significance. In:

E. Pell & K. Steffen (eds.), Oxygen/Oxidative Stress and Plant Metabolism, American Society of Plant Physiologists, USA, pp. 131-144.

Foyer, C.H., P. Descourvieres & K.J. Kunert, 1994. Protection agaist oxygen radicals: an important defence mechanism studied in transgenic plants. Plant, Cell and Environment, 17: 507-523.

Freytag, A.H., J.A. Wrather & A.W. Erichsen, 1990. Salt-tolerant sugarbeet progeny from tissue cultures challenged with multiple salts. Plant Cell Reports, 9: 647-650.

Fridovich, I., 1976. Oxygen radicals, hydrogen peroxide and oxygen toxicity. Free Radicals in Biological Systems, 1: 239-277.

Fuentes, S.I., R. Suarez, T. Villegas, L.C. Acero & G. Hernandez, 1993. Embryogenic response of Mexican alfalfa (*M. sativa*) varieties. Plant Cell Tissue and Organ Culture, 34: 299-302

Galiba, G., L. Simon-Sarkadi, A. Salgo & G. Kocsy, 1989. Genotype dependent adaptation of wheat varieties to water stress *in vitro*. Journal of Plant Physiology, 134: 730-735.

Gartner, A. 1965. Ph.D. Thesis, Purdue University. In: K.H. Evans, R.L. Davis & J.M. Nyquist, 1966. Intraction of plant spacing and combining ability in an eight clone diallel of *Medicago sativa* L., Crop Science, 6: 451-454.

Good, A.G. & S.T. Zaplachinski, 1994. The effects of drought stress on free amino acid accumulation and protein synthesis in *Brassica napus*. Physiologia Plantarum, 90: 9-14.

Gorham J, R.G. Wyn-Jones & E. McDonnel, 1985. Some mechanisms of salt tolerance in crop plants. Plant and Soil, 89: 15-40.

Gosal, S.S. & Y.P.S. Bajaj, 1988. Pollen embryogenesis and chromosomal variation in anther of three food legumes- *Cicer arietinum*, *Pisum sativum* and *Vigna mungo*. SABRAO Journal, 20: 51-58.

Gossett, D.R., E.P. Millhollon, M.C. Lucas & M.M. Marney, 1993. Antioxidant enzymes and salt stress in cotton. Beltwide Research Conference of National Cotton Council, Memphis, TN, pp. 1262-1266.

Gossett, D.R., E.P. Millhollon, M.C. Lucas, S.W. Banks & M.-M. Marney, 1994. The effects of NaCl on antioxidant enzyme activities in callus tissue of salt-tolerant and salt-sensitive cotton cultivars (*Gossypium hirsutum* L.). Plant Cell Reports, 13: 498-503.

Greenway, H. & R. Munns, 1980. Mechanisms of salt tolerance in nonhalophytes. Annual Review of Plant Physiology, 31: 149-190.

Gregory, E.M. & I. Fridovich, 1973. The induction of superoxide dismutase by molecular oxygen. Journal of Bacteriology, 114: 543-548.

Groose, R.W. & E.T. Bingham, 1984. Variation in plants regenerated from tissue culture of tetraploid alfalfa heterozygous for several traits. Crop Science, 24: 655-658.

Groose, R.W. & E.T. Bingham, 1986. An unstable anthocyanin mutation recovered from tissue culture of alfalfa (*Medicago sativa*). 1. High frequency of reversion upon reculture. Plant Cell Reports, 5: 104-107.

Guha, S. & S.C. Maheshwari, 1964. *In vitro* production of embryo from anther in Datura. Nature, 204: 497-502.

Hall, M.H. 1993. Alfalfa growth following release from drought stress. Agronomy Journal, 85: 991-994.

Halliwell, B. & C.H. Foyer, 1978. Properties and physiological function of a glutathione reductase purified from spinach leaves by affinity chromatography. Planta, 139: 9-17.

Halliwell, B. & J.M.C. Gutteridge, 1985. The importance of free radicals and catalytic metal ions in human disease. Molecular Aspects of Medicine, 8: 89-193.

Halliwell, B., 1982. The toxic effects of oxygen on plant tissues. In: L.W. Oberley (ed.), Superoxide Dismutase. Vol.1, CRC Press, Boca Raton, FL, pp.89-124.

Handa, S., R.A. Bressan, A.K. Handa, N.C. Carpita & P.M. Hasegawa, 1983. Solutes contributing to osmotic adjusment in cultured plant cells adapted to water stress. Plant Physiology, 73: 834-843.

Hanks, R.J., T.E. Sullivan & V.E. Hunsaker, 1977. Corn and alfalfa production as influenced by irrigation and salinity. Soil Science Society of America. Journal, 41: 606-610.

Hanson, A.D. & W.D. Hitz, 1982. Metabolic responses of mesophytes to plant water deficits. Annual Review of Plant Physiology, 33: 163-203.

Hanson, A.D., B. Rathinasabapathi, J. Rivoal, M. Burnet, M.O. Dillon & D.A. Gage, 1994. Osmoprotective compounds in the *Plumbaginaceae*: A natural experiment in metabolic engineering of stress tolerance. Proceedings National Academy of Sciences USA, 91: 306-310.

Harms, C.T. & J.J. Oertli, 1985. The use of osmotically adapted cell cultures to study salt tolerance *in vitro*. Journal of Plant Physiology, 120: 29-38.

Harper, D.B. & B.M.R. Harvey, 1978. Mechanisms of paraquat tolerance in perennial ryegrass II. Role of superoxide dismutase, catalase, and peroxidase. Plant, Cell and Environment, 1: 211-215.

Hasegawa, P.M., R.A. Bressan & A.K. Handa, 1986. Cellular mechanisms of salinity tolerance. Horticulture Science, 21: 1317-1323.

Hasegawa, P.M., R.A. Bressan, D.E. Nelson, Y. Samaras & D. Rhodes, 1994. Tissue culture in the improvement of salt tolerance in plants. In: A.R. Yeo & T.J. Flowers (eds.), Soil Mineral Stresses, Approaches to Crop Improvement, Monographs on Theoretical and Applied Genetics, Vol. 21, Springer-Verlag Berlin, pp. 83-125.

Hassan, H.M. & I. Fridovich, 1979. Intracellular production of superoxide radical and hydrogen peroxide by redox active compounds. Archives of Biochemistry and Biophysics, 196: 385-395.

Hassan, N.S. & D.A. Wilkins, 1988. *In vitro* selection for salt tolerant lines in *Lycopersicon peruvianum*. Plant Cell Reports, 7: 463-466.

Hendriks, T. & S.C. De Vries, 1995. The role of secreted proteins in carrot somatic embryogenesis. In: M. Terzi, R. Cella & A. Falavigna (eds.), Current Issues in Plant Molecular and Cellular Biology, Kluwer Academic Publishers, Netherlands, pp. 359-368.

Henry, Y., P. Vain & J. De Buyser, 1994. Genetic analysis of *in vitro* plant tissue culture responses and regeneration capacities. Euphytica, 79: 45-58.

Hernandez, L.D. & E. Vierling, 1993. Expression of low molecular weight heat-shock proteins under field conditions. Plant Physiology, 101: 1209-1216.

Heszky, L.E., 1992. Fundamentals of plant biotechnology. University of Agricultural Sciences, Gödöllö-Hungary.

Heuer, B., 1994. Osmoregulatory role of proline in water- and salt- stressed plants. In: M. Pessarakli, (ed.), Handbook of Plant and Crop Stress, Marcel Dekker, Inc., New York, pp. 363-383.

Hewitt, E.J., 1966. Sand and water culture methods used in the study of plant nutrition. Commonwealth Agricultural Bureaux. Technical Communication, No. 22.

Heyser, J.W. & M.W. Nabors, 1981. Growth, water content and solute accumulation of two cell lines cultured on sodium chloride, dextran and polyethylene glycol. Plant Physiology, 68: 1454-1459.

Hoffmann, F., E. Thomas & G. Wenzel, 1982. Anther culture as a breeding tool in rape. II. Progeny analysis of androgenetic lines and of induced mutants from haploid cultures. Theoretical and Applied Genetics, 61: 225-232.

Holland, D., G. Ben-Hayyim, Z. Faltin, L. Camoin, A.D. Strosberg & Y. Eshdat, 1993. Molecular characterization of salt-stress-associated protein in citrus: protein and cDNA sequence homology to mammalian glutathione peroxidases. Plant Molecular Biology, 21: 923-927.

Hu, C.-A.A., A.J. Delauney & D.P.S. Verma, 1992. A bifunctional enzyme (Δ^1-pyrroline-5-carboxylate synthetase) catalyzes the first two steps in proline biosynthesis in plants. Proceeding of the National Academy of Sciences, USA, 89: 9354-9358.

Huang, B., 1990. Wheat anther culture: Effect of temperature. In: Y.P.S. Bajaj (ed.), Biotechnology in Agriculture and Forestry, Vol. 13, Wheat. Springer-Verlag Berlin, pp. 403-415.

Hurkman, W.J. & C.K. Tanaka, 1987. The effects of salt on the pattern of protein synthesis in barley roots. Plant Physiology, 83: 517-524.

Hurkman, W.J., C.S. Fornari & C.K. Tanaka, 1989. A comparison of the effect of salt on polypeptides and translatable mRNAs in roots of a salt-tolerant and salt-sensitive cultivar of barley. Plant Physiology, 90: 1444-1456.

Hurkman, W.J., H.P. Tao & C.K. Tanaka, 1991. Germin-like polypeptides increase in barley roots during salt stress. Plant Physiology, 97: 366-374.

Ibrahim, K.M., 1990. Prouction of variation in salt tolerance in ornamental plants. Ph.D. Thesis, University of Liverpool, Liverpool, U.K.

Irigoyen, J.J., D.W. Emerich, & M. Sanchez-Diaz, 1992. Water stress induced changes in concentration of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants. Physiologia Plantarum, 84: 55-60.

Jain, R.K., S. Jain, H.S. Nainawatee & J.B. Chowdhury, 1990. Salt tolerance in *Brassica juncea* L. I. *In vitro* selection, agronomic evaluation and genetic stability. Euphytica, 48: 141-152.

Jakowec, M.W., L.T. Smith & A.M. Dandekar, 1985. Recombinant plasmid conferring proline overproduction and osmotic tolerance. Applied and Environmental Microbiology 50: 441-446.

Jensen, C.R., 1982. Effect of soil water osmotic potential on growth and water relationship in barley during soil water depletion. Irrigation Science, 3: 111-121.

John, I., H. Wang, B.M. Held, E.S. Wurtele & J.T. Colbert, 1992. An mRNA that specifically accumulates in maize roots delineates a novel subset of developing cortical cells. Plant Molecular Biology, 20: 821-831.

Johnson, D.W. & S.E. Smith, 1992. Response to NaCl of alfalfa regenerated from non-saline callus cultures. Plant and Soil, 143: 311-315.

Johnson, D.W., S.E. Smith & A.K. Dobrenz, 1992. Genetic and phenotypic relationships in response to NaCl at different developmental stages in alfalfa. Theoretical and Applied Genetics, 83: 833-838.

Johnson, L.B., S.E. Schlarbaum & D.Z. Skinner, 1984. Variation in phenotype and chromosome number in alfalfa protoclones regenerated from nonmutagenized calli. Crop Science, 24: 948-952.

Jose-Estanyol, M., L. Ruiz-Avila & P. Puigdomenech, 1992. A maize embryo-specific gene encodes a proline-rich and hydrophobic protein. The Plant Cell, 4: 413-423.

Kapulnik, Y., L.R. Teuber & D.A. Phillips, 1989. Lucene (*Medicago sativa L.*) selected for vigor in a nonsaline environment maintainned growth under salt stress. Australian Journal of Agricultural Research, 40: 1253-1259.

Kavi-Kishor, P.B., 1988. Effect of salt stress on callus cultures of *Oryza sativa* L.. Journal of Experimental Botany, 39: 235-240.

Kavi-Kishor, P.B., 1989. Salt stress in cultured rice cells: effects of proline and abscisic acid. Plant, Cell and Environment, 12: 629-633.

Kawasaki, T., T. Akiba & M. Moritsugu, 1983. Effect of high concentration of NaCl and polyethylene glycol on growth and ion absorption in plants. Plant and Soil, 75: 75-85.

Kazzazian, H.H. Jr. 1989. Use of PCR in the diagnosis of monogenic disease. In: H.A. Erlich (ed.), PCR Technology. Stockton Press, New York, pp. 153-169.

Kendall, E.J. & B.D. McKersie, 1989. Free radical and freezing injury to cell membranes of winter wheat. Physiologia Plantarum, 76: 86-94.

Ketchum, R.E.B., R.S.Warren, L.J., Klima, F. Lopez-Gutierrez, & M.W. Nabors, 1991. The mechanism and regulation of proline accumulation in suspension cell cultures of the halophytic grass *Distichlis spicata* L., Journal of Plant Physiology, 137: 368-374

Kidwell & Osborn, 1993. Variation among alfalfa somaclones in copy number of repeated DNA sequences. Genome, 36: 906-912.

Kim, Y.H. & J. Janick, 1991. Abscisic acid and proline improve dessication tolerance and increase fatty acid content of celery somatic embryos. Plant Cell Tissue and Organ Culture, 24: 83-89

King, G.J., V.A. Turner, C.E. Hussey, E.S. Wurtele & S.M. Lee, 1988. Isolation and characterization of a tomato cDNA clone which codes for a salt induced protein. Plant Molecular Biology, 10: 401-412.

Kingsbury, R.W., E. Epstein & R.W. Pearcy, 1984. Physiological responses to salinity in selected lines of wheat. Plant Physiology, 74: 417-425.

Kurth, E. & G.R. Cramer, A. Läuchli & E. Epstein, 1986. Actual cytokinin concentrations in plant tissue as an indicator for salt resistance in cereals. Plant and Soil, 123: 243-250.

Landsmann, J. & H. Uhrig, 1985. Somaclonal variation in *Solanum tuberosum* detected at the molecular level. Theoretical and Applied Genetics, 71: 500-505.

Larkin, P.J. & W.R. Scowcroft, 1981. Somaclonal variation: a novel source of variability from cell cultures for plant improvement. Theoretical and Applied Genetics, 60: 197-214.

Larkin, P.J., S.A. Ryan, R.I.S. Brettell & W.R. Scowcroft, 1984. Heritable somaclonal variation in wheat. Theoretical and Applied Genetics, 78: 443-456.

Latunde-Dada, A.O. & J.A. Lucas, 1983. Somaclonal variation and reaction to *Verticillium* wilt in *Medicago sativa* L. plants regenerated from protoplasts. Plant Science Letters, 32: 205-211.

Lazar, M.D., T.H.H. Chen, G.J. Scoles & K.K. Kartha, 1987. Immature embryo and anther culture of chromosome addition lines of rye in Chinese Spring wheat. Plant Science, 51: 77-81.

Le Rudulier, D., S.S. Yang, & L.N. Csnoka, 1982. Nitrogen fixation in *Klebsiella pneumoniae* during osmotic stress: effect of exogenous proline or a proline overproducing plasmid. Biochimica Et Biophysica Acta, 719: 273-283.

Lesko, S., A. Lorentarn & R. Tso, 1980. Involvement of polycyclic aromatic hydrocarbons and reduced oxygen radicals in carcinogenesis. Biochemistry, 19: 3023-3028.

Levitt, J., 1980. Water, radiation, salt and other stresses. In: Responses of plants to environmental stresses. Physiological Ecology, Vol. 2. Academic Press, New York.

Lindsey, K. & M.G.K. Jones, 1989. Consequences of tissue culture variability and instability; In: Plant Biotechnoloy in Agriculture, Open University Press, Milton Keynes, pp. 78-93.

Maas, E.V. & G.J. Hoffman, 1977. Crop salt tolerance current assessment. Journal, Irrigaion and Drainage Division. Proceedings of the American Society of Civil Engineers, 103: 115-122.

Maas, E.V. 1986. Salt tolerance of plants. Applied Agricultural Research, 1: 12-26.

Maas, E.V., 1987. Salt tolerance of plants. In: C.B. Johnson, (ed.), Environmental factors limiting plant productivity. Butterworths, London, pp. 271-290.

Maiti, R.K., M. de La Rosa-Ibarra & N.D. Sandoval, 1994. Genotypic variability in glossy sorghum lines for resistance to drought, salinity and temperature stress at the seedling stage. Journal of Plant Physiology, 143: 241-244.

Malan, C., M. Greyling & J. Gressel, 1990. Correlation between CuZn superoxide dismutase and glutathione reductase and environmental and xenobiotic stress tolerance in maize inbreds. Plant Science, 69: 157-166.

Massoud, F.I., 1974. Salinity and alkalinity as soil degredation hazards, FAO/UNDP Expert Consultation on Soil Degradation, FAO, Rome, 21.

Matheson, S.L., J. Nowak & N.L. Maclean (1990) Selection of regenerative genotypes from highly productive cultivars of alfalfa. Euphytica, 45: 105-112.

Mathias, R.J. & E.S. Simpson, 1986c. The interaction of genotype and culture medium on the tissue culture responses of wheat (*Triticum aestivum* L. em. Thell) callus. Plant Cell, Tissue and Organ Culture, 7: 31-37.

Matsuda, K. & A. Riazi, 1981. Stress induced osmotic adjustment in growing regions of barley leaves. Plant Physiology, 68: 571-576.

Maxwell, S.E. & H.D. Delaney, 1990. Designing experiments and analyzing data, A model comparison perspective, Wadsworth Publishing Company, Belmont, California, p. 893.

McCoy, T.J., 1987a. Tissue culture evaluation of NaCl tolerance in *Medicago* species: cellular versus whole plant response. Plant Cell Reports, 6: 31-34.

McCoy, T.J., 1987b. Characterization of alfalfa (*Medicago sativa* L.) plants regenerated from NaCl-tolerant cell lines. Plant Cell Reports, 6: 417-422.

McCoy, T.J., R.L. Phillips & H.W. Rines, 1982. Cytogenetic analysis of plants regenerated from oat (*Avena sativa*) tissue cultures: high frequency of partial chromosome loss. Canadian Journal of Genetics and Cytology, 24: 37-50

McCue, K.F. & A.D. Hanson, 1990. Drought and salt tolerance: towards understanding and application. Trends of Biotechnology, 8: 358-362.

McHughen, A. 1987. Salt tolerance through increased vigour in flax line (STS-11) selected for salt tolerance *in vitro*. Theoretical and Applied Genetics, 74: 727-32.

McNeilly, T. 1990. Selection and breeding for salinity tolerance in crop species: A case for optimism? Acta Ecologica, 11: 595-610.

Melchinger, A.E., M.M. Messmer, M. Lee, W.L. Woodman & K.R. Lamkey, 1991. Diversity and relationships among U.S. maize inbred revealed by restriction fragment length polymorphisms. Crop Science, 31: 669-678.

Michaud, R., W.F. Lehman & M.D. Rumbaugh, 1988. World distribution and historical development. In: Alfalfa and Alfalfa Improvement-Agronomy Monograph, no. 29, ASA-CSSA-SSSA, USA, pp. 25-91.

Miles, D., 1991. Increasing salt tolerance of plants through cell culture requires greater understanding of tolerance mechanism. Australian Journal of Plant Physioloy, 18: 1-15.

Ming, G., D. Nian-Cheng, H. Zi-Yi & L. You-Liang, 1989. Correlation between lipid peroxidation damage and ultrastructural changes of mesophyll cells in barley and wheat seedlings during salt stress. Acta Botanica Science, 31: 841-846.

Misra, H.P. & I. Fridovich, 1972. The role of superoxide anion in the autooxidation of epinephrine and a simple assay for superoxide dismutase. Journal of Biological Chemistry, 247: 3170-3175.

Mittal, R. & R.S. Dubey, 1991. Behaviour of peroxidases in rice: changes in enzyme activity and isoforms in relation to tolerance. Plant Physiology and Biochemistry, 29: 31-40.

Mitten, D.H., S.J. Sato & T.A. Skokut, 1983. *In vitro* regenerative potential of alfalfa germplasm sources. Crop Science, 24: 943-945.

Mitten, D.H., S.J. Sato & T.A. Skokut, 1984. *In vitro* regeneration potential of alfalfa germplasm sources. Crop Science, 24: 943-945.

Moftah, A.E. & B.E. Michel, 1987. The effect of sodium chloride on solute potential and proline accumulation in soybean leaves. Plant Physiology, 83: 238-240.

Mokhtarzadeh, A. & M.J. Constantin, 1978. Plant regeneration from hypocotyl- and anther-derived callus of berseem clover. Crop Science, 18: 567-572.

Monk, L.S. & H.V. Davies, 1989. Antioxidant status of the potato tuber and Ca^{2+} deficiency as a physiological stress. Physiologia Plantarum, 75: 411-416.

Moons, A., G. Bauw, E. Prinsen, M.V. Montagu, & D.V. Der Straeten, 1995. Molecular and physiological response to abscisic acid and salts in roots of salt-sensitive and salt-tolerant Indica rice varieties. Plant Physiology, 107: 177-186.

Mordhorst, A.P., S. Stirn, T. Dresselhaus & H. Lörz, 1995. Controlling factors and markers for embrogenic potential and regeneration capacity in barley (*Hordeum vulgare* L.) cell cultures. In: M. Terzi, R. Cella & A. Falavigna (eds.), Current Issues

in Plant Molecular and Cellular Biology, Kluwer Academic Publishers, Netherlands, pp. 383-388.

Morgan, J.M. 1984. Osmoregulation and water stress in higher plants, Annual Review of Plant Physiology, 35: 299-319.

Mullis, K.B. & F. Faloona, 1987. In: Methods in enzymology, Vol. 155. Academic Press, New York, pp. 335-362.

Munns, R. & A. Termaat, 1986. Whole-plant responses to salinity. Australian Journal of Plant Physiology, 13: 143-160.

Munns, R. & B. Weir, 1981. Contribution of sugars to osmotic adjustment in elongation and expanded zones of wheat leaves during moderate water deficits at two light levels. Australian Journal of Plant Physiology, 8: 93-105.

Murashige, T. & F. Skoog, 1962. A revised medium for rapid growth and bio-assay with tobacco tissue cultures. Plant Physiology, 15: 473-573.

Myers, R.M., V.C. Sheffield & D.R. Cox, 1989. In: H.A. Erlich (ed.), PCR Technology. Stockton Press, New York, pp. 71-79.

Nabors, M.W. & T.A. Dykes, 1985. Tissue culture of cereal cultivars with increased salt, drought, and acid tolerance. In Biotechnology in International Agricultural Research. International Rice Research Institute, Manila, Philippines. ISBN 97-1104-124-3, pp. 121-138.

Nabors, M.W. 1990. Environmental stress resistance. In: P.J. Dix (ed.), Plant Cell Line Selection, Procedures and Applications, VCH Publishers, Weinheim, pp. 167-186.

Nabors, M.W., A. Daniels, L. Nadolny & C. Brown, 1975. Sodium chloride-tolerant lines of tobacco cells. Plant Science Letters, 4: 155-159.

Nabors, M.W., S.F. Gibbs, C.S. Berstein & M.E. Meis, 1980. NaCl tolerant tobacco plants from cultured cells. Zeitschrift fuer Pflanzenphysiologie, 97: 13-17.

Naidu, B.P., L.G. Paleg & G.P. Jones, 1992. Nitrogenous compatible solutes in drought-stressed *Medicago spp*. Phytochemistry, 31: 1195-1197.

Nakano, Y. & K. Asada, 1981. Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. Plant and Cell Physiology, 22: 867-880.

Nam, L.S. & L.E. Heszky, 1986. Testing of salt /NaCl/ tolerance and regeneration in callus culture /n, 2n/ of rice. In: Walter de Gruyter & Co, Berlin. New York-Printed in Germany. pp. 617-619.

Nash, D., L.G. Paleg & J.T. Wiskich, 1982. Effect of proline, betaine and some other solutes on the heat stability of mitochondrial enzymes. Australian Journal of Plant Physiology, 9: 47-57.

Neale, A.D., J.A. Wahleithener, M. Lund, H.T. Bonnett, A. Kelly, D.R. Meeks-Wagner, W.J. Peacock & E.S. Dennis, 1990. Chitinase, ß-1,3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. The Plant Cell, 2: 673-684.

Nestiky, M., F.J. Novak, A. Piovarci & M. Dolezelova, 1983. Genetic analysis of callus growth of maize (*Zea mays* L.) *in vitro*. Zeitschrift fuer Pflanzenphysiologie, 91: 322-328.

Neumann, P.M. 1995a. The role of cell wall adjustment in plant resistance to water deficits (Review and Interpretation Article). Crop Science, 35: 1258-1266.

Neumann, P.M. 1995c. Selection for drought resistance in crop species: Physiological perspectives. Proceedings of Interdrought, 30th August-2th September, Montpellier-France, X-A.

Nielsen, D.C. & A.D. Halvorson, 1991. Nitrogen fertility influence on water stress and yield of winter wheat. Agronomy Journal, 83: 1065-1070.

Niu, X., M.L. Narasimhan, R.A. Salzman & P.M. Hasegawa, 1993. NaCl regulation of plasma membrane H^+-ATPase gene expression in a glycophyte and a halophyte. Plant Physiology, 103: 713-718.

Noble, C.L., G.M. Halloran & D.W. West, 1984. Identification and selection for salt tolerance in lucerne (*Medicago sativa* L.) Australian Journal of Agricultural Research, 35: 239-252.

Ockendon, D.J. & L. Currah, 1971. Self pollen reduces the number of cross pollen tubes in the styles of *Brassica oleracea* L. New Phytologist, 78: 675-680.

Olmos, E., J.A. Hernández, F. Sevilla & E. Hellin, 1994. Induction of several antioxidant enzymes in the selection of a salt-tolerant cell line of *Pisum sativum*. Journal of Plant Physiology, 144: 594-598.

Ouyang, J.W., 1986. Induction of pollen plants in *Triticum aestivum*. In: H. Hu & H. Yang (eds.), Haploids of higher plants *in vitro*. Springer-Verlag Berlin, pp. 26-41.

Paleg, L.G., G.R. Stewart & J.W. Bradbeer, 1984. Proline and glycine betaine influence protein solvation. Plant Physiology, 75: 974-978.

Panaccio, M. & A. Lew, 1991. PCR based diagnosis in the presence of 8% (v/v) blood. Nucleic Acids Research, 19: 1151.

Pasternak, D. & Y. de Malach, 1987. Saline water irrigation in the Negev Desert. In: Conference on Agriculture and Food Production in the Middle East, Athens, Greece. Beer Sheva: Publication Section, Ben-Gurion University of the Negev.

Pèrez-Alfocea, F., A. Santa-Cruz, G. Guerrier & M.C. Bolarin, 1994. NaCl Stress-induced organic solute changes on leaves and calli of *Lycopersicon esculentum*, *L. pennellii* and their interspecific hybrid. Journal of Plant Physiology, 143: 106-111.

Perry, L.J. & K.L. Larson. 1974. Influence of drought on tillering and internode number and length in alfalfa. Crop Science, 14: 693-696.

Piccirilli, M., G.J. Tanner & S. Arcioni, 1989. Polynucleate pollen formation in cultured *Medicago sativa* L. anthers. XVI International Grassland Congress, Nice, France, pp. 427-428.

Poljakoff-Mayber, A., 1982. Biochemical and physiological responses of higher plants to salinity stress. In A San Pietro (ed.), Biosaline Research: A look to the future. Plenum Press, New York, pp 245-269.

Polle, A. & H. Rennenberg, 1993. Significance of antioxidants in plant adaptation to environmental stress. In: L. Fowdwn, T. Mansfield & J. Stoddart (eds.), Plant Adaptation to Environmental Stress. ChapMan & Hall, London, pp. 263-275.

Polle, A., K. Chakrabarti, S. Chakrabarti, F. Seifert, P. Schramel & H. Rennenberg, 1992. Antioxidants and manganese deficiency in needles of norway spruce (*Picea abies* L.) trees. Plant Physiology, 99: 1084-1089.

Polle, A., K. Chakrabarti, W. Schürmann & H. Rennenberg, 1990. Composition and properties of hydrogen decomposing systems in extracellular and total extracts from needles of Norway spruce (*Picea abies* L.). Plant Physiology, 94: 312-319.

Prat, D. & R.A. Fathi-Ettai, 1990. Variation inorganic and mineral components in young *Eucalyptus* seedlings under saline stress. Physiologia Plantarum, 79: 479-486.

Price, A.H. & G.A.F. Hendry, 1991. Iron-catylized oxygen radical formation and its possible contribution to drought damage in nine native grasses and three cereals. Plant, Cell and Environment, 14: 477-484.

Quesenberry, K.H, & R.R. Smith, 1993. Recurrent selection for plant regeneration from red clover tissue culture. Crop Science, 33: 585-589.

Quiros, C.F. & G.R. Bauchan, 1988. The genus *Medicago* and the origin of the *Medicago sativa* complex. In: Alfalfa and Alfalfa Improvement-Agronomy Monograph, no. 29, ASA-CSSA-SSSA, USA, pp. 93-124.

Refoufi, A. & F. Larher, 1989. Compatible organic solutes and salt tolerance in seedlings of three annual *Medicago* species, Comptes Rendus. Academie Sciences (Paris), 308: 329.

Rhodes, D., S. Monda, & R.A. Bressan, 1986. Metabolic changes associated with adaptation of plant cells to water stress. Plant Physiology, 82: 890-903.

Robinson, J., M. Smith & M. Gibbs, 1980. Influence of hydrogen peroxide upon carbon dioxide photoassimilation in the spinach chloroplast. Hydrogen peroxide generated by broken chloroplasts in intact chloroplast preparation is a causal agent of the warburg effect. Plant Physiology, 65: 755-759.

Rumbaugh, M.D. & B.M. Pendery, 1990. Germination salt resistance of alfalfa (*Medicago sativa* L.) germplasm in relation to subspecies and centers of diversity. Plant and Soil, 124: 47-51.

Rumbaugh, M.D. & D.A. Johnson, 1980. Screening alfalfa germplasm for seedling drought resistance. Crop Science, 21: 709-713.

Rumbaugh, M.D., J.L. Caddel & D.E. Rowe, 1988. Breeding and quantitative genetics. In: Alfalfa and Alfalfa Improvement-Agronomy Monograph, no. 29, ASA-CSSA-SSSA, USA, pp. 777-808.

Safarnejad, A., 1992. Production of drought and salt resistant monocotyledonous genotypes by *in vitro* and *in vivo* methods. M.Sc. Thesis, University of Agricultural Sciences, Gödöllò, Hungary.

Safarnejad, A., H.A. Collin, K.D. Bruce & T. McNeilly, 1996. Characterization of alfalfa (*Medicago sativa* L.) following *in vitro* selection for salt tolerance. Euphytica, In press.

Safarnejad, A., H.A. Collin, T. McNeilly & W.J. Rogers, 1994a. Regeneration of Iranian alfalfa varieties from tissue culture. First Conference of Iranian Researchers on Agriculture and Hydrolics Sciences, Brussles (Belgium), March 11-13. In press.

Saiki, R.K., S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich & N. Arnheim, 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis of sickle cell anemia, Science, 230: 1350-1354.

Salin, M. 1988. Toxic oxygen species and protective systems of the chloroplast. Physiologia Plantarum, 72: 681-689.

Salts, Y., D. Kenigsbuch, R. Wachs, W. Gruissem & R. Barg, 1992. DNA sequence of the tomato fruit expressed proline-rich protein gene TPRP-F1 reveals an intron within the 3' untranslated transcript. Plant Molecular Biology, 18: 407-409.

Salts, Y., R. Wachs, W. Gruissem & R. Barg, 1991. Sequence coding for a novel proline-rich protein preferentially expressed in young tomato fruit. Plant Molecular Biology, 17: 149-150.

Santos-Diaz, M.S. & N. Ochoa-Alejo, 1994. Effect of water stress on growth, osmotic potential and solute accumulation in cell cultures from chilli pepper (a mesophyte) and creosote bush (a xerophyte). Plant Science, 96: 21-29.

SAS Institute. 1985. SAS user's guide: Statistics. SAS Inst., Cary, NC.

Saunders, J.W. & E.T. Bingham, 1972. Production of alfalfa plants from callus tissue. Crop Science, 12: 804-808.

Saunders, J.W. & E.T. Bingham, 1975. Growth regulator effects on bud initiation in callus cultures of *Medicago sativa*. American Journal of Botany, 62: 850-855.

Sayers, E.R. & R.P. Murphy, 1966. Seed set in alfalfa as related to pollen tube growth fertilization frequency, and post fertilization ovule abortion. Crop Science, 6: 365-368.

Scandalios, J.G. 1990. Response of plant antioxidant defense genes to environmental stress. In: J.G. Scandalios (ed.), Genomic Responses to Environmental Stress, Academic Press, Inc., San Diego, pp. 2-42.

Schenk, R.U. & A.C. Hildebrandt, 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Canadian Journal of Botany, 50:199-204.

Schnell, R.J., E.A. Wernsman & L.G. Burk, 1980. Efficiency of single-seed-descent vs. anther-derived dihaploid breeding methods in tobacco. Crop Science, 20: 619-622.

Schubert, S., R. Serraj, E. Plies-Balzer & K. Mengel, 1995. Effect of drought stress on growth, sugar concentrations and amino acid accumulation in N_2-fixing alfalfa (*Medicago sativa*). Journal of Plant Physiology, 146: 541-546.

Serrano, R. & R. Gaxiola, 1994. Microbial models and salt stress tolerance in plants. Critical Reviews in Plant Sciences, 13: 121-138.

Shaaltiel, Y. & J. Gressel, 1986. Kinetic analysis of resistance to paraquat in *Conyza*: evidence that paraquat transiently inhibits leaf chloroplast reactions in resistant plants. Plant Physiology, 85: 869-871.

Shaaltiel, Y., A. Glazer, P.F. Bocion & J. Gressel, 1988. Cross tolerance to herbicidal and environmental oxidants of plant biotypes tolerant to paraquat, sulfur dioxide, and ozone. Pesticide Biochemistry and Physiology, 31: 13-23.

Shah, S.H., S.J. Wainwright & M.J. Merrett, 1990. The interaction of sodium and calcium chlorides and light on growth, potassium nutrition and proline accumulation in callus cultures of *Medicago sativa*. New Phytologist, 116: 37-45.

Shalhevet, J. & L. Bernstein, 1968. Effects of vertically hetrogeneous soil salinity on plant growth and water uptake. Soil Science, 106: 85-93

Shalhevet, J., 1993. Plant under salt and water stress. In: L. Fowden; T. Mansfield & J. Stoddart (eds.), Plant Adaptation to Environmental Stress. Chapman and Hall, London, pp. 133-154.

Shannon, M.C. & C.L. Noble, 1990. Genetic approaches for developing economic salt-tolerant crops. In: K.K. Tanji (ed.), Agricultural Salinity Assessment and Management, ASCE Manuals and Reports on Engineering Practice No. 71, American Society of Civil Engineers, New York, pp. 161-185.

Shannon, M.C., 1984. Breeding, selection and genetics of salt tolerance. In: R.C. Staple & G.H. Toenniesson (eds.), Salinity Tolerance in Plants, Wiley, New York, pp. 231-254.

Sharp, R.E., W.K. Silk & T.C. Hsiao, 1988. Growth of the maize primary root at low water potentials. I. Spatial distribution of expansive growth. Plant Physiology, 87: 50-57.

Sigurbjornsson, B. & M. Malusznski, 1995. International programmes of FAO and IAEA in mutation breeding and *in vitro* technology. In M. Terzi, R. Cella & A. Falavigna (eds.), Current Issues in Plant Molecular and Cellular Biology, Kluwer Academic Publishers. Netherlands, pp. 1-3.

Singh, N.K., A.K. Handa, P.M. Hasegawa, & R.A. Bressan, 1985. Proteins associated with adaptation of cultured tobacco cells to NaCl. Plant Physiology, 79: 126-137.

Singh, N.K., D. Nelson, D. Kuhn, P.M. Hasegawa & R.A. Bressan, 1989. Molecular cloning of osmotin and regulation of its expression by ABA and adaptation to low water potential. Plant Physiology, 90: 1096-1101.

Singh, T.N., L.G. Paleg & D. Aspinall, 1973. Stress metabolism. Variations in response to water deficit in the barley plant. Australian Journal of Biological Science, 26: 65-76.

Singha, S. & M.A. Choudhuri, 1990. Effect of salinity (NaCl) stress on H_2O_2 metabolism in *Vigna* and *Oryza* seedlings. Biochemie und Physiologie der Pflanzen, 186: 69-74.

Skotnicki, M.L., 1986. Rapid regeneration of alfalfa plants from tissue culture. Cytobios, 46: 189-192.

Smith, M.K. & J.A. McComb, 1981. Effect of NaCl on the growth of whole plants and their corresponding callus cultures. Australian Journal of Plant Physiology, 8: 267-275.

Smith, M.K. & J.A. McComb, 1981. Use of callus cultures to detect NaCl tolerance in cultivars of three species of pasture legumes. Australian Journal of Plant Physiology, 8: 437-442.

Smith, M.K. & J.A. McComb, 1983. Selection for NaCl tolerance in cell culture of *Medicago sativa* and recovery of plants from a NaCl-tolerant cell line. Plant Cell Reports, 2: 126-128.

Smith, S.E., 1994. Salinity and the production of alfalfa (*Medicago sativa L.*). In: M. Pessarakli, (ed) Handbook of Plant and Crop Stress. Marcel Dekker, Inc., New York, pp. 431-448.

Stavarek, S.J. & D.W. Rains, 1984. Cell culture techniques: selection and physiological studies of salt tolerance. In: R.C. Staples & G.H. Toenniessen (eds.), Salinity Tolerance in Plants. Strategies for Crop Improvement. Wiley, New York, pp. 321-334.

Stavarek, S.J., T.P. Croughan & D.W. Rains, 1980. Regeneration of plants from long-term cultures of alfalfa cells. Plant Science Letters, 19: 253-261.

Subbarao, G.V. & C. Johansen, 1994. Strategies and scope for improving salinity tolerance in crop plants. In: M. Pessarakli, (ed.), Handbook of Plant and Crop Stress. Marcel Dekker, Inc., New York, pp. 559-579.

Subramaniam, K., J. Ranie, B.R. Srinivasa, A.M. Sinha & S. Mahadevan, 1994. Cloning and sequence of a cDNA encoding a novel hybrid proline-rich protein associated with cytokinin-induced haustoria formation in *Cuscuta reflexa*. Gene, 141: 207-210.

Sumaryati, S., I. Negrutiu & M. Jacobs, 1992. Characterization and regeneration of salt- and water-stress mutants from protoplast culture of *Nicotiana plumbaginifolia* (Viviani). Theoretical and Applied Genetics, 83: 613-619.

Sunderland, N., 1971. Anther culture: a progress report. Science Progress, London, 59: 527-549.

Takashima, S., H. Hasegawa & A. Nakamura, 1995. A simple method for chromosome doubling in tobacco anther culture - direct application of colchicine to anthers before culture. Breeding Science, 45: 107-110.

Tanksley, S.D. & T.J. Orton, 1983. Isozymes in Plant Genetics and Breeding. Elsevier, Amsterdam, pp. 253-294.

Tanksley, S.D., N.D. Young , A.H. Patterson & M.W. Bonierbale, 1989. RFLP mapping in palnt breeding: new tools for an old science. Biotechnology, 7: 257-264.

Tanner, G.J., M. Piccirilli, A.E. Moore, P.J. Larkin & S. Arcioni, 1990. Initiation of non-physiological division and manipulation of developmental pathway in cultured microspores of *Medicago* sp. Protoplasma, 158: 165-175.

Tautz, D., 1989. Hypervariability of simple sequences as a general source for polymorphic DNA markers. Nucleic Acids Research, 17: 6463-6471.

Taylor, P.W.J., T.A. Fraser, H.L. Ko & R.J. Henry, 1995. RAPD analysis of sugarcane during tissue culture. In: M. Terzi, R. Cella & A. Falavigna (eds.), Current Issues in Plant Molecular and Cellular Biology, Kluwer Academic Publishers, Netherlands, pp. 241-246.

Taylor, T.E. & R.E. Veilleux, 1992. Inheritance of competences for leaf disc regeneration, anther culture and protoplast culture in *Solanum phureja* and correlation among them. Plant Cell Tissue and Organ Culture, 31: 95-103.

Theiler-Hedtrich, R. & C.S. Hunter, 1995. Dihaploid chicory (*Cichorium intybus* L.) via microspore culture. In: M. Terzi, R. Cella & A. Falavigna (eds.), Current Issues in Plant Molecular and Cellular Biology, Kluwer Academic Publishers, Netherlands, pp. 149-154.

Thieme, R., H. Grieß & H. Tiemann, 1995. Use of somaclonal variation for the production of adapted cultivars of potato. Abstract, In: Adaptation in Plant Breeding XIV EUCARPIA Congress, University of Jyväskylä, Jyväskylä, pp. 85-86.

Tomes, D.T. & R.L. Peterson, 1981. Isolation of a dwarf plant responsive to exogenous GA3 from anther cultures of birdsfoot trefoil. Canadian Journal of Botany, 59: 1338-1342.

Vajrabhaya, M., T. Thanapaisal & T. Vajrabhaya, 1989. Development of salt-tolerant lines KDML and LPT rice cultivars through tissue culture. Plant Cell Reports, 8: 411-414.

Venekamp, J.H., 1989. Regulation of cytosol acidity in plants under conditions of drought. Physiologia Plantarum, 76: 112-117.

Vilardell, J., A. Goday, M.A. Freire, M. Torrent, M.C. Martinez, J.M. Torne & M. Pagès, 1990. Gene sequence, developmental expression, and protein phosphorylation of RAB-17 in maize. Plant Molecular Biology, 14: 423-432.

Voetberg, G.S. & R.E. Sharp, 1991. Growth of maize primary root at low water potentials. III. Role of increased proline deposition in osmotic adjustment. Plant physiology, 96: 125-130.

von Arnold, S., U. Egertsdotter & L.H. Mo, 1995. Importance of extracellular proteins for somatic embryogenesis *Picea abies*. In: M. Terzi, R. Cella & A. Falavigna (eds.), Current Issues in Plant Molecular and Cellular Biology, Kluwer Academic Publishers, Netherlands, pp. 389-392.

Walker, K.A., M.L. Wendeln & E.G. Jaworski, 1979. Organogenesis in callus tissue of *Medicago sativa*. The temporal separation of induction processes from differentiation processes. Plant Science Letters, 16: 23-30

Wan, Y, E.L. Sorensen & G.H. Liang, 1988. Genetic control of *in vitro* regeneration in alfalfa (*Medicago sativa* L.). Euphytica, 39: 3-9.

Waskom, R.M., D.R. Miller, G.E. Hanning, R.R. Duncan, R.L. Voigt & M.W. Nabors, 1990. Field evaluation of tissue culture derived sorghum for increased tolerance to acid soils and drought stress. Canadian Journal of Plant Science, 70: 997-1004.

Watad, A.A., D. Swartzberg, R.A. Bressan, S. Izhar & P.M. Hasegawa, 1991. Stability of salt tolerance at the cell level after regeneration of plants from a salt tolerant tobacco cell line. Physiologia Plantarum, 83: 307-313.

Wenzel, G. & B. Foroughi-Wehr, 1993. *In vitro* selection. In: M.D. Hayward, N.O. Bosemark & I. Romagosa (eds.), Plant Breeding: Principles and Prospects. Chapman & Hall, London, pp. 353-370.

Wenzel, G., U. Frei, A. Jahoor & A. Graner, 1995. Haploids an integral part of applied and basic research. In: M. Terzi, R. Cella & A. Falavigna (eds.), Current Issues in Plant Molecular and Cellular Biology, Kluwer Academic Publishers. Netherlands, pp. 127-135.

Williams, J.G., A.R. Kubelik, K.J. Livak, J.A. Rafalski & S.V. Tingey, 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research, 18: 6531-6535.

Williamson, C.L. & R.D. Slocum, 1992. Molecular cloning and evidence for osmoregulation of the Δ^1-pyrroline-5-carboxylate reductase (*proC*) gene in pea (*Pisum sativum* L.). Plant Physiology, 100: 1464-1470.

Wingate, V.P.M., M.A. Lawton & C.J. Lamb, 1988. Glutathione elicits a massive and selective induction of plant defense genes. Plant Physiology, 87: 206-210.

Winicov, I. & C.E. Deutch, 1994. Characterization of cDNA clone from salt-tolerant alfalfa cells that identifies salt-inducible root-specific transcripts. Journal of Plant Physiology, 144: 222-228.

Winicov, I. & J.D. Button, 1991. Accumulation of photosynthesis gene transcripts in response to sodium chloride by salt-tolerant alfalfa cells. Planta, 183: 478-483.

Winicov, I. & J.R. Seemann, 1990. Expression of genes for photosynthesis and the relationship to salt tolerance of alfalfa (*Medicago sativa*) cells. Plant and Cell Physiology, 31: 1155-1161.

Winicov, I. 1991. Characterization of salt tolerant alfalfa (*Medicago sativa* L.) plants regenerated from salt tolerant cell lines. Plant Cell Reports, 10: 561-564.

Winicov, I., 1990. Gene expression in salt tolerance alfalfa cell cultures and the salt tolerant plant regenerated from these cultures. In: H.J.J. Nijkamp, L.H.W. Van Der Plas & J. Van Aartrijk (eds.), Progress in Plant Cellular and Molecular Biology. Kluwer Academic Publishers, Dordrecht, pp. 142-147.

Winicov, I., 1993. A cDNA encoding putative zinc finger motifs from salt-tolerant alfalfa (*Medicago sativa* L.) cells. Plant Physiology, 102: 681-682.

Winicov, I., 1994. Gene expression in relation to salt tolerance. In: A.S. Basra (ed.), Stress-Induced Gene Expression in Plants, Harwood Academic Publishers, London, pp. 61-85.

Winicov, I., J.H. Waterborg, R. E. Harrington & T.J. McCoy, 1989. Messenger RNA induction in cellular salt tolerance of alfalfa (*Medicago sativa*). Plant Cell Reports, 8: 6-12.

Wyn Jones, R.G. & J. Gorham, 1983. Aspects of salt and drought tolerance in higher plants. In: R.C. Staples & G.H. Toenniessen, (eds.), Salinity Tolerance in Plants. Strategies for Crop Improvement. Wiley, New York, pp 189-294.

Yang, H.Y., H. Yan & C. Zhou, 1990. *In vitro* production of haploids in helianthus. In: Y.P.S. Bajaj (ed.), Biotechnology in Agriculture and Forestry; Legumes and Oilseed Crops I. Vol. 10, Springer-Verlag Berlin, pp. 472-484.

Yeo, A.R. & T.J. Flowers, 1982. Accumulation and localization of sodium ions within the shoots of rice varieties differing in salinity resistance. Physiologia Plantarum, 56: 343-348.

Yeo, A.R., 1994. Physiological criteria in screening and breeding. In: A.R. Yeo & T.J. Flowers (eds.), Soil Mineral Stresses, approaches to crop improvement. Monographs on Theoretical and Applied Genetics, Vol. 21, Springer-Verlag Berlin, pp. 37-59.

Yu, K. & K.P. Pauls, 1993. Rapid estimation of genetic relatedness among heterogeneous populations of alfalfa by random amplification of bulked genomic DNA samples. Theoretical and Applied Genetics, 86: 788-794.

Zagorska, N. & B. Dimitrov, 1995. Induced androgenesis in alfalfa (*Medicago sativa* L.). Plant Cell Reports, 14: 249-252.

Zagorska, N., P. Robeva, B. Dimitrov, R. Shtereva & V. Gancheva, 1984. Induction of regeneration in anther cultures in *Medicago sativa* L., Comptes Rendus Academie Bulgare des Sciences, 37: 1099-1102.

Zekri, M. & L.R. Parsons, 1990. Comparative effects of NaCl and polyethylene glycol on root distribution, growth and stomatal conductance of sour orange seedlings. Plant and Soil, 129: 137-143.

Zivy, M., J.L. Prioul, G. Cornic, P. Westhoff, B. Lacroix & D. de Vienne, 1995. Characterizing proteins induced by drought in maize and search for their QTLs. Proceedings of 1st International Meeting of Interdrought, 31th August- 2nd Septmbre, Montpellier-France, XIC.

APPENDICES

APPENDICES

Appendix 1.1 Approach to a classification of plant responses to water stress. Some traditional approaches are also shown (Galiba, G., Pers. Comm.).

little or no water stress		moderate water stress		extreme water stress
MECHANISM: drought escapers	water spenders (deep tap root)	water collectors (succulents, have CAM photosynthesis, sallow root system, salt secretion, dew absorption)	water saver (many adaptations to retain water, small leaves, sunken stomates, pubescence, deciduous leaves, etc.; increase solutes, osmoregulation)	dehydration tolerance (incompletely understood protoplasmic properties)
EXAMPLES: desert, annuals	mesquite alfalfa, palms	cacti, many others	many xerophytes including those mentioned in other groups have these characteristics	euxerophytes: seeds, certain mosses, lichens, creoste bush

OTHER TERMINOLOGIES AND CLASSIFICATIONS:

Levitt: resistance	resistance	resistance	resistance	tolerance
Schanz: escape	avoid	resist	avoid	endure
Daubenmire: annuals	nonsucculent perennials	succulents	non succulent perennials	non succulent perennials
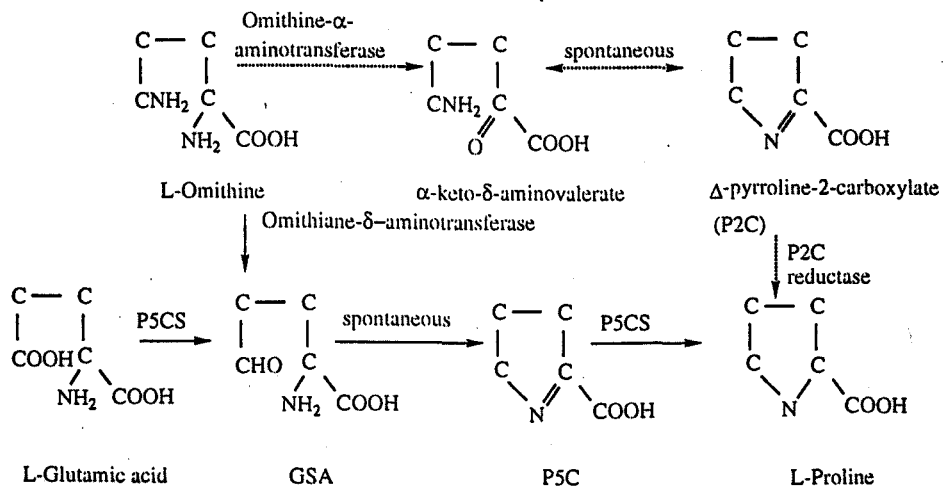
Appendix 3.1 Pathways of proline biosynthesis in plants (Delauney & Verma, 1993).

Appendix. 3.2 Composition of Rorison solution outlined in Hewitt (1966)

Nutrient sources	Concentration (g l^{-1}) in stock solutions	Add to 10 litre deionised water to give solution strength (ml)		
		Full	0.5	0.1
$Ca(NO_3)_2.4H_2O$	472.0	10	5	1
K_2HPO_4	175.0	30	15	3
$MgSO_4.7H_2O$	123.0	20	10	2
Fe-EDTA	12.5	10	5	1
Trace elements		10	5	1
$MnSO_4.4H_2O$	2.028			
H_3BO_3	2.868			
$(NH4)_6Mo_7O_{24}$	0.184			
$ZnSO_4.7H_2O$	0.440			
$CuSO_4.5H_2O$	0.390			

Appendix 3.3 Analysis of variance (ANOVA) of root and shoot lengths of *M. sativa* cv. CUF 101 and somaclones (7R1, 3R1, 4R1, 6R2IV) grown for 2 weeks in 0 and 200 g l^{-1} PEG, in half strength Rorison soloution culture plus 4 mol m^{-3} CaCl$_2$.

Root Length

SOURCE	DF	Anova SS	Mean Square	F Value	Pr>F
REP	2	138.769	69.385	5.66	0.0037
Genotype (GE)	4	516.695	129.174	10.53	0.0001
PEG	1	6288.942	6288.942	512.84	0.0001
GE*PEG	4	548.0825	137.020	11.17	0.0001
GE*REP	8	840.094	105.012	8.56	0.0001
GE*PEG*REP	7	211.996	30.285	2.47	0.0169
PEG*REP	2	101.82	50.91	4.15	0.0163
Error	505	6192.744	12.263		
Total	533	1439.141			

Shoot Length

SOURCE	DF	Anova SS	Mean Square	F Value	Pr>F
REP	2	13.97	6.986	3.60	0.0281
Genotype (GE)	4	104.739	26.185	13.48	0.0001
PEG	1	2410.041	2410.041	1240.83	0.0001
GE*PEG	4	190.77	47.693	24.56	0.0001
GE*REP	8	63.42	7.928	4.08	0.0001
GE*PEG*REP	7	26.92	3.846	1.98	0.0560
PEG*REP	2	1.374	0.687	0.35	0.7023
Error	505	980.849	1.9423		
Total	533	3792.091			
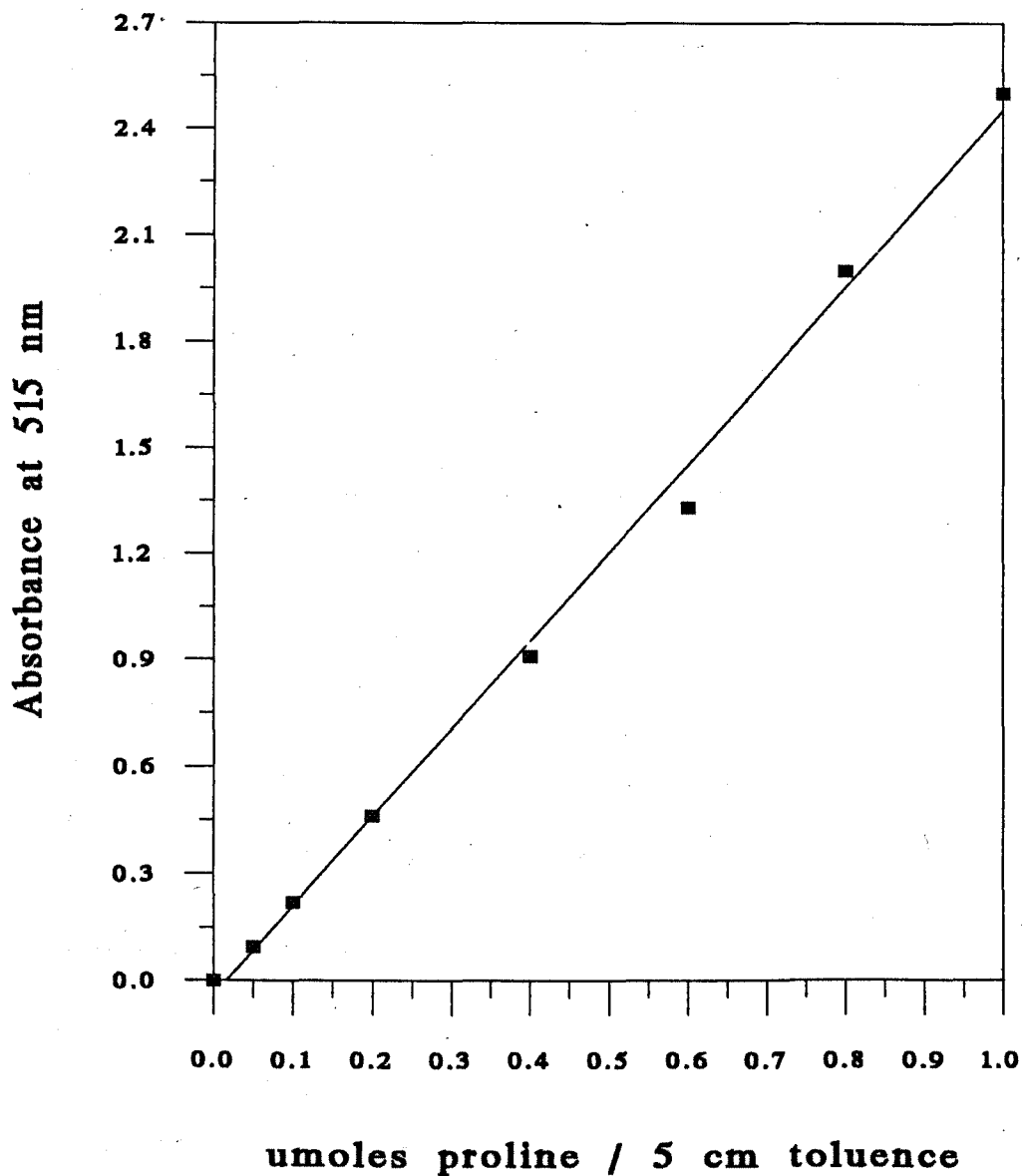
Appendix 3.4 Calibration curve for the spectrophotometric determination of proline.

Appendix 3.5 Antioxidant Enzyme Assays

Catalase-240 nm

Reaction mixture

100 µl   crude extract

  1 ml   30 mM H_2O_2

0.05 M Sodium-Potassium-Phosphate buffer $\rightarrow$ 3ml final volume

   (pH = 7.0)

Superoxide dismutase- 480 nm

 3.5 µl   crude extract

50 µl   0.18 mM adrenaline

0.05 M Sodium-Carbonate buffer $\rightarrow$ 3 ml final volume

   (pH=10.2)

Glutathione reductase - 340 nm

100 µl   crude extract

0.1 mM NADPH

0.6 mM GSSG

0.2 M   TRIS/HCl buffer $\rightarrow$ 2.5 ml final volume

   (pH=7.8)

Ascorbate peroxidase - 290 nm

25 µl   crude extract

0.25 mM ascorbate acid

0.5 mM H_2O_2

0.2 M   TRIS/HCl buffer $\rightarrow$ 2.2 ml final volume

   (pH = 7.8)

Appendix 4.2 Analysis of variance (ANOVA) of root and shoot lengths of 14-day-old seedlings of *M. sativa* cv. CUF 101 in 4 levels of NaCl, 0, 50, 100, 150 mol m^{-3} in the presence of 3 levels of CaCl$_2$, 0, 1.5, 4 mol m^{-3}, grown in half strength Rorison solution culture.

Root Length

SOURCE	DF	Anova SS	Mean Square	F Value	Pr>F
REP	2	426.497	213.249	8.93	0.0002
CaCl$_2$	2	2019.795	1009.897	42.27	0.0001
NaCl	3	4860.746	1620.249	67.81	0.0001
CaCl$_2$*NaCL	6	1237.712	206.285	8.63	0.0001
CaCl$_2$*REP	4	41.27	10.257	0.43	0.788
NaCl*REP	6	748.44	124.74	5.22	0.0001
CaCl$_2$*NaCl*REP	22	1182.587	53.754	2.25	0.0012
Error	366	8744.678	23.892		
Total	401	18472.015			

Shoot Length

SOURCE	DF	Anova SS	Mean Square	F Value	Pr>F
REP	2	41.099	20.55	4.88	0.0081
CaCl$_2$	2	144.751	72.376	17.20	0.0001
NaCl	3	1302.09	434.03	103.15	0.0001
CaCl$_2$*NaCl	6	209.866	34.988	8.31	0.0001
CaCl$_2$*REP	4	10.065	2.516	0.6	0.6643
NaCl*REP	6	46.141	7.69	1.83	0.0927
CaCl$_2$*NaCl*REP	22	121.275	5.512	1.31	0.1600
Error	366	1540.074	4.208		
Total	401	3359.155			

Appendix 4.5 Analysis of variance (ANOVA) of root and shoot lengths of *M. sativa* cv. CUF 101 and nine somaclones (1R1, 2R1, 3R1, 4R1, 5R1, 6R1, 7R1, 8R1, 9R1) grown for 2 weeks in 0 and 200 mol m^{-3} NaCl, in half strength Rorison soloution culture plus 4 mol m^{-3} CaCl$_2$.

Root Length

SOURCE	DF	Anova SS	Mean Square	F Value	Pr>F
REP	2	231.685	115.84	10.42	0.0001
Genotype (GE)	9	460.855	51.21	4.61	0.0001
NaCl	1	27676.17	2490.166	2490.44	0.0001
GE*NaCl	9	1129.264	125.474	11.29	0.0001
GE*REP	18	1591.51	88.417	7.96	0.0001
GE*NaCl*REP	17	848.72	49.925	4.49	0.0001
NaCl*REP	2	38.448	19.224	1.73	0.1778
Error	1054	11713.08	11.113		
Total	1112	43689.727			

Shoot Length

SOURCE	DF	Anova SS	Mean Square	F Value	Pr>F
REP	2	18.648	9.324	4.86	0.0079
Genotype (GE)	9	353.448	39.272	20.46	0.0001
NaCl	1	5335.308	5335.308	2779.71	0.0001
GE*NaCl	9	178.564	19.840	10.34	0.0001
GE*REP	18	240.044	13.336	6.95	0.0001
NaCl*REP	2	4.624	2.3119	1.20	0.3002
GE*NaCl*REP	17	0.0	0.0	0.0	1.0
Error	1054	2023.0183	1.9194		
Total	1112	8090.359			

Appendix 4.7 Analysis of variance (ANOVA) of root and shoot lengths of *M. sativa,* somaclonal progenies of 1st (7R1, 8R1, 9R1) and 2nd (7R2, 8R2, 9R2) tissue culture cycles, grown for 2 weeks in 0 and 200 mol m^{-3} NaCl, in half strength Rorison soloution culture plus 4 mol m^{-3} CaCl$_2$.

Root Length

SOURCE	DF	Anova SS	Mean Square	F Value	Pr>F
REP	2	444.245	222.123	16.82	0.0001
Genotype (GE)	7	151.18	21.597	1.64	0.1220
NaCl	1	26507.382	26507.382	2007.43	0.0001
GE*NaCl	7	1704.738	243.53	18.44	0.0001
GE*REP	14	1480.767	105.769	8.01	0.0001
GE*NaCl*REP	13	305.565	23.505	1.78	0.0421
NaCl*REP	2	54.494	27.247	2.06	0.1277
Error	795	10497.669	13.205		
Total	841	41146.04			

Shoot Length

SOURCE	DF	Anova SS	Mean Square	F Value	Pr>F
REP	2	41.713	20.856	9.68	0.0001
Genotype (GE)	7	80.643	11.520	5.35	0.0001
NaCl	1	3557.723	3557.723	1651.28	0.0001
GE*NaCl	7	117.368	16.767	7.78	0.0001
GE*REP	14	233.317	16.666	7.74	0.0001
GE*NaCl*REP	13	0.0	0.0	0.0	1.0
NaCl*REP	2	0.0	0.0	0.0	1.0
Error	795	1712.845	2.155		
Total	841	5651.219			

Appendix 5.2 Analysis of variance (ANOVA) of callus fresh and callus dry weights for *M. sativa* cv. CUF 101 in the presence of 6 levels of NaCl, 0, 50, 100, 150, 200, 250 mol m^{-3}. Callus derived from three explants (cotyledon, hypocotyl, root).

Callus Fresh Weight

SOURCE	DF	Anova SS	Mean Square	F Value	Pr>F
REP	2	0.0969	0.0485	0.82	0.4499
EXP	2	0.3581	0.1790	3.02	0.0615
NaCl	5	6.2529	1.2506	21.07	0.0001
EXP*NaCl	10	0.7660	0.0766	1.29	0.2723
EXP*REP	4	0.6748	0.1687	2.84	0.0381
NaCl*REP	10	0.5989	0.0597	1.01	0.4547
EXP*NaCl*REP	19	1.8441	0.0971	1.64	0.0999
Error	36	2.1366	0.0593		
Total	88	12.728			

Callus dry weight

SOURCE	DF	Anova SS	Mean Square	F Value	Pr>F
REP	2	0.0773	0.0386	3.73	0.0336
EXP	2	0.2913	0.1457	14.07	0.0001
NaCl	5	0.4836	0.0967	9.34	0.0001
EXP*NaCl	10	0.3628	0.0363	3.50	0.0026
EXP*REP	4	0.1440	0.0360	3.48	0.0168
NaCl*REP	10	0.1911	0.0191	1.85	0.873
EXP*NaCl*REP	19	0.5544	0.0292	2.82	0.0036
Error	36	0.3727	0.0405		
Total	88	2.4771			

Appendix 5.3,4 Analysis of variance (ANOVA) of regenerated callus and mean number bud callus[-1] of *M. sativa* cv. CUF 101, somaclones (6R2IV, 8R2IV, 7R2IV, 9R2IV) and parent CUF101-1S in the presence of 8 levels of NaCl, 0, 50, 100, 150, 200, 250, 300 and 350 mol m^{-3}. Callus derived from two explants (cotyledon, hypocotyl).

Regenerated Callus

SOURCE	DF	Anova SS	Mean Square	F Value	Pr>F
Genotype (GE)	5	0.6292	0.1258	13.38	0.0001
EXP	1	0.0127	0.0127	1.35	0.2595
NaCl	7	0.8261	0.1180	12.55	0.0001
GE*EXP	5	0.0445	0.0089	0.95	0.4733
GE*NaCl	31	0.6104	0.0197	2.09	0.0435
EXP*NaCl	7	0.0580	0.0083	0.88	0.5389
GE*EXP*NaCl	31	0.2389	0.0077	0.82	0.6976
Error	20	0.188	0.0094		
Total	107	0.6077			

Mean Number Bud Callus[-1]

SOURCE	DF	Anova SS	Mean Square	F Value	Pr>F
Genotype (GE)	5	12.2700	2.4540	2.59	0.0578
EXP	1	0.3320	0.3320	0.35	0.5602
NaCl	7	16.6330	2.3761	2.51	0.0502
GE*EXP	5	0.3981	0.0796	0.08	0.9939
GE*NaCl	31	12.5503	0.4048	0.43	0.9836
EXP*NaCl	7	0.8814	0.1259	0.13	0.9945
GE*EXP*NaCl	31	3.1511	0.1016	0.11	1.0000
Error	20	18.9229	0.9461		
Total	107	65.1389			

Appendix 6.3 Analysis of variance (ANOVA) of root and shoot lengths for *M. sativa* cv. CUF 101, somaclones [7R2IV (200), 9R2IV (250), 9R2IV (200), 6R2IV (250), 7R2IV (150), 9R2IV (100), 7R2IV (100)] and parent CUF101-1S in the presence 0 and 200 mol m^{-3} NaCl. Somaclones were derived from an *in vitro* screen at 100-250 mol m^{-3} NaCl.

Root Length

SOURCE	DF	Anova SS	Mean Square	F Value	Pr>F
REP	2	319.8034	159.9017	14.74	0.0001
Genotype (GE)	8	1712.8308	214.1038	19.73	0.0001
NaCl	1	27228.7584	27228.7584	2509.80	0.0001
GE*NaCl	8	937.5461	117.1933	10.80	0.0001
GE*REP	16	1650.5919	103.1620	9.51	0.0001
GE*NaCl*REP	13	0.0000	0.0000	0.0000	1.0000
NaCl*REP	2	0.0000	0.0000	0.0000	1.0000
Error	841	9124.0012	10.8489		
Total	891	40652.4918			

Shoot Length

SOURCE	DF	Anova SS	Mean Square	F Value	Pr>F
REP	2	88.7774	44.3887	26.52	0.0001
Genotype (GE)	8	150.2089	18.7761	11.22	0.0001
NaCl	1	4158.7249	4158.7249	2484.61	0.0001
GE*NaCl	8	208.2303	26.0288	15.55	0.0001
GE*REP	16	219.6897	13.7306	8.20	0.0001
GE*NaCl*REP	13	0.0000	0.0000	0.00	1.0000
NaCl*REP	2	0.0000	0.0000	0.00	1.0000
Error	841	1407.6590	1.6738		
Total	891	6019.2573			

Appendix 6.6 Analysis of variance (ANOVA) of shoot dry weight for *M. sativa* cv.

CUF 101 and somaclones (7R1, 6R2IV) mature plants in three different harvest.

| SOURCE	DF	Anova SS	Mean Square	F Value	Pr>F
REP	2	6.8704	3.4352	4.80	0.0133
Genotype(GE)	2	16.7087	8.3544	11.66	0.0001
CUT	2	20.2553	10.1276	14.14	0.0001
GE*REP	4	3.5641	0.8910	1.24	0.3071
GE*CUT	4	2.5520	0.6380	0.89	0.4779
CUT*REP	3	0.0000	0.0000	0.0000	1.0000
GE*CUT*REP	6	2.5072	0.4179	0.58	0.7414
Error	42	0.0808	0.7162		
Total	65	77.8914			

Appendix 6.7 Analysis of variance (ANOVA) of mature plant branch number and height for *M. sativa* cv. CUF 101 and somaclones (7R1, 6R2IV) in three different harvest.

Branch Number

SOURCE	DF	Anova SS	Mean Square	F Value	Pr>F
REP	2	16.5185	8.2593	0.95	0.3938
Genotype(GE)	2	66.9394	33.4697	3.86	0.0289
CUT	2	154.1032	77.0516	8.89	0.0006
GE*REP	4	6.2643	1.5661	0.18	0.9471
GE*CUT	4	1.6003	0.4001	0.05	0.9958
CUT*REP	3	32.9339	10.9780	1.27	0.2981
GE*CUT*REP	6	10.3071	1.7179	0.20	0.9755
Error	42	364.0000	8.6667		
Total	65	652.6667			

Height

SOURCE	DF	Anova SS	Mean Square	F Value	Pr>F
REP	2	248.0165	124.0083	2.14	0.1298
Genotype(GE)	2	0.0000	0.0000	0.0000	1.0000
CUT	2	142.6337	71.3168	1.23	0.3017
GE*REP	4	0.0000	0.0000	0.0000	1.0000
GE*CUT	4	0.0000	0.0000	0.0000	1.0000
CUT*REP	3	1135.4130	378.4710	6.54	0.0010
GE*CUT*REP	6	0.0000	0.0000	0.0000	1.0000
Error	42	2429.2200	57.8386		
Total	65	3955.2832			
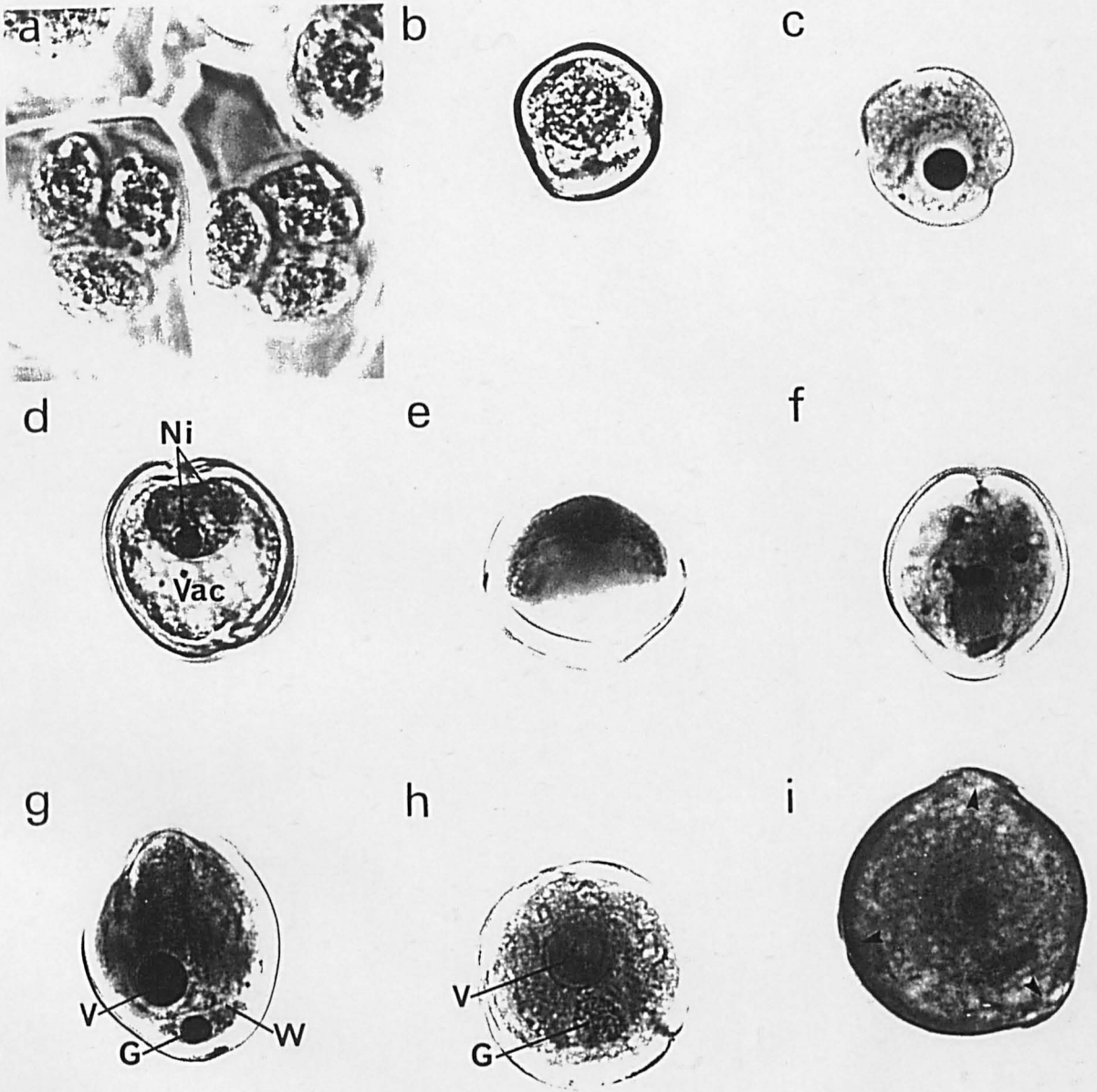
Appendix 9.1 Lucerne pollen developmental sequence *in vivo*. **a** Tetrad; **b** early unicellular; **c** mid unicellular; **d** late unicellular, note large vacuole (*Vac*), and two nucleoli (*Ni*); **e** and **f** pollen grain mitosis; **e** 'side view', **f** more common plan view; **g** early bicellular, showing vegetative nucleus (V), generative cell (G), and transient cell wall (W); **h** mid bicellular; **i** late bicellular with three pores (▲), elongated generative cell and irregular vegetative nucleus. Magnification of all pollen grains is the same; bar: 20 æm (Tanner *et al.*, 1990).