The Resistance to Salt of *Brassica sps.* and Improved Resistance by Direct Selection and Mutagenesis

by

Manal Hassan Eed

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Department of Agriculture and Food Studies

Seale-Hayne Faculty of Agriculture, Food and Land Use, University of Plymouth

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Abstract

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Salinity is one of the most serious environmental stresses influencing agriculture drastically decreasing the production of crop plants throughout the world. Due to salinity thousands of hectares of agricultural land are going out of production annually. To cope with the salinity problem, there is keen interest in the development of crop plants displaying resistance to the effects of salinity.

A series of experiments was carried out under controlled environmental conditions to determine salt resistance in Rapid Cycling *Brassica* species (RCB's) at germination early seedling and late growth stage. The effect of increasing salinity concentration was determined *in vivo* using a compost based growing system and also *in-vitro* using adapted tissue culture techniques.

At germination and during the early seedling stage, low salt concentrations (50-100 mM) stimulated germination and had no significant effect on growth in *B. rapa* and *B. rapa* appeared to have greater salt resistance than *B. napus.* There was no association between salt resistance at the early seedling stage and that at the adult stage. At later growth stages, salinity affected both the relative fresh and dry weights and tissue ion concentration with K: Na balance affected in favour of Na. The relative salt resistance in the six *Brassica* species was associated with the reduction in the total fresh weight of shoots of salt-treatment plants expressed as the percentage of control but was not associated with K, Na concentration or K/Na ratio in shoots. *B. napus* and *B. carinata* showed the greatest salt resistance, *B. juncea*, *B. rapa* and *B. nigra* were intermediate whilst *B. oleracea* was salt sensitive.

Conventional selection for salt resistance was not successful in this study because *B. oleracea, B. napus and B. carinata* were not able to complete either their vegetative or reproductive phases and died before completion of the first selection cycle. Whilst, ten percent of plants of *B. rapa*, *B. nigra* and *B. juncea* managed to complete the first selection cycle they failed to complete the second selection cycle.

Although, callus induction and maintenance were successful for all 6 RCB's, regeneration of shoots from callus was poor. Also, callus-based selection for salt resistance was unsatisfactory and had variable results and it was concluded that this was not a promising avenue for improving salt resistance in RCB's

A cauliflower curd meristem technique was adapted for *in-vitro* mutagenesis and selection. Mutagenesis was carried out using two mutagens N-nitroso-N-ethylurea or nitroso-methylurea at 1 mM and 2.5 mM. 300 green shoots were recovered from more than 1,000,000 explants mutagenised in liquid medium supplemented with 3 mM hydroxyproline as a selection agent. Of eighty *in-vitro* shoots which where measured for proline content, twelve showed higher proline level than controls. Leaf strip assays of the twelve selected *in-vitro* shoots and *in-vivo* weaned plants exposed to a 3 mM and 10 mM hydroxyproline assay showed greater resistance than controls. A few selections also had cross-resistance to salt at 550 mM NaCl and to frost at -7 °C. These results successfully indicated the existence of great opportunities for the production of stress resistance cauliflower plants via mutagenesis and hydroxyproline selection.

Contents

Abbreviations

%	Percent
°C	°Celsius
2, 4-D	2, 4-Dichlorophenoxyacetic acid
ANOVA	Analysis of variance
BAP	6-benzylaminopurine
Cl	Chloride
cm	Centimetre
dSm^{-1}	Deci Siemens per meter
EC	Electrical conductivity
g	Gram
gl^{-1}	Gram per litre
h	Hour
hyp	Hydroxyproline
IAA	Indole-3-acetic acid
IBA	Indole3-Butyric acid
K	Potassium
L	Litre
M	Molar
mg	Milligram
m	Minute
ml	Millilitre
mmhos cm^{-1}	Millimhos per centimetre
mM	Milli Molar
MPa	Mega Pascal

M&S	Murashinge and Skoog medium
Na	Sodium
NAA	Napthalenic acetic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NEU	N-nitroso-N-ethylurea
NMU	Nitroso-methylurea
no	Number
RC%	Relative conductivity percentage
s	Second

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Declaration

At no time during the registration for the Degree of Doctor of Philosophy has the author been registered for any other University award.

I declare that the work submitted in this thesis is the results of my own investigations except where reference is made to published literature and where assistance is acknowledged.

Manal Hassan Eed

Candidate

Mudad Fulle

Director of Studies

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Chapter 1: General introduction and literature review

1. Introduction

Salinity is principally regarded as an inherent problem of irrigated agriculture, restricting crop yields and sustained production in sizeable parts of the world. Soil salinity is primarily associated with arid and semi-arid regions of the world and such areas comprise 25% of the earth's surface. It has been estimated that total cultivable land has been limited by about 23% - 33% by salinity (Kumar, 1995; Gupta, 1995). Furthermore, Altman *et al.* (2000) predict that increased salinization of arable land will have devastating global effects, resulting in 30% land loss within the next 25 years and up to 50% by the year 2050.

In Egypt some 12×10^5 hectares have become saline as a result of irrigation from the Nile and a detailed soil survey of Egypt from 1957 to 1973 revealed that 49 % of the 2-4 $\times 10^6$ hectares of cultivated area is salt affected (Heakal *et al.*, 1981). Recently, El-Saidi (2000) revealed that the cultivated area in Egypt is quite limited; about 95% is desert and there is a wide gap between fresh water availability and its demand. There is about 4.4 billion cubic metes of reused drainage water with a salinity of 35 mM which is mixed with Nile freshwater and used on the soil. Moukthar *et al.* (2000) reported that crop productivity is highly affected by bad soil drainage in the north Delta-Nile in Egypt where the soils are characterised by saline clays with shallow saline water table.

In the arid and semi-arid regions there is frequently insufficient rain to leach away soluble salts. These salts occur both naturally in the soil and are added in irrigation water, rain and wind blown dust and by upward movement of ground water. Evaporation from the soil surface and transpiration from crops removes water but leaves salt in the soil. The soil salinity problem is increasing both because of inadequate irrigation and drainage practices and because the water used for irrigation, whether from a river or from wells, is never pure but always contains dissolved salt.

A strategy for coping with this problem includes:

1- Use of excess water applied periodically to leach salt from the root zone of the plant, 2- Adequate drainage, 3- Rapid crop establishment because many plant species are most sensitive to salinity during germination or early seedling development, 4- Development of tolerant agronomic species either by selective breeding from existing crops or by developing completely new crops from native halophytes.

Engineering costs and management costs of reclamation, however drainage and water control are high. Also unfortunately, efforts to breed for salt tolerance have resulted in the release of only a single variety that has high salt tolerance as a discernible character.

Such slow progress in plant breeding is due to a combination of many factors: 1- Incomplete knowledge of the effects of salinity and the interacting nature of ionic and osmotic properties of salt on plants, 2- Inadequate means of detecting and measuring salinity, 3- Ineffective selection methods, 4- A poor understanding of the interaction of salinity and the environment as it affects the plant, 5- The vague or non-specific effects other than growth at moderate salt stress.

Some directed breeding programmes and physiological studies with regard to salinity have yielded encouraging result in rice Gad El-Hak (1966) and Epstein and Norlyn (1977) selected a high tolerant barley cultivar capable of growing in seawater. Gedroiz (1971) is also optimistic that plant breeders can develop salt tolerant varieties and strains of crops. Also, improving salt tolerance in plant crops by gene transfer is a new and promising approach (Zhang *et al.*, 2000; Hasegawa *et al.*, 2000).

1. 1 The effects of salinity on plant growth

The most common adverse effects of salinity on plants include reduction in height and size, suppression of growth, yield and physiological function and deterioration in quality of the product. Rowell (1994) suggested that soluble salt could have two types of effect on the growing plant, firstly, specific effects due to particular ions they contain being harmful to the crops that fall into two classes, those operative at low and those at high concentrations. Of the former, only two salts are normally of importance, sodium carbonate and soluble borate, the former may be harmful in itself, but its harmful effect is more likely to be due to the consequences of the high pH it brings about. Thus, many nutrients, such as phosphates, iron, zinc and manganese, become unavailable to the plant at high pH, on the one hand, and the soil structure tends to become water-unstable on the other, thus bringing about the conditions of low water, poor aeration and an unkind, almost unworkable tilth. Secondly, the general effects due to the rising of the osmotic pressure of the solution around the root of the crop.

Doneen (1974) highlighted that water is osmotically held in salt solutions so that as the concentration of salt increases water become less and less accessible to the plant. Excess sodium and chloride in the protoplasm lead to disturbances in the ionic balance (K and Ca in relation to Na) as well as causing ion specific effects on enzyme proteins and membranes. As a result, too little energy is produced by photophosphorylation and by phosphorylation in the respiratory chain, nitrogen assimilation is impaired and protein metabolism disturbed. Similarly, Rains (1979) considered that plants exposed to saline environments encounter three basic problems, a reduction in water potential of the surrounding environment resulting in water becoming less available, toxic ions that can interfere with physiological and biochemical processes, and difficulty in obtaining required nutrient ions because of the predominance of other ions.

Salinity has both stimulatory and adverse effects on growth in some species (Basalah, 1991; Kumar, 1995). Sinha (1991) observed that stimulation of growth followed irrigation with water of low salinity (25-75 mM) in some plants, probably resulting from increased availability of essential nutrients but also lower crop production occurred as a result of decreased germination and early mortality of other seedlings of *Brassicas*.

Germination and early seedling growth are generally considered the most sensitive stages to saline water irrigation. Kumar (1995) indicated that *Brassica* species are highly susceptible to saline water during seed germination and early seedling growth. Francois (1984; 1994) showed that for *B. rapa*, salinity up 150 mM delayed germination but did not significantly reduce final germination percentage, however, both 200 mM and 300 mM significantly reduced final germination percentage. Bliss *et al.* (1986) found that inhibition of seed germination by salinity has been attributed to osmotic effects in barley cultivars. Other studies with cereals suggest that salinity induced reduction in germination and reductions in seedling growth are caused by specific ion toxicity (Sharma, 1983; Hampson and Simpson, 1990 a, b). Gutierrez *et al.* (1994) found that emergence percentage was significantly decreased at concentrations higher than 75 mM in *B. napus*. Waisel (1972) considered that reduced or delayed seed germination under saline conditions may be caused by osmotic effects, specific ion toxicity or a combination of both.

Greenway and Munns (1980) suggest that the deleterious effects of salinity on plant growth were attributed to specific ion toxicity and nutrient ion deficiency. Munns *et al.* (1982) suggest that inhibition of growth in barley involves a water deficit rather than a direct adverse effect of ions on metabolism under salt stress. Taleisnik-Gertel *et al.* (1983) suggest the greater inhibition by salt of the growth of shoot apices of tomato could result from an adverse water balance or from direct toxic effects of ions.

Zerbi *et al.* (1990) reported that raising solute concentrations of the solution surrounding a plant's root reduces the water potential gradient between solution and root uptake. If the external water potential is lower (more negative) than internally, water uptake will cease. In the short term this will reduce growth, in the long term it will cause wilting and ultimately death. Garcia *et al.* (1997a) indicated that NaCl reduced the growth of plants and caused chlorophyll loss in leaves in rice due to disrupted chloroplast integrity. Zeng and Shannon (2000) indicated that the reduction in seedling survival rates and growth are major causes of crop loss in salt-affected rice fields.

Yeo and Flowers (1982) and Criddle *et al.* (1989) reported that NaCl reduces a variety of activities essential for respiration and photosynthesis due to dehydration, which denatured many proteins or membranes and ion displacement, in which the accumulating chemical compound replaces inorganic cofactors needed for some enzymes to work efficiently. Yeo (1983) proposed that the growth of plants is affected by a number of factors under saline condition: 1- osmotic adjustment with organic solutes competes with growth processes for carbohydrate supply, 2- photosynthesis is reduced due to increased stomatal resistance, 3- additional active ion transport and other maintenance processes compete with growth process for carbohydrate supply, 4- ion transport may compete with other processes for energy, 5- ion transport capacity may be inadequate.

1. 2 Salt resistance

Salt resistance means the ability of plants to grow satisfactorily in saline soil. However, Levitt (1980) used the term 'salt resistance' in a broader sense, which includes: 1- salt tolerance, when plants respond to salinity stress either by accumulating salts generally in their cells or in specific cells such as salt glands. 2- salt avoidance, when plants avoid salt stress by maintaining their cell salt concentration unchanged either by water absorption or salt exclusion. Nonetheless, the term 'salt resistance' remains in use to

describe the capacity of plants to grow on salty soils, whether they accumulate ions in their cells or exclude the salts.

1.2.1 The physiological and molecular basis of salt resistance

Malkin and Waisel (1986) reported that salt resistance depends upon a variety of plant traits and involves the mechanisms of uptake, transport and excretion of ions. It also depends upon the various responses of plant metabolism to specific ions. Ashraf *et al.* (2001) demonstrated that salt tolerance is very complex in most plant species because there are numerous mechanisms, at cellular, tissue, organ, or whole plant levels. Some traits may only be functional at one time in a particular species. In addition, the effect of one mechanism may mutually exclude the effect of others at certain stages of development. Garcia *et al.* (1995) reported that physiological characteristics contributing to the resistance of salinity include: 1- reduced salt transport to the shoot which may be a consequence of low transpiration-bypass flow or of high water–use efficiency, 2- plant vigour which acts to dilute, through growth, the salt within the tissue, 3- compartmentation of salt away from young expanding or photosynthesising leaves (leaf to leaf distribution), 4- tolerance of salt within the tissue (tissue tolerance) which reflects differences in the distribution of salt between apoplast and protoplast or cytoplasm and vacuole.

Generally, two types of salt tolerance mechanism operate in higher plants. In the first, tolerance is related to specific ion effects (Schubert and Launchli, 1986; Cheeseman, 1988) wherein the plant can exclude deleterious ions such as Na and Cl from the leaves by various means. In the second mechanism, the plants allow entry of ions into the cells but direct their accumulation in the vacuoles. The vacuolar ions are known to provide energy cheap solutes for osmotic adjustment (Flowers *et al.*, 1977; Yeo, 1983). Similarly, Foolad (1997) proposed that two physiological mechanisms by which plants

respond to salt stress are: 1, inclusion and use of inorganic ions as osmotica to maintain a favourable water balance (a halophytic response) and 2, partial exclusion of ions and synthesis of organic solutes for osmotic adjustment (a glycophytic response). Ashraf (1994 a) reported that salt plant includers take up large quantities of salt and store it in the shoot. The high amounts of salts in the cytosol present problem for many physiological and biochemical events taking place there. Thomson (1974) suggested that many salt includers carry out compartmentation of salts into the vacuole and become succulent. Other salt includer species possess special glands on their leaf surface to excrete high concentrations of salts.

Greenway and Munns (1980) considered that a major characteristic of solute transport of plants in saline conditions is the degree of selectivity between K and Na, ranging from virtual exclusion of Na (glycophytes) to preferential accumulation of Na (halophytes). For many halophytes however, uptake of Na serves a useful function by providing osmotic solutes. Similarly many non-halophytes exclude Na and there seems to be an adaptive choice in plant evolution between use of ions as osmotic solutes with their potential toxic effects and the exclusion of solutes. This solves the toxicity problem but requires the plant to produce organic solutes to withstand the associated water stress.

Mahmood (1991) suggested that for survival in high salt levels a plant has to overcome two main problems: a- the solute potential of saline water is very low, and to take in water from saline soils a resistant plant must achieve an even lower intracellular water potential. b- the plant should be able to overcome specific ion effects, since high concentrations of ions, particularly Na and Cl, are toxic and after a certain level can be lethal. Kwon (1997) reported that plants under saline conditions establish physiological mechanisms of salinity tolerance such as osmotic adjustment (osmoregulation) against tissue water loss and ion uptake, and transport control against ion toxicity.

The synthesis of compatible organic solutes or accumulation of inorganic solutes achieves osmotic adjustment in the cells, which prevents internal water loss, resulting in the maintenance of water relations. Ion toxicity can be avoided by ion compartmentation, exclusion, retranslocation and control of uptake and transport.

Oertli (1976) proposed that all aspects of the hydration of a plant including water content, rate of transpiration and degree of hydration of the cytoplasm or of specific chemical compound, contribute to the determination of salt resistance. Hasegawa *et al.* (2000) defined determinants of salt stress tolerance as effector molecules (metabolites, proteins, or components of biochemical pathways) that control the amount and timing of resistance molecules. Stress adaptation effectors are categorised as those that mediate ion homeostasis, osmolyte biosynthesis, toxic radical scavenging, water transport and transducers of long-distance response co-ordination.

1.2.1.1 Resistance to salinity-induced ion toxicity

As salt is injurious to the plant if absorbed to high concentration, ion exclusion at the root would be an effective mechanism for avoiding injury. Winter and Preston (1984) reported that salt excluders have the ability to restrict the uptake of salt into the shoot. This may be due to toxic ions such as Na being absorbed in considerable amounts, but it is reabsorbed from the root or the shoot and is either stored or retranslocated to the soil. Most plants do not have the ability to exclude toxic ions and they remove high concentrations of absorbed ions from circulation by accumulating them in some particular compartment within the cell such as the chloroplast (Larkum, 1968), vacuoles (Sacher and Staples 1984) and rough endoplasmic reticule (Kramer, 1984). Excessive ion load can also be reduced by excretion through salt glands, which are found on the leaf surface (Osmond *et al.*, 1969; Thomson, 1974; Lawton *et al.*, 1981).

In non-halophytes most active excretion also occurs. In this case Na is actively withdrawn from the xylem back into the xylem parenchyma (Yeo *et al.*, 1977) and then retranslocated from shoots to root if loaded into the leaf apoplast (Lessani and Marschner, 1978) and then possibly extruded from the root back to the medium (Winter and Preston, 1982). Also, old leaves accumulate much higher salt levels than young leaves (Yeo *et al*; 1985) and shedding of old saturated leaves is considered to be the main strategy for salt regulation in many halophytes (Albert, 1975). Semikhatova *et al.* (1993) suggested that salinity tolerance is a property of whole plants. A large role in the high tolerance of halophytes to salinity is played not only by cellular mechanisms, but also by strict synchronisation of this process as with the transition of the cells of all organs from growth to maturity. Yeo and Flowers (1986) suggested that selecting separately for physiological traits in association with tolerance and then pyramiding these together could increase salt tolerance in rice. The reason is that salinity tolerance is conferred by several factors, and is the sum of a number of contributory traits at the cellular and whole plant level.

There is much evidence to show that the regulation of salt accumulation by plants, and thus their reaction to salinity, can be controlled at different levels of cellular organisation (Gorham *et al.*, 1985; Cheeseman, 1988; Wyn Jones and Gorham, 1989). These include compartmentation of ions in vacuoles and other organelles of root cells, control of translocation from root to shoot and compartmention in and retranslocation from leaf cells. More tolerant plants, such as barley, are able to take up Na as a mechanism of osmotic adjustment, allowing growth to proceed at higher saltiness than occurs for strict Na-excluders (Edward *et al.*, 1997).

1.2.1.2 Resistance to salinity –induced water stress

Osmoregulation is a common response to salinity stress, which allows maintenance of turgor and thus avoidance of leaf desiccation and all associated repercussions of turgor loss. The plants usually accumulate specific types of inorganic and organic molecules (compatible solutes). They serve the primary function of maintaining the cytoplasmic osmotic balance and can accumulate to high concentrations without impairment of normal physiological function.

Proline

Ahmad *et al.* (1981) found that salinity-resistant ecotypes of *Agrostis stolonifera* accumulated more proline and several other amino acids than the susceptible ecotypes under salinity stress. Voetberg and Stewart (1984) found that proline accumulated in barley leaves in response to salinity and its maximum concentration was linearly related to Na concentration. Bar-Nun and Poejakoff-Mayber (1979) suggested that proline accumulation in pea roots was associated with salinity resistance and Pandey and Ganapathy (1985) found that NaCl-tolerant cell lines of *Cicer arietinum* accumulate more proline than NaCl-susceptible lines, when grown on NaCl- containing medium. The significance of proline accumulation has been attributed to the ability of proline to act as a protective agent for cytoplasmic enzymes (Aspinall and Paleg, 1981) and Samaras *et al.* (1995) reported that proline accumulate in the cytoplasm to balance the osmotic potential of the vacuole where toxic solutes such as Na and Cl were found. Ashraf (1994 a) found that proline can effectively regulate the accumulation of essential N and it is osmotically very active. It also is compatible with other cytoplasmic components and can be easily converted to glutamate. This conversion is very important because glutamate takes part in the synthesis of other essential amino acids. Thus, proline in a plant under salt stress could act as both a nitrogen reserve and in osmoregulation.

Bhaskaran *et al.* (1985) highlighted the possible mechanisms of proline accumulation under stress condition: 1- stimulated synthesis from its precursors, 2- low rates of proline oxidation, 3- slow incorporation into protein due to impaired protein synthesis, or 4- accelerated protein breakdown. They considered that a stimulated synthesis might indicate a useful role of proline as an osmoprotectant and a low rate of proline oxidation may indicate a secondary effect of stress.

Delauney and Verma (1993) reported that proline is synthesised in two ways from glutamate. Where the Υ-carboxyl group of the glutamate molecule is phosphorylated by the ATP-dependent Υ glutamyl kinase (ΥGK), followed by a reduction of the resulting Υ-glutamyl phosphate to glutamic Υ-semialdehyde (GSA) via GSA dehydrogenase. GSA is then spontaneously cyclized to form Δ^1-pyrroline-5-carboxylate which is then reduced to proline by P5C reductase. Increased proline synthesis during stress involves a loss of feedback inhibition on the first enzyme, GK, leading to the formation of P5C. This is the rate-limiting step.

Singh *et al.* (1996) proved that proline over accumulation resulting from either loss of the proline oxidase-mediated pathway of proline catabolism or salinity-inducible uptake of exogenous proline confers protection against the lethal effects of salinity of 150 mM NaCl in the cyanobacterium *Nostoc muscorum*. Rhodes and Handa (1989) and Bartels and Nelson (1994) reported that tobacco cells adapted to NaCl accumulate proline to 80-fold higher levels and this is largely accounted for by increased synthesis and this accounted for almost 50% of the total osmotic adjustment. They considered that the accumulation to such level is consistent not only with its role as an adjusting solute but also its role as a compatible solute. Blum (1976) found that accumulation of proline in water-stressed leaves of sorghum was related to the ability of the plant to recover upon

relief of stress. Schobert (1977) proposed that proline protects the hydration of proteins by hydrophilic binding of water rather than acting as an osmotic solute under salt stress.

In response to drought and salinity stress, many plant species accumulate high levels of proline, which is thought to function in stress adoption (Greenway and Munns, 1980; Delauney and Verma, 1993). Stewart and Lee (1974), Smirnoff and Cumbes (1989) and Serrano and Gaxiola (1994) suggested that proline protect plant tissues against osmotic stress because it is an osmosolute, a source of nitrogen compounds, and protectant for enzymes and cellular structures and a scavenger for hydroxyl radicals. Kishor *et al.* (1995) reported that constitutive production of proline could confer osmotolerance in transgenic tobacco plants.

Naylor (1972) and Kramer (1983) proposed that a decrease in growth under stress can cause proline accumulation and water stress may also cause increasing proteolysis, thus increasing the pool of free amino acids, including proline. Accumulation of significant amounts of proline at a time when metabolic processes are going downhill as indicated by decrease in both fresh and dry weights rules out an active role for proline in combating water stress. Wyn Jones (1981) noted that proline is only accumulated at concentrations of NaCl which inhibit growth and which decrease the tissue succulence in *Atriplex spongiosa.*

Because proline accumulates in plants subject to severe conditions of both drought and salt, it may be that the synthesis of proline is a non-specific response to low water stress (Greenway and Munns, 1980; Wyn Jones, 1981). Ashraf (1989) found a negative correlation between proline content and salt tolerance in *Vigna mungo* L. Hepper inasmuch as the salt-sensitive cultivar Mash 654 had a greater leaf proline content than the more tolerant cv. Candhari Mash at different NaCl concentrations.

Similarly, Moftah and Michel (1987) found that the proline content could not be used as an indicator of salt tolerance in soybean.

Wyn Jones (1981) was of the view that proline may not play an adaptive role in plants in response to stresses because it accumulates at the extremes of stress. This may help plants to temporarily override highly damaging stress and thus its use as a selection criterion for salt tolerance does not seem plausible. However, Richards and Thurling (1979) and Lui and Zhu (1997) concluded that proline accumulation is merely a consequence of stress and does not lead to salt tolerance because the lack of correlation between proline level and salt tolerance in certain plant species. Gibon *et al.* (2000) concluded that proline accumulation was not involved in the maintenance of turgor and it appeared to be more of a consequence of the relative dehydration of stressed tissues in canola. There was no evidence that proline itself had a protective effect against the consequence of dehydration. Hasegawa *et al.* (2000) reported that proline is believed to facilitate osmotic adjustment by which the internal osmotic potential is lowered and may then contribute to tolerance. Proline as a compatible solute is typically hydrophilic, which suggest it could replace water at the surface of proteins, protein complexes, or membrane, thus acting as a nonenzymatic osmoprotectant.

Glycine betaine

Glycine betaine is another common compatible solute found in many different plant species. Rhodes and Hanson (1993) considered that plants accumulate betaine in response to drought and salinity. Rhodes *et al.* (1989) found that betaine level in *Poacea* species are correlated with salt tolerance: highly tolerant *Spartina* and *Distichlis* accumulate the highest levels, moderately tolerant species accumulate intermediate levels, and sensitive species accumulate low level or no glycine betaine.

Bartels and Nelson (1994) reported that glycine betaine is synthesised from choline in two steps, first being converted by choline mono-oxygenase to betaine aldehyde and then further oxidised by betaine aldehyde dehydrogenase. They also suggest that salinity stress induces both enzyme activities. Robinson and Jones (1986) reported that betaine is thought to protect the plant by acting as an osmolyte maintaining the water balance between the plant cell and the environment and by stabilising macromolecules during cellular dehydration and at high salt concentrations. Holmstrom *et al.* (2000) suggested that betaine-production in non-accumulating plants appears to enhance crop tolerance to different abiotic stresses (drought, salinity, freezing).

Potassium

The high concentration of potassium (an essential element) in leaf tissues may also contribute to the salt resistance capability of plants. Gupta (1995) reported that potassium has a special role under moisture stress conditions and it keeps the turgor pressure constant so that metabolic processes are unimpaired. Potassium is also involved in the mechanism of stomatal regulation, enabling the plants to use their water economically. Weimberg *et al.* (1982) suggested that potassium in the vacuole is the main cellular osmoticum and proline accumulation in the cytoplasm allows maintenance of a balance across the tonoplast. Kumar (1995) suggested that the ability of plants to maintain sufficient potassium might impart salt tolerance through osmotic adjustment. Singh and Tripathi (1979) proposed that the role of potassium in salt tolerance appears to depend on uptake by the cell sap and protoplasmic changes leading to increased water retention in stressed plants. Csonka (1989) reported that potassium ions are the most prevalent cations in the cytoplasm of bacteria and consequently they serve as one of the major intracellular osmolytes that maintain turgor under salt stress. He and Cramer (1993b) showed that salt tolerant *B. napus* had a greater concentration of potassium than salt sensitive *B. carinata* following irrigation with seawater.

1.2. 2 The genetic basis of salt resistance

Salt resistance is a quantitative characteristic of plants, which is determined by a large number of genes (Malkin and Waisel, 1986). Kueh and Bright (1982) identified three genes for enhanced proline content in barley. Lines with these genes were characterised by better salt tolerance. Cullis (1991) reported that there is no single mechanism by which multiple stresses are alleviated. All indications are that the ability to withstand stress environments is controlled by a number of genes and the multigenic character imposed limits subsequent genetic manipulation. Sacher *et al.* (1982) suggested that only one gene might be involved in the inheritance of Na accumulation whereas Tal (1984) stated that a few major genes might control salt resistance. Koval and Koval (1996) identified three genes for salt tolerance with intermediate inheritance and additive interaction in barley. Saleki *et al.* (1993) showed that three recessive genes were responsible for germination in solutions of NaCl in *Arabidopsis*. Bernstein (1977) found a single gene controls sodium and chloride uptake under salinity in grapevine and Scales and Widic (1991) confirmed that a single gene in soybean caused a decrease of salt transport to stem and leaves. It was shown that the tolerant allele was completely dominant over the sensitive allele. Shannon (1997) reported that in citrus multiple genes are involved to adequately regulate salt transport from the root to shoots.

Mahmood (1991) showed clear evidence that genes are present within wheat varieties and in some wild relatives of wheat which affect Na uptake and consequently salt tolerance of individuals and found a non-additive gene effect for shoot dry weight and water potential, but an additive effect for shoot fresh weight and Na, K and Cl content. Singh *et al.* (1989) reported that salinity affects the plant by a series of unstable and stable changes. These changes could be controlled by genetic regulatory mechanisms, which result in both the co-ordinate expressions of several genes and in multiple stable genetic changes by perhaps the simultaneous rearrangement of several genes.

Schachtman *et al.* (1989) added chromosomes from a wild salt tolerant species into a hexaploid salt sensitive wheat cultivar, resulting in alteration of ion accumulation, growth rate and ion transport rates from root to shoot. Carbonell *et al.* (1992) considered that the genetic components were involved in salt tolerance, with not only additive but also non-additive effects. Asins *et al.* (1993) demonstrated that salt tolerance in tomato is a quantitative trait where different types of gene action, a different set of genes, differently regulated were involved in the expressions of salt tolerance. Zhu *et al.* (1998) revealed that the SOS genes (for salt overly sensitive) are postulated to encode regulatory components controlling plant potassium nutrition that in turn is essential for salt tolerance in *Arabidopsis*.

1. 3 Breeding and selection for salt resistance

The association between heritability and stress in plants has attracted considerable attention because of the implication for plant breeding experiments aimed at obtaining rapid selection responses. As salinity resistance is complex, its exact nature not well understood and with its effect changing with plant age and possibly with acclimation then the inheritance of resistance is difficult to predict and measure.

Breeding strategies for salinity resistance therefore depend on screening techniques, tolerance mechanism, genetic diversity, genetic mode and heritability (Chaubey and Senadhira, 1994). The evidence from the few studies that do exist, suggests that salt tolerance is a heritable character and genetic variance decreases with increasing stress levels (Allen *et al.*, 1997; Ashraf *et al.*, 1986; Blum 1988). Johnson and Frey (1967) compared the growth of 27 oat cultivars under different nutritional conditions and different planting dates. These treatments affected several measurements of plant performance. Genotype variances generally increased, as environments became more favourable, although environmental variances also increased so that heritabilities were

not always higher under more favourable conditions. Similarly, Rumbaugh *et al.* (1984) found that genetic variance and broad sense heritabilities for shoot dry weight in alfalfa and wheat and grass seedlings declined as the amount of supplemental water was reduced. Conditions were varied so that there was almost no growth in the most extreme environment. Ramage (1980) considered that salt tolerance is a complex character and its expression largely depends on genetic background but suggested recurrent selection as a method of achieving salt tolerance lines. Subbarao *et al.* (1990) indicated that salt tolerance in pigeon pea is a heritable character controlled by a dominant gene or genes. These workers did not observe significant differences for growth between F1 reciprocals, suggesting the absence of cytoplasm-related factors and suggested that salinity tolerance could be improved by a simple backcrossing procedure.

1. 3.1 Criteria for selection

Seed germination in saline media is often used as a singular criterion or in combination with other criteria during selection. Noble (1983) considered that plants may be either more resistant or more susceptible to salinity at germination than at subsequent growth stages and is not a reliable indicator of salt resistance. It has therefore been proposed that the use of germination in saline media is an inappropriate criterion for species in which germination is more resistant to salinity because the agricultural problem lies at subsequent susceptible stages. On the other hand, Noble (1983) suggested that selection at germination is important in species that are relatively susceptible at this stage, in order to correct their deficiency in this respect.

While the improvement of germination under salinity stress may be important and possible, there is no good physiological evidence why resistance at seed germination would be associated with resistance at subsequent plant growth stages. Allen (1984) did not find an association between seed germination in saline media and seedling growth

response to salinity in alfalfa. Blum (1988) illustrated that the effect of salinity on germination is limited largely to the stage of seed imbibition and there is no reason to assume an association between factors that affect seed imbibition and factors that affect plant growth under salinity stress.

Dobrenz *et al.* (1981) obtained increased salt tolerance of *Lucerne* by selection at germination within a cultivar. Allen *et al.* (1985) also reported significant progress in increasing salt tolerance at germination in *Lucerne* after cycles of recurrent selection. They found 50% broad-sense heritability for this character, suggesting a high genetic component of salt tolerance in this species. Rawsown *et al.* (1988) suggested that germination and survival in salinized nutrient solution may bear no relationship to growth and yield of plants in saline soils but Madder (1976) suggested an association between tolerance at germination and early growth, could contribute to more vigorous seedling establishment and subsequent growth.

Al-Shamma (1979), Madder (1976) and Norlyn (1980) reported that reaction to salt stress varies with the stages of plant development and a given cultivar may be tolerant at one stage and sensitive at another. Johnson *et al.* (1992) reported that salt tolerance during germination did not correlate well with tolerance of seedlings or with mature plant re-growth yield in alfalfa. Rush and Epstein (1976) and Yeo and Flowers (1986) both used survival of seedlings at high salt concentrations as a fundamental selection criterion.

Bogemans *et al.* (1990) considered that selection during vegetative growth as seedling or plant fresh weight and dry weight as an expression of total growth could be an important selection criterion. However, Mahmood (1991) disapproved of the use of growth measurements as selection criteria for many reasons including: 1, this was a destructive

method which was unacceptable for selection at early generations; 2, sometimes varieties show different responses at different salinity levels or a variety may show a changed response in different environmental conditions at the same salinity level; 3, a genotype recognised as tolerant may already be vigorous or high yielding under normal conditions and under salinity conditions its yield, although reduced drastically will remain better than other low yielding genotypes. Salim (1989) believed that salt tolerance might be facilitated by using ion exclusion from certain tissue as selection criterion. However, Yeo and Flowers (1983) and Lauchli (1984) showed that ion exclusion should not be used as a selection criterion for salt tolerance. Lauchli and Epstein (1990) believed salt exclusion to be of limited usefulness as a selection criterion for salt tolerance in whole plants.

Selection and breeding approaches to increase resistance might be more successful, with respect to achieving maximum attainable resistance, if selection is based directly on the relevant physiological mechanism but as the relevant physiological mechanisms have yet to be agreed upon, this makes this approach difficult.

1. 4 Salt resistance in tissue culture

Plant cell culture techniques have tremendous potential for crop improvement. This collection of techniques can be directed either towards the production of identical plants (cloning) or to induce variability (soma clonal variation & mutation induction). Plants can be propagated from numerous explants including leaf sections, anthers, meristems, or even isolated single cells and protoplasts and whole plant, callus or liquid cell suspension cultures can be established.

Techniques of cell culture are generally useful in crop improvement programmes through: 1- propagation in vitro, 2- meristem culture for virus elimination, 3- secondary

product synthesis, 4- production of haploid plants from cultured anthers, and 5- development of new varieties via cellular or molecular genetics (Evans *et al.*, 1984). Callus cell culture may be uniquely useful for the study of the mechanism of salt tolerance (Hasegawa *et al.*, 1994). Dix and Street (1975) have shown that it is possible in *Nicotiana sylvestris* and *Capsicum annum L.* to select salt tolerant cell lines that were capable of growing in 340 mM NaCl. Stavarek and Rains (1984) reported selection of rice cells in media containing as high as 2 to 3 % NaCl, but regeneration of plants from the salt tolerant cells were limited. Fitch and Moore (1980) have reported selection for salt tolerance in sugarcane where plants were regenerated from callus cultured on modified MS medium containing 310 or 340 mM NaCl (sea water is approx. 500 mM NaCl). The surviving plantlets when subcultured to medium with 200 - 340 mM NaCl were salt tolerant and proved to be salt resistant in field tests.

The close relationship in response to salinity between cellular growth and whole plant performance has been found in several species. Orton (1980) compared the salt tolerance of *Hordeum vulgare* and *H. jubatum* in whole plant and callus culture and found the same response for salt tolerance. Jain *et al.* (1991) and He and Cramer (1993a) confirmed that a mechanism for whole plant salt tolerance might reside at cellular level in *Brassica* species.

However, growth response of callus and the whole plant are not clearly correlated in some instances because the cellular mechanism of tolerance to salt is associated with a number of factor such as ion inclusion or exclusion, ion compartmention and favourable ion balance (Kumar, 1995). Flowers and Yeo (1989) found that individual cells show more tolerance to salinity than intact plants as cells of rice can survive up to 500 mM NaCl while intact plants can tolerate only up to 100 mM NaCl.

Flowers *et al.* (1985) have also shown a very poor correlation between the *in vitro* performance of cells and *in vivo* growth of plants. In sorghum, callus tissue accumulated more Na and Cl than leaves of the whole plant. Tal *et al.* (1978) suggested that the better osmotic adjustment in the wild species functions at the cellular level and is independent of the organisation of the intact plant in tomato. Yang *et al.* (1990b) suggested that Na exclusion, operative in the whole plant was not expressed in cell culture and that specific anatomical and tissue mechanisms, perhaps in the root as well as in the leaves, are required to exclude Na. Dracup (1991; 1993) concluded that selection for salt tolerance by selection of cultured cells which grow at high NaCl have been largely unsuccessful, probably due to erroneous assumptions that mechanism of salt tolerance in cultured cell and whole plants are similar.

1. 5 Mutagenesis and selection for salt resistance *in-vitro*

Direct mutation through chemical mutagens or through physical factors such as gamma irradiation provides opportunities to enhance genetic variability. The mutagenic agent can be applied to the plant, to individual tissues, to organs (seeds, ovaries) or to cells. After exposure of the target material a selection screen is necessary if the desired character is to be isolated from the treated population. Identification of mutants will provide markers for cellular genetic manipulations and these selected lines will be available for physiological research and germplasm development (Gottschalk, 1981).

Selection for proline as a selective marker for NaCl tolerance and proline-over-producing mutants might especially be more tolerant to salt (Nabors, 1990). Gengenbach (1984) considered that mutants could be useful material, not only for the analysis of amino acid metabolism but also for breeding to improve nutritional quality, because the corresponding free amino acid accumulates in the mutants.

One approach to obtain proline-accumulating mutants is to select for proline analogue resistance. Dix (1986; 1993) and Deane *et al.* (1995) demonstrated that an amino acid analogue is generally toxic either by being incorporated into proteins in place of the normal amino acid and leading to dysfunctional proteins, or by false feedback inhibition which results in starvation for the amino acid, or both. A mutation resulting in feedback insensitivity of the regulatory enzyme prevents false feed back inhibition and allows the naturally occurring amino acid to accumulate to levels which can successfully compete with the analogue. Such a mutant results in resistance to the analogue and overproduction of the naturally occurring amino acid. This approach was first applied to plants by Widholm (1972 a, b) who isolated tryptophan-producing lines of tobacco by selecting for resistance to 5-methyltyptophan. Subsequently amino acid resistant mutants have been isolated for other plant species.

The potential for success in the area of selection for proline overproduction is indicated by the research of Van Swaaij *et al.* (1986) and (1987) who selected a number of hydroxyproline resistant cell lines of potato (*Solanum tuberosum L.*). Resistant lines were proline overproducers and retained this on non-selective media and some lines showed increased tolerance to NaCl. Similarly, Chandler and Thorpe (1989) selected for proline over-production and this technique appears to be a promising approach to increase salt tolerance. Efficiency of selection for proline over-production could be enhanced using single cells to reduce any effects of sectoring and specific metabolic blocks (Dix *et al.*, 1984) and to remove the necessity for proline determination at each stage of selection. Werner and Finkelstein (1995) isolated 6 mutant lines of *Arabidopsis* that expressed reduced sensitivity to salt and osmotic stress at germination and later stages of vegetative growth.

Singh *et al.* (1996) found that a mutant strain (Ac^r) of *Nostoc muscorum* resistant to growth inhibition by L-azetidine-2carboxylate (AC) was a proline overacumulator and exhibited enhanced salinity tolerance. Tantau and Dorffling (1991) and Dorffling *et al.* (1993) selected stable hydroxyproline resistant cell lines from a spring wheat cell culture that proved to be more frost tolerant than the wild type and had increased levels of free proline and had decreased osmotic potentials. Bright *et al.* (1979; 1981) demonstrated that hydroxyproline resistant mutants were capable of much greater proline accumulation under stress conditions. Kueh and Bright (1981; 1982) and Kueh *et al.* (1984) selected four barley mutants resistant to 4 mM hydroxyproline from a sodium azide mutagenized M2 population and showed that mutant leaves accumulated free proline. However, Hasegawa and Inoue (1983) and Hasegawa and Mori (1986) reported that over 20 rice mutants resistant to hydroxyproline were selected from a M2 population mutagenized by several chemical mutants and demonstrated that free proline did not accumulate in this hydroxy proline mutant lines. Chauhan and Prathapasenan (1998; 2000) selected hydroxyproline resistant cell lines of rice are tolerant to NaCl.

Other mutagenesis experiments have reported improved resistance in the absence of proline over accumulation. Hickok *et al.* (1991) reported that mutation *stl1* was irradiated and selected for tolerance to NaCl and showed that the mutant conferred high NaCl tolerance in gametophytes of *Ceratopteris.* Muller and Grafe (1978) showed that treatment of cell suspension cultures of *N. tabacum* with 0.25 M NEU (N-nitroso-N-ethylurea) increased the frequency of chlorate resistant mutants from 6.6×10^{-8} to 2×10^{-7}. Swanson *et al.* (1988) selected herbicide resistant mutants of *B. napus* from microspore derived embryos.

Widholm (1976) selected a hydroxyproline resistant carrot cell line from mutagen treated suspension cultures that produced 15-30 times the normal free proline levels and displayed cross-resistance to their proline analogues. Resistant cells were approximately one hundred times more resistant to hydroxyproline. A number of auxotrophs have been recovered from mutagenized plant cells. Such cells require various amino acids, nucleic acid and vitamins for growth. The significance of these mutants is the isolation of cell lines with markers that can be used to identify genetic traits. A linkage between the nutrient requirement and a desired trait that is expressed at the cellular level provides an effective screen for selection of whole plant characteristics at the cellular level (Maliga, 1984).

1. 6 Improving plant resistance to environmental stresses using molecular genetics

Salt, drought and freezing are stresses that cause adverse effects on the growth and the productivity of plants. Plants respond to these stresses at morphological, anatomical, cellular and molecular levels. Particular morphological adaptation may be vital in specific plant species, but are not commonly used in all plants. The two major abiotic stresses, drought and freezing, are intimately linked with salt stress and many genes that are regulated by salt stress are also responsive to drought or freezing stress (Zhu *et al.*, 1997). Recently, the isolation and analysis of plant genes has become a standard technique and the methodology to generate transgenic plants is available for many crop plants. Therefore, genetic engineering has the potential to rapidly improve the tolerance of plants (Zhang *et al.*, 2000).

Many organisms have evolved traits that enable them to survive in extreme environments and thus the genes that confer these properties can potentially be introduced into higher plants. Holmberg and Bulow (1998) reported that improving freezing, drought, and salt tolerance in plants by the transfer of genes encoding protective proteins or enzymes from

other organisms is possible. Shinozaki and Shinozaki (1997) demonstrated that the physiological response to environmental stresses arises out of changes in cellular gene expression. The products of the genes can be classified into groups: those that directly protect against environmental stresses and those that regulate gene expression and signal transduction in the stress response. The first group includes proteins that function by protecting cells from dehydration, such as the enzymes required for biosynthesis of various osmoprotectants, late-embryogenesis-abundant (LEA) proteins, antifreeze proteins, chaperonins and detoxification enzymes. The second group of gene products includes transcription factors, protein kinases and enzymes involved in phosphoinositide metabolism. Zhang *et al.* (2000) reported that improving drought resistance in plants by the transfer of genes or QTLs controlling osmotic adjustment might be possible. Holmstrom *et al.* (2000) concluded that improved tolerance to salinity and low temperature in transgenic tobacco producing glycine betaine was probably partly due to improved protection of the photosynthetic apparatus.

Several different gene transfer approaches have been employed to improve the stress tolerance of plants. The transferred genes included those encoding enzymes required for the biosynthesis of various osmoprotectants or those encoding enzymes for modifying membrane lipids, LEA protein and detoxification enzymes (Ishizaki *et al.*, 1996; McKersie *et al.* 1996; Xu *et al.*, 1996; Hayashi *et al.*, 1997). Kasuga *et al* (1999) produced transgenic plants that were highly salt, drought and freezing tolerant by overexpressing a single gene for a stress-inducible transcription factor, DREB1A. DREB1A binds to the *cis*-acting DRE and regulates the expression of many stress-inducible genes under drought, salt, and cold stress in *Arabidopsis*. Xu *et al.* (1996) showed that transgenic rice expressing *HVA1*, a gene encoding a late embryogenesis abundant protein from barley, has increased tolerance to drought and NaCl.

Smirnoff (1998) believed that the use of transgenic plants with improved antioxidant systems increases compatible solutes and improves drought resistance. Murata *et al.* (1992) and Tarczynski *et al.* (1993) showed that many transgenic plants which overproduce osmolytes improved and increased resistance to cold and high salt. Bartels and Nelson (1994) revealed that a group of low-temperature induced genes are homologous to LEA genes which are preferentially expressed during embryo maturation and encode mainly hydrophilic proteins and may be involved in the osmotic stress response which is common to cold, water and salt stress. Zhang *et al.* (2000) reported that transgenic plants have improved resistance to drought, salinity and cold that expressed /overexpress genes regulating osmolytes, specific proteins, antioxidants, ion homeostasis, transcription factors and membrane composition.

Zhang *et al.* (1999) found that it is not clear how up-regulated osmolytes confer salt, drought, cold stress resistance because the targeted osmolytes in transgenic plants did not accumulate in large enough amounts to play a role in osmotic adjustment. Zhu (2000) believed that the limited success of the molecular approach in elucidating salt tolerance mechanism is primarily due to two factors. First, the approach is only correlative (it is now considered that many salt-responsive genes do not contribute to tolerance, rather, their induction reflects salt stress damage). Second, the approach has identified genes or gene products based only on their expression, but many genes that are important for salt tolerance may not actually be induced by salt stress. He concluded that *Arabidopsis* should be used in genome scale gene expression profiling, to increase the understanding of salt tolerance. Similarly, Hasegawa *et al.* (2000) reported that plant molecular genetic models, in particular *Arabidopsis*, have provided inroads through the investigative power and causal demonstration of gain- and loss-of function molecular genetics to elucidate both cellular and organism salt tolerance.

1. 7 Rapid Cycling *Brassica* (RCB's)

Brassica species are important crops for oil production animal fodder and vegetables all over the world. From a world collection of over 2000 genotypes Rapid-Cycling *Brassica* (RCB's) populations of the six basic species *(B. rapa, B. nigra, B. olereacea, B. juncea, B. napus, B. carinata)* (Fig. 1.1) have been developed by selecting and combining the genes of diverse early flowering types (Williams and Hill, 1986). These plants are capable of cycling from seed to seed 10 times per year and their rapid growth and relatively small genome has meant that they have emerged as model plants for research in genetics, molecular biology and physiology. Furthermore these RCB's can be easily grown in cell and protoplast culture, and various nuclear genes and cytoplasm components of these plants have been well-characterised (Williams and Hill, 1986). Moreover, absence of seed dormancy and high female fertility ensures high seed yields and rapid turnaround (Fig. 1.2).

Tomkins and Williams (1990) considered that rapid cycling *Brassicas* have a valuable place in the biological context of the genus *Brassica* and the family Cruciferae. The relationship among cultivated *Brassica* species has been established by cytogenetic studies (U, 1935) (Fig. 1.1). *Brassica* crops consist of three elementary diploid species and three amphidiploid species that originated from inter-specific hybridisation between each two of the three elementary species. The elementary species are genomically described as genomes AA, BB, and CC respectively. The three inter-specific hybrids are fertile because chromosome doubling has occurred to make accurate meiotic pairing a possibility. These are then diploid for both chromosome sets and therefore referred to as amphidiploids (Fig. 1.1) with genomes of AABB, AACC and BBCC.

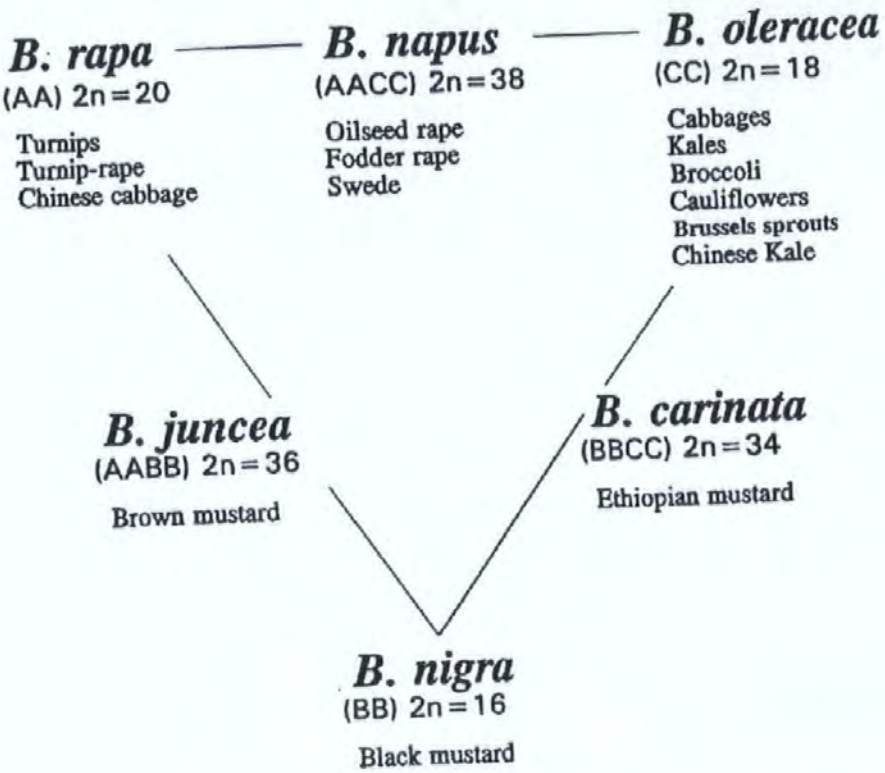


Figure 1.1: The *Brassica* triangle, showing the origin of the three amphidiploid species from the three pairs of contributing diploid genomes (After Hemingway, J, S., in Simmonds, 1976)

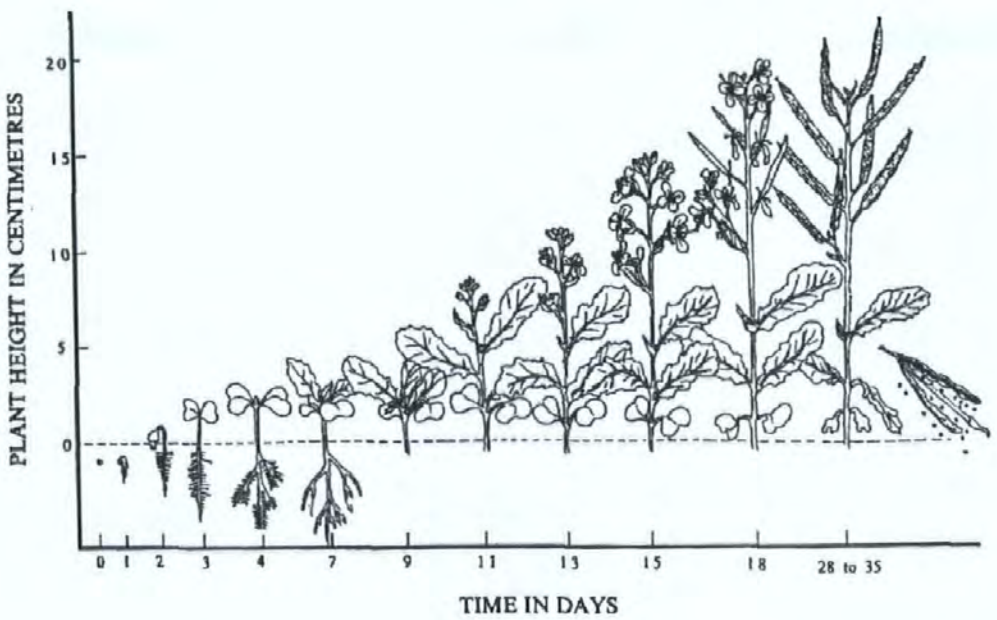


Figure 1.2: The life cycle of rapid-cycling *B.rapa* (Tomkins and Williams, 1990).

Little information has been obtained to date concerning the influence of salt on RCB's but they offer a useful system for the study of physiological and cellular traits (Williams and Hill, 1986; Williams, 1990; Tomkins and Williams, 1990; Millam *et al.*, 1991; Price, 1991; Fuller and Fuller, 1995; Fuller, 2000). He and Cramer (1992 and 1993a,b) concluded that the growth of six rapid-cycling *Brassica* species was reduced by seawater salinity. The growth reduction was associated with nutritional disturbance of these plants by salinity. *B. napus* and *B. carinata* were the most tolerant and the most sensitive species, respectively, while *B. rapa*, *B. juncea*, *B. nigra* and *B. oleracea* were moderately tolerant in their studies. They suggested that the difference in salt tolerance between species were not related to difference in specific-ion effects and also suggested that the maintenance of a high K/Na ratio as a mechanism for salt tolerance was not operative.

Ashraf and McNeily (1990) reported that *B. napus* and *B. carinata* were relatively salt tolerance and *B. rapa* and *B. juncea* were relatively salt sensitive species. However, Kumar (1995) and Ashraf *et al.* (2001) suggested that the amphidiploid *Brassica* species, *B. napus* (AC genome), *B. carinata* (BC genome) and *B. juncea* (AB genome) were more tolerant of salinity than their respective diploid progenitors, *B. rapa* (A) and *B. nigra* (B genome) and *B. oleracea* (C genome). They suggested that salt tolerance has been obtained from A and C genomes.

1. 8 The units of measurement of salts dissolved in soil and irrigation water

All irrigation water contains dissolved salts in varying amounts and the total concentration and the important constituents determine the quality of the water. When salts go into solution they separate into ions, cations and anions. The principle cations of irrigation water are sodium and potassium and the anions are chloride and sulphate. Ion concentrations have been reported in several different units, but by general consensus, most analyses are now reported as milligram equivalents (me) or milliequivalents per litre (mel^{-1}) or as milligrams per litre (mgl^{-1}). Water analyses published before 1900 are practically all expressed in terms of concentrations of combined salts such as calcium sulphate, and sodium chloride (Doneen, 1974). In the USA, parts per million (ppm) have been in common use for certain analyses and is the same as mgl^{-1}. However, the USA is gradually shifting to the metric system. In the Imperial system of units, analyses are sometimes expressed in grains per gallon. To convert grains gal^{-1} to mgl^{-1} multiply by 17.1 for the US gallon and by 14.3 for the British gallon. In the older analyses the total salt concentration was reported as total dissolved solids (TDS) in ppm by evaporating to dryness an aliquot of water and weighing the dry residue. In general, this method has been replaced by electrical conductivity (EC) which measures the conductance of a solution. Conductance is the ability of a solution to carry electricity and it is the reciprocal of resistance and has the SI Unit Siemens (S) which is equivalent to the Imperial Unit of ohm^{-1} or mho.

Electrical conductivity (EC) is the conductance of a solution filling the space between two metal surfaces 1 m apart, each with an area of 1 m^2. It has the symbol K and the Unit Sm^{-1}. Most of the salinity literature uses the old Unit, millimhos cm^{-1} for electrical conductivity, where mho=S. For convenience therefore data are often expressed as dSm^{-1} (deciSiemens per meter) because 1 dSm^{-1} = 1 mmho cm^{-1} = the Unit dS which is 10^{-1} S.

Most conductivity meters will give values in mS cm^{-1} or Sm^{-1} and these units are still commonly used. There is a direct conversion between units because 1mS cm^{-1} = 1 dSm^{-1}.

Obviously EC can vary with the amount of salts other than NaCl that are present. The yield of plants sensitive to salt stress is generally reduced if the EC values exceed 4 mS cm^{-1} and for this reason irrigation water should not have EC values above 2 $mScm^{-1}$. Similarly, Rowell (1994) points out that if the specific conductivity of a saturated extract at 25 °C is less than 4 mmhos cm^{-1} (4 mS cm^{-1}) which corresponds roughly to less than 3000 ppm salt in solution, no crop is likely to suffer from salinity trouble, whereas if the conductivity exceeds, 8 mmhos cm^{-1} (8 mS cm^{-1}), corresponding to about 5000 ppm salts, only salt tolerant crops will grow and even their yields are likely to be reduced. If it exceeds 15 mmhos cm^{-1} (15 $mScm^{-1}$) or 10000 ppm, no agricultural crops are likely to give an economic yield.

The electrical conductivity of sea water is at least 44 mS cm^{-1} which means the concentration of NaCl in sea water is approximately 500 mM or 0.5 M (Norlyn, 1980). In the open ocean in temperate latitudes the salt concentration remains relatively constant (on average 480 mM Na^+ and 560 mM Cl^{-1}; Epstien,1979). In the inter-tidal zone the salinity fluctuates over a very wide range, 290-810 mM Na^+ and in mangrove belts, and in salt marshes the Na concentration can increase at times to as much as 600-1000 mM. Sodium chloride accounts for about 80% of the dissolved salts in sea water (Rains *et al;* 1979). The USA salinity laboratory (1954) suggested the use of EC $x10^{-6}$ (mS) for irrigation water and EC $x10^{-3}$ (mS) for soil solution extracts. However, many agencies

use EC x 10^{-3} for both water and soil extracts. The EC and osmotic potential are linearly proportional (1 mS cm^{-1} = - 0.036 MPa).

1. 9 Project Aims and Objectives

Aim

To examine the resistance to salt in the *Brassica* genome and develop salt resistant and tolerant genotypes

Objectives

1- To investigate the degree of salt resistance in *Brassica* using the 6 rapid cycling *Brassica* representing the *Brassica* triangle.

2- To select survivor plants following selective salt stress and to pollinate, produce seed and reselect.

3- To examine the physiological basis of salt resistance in selected genotypes.

4- To direct NaCl selection in tissue culture

5-To select for hydroxyproline resistance in *Brassica oleracea* var. *botrytis* (cauliflower) using the mass fractionation tissue culture method in conjunction with mutagenesis.

6- To evaluate the salt and frost resistance of any mutants produced.

Chapter 2: Investigation of salt resistance in Rapid Cycling *Brassica*

2.1 Introduction

2.1.1 Investigation of salt resistance at germination and early seedling growth stage

Seed germination and seedling growth are crucial and decisive phases for plant establishment. The ability of a seed to germinate and emerge under salt stress indicates that it has some genetic potential for salt tolerance, at least at this stage in the life cycle. This does not necessarily indicate, however, that a seedling, which starts under salt stress, can continue to grow under salt stress and complete its life cycle. Tolerance of salinity at germination and emergence is, however, a highly desirable trait. The use of germination and emergence as a first indicator of salt tolerance in a species seems valid (Norlyn and Epstein, 1984). Shannon (1984) recommended screening and selection during germination as a method to begin genetic improvement of salt tolerance and to identify valuable gene resources for tolerance testing at later growth stages. A safe method might be to select for salt tolerance during germination and emergence independently from seedling or later growth stages. It is likely that different genes will become involved in various mechanisms of salt tolerance as a plant develops.

It has been shown repeatedly that no relationship exists between tolerance at germination and later growth. Kumar *et al.* (1981) also found that the effect of salinity on plant growth depends on its state of development and responses might be quite different at the germination stage compared to later stages of development. Johnson *et al.* (1992) reported that salt tolerance during germination did not correlate well with tolerance of seedlings or the mature plant re-growth yield in Alfalfa (*Medicago sativa*). Ashraf (1994b) found that increasing salt concentration had no significant effect on total germination percentage and there was no positive correlation between salt tolerance at early growth stage and that at the adult stage in pigeon pea. Kumar and Kumar (1990) and Kumar (1995) reported that the seed germination stage was more salt sensitive than later seedling stages in *Brassicas*. Kwon (1997) found that *B. juncea* 'common green'

one of the most salt-sensitive cultivars at the germination stage was the most salt tolerant at the seedling stage. Shannon (1997) reported that efforts to evaluate salt tolerance in a species on the basis of tolerance during germination and emergence had not generally been successful.

The deleterious effects of salinity on plant growth are attributed to the decrease in osmotic potential of the growing medium and specific ion toxicity leading to nutrient ion deficiency (Greenway and Munns, 1980). The extent of growth depression of young plant under saline conditions varies with salt composition, salt concentration, the physiological stage of the plant when it is exposed to salinity and the plant species (Sharma, 1983). Interestingly seeds of highly adapted halophytes still usually germinate to a higher percentage in distilled water than in NaCl solutions, and transferring such seeds from NaCl solutions to fresh water improves germination (Williams and Ungar, 1972; Rajput and Sen, 1990).

2.1.2 The effect of salinity on plant growth *in-vivo*

The mechanism of salt tolerance in *Brassicas* has been extensively studied including the three main components of salt stress, osmotic effects, ion toxic effects and nutritional effects and all three have been found to be partially responsible for the reduction of growth of plants in saline conditions. The integrated salt metabolism of plants is essentially a problem in mega-nutrient physiology and two critical aspects of cellular and organism metabolism have been given particular attention; those involved in the control and integration of sodium (Na) acquisition and allocation in plants and those involved in readjustment of other aspects of metabolism (Cheeseman, 1988). Chauhan and Prathapasenan (2000) considered that some of the detrimental effects of high salinity on plant cells might result from an ion imbalance (especially the K/Na ratio), water deficit and ion toxicity (elevated cytoplasmic Na and Cl concentrations) which lead to reduced

growth rates and eventually to plant death. Munns *et al.* (1982) and Taleisnik-Gertel *et al.* (1983) suggest that the primary cause of reduced shoot growth under NaCl salinity is located in the growing tissues. Salt may affect growth indirectly by decreasing the amount of photosynthate, water, or growth factors reaching the growth region. The amount of photosynthates reaching the growing region may decrease because of inhibition of photosynthesis due to stomata closure or by direct effects of salt on the photosynthetic apparatus. Water deficit in the growing region may occur by insufficient osmotic adjustment or increased resistance to water flow (Ownbey and Mahall, 1983). The inhibition by salt of cell division or enlargement (or both) in the growing region may be indirect or direct (Kulieva *et al.*, 1975; Mass and Nieman, 1978; Nieman and Shannon, 1976). Mass and Nieman (1978) suggested that salt ions could damage growing cells indirectly, by depriving them of essential substances or conditions. Leopold and Willing (1984) list various symptoms of salt damage, which may result from membrane damage by salt ions.

The cause of reduction in shoot growth under saline conditions has been located in the growing tissue and was the consequence of a reduction in the number of leaves and an inhibition of lateral bud initiation due to water deficit (Delane *et al.*, 1982; Shannon *et al.*, 1994). Procelli *et al.* (1995) found that K/Na ratio did not affect plant growth to salinity in B. *napus*, whilst, Cramer *et al.* (1994) found that salinity increased Na and decreased K accumulation in roots and transport of these ions to leaves. Furthermore, Na could increase K efflux into the growth medium (Cramer *et al.*, 1985; Hajji and Grignon, 1985), possibly due to a decrease in membrane integrity. Kwon (1997) indicated two simultaneous causes of growth reduction within a plant in the presence of salinity; firstly NaCl reduced plant growth by excess Na accumulation in and inadequate K transport to, fully expanded mature leaves, which was not reversible after removal of NaCl; secondly by water stress induced by the external salinity.

Thakral *et al.* (1997) found that the K: Na ratio was more or less the same in parents and hybrids of Indian mustard species in a normal environment, whereas it decreased considerably in saline environments. It is clear from the literature that shoot growth is depressed in salt affected soils through a preferential uptake of Na at the expense of K. The Na is then not metabolised and accumulates in the leaves where it exerts an osmotic effect leading to the dehydration of cells, cell death and leaf shrivelling.

In general, there are three main hypotheses that are associated with the effect of K/Na ratio on plant growth; first, Na may cause a K deficiency, which limits plant growth. Muhammad *et al.* (1987) reported that both shoot and root growth of rice changed with the alteration of K/Na ratio in the external medium. Furthermore, concentrated nutrient solution can inhibit plant growth to about the same extent as iso-osmotic NaCl solutions, indicating that K deficiency may not be limiting growth (Termaat and Munns, 1986; Cramer *et al.*, 1990). The second major hypothesis is that a high concentration of Na may inhibit biochemical processes such as protein synthesis (Hall and Flowers 1973) and thus limit growth. The observation that decreased leaf longevity is positively correlated with the leaf Na concentration supports this hypothesis (Schachtman and Munns, 1992). The third hypothesis is that the relationship between the K/Na ratio and growth is simply a correlation with no causal effect. Correlation is a measure of the degree to which parameters vary together and does not necessarily reflect any causal effect and reduced growth may result from other non-ionic factors such as osmotic and hormonal effects. Several reports (He and Cramer, 1992, Gorham *et al.*, 1990, Wolf and Jeschke, 1987; Termaat and Munns, 1986; Cramer *et al.*, 1990) show a lack of a positive correlation of K/Na ratio with growth which supports this hypothesis.

The ion relations of plants and K maintenance or an efficient K retransloction under salinity is an important physiological mechanism of salinity tolerance because a salt-induced change in potassium uptake by roots is associated with changes in photosynthesis and plant growth (Greenway and Munns, 1980; Bogemans *et al.*, 1990; Chow *et al.*, 1990; Gorham, 1993). Flowers *et al.* (1977) suggested that a preference for K at high salt concentrations is a useful attribute in salinity stress and a good criterion for selection for salt tolerance. Wyn Jones *et al.* (1984) showed that a positive correlation between salt tolerance and exclusion of Na in wheat and suggested that this is an efficient selection criterion for this crop. Storey and Wyn Jones (1977) and Shannon (1979) reported the correlated tolerance of a plant to sodium chloride with the ability to exclude Na from shoots. Sharma and Gill (1994) concluded that salt tolerance in *B. juncea* is associated with relatively lower Na and higher potassium (K) accumulation in leaves. Despite this, Yeo and Flowers (1983) showed that Na exclusion alone should not be used as an indicator of salt resistance.

It has been evidenced that selective ion absorption and transport in plants does not reside only in the roots. Transfer of the ions into the conducting tissue, subsequent movement in the xylem and phloem, absorption by cell in the stem and leaves and re-export therefrom –all these and other processes are implicated. Lynch and Lauchli (1984) reported that Na: K ratios were typically higher in the roots than in the shoots in stressed barley plants and the salt sensitivity of K transport into the xylem stream might be different from that of K uptake by the root cortex. Smart *et al.* (1996) demonstrated that potassium uptake by roots is believed to involve inward-rectifying potassium channels, allowing potassium to enter along an electrochemical gradient when potassium in the soil is relatively abundant. However a collection of inward-rectifying potassium channels (called KAT1, AKT1, KST1) have been identified from *Arabidopsis* and *Solanum*. Yeo (1998) reported the limited knowledge of how channels/transporters regulate intracellular

potassium levels and suggested that potassium transport is via channel activity rather than through altered gene expression or regulation. Garcia *et al.* (1995) highlighted that sodium transport to the shoot could involve the interaction of several biochemical and physiological processes. The composition and structure of the cell membrane can affect passive leakage through the membrane bilayer either into the root or into the xylem. The nature and relative affinities of transport proteins and ion channels will affect ion uptake and selectivity. Root anatomy and development would be expected to affect leakage of salt with the transpiration-bypass flow. Hasegawa *et al.* (2000) concluded that the precise transport system responsible for Na uptake into the cell is still unknown. The physiological data indicates that Na competes with K for intracellular influx because these cations are transported by common proteins. But K and Na influx can be differentiated physiologically into two principle categories, one with high affinity for K over Na and other for which there is lower K/Na selectivity. Gregorio and Sendhira (1993) suggested that sodium transport is likely to result from the interaction and combination of several genes and there is evidence that both sodium content and K: Na selectivity in rice are governed by both additive and dominant gene effects. Garcia *et al.* (1997b) found that genes influencing potassium transport as well as genes affecting sodium transport segregated in rice, there was no correlation between sodium transport and potassium transport into the shoot. Schachtman and Liu (1999) reported recent cloning and electrophysiological characterisation of several genes encoding different types of molecules that are involved in K and Na transport but it is not yet known which is most critical in determining the K: N ratios in whole plants.

2.1.3 Selection for salt resistance

Selection for improvement in NaCl tolerance may be made at a number of life cycle stages. However, selection at the seedling stage would clearly be easier and more economical, provided it had a consistent relationship to whole plant response.

Ashraf (1994b) reported that if a plant maintained its degree of salt tolerance consistently at all developmental stages, selection at any growth stage could be employed to identify tolerant individuals. Jones and Cassells (1995) reported that the increases in selection efficiency can be achieved by the use of selection indices, based on phenotypic traits which are components of, or correlated with, the complex target character. They highlighted that difficulties in weighting the value of individual phenotypic traits (such as high nitrate reductase activity as a possible component of the high grain protein content trait) had limited the use of these indices in breeding programmes. Noble and Rogers (1993) demonstrated that agronomic characters, such as yield, survival, leaf damage and plant height, have been the most commonly used selection criteria for identifying tolerance. This is largely due to their ease of measurement and these characters represent the combined genetic and environmental effects on plant growth and include the integration of the physiological mechanisms that confer tolerance. Epstein and Norlyn (1977) achieved a significant improvement in salt tolerance of barley after a single cycle of selection. The selected line survived until maturity and gave a reasonable seed yield when irrigated with undiluted seawater. Noble *et al.* (1984)) found that salt tolerance in alfalfa was increased through recurrent selection at the adult stage. Allen *et al.* (1985) found that alfalfa salt tolerance at the germination stage was improved after five cycles of mass selection. Jain *et al.* (1991) reported that conventional selection had met with some success in improving the response of the crop species to salt stress. Such breeding progress, however, has been slow, particularly due to limited sources of genes for salinity tolerance, lack of efficient screening procedures and precise evaluation method and limited land, labour and economic resources. Holmberg and Bulow (1998) found that the problem with traditional plant breeding for achieving crops more tolerant to abiotic stress, such as salinity, drought, chilling and freezing, is that it is difficult to modify single traits; and it relies on existing genetic variability.

Aim

The aim of these experiments was to evaluate salt resistance of *Brassica* species during early growth stage and to establish a comprehensive evaluation method for salt tolerance of Rapid Cycling *Brassica* species (RCB's) in sodium chloride modified growth conditions *in-vivo*

Objectives

1- To examine sand and peat based compost to determine a suitable growth medium for RCB's.

2 - To examine the effect of different salt concentrations at germination

and during the seedling growth stage.

3-To screen RCB's over a range of NaCl concentrations to determine lethal concentrations leading to concentrations suitable for future screening experiments.

4 -To measure tissue concentrations of Na & K in order to determine Na: K ratios of plant grown under varying NaCl concentrations.

5 -To compare the relative resistance/tolerance of different RCB's.

6- To develop a seed multiplication system for RCB's.

2.2 Material and Methods

2.2.1 Mass production of seeds

Initially, large populations of seeds needed to be produced. Seeds of rapid cycling populations of six *Brassica* species (*B. rapa* cv. 1-1, *B. nigra* cv. 2-1, *B. oleracea* cv. 3-1, *B. juncea* cv. 4-1, *B. napus* cv. 5-1 and *B. carinata* cv. 6-1) (RCB's) (Williams & Hill, 1986) were obtained from The Cruciferae Genetics Co-operative, University of Wisconsin-Madison, USA (Cruciferae Genetics Co-operative, 1997). Since only small amounts of seeds were available, it was necessary to bulk-up seed stocks in the glasshouse. Furthermore, a system needed to be developed which could be used later to ensure the controlled mass pollination of seed cohorts. In mid-March 1998, 100 seeds from each species were sown in plastic module trays containing 10 ml compost per plug (SHL peat based and John Innes potting compost 1:1 mixture by volume). Irrigation water was regularly applied by base watering onto capillary matting. At the bud development stage, an entire module was covered by a cloth net covered frame (42 x 50 x 80 cm) to allow controlled mass pollination by blowflies to take place to produce a base population of seeds for each species. Blowflies were reared from chrysalises (casters) purchased from a fishing tackle shop. At maturity, irrigation was terminated for 2-4 weeks and then plants harvested and measurements of grain yield taken after rubbing out seeds from seedpods. The greenhouse temperature was 15-20°C and a 24 hour day was maintained by supplementary lighting (sodium vapour lamps) during the growth period.

2.2.2 Controlled environment growth methods

Four growing experiments were evaluated. Experiments 1, 2 and 3 firstly tested *B.rapa* and *B. napus*, which were the species available with greatest seed stocks, and then experiment 4 used all six *Brassica* species. Throughout the study, pots were arranged in a completely randomised design in plastic trays under a light bank. Plants were grown in

a controlled environment room, temperature 25 ± 2 °C, photon irradiance at 160 μ mole $m^{-2}s^{-1}$, relative humidity 35± 3%. The light source used was a mobile light bank fitted with 4 cool white fluorescent tubes with a 24-h photoperiod. The Rapid Cycling *Brassicas* (RCB's) had been selected to grow rapidly under similar conditions (Williams & Hill, 1986) and are those advocated for classroom experimentation with RCB's (SAPS 1992).

Salinization

A series of NaCl concentrations was prepared and their Electrical conductivity (EC) measured using a WP4 conductivity meter which established a linear relationship (Fig. 2.1). A sample of sea water (EC = 45.0 mS approximately 500 mM) was collected from the beach at Teignmouth, Devon, UK was also measured as a reference standard.

The NaCl solution and distilled water control were applied as watering treatments five days after sowing and were subsequently added every 5 th day throughout the experiments so that plants were consistently challenged with salt stress.

2.2.2.1 Germination and seedling experiments to establish a suitable growing system

Germination plates have been criticised because moisture may condense on the under-surface of the top of dishes and drip back onto the filter paper, creating non-saline micro-environments (Shannon *et al.*, 1984). Therefore this study preferred to use *in-vitro* germination sand culture or compost culture.

Experiment 1: *in-vitro* germination

B. napus and *B. rapa* seeds were surfaced sterilised using a three step procedure comprising: immersion in 70% ethanol for 30 s followed by immersion in 10 % commercial bleach for 15 m and finally three washes in sterile distilled water. Surface sterilised seeds were sown into plastic pots (9: 0 cm inner diameter at the top and 5: 0 cm

at the bottom) containing 20 ml of M&S medium (Murashige and Skoog, 1962) (Appendix 1) solidified with 7.0 gl^{-1} agar supplemented with concentration 0 mM, 50, 100, 150, 200, 250, 300, 350, 400, 450 mM of NaCl and grown at 22 °C in a 16-h. light, 8-h. dark regime with a radiant flux density of 70 μ mole m^{-2} sec^{-1}. Five replicates of ten seeds per pot were used for each treatment. Data recorded were % germination, fresh weight of shoot and dry weight after seven days.

Experiment 2: Sand culture and salt stress

Seeds of rapid cycling *B. rapa* were sown in Silvaperl-Sharp sand (50 ml/ pot) in clear plastic pots (9.0 cm inner diameter at the top and 5.0 cm at the bottom). Four replicates of ten seeds per pot were used for each treatment. Each pot in the experiment was irrigated daily with 5 ml of water. The NaCl treatments (over the range 50 to 250 mM) were applied instead of water five days after sowing and every five days thereafter throughout the experiment. After ten days from sowing, data was collected on the width of cotyledons, length of hypocotyl, length of true stem, number of true leaves, and shoot fresh weight and analysed by ANOVA (Minitab Version 12)

Experiment 3: compost and sand plus nutrient solution.

Seeds of rapid cycling *B. rapa* were planted in plastic pots (as in Expt. 2) which contained Silvaperl-Sharp sand and other pots that contained a mixture of SHL potting compost and John Innes potting compost (1:1 by volume) (50 ml/pot). Four replicates of ten seeds per pot were used for each treatment. Germination percentage was determined from the number of seeds germinated five days after sowing. At fourteen days after sowing, the fresh weight was determined for plants per pot and then the dry weight determined after three days at 75°C and data analysed by ANOVA (Minitab Version 12).

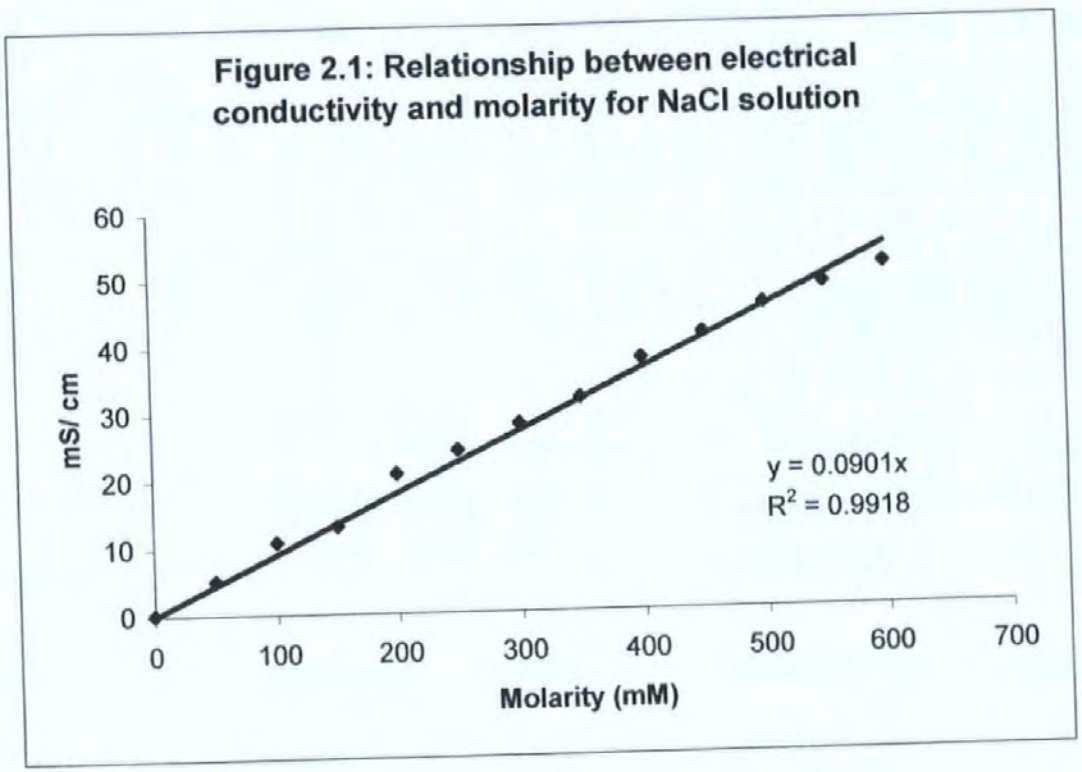


Figure 2.1: Relationship between electrical conductivity and molarity for NaCl solution
mS/ cm
60
50
40
30
20
10
0
0
100
200
300
400
500
600
700
Molarity (mM)
y = 0.0901x
R2 = 0.9918

Nutrient solution treatments

Ten ml Rorison 's nutrient solution (Hendry & Grime, 1993) (Appendix 2) was added at different intervals to the sand treatment (every 1, 2, 3, 4, 5, 6, or 7 days). In the control sand treatment, nutrient solution was replaced by distilled water. Distilled water only was added to the compost treatments.

2.2.2.2 Comparative growth experiments

Seeds of six *Brassica* species were germinated in clear plastic pots (9.0 cm inner diameter at the top and 5.0 cm at the bottom) which contained a mixture of SHL and John Innes potting compost (1:1 by volume) (50 ml/pot). Eight replicates of ten seeds per pot were used for each treatment. The NaCl treatments (over the range 250 to 600 mM) were applied instead of water five days after sowing and every five days thereafter throughout the experiment. Plants were harvested thirty days after sowing. Fresh weight was recorded for every plant per pot and dry weight determined after 72 h at 75 °C. This experiment was repeated three times and the data were pooled (24 replicates) for analysis by ANOVA used Minitab Version 12.

Potassium and sodium analyses

Dried shoots of each treatment were pooled and placed in a draft oven at 550 °C for 24 h to obtain ash samples. Five ml of concentrated hydrochloric acid was added to the ash samples and the mixture boiled for 5 min then transferred to a beaker and the volume adjusted to about 40 ml with distilled water and boiled for 10 m. This was cooled, then filtered through glass wool into a flask and the beaker rinsed with distilled water into a 50 ml volumetric flask. The concentrations of Na and K were then determined by atomic absorption spectrophotometry using a Varian Model AA-200 (Johnson and Ulrich, 1959). Data were analysed using Analysis of Variance used Minitab Version 12.

2.2.3 Selection for salt resistance

2.2.3.1 General response to sodium chloride

The experiment was carried out to determine the NaCl concentration at which maximum inhibition of growth occurred. Seeds of six *Brassica* species were sown in plastic pots. Ten seeds per pot and eight pots were used for each treatment and pots were arranged in a completely randomised design in plastic trays under controlled condition.

NaCl concentrations (0, 500, 550, 600, 650, 700 mM) were applied. Selected level of NaCl concentrations for each six *Brassica* species were presented in Table 2.1.

Table 2.1: Selected level of NaCl concentrations for each six *Brassica* species

Brassica species	% Survival seedlings	Selected level of NaCl
B. rapa	10%	650 mM
B. nigra	7.5%	650 mM
B. oleracea	0%	500 mM
B. juncea	12.5%	600 mM
B. napus	2.5%	700 mM
B. carinata	11.3%	700 mM

2.2.3.2 Selection for resistance to NaCl

The levels of NaCl used for selection were determined from the first experiment as those giving inhibition of shoot growth (Table 2. 1). Two thousand seeds of each species, one hundred seeds were sown in plastic module trays containing 10 ml compost per plug (SHL and John Innes potting compost 1:1 mixture by volume). Irrigation water and saline solution were regularly applied by base watering onto capillary matting. Very few (10 %) individuals grew well out of the two thousand seeds in each species of *Brassica*

species except *B. oleracea* which had no seedlings after four irrigations with NaCl solution.

Surviving seedlings were transplanted singly into compost filled plastic pots. At the bud development stage, plants were covered by a cloth net cover frame (42 x 50 x 80 cm) to allow controlled pollination by hand using a fine camel hair brush (blowflies were unavailable at this time). At maturity, water irrigation was terminated for 2-4 weeks and then plants harvested and measurements of grain yield taken after rubbing out seeds from seedpods. The growth room temperature was 15-20°C and a 24 h day was maintained by supplementary lighting (sodium vapour lamps) during the growth period.

2. 3 Results

2.3.1 Mass production of seeds

Fertilisation and seed set was not uniform over all of the species with *B. nigra* and *B. carinata* in particular giving poor seed yield (Table 2.2). This necessitated repeat growth cycles of these species in order to generate sufficient seed populations.

Table 2.2 Grain yield for *Brassica* species per 100 seeds sown

Species	Weight of seeds (g)	Weight of 100 seeds(g)	Approximate No. of seeds
B. rapa	6.43	0.146	4404
B. nigra	0.60	0.20	498
B. oleracea	4.36	0.12	3657
B. juncea	10.32	0.18	5863
B. napus	2.73	0.15	1796
B. carinata	0.06	0.10	61

2.3.2 The effect of salinity on germination and seedling growth

Experiment 1: *in-vitro* germination

Low salt concentrations (50-100 mM) appeared to stimulate germination percentage but were not statistically significant and above 100 mM the % germination declined with increasing NaCl concentrations. Percentage germination of *B. napus* was more sensitive to NaCl than *B. rapa* with a pronounced fall between 100 and 200 mM (Fig.2.2). Analysis of fresh and dry weight also indicated that *B. napus* was more sensitive than *B. rapa* (Fig. 2.3 & Fig. 2.4). At low concentrations of NaCl (50-100 mM) germination % was not affected but subsequent plant growth was reduced. The critical NaCl level for *B. rapa* was determined as 250 mM whereas for *B. napus* it was 150 mM NaCl.

Figure 2. 2: % of germination of *B. rapa* and *B. napus* at different NaCl concentrations (bars = SE)

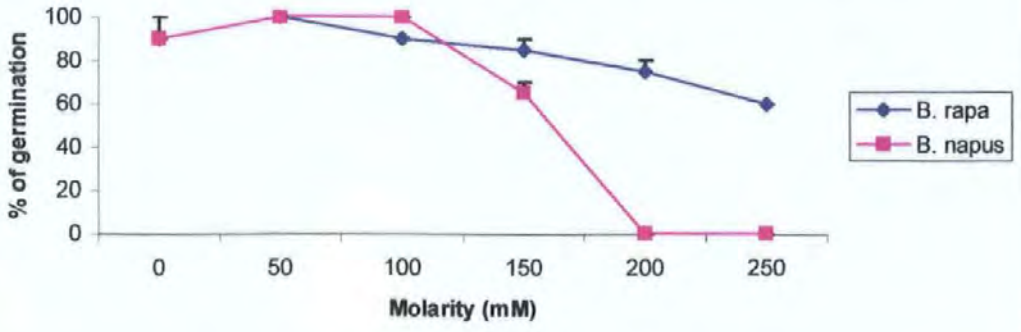


Figure 2. 3: % of fresh weight relative to control per plant of *B. rapa* and *B. napus* at different NaCl concentrations

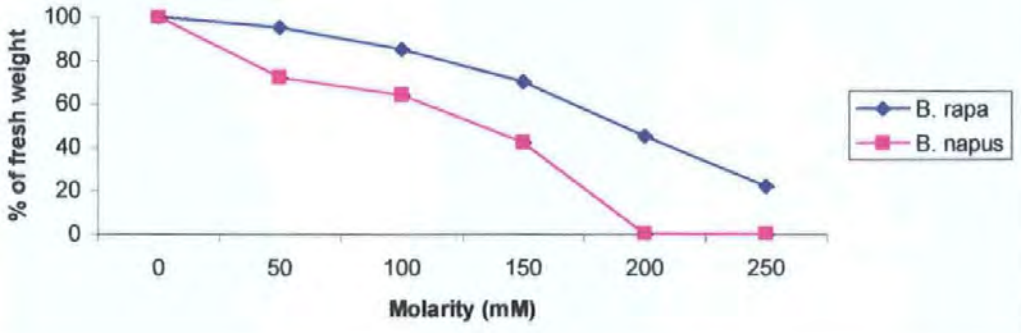
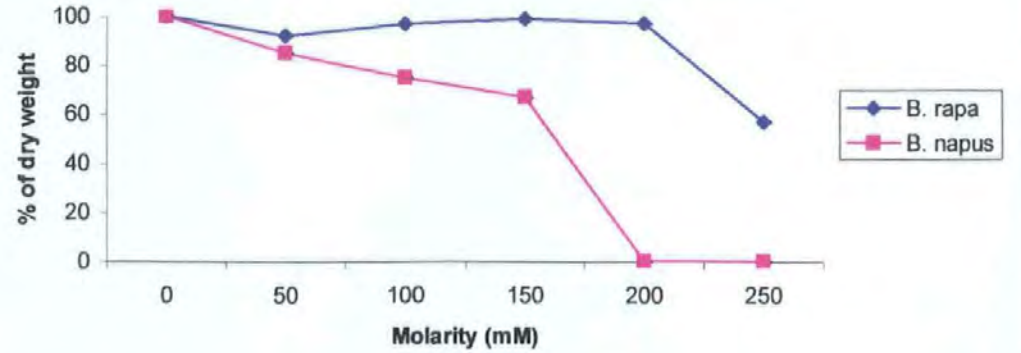


Figure 2. 4: % of dry weight relative to control per plant of *B. rapa* and *B. napus* at different NaCl concentrations



Experiment 2: Sand culture and salt stress

In sand culture all of the plants looked weak and the leaves were withered and yellow in colour even at 0mM NaCl. Measurements of growth showed no consistent effects of salt on cotyledon, hypocotyl length, and stem length, number of leaves and fresh weight (Fig.2.5, 2.6, 2.7 & 2.8). Analyses of Variance showed no significant differences among the different NaCl concentrations although there was a tendency for a depression in stem length and an increase in fresh weight with increasing salt concentration (Fig. 2.6 & 2.8).

Experiment 3: compost and sand culture

As in exp. 2, sand-grown plants were weak, small and yellow. Analysis of data for germination percentage indicated no significant differences between compost and sand culture (Fig. 2. 9) but comparison of fresh and dry weight (Fig. 2.10 &11) showed that compost grown plants had a significantly greater fresh weight of 3 to 4 times that of sand grown plants and a dry weight nearly 2 times greater (Plate 2.1.). This indicated a major physiological effect of compost culture on RCB's i.e. a raising of plant water content. There were no significant differences between the varying nutrient applications in sand culture. The results clearly showed that the addition of Rorisons nutrient solution could not compensate plant growth as well as compost (Plate 2.1).

Figure 2. 5: Left cotyledon and right cotyledon width per plant of *B. rapa* grown in sand culture at different NaCl concentrations (bars =SE)

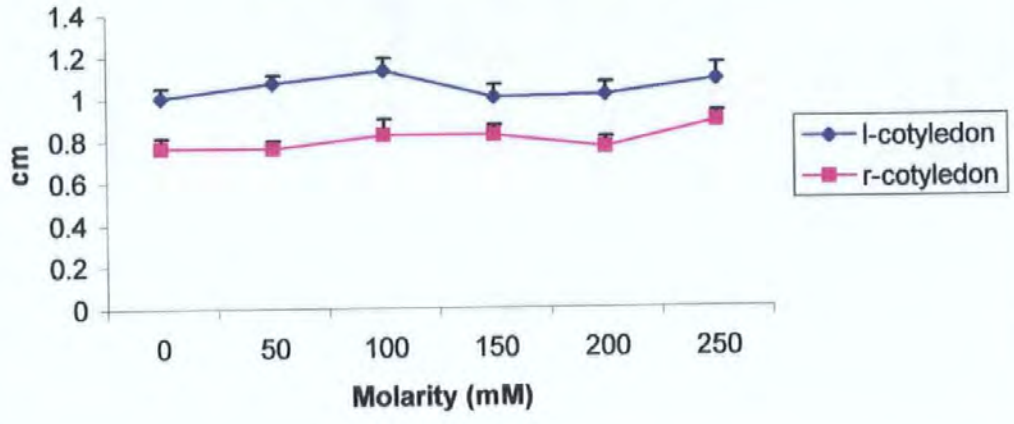


Figure 2. 6: Hypocotyl and true stem length per plant of *B. rapa* grown in sand culture at different NaCl concentrations (bars = SE)

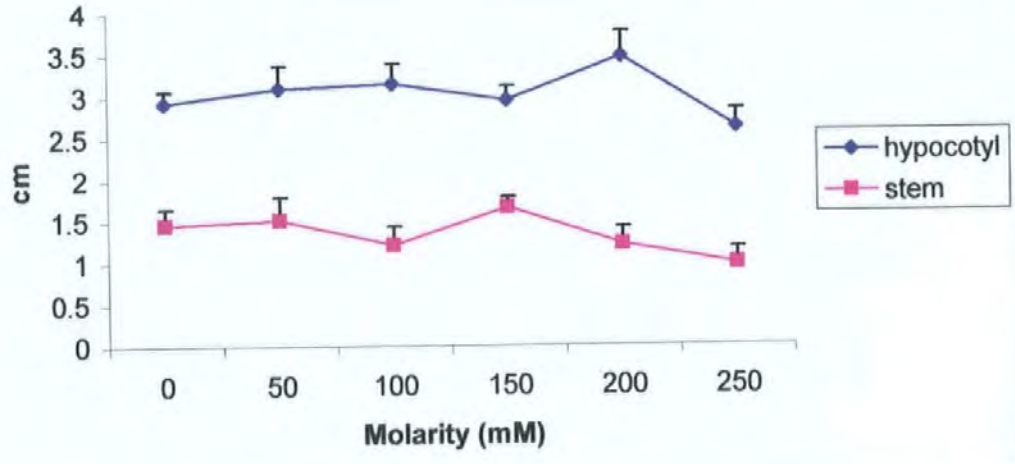


Figure 2. 7: Number of leaves per plant of *B. rapa* grown in sand culture at different NaCl concentrations (bars = SE)

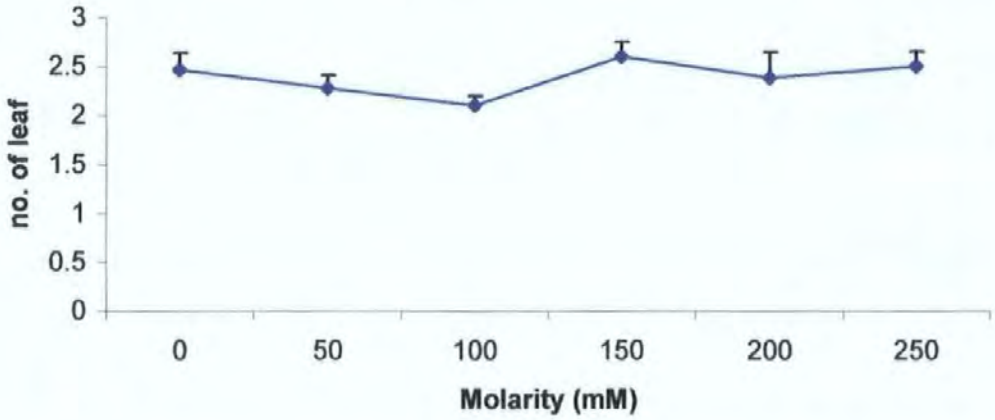


Figure 2.8: Fresh weight per plant of *B. rapa* grown in sand culture at different NaCl concentrations (bars = SE)

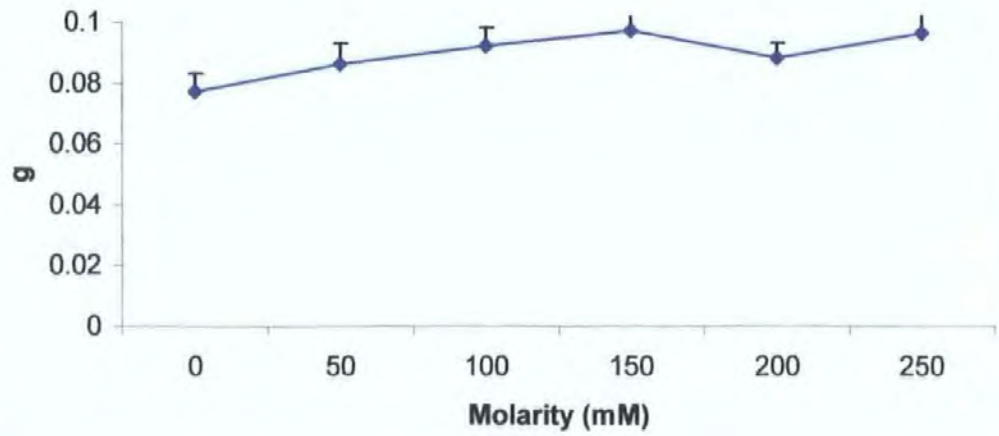


Figure 2. 9: % of germination of *B. rapa* grown in sand with various additions of Rorison's solution and in compost (bars = SE)

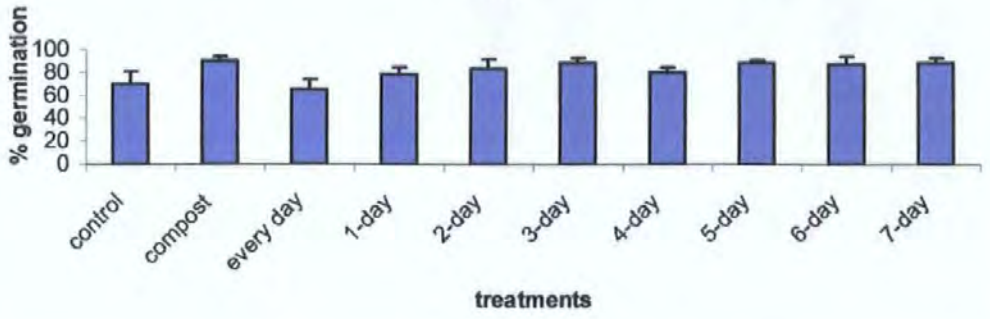


Figure 2. 10: Fresh weight per plant of *B. rapa* grown in sand with various additions of Rorison's solution and in compost (bars = SE)

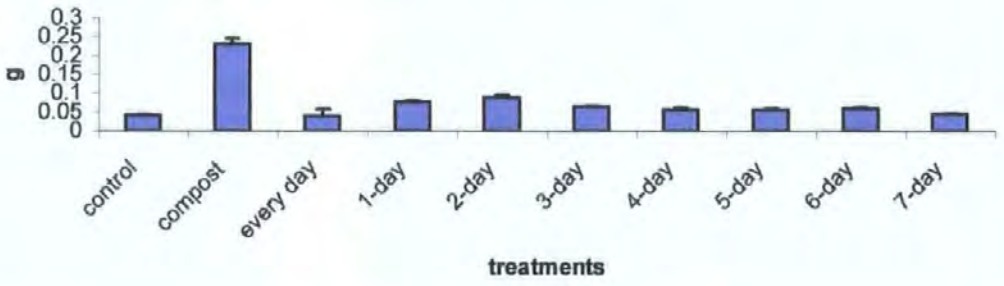


Figure 2. 11: Dry weight per plant of *B. rapa* grown in sand with various additions of Rorison's solution and in compost (bars = SE)

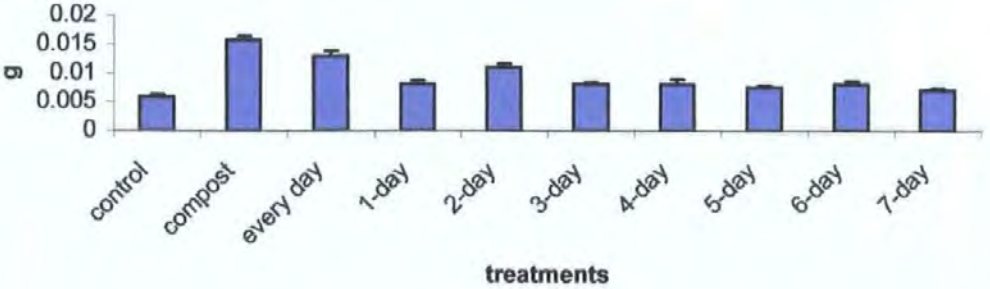
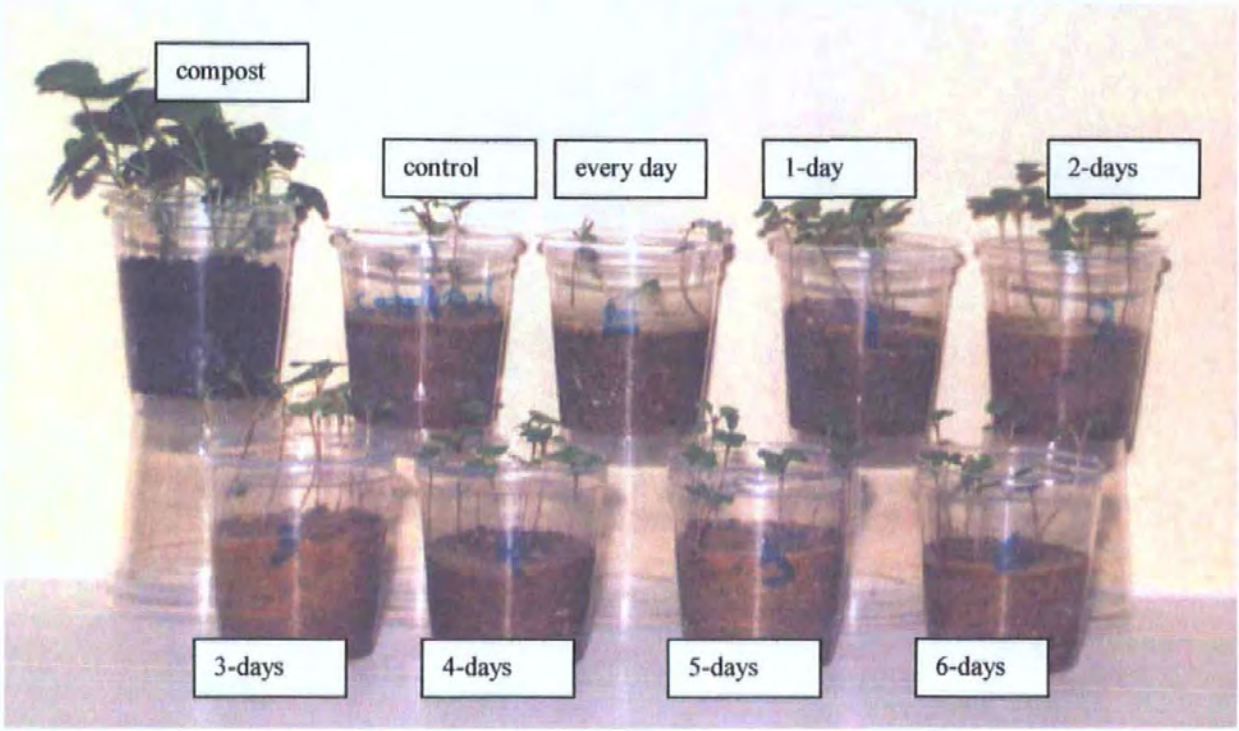


Plate 2.1: Plants of *B. rapa* grown in compost and in sand culture plus nutrient solution



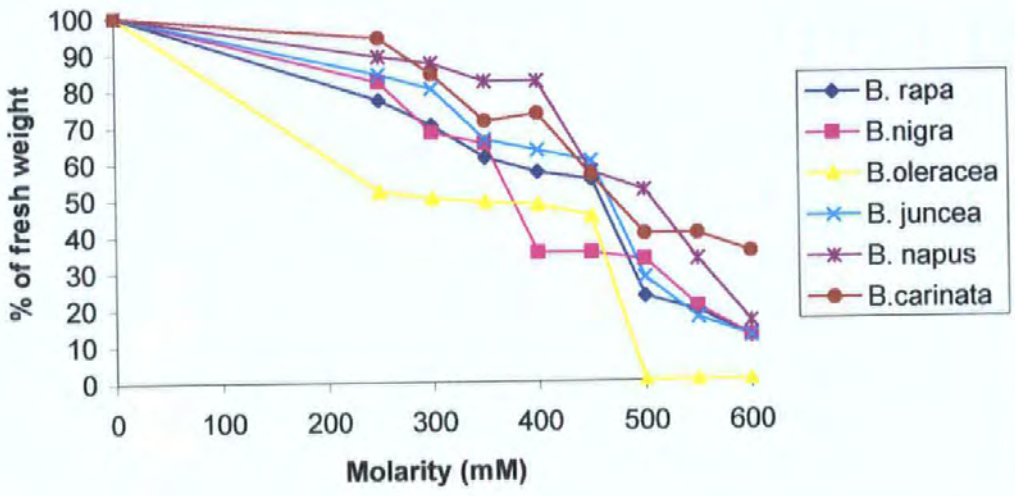
2.3.3 The effects of salinity on plant growth *in-vivo*

2.3.3.1 Fresh weight and dry weight

Analysis of variance of data showed that species differed significantly ($p \leq 0.05$) in their responses to different NaCl concentration and that increasing NaCl concentration reduced the fresh weight. Overall the inhibitive effect of NaCl on the fresh weight was almost proportional to the concentration of NaCl applied (Fig. 2.12). Fresh weights of shoots of *B. napus* and *B. carinata* were significantly greater ($p \leq 0.05$) than those of *B. rapa, B. oleracea* and *B. juncea*.

In order to take this into account comparative species data was compared on a percentage basis relative to controls (Fig. 2.12). At 250 mM NaCl, there was a great reduction in relative fresh weight of *B. oleracea* and there were proportionately smaller reductions in fresh weight relative to control in all other five species (Fig. 2.12). With the increase in the concentration of NaCl, the relationship between the salinity and the relative fresh weight of *B. rapa, B. juncea* and *B. oleracea* was unchanging until 450 mM. The relative fresh weight of *B. nigra* declined dramatically at 400 mM NaCl whereas those of the relative fresh weight of *B. napus* and *B. carinata* declined dramatically at 450 mM. *B. oleracea* showed that a decline in the relative fresh weight was slightly increased at all treatments but it was completely inhibited above 450 mM. At the highest NaCl concentration used (600 mM), the relative fresh weight of *B. carinata* was the highest of all species tested (Fig. 2.12).

Figure 2.12: % of fresh weight relative to control per plant of 6 *Brassica* species at different NaCl concentrations



The results for relative dry weights of shoots of the 6 *Brassica* species were very similar to the fresh weight (Fig. 2.13). Analysis of Variance of data for relative dry weight indicated that there were significant ($p<0.05$) differences between control and all NaCl treatments and significant differences among the six *Brassica* species, but no significance difference for the interaction between species and treatments.

The response patterns of plant growth to NaCl treatment were complex and made it difficult to distinguish the difference between the species in salt resistance since the response of a given species could be higher than another species at one NaCl treatment but lower at another. However, these six *Brassica* species could be roughly categorised into three groups according to their relative resistance 1- the most salt resistant species were *B. napus* and *B. carinata*; 2- the moderately salt resistant species were *B. rapa*, *B. nigra*, *B. juncea;* 3- the least salt resistant species was *B. oleracea.*

2.3.3.2 Potassium and sodium analyses

The data showed that salinity had profound effects on ion concentration in the shoots of the six *Brassica* species tested. Potassium concentration tended to reduce with increase in the NaCl concentration applied up 450 mM but then became erratic (Fig. 2.14). It is possible that below 450 mM K determination is confounded by the very small plant sample sizes as a result of the high NaCl concentrations. Sodium concentration showed the opposite trends, generally increasing but then also becoming more erratic (Fig. 2.15). Analysis of Variance of data for K showed that there were significant ($p\leq0.05$) differences between the control and all NaCl different treatments and significant differences between species but no significant differences for the interaction between species and treatments. Similarly, Analysis of Variance of data for Na showed significant ($p\leq0.05$) differences between the control and all NaCl treatments and significant

Figure 2. 13: % of dry weight relative to control per plant of 6 *Brassica* species at different NaCl concentrations

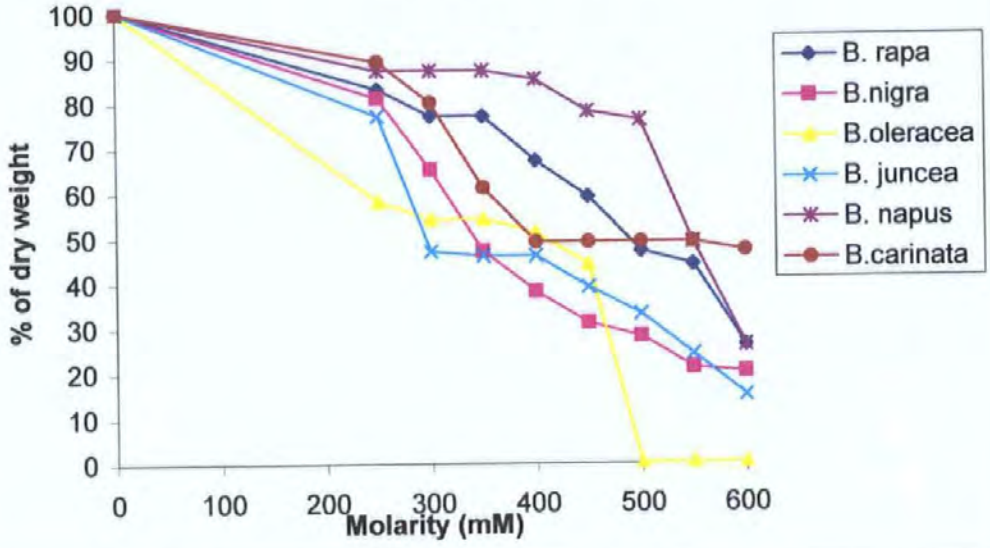


Figure 2. 14: Potassium concentration of plant tissue of 6 *Brassica* species at different NaCl concentrations

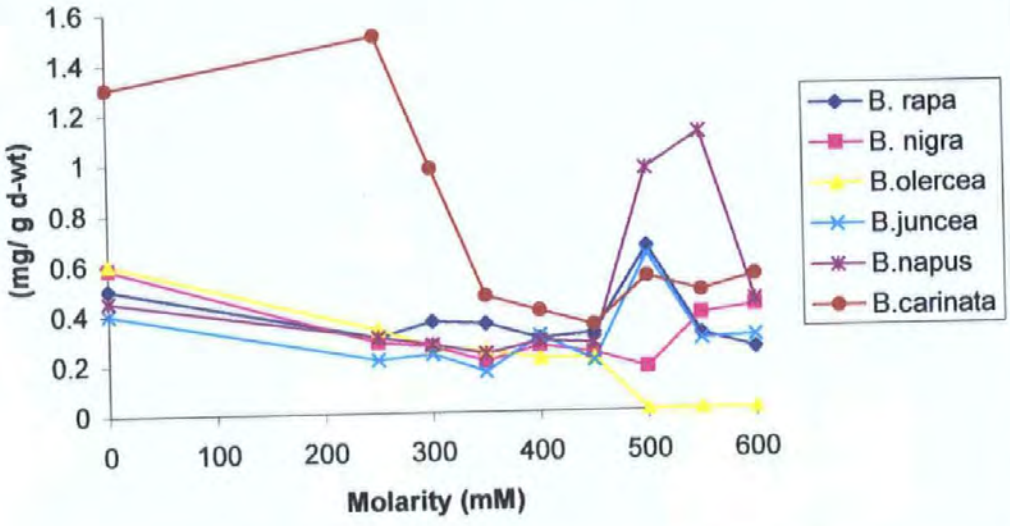
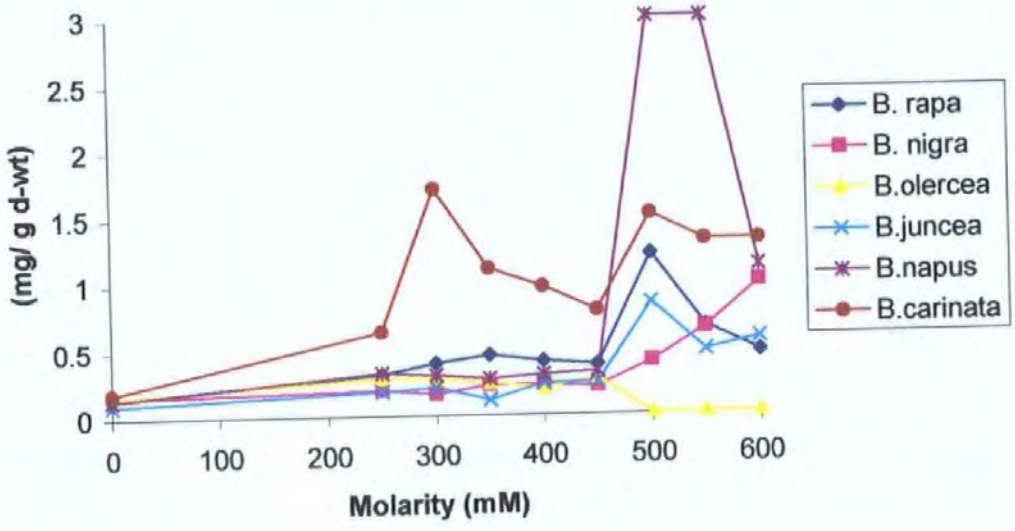


Figure 2. 15: Sodium concentration of plant tissue of 6 *Brassica* species at different NaCl concentrations



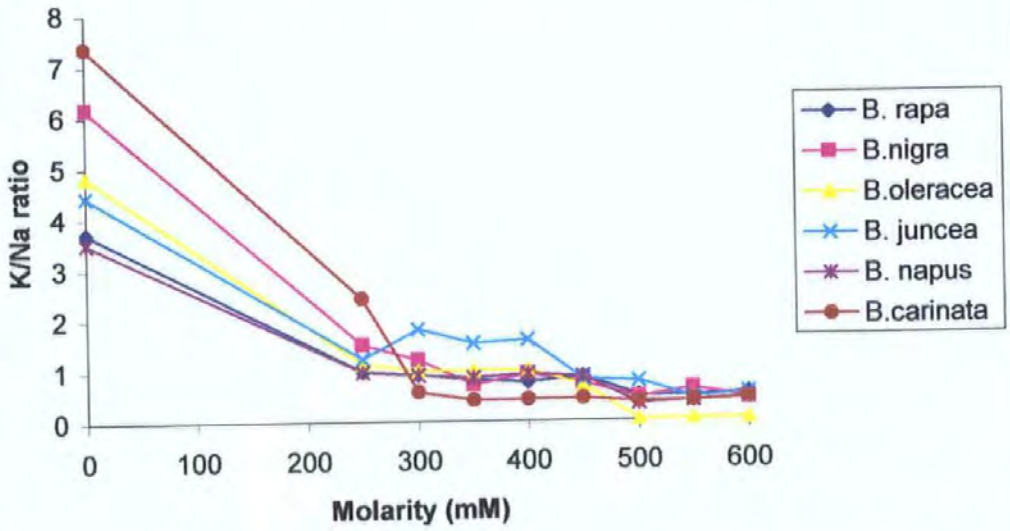
differences between *B. caranita* and the other five *Brassica* species. Overall correlation of K concentration and Na concentration was r= -0.5 (df = 100).

When the varying K and Na concentration was expressed as a K/Na ratio the apparent erratic behaviours of these compounds reduced and K/Na ratio was inversely correlated with increased levels of NaCl concentration (Fig.2.16). K/Na ratio was reduced by approximately 76 to 91 % relative to respective controls and most of this reduction occurred between 0 and 250 mM. Analysis of Variance of data for K/Na ratio showed a significant difference between the control and all NaCl treatments but no significant difference among the six species.

2.3.4 Selection for salt resistance

The results showed that ten percent of *B. rapa, B. nigra; B. juncea* completed their first selection cycle through to seed yield stage after moving to fresh compost. In the second selection cycle, they responded to selection pressure in the same manner as the control population and were unable to complete their reproductive stage. *B. oleracea* had no surviving seedlings at the selection pressure of 500 mM NaCl. *B. napus* and *B. carinata* were unable to complete their first selection cycle after moving to fresh compost culture.

Figure 2. 16: K/Na ratio of plant tissue of 6 *Brassica* speciesat different NaCl concentrations



2.4 Discussion

2.4.1 The effect of salinity on germination and seedling growth

Experiment 1 *in-vitro* culture

The experiment showed that low salt concentrations (50-100 mM) did not affect germination although fresh and dry weight declined. This could indicate that there is no association between factors that affect seed imbibition and factors that affect seedling growth under NaCl concentrations. This was also found in Rice (Zeng and Shannon, 2000). Zeng and Shannon (2000) reported that seed germination was not significantly affected up to 16.3 dSm^{-1}, but was severely inhibited when salinity increased to 22 dSm^{-1} in rice. The suppression of germination at high salt levels might be mainly due to osmotic stress. Whereas the reductions in fresh weight and dry weight of plant are caused by specific ion toxicity an explanation supported by Allen (1984), Blum (1988) and Chauhan and Prathapasenan (2000).

The apparent greater tolerance of *B. rapa* may be due to more growth than *B. napus* or differential uptake of salt at this stage. Francois (1984, 1994) found that *B. rapa* was more salt tolerant at germination than at later stages of growth. In this study, *B napus* was more sensitive to NaCl than was *B. rapa* despite previous reports that *B. napus* is a relatively less salt tolerant species (Ashraf *et al.* 1987; He and Cramer, 1992). Such differences may be cultivar specific and / or growth stage based and RCB's may differ to commercial varieties of *Brassicas* (Madder, 1976; Norlyn, 1980).

Experiment 2: Sand culture and salt stress

There were no significant differences among NaCl treatments for all growth parameters in sand culture. The data showed that NaCl treatments sometimes out-performed the control in *in-vivo*. Perhaps sand culture might actually buffer the growth response of salt-tolerant plant, whereas plants that happen to be located in slightly less saline areas might

appear more tolerant, but in reality are not. Another explanation is offered by Shannon (1984) who recommended that saline solutions for screening studies should be composed of a realistic mixture of salt, not just NaCl. Salt imbalance in the nutrient solution is the most frequent deficiency in screening studies. In this study, NaCl only was used as the saline solution.

This result was the same as that of Basalah (1991) who found that the length, and the fresh and dry weight of root and shoot of squash seedlings increase as the salinity level increased up to 8 mS/ cm^{-1} EC (equivalent to about 100 mM NaCl). Williams and Ungar (1972) reported that seedlings of *Suaeda depressa* (push) grew better at 170 mM than 0 mM NaCl, when nitrogen was not limiting. The experiment clearly showed that pure sand culture was a poor medium for RCB's.

Experiment 3: compost and sand culture

The lack of significant differences for germination percentage between compost and sand culture and nutrient solution indicated that the stress imposed did not interfere with germination processes. Fresh weight and dry weight of plants provide an estimate of the level of investment of photosynthetic products required for growth. Plants grew more successfully in compost when compared with either control (water only) or where nutrient solution was applied to sand culture. This suggested that the compost provided better (though unspecified) supplies of nutrients and formed a good substrate for rooting. Other workers have also reported problems with sand culture (Booth *et al.*, 1993). Herrera *et al.* (1997) investigated the effect of compost on the growth of seedlings of *Angelica arch angelica* and concluded that plant shoot dry weight and mineral content increased with increasing percentage of compost in the media. With regard to the sand culture system used here, it appeared to have a good drainage rate and low water retention and leaves may have been damaged by the daily addition of nutrient solution

that may have accumulated as osmotically active salts present in the sand. These salts may be as potassium, nitrate and chloride resulting directly from the accumulation of residues of the liquid Rorison nutrient solution in excess of plant needs (Rowell, 1994) analogous to irrigation induced salinization in the field. The important physical properties of the compost will be its total pore space, air capacity, the amount of water available at low tension and its bulk density and its cation and anion exchange capacity that helps remove osmotically active nutrients from the soil solution reducing the Electrical conductivity and osmotic effect.

In conclusion the compost gave the most optimum growing medium for Rapid Cycling *Brassica* species and was chosen as the growing medium for all subsequent *in vivo* experiments.

2.4.2 The effects of salinity on plant growth *in-vivo*

Fresh weight and dry weight

Data for mean fresh weights and dry weight of shoots of the six *Brassica* species showed that increasing NaCl concentration reduced the fresh and dry weight. The reductions in fresh weight and dry weight might result from the excessive salt uptake inducing nutritional imbalances or deficiencies (K relation to Na) which has effects on enzyme proteins and membranes. Under salt stress, little energy is produced by photophosphorlation and by phosphorylation in the respiratory chain, nitrogen assimilation is impaired and protein metabolism disturbed causing impaired growth. This interpretation is supported by Yang *et al.* (1990) who demonstrated that the reduction in dry weight may reflect the metabolic energy cost associated with salt adaptation, reduced photosynthetic rates, reduced C gain, salt injury to tissue and attainment of maximum salt concentration tolerated by full expanded leaves.

B. oleracea was killed completely at 500 mM (similar to the concentration of seawater) which might result from a rapid rise in salt concentration in the cell walls or cytoplasm when the vacuoles could no longer sequester incoming salt which caused plant die. Another explanation by Huang and Redmann (1995) proposed that the death of old leaves due to build up of salt in the tissue would prevent the supply of nutrients to emerging leaves, leading eventually to death of the plant. Kingsbury *et al.* (1984) found that sensitive species were more impaired by salt stress than that of resistant ones, due to a greater osmotic shock, reduced photosynthesis and C gain resulting in lower growth in these species.

Among the six *Brassica* species tested, *B. napus* and *B. carinata* had the smallest growth reduction, indicating that these species were more salt-tolerant, as confirmed in other studies (Ashraf and McNeilly, 1990; Chandler *et al.*, 1986). He and Cramer (1995) considered that the most salt tolerant *Brassica* species was *B. napus*; the moderately salt sensitive species were *B. rapa, B. nigra, B. juncea* and *B. oleracea;* the most salt sensitive species was *B. carinata* whilst Kumar (1995) suggested that the amphidiploid *Brassica napus, B. carinata* and *B. juncea* are more tolerant of salinity than their respective diploid progenitors, *B. rapa, B. oleracea* and *B. nigra.* The experiments reported here tended to support the findings of Kumar more than He and Cramer.

Potassium and Sodium analyses

Salinity caused changes in ion concentrations in the shoots of the six *Brassica* species. Data showed that Na concentration in the shoot increased with increasing concentration of NaCl applied and was accompanied by a decline in K concentration. This decline in tissue K concentration may result from the direct competition between K and Na at sites of uptake at the plasmalemma, an effect of Na on K transport into the xylem (Lynch and Lauchli 1984) and/ or a Na-increased K efflux from the root (Cramer *et al.* 1985) or

indirect inhibition of the uptake process in other aspects, for example, H-ATPase activity (Gronwald *et al.*, 1990; Suhayda *et al.*, 1990). It is a common finding that high concentration of Na in external solutions causes decreases in K in the tissue of the plant (Greenway and Munns, 1980; Rathert, 1983).

Ion effects have been considered to be related to salt tolerance (Cheeseman, 1988; Gorham *et al.*, 1985; Greenway and Munns, 1980). One hypothesis associated with the relationship between salt tolerance and ion effects is that there is a difference between plant species in degree of toxicity of Na and Cl to growth but excessive accumulation of Na will always lead to the reduction of growth in the long term (Munns and Termaat, 1986).

In these results, the concentration of Na in the shoot of the resistant *B. napus* did not appear to be lower than that of other *Brassica* species. It could be that the measurement of bulk tissue ion concentration masks spatial differences that may actually occur and certainly ions can be preferentially accumulated in certain cells or cellular compartments. Ashraf (1994a) reported that salt tolerant species or cultivars accumulate considerable amounts of salt in their leaves (rice and maize). Van Steveninck *et al.* (1982) demonstrated that *Lupinus luteus* was more salt resistance more than *L. angustifolius*, but the former accumulated more Na than the latter.

In this study, the increase in K concentration in the shoots of salt treated plants of *B. napus* was significantly different from the other species, indicating that salt tolerance of *B. napus* was possibly due to its higher concentration of K in the shoot. The higher concentration of K in *B. napus* might indicate that favourable cytoplasm Na: K ratios are maintained by a combination of K: Na uptake selectivity, Na extrusion, vacuolar Na

compartmentation and partitioning of Na away from growing tissues at the expense of old organs (Lynch and Lauchli, 1984).

He and Cramer (1993b) support the argument that ion effects are not responsible for the differences in salt-tolerance among *Brassica* species. Another hypothesis associated with the relationship between salt tolerance and ion effects is that there is a difference among species in capacity to maintain sufficient nutrient concentrations, like K for growth of plants under salt stress.

The results for K/Na ratio showed a decrease with increasing concentration of NaCl applied. Clearly tissue K concentration decreased whilst tissue Na concentration increased suggesting an overall replacement of K by Na in *Brassicas* under salt stress. This decreased K/Na ratio in the shoot has been explained in two conventional ways:
1- A substantial passive Na transport to the shoot is combined with the regulation of Na + K concentration as follows; passive Na transport→ increased Na in shoots→replacement of K by Na in vacuoles (Jeschke, 1979). →High K in cytoplasm →K export in the phloem (Jeschke, 1979) and/ or signal to roots for reduction of K uptake. 2- Na + K transport to the shoot becomes saturated, and at high Na rapid mass flow through the outer cortex results in low K/Na at the uptake sites near the stele (Pitman, 1965 & 1966). These alternatives can not be distinguished in this study because the only measured data on K and Na are for the shoot.

Under saline conditions, a decreased K/Na ratio is found in plant species such as cotton (Lauchli and Stelter, 1982), wheat (Gorham *et al.*, 1990) and sorghum (Yang *et al.*, 1990). Seawater salinity reduced the K/Na ratio in the shoots of six rapid cycling *Brassica* species (He and Cramer, 1993b). This reduction directly resulted from a decrease in shoot K concentration and an increase in shoot Na concentration and shoot

K/Na ratio was positively correlated with growth reduction within the *Brassica* species (He and Cramer, 1993b; Lauchli and Stelter, 1982; Rathet, 1983).

The interspecific difference in the shoot K/Na ratio was not found to be correlated with the relative salt tolerance of Brassica species, although preference for K over Na is correlated with salt tolerance for many species (He and Gramer, 1993b; Lauchi and Stelter, 1982; Rathert , 1983; Yang *et al.*, 1990.) Variety differences in salt tolerance with similar ion concentration may be due to relative ion distribution (Greenway and Munns 1980). Both ion exclusion and ion inclusion mechanism to resist salt stress are dependent on many other physiological process such as ion redistribution from one leaf to another or ion compartmentation with the cell, i.e., the ions are not contained in compartment, such as vacuoles, separated from cellular sensitive sites.

2.4.3 Selection for salt resistance

Results showed that ten percent of plants of *B. rapa*, *B. nigra*, *B. juncea* managed to complete the first selection cycle through to seed yield stage but they failed to manage their second selection cycle. Perhaps these original survivors were just escapes from the selection pressure for unknown reasons and no true selection for salt resistance occurred. *B. napus* and *B. carinata* grew well under high level of NaCl but also were not able to complete their life cycle. It is possible that *B. napus* and *B. carinata* plants are able to tolerate salt stress for longer duration before significant reduction in growth and survival occurs. Alternatively roots of *B. napus* and *B. carinata* seedlings may still have had high sodium concentration which caused late ion toxicity which led to reduced growth rates and eventual plant death an explanation supported by Chauhan and Prathapasenan (2000) and Zeng and Shannon (2000). Lazof and Berntens (1999) demonstrated that high salinity could cause disturbance in mineral supply to the shoot, either excess (Na excess) or deficiency that might directly affect plant growth.

2.5 Conclusion

In the study reported here, salt stress was effectively applied to plants to cause reductions in growth and alteration in the K: Na ratio in shoots in accordance with other workers. Salt stress over the range 250-600 mM had similar effects on growth for most of the species although *B. napus* and *B. carinata* were reduced in growth proportionally less in response to increasing salt concentration. *B. oleracea* had the clearest cut-off point for NaCl tolerance being totally killed above 450 mM. The other species showed some continued survival and growth above this point. This may indicate more genetic variability in the species other than *B. oleracea*. Since RCB's are maintained by open pollination then such variability is to be expected. However conventional selection for salt resistance was not achieved in these experiments.

Chapter 3: Investigation of salt resistance in tissue culture

3.1 Introduction

Cell and tissue culture techniques offer a number of advantages not found in the conventional selection and breeding procedures currently used to enhance tolerance of plants to saline environments. They also offer opportunities to help to quantify compartmentation leading to a better understanding of the process of osmoregulation. Tal (1984) and Epstein and Rains (1987) discussed some of the advantages in using tissue culture for physiological studies for salt resistance and these have been adapted and summarised as: 1- experiments can be performed year- round since the growth of tissue culture is independent of seasonal fluctuations, 2- tissue culture can be treated uniformly in a controlled environment with controlled nutrient conditions for manipulation, 3- relatively homogeneous populations of cells can be developed in tissue culture as compared with heterogeneous whole plants, thus giving a potential tool to study the effect of stress on various components of growth, 4- a relative lack of differentiated tissues which reduce complications arising from morphological variability, 5- characterisation, at the cellular level, of physiological markers associated with salt tolerance, which can be used for studying mechanisms of salt tolerance on both cellular and whole plant levels, 6- the contribution of different parts of the plant to the response of the whole plant can be determined by studying their response in culture, 7- in cell culture systems millions of cells can be screened and evaluated for their performance in a relatively small area and increase the probability of obtaining variants with desired characters 8- utilisation of new procedures have been developed such as haploid production, somatic hybridisation, gene transfer and mutant induction and selection, 9- protoplasts can be created which are especially useful for studying the involvement of the surface membrane in stress injury.

More recently, Hasegawa *et al.* (1994) have suggested that the value of *in-vitro* experiments is to further the understanding of the cellular basis of tolerance in plants. There are numbers reports of plants regenerated from *in-vitro* cultures that exhibit greater salt tolerance than the original genotype. There are, however, a number of difficulties in applying cell culture procedures to the selection and genetic manipulation of plant cells for salt tolerance. Deane (1994) referred to the disadvantages that surviving callus may consist of a mixture of resistant and sensitive cells which escaped the selection pressure (chimeric callus). Furthermore, cells of the required phenotype may be surrounded by dead or non-growing cells and will be difficult to detect and also cells may acquire resistance through the production of a compound which may be transferred to adjacent sensitive cells, conferring temporary resistance leading to "false positives". Epstein and Rains (1987) illustrated that the character under selection isolated at the cellular level will have to be expressed at the whole plant level. This will require regeneration of the cells to whole plants and although the list of plant species capable of regeneration continues to increase not all species under all conditions can be regenerated. Stavarek *et al.* (1980) and Stavarek and Rains (1984) considered that a major potential problem with cell culture systems is that plants must be regenerated from the selected cells. One difficulty is the effect of culture time on regeneration frequency; the longer cells are maintained in culture the more difficult it is to regenerate these cells to plants. Concomitantly long-term cultures show enhanced variability in characters unrelated to the selected character resulting in a very heterogeneous population of regenerated plantlets with the target trait potentially masked by the non-targeted traits.

It is essential that the regenerated plants maintain the characteristics of the original plant genotype while incorporating the desired genetic character but without incorporating undesirable genes. This is not always the situation using cell culture techniques (Meins, 1983). Rowland *et al.* (1989) and Winicov (1991) concluded that there are no known

instances of increased salt tolerance being expressed unequivocally in regenerated plants and no published evidence of improvements being expressed in the field, but improvements could be demonstrated under controlled environmental conditions. Dracup (1993) considered that the lack of success of *in-vitro* selection and the frequent survival of cultures at relatively high NaCl concentrations might be due to many reasons such as; 1- many responses of plants to high NaCl are associated with the functional integrity of the whole plant, 2- the nature and availability of solutes is very different for cultured cells than for cells in whole plants, 3- slow growth of cultured cells would make them less sensitive to water deficit than growing cells and less sensitive to ion toxicity than non-growing cells in whole plants, 4- cell culture media have lower water potential than culture solutions used for whole plants, although the transpiration-induced difference in water potential between leaves and roots is often of a similar magnitude.

Despite these concerns, the literature does contain evidence of apparent successful selection for salt resistance via tissue culture. Nabors et *al.* (1980) found that tobacco plants regenerated from selected cell lines showed greater survival rate when watered with saline water. Croughan *et al.* (1987) demonstrated that sodium chloride selected alfalfa cells have been regenerated to plants but with no improvement in tolerance to salinity. Woo *et al.* (1985) and Wong *et al.* (1986) reported successful attempts by selecting callus with up to 257 mM NaCl and some of their regenerates showed increases in fresh weight, height and survival rates compared to controls under salt stress. They found different sorts of variants arising from the same callus possibly because their selection period was only 30-40 days but stability and heritability of the increased tolerances was not reported. Pandey and Ganapathy (1984) selected NaCl tolerant calli of *Cicer arietinum* and found that tolerance persisted in the absence of stress for 12 weeks. Salgado *et al.* (1985) reported similar results for callus of sweet potato. Chandler and Vasil (1984) selected *Napier* grass callus for 30-40 weeks in medium containing 214

mM NaCl which was gradually increased to 343 mM, when a tolerant callus became necrotic, it was moved to salt-free medium where it recovered. But in these experiments the recovered callus showed no retention of tolerance and regenerated plants were actually less tolerant to salt than controls. Pua and Thorpe (1986) and Chandler *et al.* (1986) has reported experiments to select Na_2SO_4 and NaCl tolerant callus of *Beta vulgaris* L, *B. napus* and *B. campestris* and tolerant callus for the first two species were successful.

Plant cell lines from a number of species have been selected for tolerance to salinity. In most of the examples the enhanced tolerance was documented at the cellular level but not at whole plant level. Minimal success has been achieved however in demonstrating the expression of salt tolerance by the plants regenerated from these salt selected cell lines. Flowers and Yeo (1995) concluded that selection in tissue culture is not a process that can be used simply to generate salt resistant plants in spite of the fact that salt resistant cell lines can readily be selected. This opinion was borne out of their search for Patents registered between 1982-1993, which revealed only a small number of claims (10) for increasing salt resistance. There are just two Patents for *in-vitro* selection of salt tolerant cell lines and regenerated plants with enhanced salt resistance: one for flax (Patent, 1986) and one for alfalfa (Patent, 1991).

So the use of cell culture in NaCl resistance studies sometimes leads to putative resistance callus or lines that lose their tolerance subsequently. It would appear that rigorous testing and retesting to establish stable and heritable tolerant line must always follow selection.

Aim

The aim of these experiments was to establish an evaluation method for salt tolerance of RCB species *in-vitro*.

Objectives

1- To establish a callus system

2- To evaluate a callus system to salt tolerance

3-To examine callus culture grown under saline conditions leading to regeneration.

3.2 Material and Methods

3.2.1 Plant material

Seeds of six Rapid Cycling *Brassica* species (*B. rapa, B. nigra, B. oleracea, B. juncea, B. napus* and *B. carinata*) (Williams and Hill, 1986) were surfaced sterilised using a three step procedure comprising: immersion in 70% ethanol for 30 s followed by immersion in 10 % commercial bleach for 15 m and finally three washes in sterile distilled water.

Surface sterilised seeds were sown into plastic pots (9.0 cm inner diameter at the top and 5.0 cm at the bottom) containing M&S (Murashige and Skoog, 1962) medium, 30.0 gl^{-1} sucrose and solidified with 7.0 gl^{-1} agar and maintained in a growth room at 22 °C in a 16 h light, 8 h dark regime with a radiant flux density of 70μ mol m^{-2} sec^{-1} for 7 days. All subsequent cultures were maintained under the same growing conditions.

3.2.2 Culture media for callus induction and maintenance

The media used were the same as the initial germination medium (M&S, 30.0 gl^{-1} sucrose and 7.0 gl^{-1}) agar and supplemented with auxins either 1.0, 2.0, 5.0, mgl^{-1} 2,4 D (diohlorophenoxyacetic acid) or IBA (indol butyric acid). The media were adjusted to pH = 5.8 and autoclaved at 120 °C for 15 m.

The 7-day-old seedlings were dissected in a sterile laminar flow cabinet to obtain cotyledon, hypocotyl and root explants which were then transferred to the respective culture media following the technique described by Fuller and Fuller (1995). Once callus was obtained it was subcultured every 4 weeks onto the same induction medium.

3.2.3 Regeneration media

In a more detailed study on one of the six *Brassica* species, (*B. nigra*) that initiated callus. investigations to determine the effect of media and cultural conditions on regeneration were carried out. Regeneration media were the germination medium supplemented with 16 various combinations of auxin; 0, 0.1, 1.0, 10 mgl^{-1} IBA (indol butyric acid) and cytokinin; 0, 0.01, 0.1, 1.0 mgl^{-1} Kinetin or BAP (6-benzyl amino purine).

3.2.4 Callus growth in NaCl culture media

Once callus had been obtained, it was used to determine NaCl resistance.

The inhibitory concentration of NaCl in callus culture was determined by fresh weight (fw.s) measurement of 5 mm diameter callus pieces after 4 weeks in Petri dishes (5 per dish) containing germination medium supplemented with 0, 250, 300, 350, 400, or 450 mM of NaCl. Twelve replicates were used for each treatment.

The callus was then sub-cultured back to salt-free medium for a further 4 weeks and the fresh weight re-recorded (fw.r). Mean salinity index was calculated as the average performance for fresh weight for all salinity levels, divided by its control value and expressed as a percentage as suggested by Rana (1986a).

3.3 Results

3.3.1 Initiation and maintenance of callus

Different explants, cotyledon, hypocotyl and root responded differently to the auxin growth regulators (2,4-D and IBA). Callus was induced using 2,4-D at all concentrations whereas IBA had no callus inducing effect and only induced shoots. 2,4-D at 2.0 mg l^{-1} gave the highest frequency (100%) of callus formation and as consequence 2,4 -D was used in the subsequent study for all callus experiments.

3.3.2 Regeneration from callus of *B. nigra*

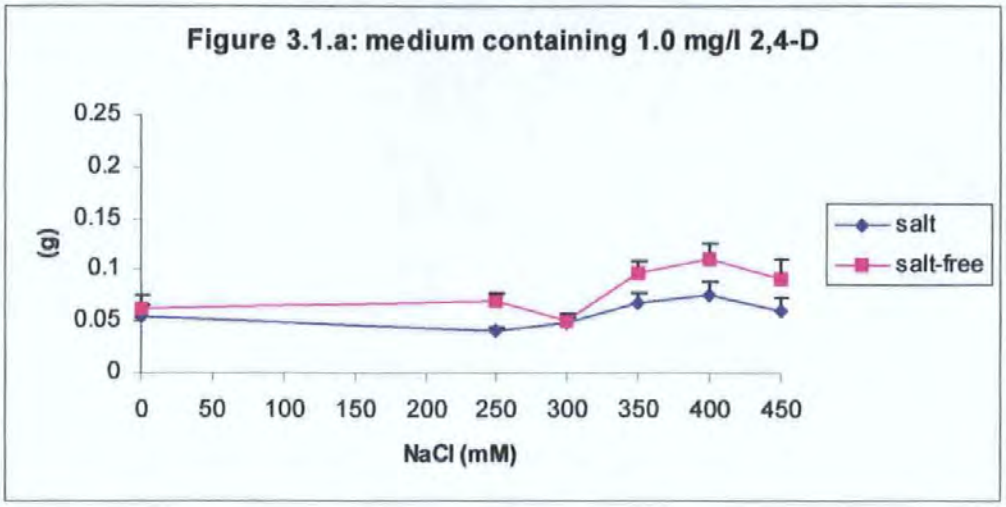
No roots were formed on calli derived from cotyledons over all 16 different combinations of both of growth regulators and the colour of calli was dark brown expect at concentrations of 0.1-0.01 (IBA and BAP) where it was green. Roots were formed on more calli derived from roots than from hypocotyls and the colour of calli was light brown except at concentrations of 0.1-0.01 (IBA and BAP) where it was dark brown in colour. No roots were formed on calli derived from hypocotyls at the concentration of 10.0 mgl^{-1} IBA. Kinetin had no inhibitory effect on the induction of roots in calli derived from hypocotyls and roots. Only 2 calli from roots exhibited shoot formation at concentration of 0.1-0.1 and 0.1-1.0 (IBA and Kinetin).

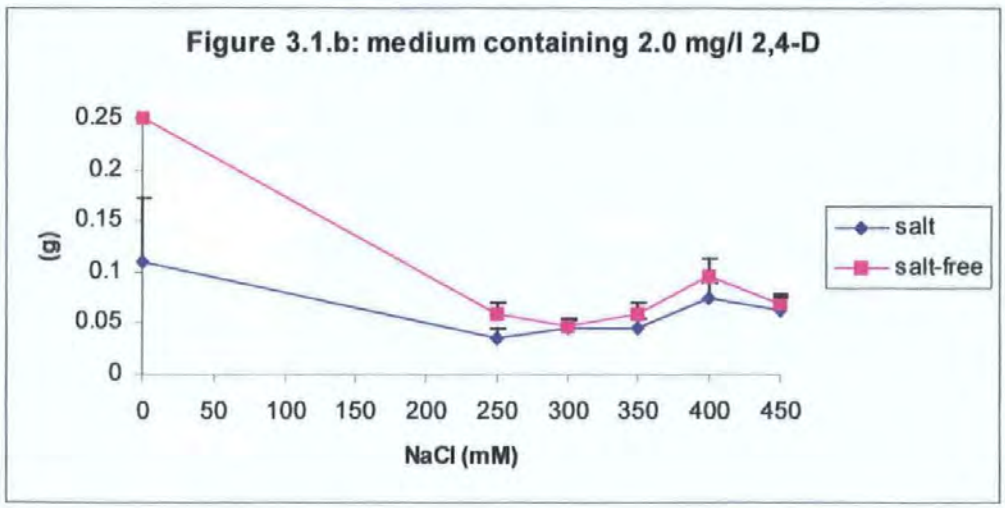
3.3.3 Callus growth measurement in NaCl culture media

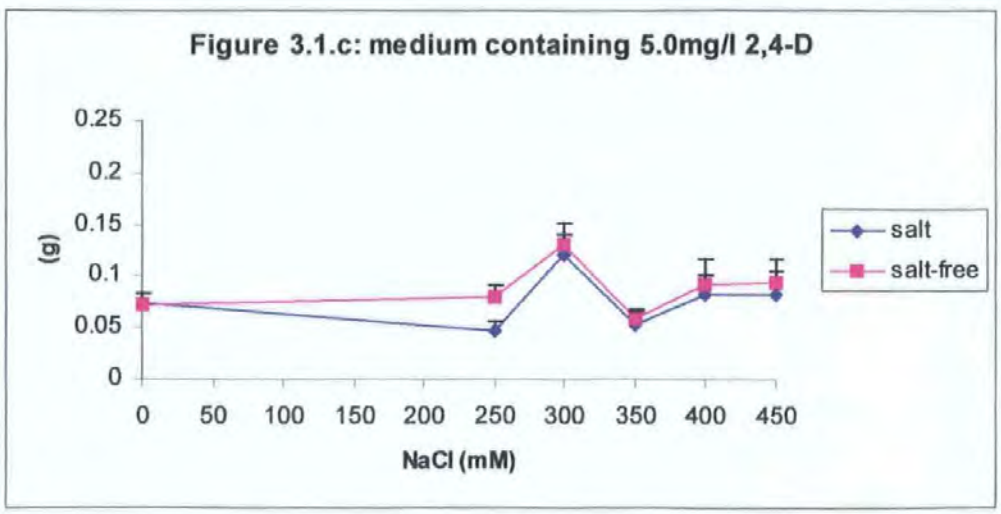
NaCl did not affect callus growth in an expected manner (Fig. 3.1) with no incremental effect on growth with increases in NaCl concentrations. Analysis of Variance of data for fresh weight of callus under salt treatments indicated no significant differences between any salt treatment and the control. Similarly, analysis of variance of data for fresh weight of callus after the recovery period in control media indicated no significant differences among all treatments except for those on recovery media (1.0 mgl^{-1} 2,4-D).

All treatments showed recovery. Salinity index values of 1.079 %, 0.49%and 1.036% were calculated for the three media test.

Figure 3.1: Fresh weight of callus of *B. nigra* grown on media containing different NaCl concentrations and NaCl free media







3.4 Discussion

3.4.1 Initiation and maintenance of callus

Callus was induced using 2,4-D at all concentration whereas IBA only produced shoots. This agreed with Harms *et al* (1976) who suggested that 2,4-D is the best hormone to use for callus initiation. 2, 4-D is required for inducing cell division *in-vitro*.

3.4.2 Regeneration from callus of *B. nigra*

Despite, anticipating that many shoots would regenerate from callus with 16 hormone combinations of IBA and Kinetin /· BAP, only 2 shoots per 5 pieces of callus were obtained in only 2 of 16 media in each case (IBA+ Kinetin/ BAP). The inability of cell callus to regenerate shoots may be a result of increase in somatic age (Murata and Orton, 1987) or accentuation of genetic abnormalities that accompanied regeneration (McCoy, 1987). It may also be that the hormone combinations need to be repeated using a stronger concentration of IBA + Kineten /BAP although the range of concentrations used was vary wide. The result agreed with other published work by Jain *et al.* (1991) who, found that all the callus and cell suspension cultured-based selections failed to regenerate shoots on MS media containing Kinetin and IAA. Murata and Orton (1987) used the common regeneration media composed of MS salts supplemented with 0.5-5.0 mgl^{-1} Kinetin and a low concentration of IAA or NAA. They obtained no satisfactory results in their study with *Brassica* species and attributed this to explant source and or genotype used. Furthermore, many previous researchers (Dunwell, 1981; Pua *et al.*, 1987; Millam *et al.* 1991; Fuller and Fuller, 1995; Fuller, 2000) have dealt with a number of *Brassica* species and no single one of their regeneration media was optimal to regenerate plants of the diverse range of *Brassica* species and varieties.

3.4.3 Callus growth in NaCl culture media

The data in this study showed that NaCl treatments did not affect callus growth in an expected manner and sometimes even the control treatment was lower than NaCl treatments. An explanation for this may be that cells within a callus piece may not come in contact with NaCl agent and are not uniformly exposed to the NaCl. This may lead to escapes from NaCl stress. It is possible that the uptake of NaCl only occurs in the peripheral cells of the callus and central cells have no exposure and continue to multiply and expand. Also, it is difficult to apply salt tolerance to calli due to the nature of the callus which acts as a sponge on the medium with no control of diffusion of the medium into the intercellular spaces, an explanation supported by Smith and McComb (1981), Meredith (1984) and Deane (1994). Jain *et al.* (1991) suggested that callus-based selection was unsatisfactory in *Brassica juncea* for the same reasons as the work of Meredith (1984), but they suggested that selection made via planted cell suspension were found to be more stable for salt tolerance.

This result is different from those of He and Cramer (1993a) with two rapid-cycling *Brassica* species, *B. napus* and *B. carinata.* They found that the callus growth was affected by seawater salinity. The difference may be attributed to the different salinity source used in the experiment. Seawater rather than a single salt (NaCl) may be more representative of a generalised cell response to salinity.

The literature contains several reports of problems using direct selection of callus. It has also been observed that the growth response of callus and whole plants are not correlated in some instances and that plants regenerated from salt tolerant cell lines of some plant species do not show an improvement in salt tolerance (Croughan *et al.*, 1987; Dix and Street, 1975; Stavarek and Rains, 1984). Dracup (1993) suggested that cultured cells are much more tolerant of high NaCl than is the whole plant because cultured cells grow

slower than cells in the whole plant (such as root meristem) so, assuming similar uptake mechanism and treatment, cultured cells should be less sensitive than roots to water deficit.

Dix and Street (1975) selected lines of tobacco and pepper cells that were capable of growth in liquid medium containing up to 342.5 mM NaCl and measured growth as an increase in cell number or change in packed cell volume and observed that packed cell volume tended to decrease after exposure to salt despite an increase in cell number. This was attributed to a decrease in cell size and confounds selection based on size criteria. With regard to the cell culture methods reported here it is clear that problems exist in callus culture, which lead to variable results.

3.5 Conclusion

Callus culture did not appear to be a good system to apply to selection of salt resistant in Rapid Cycling *Brassica*. It may be necessary to retest this system using suspension culture to overcome the problem of exposure to the selective agent. In this system if the suspension is fine (i.e. not clumpy) then every cell will be equally exposed to the stress and if the cell population contains several variants of the desired phenotype, the resulting population will consist of a mixture which can be regenerated and retested. Within the time scale of the reported project it was not possible to undertake such an investigation.

Chapter 4: Mutagenesis and selection for hydroxyproline resistance and salt resistance using a curd meristem technique

4.1 Introduction

4.1.1 Mutation and selection

The idea that plant cells could be grown and manipulated like micro-organisms and that various kinds of agriculturally useful mutations could be selected and expressed in regenerated whole plants was realised and developed by a number of investigators beginning in the late 1970's (Nabors, 1976). The underlying technologies making the idea plausible were (1) the ability to regenerate whole plants from single cells found in cell cultures and (2) the ability to select defined mutant cell lines from cultures (Carlson, 1970). Progress in mutant selection in cultured plant cells up to 1980 has been reviewed in great detail (Maliga, 1980 & Chaleff, 1983).

Specific problems have also been reviewed, including the sources and nature of heritable variation in culture (Meins, 1983), the use of tissue culture-induced variability for crop improvement [somaclonal variations] (Larkin and Scowcroft, 1981; 1982), the application of protoplast cultures in mutant selection (Bourgin, 1983; Maliga, 1983) and the isolation of agronomically useful mutants from plant cell cultures (Chaleff, 1983).

The post-1980 period is marked by three developments: 1. Haploid systems have been introduced; 2. The number of attempts to isolate mutants with practical value (that is amino acid overproduction, herbicide resistance, and salt tolerance) has increased significantly; 3. Tissue culture-induced genetic changes are no longer viewed only as an undesirable consequence of the culture system but as an opportunity to obtain valuable genetic variations (Maliga, 1984).

Mutations can be induced by the use of mutagens which may be either physical or chemical, and both have been used in conventional plant breeding programmes as well as in conjunction with *in vitro* selection methods. The majority of chemicals used to induce

mutations in plant cell cultures can be placed into two groups, base analogues and alkylating agents. Base analogues are similar in structure to the DNA bases and can be incorporated into DNA and result in errors (Heslot, 1977). Alkylating agents include alkyl sulfates and sulfonates, nitroso compounds, and sulfur or nitrogen mustards, Negrutiu (1990) has discussed the modes of action of these compounds.

Detrimental effects due to osmotic stress are the main factors causing salt damage in plants (Flowers *et al.*, 1977). High intracellular proline content as observed in halophytes (Stewart and Lee, 1974) and its increase in some plant species upon exposure to high sodium chloride concentration could play a major role in osmoregulation (Greenway and Setter 1979). Proline appears to be a suitable osmoregulator because of its high solubility in water and its low inhibition of enzyme activities even at high concentration. Hasegawa *et al.* (2000) considered that proline was one of the common osmolytes involved in either osmotic adjustment or in the protection of structure. In all cases, protection has been shown to be associated with accumulation of these metabolites, either in naturally evolved systems or in transgenic plants.

Proline levels vary naturally depending on stress levels but mutants over-expressing proline can be defined in the lab and may be useful in developing resistant varieties. One way to obtain a proline–accumulating mutant is to select for proline analogue resistance (Widholm 1972.a, b). Such mutants are considered to be useful in studying proline biosynthesis and stress resistance in higher plants (Miflin *et al.*1983). The mechanism thought to confer amino acid analogue resistance involves a mutation which results in an enzyme becoming feedback-insensitive resulting in the over-production of the corresponding amino acid and /or preventing false feed back inhibition by the analogue (Dix, 1993).

This system appears to be a fundamental mechanism in both prokaryotes and eukaryotes. One of a number of bacterial mutants selected for proline accumulation was found to be more salt tolerant than its parent and to transmit the salt tolerance concomitantly with proline accumulation via a plasmid to other bacteria (Anderson *et al.* 1980). Deane *et al.* (1995) selected one hydroxyproline resistant shoot from shoots arising from mutagenised cauliflower curd by N-nitroso-N ethylyurea (NEU) but found that despite slightly higher proline levels, no increase in stress tolerance over the controls was observed. Herbicide tolerant *B. napus* plants were obtained after treatment of microspores with NEU. NEU has also been shown to increase the frequency of chlorate resistant *N. tabacum* callus (Muller and Grafe, 1978). Treatment of strawberry shoot cultures with N-nitroso-N-methylurea (NMU) resulted in chlorophyll deficient shoots arising from axillary buds (Malone and Dix, 1990).

Widholm (1976) reported that screening for mutants which over-accumulate proline, involves growing plants or cells in media containing proline analogues such as trans-4-hydroxy-L-proline (HP) or azetidine-2-carboxylate (AZC) which are normally toxic to plants because they switch off proline production by acting as feedback inhibitors of the P5CS gene. Cultured carrot and tobacco cells resistant to various amino acids and proline analogues have been selected and characterised by Widholm (1976). Hasegawa and Inoue (1983) treated rice seeds with various chemical and physical mutagens and selected 24 hydroxyproline resistant seedlings. The seeds of some of these lines had a proline content more than 20 times the control (Mori *et al.* 1989). After mutagenesis of barley seeds with sodium azide, 20,000 mature embryos were screened for growth in 4 mM hydroxyproline and 4 plants were selected with good growth and leaves which contained more proline initially and accumulated proline more rapidly than the parental leaves (Kueh and Bright, 1981). A hydroxyproline resistant carrot line containing 15-30

times the normal free proline levels was reported by Riccardi *et al* (1983) and also showed increased resistance to stresses such as salt.

The genetic basis of stress tolerance in higher plants is still largely unknown and is probably polygenic and multi-allelic. Most direct selection techniques do not require knowledge of the location and exact phenotypic manifestations of the gene or genes involved. There is a possibility that mutant cell lines and regenerated plants selected in this way will be genetically unstable with tolerance not retained in subsequent cell or whole plant generations in the absence of stress but often this is not the case (Nabors, 1990). Bressan *et al.* (1985) found that selection at low levels of salt (171 mM) resulted in physiological adaptation and unstable tolerance in *Nicotiana tabacum* cv. Wisconsin38 whilst selection at higher levels (428 mM) resulted in stable mutants. Nabors *et al.* (1980) however found that in the cultivar Samsum, stable mutants could be obtained by selecting at salt concentrations considerably below 171 mM with maximum selectable tolerance at 150 mM.

The use of mutagensis and selection for hydroxyproline resistance appeared to be a promising line for experimentation in the current investigation provided that an effective cell or tissue culture method was available and could be adapted.

4.1.2 Cauliflower curd microshoot technique

Meristems are the basic unit used in plant micropropagation, they are the most genetically stable part of a plant and in consequence the most suitable for production of true-to-type propagules. Clonal multiplication is of paramount importance in modern cauliflower breeding programmes and is most needed for the maintenance of elite lines such as the parents of F1 hybrids that are usually strongly self incompatible or male sterile. Maintenance of these lines using seed requires time consuming manual self-

pollination of immature flower buds, or costly equipment (gas-proof isolation chamber for carbon dioxide treatment) to overcome self incompatibility, or maintainer lines that are not always available for male sterile lines.

Micropropagation using curd meristems has been used for some time to maintain cauliflower parent lines and bulk up plants prior to seed production (Crisp and Walkey 1974). It has also been used for the production of virus-free cauliflower (Walkey *et al.* 1974), and for the early screening of curd quality (Crisp and Gray 1979). More recently this tissue has been used as a source of protoplasts (Yang *et al.* 1994) and for chemical mediated mutagenesis (Deane *et al.* 1995).

Ideally, the culture system used should enable a high number (10^5-10^6) of individuals to be screened using relatively limited space. One problem in developing mutants is that the mutation rate in cell cultures is too low to result in the occurrence of stress tolerant mutants in a high enough frequency to be located by selection (Nabors, 1990). However for cauliflower, Kieffer *et al.* (1995 & 2001) have recently described a system of mass production of propagules involving homogenisation of curd tissues. The technique produced tens of thousands of meristematic pieces in liquid culture in relatively small volumes (e.g. 1-2000 propagules in 20 ml) and offers unique and exciting possibilities for mutagenesis and rapid screening in culture.

Aim

To establish the cauliflower microshoot technique as a system to be used in conjunction with mutagenesis for selection for hydroxyproline resistance and concomitant salt resistance.

Objectives

1. To evaluate the critical level of resistance to salt stress of cauliflower microshoots.

2. To determine the hydroxyproline sensitivity of cauliflower microshoots

3. To carry out mutagenesis of cauliflower microshoots and select for hydroxyproline resistance.

4. To measure proline content in control and selected shoots and plants

4.2 Material and Methods

4.2.1 Cauliflower curd microshoot technique

Plant material

One variety of cauliflower was used: January heading Roscoff type F1 Medaillon bred by Elsoms Seeds Ltd. The plants were grown according to good commercial practice (MAFF 1982) in the field at Seale-Hayne as part of the MAFF HORTLINK project 192 (1998/9).

Experimental protocol

Large pieces of curd (1-5 cm) were surface sterilised firstly by rinsing for 30s in 70% ethanol, then 15 m in 10% commercial bleach (sodium hypochlorite 0.06 % active chlorine), followed by 3 washings in sterile distilled water. Following surface sterilisation, explants were produced manually (Plate 4.1). The first step (predisruption) eliminates the mass of non-responsive tissue (stem branches) by shaving off the upper meristematic layer using a scalpel under sterile conditions in a laminar flow hood. The second step is a mechanical partial homogenisation of the selected tissue using a commercial blender (Waring Model 800) at approximately 17000 rev. min^{-1} for 25 s followed by the use of precision sieves (600, 300, 100 μm) (Endecotts Ltd., London) to rank the explant into the size classes 1-300μm and 300-600 μm. Blending and sieving were made in an osmotic protection solution W5 to limit cell stress (Appendix 3) (Menzel *et al.* 1981). The explant culture density used was a constant volume (240 μl) per container containing 20 ml of culture medium. Seventy to eighty containers were used per experiment and incubated on a shaker (< 50 revs' min^{-1}) (Plate 4.2). Culture media were derived from Murashige and Skoog (1962), according to Anderson and Carstens (1977), and supplemented with Kinetin (2 mgl^{-1}) and IBA (Indol-3-Butyric Acid)

(1 mg l^{-1}) for the shoot development medium, and 1BA only (2mg l^{-1}) and agar (7g l^{-1}) for the rooting medium. Culturing was carried out in a growth room with adjacent lighting provided by a mixture of cool and warm white fluorescent tubes with a photoperiod of 16 hrs supplied light in a 23 °C culture room. The overall method was perfected by 3 practice attempts to help reduce contamination. The addition of the antibiotic Plant Preservative Mixture (PPM) 0.3 ml l^{-1} was routinely added to further reduce contamination risk (Appendix 4) (Fuller and Pizzey, 2001).

4.2.2 Selection

4.2.2.1 Evaluation of the critical level of resistance of microshoots to salt stress

In the first experiment explants were cultured for 7 days prior to stressing to ensure lack of contamination and then NaCl was added directly to each container to achieve different final concentrations (0 mM, 128 mM, 214 mM, 299 mM, 385 mM, 470 mM, 550 mM) as used by Dix and Street (1975).

After three weeks growth, data recorded was the number of growing propagules in each of 3 categories; with shoots and roots, with shoots only and with bracts (green leafy fragments). The fresh weight and dry weight of the total explants per container were also determined.

4.2.2.2 Determination of hydroxyproline sensitivity of cauliflower microshoots

Different concentrations of hydroxyproline (hyp) were added (0 mM, 0.03 mM, 0.3 mM, 3 mM, 30 mM) after microshoot culturing for 7 days.

After three weeks growth, data recorded was the number of growing propagules in each of 3 categories; with shoots and roots, with shoots only and with bracts (green leafy

fragments). The fresh weight and dry weight of the total explants per container were also determined.

4.2.3 Mutagenesis with N-nitroso-N-ethylurea (NEU) and N-Nitroso-N-methyl-urea (NMU)

NEU and NMU are mutagenic and carcinogenic chemicals and great care must be taken during handling. Procedures for the safe handling of the mutagens were followed as described by MaCabe *et al* (1990). The mutagens were obtained from Sigma in sealed 100 ml containers to avoid direct handling and arrived in powder form. This was dissolved in dimethylsulphoxide (DMSO) as recommended by the manufacturer. To this, liquid medium (S23) (Appendix 5) was added to make up a stock solution. Mutagen solutions were dropped with a micropipette into each container of microshoots (20 ml medium) to achieve final mutagen concentrations of 1 and 2.5 mM. After 90 minutes, mutagens were removed by washing three times with liquid culture medium using a decanting technique. All glassware was decontaminated in a fresh 5 M NaOH solution in which after 1-2 days, geneotoxic compounds are destroyed.

A post-mutation selection of hyp (3 mM) was imposed immediately rather than delaying for 7 days. These experiments were maintained in the same controlled growth condition as described earlier. Cultures were incubated for 3 weeks and then all green shoots were subcultured onto solid medium (S23) to develop further. The experiment was repeated seven times and all the experiments produced about 1500000 microshoots. About 300 microshoots only survived after mutagensis and hyp selection (Plate 4.2 and Plate 4.3) approximately 0.03 % of explants tested. This success rate is similar to that found by other workers, Hasegawa and Inoue, (1983) (0.025% in Rice) and Deane (1994) (0.05% in cauliflower).

Plate 4.1: Protocol for mass production of cauliflower propagules from fractionated and graded curd (Kieffer *et al*, 2001)

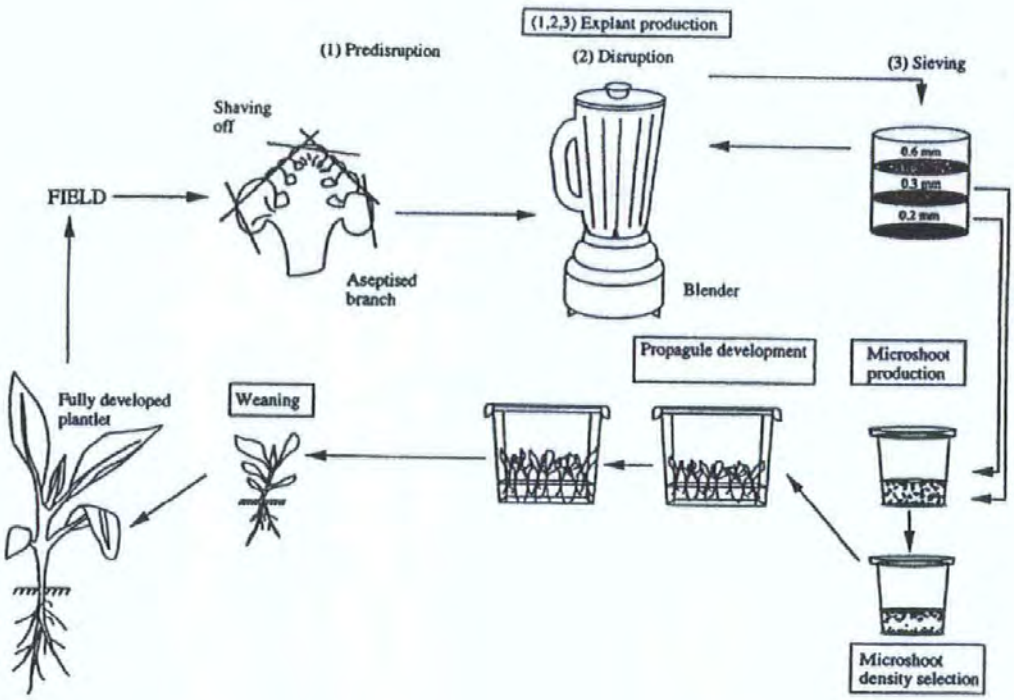


Plate 4.2: Mutagensis experiments during hydroxyproline selection. Each layer is one exp. from cauliflower curd. Each pot contains about 2000 microshoots.

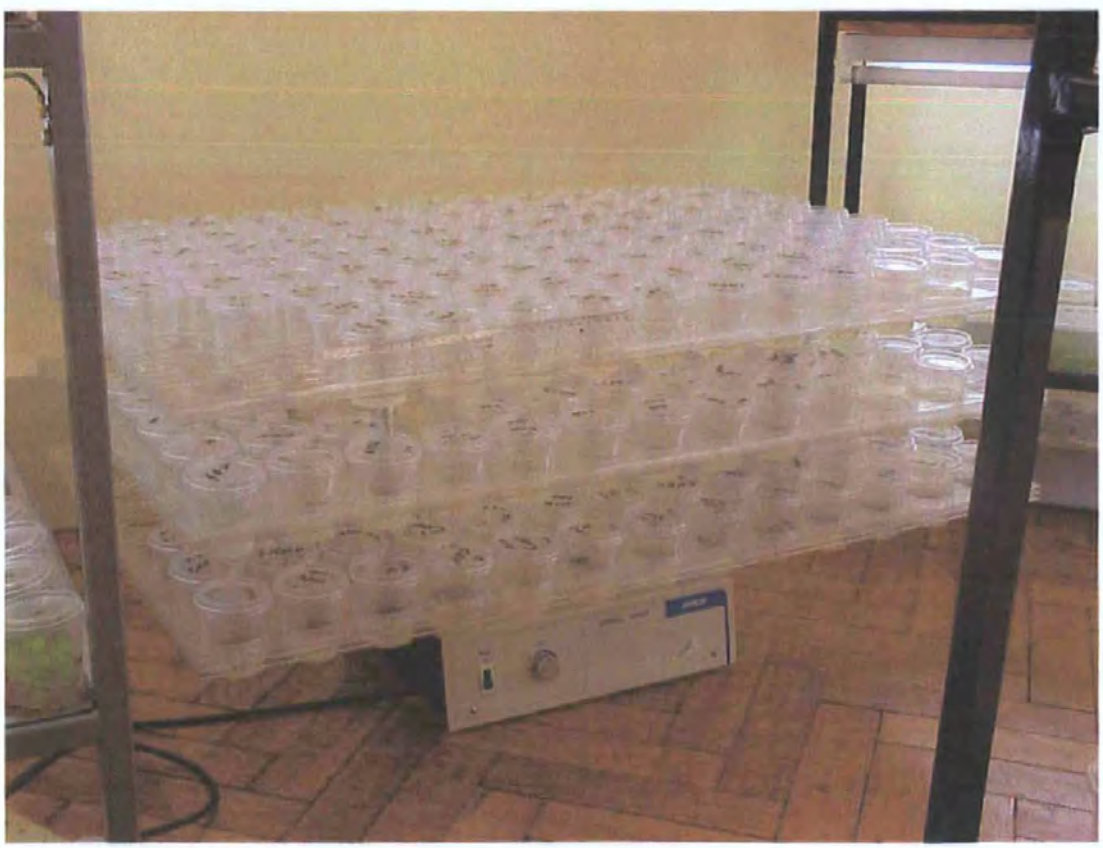
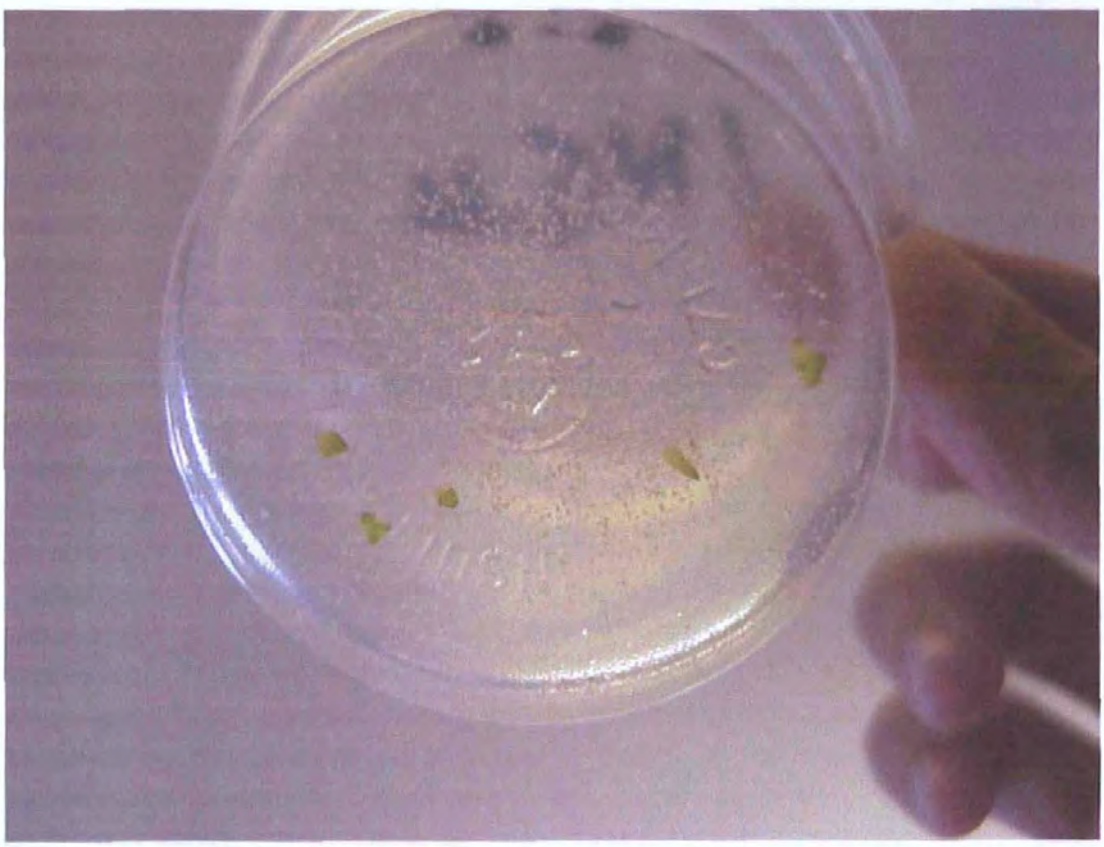


Plate 4.3: Example of successful mtagenesis and selection: 5 green shoots from approximately 2000 microshoots explants (white specks).



4.2.4 Weaning and green house-grown plants

The weaning of the plantlets into compost was carried out after 3-4 weeks subculture in rooting medium (Appendix 6). Generally conditions of high relative humidity, shade and adequate heat were required for at least 3-4 weeks in a growth room before being transferred to the greenhouse.

4.2.5 Estimation of free proline content

4.2.5.1 Ninhydrin method

Proline levels were measured by the method of Bates *et al.* (1973). Samples of leaves were collected from shoots and plants and were cooled in 2 °C for 2 h then put in freeze dryer for 72 h and then stored in a dessicator. Freeze dried samples were homogenised in 3 % sulfosalicylic acid. The filtered homogenate was allowed to react with ninhydrin and glacial acetic acid for one hour at 100 °C. The reaction mixture was extracted with toluene. The toluene was aspirated from the aqueous phase and the aqueous absorbance read at 520 nm using Philips (Pu 8625 UV/VIS) spectophotometer. The proline concentration was determined from a standard curve and calculated on a fresh weight basis.

4.2.5.2Dionex AAA-Direct Amino Acid

Late in this project a Dionex AAA-Direct Amino Acid analyser (Plate 4.4) was obtained by the Department and this was used to crosscheck the accuracy of the Ninhydrin method.

The same samples which were homogenised in 3 % sulfosalicylic acid were used in this analysis. Samples of leaf extract were initially diluted 1: 999 in 0.2 µml filtered deionised water prior to analysis. Separation of samples was achieved on a 30 cm x 2mm *i.d.* AminoPac PA 10 analytical column (Dionex Corp.). The eluent used was a combination of dionised water, 250mM sodium hydroxide with 1M sodium acetate

gradient at a constant 0.25 ml m^{-1} (Table. 4.1). Samples (50µl) were injected into the system by a Dionex AS50 Autosampler and compounds were detected by integrated amperometry using a Dionex ED50 Electrochemical Detector. Authentic reference compounds were used for calibration, identification and quantification purposes.

Table 4.1.Gradient conditions used by the Dionex AAA-Direct Amino Acid Analyser system.

Time (min)	Dionised water	Sodium hydroxide	Sodium acetate
0.0	80	20	0
2.0	80	20	0
12.0	80	20	0
16.0	68	32	0
24.0	36	24	40
40.0	36	24	40
40.1	20	80	0
42.1	20	80	0
42.2	80	20	0
62	80	20	0

4.2.6 The effect of hydroxyproline on growth of control shoots *in-vitro*

Shoots were subcultured onto solid medium supplemented with 3mM, 10 mM hyp and control medium to develop for 4 weeks. The damage was assessed every week using a five point vigour score where 0 = dead and 4 = undamaged. Also proline content was measured every week.

Plate 4.4: Dionex AAA-Direct Amino Acid Analyser.

4. 3 Results

4.3.1 Selection

4.3.1.1 Evaluation of the critical level of resistance of microshoots to salt stress

The results for the 1- 300 μm sized microshoots showed that shoot numbers and the relative proportions of 3 shoot categories (shoot+root, shoot only, bract) were affected by increasing salt concentrations (Fig.4.1a). Above a concentration of 214 mM NaCl there was practically no shoot+root survival. At concentrations of both 128 & 214 mM the proportion of shoots+root declined dramatically with a consequent rise in shoot only and bracts as the concentration of NaCl increased. Both fresh and dry weight measurements reflected the effects seen with shoot number (Fig 4.1.b, c).

The result for the 3-600 μm sized microshoots contrasted with those for 1- 300 μm. The inhibition observed for 1- 300 μm above 214 mM was not evident for 3-600 μm and the cut off point for complete inhibition of shoot growth was above 470 mM NaCl (Fig. 4.2a). Bract proportions did appear to increase with increasing salt concentration but there were still plenty of shoots at the higher salt concentrations. Data for fresh and dry plant weight (Fig. 4.2 b, c) showed that whilst intermediate levels of salt did not kill the shoots, it did severely limit their growth.

Figure 4.1, a: % of propagules of microshoots (size 1-300 mm) at different NaCl concentrations

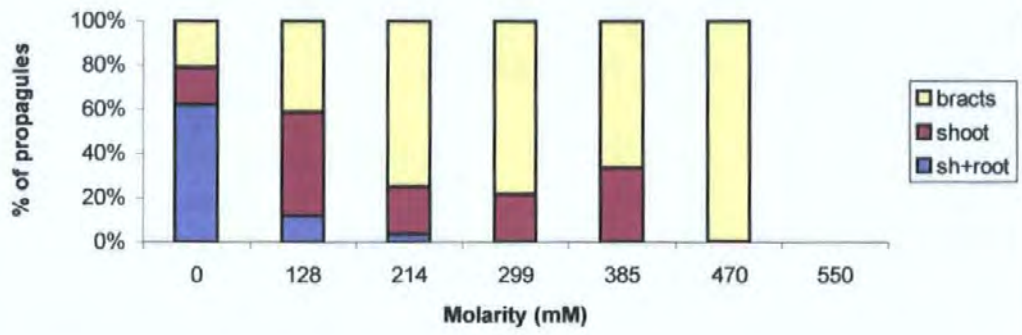


Figure 4.1, b: Fresh weight of microshoots (size 1-300 mm) at different NaCl concentrations (bars = SE)

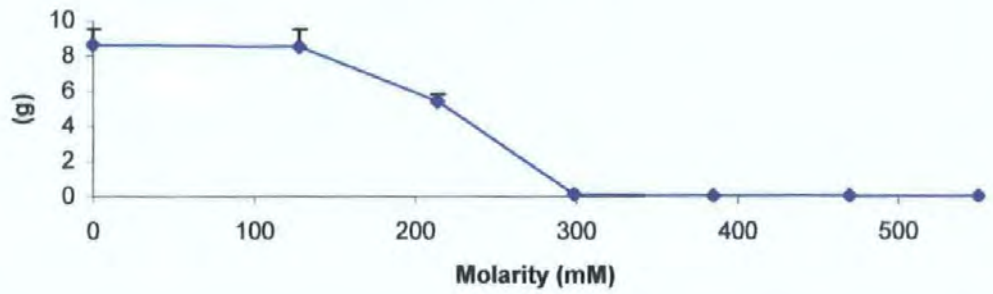


Figure 4.1, c: Dry weight of microshoots (size 1-300 mm) at different NaCl concentrations (bars = SE)

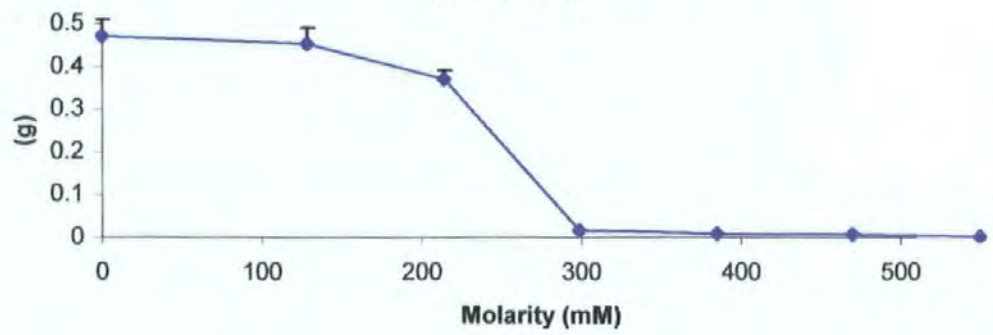


Figure 4.2,a: % of propagules of microshoots (size 3-600mm) at different NaCl concentrations

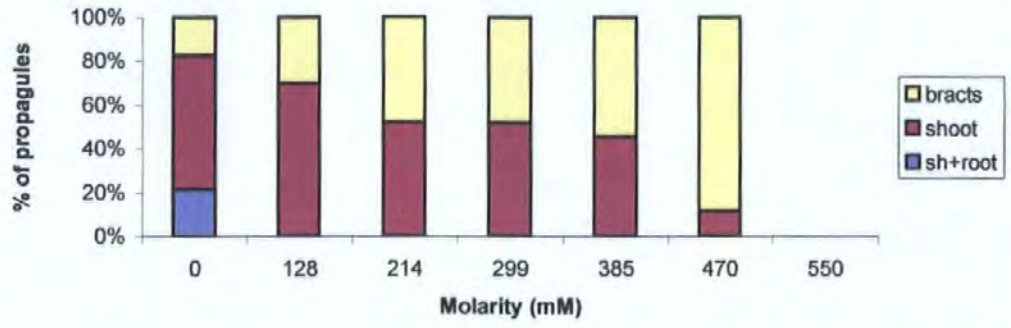


Figure 4.2, b: Fresh weight of microshoots (size 3-600 mm) at different concentrations (bars = SE)

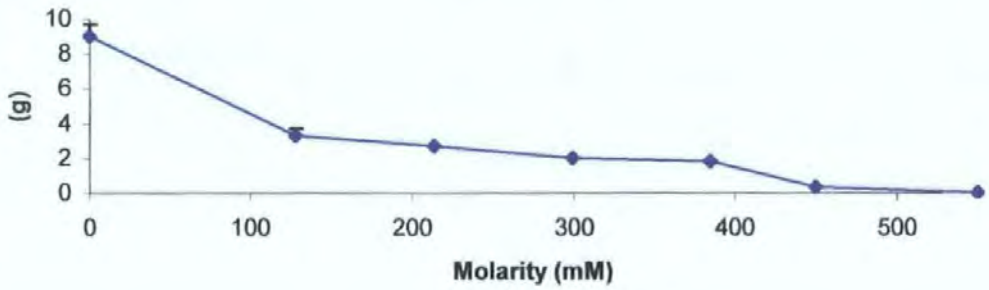
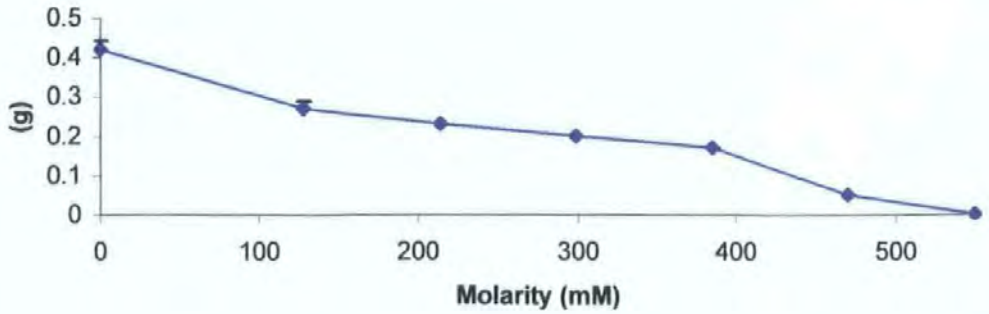


Figure 4.2, c: Dry weight of microshoots (size 3-600 mm)at different NaCl concentrations (bars = SE)



4.3.1.2 Determination of hydroxyproline sensitivity of cauliflower microshoots

The results for the 1-300 μm sized microshoots showed that shoot numbers and the relative proportions of the 3 shoot categories (shoot + root, shoot only, bract) were affected by increasing hydroxyproline concentration (Fig.4.3a). Above a concentration of 0.3 mM there was practically no shoot+root survival. At concentrations of both 0.03 & 0.3 mM hydroxyproline, the proportion of shoot + root declined with a consequent rise in shoot only and bracts as the concentration of hydroxyproline increased. Both fresh and dry weight measurements reflected the effects seen with shoot number (Fig 4.3 b & c).

The result for the 3-600 μm sized microshoots showed that the cut off point for complete inhibition of shoot+root growth was above 30 mM but not as clear a cut off as with 1-300 μm. There were plenty of shoot only and bracts at the high concentration of hydroxyproline (Fig.4.4 a). The fresh and dry weight measurements reflected the effects seen with the shoot number (Fig 4.4 b & c).

4.3.2. The effect of hydroxyproline on growth of control shoots *in-vitro*

Control shoots exposed to 0, 3 or 10 mM hyp for 4 weeks in solid culture showed a decline in vigour (Plate 4.5) with the fastest decline as expected at 10 mM hyp (Fig 4.5). Control shoots on 0 mM hyp showed a slight decline at the fourth week probably indicating the need for subculturing.

Over the same period proline content increased with a greater increase at the higher hyp stress levels (Fig 4.6). Proline content of control leaves was highest after 3 weeks on 10 mM hyp.

Figure 4. 3, a : % of propagules of microshoots (size 1-300 mm) at different hydroxyproline concentrations

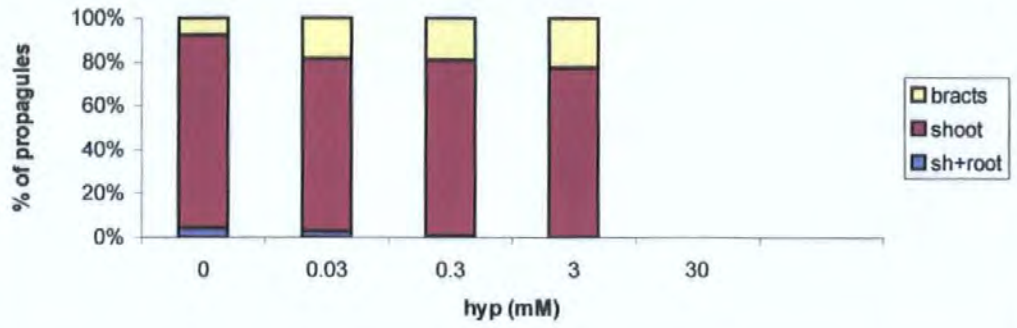


Figure 4.3, b: Fresh weight of microshoots (size 1-300mm) at different hydroxyproline concentrations (bars = SE)

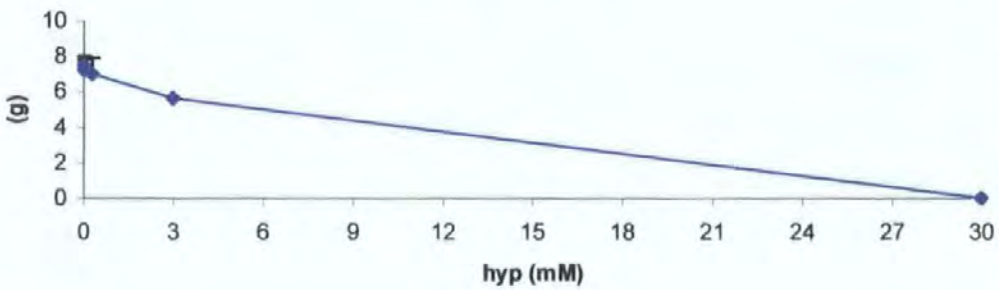


Figure 4.3, c: Dry weight of microshoots (size 1-300 mm) at different hydroxyproline concentrations (bars = SE)

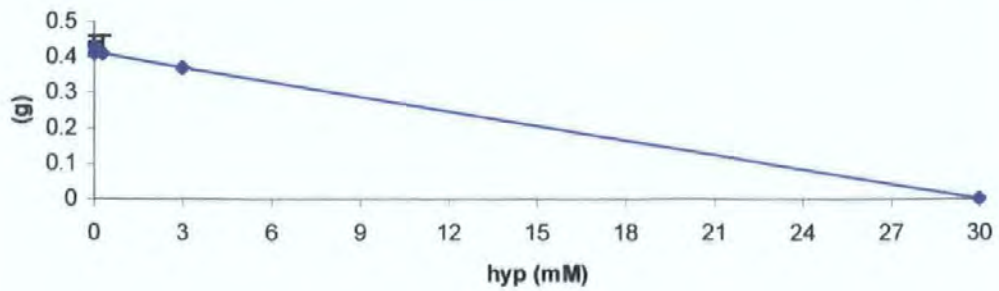


Figure 4. 4, a : % of propagules of microshoots (size 3-600 mm) at different hydroxyproline concentrations

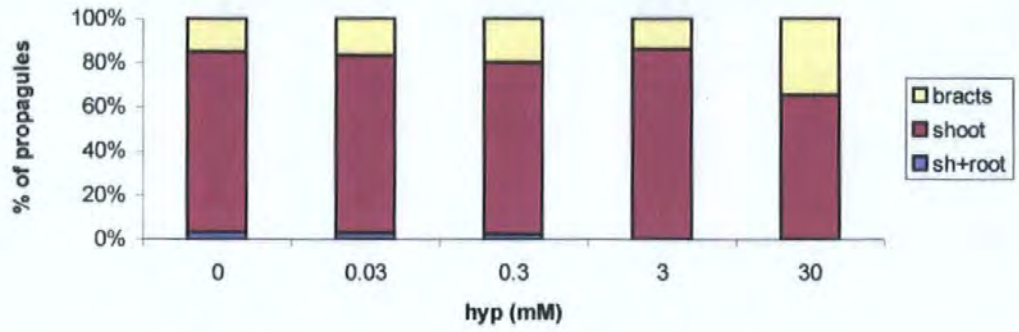


Figure 4. 4, b: Fresh weight of microshoots (size 3-600 mm) at different hydroxyproline concentrations (bars = SE)

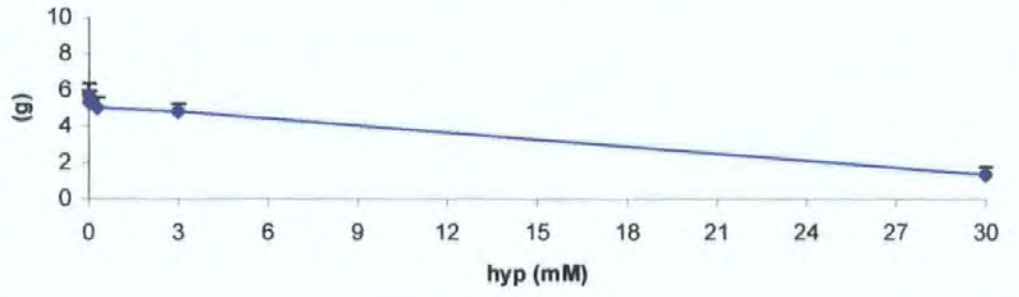


Figure 4. 4, c: Dry weight of microshoots (size 3-600 mm) at different hydroxyproline concentrations (bars = SE)

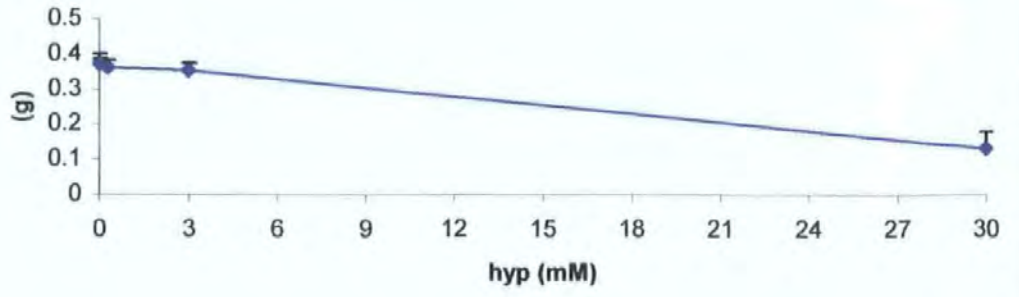


Plate. 4.5: The vigour of shoots over a sub-culture period (4 weeks) grown on media containing 0 mM (right) and 10 mM hydroxyproline (left).

Figure 4.5: The vigour of shoots over a sub-culture period (4 weeks) grown on media containing 0, 3, 10 mM hydroxyproline

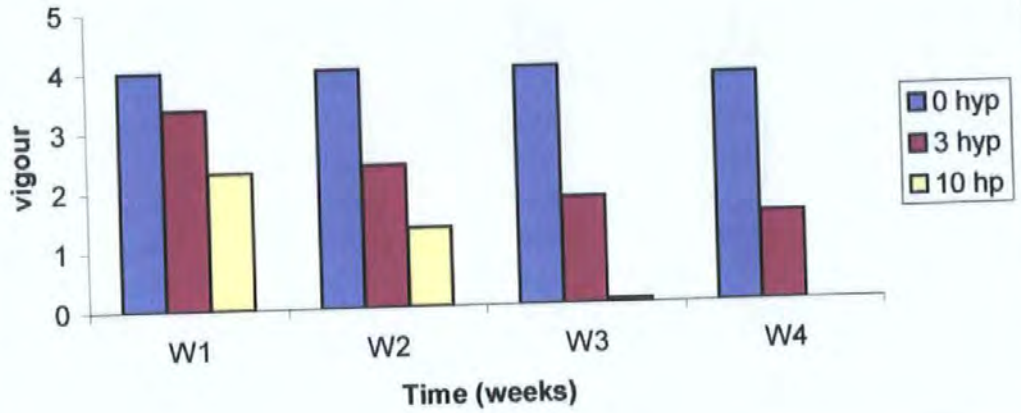
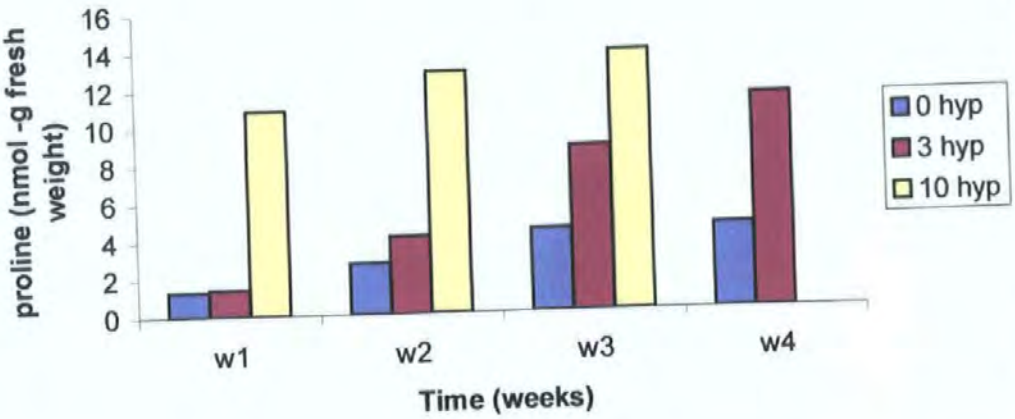


Figure 4.6: Proline content of control shoots over 4 weeks exposed to media contaning 0, 3, 10 mM hydroxyproline



4.3.3. Mutagenesis

Results showed that above 3 mM hyp there was no shoot survival for both 1-300 μm sized and 3-600 μm sized microshoots. These results were in complete contrast to those where hyp was added 7 days after initial culturing and illustrate the importance of explant development stage in resistance to hyp .

4.3.3.1 Proline content of shoots and plants selected from mutagenesis

The results of the proline assay of eighty *in-vitro* shoots showed that twelve *in-vitro* shoots were higher than control shoots. An increase in proline level was observed in all twelve mutants. Proline levels of *in-vitro* shoots were higher than *in-vivo* plants (Fig. 4.7& 4.8). The proline levels of selections S4, S6, S9, S10, and S11 were lower than the control plants. It should be noted that levels of proline were an order of magnitude lower in *in-vivo* plants compared to *in-vitro* plants. Analysis variance of data of proline content showed no significant difference ($p \leq 0.05$) between the results obtained using the ninhydrin method and those using the Dionex –AAA-Direct method and the two methods were linearly correlated (Fig. 4.9& 4.10).

Figure 4.7: Proline content of control and selected shoots *in-vitro*

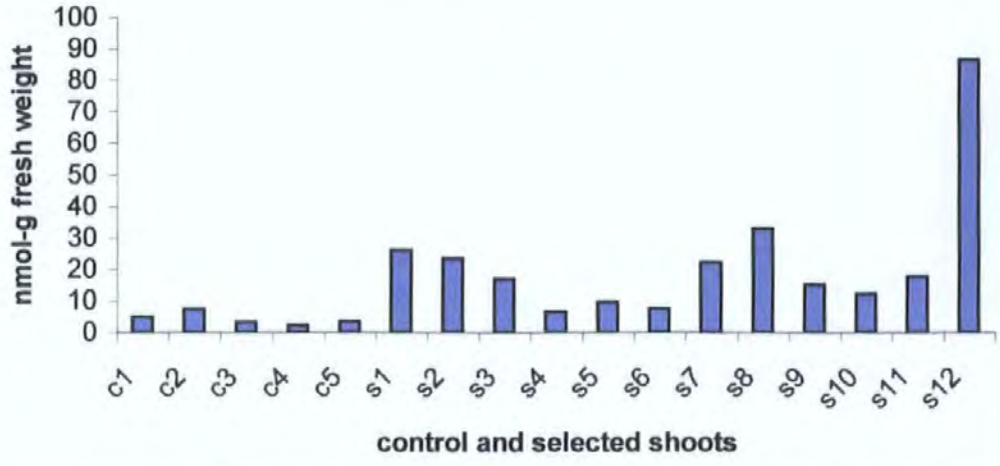
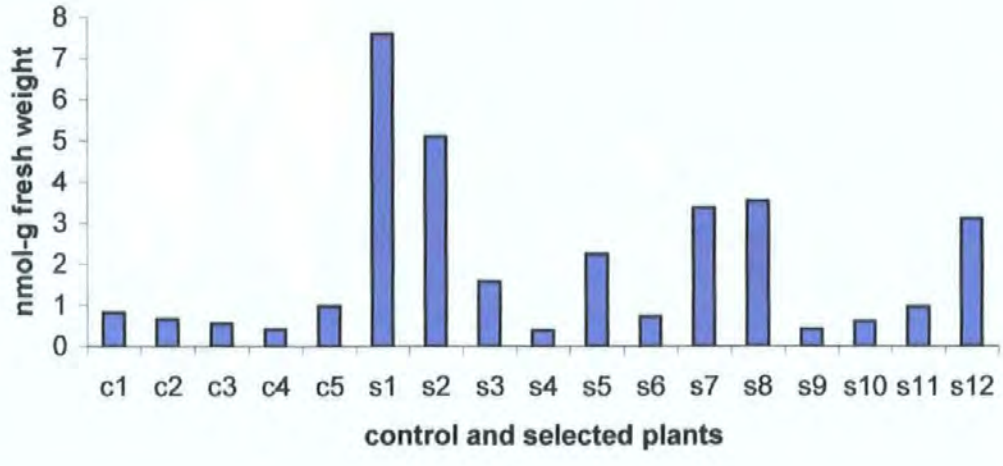


Figure 4.8: Proline content of control and selected plants *in-vivo*



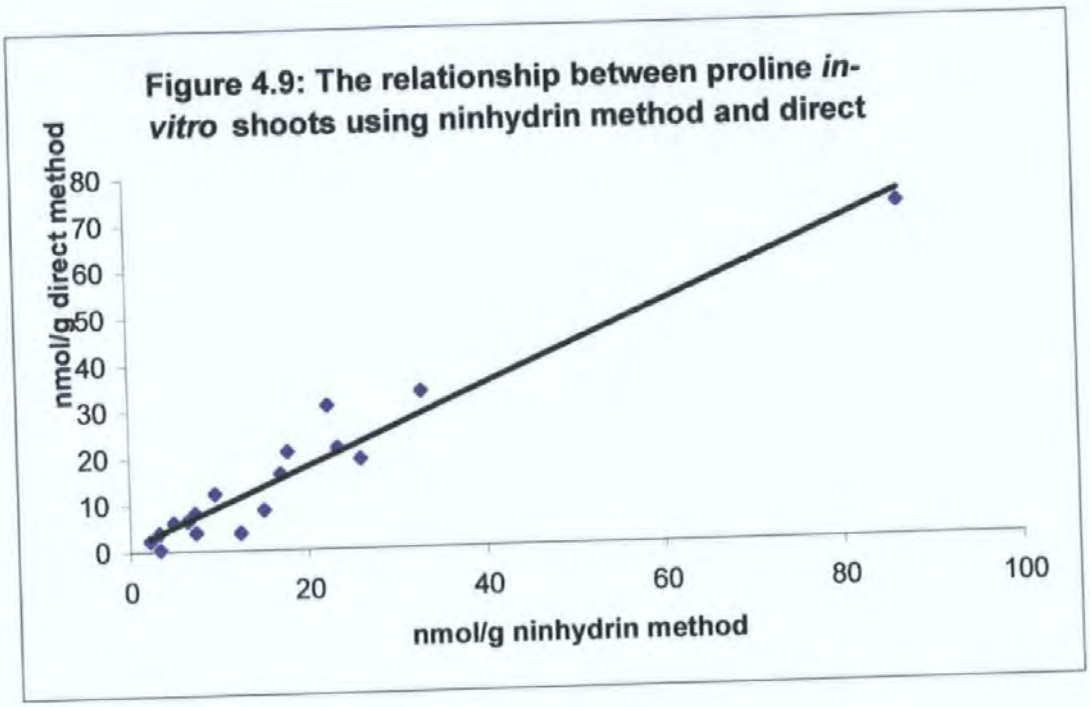


Figure 4.9: The relationship between proline *in-vitro* shoots using ninhydrin method and direct

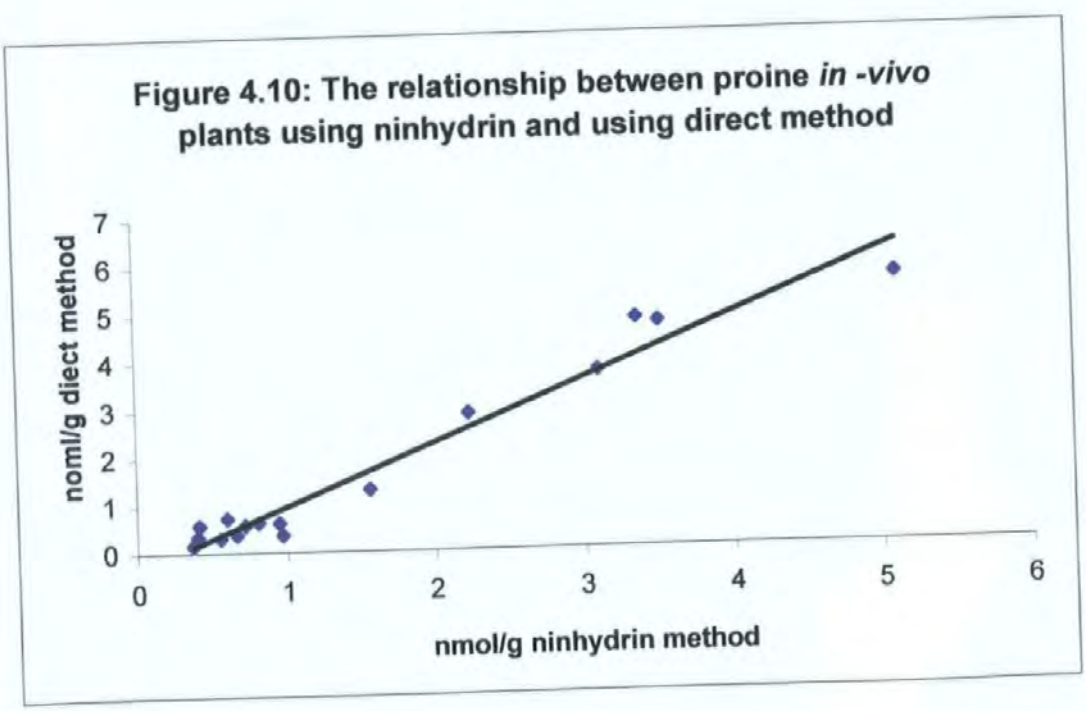


Figure 4.10: The relationship between proine *in -vivo* plants using ninhydrin and using direct method

4.4 Discussion

Evaluation of the critical level of resistance to salt stress

The results indicated that 550 mM NaCl (roughly the concentration of seawater) was lethal for both sizes of cauliflower microshoots. The critical level for selection for salt resistance appeared to be 299 mM for the small explant category but for the large explant category was 385 mM NaCl. This difference may be because the small explants were more sensitive to NaCl stress due to their size. To put this into context levels of resistance to 500 mM for *N. tabacum* have been achieved using cell suspension culture (Watad *et al.*, 1983). It should be noted that the NaCl in the present experimentation was added 7 days after initiation of explant growth when they had made some growth and were possibly more resistant.

Determination of hydroxyproline sensitivity of cauliflower microshoots

The results indicated a marked decline in shoot survival between 0.3 mM and 3 mM hyp and suggest 30 mM as a critical level for selection when added 7 days after culture initiation. However when added after one day, 3 mM hyp was seen to be the selective level, as in other published work on cauliflower (Deane *et al.* 1996). This difference appears to be a function of explant size or development as was evident in the NaCl experiment. The number of shoots in small explants (1-300 μm sized) was lower than in large explants (3-600 μm sized) at all different treatments and may be due to intense competition between the shoots for nutrients which resulted in the number of good quality shoot recovered from selection agent being low an observation also noted by Kieffer *et al.* (1995).

The effect of hydroxyproline on growth of in-vitro control shoots

The decline in shoot vigour of control shoots grown on hyp containing media probably results from the irretrievable competition for a proline-binding site thus reducing uptake

of proline leading to protein synthesis inhibition and subsequent plant death. Such perturbations of protein synthesis could also lead to proteins which have altered properties, which could be detrimental to the growth of the cell (Singh and Widholm, 1975, Widholm, 1976).

Similar observations have been reported by Hasegawa and Inoue (1983), who demonstrated that retardation of rice seedling growth was detected in culture at concentrations above 5 mM hyp. Furthermore, rice callus exhibited reduction in dry weight gain under the influence of 10 mM hyp (Chauhan and Prathapasenan, 2000).

Control shoots exposed to 0, 3, 10 mM hyp for 4 weeks showed an increase in proline content that might result from reflecting the degree of stress experienced by the plants, an explanation supported by Hanson *et al.* (1979). The rise in endogenous proline levels of control *in-vitro* shoots might result from a slight stress caused by the exhaustion of the culture medium.

Proline content of shoots and plants selected from mutagenesis

Proline levels of the twelve selected *in-vitro* shoots were higher than control shoots and might result from increasing activity of the enzymes involved in the synthesis and or inhibition of enzymes involved in the degradation of proline, an explanation supported by Aspinall and Paleg (1981). The low proline levels in the *in-vivo* state might result from mature plants having more protein synthesis and carbohydrates while in the *in-vitro* stage shoots may have made proline but had a lower protein rate. Kueh and Bright (1981) selected mutant barley seedling resistant to hyp and found that leaves of the mutant contained more proline initially and accumulated proline more rapidly than the parental leaves. Proline-accumulating carrot cell lines were also obtained by selection for growth on hyp (Widholm 1976) whilst Dix *et al.* (1984) reported the isolation of hyp

Nicotiana sylvestris cell lines overproducing proline and the subsequent regeneration into plants still showed increased proline levels.

4.5 Conclusion

The microshoot technique was successful if technically demanding. It did give different microshoot numbers from one experiment to another, and also different proportions of shoot categories for unknown reasons. It did however give a good contact between the selection medium and the microshoots through providing well dispersed cell aggregates which were uniformly exposed to the selective agent (salt, hydroxyproline or mutagen). Its main advantage is for production of large-scale micropropagation allowing the production of many shoots, which is a huge advantage in low frequency selection systems of mutagenesis.

Chapter 5: Selection, characterisation of hydroxyproline resistance mutants of cauliflower: resistance to salt and frost stress

5.1 Introduction

Breeding for resistance against salt, drought, and cold by classical methods of selection and crossing is a time-consuming and often inefficient procedure. On the basis of somaclonal variation and by using biochemical markers as selection tools, *in-vitro* selection techniques may provide an alternative way to selected new genotypes with improved properties. Many studies have been reported that free proline-accumulating cell lines resistant to proline analgues show increased resistance to stress such as salt (Riccardi *et al.*, 1983) and freezing (Van Swaaij *et al.*, 1986, 1987). Van Swaaij *et al.* (1986, 1987) were the first ones to succeed, by means of hyp, with the *in-vitro* selection of potato plants with increased frost tolerance. Tantau and Dorffling (1991) selected stable hyp resistance cell lines from a spring wheat cell culture that proved to be more frost tolerant than the wild type. There is also evidence that hyp resistant cell lines are tolerant to NaCl (Van Swaaij *et al.*, 1986, Chauhan and Prathapasenan 1998). Chauhan and Prathapasenan (2000) selected hyp resistant calli of rice that reduced the accumulation of sodium and chloride ions and enhanced the intracellular level of potassium, magnesium and calcium ions.

Zhu *et al.* (1997) and Zhu (2000) reported that the two major abiotic stresses, drought and cold, are intimately linked with salt stress. Many genes that are regulated by salt stress are also responsive to drought or cold stress. Because salt stress can be applied accurately and reproducibly, many drought stress studies in the laboratory use salt stress instead of actual drought. Brewster *et al.* (1993) discovered the Hog pathway for osmotic stress perception and signaling in yeast by using NaCl stress.

The present study had already identified a number of selected plants which had resistance to hyp and salt. An assessment of the frost resistance of these selections was

therefore under taken to determine whether any cross-resistance to other stresses existed in the selections.

5.1.1 Frost resistance

Frost stress is intimately linked with drought and salt stress, and these three major abiotic stress factors strongly limit plant productivity. For 64% of the earth's landmass the mean minimum temperature over the whole year is below 0 °C and for 48 % it is below –10 °C (Deane, 1994).

5.1.1.1Freezing injury

Thomashow (1999) explained that when temperatures drop below 0 °C, ice formation is generally initiated in the intercellular spaces due, in part, to the extracellular fluid having a higher freezing point (lower solute concentration) than the intracellular fluid. Because the chemical potential of ice is less than that of liquid water at a given temperature, the formation of extracellular ice results in a drop in water potential outside the cell. Consequently, there is movement of water vapour down the chemical potential gradient from inside the cell to the intercellular spaces. At –10°C, more than 90% of the osmotically active water in a plant typically moves out of the cells and the osmotic potential of the remaining unfrozen intracellular and intercellular fluid is greater than 5 osmolar. Steponkus *et al.* (1993) indicated that membrane systems of the cell are the primary site of freezing injury in plants and established that freeze-induced membrane damage results primary from the severe dehydration associated with freezing. Olien and Smith (1977) believed that freezing causes intercellular ice forming adhesion with cell walls and membrane which then causes cell ruption. Guy *et al.* (1998) indicated that protein denaturation occurs in plants at low temperature, which could potentially result in cellular damage. Bartels and Nelson (1994) reported that frost stress leads to a depletion of the cellular water; therefore, cellular dehydration is a consequence of this stress and

Zhu *et al.* (1997) also indicated that freezing may lead to osmotic stress due to reduce water absorption and cellular dehydration by ice formation.

5.1.1.2 Freezing avoidance and tolerance

Freezing resistance is a very desirable trait for many economically important plants but is complex, as it involves various physiological, chemical and physical processes and reactions at both the tissue and cellular levels. However, Blum (1988) defined freezing resistance in terms of the internal interactions among physiological, chemical and physical reaction during a freeze-thaw cycle of the cell and tissue and believed that the increased osmolality of the cellular solute is an effective mechanism for avoiding intracellular ice formation and cellular dehydration. Levitt (1980) defined freezing tolerance as the ability of the cell to withstand stress developed by extracellular freezing, recognising that intracellular freezing is lethal and proposed that hardening (or acclimation) and resistance be referred to freezing tolerance. Freeze hardening is the positive effect of exposure to stress of the plant or parts thereof on subsequent resistance to freezing. During the exposure of plants to low temperatures, many changes in physiological and biochemical parameters have been observed: modified levels and activities of enzymes from various metabolic pathways, accumulation of carbohydrates (e.g.sucrose, fructans) and amino acids (e.g. proline) and altered lipid composition of the cell membrane (Bartels and Nelson, 1994). Guy (1990) suggested that acclimation-induced cold tolerance is a quantitative character controlled by number of additive genes. Thomashow (1999) demonstrated cold acclimation includes the expression of certain cold-induced genes that function to stabilise membranes against freeze-induced injury. The CBF/DREB1 proteins have been identified that control the expression of a regulon of cold-induced genes that increase plant-freezing tolerance.

Several studies have shown that increases in frost resistance by *in-vitro* selection for hyp resistance. Van Swaaij *et al.* (1986; 1987) isolated hyp resistant cell lines of potato, which proved to possess increased proline and other amino acid levels as well as increased frost tolerance. Tantau and Dorffling (1991) and Dorffling *et al.* (1993) selected hyp resistant cell lines of wheat which contained increased levels of free proline and had increased frost tolerance. Dorffling *et al.* (1997) provided strong evidence on the heritability of the traits like increased frost tolerance and increased proline content by *in-vitro* selection procedures for hyp in winter wheat. Recently, several different gene transfer approaches have been employed to improve the stress tolerance of plants (Kasuga *et al.*, 1999; Zhang *et al.*, 2000; Holmstrom *et al.*, 2000).

5.1.1.3 Measurement of frost resistance using electrical conductivity

The determination of levels of frost resistance of field-grown plants is hindered by the need to freeze test plants artificially and thus potentially destroy them. Furthermore, the size of field-grown plants can make the laboratory freezing of test plants extremely difficult and introduce a large degree of environment variability in the testing chamber (Fuller, 1979). In cauliflower plants, it is possible to use just part of the vegetative material such as a leaf or leaf segment and assess the frost-hardiness of this using an electrical conductivity method (Fuller *et al*, 1989; Deane, 1994; Deane *et al.*, 1995). Dexter *et al.* (1932) described the electrical conductivity method which is based on the assumption that where there is cell membrane injury by low temperature there will be greater efflux of solutes from cells (ions, sugar and proteins). Electrical conductivity of effuse is recorded after freeze injured tissue is incubated and shaken in distilled water. Conductivity is recorded again after the samples have been heat killed in the same solution which provides a measure of total ions present in the tissue. Freezing injury is calculated as a percentage of total ions. The advantage of this method is that data can collected immediately or a few hours after freezing.

Aim

To investigate the correlation between hyp resistance, salt resistance and frost resistance of twelve selected plants following mutagenesis.

Objectives

1-To estimate hydroxyproline resistance of selected shoots and plants

2-To estimate the salt resistance of selected plants

3- To estimate the frost resistance of selected plants

5.2 Material and Methods

5.2.1 Analysis of hydroxyproline resistance in leaf strips

To determine toxic levels of hyp a leaf strip assay was devised similar to that used by Deane (1994). Leaf strips approximately 5 mm wide, were cut from leaves of *in-vitro* shoot cultures and *in-vivo* shoots of 5 control plants. Five leaf strips were placed on each petri dish of solid S23 medium (Appendix 5) supplemented with range of hyp concentrations (0mM, 1mM, 3mM, 4mM, 5mM, 10 mM). After 2 and 4 weeks the leaf strips were scored for resistance categorised according to whether they displayed total, partial or no resistance evidenced by bleaching (disappearance of chlorophyll) and the percentage of leaf strips in each category was calculated. After four weeks exposure, the maximum tolerance level to hyp of *in-vitro* shoots and *in-vivo* plants was 3mM and 10 mM hyp respectively and these levels were subsequently used to assay all selected and control.

5.2.2 Assessment of salt resistance in leaf discs

To test the salt resistance of control and selected plants a leaf disc assay was devised. One centimetre diameter leaf discs were cut from leaves of *in-vivo* control plants (*in-vitro* plants were too small to be assessed in this manner). Five leaf discs were placed per container of 20 ml M&S liquid medium supplemented with range of NaCl concentrations (0mM, 250 mM, 350 mM, 450 mM, 550 mM, 650 mM, 750 mM).

Salt damage was assessed after 7 days using a five point % score where 0 = white (no chlorophyll) and 4 (100%) = green.

The medium containing 550 mM NaCl was subsequently chosen as the screening level for salt resistance.

5.2.3 Assessment of frost resistance

The use of an electrical conductivity assessment for indicating frost resistance in leaves of cauliflower has been established (Fuller *et al.* 1989 and Deane *et al.* 1995). This technique can be applied to cauliflower plants without the need to freeze whole plants and kill them. Given the sizeable leaf area of an individual mature plant, repeated assessment of leaf freezing is possible without substantial damage to the plants.

Leaf discs, 1 cm in diameter, were cut from the newest fully expanded leaf of control and selected plants both before and after hardening at 2 °C for 14 days. Selection S11 was not included in this experiment because it only had very small leaves. The main veins and leaf margins were avoided. Forty discs from each plant were divided randomly into sets of five discs and placed into glass test tubes. The tubes were chilled to 2 °C and an ice added to each tube to ensure nucleation of freezing. The tubes were placed in a copper freezing tank filled with ethylene glycol similar to that described by Fuller and Eagles (1978). The freezing tank was programmed to run to –3, -5 and –7 °C (freezing rate 2 °C h^{-1}) with a 2-hour hold at each temperature. Duplicate samples were removed at 2 °C and at the end of each 2 hours subzero hold. Tubes were allowed to thaw over night at + 4°C and then 12.5 ml of distilled water was added to each tube using an automatic dispenser. After 5- 6 hours incubation at 20 °C, the electrical conductivity of the water in each tube was determined using a Walden Precision conductivity meter fitted with a platinum electrode (Fuller *et al.*, 1989) and a test reading was established. The tubes were then autoclaved at 15 psi for 5 minutes and cooled to 20 °C before the conductivity was measured to give the final reading. The degree of damage caused by freezing was expressed as a relative conductivity percentage after correcting all conductivity readings for the background conductivity of distilled water and RC % analysed by ANOVA.

Relative conductivity percentage (RC%) = (Test reading ÷Final reading) × 100 %

5.3 Results

5.3.1 Analysis of hyp resistance in shoots and plants selected from mutagenesis

The results of leaf strip assays of the control *in-vitro* shoots (Plate 5.1) and *in-vivo* plants (Plate 5.2) showed that no resistance to hyp existed in control plants with complete damage after 4 weeks exposure (Fig.5.1). The twelve selected shoots all showed greater resistance than controls at 2 weeks but selections S3, S4, S9, S10 showed some decline after 4 weeks. The rest of the selected shoots however retained strong resistance over the four weeks (Fig.5.1.a).

The twelve selected plants can be divided into 3 groups according to hyp resistance: group one (S3, S4, S9, S10) showed hyp resistance and high proline only in the *in-vitro* stage and no hyp resistance and low proline level in the *in-vivo* stage; group two (S6 and S11), showed hyp resistance in both of *in-vitro* and *in-vivo* stages but they have low proline levels in the *in-vivo* stage; group three, (S1, 2,5, 7, 8,12) have a positive relationship between hyp resistance and proline content in both *in-vitro* and *in-vivo* stages (Fig. 5.1. & 5.2).

Plate 5.1: The leaf strips of control shoots exposed to 0, 3 mM hyp and those of a selected *in-vitro* shoot exposed to 3 mM hyp.

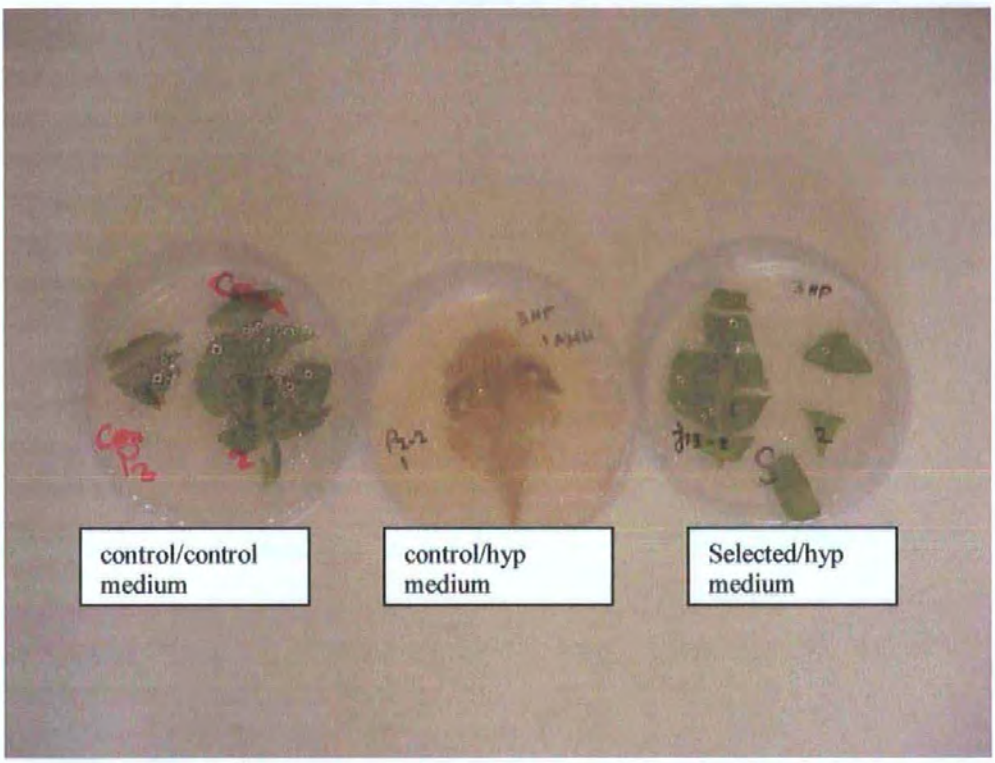
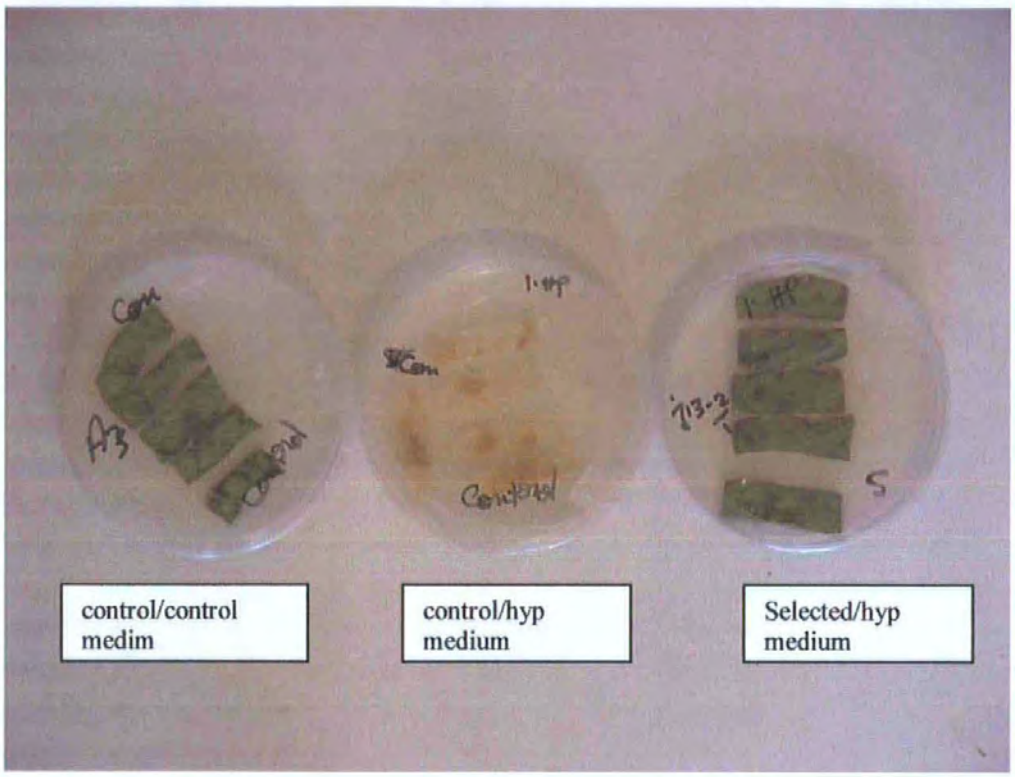


Plate 5.2: The leaf strips of control plants exposed to 0, 10 mM hyp and those of selected *in-vivo* plants exposed to 10 mM hyp.



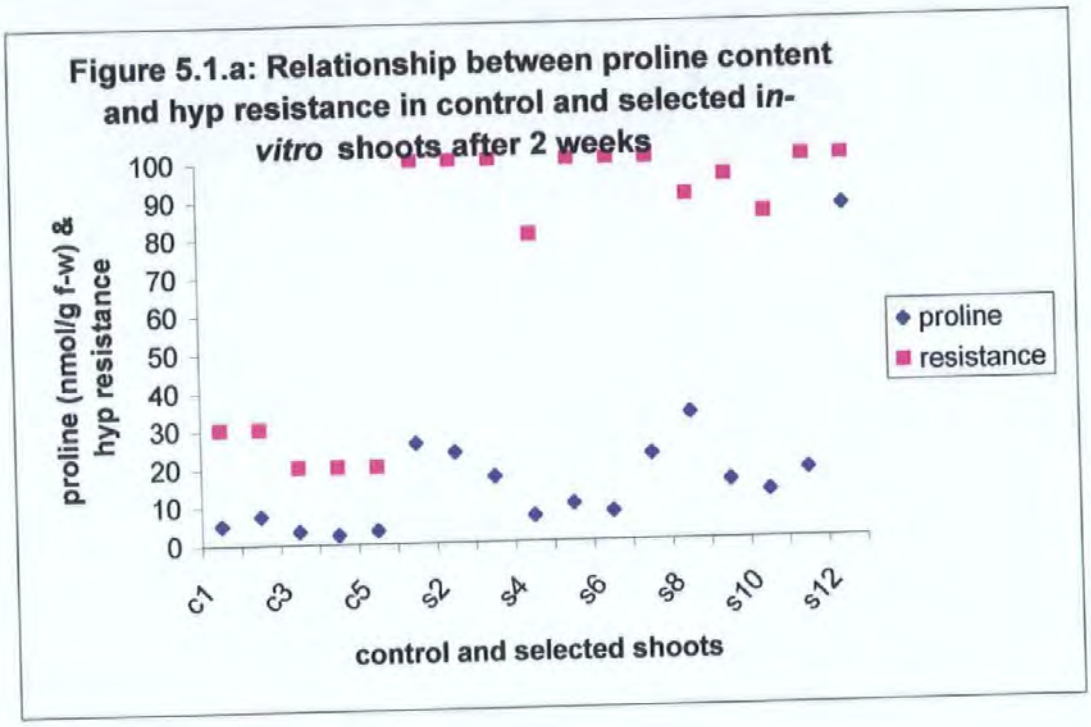


Figure 5.1.a: Relationship between proline content and hyp resistance in control and selected in-vitro shoots after 2 weeks
proline (nmol/g f-w) & hyp resistance
100
90
80
70
60
50
40
30
20
10
0
c1
c3
c5
s2
s4
s6
s8
s10
s12
control and selected shoots
proline
resistance

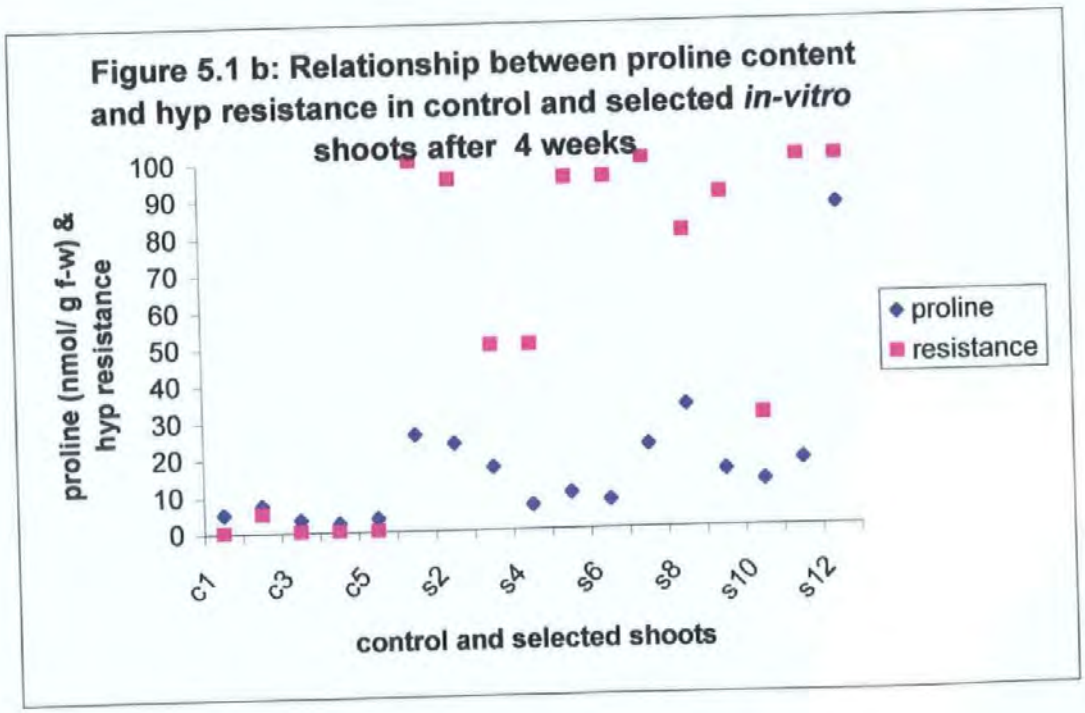


Figure 5.1 b: Relationship between proline content and hyp resistance in control and selected in-vitro shoots after 4 weeks
proline (nmol/ g f-w) & hyp resistance
100
90
80
70
60
50
40
30
20
10
0
c1
c3
c5
s2
s4
s6
s8
s10
s12
control and selected shoots
proline
resistance

Figure 5.2.a: Relationship between proline content and hyp resistance in control and selected *in-vivo* plants after 2 weeks

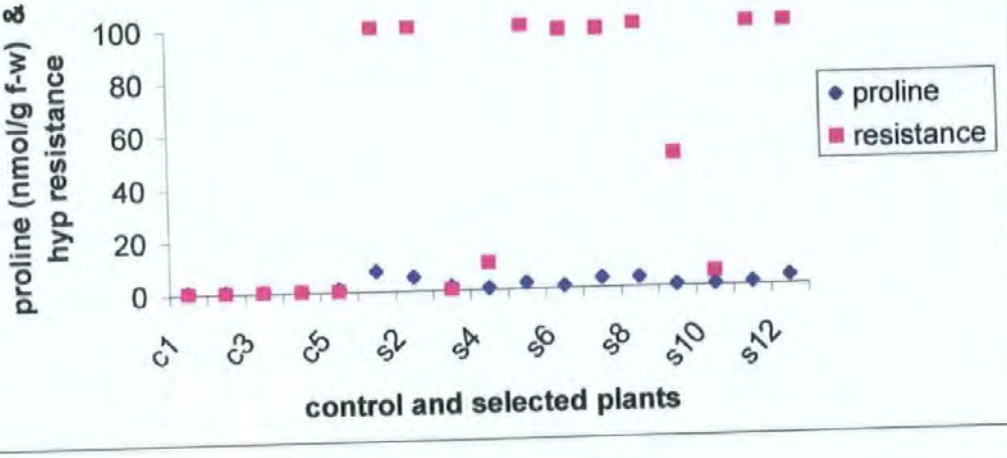
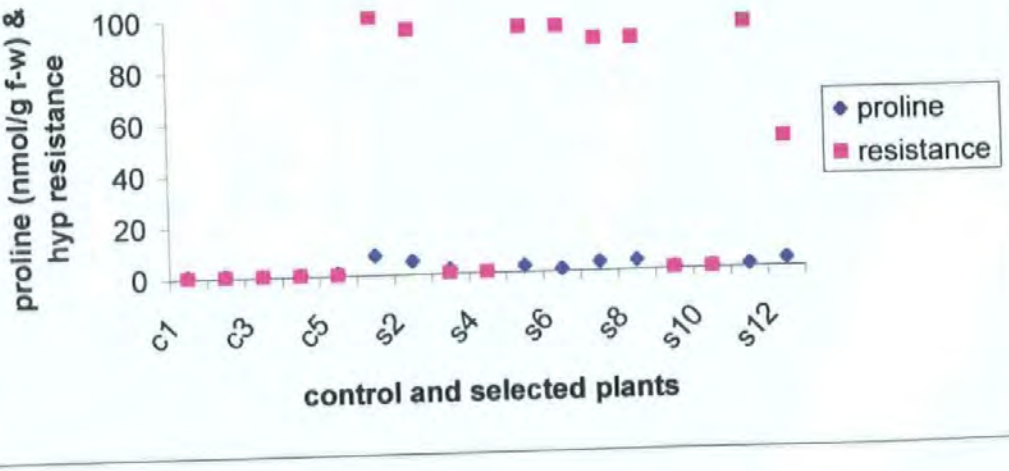


Figure 5.2.b: Relationship between proline content and hyp resistance in control and selected *in-vivo* plants after 4 weeks



5.3.2 Assessment of salt resistance in leaf discs

The results showed that control plants had no salt resistance and that selected plants had varying degrees of salt resistance (Fig. 5.3) and (Plate 5.3). There was a positive correlation between salt resistance and hyp resistance with the exception of selections S3, S4, S10 which had no hyp resistance but showed slight to moderate salt resistance (Fig. 5.4). S12 had a high proline level but only had partial salt and hyp resistance (Fig. 5.5).

5.3.3 Assessment of frost resistance in leaf discs

The results for unacclimated plants showed that damage increased with increasing sub-zero temperature and was only slight at –3 °C (2-10 % RC) but was substantial at –7 °C (32-80 % RC) (Fig. 5.6). The control plants all showed high degrees of frost damage and at –7 °C were clustered between 70 and 80 % RC. The selections demonstrated a wide range of frost resistance expression. At –5 °C S2, S4, S6, S8, S9 all had RC % 's lower than the lowest control (C1) and S2, S6 and S9 were significantly lower ($p \leq 0.05$). At –7 °C, all of the selections had lower RC % 's than the lowest control and S2, S4, S6 and S9 were significantly lower ($p \leq 0.05$). Indeed, S2 had a frost resistance at –7 °C equivalent to the best control (C1) at –5 °C or alternatively, a 2 °C better frost resistance.

Plants that were acclimated failed to show improved hardiness compared to non-acclimated plants (data not shown).

When the data for frost resistance was compared with the data for hyp resistance, salt resistance and proline accumulation a profile of resistance expression could be constructed (Table 5.1).

Table 5.1: Profile of resistance expression in each of the selections

Groups	Selected plant	salt	frost	hyp	Proline/ *In-vitro*	Proline/ *In-vivo*
Triple Resistance	**S2**	+++	+++	++	+++	++
	S6	+++	+++	+++	++	+
Double Resistance	**S1**	+++	_	++	+++	++
	S4	++	+++	-	++	+
	S5	+++	-	+++	++	+
	S7	++	-	++	++	++
	S8	+++	-	+++	+++	++
	S9	+++	+++	-	++	+
	S11	+++	-	+++	++	+
	S12	+	-	++	+++	++
Single Resistance	**S3**	++	-	-	++	+
	S10	-	+	-	++	+

Code: +++ = strong; ++ moderate; + weak; – same as control

Plate 5.3: The leaf discs of control plants exposed to 0, 550 mM NaCl and those of selected plants exposed to 550 mM NaCl for 7 days.

Figure 5.3: Relationship between proline content and salt resistance in *in-vivo* plants

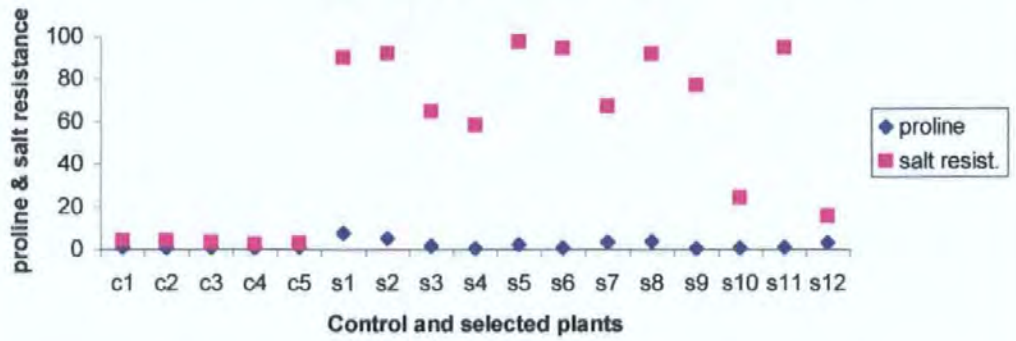


Figure 5.4: Relationship between salt and hydroxyproline resistance in *in-vivo* plants

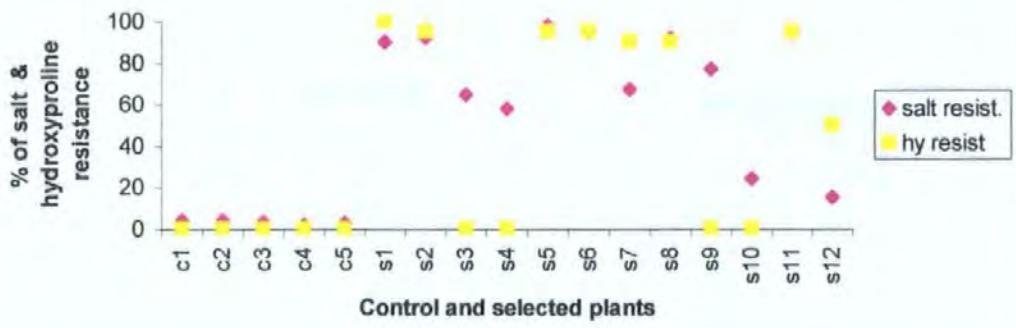


Figure 5.5: Relationship among proline , salt resistance and hydroxyproline in *in-vivo* plants

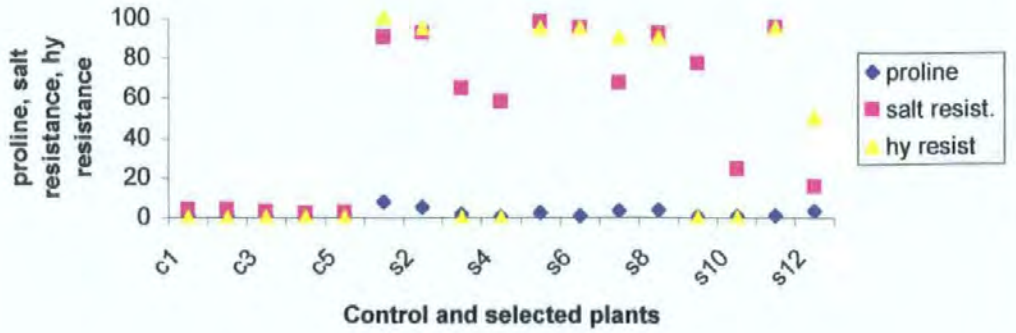
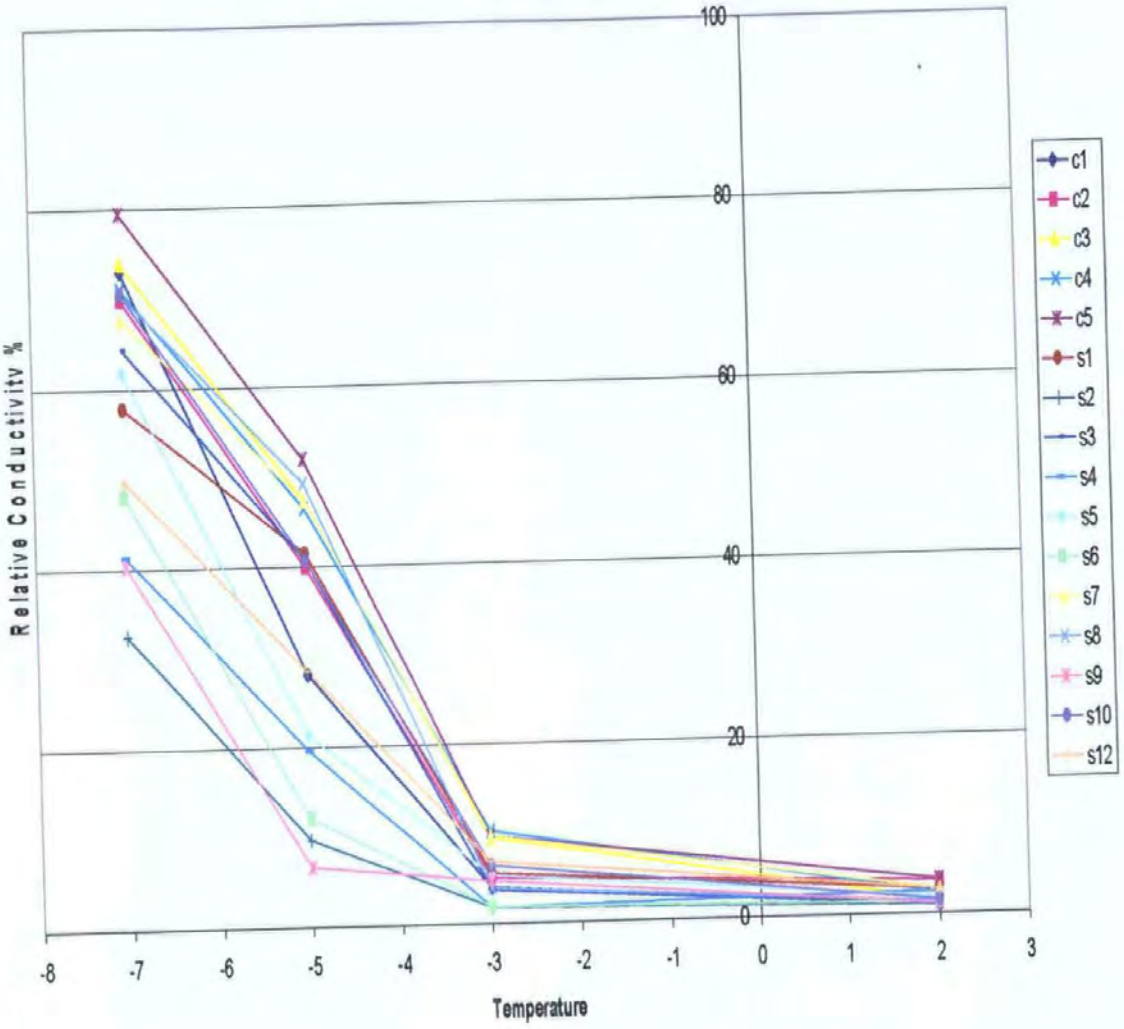


Figure 5.6: Frost resistance in control and selected leaves of in-vivo plants



5.4 Discussion

With the leaf strip assays, in control shoots hydroxyproline led to a lack of chlorophyll probably through loss of maintenance of chloroplast protein including chlorophyll binding protein leading to subsequent loss of chlorophyll resulting in the bleaching effect. The resistance to hyp shown by the selected shoots from mutagenesis might occur by a number of pathways, e.g. the relaxed control of the proline biosynthetic pathway or decreased uptake or selective discrimination against hyp.

S3, S4, S9, S10 showed hyp resistance and high proline only in the *in-vitro* stage and no hyp resistance and low proline level in-*vivo*. This could be due to the instability of the genome in *in-vitro* culture or loss of genes responsible for hyp resistance as proposed by (Karp and Bright, 1985; Van Swaaij *et al.*, 1986). Alternatively the resistance may just be an epigenetic effect only manifested *in-vitro*.

Proline accumulation might result from increasing activity of the enzymes involved in the synthesis and or inhibition of enzymes involved in the degradation of proline (Aspinall and Paleg, 1981).

In rice more than 20 mutants resistant to hydroxy-l-proline have been selected (Hasegawa and Inoue 1983). Three of these do not accumulate free proline either in the seeds or in the seedlings and their hyp resistance is controlled by a single recessive nuclear gene (Hasegawa *et al.* 1985). Mori *et al.* (1985; 1986; 1989) demonstrated that the characteristics of all the hyp resistance mutants were expressed in callus as well as in the seedling. Hyp resistance mutants were divided into two groups: the first group was classified as the levels of free proline similar to that of the original variety; in the second group a remarkable increase in free proline content is observed.

The resistance to salt shown by the selected shoots from mutagenesis might result from an ability to maintain low Na/K ratio or proline and other amino acids which could affect salt resistance.

It has been reported that in higher plants, free proline-accumulating cell lines resistant to proline analogues show salt or freezing tolerance (Riccardi *et al.* 1983; Van Swaaij *et al.*, 1986; 1987). The lack of salt – or drought-resistance in barley mutant resistance to hyp (Kueh and Bright 1981; 1982) was, on the contrary, attributed to its low content of free proline (Miflin *et al.* 1983). Proline acts in an adaptive role related to survival seedlings rather than to maintenance of growth (Greenway and Munns, 1980). Hanson *et al.* (1979) suggested that the amount of free proline reflected the degree of stress experienced by the plant.

The results showed that some selections (S2, S4, S6, S9) had high frost resistance compared with controls. This is a highly significant finding illustrating further cross-resistance to stress in some selections. S2 showed frost resistance, accompanied by a higher leaf proline content which might indicate that proline is involved in protection mechanisms against freezing injury. Van Swaaji *et al.* (1986; 1987) isolated hyp resistance lines of potato, which proved to posess increased proline levels as well as increased in frost tolerance. S4, S6, S9 showed frost resistance, accompanied by salt resistance or resistance of both salt and hyp but without increased proline. These plants could have other factors conferring greater frost resistance and is consistent with work on cauliflower by Deane *et al.* (1995). The positive correlation between frost resistance and salt and hyp resistance agrees with many other reports (Tantau and Dorffling, 1991; Dorffling *et al.*, 1993; 1997; Van Swaaji *et al.*, 1986; 1987). This positive correlation confirms the suggestion of Zhu *et al.* (1997) who reported that many genes regulated by salt stress are also responsive to freezing stress. This cross-resistance suggests a

common resistance mechanism in these plants, which requires further investigation. Based upon the resistance groupings given in Table 5.1, the plants were flowered and seeded in isolation chambers to obtain seed populations for future characterisations.

In this investigation the frost hardening treatment did not induce an acclimation response in either the control plants or the selected plants. This is probably because of the relatively poor health of the plants which only had a few leaves and also because the plants were in the early flowering stage and plants are often incompetent to acclimate during flowering. It is known that the ability to harden depends on gene expression and also the health of the plant, demanding on excess of photosynthate (Levitt, 1980). Whilst cauliflower is known to acclimate in the field (Fuller *et al.*, 1989) other workers have reported difficulties in inducing cold hardening in this species (Deane *et al.*, 1995). Despite the lack of acclimation the results following hardening did support the frost hardiness rankings of the non-hardened plants leading to confidence in the findings.

5.5 Conclusion

Hydroxyproline is generally toxic and impairs plant cells. Resistant cells and plants were selected from large populations by growing them in the presence of inhibitory concentrations of hydroxyproline and this resistance was retained in some selections when weaned *in-vivo*. Some hydroxyproline resistant plants were also salt resistant and frost resistant. Other factors than high proline level, possibly the content of other amino acids, could affect these resistances since proline levels were not uniformly high in all selected plants.

Chapter 6: General discussion and conclusion

6.1 The effects of salinity on plant growth

Although, germination and early seedling stages are generally considered the most sensitive stages to saline water in *Brassica* species, the result of *in-vitro* experimentation showed that low salt concentrations (50-100 mM) did not affect germination but subsequent fresh and dry weight of plants were adversely affected. This could indicate that there is no association between factors that affect seed imbibition and factors that affect seedling growth under low NaCl stress. Blum (1988) illustrated that the effect of salinity on germination is limited largely to the stage of seed imbibition and the inhibition of germination at high NaCl concentrations is likely to result from osmotic stress. In contrast, the reduction in plant fresh and dry are probably caused by specific ion toxicity (Chauhan and Prathapasenan, 2000). These results are similar to those of Zeng and Shannon (2000) who found that seed germination in rice was not affected up to 200 mM NaCl but was severely inhibited when salinity increased to 250 mM but even at 25 mM seedling dry weight was reduced. In the experiments reported here *B. rapa* appeared to have greater tolerance than *B. napus* possibly due to different uptake of salt at this early stage. Francois (1984; 1994) also found that *B. rapa* was salt tolerant during germination and early seedlings stages.

The data in the sand culture experiment showed no significant differences among NaCl concentrations for all growth parameters and sometimes NaCl treatments out-performed the control. This result agrees with that of Sinha (1991) who found that stimulation of growth followed irrigation with low salt concentrations (25-75 mM) in *Brassica* seedlings. For squash seedlings, Basalah (1991) found that the root and shoot length and fresh and dry weight increased as the salinity level increased up to 100 mM and Williams and Ungar (1972) reported that seedlings of *Suaeda depressa* grew better at 170 mM than 0 mM NaCl, when nitrogen was not limiting.

For most experiments compost was chosen as the growing medium for RCB's since it clearly produced better plants by providing better supplies of nutrients and a good substrate for rooting. Herrera *et al* (1997) similarly found that compost was the optimum growing medium for experiments with *Angelica arch angelica.* With regard to the sand culture system used here, it is possible that leaves of plants may have been damaged by the daily addition of the Rorison nutrient solution which may have accumulated as osmotically active salts present in the sand (Rowell, 1994).

The growth of six RCB's grown in NaCl treatments was reduced relative to untreated control plants at all concentrations tested (250-600 mM) at the adult stage. The reduction in fresh and dry weight relative to control (as percentage of control) was greater with increasing NaCl concentration. It is probable that with increasing NaCl concentration in the external medium water becomes less and less accessible which causes osmotic effects in the plants. Also excess sodium and inadequate potassium in the protoplasm lead to disturbance in the ionic balance as well as causing ion specific effects on enzymes and membranes. As a consequence, many physiological mechanisms such as stomatal movement, photosynthesis and transpiration are affected by these responses ultimately leading to the reductions in plant growth. Greenway and Munns (1980) suggest that the deleterious effects of salinity on plant growth were attributed to specific ion toxicity and nutrient ion deficiency. Kwon (1997) also found that both ion and osmotic effect responses were the cause of growth reduction by salinity in *Brassica* species.

In this study, the relative salt resistance in the six RCB's was evaluated based on the reduction in the total fresh weight of shoots of salt-treatment plants expressed as the percentage of control. It was clear that the amphidiploids *B. napus* and *B. carinata* showed the greatest salt resistance, followed by *B. juncea*, which was intermediate in salt

resistance. The diploids, *B. rapa* and *B. nigra* were intermediate in salt resistance whilst *B. oleracea* was salt sensitive with all of the plants killed when grown at 500 mM NaCl. High relative salt resistance of *B. napus* and *B. carinata* to NaCl salinity has also been found in other studies (Ashraf and McNeilly, 1990; Ashraf *et al.*, 2001; Kumar, 1995). However, *B. oleracea* was considered a moderately salt tolerance in the studies of Ashraf *et al.* (2001). This difference may be due to the index chosen for the evaluation of salt resistance and the time and the NaCl concentration chosen for applying salt stress. In their studies, evaluation of the relative salt tolerance was based on the plant growth performance in the saline medium as absolute shoot dry weights of each species, while in the present study the reduction in fresh weight relative to control was used to evaluate the salt resistance. Also, additions of NaCl treatment, 0, 100, 200 mM in full-strength Hoagland nutrient solution were begun 23 days after the start of their experiment whereas in this study, additions of NaCl treatment (250-600 mM) in distilled water were begun 5 days after the start the experiment. A further difference could be in the genetic background of the varieties used. Here RCB's were used whilst Ashraf *et al.* (2001) used commercial varieties. In order to cross-compare directly both sets of plants would need to be evaluated under the same experimental conditions.

Numerous reports exist that there is no relationship between tolerance at germination and at later stages of growth (Mahmood, 1991; Blum, 1988, Ashraf, 1994a). The results presented here showed that the most salt resistance species *B. napus* was susceptible at the germination stage and *B. rapa* was relatively salt resistant at germination but was intermediate in salt resistance at later stages of growth. Practically, it is difficult to compare resistance during germination with later stages of growth because of dissimilarities in the experimental conditions. In the germination test, *in-vitro* culture was used whereas during later growth stages, the seedlings were grown in compost culture. However, there is no reason on physiological grounds why resistance at

germination and during early stages would be associated with resistance at subsequent plant growth stages.

Salt resistance is very complex in most plant species because salt stress is known to cause tissue dehydration, ion toxicity, nutritional imbalance, or a combination of these effects. There are numerous mechanisms to be affected, at cellular, tissue, organ, or whole plant levels. Some traits may only be functional at one time in a particular species. In addition, the effect of one mechanism may mutually exclude the effect of the others at certain stages of development (Yeo, 1998; Ashraf *et al.*, 2001). The situation becomes even more complex with rapid cycling *Brassica* species (RCB's) which are maintained by open pollination and where genetic variability is to be expected.

The data showed that salinity had profound effects on ion concentrations in the shoots of the six *Brassica* species tested. Potassium concentration tended to reduce with increasing NaCl concentration applied up 450 mM but then became erratic. It is possible that below 450 mM potassium determination is confounded by the very small plant sample size as a result of the high NaCl concentrations. Sodium concentration showed the opposite trends generally increasing but also becoming more erratic at NaCl concentrations over 450 mM. In general, the initial decline in K concentrations in the shoots of all six *Brassica* species was associated with the initial rapid increase in Na having an antagonistic effect on K uptake (Ashraf and McNeilly, 1990). Na concentration in the shoot increased with increasing external concentration of salt. This increase was accompanied by a corresponding decline in K concentration showing an apparent antagonism between K and Na. This antagonism could be due to the direct competition between K and Na at sites of uptake at the plasmalemma. Also Na could increase K efflux into the growth medium, possibly due to a decrease in membrane integrity (Cramer *et al.*, 1985; Hajji and Grignon, 1985; He and Cramer, 1992). However, the exact reasons why potassium

and sodium concentrations tend to become erratic at high NaCl concentrations are difficult to determine from such a limited study where chemical analysis was of whole shoots not individual organs. The sudden rise in shoot Na concentrations was possibly due to a sudden increase in root permeability or when the leaf cells accumulated a maximum concentration of Na and could no longer withdraw Na from the xylem stream as suggested by Schachtman and Munns (1992). *B. napus* showed an unusual pattern of accumulation of Na and K with a maximum at 500 mM and a decline in the concentration of both ions thereafter which might be due to several reasons. It is possible that a high NaCl concentration in the external medium may have a severe adverse effect on root permeability as a consequence of which ions absorbed were leaked out into the external medium. Possibly a reduction in transpiration at a high external salt concentration was greater in *B. napus* at these levels of NaCl concentration. Whilst the rate of transpiration was not measured in this study this explanation was put forward by Ashraf and McNeilly (1990) who found that maximum accumulation of Na in shoots of *B. campestris* at 100 mM NaCl and a rapid decline in the concentrations of this ion thereafter at all salt treatment. Another explanation is that the decline in Na concentration was probably due to the reduced shoot capacity to remove Na from the xylem stream and then the shoot may more quickly reach the same lower maximum Na concentration that was measured, making the rise in Na concentration less sudden and conspicuous (Schachtman and Munns, 1992).

In these results, the concentration of Na in the shoots of the resistant *B. napus* and *B. carinata* did not appear to be lower than that of other *Brassica* species. It could be that the measurement of bulk tissue ion concentration masks spatial differences that may actually occur and certainly ions can be preferentially accumulated in certain cells or cellular compartments. Perhaps the ability to tolerate high Na concentration in the shoots was greater in both of *B. napus* and *B. carinata* (Schachtman and Munns, 1992).

He and Cramer (1993b) also found that the concentration of Na in the shoot and root of B. *napus* was not lower than other *Brassica* species. Indeed Van Stevninck *et al.* (1982) demonstrated that *Lupinus luteues* was more salt resistant than *L. angustifolius*, but the former accumulated more Na than the latter. Ashraf (1994a) also reported that some salt resistant species accumulated considerable amount of salt in their leaves (rice, maize). Mahmood (1991) found that some salt tolerant varieties of wheat had an unusual combination of high Na in leaves and high salt tolerance and suggested that genotypes within a species may have different mechanisms of salt tolerance. Ashraf *et al.* (2001) believed that salt exclusion is not a universal mechanism to resist salt stress but simply another mechanism, ions absorbed by cells are accumulated in the vacuoles of plants. Similarly, Akita and Cabuslay (1990) report that entry restriction of sodium ions is a major contributing factor to salt resistance, but initial salt uptake is responsible for only a certain amount of the variability. Other factors include differences in tissue tolerance to absorb Na ions and synchronisation of ion compartmentation by the leaf cells with a high rate of ion transport to the shoot (Greenway and Munns, 1980). Also, it was clear that *B. carinata* had the highest K concentrations among all six *Brassica* species over NaCl concentrations applied up to 450 mM. It is possible that favourable cytoplasm K: Na ratios are maintained by a combination of K: Na uptake selectivity, Na extrusion, vacuole Na compartmentation and partition of Na away from growing tissues at the expense of old organs (Lynch and Lauchli, 1984). The results for K/Na ratio showed a decrease with increasing concentration of NaCl applied in *Brassica* species. This decrease directly resulted from a decrease in shoot K concentration and an increase in shoot Na concentration. He and Cramer (1993a) found that seawater salinity reduced the K/Na ratio in the shoots of rapid cycling *Brassica* species and that correlated with a decrease in shoot K concentration and an increase in shoot Na concentration.

In this study, the relative salt resistance in the six RCB's was not associated with K or Na concentration nor K/Na ratio in shoots, agreeing with results of He and Cramer (1992; 1993a) who support the argument that ion concentrations and K/Na ratio in shoots were not responsible for the difference in salt-tolerance among the six RCB's. Another hypothesis associated with the relationship between salt tolerance and ion effects is that there is a difference among species in the capacity to maintain sufficient nutrient concentrations, like K for growth of plants under salt stress (Ashraf and McNeilly, 1990). Ashraf *et al.* (2001) found that salt tolerance of three amphidiploid *Brassica* species accumulated lower Na but higher K in their shoots and roots, the K/Na ratio therefore being considerably higher than those of the diploid parents. Greenway and Munns (1980) reported that the disturbance of the ratios between ions of nutritional importance and Na could result in nutritional imbalances and thus affect plant growth. They believed that a low cytoplasm K/Na ratio disrupted the metabolism and K/Na ratio in the shoots and is correlated with salt tolerance. Ashraf (1994a) considered that K is required in the external growth medium to maintain the selectivity and integrity of the cell membrane and plays a role for selective transport of ions across membranes. As a consequence, a low K/Na ratio in saline environments may impair the selectivity of root membranes and account for passive accumulation of Na in the roots and shoots. Other physiological mechanisms such as stomatal movement, photosynthesis, and transpiration are also affected by the low K/Na ratio. Ashraf (1994a) argued that K/Na ratio in the external environment does not have the same effect in different species and may be a salt tolerance criterion for many species but not for all.

How sodium enters the plant under conditions of high salinity or what controls the accumulation of Na in shoots is not known. Na concentrations in leaves may be influenced by the rate of leaf expansion or may be co-regulated with leaf growth rates (Schachtman and Munns, 1992). In order to determine whether *Brassica* species

differences in sodium and potassium concentrations are linked to the salt tolerance a precise estimation is needed such the one described by Flowers and Hajibagheri (2001). They used X-ray microanalysis to estimate the ion concentration in the cytoplasm of two barley cultivars differing in salt tolerance and hypothesise that ion transport to the shoot reflects cytosolic ion concentrations, with a more sensitive cultivar having a higher sodium concentration in its cytoplasm than a more resistant variety. In this investigation the K/Na ratio in the shoots of the resistant variety was about twice that in the sensitive variety.

Conventional selection for salt resistance in RCB's in the present study was not achieved for many reasons. Plants of the most sensitive species, *B. oleracea* died during the vegetative stage while plants of the most salt resistance species, *B. napus* and *B. carinata* died during the reproductive phase, when they had been grown in selection pressure (500 and 700 mM NaCl respectively). Perhaps, plants of *B. napus* and *B. carinata* could resist salt stress for long durations before significant reduction of survival rate occurred. Another explanation supported by Chauhan and Prathapasenan (2000) and Zeng and Shannon (2000) that roots *of B. napus* and *B. carinata* had high sodium concentration which caused ion toxicity in cytoplasm and led to reduced growth rates and eventually plant death. Ten percent of plants of *B. rapa, B. nigra* and *B. juncea,* managed to complete the first selection cycle through to seed yield stage. In the second selection cycle, they responded to selection pressure in the same manner as the control population and were not able to complete their reproductive stage. This suggests that the original survivors were escapes and no true selection for salt resistance occurred. In this study, 2000 seeds of each species were used in selection and this might be too small a number of plants for this type of selection pressure. This is supported by Ashraf (1994a) who recommended that for successful selection, up to 30,000 seeds of a species should be screened. Allowing for less than 1% survival such selection has successfully led to the

development of highly salt resistance lines. The results suggest that within the out breeding RCB's the genetic variation for salt resistance is very small and therefore not an easy trait for mass selection improvement.

6.2 Tissue culture

Plant tissue culture showed that 2,4-D was the best hormone to use for callus initiation and maintenance in *Brassicas*. Harms *et al.* (1976) suggested that 2,4-D was required for inducing cell division and formation *in-vitro* and 2,4-D is commonly used for many species (Murata and Orton, 1987; Fuller and Fuller, 1995).

Regeneration of shoots from callus was poor and it may be that the range of concentrations of the hormone combinations of IBA and Kinetin/BAP was incorrect, but a very wide range of concentrations was tested. Murata and Orton (1987) and McCoy (1987) believed that the inability of cell callus to regenerate shoots might be a result of increase in somatic age or the accentuation of genetic abnormal that accompany callus growth and subsequent regeneration. Jain *et al.* (1991) similarly found that all callus and cell suspension failed to regenerate shoots on MS media containing Kinetin and IAA in *Brassica* whereas Murata and Orton (1987) also used MS media supplemented with Kinetin and IAA or NA and obtained regeneration from callus in *Brassica* species.

The data from stress experiments with callus showed that NaCl treatments did not affect callus growth in an expected manner and sometimes even the control treatment growth was poorer than NaCl treatments. An explanation for this may be that the uptake of NaCl only occurs in the peripheral cells of the callus and central cells have no exposure and continue to multiply and expand. Also, the callus nature which acts as a sponge on the culture medium with no control of diffusion from this medium into the callus causes difficulties to apply salt stress uniformly. Jain *et al.* (1991) found that callus-based

selection for salt tolerance was unsatisfactory in *B. juncea* for the same reasons as the work of Meredith (1984) who believed that callus selection is likely to be inefficient and uncertain as all the cells in the callus piece were not uniformly exposed to the selective agent and this can result in stress avoidance due to cross-feeding between the cells in close contact with each other.

Several salt tolerant somatic cell lines have been developed for a variety of species, including *N. sylvestris* and *C. annuum* (Dix and Street, 1975), alfalfa (Croughan *et al.*, 1987) and Citrus (Pandey and Ganapathy, 1985). But only in a few cases was it possible to regenerate salt tolerant plants from salt tolerant cell lines, e.g. Watad *et al.* (1985; 1991) who indicated that resistance to salt is operating and stable at the cellular level before and after plant regeneration in *N. contiana.*

With regard to the cell culture methods reported here it is clear that problems exist in callus culture, which lead to variable results. The literature contains several reports of problems using callus culture for selection for salt resistance (Dracup, 1991; 1993; Flowers and Yeo, 1995) and it is concluded that callus cell culture selection is not a promising avenue for increasing salt resistance in RCB's.

6.3 Mutagenesis and selection for hydroxyproline, salt and frost resistance

The cauliflower curd meristem technique gave a good opportunity to evaluate the critical level of salt resistance and hydroxyproline resistance. With a huge production of thousands of shoots and a good contact between the selection medium and the explants. The results indicated that 550 mM NaCl (roughly the concentration of seawater) was lethal for small and large sizes of cauliflower microshoots. The critical level for selection for salt resistance was determined as 299 mM for the small explant category but for the

large explant category was 385 mM NaCl. This difference must be due to size indicating the NaCl uptake is probably by diffusion.

As for hydroxyproline selection the results indicated a marked declined in shoot survival at 3 mM and suggested 30 mM as a critical level selection when added 7 days after culture initiation. However, 3 mM hydroxyproline was seen to be an adequate selective level when added after one day, as in other published work on cauliflower (Deane *et al.* 1996). This difference also appears to be a function of explant size as was evident with NaCl.

Hydroxyproline had generally toxic effects on growth of *in-vitro* control shoots and caused a decline in shoot vigour of control shoots when grown on media containing this analogue. This probably results from the irretrievable competition for proline-binding sites thus reducing uptake of proline which in turn leads to protein synthesis inhibition and subsequent plant death. Such perturbations of protein synthesis could also lead to proteins which have altered properties that could be detrimental to the growth of the cell (Singh and Widholm, 1975; Widholm, 1976). Similar observations were found in other studies (Hasegawa and Inoue, 1983) who demonstrated that retardation of rice seedling growth was detected in culture at concentrations above 5 mM hydroxyproline. Furthermore, rice callus exhibited reduction in dry weight gain under the influence of 10 mM hydroxyproline (Chauhan and Prathapasenan, 2000).

Control shoots exposed to 0, 3, 10 mM hydroxyproline for 4 weeks showed an increased in proline content possibly reflecting the degree of stress experienced by the plants, an explanation supported by Hanson *et al.* (1979). Similarly a rise in endogenous proline levels of control *in-vitro* shoots might result from a slight nutrient stress caused by the exhaustion of the culture medium. Despite elevated control levels, proline levels of

selected shoots were higher than controls and might result from increasing activity of the enzymes involved in the synthesis and or inhibition of enzymes involved in the degradation of proline, an explanation supported by Aspinall and Paleg (1981). Widholm (1976), Kueh and Bright (1981) and Dix *et al.* (1984) succeeded to select mutant cell lines resistance to hydroxyproline and found that these mutant cell lines overproduced proline. When plants were subsequently weaned and established *in-vivo*, proline levels dropped which might be a result of mature plants having more protein synthesis and carbohydrates than at the *in-vitro* stage.

Several authors question whether data obtained with leaf disc and leaf strip of excised tissues are valid because of the possibility of artifacts, such as the consequences of wounding when plant tissues are excised (Ghosh *et al.* 1993; Davies and Zhang, 1991). This experimental model however provides several advantages over the study of intact plants because the reaction takes place over a shorter time, under well controlled experimental conditions and provides excellent repeatability of results. Control shoots showed no resistance to 3 mM and 10 mM hydroxyproline (*in-vitro* stage and *in-vivo* stage respectively) with severe bleaching effects. It is possible that hydroxyproline leads to a lack of maintenance of chloroplast protein including chlorophyll-binding protein and subsequent loss of chlorophyll resulting in the bleaching. The greater sensitivity of the *in-vitro* leaves emphasises their relative weakness and probably reflects a thinner cuticle which would increase hydroxyproline uptake to lethal levels at lower concentrations.

Mutagenesis and hydroxyproline selection proved successful yielding about 300 green putatively resistance lines from 7 experiments (0.03 % recovery). The resistance to hydroxyproline shown by the selected shoots from mutagenesis may be due to several reasons. Perhaps mutation resulting in feedback insensitivity of the regulatory enzyme prevents false feedback inhibition and allows the naturally occurring proline to

accumulate to levels at which it can successfully compete with hydroxyproline. The resistance to hydroxyproline may also be due to decreased uptake of the hydroxyproline (Widholm, 1976) or preferential incorporation of the naturally occurring proline (Negrutiu *et al.* 1978). Another explanation of resistance to hydroxyproline is the partial conversion of hydroxyproline into the naturally occurring proline (Van Swaaij *et al.* 1986). These mechanisms of hydroxyproline resistance are not mutually exclusive and more than one may operate in a variant cell line.

Some selected shoots (30 % of those tested) from mutagenesis showed hydroxyproline resistance in *in-vitro* stage but not in *in-vivo* stage. This could be due to instability of the genome in *in-vitro* culture or loss of genes responsible for hydroxyproline resistance as proposed by Karp and Bright (1985) and Van Swaaij *et al.* (1986). Alternately the resistance may just be an epigenetic effect only manifested *in-vitro*. Many selected shoots (70%) carried the hydroxyproline resistance forward from *in-vitro* to the *in-vivo* phase.

The leaf disc assay developed was able to discriminate between control and salt resistant plants after only 7 days incubation. The results showed that control plants had no salt resistance and that selected plants had varying degrees of resistance. Leaf discs of control plants lost their chlorophyll resulting in a bleaching effect under salt stress. This may suggest that leaf discs of control plants lost the chlorophyll as a symptom of salt stress injury or that the plasmalemma is damaged, the cell contents leak out and the cells then die. However, Gibon *et al.* (2000) hypothesised that the loss of chlorophyll was a result of stress-induced senescence or could contribute to avoidance of photoinhibitory damage under stress. The resistance to salt shown by the selected plants from mutagenesis might result from an ability to maintain low Na/K ratio or proline and other

amino acids which affect salt resistance, but the precise mechanism remains to be determined.

The results in this study showed that selected plants following mutagensis appeared to show high resistance to hydroxyproline and salt despite some plants having low levels of proline. This suggests that other mechanisms may be controlling resistance to hydroxyproline and salt stress besides proline over-production. This agrees with findings of Mori *et al.* (1985; 1986; 1989) and Haesgawa *et al.* (1985) who found that hydroxyproline resistant mutants were divided into two groups: one group with free proline at a similar level to the original variety; and a second group with a remarkably increased free proline content. It has been reported for several species, that free proline accumulating cell lines resistant to proline analogues show salt or freezing tolerance (Riccardi *et al.*, 1983; Van Swaaij *et al.*, 1986; 1987 ; Chauhan and Prathapasenan, 1998; 2000) and this is supported by the present study. Although, numerous investigation exist on biochemical processes and their regulation under stress conditions, the function of proline remains a matter of debate. There no definite evidence for the adaptive value of proline itself under stress conditions. Furthermore, the various roles suggested for this stress-induced response include osmotic balancing, protection of subcellular structures, or metabolic regulation or deregulation (Gibon *et al.* 2000). Kishor *et al.* (1995) observed increased resistance to water deficit and salinity stress in transgenic tobacco plants overexpressing Δ^1-pyrroline-5-carboxylate synthetase, but could not conclude whether stress resistance was enhanced by proline over-accumulation or by some other mechanism (Sharp *et al.*, 1996). Dorffling *et al.* (1997) provided strong evidence on the heritability of the traits of frost tolerance and increased proline content by *in-vitro*-selection procedures for hydroxyproline in winter wheat but the authors could not distinguish whether the increase in frost tolerance was due to proline over-accumulation or to the metabolic disturbances induced by this accumulation.

The results here showed that the positive correlation between frost resistance and salt, hydroxyproline resistance agrees with many other reports (Tantau and Dorffling, 1991; Van Swaaji *et al.*, 1986; 1987). This positive correlation confirm the suggestion of Zhu *et al.*, (1997) who reported that many genes regulated by salt stress are also responsive to freezing stress. Frost resistance of selected plants was not improved by cold acclimation because the plants were in the early flowering stages and plants were often incompetent to acclimate during flowering.

6.4 Conclusion

Low NaCl concentrations did not affect germination although fresh weight and dry weight declined in *B. rapa* at germination and early seedlings stage and there was no association between factors that affect seed imbibition and factors that affect seedling growth under NaCl concentrations.

B. rapa and *B. napus* appeared to have different responses to salt resistance between early and late growth stage and there was no positive correlation between salt resistance at early growth stage and that at the adult stage.

The relative salt resistance in the six *Brassica* species was associated with the reduction in the total fresh weight of shoots of salt-treatment plants expressed as the percentage of control but was not associated with K, Na concentration and K/Na ratio in shoots. *B. napus* and *B. carinata* were the greatest salt resistance, *B. juncea*, *B. rapa* and *B. nigra* were intermediate in salt resistance whilst *B. oleracea* was salt sensitive.

Compost gave the most optimum growing medium for Rapid Cycling *Brassica* species (RCB's) in *in-vivo* experiments.

Conventional selection for salt resistance was not achieved in Rapid Cycling *Brassica* species (RCB's).

Callus culture was not a good system to apply to selection of salt resistance in Rapid Cycling *Brassica* species (RCB's).

The cauliflower microshoot technique was a highly successful system for mass production of thousands of shoots, which gave an excellent advantage in the low frequency selection system of mutagenesis. This technique gave a good contact between the selection medium and the explant through providing well-dispersed cell aggregates, which were uniformly exposed, to the selective agent.

Leaf strip and leaf disc resistance assays provided several advantages over the study of intact plants because the reaction takes place over a shorter time, under well-controlled experimental conditions and provides excellent repeatability of results.

Chemical mutagenesis of cauliflower plants, resulted in a few mutant selected plants that show promise for salt, hydroxyproline and freezing resistance. Selected plants from mutagenesis fell into 3 groups; the first group appeared resistance to hydroxyproline and salt and were associated with over-production of proline, the second group had resistance to hydroxyproline and low level of proline and third group had no resistance and very low level of proline.

It was clear in this study that selection for salt resistance using conventional methods and callus culture were not good and easy ways to achieve salt resistant Rapid Cycling *Brassica* species (RCB's). The most promising method was using mutagenesis.

Cauliflower was the only species of *Brassica* that can be used in this technique because of the need for curd meristems to use the microshoot technique.

Further Work

The mechanism of salt resistance is very complex and is a quantitative trait. More biochemical and physiological investigations are needed to understand and to confirm the mechanism (s) of resistance in the selected plants. This study has provided a number of mutant selected plants that have been allowed to complete their reproductive stage yielding seeds. This provides a great opportunity for more genetic and cytogenetic investigations to determine the inheritance of stress resistance in cauliflower.

Due to limitations of time only a fraction (12) of the total number of mutants created (300) were tested, it will be necessary to characterise the remaining plants and also to evaluate their agronomic characters to ensure no loss of crop competence.

Appendix 1

M&S medium

Contents	mg/l
Ammonium Nitrate	1650.0
Boric Acid	6.2
Calcium Chloride Anhydrous	332.2
Cobalt Chloride Hexahydrate	0.025
Cupric Sulphate Pentahydrate	0.025
Disodium EDTA Dihydrate	37.26
Ferrous Sulphate Heptahydrate	27.8
Glycine (Free Base)	2.0
Magnesium Sulphate Anhydrous	180.7
Manganese Sulphate Monohydrate	16.9
Myo-Inositol	100.0
Nicotinic Acid (Free Acid)	0.5
Potassium Iodide	0.83
Potassium Nitrate	1900.0
Potassium Phosphate Monobasic	170.0
Pyridoxine Hydrochloride	0.5
Sodium Molybdate Dihydrate	0.25
Thiamine Hydrochloride	0.1
Zinc Sulphate Heptahydrate	8.6

This powder is extremely hygroscopic and must be protected from atmospheric moisture. 4.4 g of powder required preparing 1 L of medium.

Appendix 2

Rorison nutrient solution

Contents	g/l
Calcium Nitrate Hydrated	476.1
Magnesium Sulphate Hydrated	248.0
Ethylene Diamine Tetra Acetic Acid Ferric Monosodium	25.0
Manganese Sulphate Hydrated	2.028
Boric Acid	2.863
Ammonium Molybdate	0.184
Zinc Sulphate Hydrated	0.44
Copper Sulphate Hydrated	0.393
Sulphuric Acid (Normal solution)	0.028
Potassium Hydrogen Orthophosphate (anhydrous)	176.04

The pH = 6

The pH is adjusted with N. H_2SO_4 or N. NaOH as required

Appendix 3

W5 solution

Contents	g/l
Calcium Chloride	18.38
Sodium Chloride	9.0
Potassium Chloride	0.9

Appendix 4

Plant Preservative Mixture (PPM)

Contents	g/l
MethylChloro-isothiazolinone	1.25
Methyl-isothiazolinone	0.35
Magnesium Chloride	10
Magnesium Nitrate	10
Potassium Sorbate	10
Sodium Benzoate	10

Appendix 5

S 23 Medium

Contents	g/l
M & S	4.4
Thiamine	0.004
Adenine	0.080
Sodium Phosphate	0.170
Sucrose	30
Indol-3-Butyric Acid (IBA)	0.001
Kinetin	0.002
Agar	7

Appendix 6

Rooting medium

Contents	g/l
M & S	4.4
Thiamine	0.004
Adenine	0.080
Sodium Phosphate	0.170
Sucrose	30
Indol-3-Butyric Acid (IBA)	0.002
Agar	7

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