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The use of tissue culture for the improvement of salt tolerance in Atriplex SPP

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THE USE OF TISSUE CULTURE

FOR THE IMPROVEMENT OF SALT TOLERANCE

IN *ATRIPLEX* SPP.

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ABSTRACT

Soil salinity is widespread throughout the world, and human activity is responsible for increases in the area of land affected by salt. Replanting saline areas using salt-tolerant, or halophytic, species is one method of reclaiming this land. This project investigated the possibility of using in vitro methods to select for increased salt tolerance in halophytic plants. By establishing clonal lines of halophytes in culture and screening those clones for cells exhibiting variation in their capacity to tolerate salt, it may be possible to regenerate plants with elevated salt tolerance.

Clonal lines of six species of *Atriplex* (saltbushes) were obtained. Two clones each of *A. amnicola* and *A. cinerea*, and one clone of *A. nummularia* were established as shoot cultures. Explants were induced to form multiple shoots or roots in tissue culture, by the addition of 1μM of the cytokinins kinetin or 2iP, or the auxins NAA, IAA or IBA to M&S basal medium. Callus was readily initiated from leaves of *A. nummularia*, by the use of M&S medium containing 9 - 18μM NAA or IAA, and 9μM kinetin or 2iP. Suspension cultures of cells from *A. nummularia* callus were established in M&S medium without gelling agents or hormones. Regeneration of organs from callus was observed infrequently. The medium giving highest rates of organogenesis could not be defined. Shoots were formed on callus cultures on M&S medium containing 9μM NAA and 9μM kinetin; roots were formed on medium containing a variety of hormone concentrations. Under the conditions imposed here, shoot regeneration can take up to four months to become evident.

Callus and suspension cultures of *A. nummularia* were exposed to up to 342mM NaCl. Callus cultures were not affected by the addition of 43mM NaCl, but growth was depressed at higher salt concentrations. Cell density in suspension cultures were lower when NaCl was present in the medium. These results suggest that although *Atriplex nummularia* plants possess salt tolerance, this is not expressed or not effective at the cellular level.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma an any institution of higher education; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

AAM	*Atriplex amnicola*
ACI	*A. cinerea*
ANU	*A. nummularia*
ASE	*A. semibaccata*
AST	*A. stipitata*
AVE	*A. vesicaria*
BAP	6-benzylaminopurine
2,4-D	2,4-dichlorophenoxyacetic acid
EDTA	ethylenediamine tetraacetic acid
FAO	Food and Agriculture Organisation
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
2iP	N-isopentenylaminopurine
kinetin	6-furfurylaminopurine
M&S medium	Murashige & Skoog basal medium
NAA	1-naphthaleneacetic acid

1. INTRODUCTION

1.1 SOIL SALINITY

Authorities have estimated that around one billion hectares of land (or between eight and ten percent of the earth's land surface) can be classified as salt affected (F.A.O., 1971 - 1981; Malcolm, 1993), with about 12 million hectares occurring in Western Australia (Selected Committee on Salinity, 1988). Estimates of soil salinity vary according to the criteria used, but all are alarming in view of the expanding world population and the associated increase in world agricultural demand. Naturally occurring saline soils are widespread in arid areas where factors including sporadic rainfall, high evaporation rates and advanced weathering of the parent rock combine to give increased soil salinity (Strahler & Strahler, 1973, pp. 288-318). Human activities are responsible for increases in the level of salinity of land under cultivation, and in the area of land affected by soil salinity. Around half the world's farms have been damaged by salt, greatly reducing their productivity (Pearce, 1987). Mudie (1974) has estimated that 53 million hectares of the world's irrigated land is salt-affected, with the Punjab in India being perhaps the worst-affected area of the world. The land here has been irrigated since the Indus civilisation, around 5 000 years ago (Stoner, 1988). In 1984 it was estimated that 25 million hectares of agricultural land in India was affected by salinity and no longer fit for conventional agriculture (Gill & Abrol, 1993).

The increase in the world's saline soils due to human activity is largely a result of poor or inappropriate agricultural practices. Much of the salinisation now evident in Asia and Northern Africa is due to the use of poor-quality water supplies for irrigation. This results in deposition of salts in the soil, and a concentration of these salts following evapotranspiration. It has been claimed that the spread of salinisation due to irrigation is so severe, and so difficult to ameliorate that in the long term, irrigation schemes are not viable (Casey, 1972).

In southern Australia, induced salinisation had affected 4.2 million hectares of previously productive land by 1982, and this area was estimated to be increasing at a rate of 5% per annum (Soil Conservation Standing Committee, 1982). In the south-east region of Australia irrigation schemes used to grow citrus, grape and other fruit crops have led to increases in soil salinity (Hall, Baden, Christian, London, Dale, Hart, Leigh, Marshall, McArthur, Russell &

Turnbull, 1972, pp. 415-419). In the south-west region irrigation is not widespread, and salinity has resulted from other dryland agricultural practices. Here 5.4% of all salt-affected land (650 000 ha of the total 12 million affected by salt), can be attributed to human activity (Malcolm, 1983; Selected Committee on Salinity, 1988), and around 440 000 hectares of previously productive land has become too saline for conventional agriculture (George, 1990). These estimates are likely to rise as the consequences of past clearing become evident, and as geological and pedalogical knowledge improves.

The causes of salinity in Western Australia have been summarised by Mulcahy (1978). Here replacement of the perennial, deep-rooted native plant communities by shallow-rooted crops and pastures with a limited growing season has been the predominant cause of salinity. Tree-dominated native plant communities utilise groundwater supplies throughout the year, including the hot dry summer months when shallow-rooted crop and pasture species do not grow. Native plant communities use far more water over the whole year than do agricultural plants. The result of clearing land for agriculture is a decrease in the amount of water returned to the atmosphere via evapotranspiration, and thus an increase in soil water reserves through groundwater recharge, stream discharge and surface flow. Through these increases in water movement, soluble salts are leached from the soils and are carried to low-lying areas of the landscape where subsequent evaporation leaves a concentration of salts in the soil. Dissolved salts are also brought to the surface from deep within the soil profile by rising groundwater. Native plant communities have adapted to the hot dry summer conditions using deep sinker roots to utilise underground aquifers: clearing of vegetation reduces water consumption from these aquifers and increases water recharge. Underground water levels can rise to the surface in low-lying areas, bringing dissolved salts with them (Hall et al., 1972, pp. 415-419).

A further cause of salinity in the south-western part of the state has been the use of agricultural fertilisers (Mulcahy, 1978). Due to the inherent nutrient deficiencies of soils of this region, phosphates were added in high quantities to improve yields. In the past rock phosphate (obtained from seabird guano, which is highly saline), was used as a fertiliser. With the advent of manufactured low-salt chemical fertilisers, this source of salt to the landscape is no longer significant.

The problems resulting from such mobilisation of salt in the landscape are widespread, and combine to reduce the productivity of agriculture. Water quality in streams, rivers and dams can be affected, to the extent that the water becomes unusable for humans, stock or even as irrigation water. Salt encroachment has serious effects on soil quality, leading to a loss of soil structure, increases in erosibility, the development of water repellance and calcic crust formation (Malcolm, 1993). Salinisation is detrimental to plant growth including crops and pastures, leading to a decrease in agricultural productivity. Of equal importance is the impact that salinisation has on human land values such as conservation and recreation.

1.2 SALINITY TOLERANCE IN PLANTS

Various forms of salt, including chlorides, carbonates and sulphates of sodium, calcium, magnesium and potassium affect plants in different ways, but the predominant salt in the environment is NaCl. In addition to high Na^+ and Cl^-, waterlogging and the presence of other toxic elements (such as boron) also retard the growth of plants in saline soils. Halophytes are defined as plants capable of growing on soils with elevated salinity, as opposed to glycophytes which cannot grow and survive on saline soils. Smith (1981) has suggested that for the purposes of defining halophytes, soil salinity can be considered as "high" if salt concentrations exceed 300mM. For the purposes of this thesis, the terms "salt" and "saline", refer to NaCl, and environments with relatively high NaCl concentrations, respectively.

Salinity is a problem in arid and semi-arid regions, reducing the productivity of both dryland and irrigated agriculture, decreasing water quality and affecting soil properties. It also restricts the rehabilitation of many areas of degraded land such as mineral residue sites and agricultural saltpans. Considerable research and development is being undertaken in order to develop effective rehabilitation strategies for such areas in Western Australia. The selection of salt tolerant, or halophytic, species and particularly tolerant clones or lines of these species has considerable potential for use in replanting programs (Van Der Moezel, Bell, Bennett, Strawbridge & McComb, 1990). Plants selected for their salt tolerance could be of use in landcare and soil conservation projects to halt land degradation (such as the replanting of saline farmlands in the eastern wheatbelt region), and for the rehabilitation of mined lands and other saline areas.

Replanting programs on salt-affected !   .ising specially-selected salt tolerant species appear to be currently the most efficient and least expensive method of reclaiming saline land, and have been applied to many areas in Western Australia (Malcolm, 1993). The growth of evergreen trees and shrubs such as *Eucalyptus* spp. may lower the groundwater table by using more water than crops and by taking water from deeper in the soil profile, hence facilitating the removal of surface salts by leaching (Greenwood & Beresford, 1979). Plantations of halophytes such as *Atriplex* spp. may have a similar effect, and can be used in more saline landscapes because of their greater salt tolerance (Greenwood & Beresford, 1980). Replanting programs have the added benefit of limiting erosion by stabilising the soil surface. In agricultural areas, *Atriplex* spp. have long been used for pasture on marginal soils because of their drought-resistant and halophytic properties, and their suitability as sheep fodder (Malcolm, 1993). Some selection of successful lines has been attempted to improve these qualities.

In order to survive on saline soils halophytes must overcome several problems:

(i) Osmotic effects - the acquisition of water is more difficult since the high solute concentration in the soil results in a low external water potential. There is therefore no water movement into plant roots unless the internal water potential is lowered through osmoregulation. This lack of water can inhibit cell division and growth in the root zone.

(ii) Specific ion effects - high Na^+ and Cl^- concentrations are toxic to plant cells, as are other elements often found in saline soils such as boron and sulphur. Ions of these elements can affect the integrity of cell membranes. Sodium and chloride ions can also affect the acquisition of other mineral nutrients; for example Na^+ can competitively inhibit K^+ uptake. High Cl^- concentrations are inhibitory to most enzymes, thus affecting metabolism.

(iii) Habitat effects - soils affected by salts tend to be either very dry, with low sporadic rainfall; or very wet, being frequently inundated by seawater or waterlogged by groundwater (Fitter & Hay, 1987, pp. 223-259). Saline soils can also become sodic (pH > 8.5), resulting in the development of soil hypoxia (due to the dispersion of colloids by Na^+ ions at high pH), and deficiencies in Ca^+ and Mg^+ (Marcar, Crawford and Leppert, 1993).

The indirect effects of these salt stresses may be observed as an inhibition of growth and development (as energy is redirected towards osmoregulation), alterations in morphology, plant development and reproductive activity, and

metabolic disturbances of photosynthesis, respiration, protein synthesis, amino acid synthesis, nucleic acid synthesis, and enzyme activity (Levitt, 1980, pp. 365-488).

Plants exhibit a variety of mechanisms to reduce the effects of salt on their life cycle and these can be grouped into categories of avoidance, osmoregulation and adaptation (Table 1.1). Many of these mechanisms utilise a series of cells and tissues in a co-ordinated process, to confer salinity tolerance. These include transport and ion exchange mechanisms which remove Na^+ and Cl^- from actively growing cells and, for example, extrude them via ion pumps (Greenway & Munns, 1980). Many halophytes, including species of *Atriplex* (Osmond, Troughton & Goodchild, 1969; Mozafar & Goodin, 1970) possess specialised structures such as salt glands, bladders and trichome cells. These act to sequester salt so that metabolic processes are not affected (Flowers, Troke & Yeo, 1977). All these mechanisms require the anatomical organisation which exists in entire plants (Binzel, Hasegawa, Handa & Bressan, 1985).

Other mechanisms of salinity tolerance involve properties intrinsic to individual cells. Within cells, salts appear to be accumulated in the vacuole (Flowers & Läuchli, 1983). Metabolic investigations (for example Greenway & Osmond, 1972; Heimer, 1973) show that a range of enzymes from several glycophytes and halophytes have similar sensitivity to NaCl. This supports the suggestion that intracellular salts are mainly localised in the vacuole, and that halophytes have an increased capacity to recognise and transport salt ions across a concentration gradient into the vacuole, as opposed to an increased enzymatic tolerance of internal salinity (Flowers et al., 1977).

Some halophytes can accumulate organic solutes (primarily organic acids, nitrogen compounds and carbohydrates) to concentrations high enough to affect osmotic balance. High external Na^+ concentrations can be balanced by high organic solute levels such as oxalate in *Atriplex* spp. (Osmond, 1967), malate (Oaks & Bidwell, 1970) and proline (Demmig & Winter, 1986) in *Mesembryanthemum crystallinum*, betaine in *Suaeda monoica* (Storey & Wyn-Jones, 1978), and a variety of carbohydrates including glycerol and mannitol in algae (Wegmann, 1971; Hellebust, 1976). Such compounds are also accumulated by plants under other conditions of osmotic stress including drought, heat and extreme cold.

TABLE 1.1 : Biochemical and physiological mechanisms
conferring salt tolerance in plants

METHOD	MECHANISMS	EXAMPLE	REFERENCES
AVOIDANCE			
Passive exclusion	Low NaCl permeability	*Glycine wightii* (soybean)	Gates, Haydock & Robins (1970)
Extrusion	Active ion pump (Na^+:K^+)	*Distichlis spicata* (saltmarsh grass)	Hansen, Dayan-adan, Kaufmann & Brotherson (1976)
Excretion	Localised salt glands	*Avicennia marina* (mangrove)	Shimony, Fahn & Reinhold (1973)
Secretion	Epidermal bladder cells	*Atriplex* spp. (saltbush)	Osmond et al. (1969)
Dilution	Adjustment of internal fluid levels; succulence	*Atriplex* spp. (saltbush)	Greenway, Gunn & Thomas (1966)
OSMOREGULATION			
Ion accumulation	Salt concentrated internally (in leaves)	*Atriplex* spp. (saltbush)	Wallace, Mueller & Romney (1973)
Organic solute accumulation	Synthesis of solutes, e.g. proline	*Mesembryanth-emum* sp. (ice plant)	Ostrem, Vernon, Olson & Bohnert (1987)
ADAPTATION			
Metabolic adaptation	alternative metab-olic pathways, (CAM photosynthesis)	*Mesembryanth-emum* sp. (ice plant)	Adams, Thomas, Vernon, Bohnert & Jensen (1992)

Studies on a variety of glycophytes and selected salt-tolerant plants have suggested that one mechanism of salt tolerance involves the selective absorption and/or exclusion of ions. Such cellular ionic regulation mechanisms have been summarised by Stavareck and Rains (1984). Liu and Yeh (1982) found that Na^+ and Cl^- content was increased in salt-tolerant sugar cane cells. Conversely salt levels are lower, and Ca^{2+} levels higher, in salt-tolerant cell lines of *Citrus sinensis* (Ben-Hayyim & Kochba, 1983). Salt-selected alfalfa (*Medicago sativa*) strains also show higher levels of intracellular Ca^{2+} as well as K^+ levels (Stavareck & Rains, 1984). It appears that salt tolerant cells have the ability to regulate the entry of ions into the cell to effect osmotic balance. These cellular mechanisms are especially important to non-halophytes which lack anatomical structures such as salt glands (Binzel et al., 1985).

1.3 GENETICS OF SALT TOLERANCE

Salt tolerance is widespread among the orders of higher plants, and is conferred by a range of slight modifications to plant physiology. Flowers et al. (1977) claim that there is a conspicuous absence of dramatic adaptation among plants, and such a widespread occurrence (of salt tolerance) among higher plants is indicative of a polyphyletic origin involving relatively few changes in the genome. Such changes may be quantitative rather than qualitative, involving the regulation of ion transport, ion uptake, ion exclusion from the cytoplasm, ion accumulation in the vacuole, and the balancing of cytoplasmic and vacuolar water potentials. Evidence presented by Flowers et al. (1977) suggests that in most species, salt tolerance results from several simple genetic characters acting either in concert or alone. Later studies of the genetics of salt tolerance have suggested that it is a polygenic response to environmental triggers. Breeding for salt tolerance is difficult because of the complex interactions and environmental effects on the expression of tolerance (Johnson and Smith, 1992; Tal, 1985).

Several researchers have attempted to screen for salt tolerant cells in vitro (for example Smith and McComb, 1983; Binzel et al., 1985; McCoy, 1987). Few selection programs have resulted in the inheritance of a stable form of salt tolerance. In *Nicotiana tabacum* (tobacco) plants, salt tolerance has been selected for and inherited in two subsequent generations of regenerated plants (Nabors, Gibbs, Bernstein & Meis, 1980). Salt tolerance of these plants was found to be a dominant or co-dominant phenotype, but not to follow Mendelian-based inheritance patterns. It was suggested that the step-wise

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selection process of stress application and recovery time resulted in a multiallelic or multigenic system of salt tolerance. Nabors et al. (1980) also found that the cell lines of NaCl-tolerant tobacco plants had somewhat different growth habits and different regeneration rates, and proposed that a different set of mutations in the original screening process had resulted in the observed salt tolerance and in the phenotype differences. These observations further support the hypothesis that salt tolerance is a polygenic character and the transmission of tolerance from generation to generation is complex, and environmental variables can influence their expression.

While numerous authors have selected salt-tolerant cell lines and demonstrated the superior tolerance of these lines (Stavareck & Rains, 1984), the physiological differences between lines have rarely been considered. Blascheck and Franz (1983) reported a change in the polysaccharide composition of the cell wall in plants exposed to salts. Winicov and Button (1991) recorded an increase in the accumulation of photosynthetic gene transcripts in a salt-tolerant alfalfa cell line. The halophyte *Mesembryanthemum crystallinum* responds to salt stress by switching to crassulacean acid metabolism (CAM) photosynthesis to conserve water, and by accumulating proline as an osmotic adjusting agent. Salts have been shown to induce transcription activation of genes coding for CAM enzymes (Thomas, De Armond & Bohnert, 1992) and for proline synthesis (Thomas, McElwain & Bohnert, 1992). These biochemical investigations indicate that halophytes possess several genes or groups of genes that code for responses to external salinity at the level of both the whole plant and the individual cell.

1.4 IN VITRO SELECTION FOR SALT TOLERANCE

The use of tissue culture raises the possibility of intensive screening and selection for more salt-tolerant genotypes. Given that there is a strong genetic basis to salt tolerance, selection programs using tissue culture methods can be used to screen for naturally occurring genetic variability and to develop clonal lines incorporating this genetic salt tolerance. Individual plants may exhibit superior genetic ability (compared to other plants in the species) to tolerate higher salinities through any of the mechanisms of salt tolerance. These plants could be used as the basis for selection programs, using tissue culture to screen for more tolerant genomes in vitro. In the selection of salt tolerance, as in any selection program, the important emphasis is on genetic, not acquired, salt tolerance. The selected character must always be expressed in the whole plant,

and expressed in the progeny of the selected plants so that revegetation programs have long-term viability.

Somaclonal variation (the range of responses exhibited by cells in tissue culture due to their genetic variation, and to mutations induced by normal culture conditions) can also be exploited as a source of genetic variation within plants in tissue culture. Variant cells may have, among other functional differences, a higher degree of salt tolerance. Media manipulation can be used to select the cells that are tolerant, and these cells can be regenerated and cloned to produce plants of the same genotype. Tissue culture methods can be used to screen millions of cells for tolerance to salt at the cellular level, and these cells then have the potential to regenerate whole plants with the salt tolerant genome. To be successful, such a selection process must result in the expression of salt tolerance at the cellular level in culture, and the retention of that tolerance when cell lines are regenerated to whole plants.

There have been many published reports of successful selection of salt-tolerant cells from both glycophytes and halophytes. Direct in vitro selection for salt tolerant genotypes of alfalfa (*Medicago sativa*) has involved the addition of up to 250mM NaCl to the media to select for the more tolerant cells (Croughan, Stavareck & Rains, 1978; Smith & McComb, 1983; McCoy, 1987). Tobacco cells which are tolerant of 151mM (Nabors et al., 1980), 342mM (Dix & Street, 1975) and 599mM NaCl (Binzel et al., 1985) have been produced. Cell lines from a variety of plants have been selected for their tolerance to fairly high levels of NaCl:

- Pepper (*Capsicum annuum*), to 342mM (Dix and Street, 1975);
- Rice (*Oryza sativa*), to 342mM (Croughan, Stavareck & Rains, 1981);
- Jojoba (*Simmondsia chinensis*), to 274mM (Mills & Benzioni, 1992).

Reports on selection of salt tolerant cell lines in a variety of plants indicate that in vitro breeding programs could be a viable method of screening and selecting for genotypes with a greater tolerance to salt. There have however, been difficulties in obtaining lines of cells which retain this salt tolerance through passages of in vitro culture, and which result in the regeneration of more salt-tolerant plants. Some reports have claimed that the salt tolerant trait is stable through prolonged periods of culture in the absence of salt (Dix & Street, 1975; Nabors, Daniels, Nadolny & Brown, 1975; Nabors et al., 1980; Ben-Hayyim & Kochba, 1983), while other authors have reported that salt

tolerance is lost from the cell line following extended periods in culture or growth in NaCl-free media (Hasegawa, Bressan & Handa, 1986; Binzel et al., 1985; Chandler & Vasil, 1984).

There have been few reports of salt selected cell lines being successfully regenerated into plants with superior salt tolerance. Nabors et al. (1980) regenerated tobacco plants from cells tolerant to NaCl. The plants regenerated from these cells showed higher survival rates than those from unselected origins, when watered with 571.5mM NaCl. However most authors report that regeneration from salt tolerant callus is not successful, and the survival of regenerated plants is marred by adverse genetic or epigenetic effects (Stavareck & Rains, 1984). Smith and McComb (1983) found that regeneration of alfalfa was severely depressed in salt-tolerant cell lines, and plants could only be regenerated from one callus. These regenerated plants did not show an improved tolerance to salt when compared to unselected parent seedlings. Plants regenerated from salt-tolerant alfalfa cells selected by Croughan et al. (1981) were later found to be weak and stunted, and did not demonstrate increased salt tolerance (Stavareck & Rains, 1984). Wong, Ko and Woo (1983) found that rice plants regenerated from salt tolerant cell lines showed a high rate of albinism (39 out of 44 plants), and were partly sterile.

Cell culture systems, as they have been used to select cells tolerant to elevated levels of salt, have several advantages over conventional, in vivo breeding techniques. The environmental and nutrient conditions can be controlled uniformly and precisely, and a large number of cells (up to 10^6 cells per 250mL flask) can be screened rapidly in a relatively small area (Smith, 1981). The relatively undifferentiated nature of the cultured cells reduces the complications of differences in the morphology and developmental stage. The use of in vitro cultures, such as callus or cell suspensions, offers a means to focus only on the biochemical and physiological processes inherent to the cell which contribute to salinity tolerance (Stavareck & Rains, 1984).

Studies using cell and callus cultures have indicated that correlations of salinity tolerance of a plant with that of its cultured cells, occurs if the tolerance is due to predominantly cellular-based mechanisms (Tal, Heiken & Dehan, 1978; Orton, 1980; Smith & McComb, 1981a, 1981b, 1983; Warren & Gould, 1982). The application of cell-based selection programs to halophytic plants such as *Atriplex* spp. raises the possibility of increasing their cellular salt tolerance on

top of, or in addition to, their halophytic properties. Conventional in vivo breeding programs can only be used to select for plants with improved whole-plant tolerance mechanisms.

There are several practical problems in the application of cell culture systems to selection programs. It is possible, given the right conditions, to regenerate plants from callus cells, but regeneration from salt-selected callus cells has had limited success to date. Selection programs require callus to be maintained in culture for long periods of time, and the capacity for regeneration often decreases rapidly with time. The development of procedures and media sequences for each species under investigation may improve the success of regeneration attempts. The expression of desired traits has not always been seen in regenerated plants: selection programs must aim for a high degree of heritability of the desired trait. Regenerated plants should retain the desirable characters of their variety or species (such as high yield or palatability), without incorporating any deleterious mutations through the selection process (Stavareck & Rains, 1984).

Although also an advantage, one of the major limitations of in vitro screening programs is that physiological features of plants which rely on the differentiation of cells into tissues and organs, cannot be observed since cells in culture exist in a dedifferentiated form. In vitro programs for selecting salt tolerance have a limited application to screening for the ability of halophytes to minimise the effects of salinity with specialised tissue such as salt glands, bladder cells or the succulence mechanism, because of the unorganised nature of the callus. Salt tolerance is generally held to be a polygenic character, and selection programs can only apply to some aspects of the tolerance polygenes at any one time (Tal, 1985). In vitro selection programs can only apply to those polygenes which are expressed at the level of the dedifferentiated cell.

It is important to realise that it is possible to embark on screening and selection programs for salt tolerance, even though the exact functioning of the mechanisms of salt tolerance, and the genetics and heritability of salt tolerance are not fully understood. Successful selection programs may elucidate further information on the modes of inheritance of salt tolerance.

The objective of this research is to investigate the possibility of using in vitro methods to select for increased salt tolerance in species of *Atriplex*. Given that

Atriplex spp. can be established in tissue culture, it should be possible to manipulate culture conditions in order to select for more salt tolerant cells. If the mechanisms of salt tolerance operate at both the cellular and whole-plant levels, then both levels of salt tolerance can be selected for independently. These two forms of salt tolerance could then be combined in the same genotype, and cloned through tissue culture. If both types of salt tolerance are expressed additively in the whole plant when soil salts are present, then plants with this genotype may have "supertolerant" properties, and would be very useful in rehabilitation and revegetation projects. The study examines the following:

(i) The conditions necessary for shoot and root initiation and growth in culture in a range of *Atriplex* species.

(ii) The conditions necessary for callus initiation, multiplication and regeneration in *Atriplex nummularia*.

(iii) The conditions necessary for suspension cell culture in *Atriplex nummularia*.

(iv) The possibility of screening for salt tolerance in *Atriplex* callus.

2. MATERIALS AND METHODS

2.1 PLANT MATERIAL

Cuttings of *Atriplex nummularia* Lindley in T. Mitch. (ANU 001) were obtained from a mature plant at Murdoch University. *Atriplex vesicaria* Heward ex Benth. (AVE 001) cuttings were collected from a plant growing at the north-east shore of Baandee Lakes, near Kellerberrin, W.A. *Atriplex stipitata* Benth. (AST 001) and *Atriplex semibaccata* R. Br. (ASE 001) cuttings were collected from plants growing on saline tailings dumps at Poseidon Gold, Kalgoorlie operations. *Atriplex cinerea* Poir. in Lam. cultivars C2 and C3 (ACI 002 and ACI 003) and *Atriplex amnicola* Paul G. Wilson prostrate cultivar C2 and erect cultivar C3 (AAM 002 and AAM 003) were provided as potted specimens by the Agriculture Department of Western Australia.

Stock plants were grown in pots containing free-draining soil in a glasshouse. Tissue culture explants were taken from fresh plant material cut from glasshouse grown stock plants, or from cuttings from mature plants growing in situ when necessary.

2.2 STERILE TECHNIQUE

Tissue culture techniques are designed to allow plant material to be grown under a defined set of conditions (nutrients, growth regulators, carbon source, light, temperature, humidity) in a sterile environment.

Sterile conditions were achieved by autoclaving any materials that were to contact either plant material or the inside surfaces of media containers (such as glassware, metal instruments, plastic plates used as cutting surfaces, and rinsing water), for 15 minutes at 121°. Disposable equipment such as petri dishes and scalpel blades was purchased as pre-sterilised units.

Once plant material was surface sterilised, (refer to Section 2.5), it was handled aseptically in a laminar flow unit which had been exposed to ultra-violet radiation for approximately 20 minutes prior to use, and had been swabbed with 70% ethanol. Instruments were regularly re-sterilised by exposure to heat, using a Bacticinerator sterilising unit (Sigma-Aldrich, Castle Hill NSW).

2.3 CULTURE CONTAINERS AND GROWTH ENVIRONMENT

Experimental cultures were grown in 8 × 2.5cm screw-top polycarbonate vials containing 10mL of solid medium. Suspension cultures were grown in 100mL Erlenmeyer flasks containing 25mL of liquid medium. Flasks were stoppered with cotton wool wrapped in gauze, and loosely covered with aluminium foil. Shoot cultures were maintained in 7cm square Magenta™ vessels (Sigma-Aldrich, Castle Hill, NSW) containing 50mL of solid medium. Plastic Petri dishes (9 × 1.5cm) containing 20mL of solid medium were also used for callus initiation experiments.

Cultures were maintained in growth cabinets at $25°C \pm 1°C$, with a 12h photoperiod. Some shoot cultures were maintained in a culture room at $25°C \pm 4°C$, with a 12h photoperiod. Light was provided by cool white fluorescent tubes, and irradiance at the culture surface was approximately $24\mu mol.s^{-1}.m^{-2}$ in growth cabinets and $36\mu mol.s^{-1}.m^{-2}$ in the culture room.

2.4 CULTURE MEDIA

2.4.1 Stock Solutions

Stock solutions of the plant growth regulators IAA, NAA, IBA, 2,4-D, kinetin, 2iP and BAP were used for media preparation. Stock solutions were prepared by dissolving powdered auxins and cytokinins (Sigma-Aldrich, Castle Hill NSW) in absolute ethanol or 1M NaOH respectively, and making up the required volume with ultrapure water (ion-exchange filtered to 15 milliohms of electrical resistance). Stock solutions were stored at 5°C. NAA was stored in dark bottles to minimise deactivation by light.

2.4.2 Media Composition

Culture media were prepared using Murashige and Skoog (M&S) Basal Medium Powder (Sigma-Aldrich, Castle Hill NSW; Product number M5519), containing macro- and micro-nutrients and vitamins (Table 2.1). Agar (High Purity Agar, Coast Biologicals, Auckland NZ), gellan gum (Phytagel™, Sigma-Aldrich, Castle Hill, NSW) plant growth regulators and sucrose (CSR Ltd, North Sydney, NSW) were also added (Table 2.1). For salt tolerance experiments, analytical grade NaCl (Ajax Chemicals, Auburn NSW) was added to the medium in relevant concentrations as discussed in Section 4.2.4.

TABLE 2.1: Composition of Murashige and Skoog (1962) Media

COMPONENTS	CONCENTRATION (mg.L^{-1})
Macronutrients:	
Ammonium Nitrate	1 650.0
Calcium Chloride (anhydrous)	332.20
Magnesium Sulphate (anhydrous)	180.70
Potassium Nitrate	1 900.0
Potassium Phosphate Monobasic	170.0
Micronutrients	
Boric Acid	6.20
Cobalt Chloride.6H$_2$O	0.0250
Cupric Sulphate.5H$_2$O	0.0250
Na$_2$.EDTA	37.260
Ferrous Sulphate.7H$_2$O	27.80
Manganese Sulphate.H$_2$O	16.90
Potassium Iodide	0.830
Sodium Molybdate.2H$_2$O	0.250
Zinc Sulphate.7H$_2$O	8.60
Vitamins	
Glycine (free base)	2.0
Myo-Inositol	100.0
Nicotinic Acid (free acid)	0.50
Pyridoxine.HCl	0.50
Thiamine.HCl	0.10
Sucrose	20 000.0
Hormones for shoot culture:	(μM)
Kinetin	1.0
Hormones for callus culture:	
NAA	9.0
Kinetin	9.0
For solid media:	(g.L^{-1})
Agar	2.5
Phytagel	2.5

2.4.3 Media Preparation

Media were prepared using analytical grade reagents and ultrapure water. Glassware and culture vessels were washed in phosphate-free detergent and hot water, rinsed twice in cold tap water and given a final rinse in deionised water before being oven-dried.

Media components were weighed and dissolved in ultrapure water. Stock solutions of hormones were added to the medium, the solution made up to its final volume, and the pH adjusted to 5.75 ± 0.05 units, with 1M KOH. Powdered gelling agents were then added to solid media, and dissolved by heating in a microwave oven. Media was dispensed into culture vessels while hot, then autoclaved. Media for petri dishes was first autoclaved in glass bottles, then dispensed under sterile conditions. After solidification, media was stored in darkness at $5°C$ until required.

2.5 STERILISATION OF PLANT MATERIAL

Plant material was cut from plants and trimmed to 5 - 8cm lengths (Figure 2.1). Sections were washed under running water, shaken briefly (approximately 30 seconds) in 70% ethanol, and rinsed in deionised water to remove residual anol before being immersed in zephiran solution (consisting of 2% benzalkonium chloride in 10% ethanol) for 15 minutes. After three rinses in sterile ultrapure water, plant material was excised and placed onto culture media as required.

2.6 INITIATION AND MAINTENANCE OF CULTURES

2.6.1 Nodal Cultures

After sterilisation, leaves were removed from 20 - 30mm nodal sections of young stems (Figure 2.2), and the sections embedded upright in shoot culture medium containing 1µM kinetin. Following initiation, plantlets were subcultured onto fresh media every six to eight weeks depending on growth. The response of plants to various auxins and cytokinins was examined by initiating nodal sections on media containing a range of hormone concentrations (refer to Section 3.2.2).

Non-destructive measurement methods were used wherever possible when assessing culture growth. For nodal cultures, the number of new shoots, height of tallest shoot, and number of roots per shoot,were measured with the aid of a dissecting microscope.
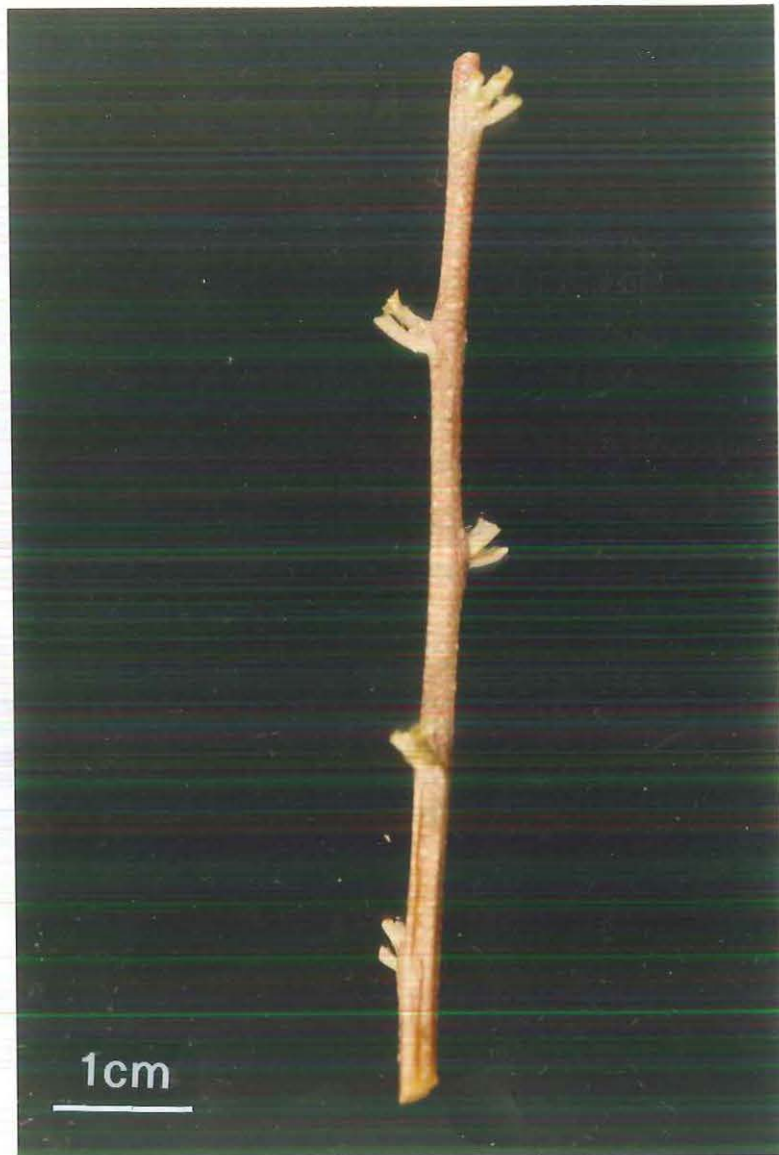
FIGURE 2.1: Stem sections (with leaves removed) used for initiation of nodal (shoot) cultures of *A. nummularia*
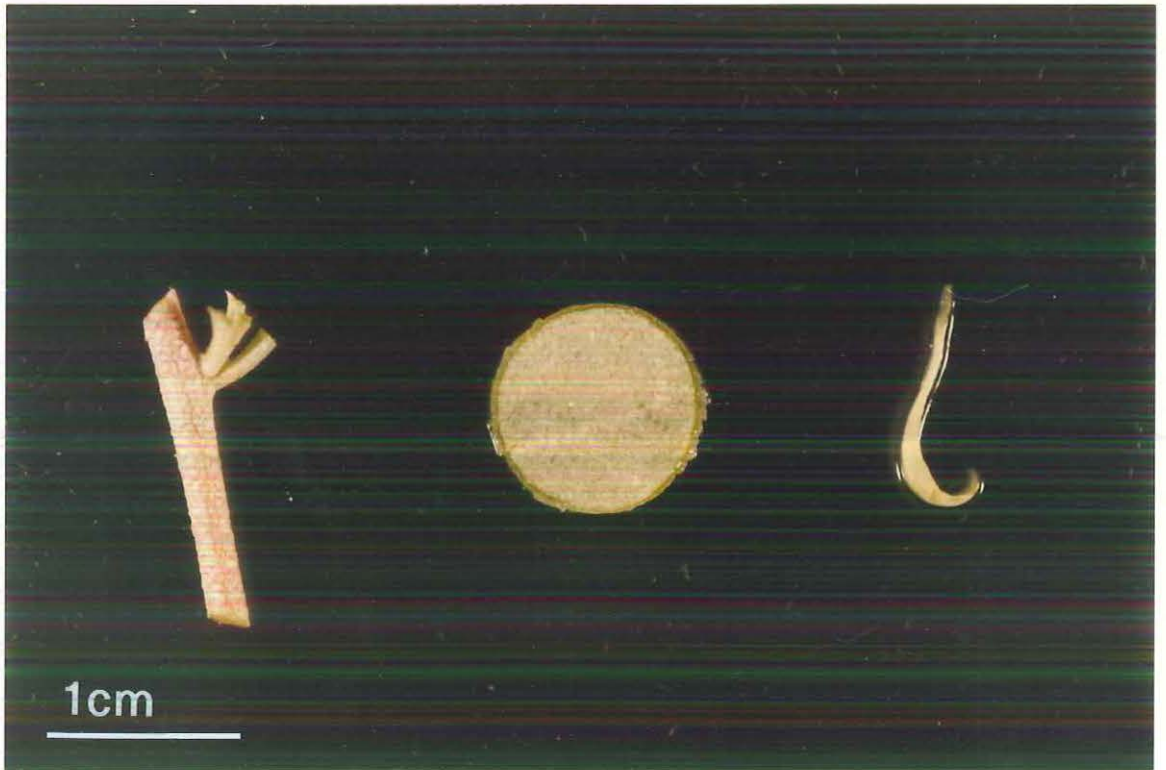
FIGURE 2.2: *A. nummularia* explant material.
Left: nodal section used for initiation of shoot culture
Centre: leaf disc from whole plant, used for intiation of callus
Right: leaf half from cultured shoot, used for initiation of callus

2.6.2 Callus Cultures

For most experiments, callus cultures were initiated from leaf halves excised from shoot cultures. Initially, leaf discs from field-grown plants were also used prior to the establishment of a sizeable culture base. Leaf sections (Figure 2.2) were embedded in callus induction medium (adapted from Smith and McComb, 1983) containing $9\mu M$ NAA and $9\mu M$ kinetin, in either $8 \times 2.5cm$ vials or $9 \times 1.5cm$ petri dishes. Experimental treatments for callus initiation, multiplication and later regeneration involved the addition of a range of concentrations of auxins and cytokinins as detailed in Section 4.2.

Callus was assessed both qualitatively and quantitatively. Qualitative assessment was by means of a scoring system based on proportions of callus development. Callus was examined under a dissecting microscope and allocated to one or more categories on the basis of the proportion of explant that had become callused (refer to Section 4.2.1; Figure 4.1). Quantitative assessment was made by weighing callus under aseptic conditions. Following this procedure callus could be subcultured onto fresh medium for further growth, or used for subsequent experiments.

2.6.3 Suspension Cultures

Suspension cultures were initiated from approximat ' ˙ 250mg of callus (from leaf explants). Suspensions were maintained in solutions of M&S medium (Table 2.1) without gelling agents, containing $9\mu M$ NAA and $9\mu M$ kinetin, in an orbital shaker at 100rpm. Culture conditions were otherwise identical to those in culture rooms (refer to Section 2.3).

Suspension cultures were assessed by measuring cell density using an haemocytometer (Weber and Sons, Lancing, UK). Five aliquots of $10\mu L$ were removed from each suspension, and the number of cells in each of five $0.0025mm^2$ grids of the haemocytometer were counted from each aliquot. The number of cells per mL was calculated from the average of these counts (adjusted for volume).

2.7 ASSESSMENT AND ANALYSIS

Where possible, assessments of growth and development were made without compromising the survival or sterility of the plantlet. Measurements of root and shoot length and number, and qualitative measurements of callus production and regeneration were made without removing the plant material

from its culture container. Callus was generally weighed under sterile conditions, by first irradiating and then swabbing a balance with 70% ethanol under a laminar flow cabinet.

In most instances results were compared by analysis of variance, with a 5% level of significance. Where appropriate, two-way analysis of variance was used, and if this indicated significant differences then one-way analysis of variance was used to define those experimental treatments that gave different results. Multiple range tests such as Tukey's comparison of difference (Steel & Torrie, 1981, pp. 172 - 186) were used when one-way analysis of variance indicated significant differences. Friedman's non-parametric analysis of variance by rank (Siegel and Castellan, 1988, pp 166-172) was also used.

3. SHOOT CULTURE AND ROOT INITIATION IN *ATRIPLEX SPP.*

3.1 INTRODUCTION

A collection of shoot cultures of various species of *Atriplex* was established as the base for experiments on shoot growth and root induction. Preliminary trials were conducted to investigate variables such as the effectiveness of sterilisation procedures, and the suitability of media formulations and of explant types. The effects of auxins and cytokinins in the growth medium on shoot multiplication and root initiation were examined in a series of experiments. Differences in growth responses between species and clones were also examined.

3.2 MATERIALS AND METHODS

3.2.1 Culture Initiation

Shoot cultures of *Atriplex amnicola* (clones AAM 002 and AAM 003), *A. cinerea* (ACI 002 and ACI 003), *A. nummularia* (ANU 001), *A. semibaccata* (ASE 001), *A. stipitata* (AST 001), and *A. vesicaria* (AVE 001) were established according to the procedure outlined in Chapter 2. Young stems from each clone were surface sterilised with benzalkonium chloride and grown in M&S shoot medium, containing 1μM kinetin. Survival and contamination rates of shoots sterilised in 2% and 0.2% solutions of benzalkonium chloride for five, ten and 20 minutes were recorded. Differences in survival and contamination rates of *A. nummularia* cultures from field-grown and glasshouse-grown plant material were also recorded. The numbers of replicates in each treatment varied (Table 3.1).

3.2.2 The Effects of Auxins and Cytokinins

Cultured shoots of clone ANU 001 were subcultured onto media containing 1μM of the auxins NAA, IAA or IBA, or the cytokinins kinetin, 2iP, or BAP. Shoots were also initiated from mature, field-grown plant material, and from glasshouse-grown cuttings. The experiment was replicated 25 times. Ten replicates of surface sterilised shoots of clones AAM 002, AAM 003, ACI 002 and ACI 003 were initiated on media containing 0, 1 or 4.5 μM kinetin, 2iP or BAP.

Shoot cultures were grown under standard conditions (Section 2.3), and assessed after two and eight weeks. Growth was assessed quantitatively by measuring the number of new shoots, the height of the tallest shoot, and the number of roots.

3.3 RESULTS

3.3.1 Culture Initiation

Survival of new shoots varied from zero to 65% (Table 3.1). Shoots sterilised in a 0.2% solution of benzalkonium chloride had very high contamination rates (72% and 100% for ANU 001 and AVE 001 respectively). Increasing the time of sterilisation (in full strength, 2% solution) from five to 20 minutes lowered the contamination rate of new shoots, but also greatly increased the death rate (and consequently lowered the survival rate) of those shoots. Doubling the sterilisation time from five to 10 minutes did not affect the overall survival rate of ANU 001 shoots (from 64% to 65%), but halved the contamination rate (from 22% to 9%).

TABLE 3.1: SURVIVAL AND CONTAMINATION SIX WEEKS AFTER INITIATION OF *ATRIPLEX* SHOOTS

CLONE (replicates)		STERILIS-ATION[1]	SURVIVAL[2]	CONTAM-INATION[3]	DEATH[4]
ANU 001	(50)	2%, 5min	64	22	14
	(100)	2%, 10min	65	9	26
	(50)	2%, 20min	24	8	68
	(65)	0.2%, 10min	28	72	0
field	(150)	2%, 10min	14	57	29
glasshouse	(25)	2%, 10min	56	13	31
AVE 001	(40)	0.2%, 10min	0	100	0
AST 001	(25)	2%, 10min	0	92	8
ASE 001	(25)	2%, 10min	0	84	16
AAM 002	(25)	2%, 10min	52	36	12
AAM 003	(25)	2%, 10min	24	72	4
ACI 002	(25)	2%, 10min	56	32	12
ACI 003	(25)	2%, 10min	36	48	16

NOTE   1: Sterilisation: Concentration of benzalkonium chloride solution and

time of immersion in solution

2 Survival: Percentage of shoot explants surviving uncontaminated

after six weeks

3: Contamination: Percentage of shoot explants becoming

contaminated within six weeks

4: Death: Percentage of shoot explants becoming discoloured after

six weeks (excluding contaminated shoots)

The contamination rates of shoots sterilised for 10 minutes in 2% solution varied widely, with clones ANU 001, AAM 002 and ACI 002 having comparatively lower contamination rates (9%, 36% and 32% respectively), while clones AST 001, ASE 001 and AAM 003 had very high contamination rates (92%, 84% and 72% respectively). Contamination rates of glasshouse-grown shoots of ANU 001 were much lower (13%) than those of shoots from field-grown plants (57%). Death rates of shoots due to the sterilisation and culture process were similar for all clones (excluding those which had been sterilised for 20 minutes), ranging between 4% and 31% .

3.3.2 Effects of Explant Source

On both measurement dates, growth of ANU 001 plantlets (in terms of mean number of shoots, mean height of shoots, and mean number of roots) was significantly greater for subcultured shoots (Figures 3.1 and 3.4) than for shoots initiated from field-grown or glasshouse-grown material (Figures 3.2, 3.3, 3.5 and 3.6). Although plantlets initiated from glasshouse material were only grown on medium containing kinetin, the production of shoots and roots from those cultures was very low compared to subcultured plant material.

3.3.3 Effects of Auxins and Cytokinins
3.3.3a: Clone ANU 001

The differences observed in root and shoot growth with each hormone treatment were not significant (at the 95% confidence level), when compared to the differences within treatments. Subcultured shoots produced, on average, 2.7 shoots which were 23mm tall, and 2.6 roots per explant. In general, low concentrations of IAA and of 2iP promoted the development of more shoots (Figures 3.1 to 3.6 a); IAA and IBA (and to a lesser extent NAA) promoted the growth of taller shoots (Figures 3.1 to 3.6 b); and IAA and IBA also promoted the development of more roots (Figures 3.1 to 3.6 c), in cultures of ANU 001. It appears that low concentrations of auxins were slightly more effective in promoting root and shoot growth and development, than were low concentrations of cytokinins, however the differences were not significant.

3.3.3a: Clones AAM 002, AAM 003, ACI 002 and ACI 003

Shoot production and mean shoot height were higher on media containing 1μM cytokinins, but these differences were not statistically significant (Figures 3.7 and 3.8). The mean numbers of roots and new shoots did not appear to be affected by the cytokinin in the medium.

FIGURE 3.1: The effect of auxins and cytokinins on shoot growth and root initiation in established (subcultured) *A. nummularia* cultures, after 2 weeks.

FIGURE 3.1a: Mean number of shoots produced per node.

FIGURE 3.1b: Mean height of the tallest shoot produced.

FIGURE 3.1c: Mean number of roots produced per node.

FIGURE 3.2: The effect of auxins and cytokinins on shoot growth and root initiation in *A. nummularia* cultures from field-grown plants, after 2 weeks.
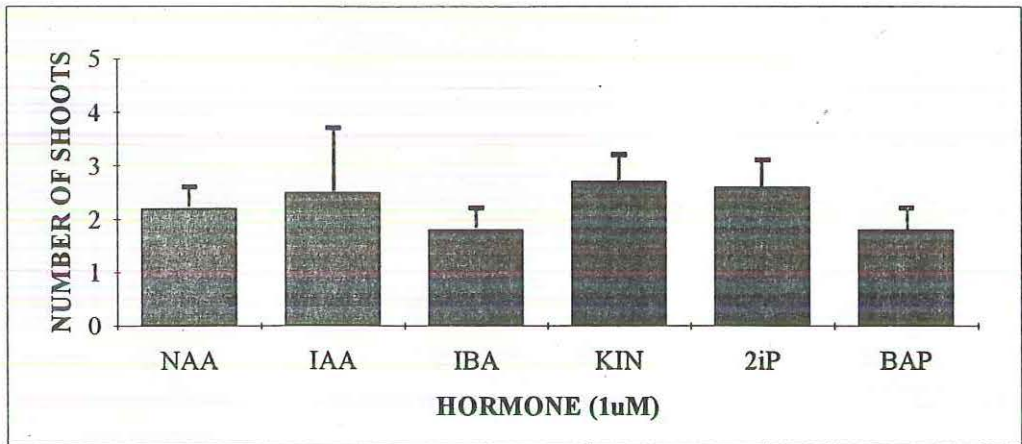

FIGURE 3.2a: Mean number of shoots produced per node.

FIGURE 3.2b: Mean height of the tallest shoot produced.

FIGURE 3.2c: Mean number of roots produced per node.

FIGURE 3.3: The effect of kinetin on shoot growth and root initiation in *A. nummularia* cultures from glasshouse cuttings, after 2 weeks.

FIGURE 3.3a: Mean number of shoots produced per node.

FIGURE 3.3b: Mean height of the tallest shoot produced.

Note: Shoots initiated from glasshouse cuttings
did not form roots within 2 weeks

FIGURE 3.4: The effect of auxins and cytokinins on shoot growth and root
initiation in established *A. nummularia* cultures, after 8 weeks.

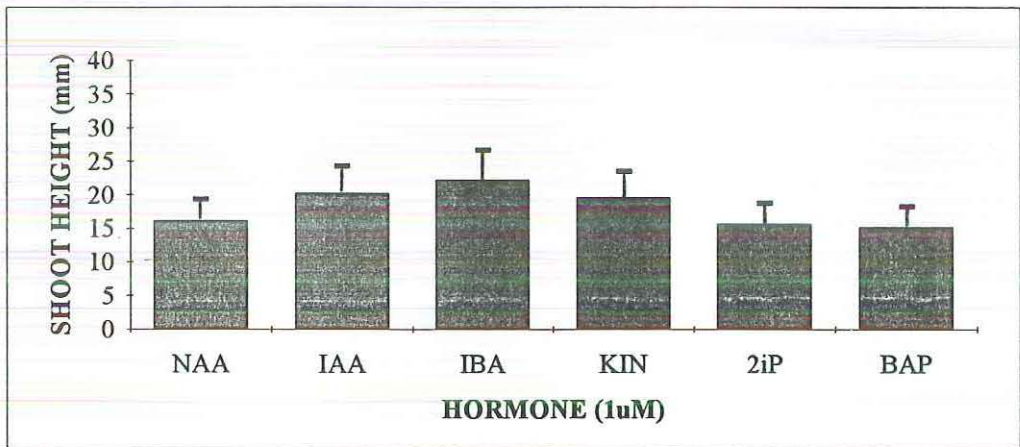FIGURE 3.4a: Mean number of shoots produced per node.
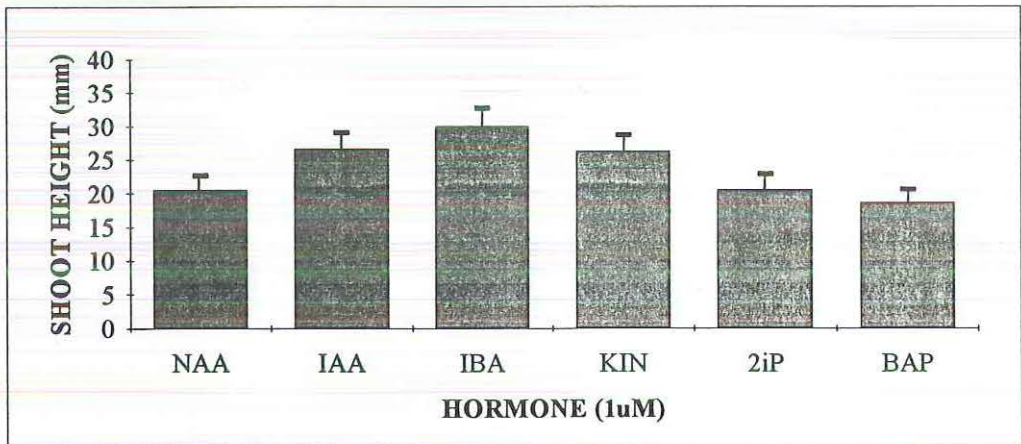
FIGURE 3.4b: Mean height of the tallest shoot produced.
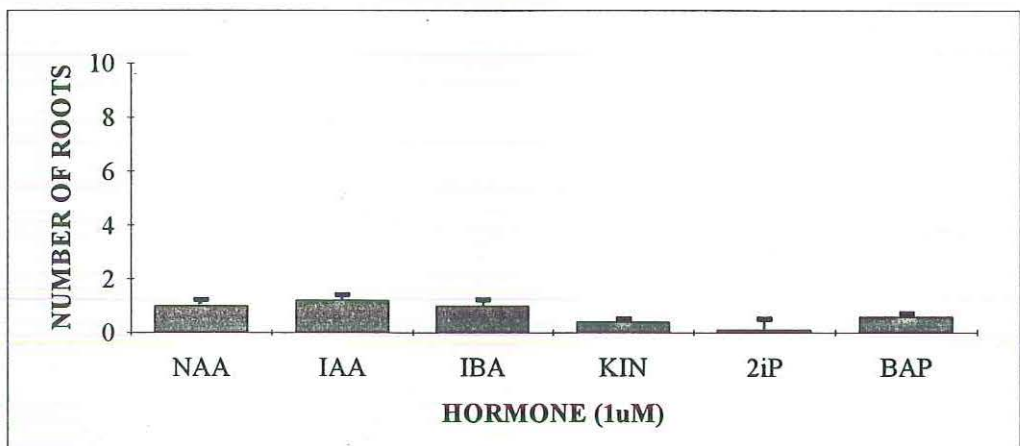

FIGURE 3.4c: Mean number of roots produced per node.

FIGURE 3.5: The effect of auxins and cytokinins on shoot growth and root initiation in *A. mummularia* cultures from field-grown plants, after 8 weeks.
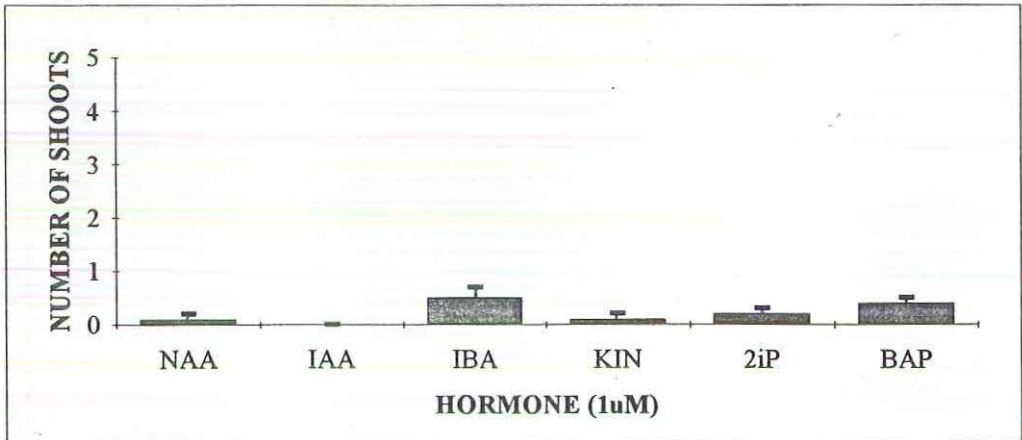

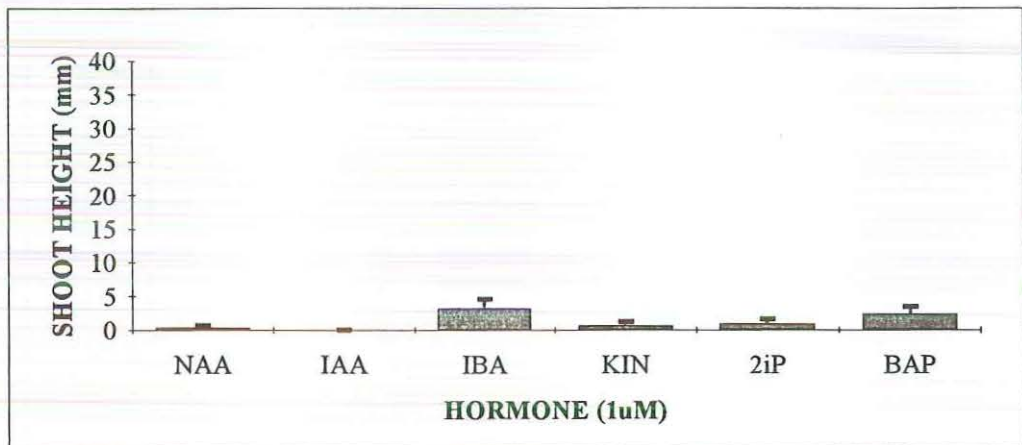FIGURE 3.5a: Mean number of shoots produced per node.

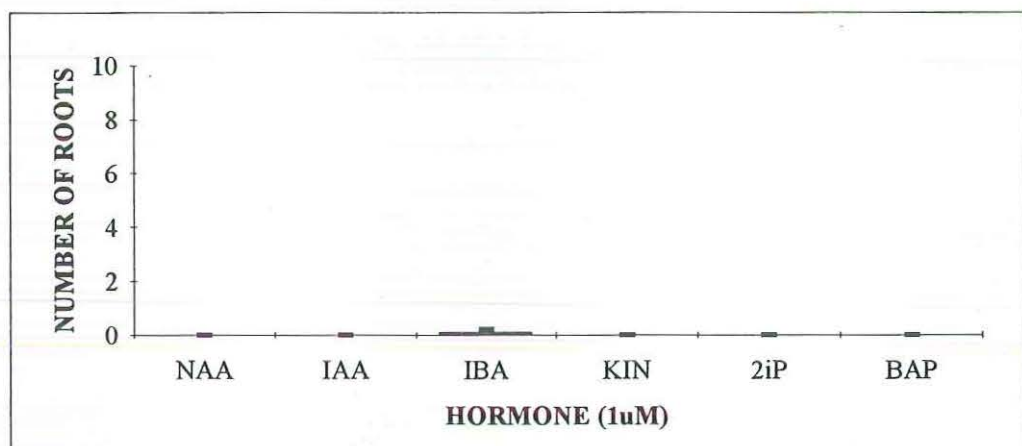FIGURE 3.5b: Mean height of the tallest shoot produced.

FIGURE 3.5c: Mean number of roots produced per node.

FIGURE 3.6: The effect of kinetin on shoot growth and root initiation in *A. nummularia* cultures from glasshouse cuttings, after 8 weeks.
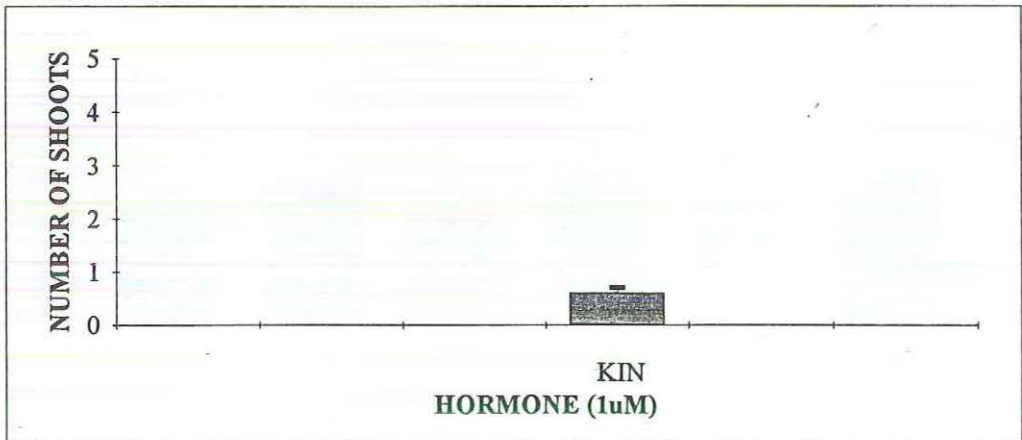

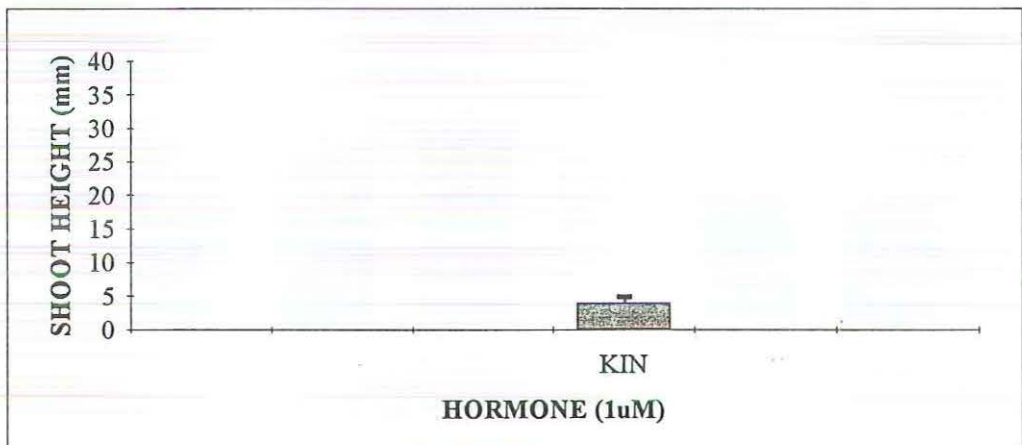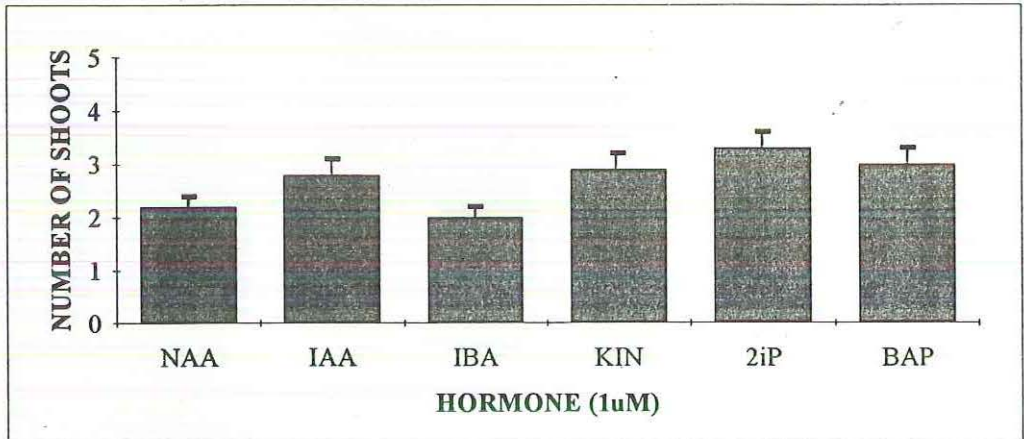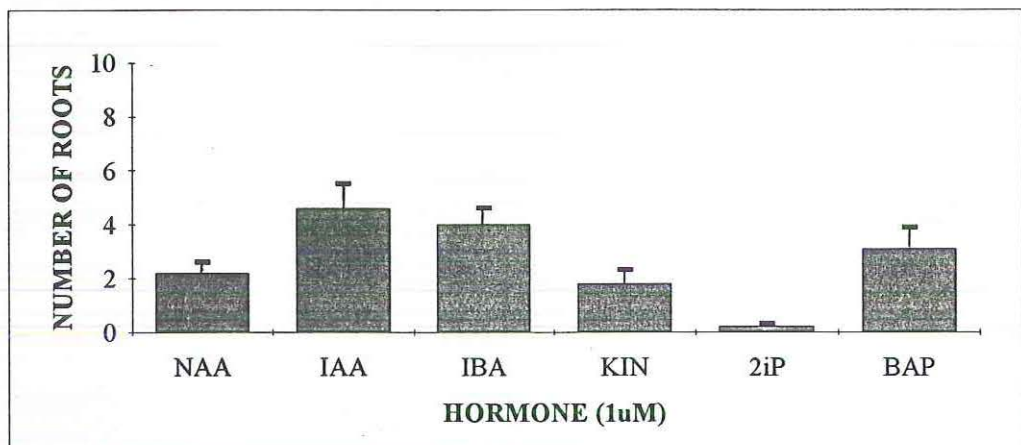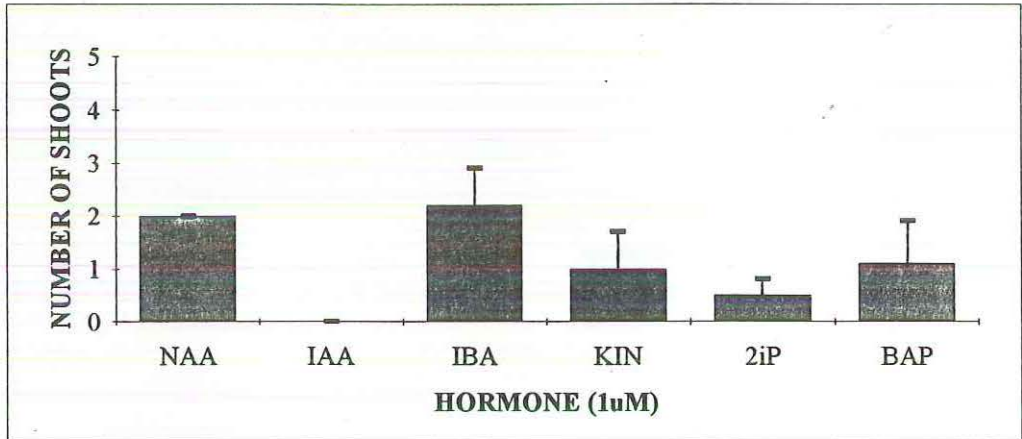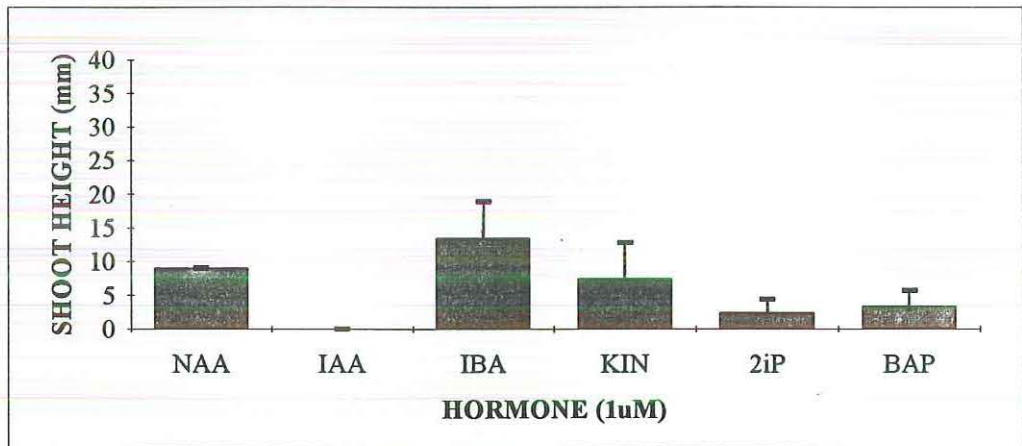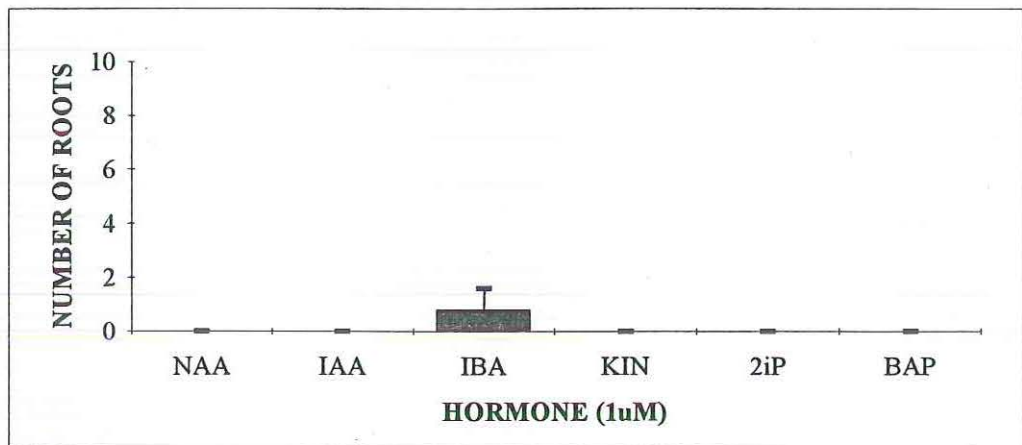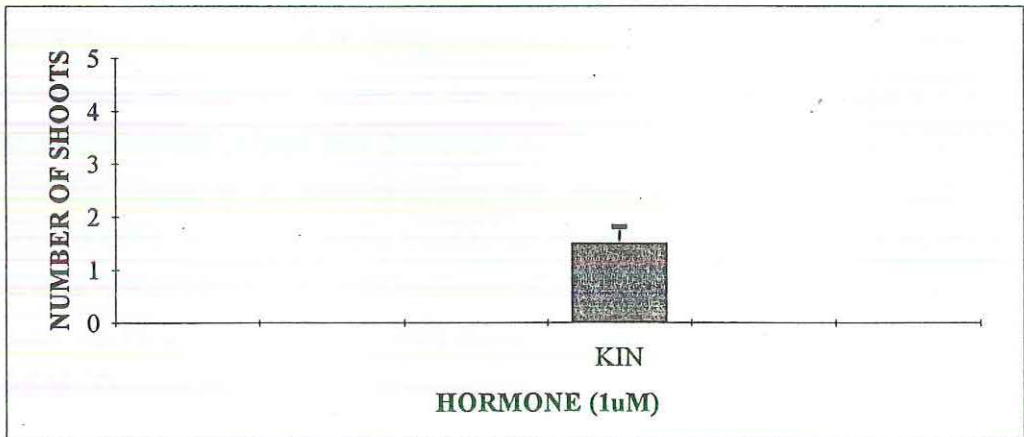FIGURE 3.6a: Mean number of shoots produced per node.
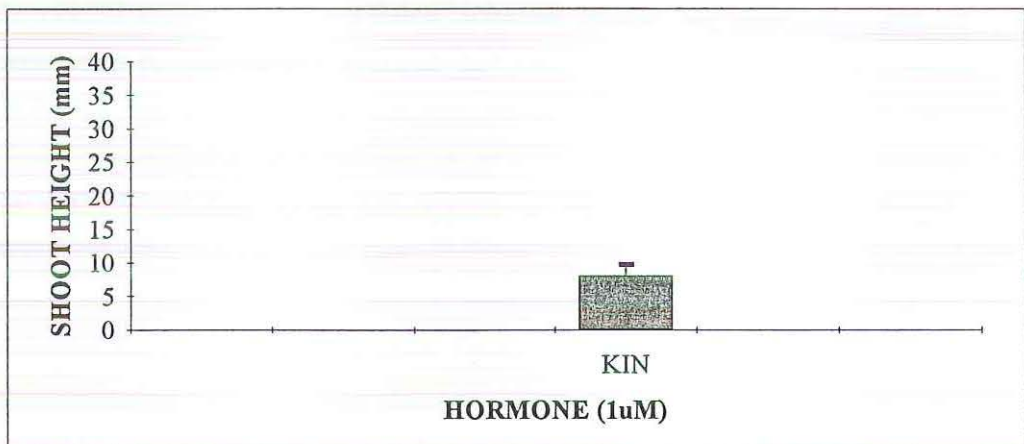
FIGURE 3.6b: Mean height of the tallest shoot produced.
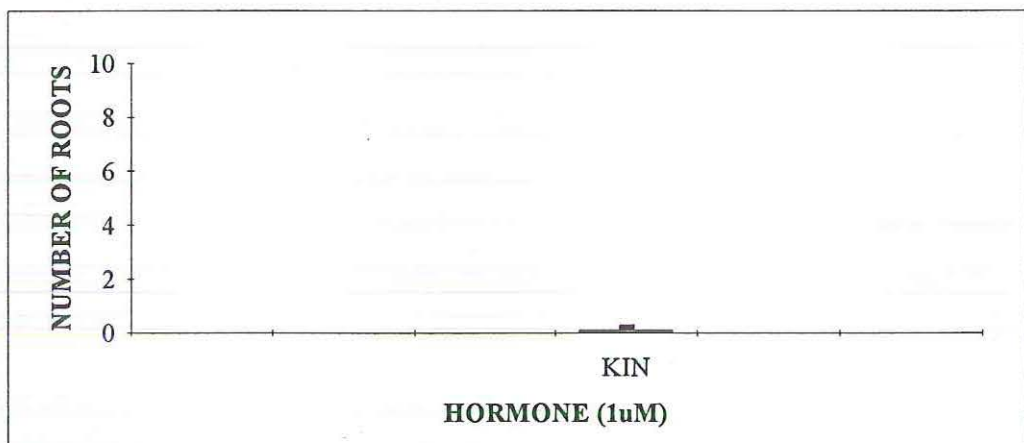
FIGURE 3.6c: Mean number of roots produced per node.

Mean shoot production over eight weeks was higher in clones of *A. amnicola* than *A. cinerea* for all media treatments (Figure 3.7), however these differences were not statistically significant (at 95% confidence level). Overall mean shoot production for clones of *A. amnicola* and *A. cinerea* respectively, was 0.9 and 0.1 shoots per explant. Mean height of shoots was significantly higher for *A. amnicola* clones (42mm and 39mm for AAM 002 and AAM 003 respectively) than for clones of *A. cinerea* (3mm and 10mm for ACI 002 and ACI 003 respectively), across all media treatments (Figure 3.8). Roots were produced in very low numbers, and only in clones of *A. amnicola* (Figure 3.9). The three most vigorous clones overall, were ANU 001 (Figure 3.10) and AAM 002 and AAM 003 (Figure 3.11).

3.4 DISCUSSION
3.4.1 Culture Initiation

Increased exposure to sterilising solution (either for increased time or at increased concentration) reduced the rate of subsequent contamination in cultures, but also reduced the rate of survival of uncontaminated cultures. Sterilising *Atriplex* plant material in benzalkonium chloride for five minutes, or in a 0.2% solution, does not appear to be effective in killing fungal and bacterial cells, but does not damage the plant cell structure so consequent death rates of explants due to cellular disruption, are low. Sterilisation in 2% solution for 20 minutes kills most fungal and bacterial cells resulting in a low contamination rate, but also damages plant cells and reduces the subsequent survival of explants. Exposure to a 2% solution of benzalkonium chloride for 10 minutes appears to represent a good compromise between effective surface sterilisation of plant material, and subsequent survival in culture.

Benzalkonium chloride is a quaternary ammonium compound, with surface cationic activity on cells (Stecher, 1968, pp. 126-127). Its main effect is through disrupting the integrity of the cell membrane. Cationic agents are more effective on prokaryotic cell membranes than on plant and fungal cells, which have complex cell walls protecting the cell membrane (Salisbury & Ross, 1992, pp. 6-12).

Contamination rates of new cultures varied between species and clones, and within clones where plant material was obtained from different sources: this may be attributed to the source and treatment of plant material prior to culturing. Cuttings of clones AVE 001, AST 001 and ASE 001 were

FIGURE 3.7: The effects of cytokinins on number of shoots produced by
Atriplex spp. cultures, over 8 weeks
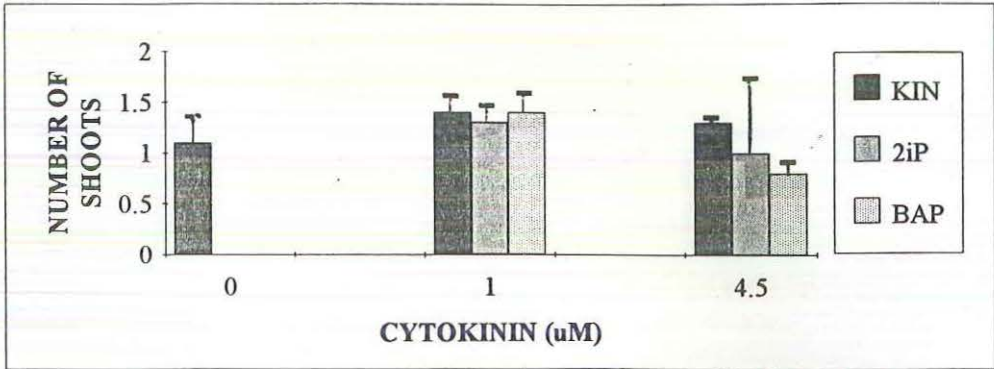
FIGURE 3.7a: Mean number of shoots produced by AAM 002 cultures
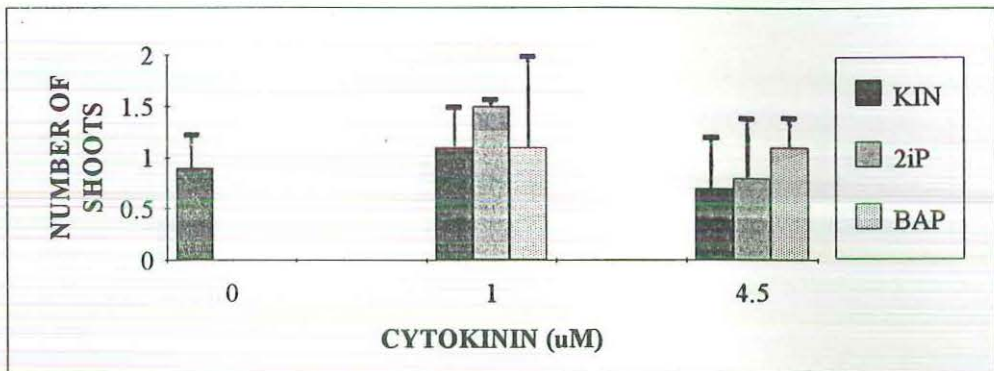
FIGURE 3.7b: Mean number of shoots produced by AAM 003 cultures
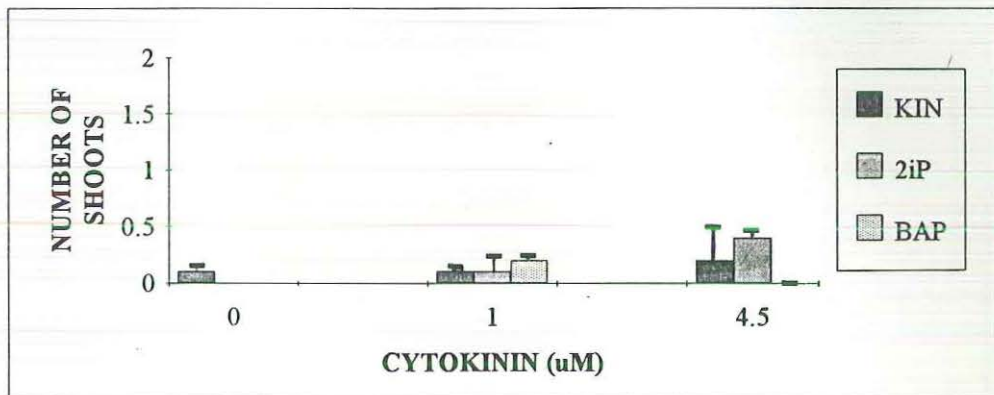
FIGURE 3.7c: Mean number of shoots produced by ACI 002 cultures
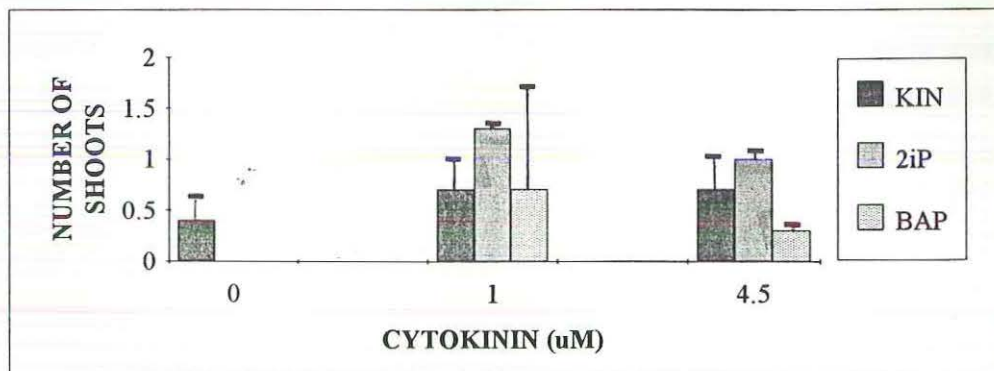
FIGURE 3.7d: Mean number of shoots produced by ACI 003 cultures

FIGURE 3.8: The effects of cytokinins on height of shoots produced by
Atriplex spp. cultures, over 8 weeks
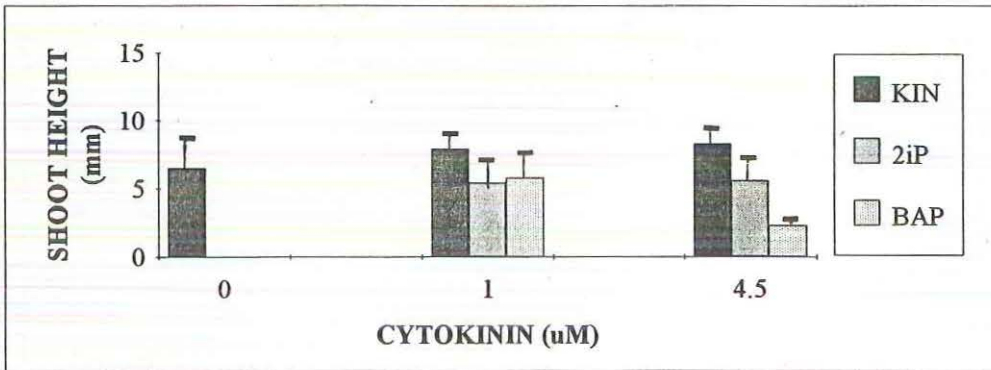
FIGURE 3.8a: Mean height of shoots produced by AAM 002 cultures
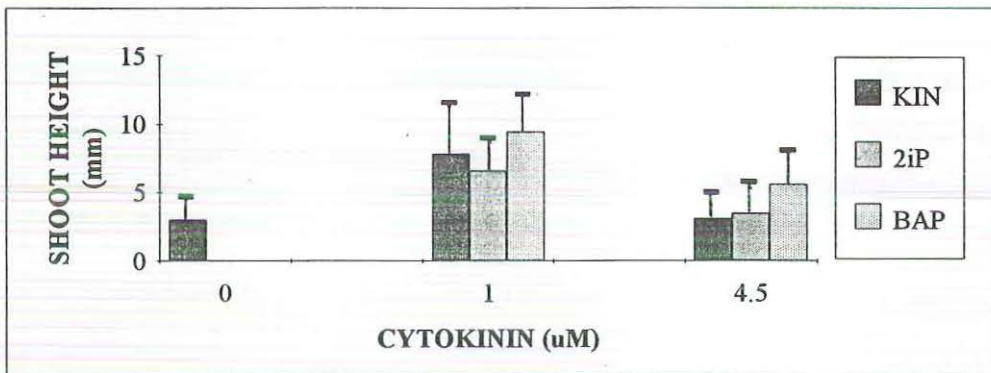
FIGURE 3.8b: Mean height of shoots produced by AAM 003 cultures
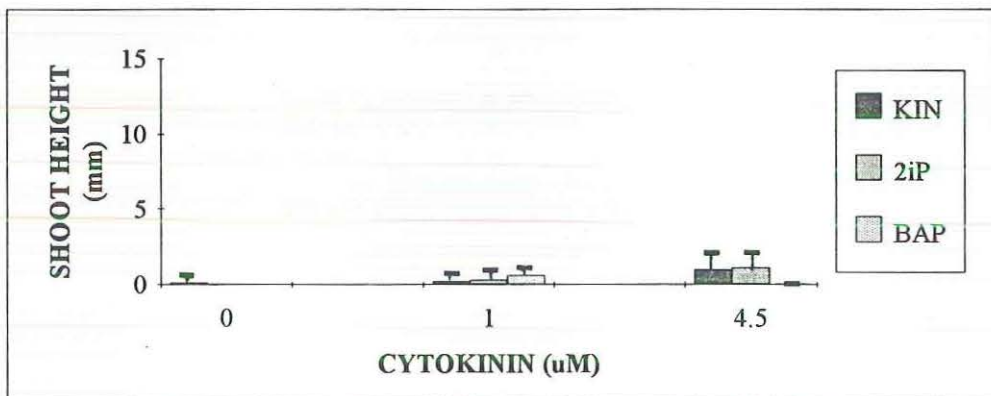
FIGURE 3.8c: Mean height of shoots produced by ACI 002 cultures
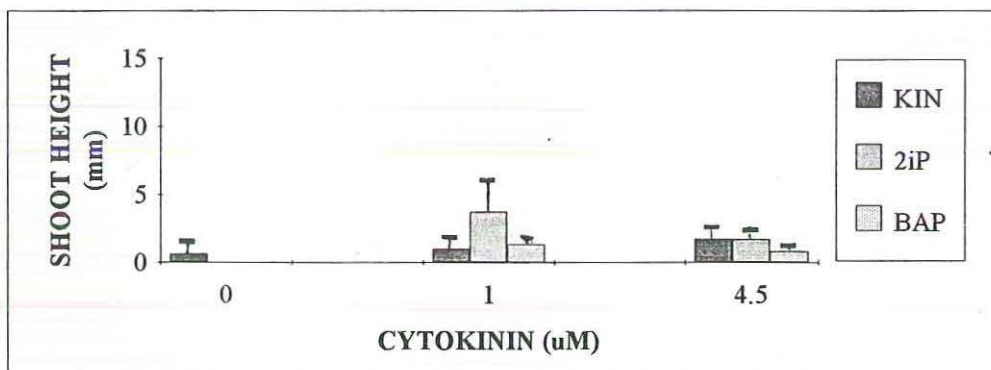
FIGURE 3.8d: Mean height of shoots produced by ACI 003 cultures

FIGURE 3.9: The effects of cytokinins on number of roots produced by
Atriplex spp. cultures, over 8 weeks
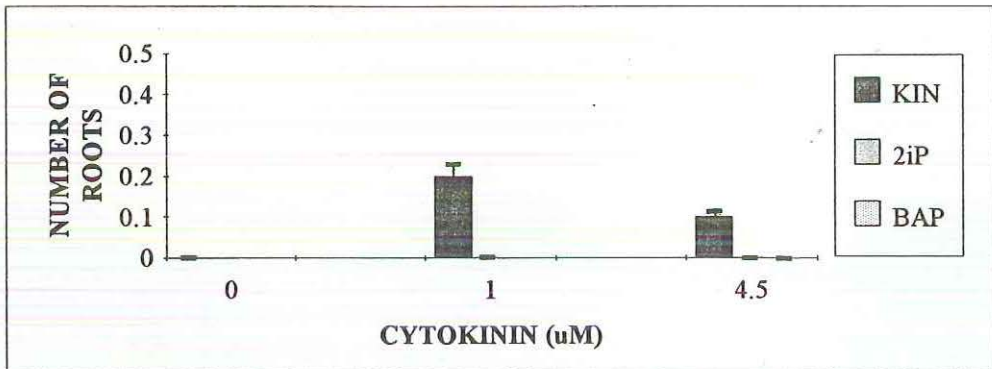
FIGURE 3.9a: Mean number of roots produced by AAM 002 cultures
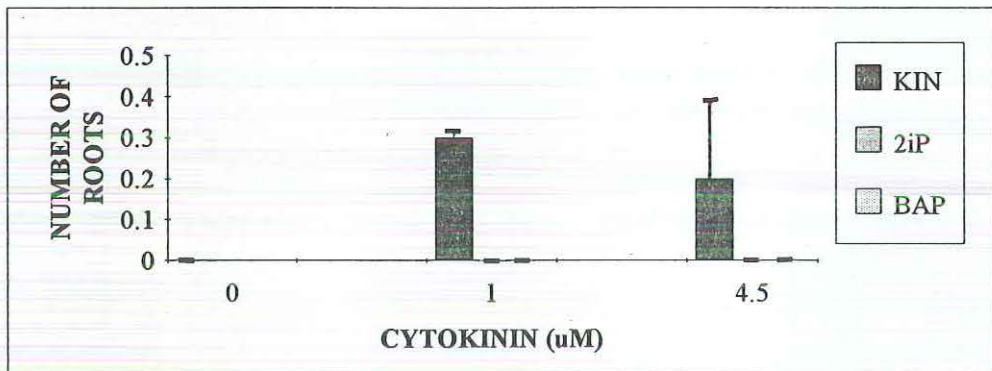
FIGURE 3.9b: Mean number of roots produced by AAM 003 cultures

Note 1: Shoots from clones of AAM 002 and AAM 003
did not form roots if the cytokinin source was 2iP or BA

2: Shoots from clones ACI 002 aand ACI 003
did not form roots within 8 weeks

FIGURE 3.10: Shoot Cultures of Clone ANU 001
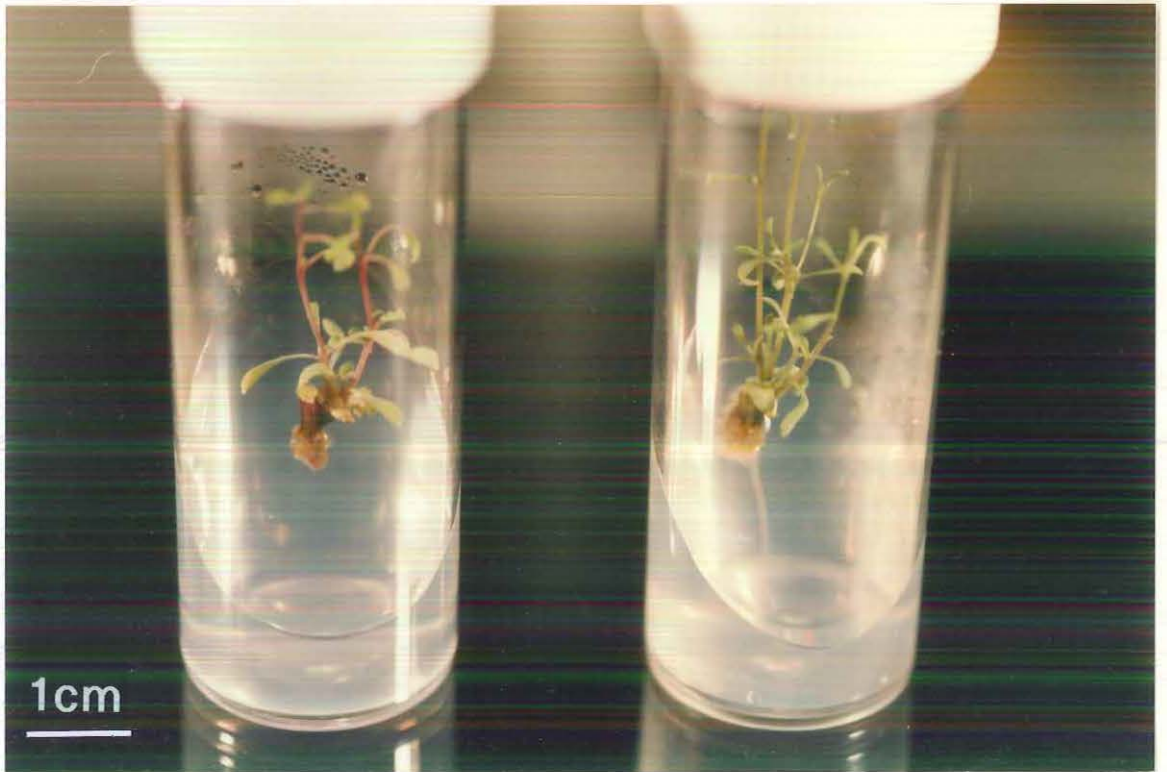
FIGURE 3.11: Shoot Cultures of Clones AAM 002 (left) and Clone AAM 003

transported from Merredin or Kalgoorlie (distances of over 500km) in plastic bags, whilst other plant material was transported less than 50km. Similarly, plant material of these three clones was collected from saline areas, whilst other clones were growing under cultivation, without salt stress. It is likely that this combination of growing conditions and transportation, favoured the growth of fungi and bacteria on and in the plant material. Because of the physiological stress of growing in saline soils, plants may harbour more internal contaminants, and the process of transporting plants sealed in warm, moist conditions may increase the presence of external contaminants. However contamination of plant material also occurred regardless of these factors, as seen with cultures of clone AAM 003.

3.4.2 Effects of Explant Source

Growth in culture (when measured by root and shoot production), showed differences between explants of the same clone, from different sources. Plant material subcultured from previous tissue culture plantlets exhibited the highest growth rates of roots and shoots. Plant material from in vivo sources (glasshouse and field plants) produced fewer roots and shoots. After eight weeks however, the magnitude of these differences between subcultured and new shoots was much less: the in vivo plants were starting to show similar growth responses to that of subcultured material.

It is reasonable to assume that growth of *Atriplex nummularia* shoot cultures, exhibit the typical sigmoidal curve of most plant organs, with log, linear and senescent (stationary) growth phases (Lindsay & Jones, 1990, pp. 15-34; Salisbury & Ross, 1992, pp. 340-341). Established cultures would exhibit linear growth, and when subcultured would show a rapid log phase before passing into linear phase again. The log phase may be expected to take longer in new cultures, as cells recovered from the sterilisation and culturing processes and adjusted to the new culture conditions. The differences observed here between cultures from different sources at each measurement date are as predicted by this model. After two weeks, established cultures would be in linear phase, while new cultures would be still in log phase. At eight weeks, established cultures would have neared stationary or senescence phase, while new cultures would be in linear phase. The differences between cultures, would be greater initially than at eight weeks.

The sigmoidal growth curve appears to provide a good model for the growth kinetics of *A. nummularia* shoot cultures if the assumptions about length of log phase are correct. In order to test the application of this model, similar experiments with more frequent measurement dates (i.e. weekly recordings) over a longer period would be useful.

3.4.3 Effects of Auxins and Cytokinins

At high concentrations, auxins generally promote root initiation, while cytokinins promote shoot formation in plant tissue cultures (Wareing & Phillips, 1981, pp. 151-168; Hussey, 1983). At the low concentration tested, the use of either auxins or cytokinins did not result in any differences in growth response in *A. nummularia* culture. The use of hormones at higher concentrations (5 - 10μM) may be necessary in order to obtain enhanced rates of root or shoot formation.

Varying the type and concentration of cytokinin present in the medium did not affect root and shoot production in clones of *A. amnicola* or *A. cinerea*. The addition of cytokinins to cultures of plant material containing shoot meristems (such as nodal explants), usually enhances the production of shoots from these meristems. Since such an enhancement was not observed here, it appears most likely that *Atriplex spp.* have a greater requirement for cytokinins. The use of cytokinins at concentrations of five to ten micromolar, or the use of more active forms of cytokinin such as zeatin (Wareing & Phillips, 1981, pp. 64-67), may result in an enhancement of shoot production in these species.

3.4.4 Comparison of Species and Clones

Since explants of the five clones AAM 002, AAM 003, ACI 002, ACI 003 and ANU 001 were initiated from similar source material (glasshouse-grown rooted cuttings), under identical conditions, their growth responses can be compared. Cultures of ANU 001, AAM 002 and AAM 003 exhibited more vigorous growth when compared to *A. cinerea* clones. Provenance trials of seedling plants have ranked plantings of *A. amnicola* and of *A amnicola* x *A. nummularia* hybrids, more highly than *A. cinerea* and other species in terms of plant volume and biomass productivity. Accessions of these and other *Atriplex* species, were found to be very promising for use in revegetating degraded pastoral land in Pakistan, and possibly in other saline areas (Ahmad & Ismail, 1993). The differences in growth and vigour of these species in vivo, appears to have been reflected in vitro, in the clones used here. Differences in shoot

growth and morphology were not observed between the prostrate and erect cultivars of *A. amnicola*. Such differences may develop over a longer period than the plants were retained in culture.

Roots were produced only in very low numbers in clones of *A. amnicola*, while clones of *A. cinerea* produced no roots in culture. These results suggest that further experimentation is necessary to determine the exact requirements of root initiation in *Atriplex spp*. The inclusion of auxin in the culture medium may result in higher rates of root initiation.

The failure to establish cultures of *A. semibaccata*, *A. stipitata* or *A. vesicaria* also suggests that further work is necessary with these species, to determine the best tissue culture procedures. The use of alternative explants such as leaves or floral parts, and of sterilisation procedures, may give better establishment rates. The use of cotyledons or hypocotyls from seedlings germinated under sterile conditions may prove to be more effective.

Generally, root initiation, and shoot multiplication and growth can be obtained for most *Atriplex* spp. tested using common techniques and media, although some refinements of hormone concentrations are necessary. The results indicate that *Atriplex* spp. are amenable to manipulation in tissue culture and thus can be used in selection programmes.

4.   CALLUS CULTURE OF *ATRIPLEX NUMMULARIA*

4.1 INTRODUCTION

The use of cell and callus cultures in salt tolerance investigations has been reviewed by Binzel et al. (1985). Such systems are useful for the selection of cells that have enhanced cellular-based salt tolerance mechanisms. Selection of salt tolerant cells from callus or suspension cell cultures has been achieved for a range of glycophytic and halophytic species (Hasegawa et al., 1986).

A prerequisite for selection processes using callus and suspension cultures, is a bank of knowledge about the culture requirements and responses of the species and clones used. This chapter describes a series of experiments examining the initiation and growth characteristics of *Atriplex nummularia* callus.

Callus cultures were initiated and grown on media containing a range of auxins and cytokinins in varying concentrations, to determine which media gave the highest rates of callus initiation, multiplication and regeneration. Callus was also exposed to NaCl through solid medium culture, and through suspension culture.

4.2 MATERIALS AND METHODS
4.2.1 Callus Initiation

Shoot cultures of *Atriplex nummularia* (clone ANU 001) were used as a source of leaf explant material for callus initiation. The petiole was removed from whole surface sterilised leaves, and the lamina cut along the mid-vein to yield two explants, which were placed on the surface of the medium in Petri dishes or small polycarbonate vials containing M&S medium (Section 2.4).

Three separate experiments were conducted to examine callus initiation on media containing the auxins NAA, IAA, IBA, and 2,4-D (at concentrations of 2.25, 4.5, 9.0 and 18.0μM) and cytokinins BAP, 2iP and kinetin (at concentrations of 0, 4.5 and 9.0μM). Some combinations of auxin and cytokinin were duplicated in all three experiments, whilst others were not included. The total number of replicates for each treatment varied from five to 45 (refer to Table 4.2 for the treatment combinations and replicate numbers). After five weeks, callus cultures were examined with the aid of a dissecting microscope and allocated a score of zero to five based on the following scale:

X	Dead
0	No change in appearance
1	Minimal growth
2	Callus developed on less than 50% of margins
3	Callus developed on more than 50% of margins
4	Three-dimensional callus growth on less than 50% of explant surface
5	Three-dimensional callus growth on more than 50% of explant surface

Representative photographs of each classification were used as a reference (Figure 4.1).

4.2.2 Effects of Auxins

Callus cultures from leaf halves were initiated on media containing a range of auxins and cytokinins as described in Section 4.2.1. Calli were removed from these media, trimmed to a uniform mass of around 50mg and transferred aseptically to fresh M&S media containing 9μM kinetin and 9μM NAA, IAA, IBA or 2,4-D. Fifty-two calli from different initiation media were randomly allocated to each of the four auxin treatments, and grown under standard conditions (Section 2.3). After five weeks, calli were aseptically weighed, trimmed to a uniform mass of around 100mg, and subcultured onto fresh media of the same experimental treatment. After a further five weeks growth, calli were weighed again. The relative growth rate (%) for each culture passage was calculated using the following formula:

$$\frac{\text{final callus mass (mg) - initial callus mass (mg)}}{\text{initial callus mass (mg)}} \times 100\%$$

4.2.3 Callus Regeneration

Callus was initiated from leaf explants on M&S medium containing 9μM kinetin and either 9μM NAA or 9μM 2,4-D as described in Section 4.2.1. Ten calli were randomly allocated to each of 14 experimental treatments with varying auxin to cytokinin ratios (Table 4.1). Callus explants were trimmed to a uniform size of around 250mg and placed on experimental media. Calli were assessed after eight weeks growth under standard conditions (refer to Section 2.3). Callus mass and colour, and the presence of roots, shoots and dark green nodules were recorded. The proportions of calli regenerating organs was calculated for each treatment.
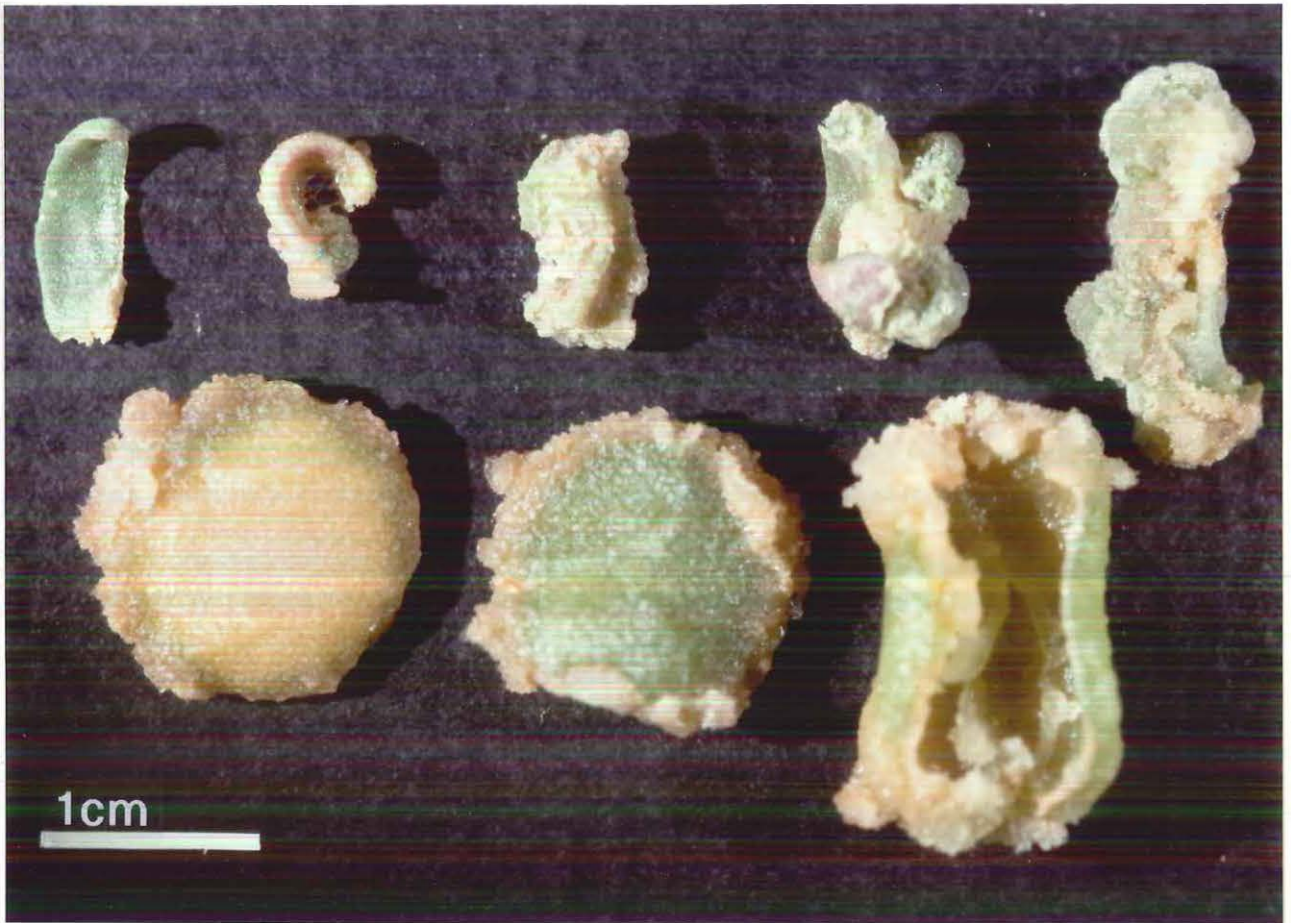
FIGURE 4.1: Representative examples of callus score categories
(from left to right)

1 = Minimal growth

2 = Callus developed on less than 50% of margins

3 = Callus developed on more than 50% of margins

4 = Three-dimensional callus growth on less than 50% of explant surface

5 = Three-dimensional callus growth on more than 50% of explant surface

TABLE 4.1: Experimental ratios of auxins to cytokinins for regeneration trials

RATIO	NAA (µM)	KINETIN (µM)
0 : 0	0	0
1 : 1	9	9
1 : 2	9	18
	4.5	9
	2.25	4.5
	1.125	2.25
1 : 3	6	18
	3	9
	1.5	4.5
	0.75	2.25
1 : 4	4.5	18
	2.25	9
	1.125	4.5
	0.5625	2.25

4.2.4 Callus Salt Tolerance

Callus was initiated from leaf explants on M&S medium containing 9µM kinetin and either 9µM NAA or 9µM 2,4-D (as described in Section 4.2.1). Ten calli were randomly allocated to each of nine experimental treatments with different salt levels. Calli were weighed and trimmed to a standard mass of 100mg and transferred to the experimental media. The nine experimental treatments consisted of M&S media with 9µM NAA and 9µM kinetin, and a range of NaCl concentrations from 0 to 342mM, in 43 mM increments. The basal medium contains 0.2mM Na^+ and 6.0mM Cl^- as macronutrients, but was designated as 0 NaCl for convenience (Smith & McComb, 1981a).

Cultures were harvested after five weeks under standard conditions (Section 2.3). Fresh weight was used to calculate relative growth rate.

4.2.5 Suspension Culture

Callus was initiated from leaf explants on M&S medium containing 18µM NAA and 9µM kinetin (as described in Section 4.2.1). Ten calli were trimmed to a uniform mass 250mg and randomly allocated to each of three experimental treatments with either 0, 171, or 342mM NaCl (as with solid medium, the zero

treatment actually contains 0.2mM Na$^+$ and 6.0mM Cl$^-$ as macronutrients). Cultures were grown for four weeks on an orbital shaker platform at 100rpm (as described in Section 2.3), before being harvested. An haemocytometer was used to count cells and cell aggregates, to estimate cell concentrations. This value was assumed to be a good indicator of the development of a cell suspension, and of the survival of callus cells in saline conditions.

4.3 RESULTS
4.3.1 Callus Initiation

Initiation of callus from leaf explants was successful, with 72% of explants (335 from 468) producing callus on half of the leaf margin (a score of three or higher), and 42% of explants (197 from 468) producing three-dimensional callus growth (a score of four or higher). Mean scores for callus production were higher on media containing 9µM IBA or 18µM NAA or IAA as the auxin source, and 9µM cytokinin as either kinetin or 2iP. However differences between auxins and cytokinins were not pronounced (Table 4.2), with the auxins NAA, IAA, and IBA at all concentrations (Figure 4.2a) and the cytokinins kinetin and 2iP at 9µM (Figure 4.2b) producing callus with a mean score of at least 3.0. Analysis of variance (at 95% confidence level) of the results indicated that callus initiation was not affected by the use of different hormones in the growth medium. The use of 2,4-D, BAP, or auxins and cytokinins at low (<4.5µM) concentrations, resulted in lower mean callus production scores, but these differences were not statistically significant.

Qualitative observations of callus throughout the growing period indicated that the presence of 2,4-D in the medium resulted in more rapid initiation of callus (within two weeks). Callus which had been initiated on media containing 2,4-D was noticeably more friable, less dense and had a light tan colouration. Callus initiated on other auxins tended to be compact, light green in colour and more dense (i.e. to have a greater mass for the same volume).

4.3.2 Effects of Auxins

Observations of callus size and morphology over the five week growing period suggested that the auxin treatments did not have any obvious effects on callus growth. Within each treatment however, there were differences in callus morphology that appeared to be related to the initiation medium of each callus, as discussed above.

AUXIN		CYTOKININ			BAP	2iP
		KINETIN				
		0 uM	4.5uM	9.0 uM	9.0 uM	9.0 uM
NAA	2.25 uM	1.6 (0.5)	2.8 (0.4)	2.6 (0.8)		
		16	*18*	*22*		
	4.5 uM	1.6 (0.6)	3.1 (0.6)	3.2 (0.9)	3.7 (1.1)	3.9 (1.0)
		15	*25*	*44*	*10*	*10*
	9.0 uM	2.6 (0.9)	3.6 (0.5)	3.5 (0.9)	2.8 (1.5)	4.1 (0.9)
		5	*15*	*45*	*10*	*10*
	18.0 uM	2.4 (0.7)	3.5 (0.5)	3.9 (1.0)	0.9 (0.3)	3.6 (1.6)
		24	*20*	*44*	*10*	*10*
IAA	4.5 uM			3.8 (0.9)		
				10		
	9.0 uM			3.6 (1.3)		
				10		
	18.0 uM			4.1 (0.9)		
				10		
IBA	4.5 uM			3.0 (1.9)		
				10		
	9.0 uM			3.9 (1.2)		
				10		
	18.0 uM			2.7 (1.4)		
				10		
2,4-D	4.5 uM			3.2 (1.4)		
				10		
	9.0 uM			3.4 (1.4)		
				35		
	18.0 uM			1.3 (0.8)		
				10		

TABLE 4.2: Initiation of *A. nummularia* callus from leaf half explants

Mean callus score after five weeks. Figures in parentheses indicate standard errors. Figures to lower right of each score indicate replication of each experimental combination. Scores were allocated according to the scale explained in Section 4.2.1 and the categories shown in Figure 4.1.

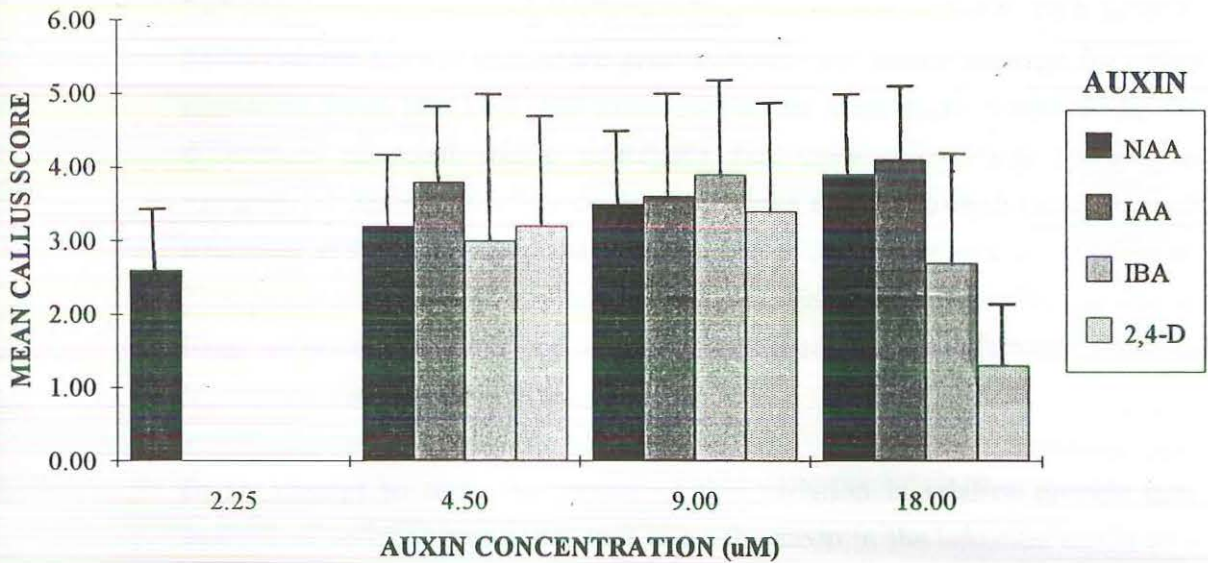FIGURE 4.2: Effects of auxins and cytokinins on callus initiation from *A. nummularia* leaf halves

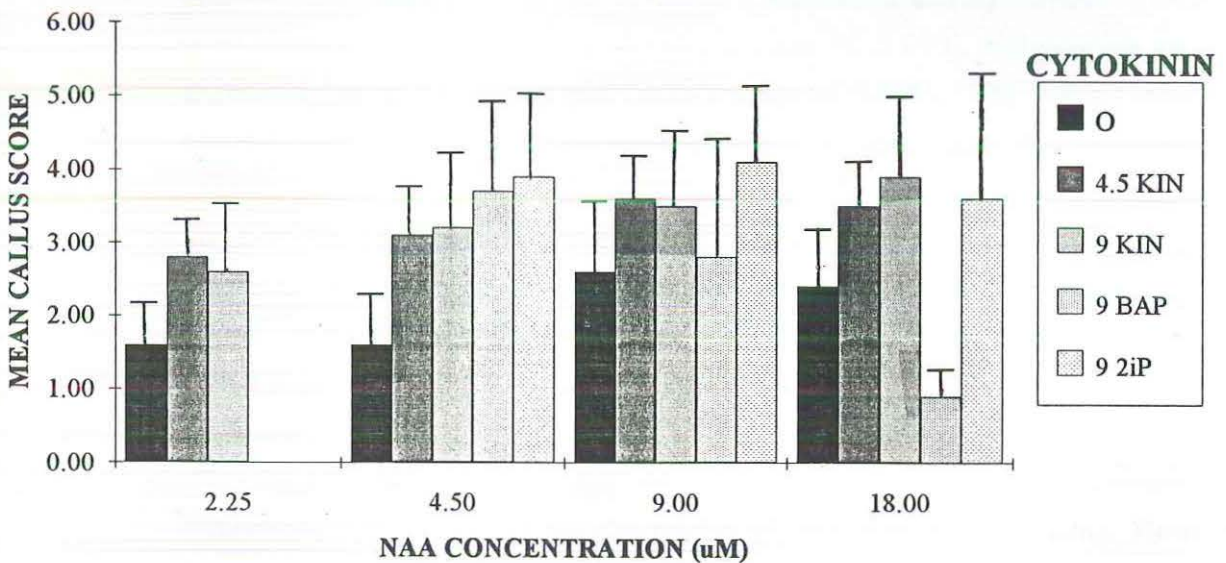Figure 4.2a: Effects of auxins on callus initiation on media containing 9μM kinetin.

Figure 4.2b: Effects of cytokinins on callus initiation on media containing NAA at four concentrations.

Quantitative measurements of relative growth rate revealed that there was a large variation in growth response within each treatment, and very little overall difference between treatments or trials (Figure 4.3). Callus grown on 2,4-D had a lower mean relative growth rate than that grown on NAA, IAA or IBA. Mean relative growth rates were greater in the first culture passage for callus grown on NAA and IAA, but these differences were slight compared to the differences observed within treatments and trials. Two-way analysis of variance (at the 95% confidence level) of mean relative growth rates for each treatment and trial revealed that the differences observed were not significant. Non-parametric analysis of variance by rank (minimising interference due to large variance measurements) also showed no significant differences between treatments and trials at a 95% confidence level.

In an attempt to trace the source of this variation in relative growth rate, analysis of variance was conducted using the auxin in the initiation media as a variable. At the 95% confidence level, no significance differences were observed between initiation media for each treatment and for each trial.

The validity of relative growth rates as an assessment method was examined by calculating the correlation coefficients of initial callus mass, and relative growth rate for ⁓ch treatment and trial (Figures 4.4 to 4.7 a and b). These values were close to ⁓ in the first trial (with a mean of -0.099), and positive and slightly higher in the second trial (with a mean of 0.408). The overall mean correlation of initial callus mass with relative growth rate, was 0.154.

Similarly, the relationship between relative growth rates for the two trials, was examined by calculating their correlation coefficients (Figures 4.4 to 4.7 c). There was positive correlation between relative growth rates for the two trials, with a mean of 0.616 for all media treatments.

4.3.3 Callus Regeneration

Regeneration of shoots was not observed in any experimental treatments. Root formation occurred in two media treatments where the ratio of auxins to cytokinins was 1:4. Roots were formed by one callus growing on media containing 1.125μM NAA and 4.5μM kinetin, and by two calli growing on media containing 0.6525μM NAA and 2.25μM kinetin.
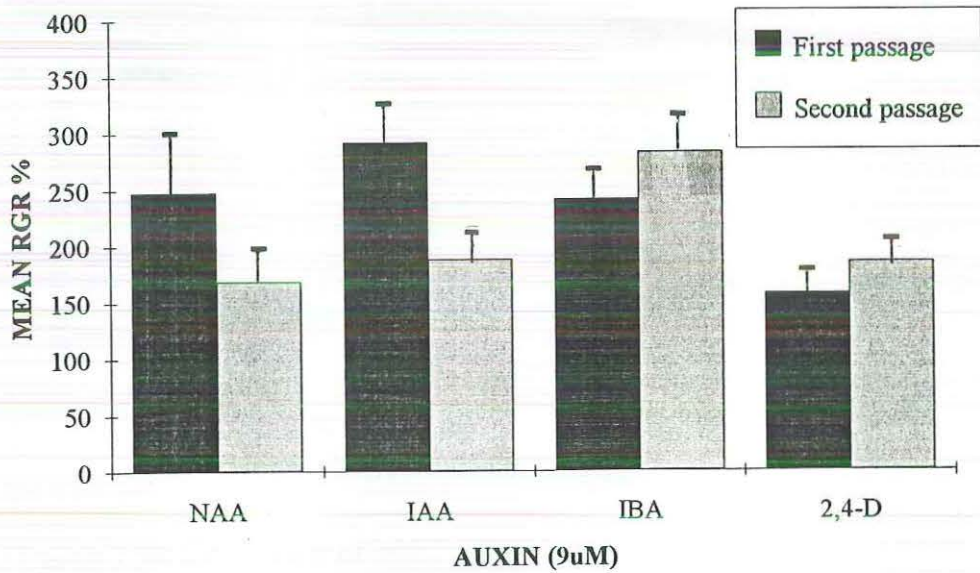
FIGURE 4.3: Effect of auxin type on mean relative growth rates of _A._
mummularia callus

Relative growth rate (RGR%) calculated from increase in callus mass ÷ initial
callus mass x 100%. Culture passage refers to the five week growth period
between subculture. N (first passage) = 52, N (second passage) = 38.

FIGURE 4.4: Correlation of growth parameters of *A. nummularia*
callus grown on M&S medium containing 9uM NAA
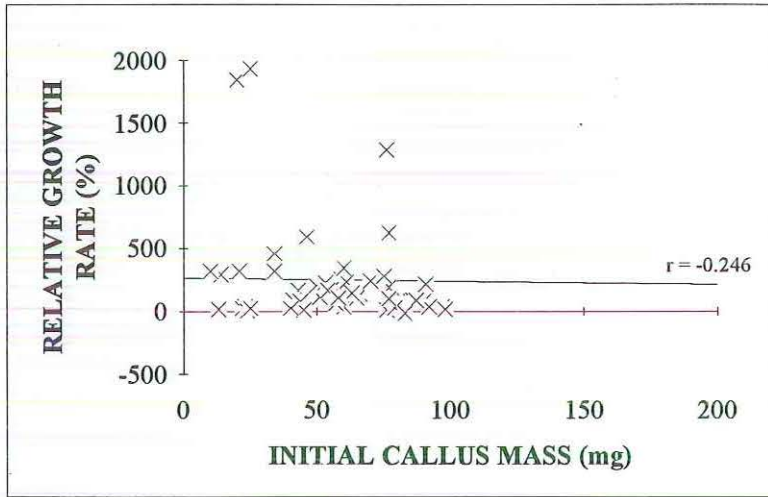
Figure 4.4a: Correlation of initial callus mass to relative
growth rate in the first culture passage
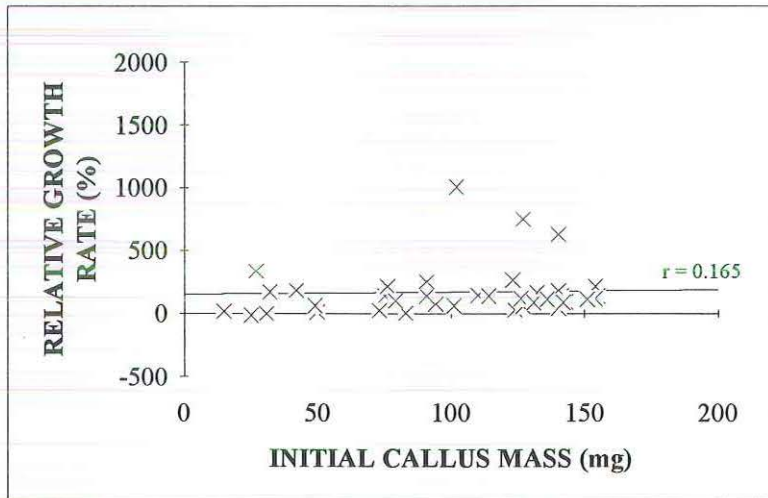
Figure 4.4b: Correlation of initial callus mass to relative
growth rate in the second culture passage
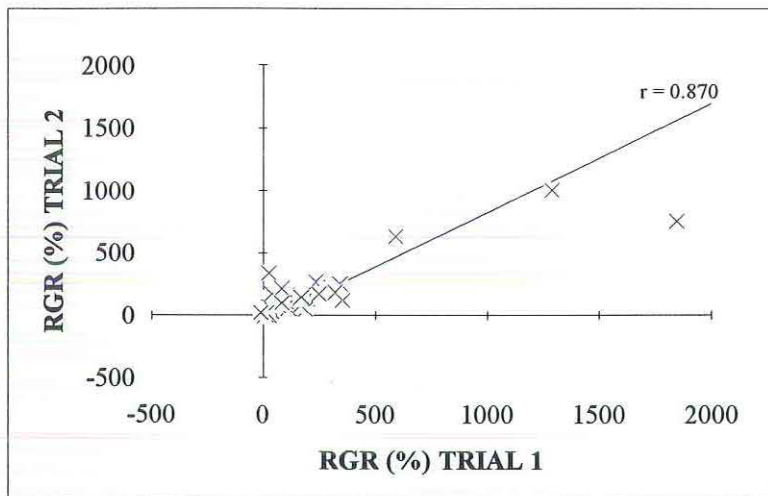
Figure 4.4c: Correlation of relative growth rate in the first culture
passage and second culture passage

FIGURE 4.5: Correlation of growth parameters of *A. nummularia* callus grown on M&S medium containing 9uM IAA
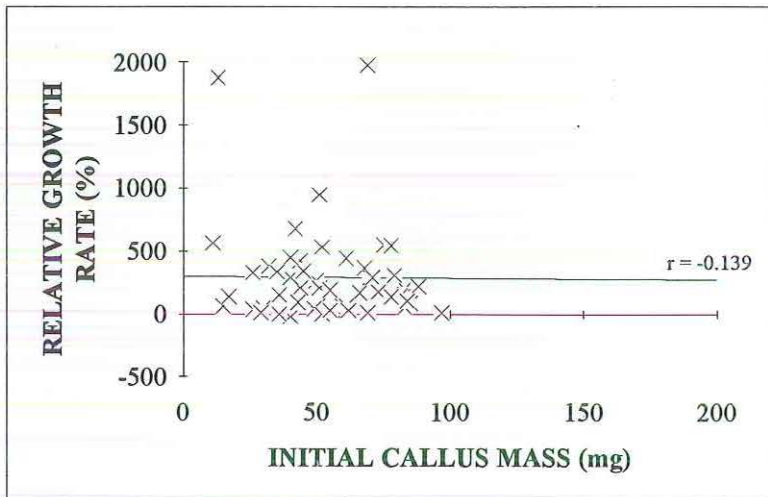
Figure 4.5a: Correlation of initial callus mass to relative growth rate in the first culture passage
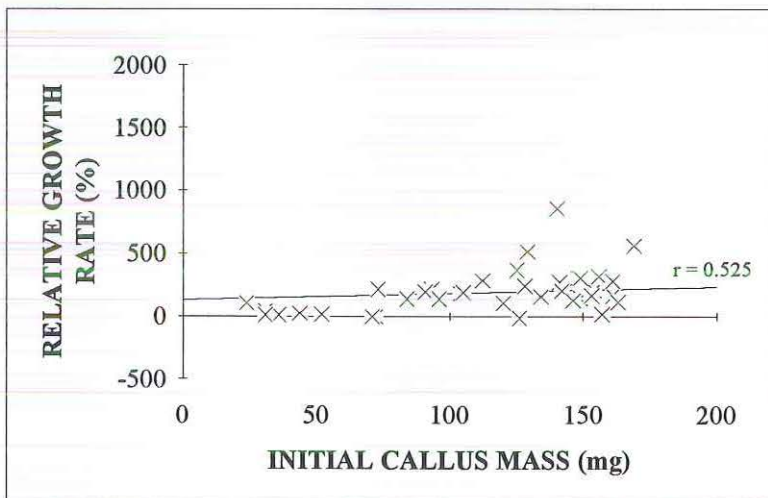
Figure 4.5b: Correlation of initial callus mass to relative growth rate in the second culture passage
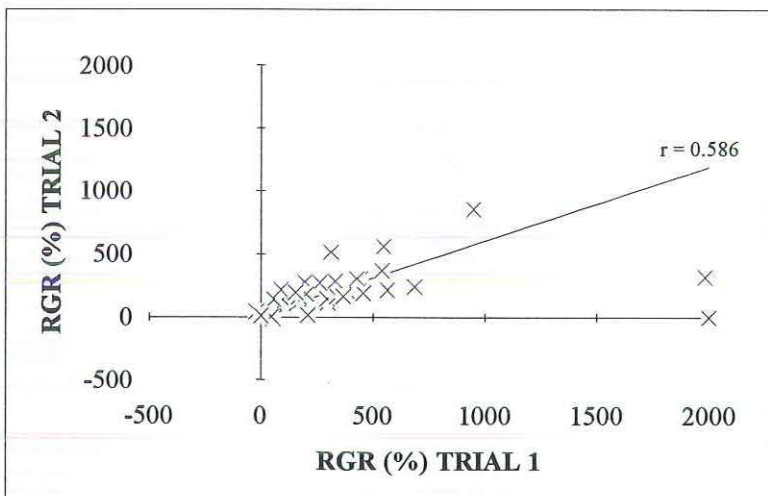
Figure 4.5c: Correlation of relative growth rate in the first culture passage and second culture passage

FIGURE 4.6: Correlation of growth parameters of *A. nummularia* callus grown on M&S medium containing 9uM IBA
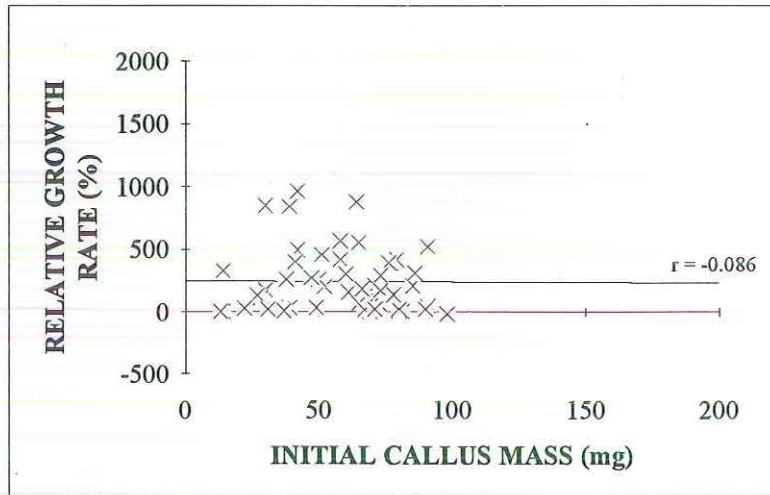
Figure 4.6a: Correlation of initial callus mass to relative growth rate in the first culture passage
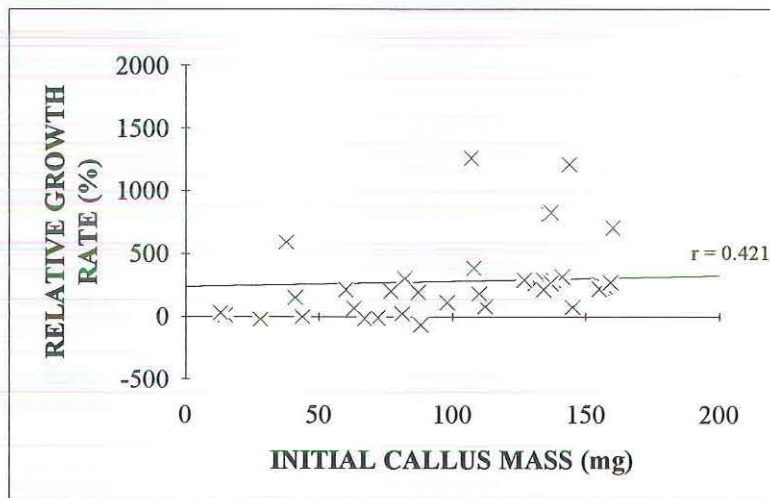
Figure 4.6b: Correlation of initial callus mass to relative growth rate in the second culture passage
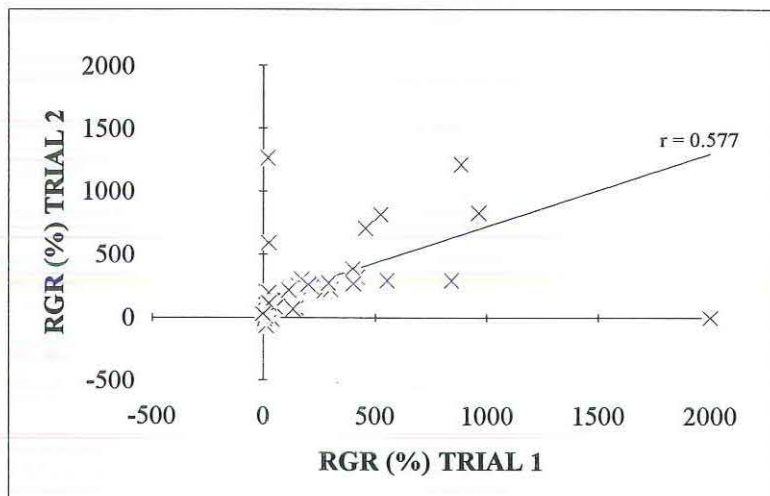
Figure 4.6c: Correlation of relative growth rate in the first culture passage and second culture passage

FIGURE 4.7: Correlation of growth parameters of *A. nummularia* callus grown on M&S medium containing 9uM 2,4-D
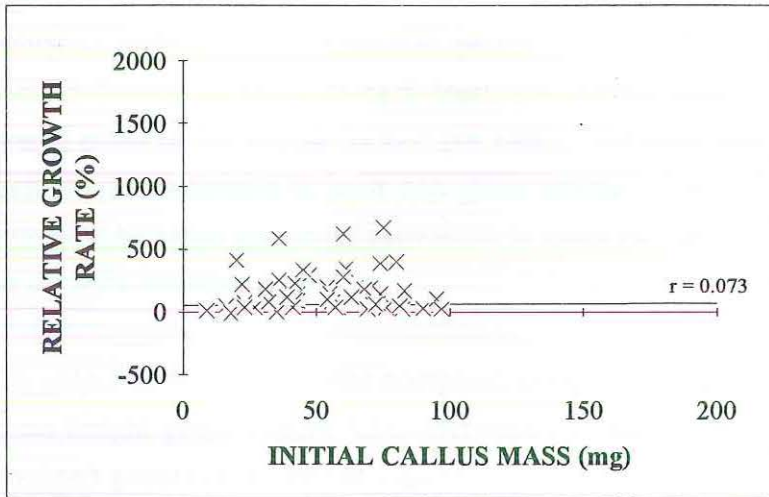
Figure 4.7a: Correlation of initial callus mass to relative growth rate in the first culture passage
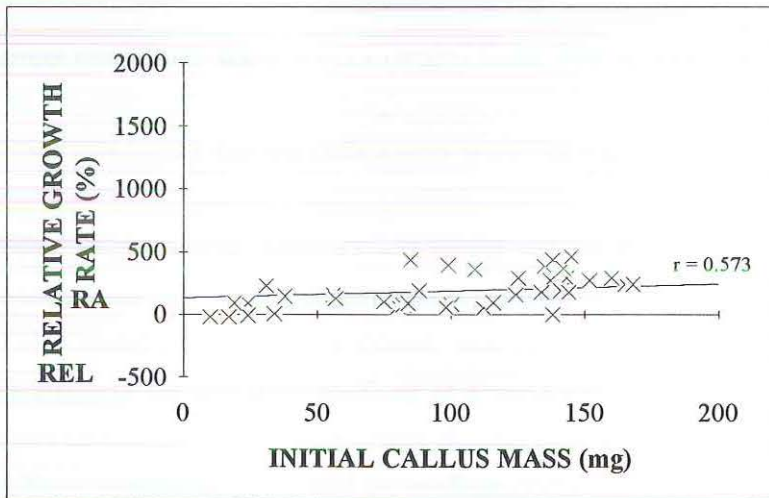
Figure 4.7b: Correlation of initial callus mass to relative growth rate in the second culture passage
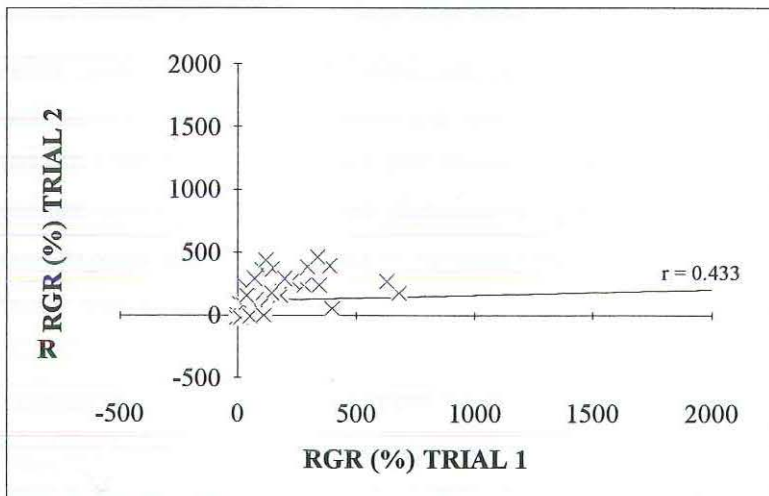
Figure 4.7c: Correlation of relative growth rate in the first culture passage and second culture passage

Many of the calli formed small dark green nodules after eight weeks on regeneration medium but these did not develop further (into shoots or embryos) within the experimental period. The average number of green nodules formed by callus in each treatment ranged from 0.6 to 2.1, with an overall mean of 1.0 nodule formed per callus. Between two and seven calli in each treatment formed at least one green nodule. The differences in nodule formation by callus grown on each auxin to cytokinin ratio, were not significant (at the 95% confidence level).

The colour of each callus was compared using a scale of one (dark brown) to seven (bright green, Figure 4.8). Between two and six calli in each treatment remained green (i.e. scored at least four), and the mean score for green colour in each treatment ranged from 2.8 to 4.3. The differences did not appear to be linked to the media treatment, and were not statistically significant.

Callus mass after eight weeks ranged from 566 to 1388mg for all treatments. The highest values (>1000mg) were recorded from media containing low auxins (<4.5µM), but the differences were not statistically significant.

Callus responses to regeneration media exhibited a wide range that did not appear to be due to the experimental treatments. In order to trace the source of this variation in callus response, analysis of variance was conducted using the auxin in the initiation medium as a variable. At the 95% confidence level, callus responses were significantly different for calli that had been initiated on medium containing NAA and on medium containing 2,4-D.

Callus that had been intiated on medium containing NAA was significantly greener (mean score = 5.1) than that from 2,4-D medium (mean score = 2.3). Callus that had been initiated on medium containing NAA produced significantly more green nodules per callus (mean = 2.0) than that from 2,4-D medium (mean = 0.4 nodules per callus). Callus that had been initiated on medium containing 2,4-D had significantly greater increase in mass from the same explant size (mean mass = 1018mg) than that from NAA medium (mean mass = 544mg).

Spontaneous regeneration of roots or shoots occurred infrequently in cultures that were being maintained for other purposes. In a total of approximately 500 callus cultures, shoot regeneration was observed on nine occasions (Figure
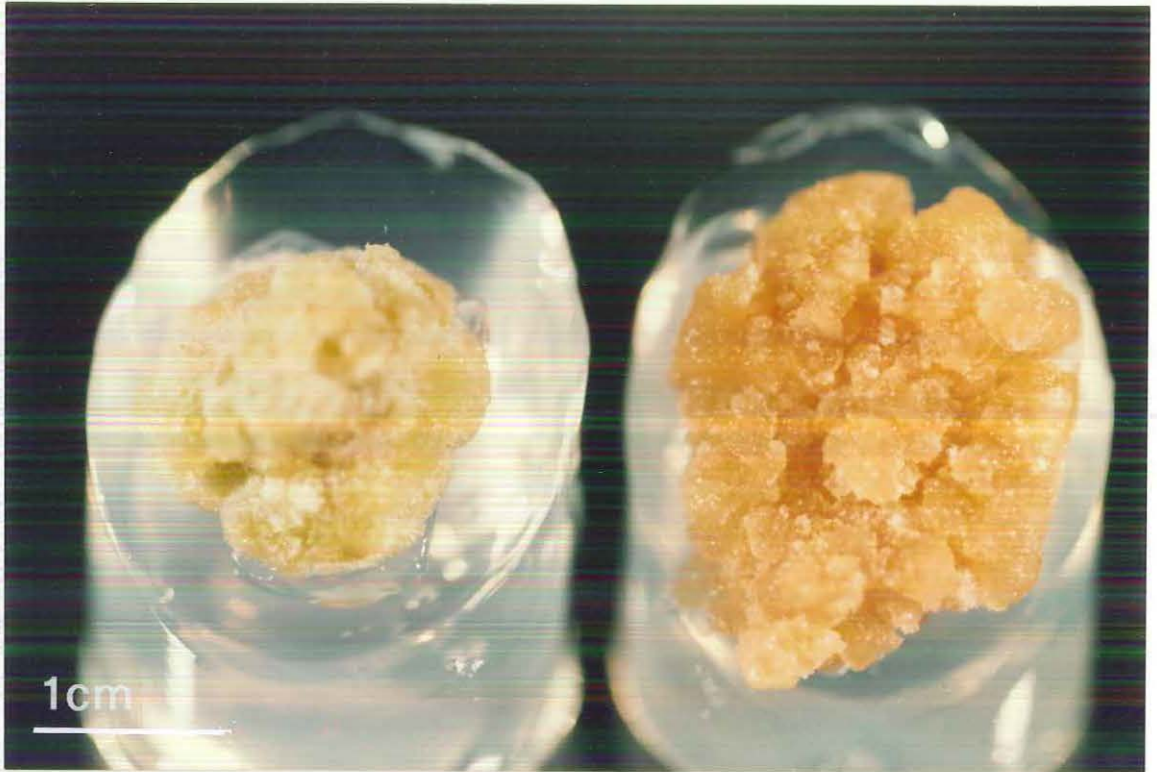
FIGURE 4.8: Colour variation in *A. nummularia* callus cultures
Callus at left scored 6, callus at right scored 1
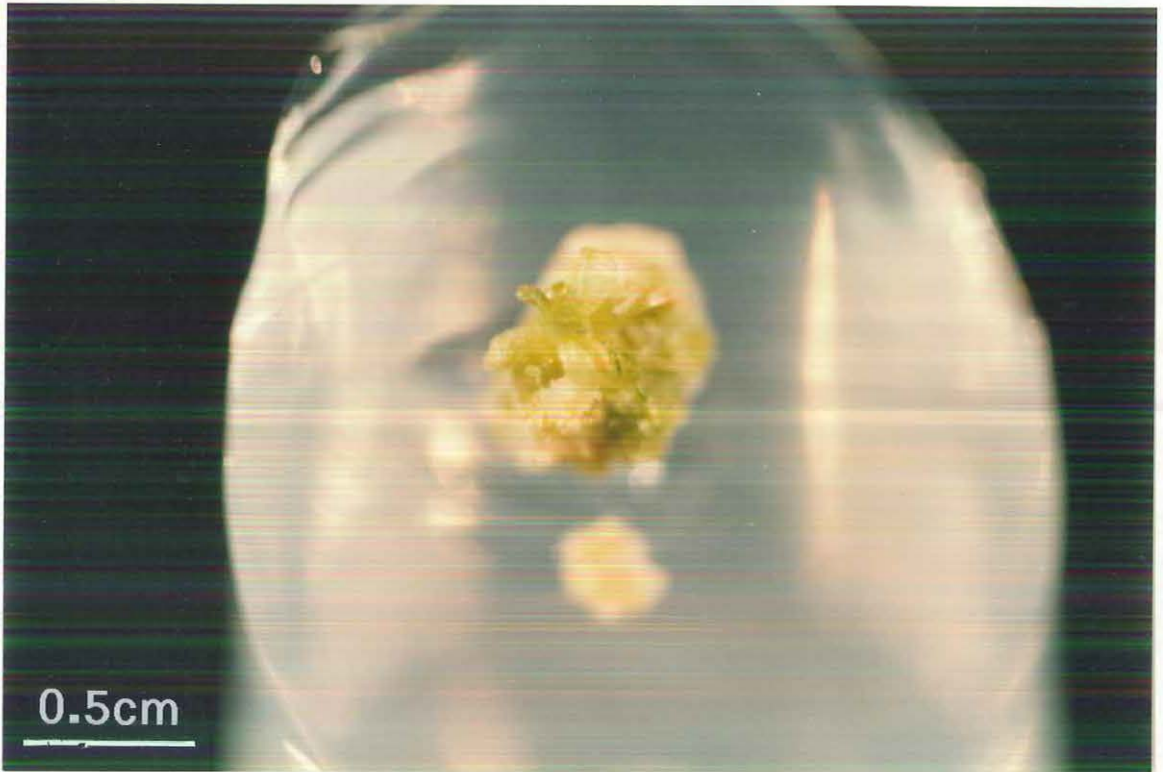
FIGURE 4.9: Shoot regeneration in *A. mummularia* callus cultures
Shoots formed after growing for 14 weeks on M&S medium containing 9μM
NAA and 9μM kinetin

4.9), and roots developed from 38 calli. Shoot regeneration was observed on cultures growing on medium containing 9µM NAA and 9µM kinetin, some three to four months after subculture. Root regeneration was observed on a variety of media. The hormone levels that resulted in more frequent root induction were 9µM IBA with 9µM kinetin, 9µM NAA with 18µM kinetin, 18 µM NAA without cytokinin, and 18µM NAA with 4.5µM kinetin.

4.3.4 Callus Salt Tolerance

Comparisons of mean relative growth rates show that, as expected the addition of NaCl to the growing medium reduces the mean relative growth rates of callus cultures (Figure 4.10). Analysis of variance and Tukey's comparisons of difference indicate that at the 95% confidence level, growth was not significantly different from the control with the addition of 43mM NaCl, but was significantly less than the control when 83mM or more NaCl was added. Addition of NaCl at concentrations of 83mM or greater, resulted in growth rates less than 50%, and in some cases negative rates were observed. Callus grown at these higher salt levels became dark brown and less friable, than the light green growth resulting on media containing 43mM or less, NaCl.

4.3.5 Suspension Culture

Cell suspensions with high densities (approximately 20×10^4 cells per mL) were produced in only two of the thirty suspension flasks: both of these were in the zero NaCl treatments. Mean cell densities for all treatments, were significantly higher when NaCl was absent from the medium, and densities were very low (less than 1×10^4 cells per mL) in media containing 171mM and 342mM NaCl (Figure 4.11).

4.4 DISCUSSION

4.4.1 Callus Initiation

Initiation of callus from *Atriplex nummularia* tissue was readily accomplished using leaf explants on M&S media. A range of auxins including NAA, IAA and IBA, and cytokinins including 2iP and kinetin, over concentrations of 4.5 to 18µM in the initiation medium produces satisfactory callus initiation. These hormone levels are within those commonly used for cereal tissue culture (Dixon, 1985), and for initiation of callus from other salt tolerant cell lines including alfalfa (*Medicago sativa* L.) clones (Winicov & Button, 1991; Johnson & Smith, 1992), and *Mesembryanthemum crystallinum* L. (Thomas, De Armond & Bohnert, 1992). Smith and McComb (1981a) used 9µM 2,4-D
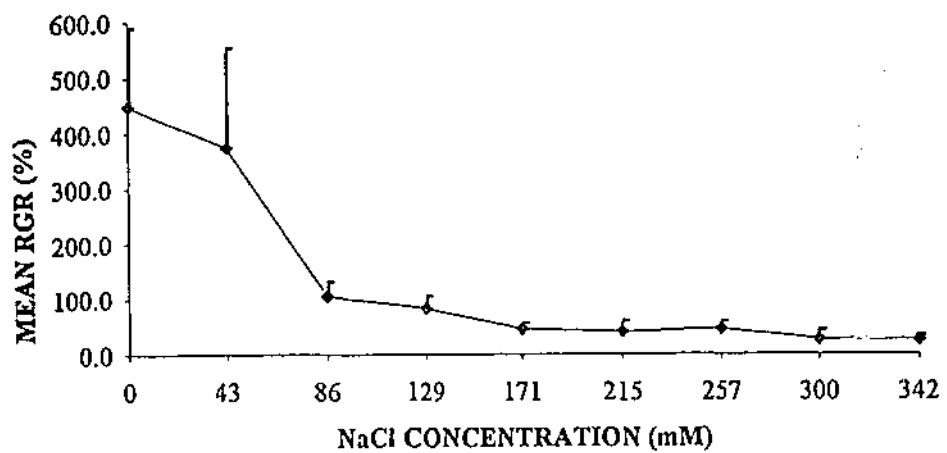
FIGURE 4.10: Effect of NaCl in the growing medium, on mean relative growth rate of callus after five weeks (N = 10).
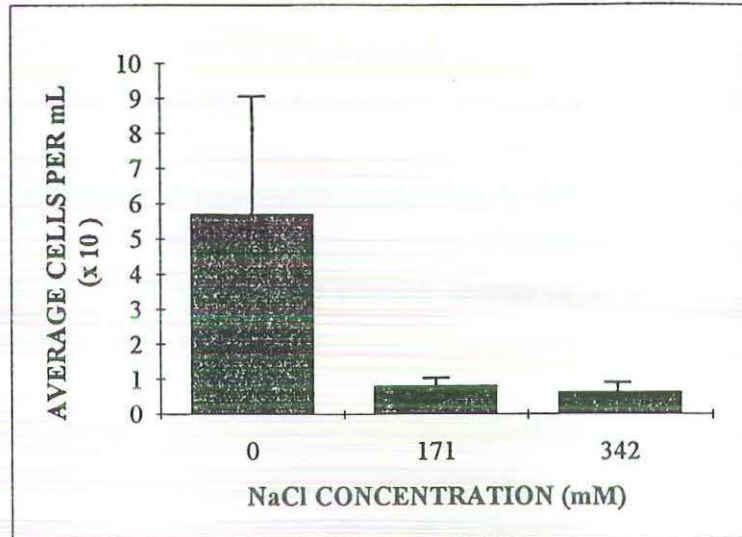
FIGURE 4.11: Mean cell density of suspension cultures with and without NaCl in the medium, after four weeks (N = 10)

and 9μM kinetin for callus initiation from hypocotyl tissue of *Atriplex undulata* D.Dietr.: it appears that the use of NAA, IAA or IBA in the place of 2,4-D gives better callus initiation for *A. nummularia* leaf explants. In addition other auxins are favoured because of their reduced environmental toxicity compared to 2,4-D (Durham, 1987).

4.4.2 Effects of Auxins

The results suggest that callus growth, when measured by the method of relative growth rate, is not significantly affected by the type of auxin used in the media, by the time in culture or by the auxins used in the initiation media. The range of conditions tested was not extensive: auxins were not tested over very high, or very low, concentrations, and the experiment was only conducted for 10 weeks. It is possible that varying responses to the four auxins would be found over a greater range of concentrations or a longer time frame. Similarly, an assessment system that took into account factors other than mass increase, may have revealed some differences.

Although no significant differences in relative growth rates could be attributed to initiation media, there was considerable variation in the morphology and physical dimensions of callus, that appeared to be related to the presence of 2,4-D in the initiation medium. 2,4-D is more persistent than other auxins, particularly IAA, which are rapidly oxidised by light and by plant cells (Hussey, 1983). It has been used extensively in callus culture because it maintains cells in a dedifferentiated state for longer (Bolwell, 1985). In *A. nummularia* callus, the inclusion of 2,4-D in the initiation medium appeared to promote the growth of a less differentiated, friable brown callus, and this effect continued for at least 10 weeks after subculture onto media without 2,4-D. Because of the randomisation of calli throughout the experimental treatments in this section, replication of calli with similar initiation media was poor (in some cases as low at two), and the effects of initiation medium on subsequent callus growth could not be investigated fully. Further experimentation designed to examine the relationship between initiation medium and subsequent growth parameters, may reveal that significant differences exist.

The low correlation observed between initial callus mass and relative growth rate, suggests that callus growth is not dependent on explant mass (within the range of 20 - 150mg). It appears that the minimum critical explant size for *A. nummularia* callus cultures is less than 20mg, and given that explants are

within this limit, their growth rates may be compared regardless of differences in original size.

Growth rates of callus in each five week culture passage showed strong positive correlation, indicating that time in culture does not affect growth rate (at least over a 10-week period). Smith and McComb (1983) found that callus growth rates are not significantly different for explants of *A. undulata* that have been in culture for up to one year, allowing comparisons of growth rates to be made between callus cultures of different ages.

Subsequent investigations of callus growth factors have made use of these findings. Nine micromolar NAA and 9μM kinetin have been used to obtain maximum callus growth rates, callus initiated on a variety of media have been used randomly in experimental treatments, and callus explants of varying sizes and ages have been used.

4.4.3 Callus Regeneration

Regeneration by organogenesis from *A. nummularia* callus did not appear to be enhanced by any of the combinations of hormones used, but was spontaneously observed when high concentrations of auxins and cytokinins were present in the medium. Few shoots were regenerated, and these occurred when 9μM auxin and 9μM cytokinin were present in the medium. This is unusual since shoot regeneration is usually favoured by a ratio of low auxin concentration to high cytokinin concentration (Tisserat, 1985). Few callus cultures developed roots, and this occurred on media containing a variety of hormones and concentration ratios including 9 - 18μM auxin and 0 - 18μM cytokinin. Again these results are unusual since adventitious root formation is usually enhanced by the inclusion of low levels of auxins and low or no cytokinin, as well as other environmental conditions such as low light intensity and reduced nutrient concentration (Tisserat, 1985).

These results do not necessarily reflect the conditions which give best organogenesis from *A. nummularia* callus. Shoot and root regeneration that was observed from stock cultures, occurred in old cultures that had been maintained without subculture for up to four months. The effective concentrations of auxins and cytokinins in the medium after this period are likely to be much lower than the original concentrations added to the medium.

In this case the results would be more in keeping with those found by other authors (Tisserat, 1985).

Regeneration of shoots was not observed during the experiment, which was limited by time constraints. In order to determine the hormone combinations and concentrations that give maximum rates of organogenesis, it appears necessary to culture callus for longer periods of time (up to four months). Since many calli developed dense (and sometimes protruding) green nodules which are considered to be the precursors of adventitious shoots during the experiment, shoots may be expected to be regenerated given adequate time.

4.4.4 Callus Salt Tolerance

The results obtained here are similar to those reported by Smith and McComb (1983), who found that growth in *A. undulata* callus was depressed by the addition of 62.5mM or more NaCl, and severely depressed by the addition of NaCl in concentrations of 125mM and higher. The results support the hypothesis that *Atriplex spp.* do not possess cellular salt tolerance (assuming that if such salt tolerance was present it would be expressed). It is however, possible that NaCl tolerance was not expressed because of the sudden transition from relatively salt-free, osmotically balanced media to media containing elevated salt levels. Galvez, Gulick and Dvorák (1993) suggested that cells of glycophytic and halophytic plants may have enhanced ability to tolerate salt stress if they have been previously exposed to low salt concentrations, allowing possible changes in gene expression.

The presence of negative growth rates (representing weight loss by the callus) in the experiment suggests that there were osmotic differences between the callus and the growth media, causing water movement out of cells and intercellular spaces. The use of alternative measurements that are not affected by callus water content, such as ashed dry weight or protein content, would give a more accurate measurement of cell growth than fresh weights.

An experiment involving step-wise introduction of NaCl to the growth environment, similar to those conducted by Winicov and Button (1991) and Binzel et al. (1985) may reduce the effects of osmotic shock, and would examine the possibility that cultures possess salt tolerance but need some form of acclimatisation time or adaption in order to express that tolerance.

4.4.5 Suspension Culture

The results obtained here agree with those of other authors, who report a decrease in cell growth or number of viable cells when NaCl is included in suspension cultures (Hasegawa et al., 1986; Spiegel-Roy & Ben-Hayyim, 1985; Smith & McComb, 1983). Inclusion of NaCl in the liquid medium selects for cells which have a greater salt tolerance, although very few cells (less than 1 x 10^4 cells per mL) survived five weeks in culture. Comparison of the cell densities obtained here, with those of other authors is difficult. The other measurements of growth in suspension culture listed above, have used more complex methods involving filtration of cells (to determine cell volume or packed cell volume), or plating cells out onto solid medium (to count colonies developing from surviving cells). The use of these and other methods is recommended by Dixon (1985), and is suggested for future experiments involving the selection of *Atriplex* cells for salt tolerance.

The low numbers of cell suspensions produced in this experiment suggest that some refinement of the method used, may be necessary. Filtration of cell suspensions following culture establishment (either with metal sieves or with fine-weave fabric such as muslin or nylon), and frequent subculture into fresh nutrient solution, are measures both recommended by Dixon (1985). The use of these methods may have improved the rate of formation of cell suspensions in this experiment. It is unlikely however, that the methods would have altered the relationship observed between cell densities, and NaCl concentration.

Regardless of the methodological difficulties, this experiment suggests that suspension cultures are a useful tool in screening programmes for *Atriplex* spp. Results obtained here, support the hypothesis that suspension culture may be used to screen for salt tolerant cells in *Atriplex* spp. callus cultures, since cell suspensions were readily formed from callus cultures, and at least some cells were capable of survival at elevated salt concentrations. This culture system may be utilised to select and isolate lines of cells which survived at the higher salt levels, and subsequently to regenerate and multiply plantlets with the same genome. It remains to be seen whether the cellular salt tolerance is stable and will be inherited by daughter cells, and whether this salt tolerance will confer additional tolerance to whole plants.

5. GENERAL DISCUSSION

Tissue culture of *Atriplex* spp., including *A. amnicola*, *A. cinerea* and *A. nummularia*, is readily achieved by the methods developed here. Shoot cultures can be initiated from plant stems, and can be induced to form multiple shoots and roots. Callus cultures can be initiated from leaf material and multiplied, and can regenerate roots and shoots. Suspension cultures of single cells can be established from callus cultures.

Shoot cultures of *A. amnicola*, *A. cinerea* and *A. nummularia* can be initiated from field-grown and glasshouse-grown plants, on M&S medium containing low concentrations of hormones. Cultures can be induced to form multiple roots and shoots within eight weeks. The use of plant material that originates from glasshouse plants, improved the efficiency of initiation of *A. nummularia* shoot cultures (when compared to cultures originating from field-grown plants). Glasshouse-grown plants tend to have far lower populations of surface micro-organisms than field-grown plants (De Fossard, 1976, pp. 36-53), and are usually grown under more favourable conditions. Explants from glasshouse-grown material can be expected to be in better physiological condition (except where deliberately exposed to stress), and to carry fewer contaminants, than field-grown plants.

Difficulties in establishing certain species in culture, were experienced. In particular, material from *A. semibaccata*, *A. stipitata* and *A. vesicaria*, which was obtained from distant field sites, could not be established. High rates of explant contamination were observed, and this was thought to be a product of growth in the field environment, and/or the transportation process. Rooted cuttings of these species that have been grown under glasshouse conditions for a period of time, may provide a better explant source. Better rates of culture initiation and survival were obtained using glasshouse-grown *A. nummularia* material, than using field-grown material. It is likely that the use of glasshouse cuttings of other species that proved difficult to establish here, would yield similarly improved results. This method would also allow the isolation of clones from plants which exhibited desirable properties in the field (such as salt tolerance or a suitable growth habit). This option could not be fully investigated here because of time restrictions.

Initiation of callus cultures of *A. nummularia* was readily accomplished using leaf explants. The inclusion of 9μM NAA, or IAA, and 9μM kinetin in the initiation medium was found to give good rates of callus formation and subsequent callus growth. The inclusion of 2,4-D in the initiation or multiplication medium, as recommended for *A. undulata* by Smith and McComb (1981a), was seen to affect subsequent callus growth: the use of other auxins such as NAA or IAA is recommended. The synthetic auxin 2,4-D has been previously used to induce callus growth and multiplication in a variety of species, since it is more active in maintaining cells in the dedifferentiated state. Transfer to a 2,4-D-free medium, and depletion of endogenous levels of 2,4-D (adsorbed into callus cells and intercellular spaces) appears to be necessary before morphogenesis or differentiation into meristems can occur (Bolwell, 1985). In callus cultures of *A. nummularia* regeneration was rarely observed if callus had been initiated or grown on 2,4-D. The use of this auxin appears to have an influence on the future growth response and regeneration potential of callus, and therefore is not appropriate in screening programmes where regeneration is an essential step in the process.

Regeneration of shoots and roots from callus of *A. nummularia* occurred in several cultures, but the combination of auxins and cytokinins in the medium giving optimum results could not be defined. Similarly, optimum media for organogenesis (the development of adventitious roots and shoots) from shoot cultures could not be precisely defined. Shoot cultures could be grown on M&S medium containing the auxins NAA, IAA and IBA and the cytokinins kinetin, 2iP and BAP. The presence of either auxins or cytokinins did not enhance adventitious shooting. It is suggested that relatively high concentrations of hormones (5 - 10μM) would have a greater effect on organogenesis from shoot cultures. There is the possibility that the use of a more active cytokinin such as zeatin, or changes to the growth environment (for example by increasing light intensity), may enhance shoot production (Tisserat, 1985; Wareing & Phillips, 1981, pp. 151-167).

Root initiation in most cultures, and particularly in clones of *A. cinerea*, occurred infrequently unless auxins were present in the medium. The use of low concentrations of auxins appears to enhance root production in these and other clones of *Atriplex* spp. The production of roots from both callus and shoot cultures of a variety of other species, including tobacco and *Begonia* is enhanced on medium without growth hormones, or with low concentrations

(<1µM) of auxins (Tisserat, 1985). Further experimentation to define the exact hormone requirements for regeneration from shoot and callus cultures of *Atriplex* spp. should allow better rates of organogenesis to be attained. In developing future experiments to determine the regeneration requirements of the genus, consideration should be given to the effects of persistent hormones such as 2,4-D, and to the length of time required for regeneration to occur.

Suspension cultures could be established from callus explants within a short time. Several refinements of the method have been suggested, but results obtained here indicate that suspension cell culture of *A. nummularia* is feasible. The further development of suspension culture as a viable method of obtaining large populations of single cells, is essential to the success of many types of selection programmes. Suspension cultures offer a source of single cells that can be exposed to selection agents (such as salt) under controlled conditions, so that surviving cells (with presumably greater tolerance to the selection agent) can be identified and isolated.

Atriplex cells exposed to salt, either in solid or liquid medium, did not appear to exhibit tolerance to moderate or high levels of salt. Some questions remain unanswered, as to whether this result truly reflects the absence of salt tolerance in *Atriplex* cells: the modification of experimental method to investigate the possibility of inducible genetic expression of salt tolerance, should be undertaken (Galvez, Gulick & Dvorák, 1993). Assuming that this is not the case, and that cells do not possess the capacity for salt tolerance, the use of tissue culture methods to screen large numbers of cells for a more salt-tolerant phenotype could be used as the basis for an improvement programme of *Atriplex* spp. Although the cells as population did not exhibit salt tolerance in suspension cell culture, low numbers of cells survived for five weeks in suspension culture in the presence of 171 or 342mM NaCl. These results suggest that there may be considerable somaclonal variation evident in *A. nummularia* cultures. Somaclonal variation can be summarised as genetic differences in individual cells, that arise either spontaneously or as a result of the culture process (Lindsay & Jones, 1990, pp. 78-93). The presence of somaclonal variants in suspension culture, allows for the use of selection processes to identify cells with potentially useful genotypes. By adding salt to the liquid medium of suspension culture, or by plating cell suspension onto solid medium containing salt, cells with the normal, salt-sensitive genotype can be eliminated while those with a tolerant genotype remain in culture.

The process of isolating and regenerating a salt tolerant genotype to result in plants with improved salt tolerance was not attempted, but this research has identified the culture requirements of *Atriplex* spp., and indicates that such a selection process is viable. There are however, numerous theoretical considerations that arise from this work, and these can be explored here. The selection of cells through tissue culture, relies on the phenotypic expression of genetic differences. In a complex character such as salt tolerance, which depends on the interaction of several physiological processes, phenotypes may be cryptic or not expressed where partial genetic selection has resulted in an organism with a particular combination of genes which confer salinity tolerance (Yeo & Flowers, 1989). The separate isolation several lines of cells, may result in the selection of clones with similar phenotypes but with differing genetic bases for this tolerance. This has advantages in the selection of plants for revegetation programs since each clonal line (and the plants regenerated from them) would have potentially different properties.

The inclusion of cellular level salt tolerance, in a halophytic plant could potentially increase the overall salt tolerance of the plant if both forms of salt tolerance are expressed additively. Little is known about the modes of genetic expression, or the physiology, of salt tolerance (Tal, 1985; Yeo & Flowers, 1989). Although it has not proven the hypothesis that salt tolerance in halophytes is not a cellular mechanism, this work has indicated that cells of *Atriplex* spp. do not possess elevated salt tolerance. There is therefore, an avenue for the genetic improvement of salt tolerance in halophytes, through the incorporation of a cellular slat tolerance genotype in these plants. Tissue culture offers a vehicle for the selection of salt tolerance at the cellular level, and its subsequent inclusion in halophytes with this genome. It remains to be seen whether this process will produce more tolerant plants than those which posses either cellular, or whole-plant tolerance mechanisms alone. The inclusion of a genetically-stable, heritable form of salt tolerance in halophytes, could produce more salt tolerant plants which would be of use in the revegetation of saline land. Preliminary work completed here, suggests that cellular salt tolerance can be selected for in *Atriplex* spp. There is potential for the genetic improvement of halophytes, with application to the rehabilitation of saline land.

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