EFFECTS OF NITROGEN NUTRITION ON SALT STRESSED *NICOTIANA TABACUM* VAR. SAMSUM *IN VITRO*

by

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PREFACE

The experimental work described in this thesis was carried out in the Department of Biology, University of Natal, Durban, from January 1991 to December 1992, under the supervision of Drs M.P. Watt and B.I. Huckett.

These studies represent original work by the author and have not been submitted in any form to another University. Where use was made of the work of others it has been duly acknowledged in the text.

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ABSTRACT

The responses of *Nicotiana tabacum* L. var. Samsun to alterations in the nitrogen (N) supply under saline conditions *in vitro* were monitored. The aim was to test the hypothesis that nitrate-nitrogen supplementation to salt stressed plants alleviates the deleterious effects of salt on plant growth.

Due to its capacity to be maintained under stringent environmental conditions, *in vitro* shoot cultures were chosen as the system of study. *Nicotiana tabacum* plantlets regenerated from callus *in vitro* were excised and rooted on solid MS culture medium containing a range of concentrations of NaCl (0 - 180 mM) and N (0 - 120 mM, as NO_3^--N, NH_4^+-N or a combination). A variety of parameters of root and shoot growth, nutrient utilisation and nitrogen metabolism were assessed over a 35 d period.

Plant growth on 40 mM NO_3^--N + 20 mM NH_4^+-N (standard MS nutrients) was inhibited by the presence of salt, with root growth being more adversely affected by salt than stem growth. Root emergence was delayed from 6 d (0 mM NaCl) to 15 d (180 mM NaCl). Similar suppression of growth for all parameters, except root mass and leaf chlorophyll content, was observed when NaCl was replaced with mannitol at equivalent osmolalities. Root mass and leaf chlorophyll were significantly improved in plantlets supplied with mannitol. The time of root emergence was unaffected by mannitol supply, with all roots emerging after 10 d in culture. Plantlet growth on NH_4^+-N only (0 - 60 mM) was severely inhibited, even in the absence of NaCl, and was inferior to growth on NO_3^--N. Nitrate additions to salt stressed plantlets could not match growth in control (0 mM NaCl) plantlets. When plantlets were cultured on NO_3^--N only (0 mM, 30 mM, 60 mM, 120 mM), the increase in nitrate supply up to 60 mM resulted in a small improvement in growth on 90 mM NaCl, but had almost no effect on growth at 180 mM NaCl. A nitrate supply of 120 mM led to growth inhibition in all parameters, even in the absence of NaCl. Plantlet growth on isosmotic concentrations of mannitol in the presence of 0 - 120 mM NO_3^--N essentially mimicked that of NaCl, except for leaf chlorophyll content which was improved on mannitol at all NO_3^--N levels.

Nitrate uptake (measured as depletion from growth medium) by plantlets grown on 0 - 180 mM NaCl was positively correlated to availability of nitrate but negatively correlated to NaCl supply. Similar results were obtained for a mannitol supply

except nitrate uptake was enhanced significantly on mannitol compared to NaCl. Sodium and chloride uptake appeared unaffected by nitrate concentration. Leaf protein content responded favourably to an increase in the NO_3^--N supply up to 60 mM and, in particular, appeared to be stimulated in the presence of 180 mM NaCl. Nitrate reductase (NR) activity was found to be inhibited drastically by salt and NO_3^--N supplementation to the salt medium had no effect on enzyme activity.

A reduction in leaf total RNA content was recorded with an increase in NaCl concentration from 0 - 180 mM. A positive response to an increase in the NO_3^--N supply from 30 mM to 60 mM was detected in the presence of NaCl. Attempts were made to assess the levels of mRNA for NR in response to the various NaCl and N regimes. The plasmid pBMC102010 containing a NR cDNA insert was isolated and purified and used in both radioactive and non-radioactive RNA slot blot hybridisation procedures. However, due to problems of non-specific binding of the probe, no quantification of the levels of NR mRNA in response to the various treatments could be made.

Nitrate supplementation to plantlets of *Nicotiana tabacum* growing *in vitro* did not appear to ameliorate the effects of salinity stress, such that growth of plantlets in the presence of NaCl was always inferior to that in the absence of NaCl. As a large portion of growth inhibition was found in this study to be a result of osmotic rather than ionic effects of salt, it is questioned whether a nitrate supply would have an ameliorating effect on plant growth under field conditions.

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LIST OF ABBREVIATIONS AND SYMBOLS

centimetre	cm
copy deoxyribose nucleic acid	cDNA
counts per minute	cpm
days (time)	d
degrees celcius	°C
deoxyribose nucleic acid	DNA
grams per litre	g/l
hour	h
kilobases	kb
kilodaltons	kD
megapascals	MPa
messenger ribose nucleic acid	mRNA
micro Einsteins per metre^2 per second	$\mu E\ m^{-2}\ s^{-1}$
microgram	μg
milligram	mg
millilitre	ml
millimolar	mM
minute (time)	min
molar (concentration)	M
nanometer	nm
number of observations	n
percentage	%
revolutions per minute	rpm
ribose nucleic acid	RNA
second (time)	sec
standard deviation	s.d.
volume by volume	v/v
weight by volume	w/v

1. GENERAL INTRODUCTION

The world is faced with an accelerating demand for food due to the continuing growth in the global human population. Agricultural production will need to increase in the range of 3% to 4% per year for the next 50 years if it is to match the nutritional requirements of the increasing human population (Toenniessen, 1984). As the earth's resources are finite and there are limits to the land that may be committed to agriculture and food production, there is a necessity to utilise more efficiently the land that is available. In particular, there is an urgency to develop an approach to management of saline soils such that they may become a source of arable land. Most agricultural crops, including staple foods such as maize, wheat and sorghum are highly sensitive to the presence of salt in the soil environment and exhibit reduced growth and productivity when grown under saline conditions (McWilliam, 1986; Broadbent *et al.*, 1988; Lupotto *et al.*, 1989; Yang *et al.*, 1989). Although there are many salts present in soil water which may have deleterious effects on plant growth, sodium chloride is by far the most abundant and is considered to be the major salt responsible for causing low productivity and yield of plants (Levitt, 1980).

Salinity remains one of the world's oldest and most serious environmental problems. Excessive irrigation and inadequate drainage are the principal causes of a build-up in soil salinity, which in turn undermine the productivity of a large portion of the world's irrigated lands (McWilliam, 1986). The mistakes made by the Sumarians over 4000 years ago in the Tigris and Euphrates basin of Mesopotamia are being repeated today in almost every major irrigation development in the world (McWilliam, 1986). It is estimated that the global proportion of all arid and semiarid lands affected by salt is in the region of about 15% or 950 x 10^6 ha., while the proportion of irrigated land affected by salt is about 3% or 77 x 10^6 ha. (Epstein, 1980). While the fertility of a saline soil can be built up by good management, so it can be destroyed by poor management. There is no single solution to the problem of increasing salinization of soils, but a number of options have been put forward by McWilliam (1986), including 1) improved water management to reduce the rise of the water table and the amount of salt introduced, 2) development of systems to drain salts away from the root zone to where they can be disposed of by evaporation, desalination or drainage into the ocean, and 3) research to develop plants that are more salt tolerant. However, these methods are expensive and labour intensive and, especially in the case of 3), provide only a short-term solution to the problem.

Evidence has accumulated over the past 40 years suggesting that increased nitrogen fertilization, in the form of nitrate, to plants growing on saline soils may result in improved plant growth and a greater tolerance to the salt (Heinmann, 1958; Ravikovitch and Yoles, 1971). Salinity adversely affects the processes of nitrate assimilation into proteinaceous compounds (Helal and Mengel, 1979; Abdul-Kadir and Paulsen, 1982; Pessarakli *et al.*, 1989) and it is largely upon these processes that the productivity of a plant is dependent. Increasing the fertility of a saline soil by nitrate fertilization is an attractive option as it is relatively simple to implement. Nonetheless, efforts to ameliorate the negative effects of salinity on plant productivity by nitrate supplementation have been hampered. There is a lack of understanding of the underlying mechanisms responsible for salt injury to plant tissues which is further complicated by inadequate knowledge of the relative contributions of the osmotic and ionic effects of salt on plant growth and metabolism (Levitt, 1980; Flowers and Yeo, 1986). It is not understood well what the salt effects are at the physiological, biochemical, and molecular levels and what, if any, is the nature of their interactions. In addition, although improved growth of salt stressed plants provided with nitrate implies a relationship between nitrate and salinity, the underlying nature of such interactions have not been elucidated.

Many studies of the effect of nitrate nutrition on salt stressed plants have employed the use of soil-grown (Shaviv *et al.*, 1990) or, more commonly, hydroponically-grown plants (Papadopoulos and Rendig, 1983; Feigin *et al.*, 1987; Cerdá and Martínez, 1988; Lewis *et al.*, 1989; Leidi *et al.*, 1992) under controlled conditions. An alternative approach to these methods is the utilisation of *in vitro* cell culture systems, which represent a simplification of the complex biological systems represented in whole plants (Dougall, 1980). This simplification is achieved by removing many aspects of organisation of whole plants and thereby facilitating investigations of plant growth and metabolism. The potentialities and not the actual performance of a specific cell or cell type located in the plant may be investigated with the conclusion that the potentialities demonstrated are those present in the whole plant (Dougall, 1980). However, when the integrated functioning of a whole plant is an essential component of its response to a particular situation, *in vitro* cell culture systems present limitations to physiological studies as processes which occur at the cellular level may not always correspond to those at the whole plant level. *In vitro* shoot and plantlet cultures, however, represent a level of organisation that is equated with that of the whole plant, while still retaining the advantages of an *in*

vitro system such as the stringent control of environmental conditions. In addition, processes such as the emergence of roots may be monitored using shoot cultures, which may not be accomplished in soil or hydroponic culture. This study was designed thus to utilise an *in vitro* shoot culture system of *Nicotiana tabacum* L. var. Samsun to investigate aspects of nitrate nutrition on growth and metabolism of salt stressed plants. It was anticipated that the use of an *in vitro* system would facilitate investigations of mechanisms operating at the physiological and molecular level in *Nicotiana tabacum*. Furthermore, it was of interest whether the responses observed with the *in vitro* system correlated to those reported for hydroponic culture.

The choice of *N. tabacum* (tobacco) as the plant of study was two-fold: firstly, tobacco is a glycophyte, ie. it is sensitive to the presence of salt in the external environment; secondly, tobacco has been used traditionally as a model system for the study of many physiological processes using both soil/hydroponic culture and *in vitro* culture systems. The most common nutrient medium employed today by researchers utilising *in vitro* culture systems (the MS medium) was originally formulated by Murashige and Skoog (1962) specifically for their studies on the growth of tobacco in culture. Subsequent to this, much work on the behaviour of plants in culture has used tobacco, with the result that the conditions required for its growth in culture are very well established (Mantell *et al.*, 1985).

With the knowledge that the deleterious effects of salt on plant growth may be alleviated by the addition of nitrate, the objectives of this study were to address the following questions:

(a) how does the presence of salt (NaCl) in the nutrient medium affect the growth of *Nicotiana tabacum in vitro*?

(b) what are the effects of salt on some of the processes involved in the assimilation of nitrate?

(c) if salt does alter nitrogen assimilation *in vitro*, are these effects located at the physiological level, molecular level, or both?

(d) what is the contribution of osmotic or ionic effects of salt to the above observations? and

(e) how does the level of nitrate in the growth medium affect (a) through (d)?

2. REVIEW OF LITERATURE

2.1 NITRATE ASSIMILATION IN HIGHER PLANTS

The acquisition and assimilation of nitrate is fundamental to higher plant survival. On a global scale, plants obtain approximately 100-fold more nitrogen through nitrate assimilation than from biological assimilation of gaseous nitrogen (Guerrero *et al.*, 1981). As a result, nitrate assimilation is estimated to produce more than 2 x 10^4 megatons of organic nitrogen per year (Guerrero *et al.*, 1981; Paul and Clark, 1988). Thus it is not surprising that the processes involved in the assimilation of nitrate have been subject to intensive research aimed at understanding the mechanisms involved and the regulation thereof. Nitrate assimilation essentially involves the flux, transport and reduction of nitrate to ammonia (Beevers and Hageman, 1969; Hewitt, 1975; Guerrero *et al.*, 1981; Jackson *et al.*, 1986a; Warner and Kleinhofs, 1992). Although the mode of nitrate assimilation is effectively a simple chemical process it is under very complex control in biological systems. The following discussion is a brief description of some of the available evidence regarding the uptake and reduction of nitrate and the regulatory processes involved. The complexity of the processes involved in nitrate assimilation will become more apparent later in this review as interactions with salt stress are discussed.

2.1.1 Nitrate uptake

Nitrate uptake across the plasmalemma and into the cytoplasm of root cells is known to be an active process requiring metabolic energy for the transport of nitrate ions against an electrochemical gradient (Higinbotham *et al.*, 1967; Jackson *et al.*, 1986a; Glass, 1988; Siddiqi *et al.*, 1989; Siddiqi *et al.*, 1990). Absorption of nitrate by plant roots has been shown to be inhibited by anaerobic conditions (Clarkson and Warner, 1979), low temperature (Glass, 1988), and metabolic inhibitors (Rao and Rains, 1976), suggesting the involvement of a nitrate-specific carrier protein located in the plasma membrane. The transport of nitrate into roots is induced upon exposure of the roots to nitrate (Jackson *et al.*, 1986a). Low concentrations of nitrate (5-10 μM) in the external environment are sufficient for the induction of nitrate transport, but the continued availability thereof is required to maintain uptake rates (Jackson *et al.*, 1973; Deanne-Drummond, 1982; Jackson *et al.*, 1986a).

The induction and maintenance of the nitrate transporter also appears to be dependent upon the concentration of nitrate in the cytoplasm, and not the total content of the root (Siddiqi *et al.*, 1989).

Due to the induction of nitrate transport by nitrate (Minotti *et al.*, 1968; Jackson, 1978; Bretelar and Luczak, 1982; Jackson *et al.*, 1986a) and the fact that this induction is prevented by inhibitors of RNA and protein synthesis (Jackson *et al.*, 1973), it appears highly likely that a membrane bound nitrate-specific carrier protein exists. Omata and coworkers in studies of the alga *Synechococcus*, have identified, characterised, and cloned a 45 kilodalton (kD) cell membrane polypeptide involved in nitrate transport (Omata *et al.*, 1986, 1989; Omata, 1991). However, limited success has been obtained in attempts to isolate membrane associated nitrate carrier proteins from higher plants. There are several reports which have identified certain membrane-bound polypeptide species induced by the presence of nitrate, but none has provided unequivocal proof that these proteins are involved with nitrate transport. For example, McClure *et al.* (1987) identified a nitrate inducible 31 kD polypeptide in tonoplast and endoplasmic reticulum membrane vesicles isolated from maize roots, but could not identify such polypeptides in other membrane fractions. Similarly, Dhugga *et al.* (1988a) isolated polypeptides of 165, 95, 70 and 40 kD from the plasma membrane, and a 47 kD polypeptide from the microsomal fractions of maize roots, but could not conclude that they were involved with nitrate transport. Warner and Kleinhofs (1992) have suggested that more sensitive techniques may be required for the detection of nitrate carrier proteins in plant membranes.

Determination of the mechanisms involved in controlling nitrate uptake is complicated by a number of factors. After nitrate is absorbed by the roots, varying proportions of it may be reduced in the roots, translocated through the xylem to the leaves and reduced there, or accumulated in the roots (Morgan *et al.*, 1985; Jackson *et al.*, 1986a). When nitrate is accumulated, it is usually found to be compartmentalised predominantly in the vacuoles of both leaves and roots (Jackson *et al.*, 1986a) a factor which may often complicate accurate assessments of uptake. Efflux of nitrate ions out of the vacuole, into the cytoplasm, and back across the plasmalemma have also been shown to occur concurrently with nitrate influx (Jackson *et al.*, 1986a; Lee and Clarkson, 1986). Furthermore, high root nitrate concentrations have been demonstrated to have negative feedback effects on nitrate influx (Siddiqi *et al.*, 1989). The complexity of nitrate transport is increased

additionally by the possibility that nitrate may also enter roots by passive diffusion through nitrate channels in the membrane (Glass *et al.*, 1990; Siddiqi *et al.*, 1990). Although the contribution of passive influx of nitrate to total nitrate uptake is not known, active transport systems appear to be responsible for the majority of nitrate uptake into roots (Glass *et al.*, 1990).

2.1.2 Nitrate reduction

Once in the plant, nitrate is reduced to nitrite via a two-electron step by the enzyme nitrate reductase (NR) (EC 1.6.6.1) (Beevers and Hageman, 1969; Hewitt, 1975; Guerrero *et al.*, 1981; Wray, 1986; Solomonson and Barber, 1990). In eukaryotes, NAD(P)H serves as the electron donor for this reaction (Solomonson and Barber, 1990; Warner and Kleinhofs, 1992). Nitrite is then rapidly converted to ammonia by a six-electron reduction step catalysed by nitrite reductase (NiR) (EC. 1.7.7.1) (Beevers and Hageman, 1969; Hewitt, 1975; Guerrero *et al.*, 1981; Wray, 1986; Solomonson and Barber, 1990). In this case, reduced ferrodoxin serves as the electron donor (Solomonson and Barber, 1990; Warner and Kleinhofs, 1992). The incorporation of ammonia into organic compounds is generally recognised to be mediated by two enzyme reactions. Glutamine synthetase (GS) converts ammonia to glutamine, followed by the conversion of glutamine to glutamate by glutamate synthase (GOGAT) (Miflin and Lea, 1982). Although nitrate assimilation subsequent to its uptake involves all of the above processes, only information pertaining to NR will be discussed further in this review.

Nitrate reductase is a complex enzyme containing two identical subunits, three prosthetic groups, and several partial activities (Solomonson and McCreery, 1986; Solomonson and Barber, 1990; Warner and Kleinhofs, 1992). Although the localisation of the NR enzyme within the cell has not yet been firmly established, evidence suggests that it is located in the cytoplasm (Solomonson and Barber, 1990). The prosthetic groups making up the enzyme molecule are flavin adenine dinucleotide (FAD), cytochrome b_{557} and molybdenum, each in an approximately 1:1:1 ratio per subunit (Redinbaugh and Campbell, 1985; Solomonson and McCreery, 1986; Solomonson and Barber, 1990). In addition to the NAD(P)H-dependent reduction of nitrate, NR enzyme also catalyses a variety of partial activities that use alternate electron donors and acceptors (Guerrero *et al.*, 1981; Solomonson and Barber, 1990; Warner and Kleinhofs, 1992). The diaphorase activity uses the electron acceptors cytochrome *c* and ferricyanide, while the nitrate-

reducing activities utilise electron donors such as reduced flavins and viologens to catalyse the reduction of nitrate (Guerrero *et al.*, 1981; Solomonson and Barber, 1990; Warner and Kleinhofs, 1992).

The regulation of nitrate reduction has been the focus of intense research. Nitrate reduction by NR generally occurs in the leaves (Jackson *et al.*, 1986a) but significant reduction in the roots has been found in species such as cotton (Radin, 1977), bean (Bretelar *et al.*, 1979) and rice (Chang and Jung, 1981). Evidence has been presented for the modulation of NR by light, oxygen, carbon dioxide and other metabolites (Beevers and Hageman, 1969). However, the primary environmental signal regulating nitrate assimilation is the presence of nitrate (Beevers and Hageman, 1969; Shaner and Boyer, 1976a; Solomonson and Barber, 1990). Like nitrate uptake, the activation of NR has been found to be substrate-inducible (Beevers and Hageman, 1969; Jackson *et al.*, 1973; Ward *et al.*, 1986; Kleinhofs and Warner, 1990). The availability of a source of energy, nitrogen, phosphorus and sulfur has been shown also to be necessary for the induction of NR by nitrate (Zink, 1981). Furthermore, the nitrate flux to the leaves from the roots has been shown also to be important in controlling the activation of NR activity (Shaner and Boyer, 1976a).

2.1.3 Molecular aspects of nitrate assimilation

Although much is known about the physiological aspects of nitrate assimilation, little information is available about the molecular aspects in higher plants, especially those concerning the nitrate transport proteins. As discussed in Section 2.1.1, the gene encoding a 45 kD cell membrane polypeptide involved in nitrate transport has been characterised in *Synechococcus*, but investigations with higher plants have succeeded only in the identification of membrane bound polypeptides that appear to be induced by the presence of nitrate. It has been demonstrated by several workers that the induction of NR activity by nitrate is due to *de novo* synthesis of the enzyme (Zielke and Filner, 1971; Somers *et al.*, 1983; Gupta and Beevers, 1984; Small and Gray, 1984). Furthermore, the development of cDNA probes for detecting NR mRNA have shown that the induction of NR by nitrate is at the transcriptional level in tobacco (*Nicotiana tabacum*) (Calza *et al.*, 1987; Galangau *et al.*, 1988a), *Arabidopsis* (Crawford *et al.*, 1988), and barley (*Hordeum vulgare*) (Melzer *et al.*, 1989). The appearance of the NR enzyme also correlated with the appearance of NR mRNA in mustard (*Sinapis alba*) (Mohr *et al.*, 1992).

The addition of nitrate resulted in a rapid increase in NR mRNA levels in N-starved barley seedlings (Melzer *et al.*, 1989). These workers showed that after a period of 12 hours of exposure to nitrate, the NR mRNA levels in the leaves declined and stabilised to 50% of the peak levels. The NR activity and nitrate levels of the barley seedlings investigated by Melzer continued to increase, however, while the NR mRNA declined, indicating that regulation of NR was not entirely due to NR mRNA production or nitrate concentration. Tobacco also showed an increase in NR mRNA levels on exposure to nitrate but the response in this case was slower due to prior growth of the plants on nitrate (Galangau *et al.*, 1988a). As it has been suggested by Warner and Kleinhofs (1992), more research is required to determine if transcription is the primary mechanism controlling NR activity in higher plants.

2.2 THE EFFECTS OF SALT ON HIGHER PLANTS

2.2.1 General aspects of salt stress injury in higher plants

Plants exhibit a wide range in sensitivity to the presence of salt in the external medium. Table 2.1 summarises some of the limits of salt stress for growth of selected glycophyte and halophyte plant species.

Table 2.1. Limits of salt stress for growth in some glycophytes and halophytes (Based on Levitt, 1980)

PLANT	SALT CONCENTRATION (mM)	OSMOTIC PRESSURE (MPa)
Glycophytes		
Citrus spp.	50	-0.252
Gossypium hirsutim (cotton)	68	-0.336
Hordeum vulgare (barley)	240	-1.186
Phaseolus vulgaris (bean)	100-200	-0.494 to -0.988
Lycospersicon esculentum (tomato)	63	-0.311

Zea mays (maize)	74	-0.366
Triticum aestivum (wheat)	60-120	-0.296 to -0.593
Nicotiana tabacum (tobacco)	30-60	-0.148 to -0.296
Solanum tuberosum (potato)	34	-0.168
Halophytes		
Distichlis spicata	>260	<-1.284
Atriplex nummularia	>400	<-1.976
Suada depressa	685	-3.385
Atriplex halimus	283	-1.4
Glaux maritima	300	-1.482

Examination of Table 2.1 reveals that growth of very salt sensitive non-halophytic plants such as fruit trees is inhibited by NaCl concentrations less than 100 mM, while less sensitive nonhalophytes such as tomato (*Lycospersicon esculentum*), bean (*Phaseolus vulgaris*), cotton (*Gossypium hirsutum*), and barley (*Hordeum vulgare*) may survive salinity levels up to 250 mM NaCl (Greenway and Munns, 1980). In contrast, salt tolerant (halophytic) plant species such as members of the genera *Suaeda* and *Atriplex* may survive anywhere in the range of 0 mM to 700 mM NaCl before exhibiting signs of salt injury (Greenway and Munns, 1980). Of particular interest to this review is the limits of tolerance of tobacco (*Nicotiana tabacum*) to saline conditions. Much of the research on tobacco under conditions of salt stress has focused on the production of salt tolerant plants and has involved thus the exposure of these plants to abnormally high concentrations of salt (Nabors *et al.*, 1980; Binzel *et al.*, 1987, 1988). As tobacco belongs to the family Solanaceae of which tomato and potato (*Solanum tuberosum*) are members, it would be expected that tobacco would share the same sensitivity to a salt stress. Using this premise, the limit of tobacco to salt might be predicted to be in the region of 30-60 mM NaCl and hence classifiable as salt sensitive.

The effect exerted by a salt dissolved in soil water on higher plant growth and metabolism is related directly to the chemical behaviour of salts in an aqueous solution. According to the laws of osmosis (as described in Morris, 1974), when a

concentrated solution of a salt dissolved in water is separated from a dilute solution by a semi-permeable membrane, the solvent molecules move across the membrane to equalise the two concentrations. Such is the situation in nature where the presence of high concentrations of salt dissolved in the soil and xylem water results in the movement of water out of plant cells where the salt concentration is more dilute. The removal of water from the cells results in a loss of cell turgor which may cause the plant to dehydrate (Oertli, 1966, 1968; Greenway and Munns, 1980). An ideal semi-permeable membrane is permeable to water only, but in nature most of the membranes such as the plasmalemma of plant cells are selectively permeable and thus also allow the movement of certain solutes both into and out of the cell (Flowers and Yeo, 1986). Salts are electrolytic compounds which means that, when dissolved in an aqueous solution, they dissociate into their component positive and negative ions (Whitten and Gailey, 1984). In contrast, many solutes present in living cells are non-electrolytes and thus stay intact as individual molecules when in solution (Morris, 1974). The movement of salt ions into a cell will disrupt thus the ionic balance of the cell. Such a disruption will have deleterious effects on plant metabolic processes which often lead to injury and a reduction in growth.

The most common salt responsible for causing injury to plants is NaCl and thus any further reference to salt in this review will be confined solely to NaCl, unless otherwise stated. If a plant is to survive a salt stress, it must employ a variety of mechanisms to cope with the ionic and osmotic effects caused by the salt. Plants may avoid or minimize toxic effects by excluding salt from the plant, excreting it from glands, or translocating it to leaves that then drop off the plant (Greenway and Munns, 1980; Levitt, 1980). Alternatively, plants may accumulate NaCl in the leaves, thereby balancing the osmotic potential, but are then required to exclude salts from the cytoplasm to avoid ionic interactions with metabolic processes (Levitt, 1980; Flowers and Yeo, 1986; Binzel *et al.*, 1987). The ability of halophytic plant species to tolerate high concentrations of salt is reported to be due to their efficiency in synchronising ion compartmentation within leaf cells with a high rate of ion transport into the leaves (Greenway and Munns, 1980; Flowers and Yeo, 1986). In the halophyte *Suaeda maritima*, ion accumulation into leaf cells was shown to potentially exceed the supply from the roots and there was little net increase in the salt load in the apoplastic compartment with time of exposure to salt (Flowers and Yeo, 1986). In addition, these authors showed that any excess in salt supply was excreted via salt glands in the leaves. Glycophytes are unable, however, to manage the influx of salt in such an efficient way with the result that NaCl entry exceeds

accumulation into leaf cells and salt damage to the tissues occurs (Greenway and Munns, 1980; Flowers and Yeo, 1986, Iraki *et al.*, 1989).

2.2.1.1 The distinction between a secondary and a primary salt-induced stress effect

Bernstein (1961) proposed that salt has both ionic and osmotic effects on plants and most of the known responses to salinity are closely linked to these effects. In terms of stress terminology, osmotic dehydration and nutrient deficiencies resulting from salt exposure are classified as secondary salt effects, while specific toxic (ionic) effects of salt may cause a primary injury to plants (Levitt, 1980). Primary salt injury usually acts directly on the external plasma membrane of plant cells, or after the salt ions have penetrated the membrane and entered the cells (Levitt, 1980). As both osmotic and ionic effects are responsible for the reduction in growth of salt stressed plants, and are usually both operative simultaneously throughout the duration of the stress, it is not always possible to determine the major cause of a salt injury. In addition, a wide variation in sensitivity to either effect exists between different plant species (Table 2.1). Thus, a clear distinction between the various described effects is not always possible and often leads to confusion between salt toxicity and osmotic effects in analyses of tissue lesions caused by salinity (Levitt, 1980).

The following two sections of this review discuss the recognised aspects of salt-induced injury to salt sensitive plants (Levitt, 1980; Abdul-Kadir, 1982; Termaat and Munns, 1986; Cramer *et al.*, 1987; Wilson *et al.*, 1992). In later sections, the effects of NaCl on specific aspects of plant nitrogen metabolism will be broached.

2.2.2 Secondary salt-induced stress effects

2.2.2.1 Osmotic stress

The nature of the osmotic stress

A direct and inseparable relationship exists between a salt and a water stress. The addition of a salt to soil lowers the soil osmotic potential which interferes with a plant's ability to extract water from the soil (Levitt, 1980; Shannon, 1984). A salt stress thus exposes the plant to an osmotic (water deficit) stress which invokes dehydration and turgor losses such as are known to occur under conditions of

drought (Levitt, 1980; Termaat and Munns, 1986). Plants exposed to a low external osmotic potential are required to reduce the internal osmotic potential to levels lower than that of the external medium if they are to obtain water and thus survive the stress. It is well documented that plants counteract osmotic imbalances caused by salt by accumulating organic or "compatible" solutes such as proline and betaine in the cytoplasm of leaves, and by taking up and compartmentalizing Na^+ and Cl^- ions in the vacuoles (Greenway and Munns, 1980; Binzel *et al.*, 1985, 1987). The rapid rates of ion uptake that often occur in plants grown at high salinity can, however, result in the buildup of high ion concentrations in the cell walls of leaves, which would cause adverse effects to the water relations of individual leaf cells (Greenway and Munns, 1980). The loss of turgor in root and leaf cells as a result of a salt-induced osmotic stress contributes to the reduction in plant growth observed in salt stressed plants (Greenway and Munns, 1980; Flowers and Yeo, 1986; Termaat and Munns, 1986).

The plant response

Wheat (*Triticum aestivum*) seedlings treated with polyethylene glycol (PEG) (a non-ionic osmoticum commonly used to simulate water stress effects in plants) showed visual wilting within a few hours of exposure to 20% PEG followed by a decline in tissue water content over a period of six days (Larsson *et al.*, 1989). Dehydration of the tissues led to a drastic decline in the relative growth rate of the plants to about half that recorded in untreated plants. Seedlings of sour orange (*Citrus aurantium*) exposed up to 48 mM NaCl and 20 mM PEG were found also to have smaller shoot and root dry weights, fewer leaves, fewer roots and were shorter than control plants grown in the absence of a salt-induced water stress (Zekri and Parsons, 1990).

A reduction in water availability to osmotically stressed plants has been shown to influence shoot growth more than root growth, resulting in reduced shoot:root ratios in barley (*Hordeum vulgare*) and Egyptian clover (*Trifolium alexandrinum*) (Munns and Termaat, 1986), wheat (Munns and Termaat, 1986; Larsson *et al.*, 1989), sunflower (*Helianthus annuus*) (Robertson *et al.*, 1990) and several other plant species (Sharp and Davies, 1989). It has been suggested by Larsson (1992) that such a response may be due to changes in allocation of resources such as nitrogen within a plant. After nitrate-N is taken up by the roots, several translocation steps are involved before it is incorporated into the tissues (Section 2.1). Any one of these

transport steps may be affected by an osmotic stress, leading to changes in dry matter distribution within the plant. Luque and Bingham (1981) demonstrated that a decrease in the osmotic potential from -0.6 MPa to -5.4 MPa of a nutrient solution supplied to barley (*Hordeum vulgare*) seedlings caused a decrease in the nitrate concentrations of roots independent of the salt species used. Wheat seedlings treated with 20% PEG showed an inhibition of specific absorption rates of nitrate-N by the roots and an increase in xylem and shoot nitrate concentrations (Larsson *et al.*, 1989). This was correlated with a decline in the activity of NR in both roots and shoots. As a consequence of this decline, nitrate ions accumulated instead of being reduced and incorporated into protein compounds (Larsson *et al.*, 1989). Further studies of osmotically stressed wheat indicated, however, that the strong inhibition of nitrate uptake and transport from root to shoot is correlated also with a deterioration in the plant's water status (Larsson, 1992).

2.2.2.2 Nutrient deficiency stress

The effect of high NaCl concentrations in the external medium on the uptake and internal concentrations of certain macronutrients has been studied extensively (Delane *et al.*, 1982; Lynch and Läuchli, 1984; Aslam *et al.*, 1984; Cramer *et al.*, 1987; Cramer *et al.*, 1991). It has been shown that uptake of, amongst others, potassium, calcium, magnesium (Termaat and Munns, 1986; Cramer *et al.*, 1991; Jeschke *et al.*, 1992) and nitrate (Aslam *et al.*, 1984) is suppressed in the presence of high concentrations of NaCl and often leads to a deficiency of these nutrients in salt-stressed plants. Typically, such disturbances in the nutritional balance are manifested as a reduction in plant growth.

Most workers agree that competition between NaCl and nutrient ions for uptake is responsible for the observed suppression of nutrient absorption by salinized plants. It was suggested by Wallace and Berry (1981) that one of the contributions to the growth reduction observed in salt stressed plants was a nitrate deficiency induced by high external chloride concentrations. Direct competition between nitrate and chloride ions for uptake has been reported widely for a number of different plant species such as Mexican wheat (Torres and Bingham, 1973), tomato (*Lycospersicon esculentum*) (Kafkafi *et al.*, 1982), melon (*Cucumis melo*) (Feigin *et al.*, 1984) and wheat (*Triticum durum*) (Shaviv *et al*, 1990) and is thus supportive of Wallace and Berry's observations. Similarly, potassium and calcium deficiencies in pumpkin (*Cucurbita pepo*) and clover (*Melilotus alba*) have been shown to be due to competition for uptake with sodium ions (Solov'ev, 1969b; Stassart *et al.*, 1981).

Reduced uptake of a nutrient will occur also as a result of a salt induced decrease in growth (Patel *et al.*, 1975). Diminished root growth due to osmotic effects of salt leads to a decrease in the capacity of roots to obtain nutrients from the external medium (Fogle and Munns, 1973). Thus, a nutrient deficiency may result from both a reduction in uptake of essential nutrients and an osmotically produced decrease in root growth.

2.2.3 Primary salt-induced stress effects

2.2.3.1 Direct effects

Direct toxic effects of salt are the most vague and least readily proven of all the recognised components of salt stress (Leopold and Willing, 1984). Primary direct salt injury is thought to be due to a specific toxic effect of salt ions on cell membranes (Levitt, 1980; Leopold and Willing, 1984; Kurth *et al.*, 1986). Swelling and distortion of the plasmalemma of salt-stressed plants has been observed and is usually found to be associated with the loss of selective permeability of the membrane (Leopold and Willing, 1984; Kurth *et al.*, 1986). Such permeability changes imply injury to the lipid and/or protein component of the membranes (Levitt, 1980) which may lead to alterations in the structural and functional integrity of membranes.

Evidence for a specific toxic effect of salt has been demonstrated by Marschner and Mix (1973) in studies of K^+ efflux from bean, barley and sugar beet leaf and root segments. Exposure of segments to 50-100 mM NaCl resulted in a strong efflux of K^+ from bean and barley, a phenomenon which was not observed in sugar beet, a salt tolerant plant. In addition, exposure of barley roots to 200 mM mannitol had no effect on K^+ retention suggesting that the observed ion efflux was not an osmotic effect but was due specifically to the presence of NaCl ions. Leopold and Willing (1984) noticed similar patterns in soybean leaf discs exposed to 200 mM NaCl. Significantly higher solute leakage occurred than from discs exposed to isosmotic concentrations of sorbitol. These workers concluded that the susceptibility of the membranes to damage was specifically exacerbated by salt and not solely due to osmotic effects. There have been indications of a reduction in the amount of Ca^{2+} bound to plasma membranes of salt stressed roots of cotton (Cramer *et al.*, 1985) and protoplasts of maize (*Zea mays*) (Lynch *et al.*, 1987) and wheat (Bittisnich,

1991). Sodium ions in particular appear to be responsible for the displacement of Ca^{2+} ions from the plasma membrane, which instantly changes the physiology and functioning of the membrane (Läuchli, 1990). A direct effect of Na^{+} on the plasma membrane is thus proposed to be accountable for the disturbance in membrane Ca^{2+} transport systems under saline conditions (Rengel, 1992).

2.2.3.2 Indirect effects

Penetration of salt ions through the plasmalemma into the protoplast of cells may lead to several metabolic disturbances. Such disturbances typically curb plant growth but do not injure the plant irreversibly (Greenway and Munns, 1980; Abdul-Kadir and Paulsen, 1982; Taleisnik, 1987; Pessarakli *et al.*, 1989; Wilson *et al.*, 1992). Any one of the disorders in cellular metabolism caused by the presence of salt ions may be expected to inhibit growth or be involved in cell injury (Levitt, 1980). Some, or all of them, may also be interrelated. A number of metabolic processes affected by salt have been identified and are described below.

Nucleic acid metabolism

Disturbances in nucleic acid metabolism by salt have been described. Tsenov *et al.* (1973) and Kabanov (1973) showed that the total DNA and RNA content of tomato and pea leaves was unaffected by low levels of salinity (0.2% - 0.4%) but was decreased at higher, injurious levels (1.6%). In some cases, total DNA has been found to be decreased while RNA has been unaltered by salinity (Tal, 1977). The structure of the chromatin material itself has been shown to be very sensitive to changes in the ionic environment (Riehm and Harrington, 1987, 1988). Certain conformational transitions which appear to be due to loosening or unfolding of the chromatin structure, such as occurs during cell division, have been observed to be dependent on the intracellular ionic strength (Riehm and Harrington, 1988). Changes in the intracellular salt concentration such as occur during salinity stress may be expected thus to influence the balance of electrostatic and hydrophobic interactions within the chromatin material (Waterborg *et al.*, 1989). There is evidence that the acetylation of histone proteins, to which DNA is bound, is associated with chromatin activation for gene transcription in eukaryotes (Matthews and Waterborg, 1985; Sterner *et al.*, 1987). Waterborg *et al.* (1989) demonstrated that exposure of salt sensitive alfalfa (*Medicago sativa*) callus to 1% NaCl led to major increases in the acetylation of several histone variants. Waterborg and his

group proposed that the observed increase in acetylation in response to a salt stress may serve as an *in vivo* reporter for alterations in the intranuclear ionic environment and hence, possible changes in transcriptional activity at the chromatin level.

Salt-induced changes in gene expression

An approach taken by many researchers in attempts to elucidate molecular changes induced by salt is to identify modifications in the levels of individual proteins in salt-stressed plant tissues. The assumption inherent in such investigations is that alterations in the pattern of protein synthesis is a result of altered gene expression. Singh *et al.* (1985) reported that SDS polyacrylamide gel electrophoresis performed on salt-adapted tobacco cells revealed an increase in eight protein bands and a decrease in four. These workers noticed that a 26 kD protein increased to the greatest degree and constituted about 10% of the total cellular protein. Ramagopal (1987) demonstrated the induction of eleven new proteins in the roots and shoots of barley seedlings supplied with 350 mM NaCl. These proteins were all in the low molecular weight range: 20 to 24 kD for shoots, 24 to 27 kD for roots, and were unique to each tissue type. He suggested that the induction of these proteins was regulated during the imposed stress by both transcriptional and posttranscriptional mechanisms. Newly synthesised proteins detected during salt shock of salt-adapted tobacco cells were shown by Singh *et al.* (1985) to be unrelated to those associated with adaptation to salt. This finding suggests that there may be differences in expression of genes induced by shock and genes where expression is altered only after adaptation to the stress. In this respect, investigations of barley roots exposed to 200 mM NaCl showed that the significant increase observed in 26 and 27 kD protein levels was probably representative of a salt shock response in protein synthesis rather than the production of proteins which may allow barley to survive better in the presence of salt (Hurkman and Tanaka, 1987).

Much of the evidence for the induction of mRNA and control of gene expression in plants under saline conditions has been generated by investigations of halophytic plant species or salt tolerant phenotypes derived and selected from salt sensitive species. Suspension cells of the halophytic grass species *Distichlis spicata* (salt grass) have been shown to accumulate substantial quantities of the organic solute proline, 8-12 hours after exposure to a salt stress (Rodriguez and Heyser, 1988). As this increase was found to be sensitive to inhibitors of transcription it was suggested by

Rodriguez and Heyser that during the time period before proline was detected, transcription of genes encoding enzymes of the proline biosynthesis pathway would occur. Transient changes in the amounts of translated polypeptides were detected over 24 hours of stress and were associated with increases and decreases in mRNA (Zhao *et al.*, 1989). This implied that salt grass exhibited a controlled response to salt stress at the level of protein expression by fluctuations in relative mRNA amounts. Studies of the facultative halophyte *Mesembryanthemum crystallinum* (common ice plant) have shown that a shift in carbon metabolism from C_3 photosynthesis to Crassulacean acid metabolism (CAM) occurs when the plant is salt stressed (Winter and von Willert, 1972). Associated with this change is an increase in the activity of phosphoenolpyruvate carboxylase (PEPCase), an enzyme which assimilates carbon dioxide via the CAM pathway. Ostrem *et al.* (1987) demonstrated that the increase in the level of PEPCase in response to a salt stress occurred via a stress-induced increase in the steady-state level of translatable mRNA for the enzyme. Furthermore, the increase in transcripts were shown to occur in the first 5 days following imposition of the stress, while CAM was only fully established after 10 days (Michalowski *et al.*, 1989). Therefore, genetic adaptation to the imposed stress occurred before physiological responses, implying that genetic changes were vital to the ability of the plant to tolerate the stress.

An enhancement in gene expression at the mRNA level in salt tolerant *Medicago sativa* (alfalfa) cells, derived from a salt sensitive line, has been described by Winicov *et al.* (1989). They showed that the mRNA composition differed between HG2-N1 (salt tolerant) and HG2 (salt sensitive) cells and selectively identified an inducible mRNA species which appeared only in the salt tolerant line when both cell types were grown in salt. In addition, the increases and decreases in specific polypeptides exhibited by HG2-N1 were different to those of HG2 following 24 hours of salt stress. Hence, Winicov and coworkers suggested that, in some aspects, the adaptive mechanisms for salt tolerance may differ from acute stress mechanisms. Visible changes in chlorophyll levels between the two cell lines led to further investigations of the activation of genes for photosynthesis in order to evaluate their contribution to salt tolerance in the salt adapted cell line (Winicov and Seemann, 1990). Elevated chlorophyll levels in HG2-N1 cells were found to be associated with increases in the level of mRNAs coding for the large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), and occurred in conjunction with increases in enzyme activity. These salt-induced changes in mRNA and protein accumulation involved with photosynthesis appeared

to be an important contribution to the salt tolerant capabilities of the HG2-N1 cells. In addition, salt tolerant suspension cultures of sugarcane (*Saccharum* spp.) were found to exhibit characteristic alterations in specific mRNAs and proteins which were not detected on comparison with unadapted cells undergoing a short-term salt stress (Ramagopal and Carr, 1991). The synthesis of the 29 salt-altered sugarcane proteins detected appeared to be subject to a multitude of complex regulatory mechanisms which led Ramagopal and Carr to propose that gene expression in salt adapted sugarcane cells was subject to control at transcriptional, post-transcriptional and post-translational levels.

Differences in levels of translatable mRNAs and polypeptides are not always detected during comparisons of salt tolerant and salt sensitive plants. Exposure of a salt sensitive and a salt tolerant cultivar of barley (*Hordeum vulgare*) to 200 mM NaCl for 6 days caused only very small changes in the number of polypeptides and translatable mRNAs, and did not induce or repress the synthesis of any unique gene products (Hurkman *et al.*, 1989). As a consequence, specific polypeptides and translatable mRNAs related to salt tolerance in barley could not be identified.

Salt stress may be recognised as having specific effects on gene expression by inducing the production or repression of mRNAs that code for unique polypeptide species (Singh *et al.*, 1985; Hurkman and Tanaka, 1987; Ramagopal, 1987; Winicov *et al.*, 1989). The mechanisms by which gene expression is altered by salt is likely to be complex, however, and influenced by a number of other factors, both physiological and environmental. Such mechanisms are still to be elucidated.

Protein metabolism

Changes in protein metabolism in response to salinity have been well documented. It is generally recognised that protein synthesis is adversely affected by salt stress (Helal and Mengel, 1979; Abdul-Kadir and Paulsen, 1982; Pessarakli and Tucker, 1985b, Pessarakli *et al.*, 1989) but it is not well established whether the reduction in synthesis is due to an increase in protein degradation (Mothes, 1956) or a disturbance in the incorporation of amino acids into protein (Kahane and Poljakoff-Mayber, 1968). In contrast, a stimulation of protein synthesis by salt has been detected in some instances. For example, the incorporation of labelled N into the protein fraction of young barley plants supplied with $^{15}NH_4{}^{15}NO_3$ was improved in the presence of 60 and 120 mM NaCl, despite a reduction in growth and uptake of

labelled N by the plants (Helal *et al.*, 1975). Furthermore, salt stress has been shown to modify the pattern of protein synthesis in cells by elevating the synthesis of certain unique proteins while depressing others. For example, maize callus (Ramagopal, 1986) and barley roots (Hurkman and Tanaka, 1987) exposed to NaCl levels up to 300 mM and 200 mM respectively, exhibited both increases and decreases in the synthesis of a number of specific low molecular weight polypeptides. Similarly, a salinity concentration of 250 mM inhibited the synthesis of a majority of shoot proteins in barley seedlings but induced five new shoot and six new root proteins which were unique to each tissue (Ramagopal, 1987).

Enzyme activity

There have been numerous reports of both increases and decreases in enzyme activity in plants exposed to saline conditions. Levitt (1980) lists a wide selection of enzymes from a variety of plant species that have been shown to be affected by salt and stresses the complex interactions which occur between them. For example, invertase and amylase in *Pennisetum typhoides* have been shown to be to be inhibited by the same level of salinity that enhanced the activity of other enzymes of carbon metabolism such as phosphorylase, aldolase and sucrose synthetase (Huber *et al.*, 1974). Certain responses may be species dependent. For example, nitrate reductase has been found to increase in activity in bean and corn (Alina and Klyshev, 1975; Sankhla and Huber, 1975) but decrease in tomato and cucumber (Martínez and Cerdá, 1989). Alterations in protein synthesis may in many cases be responsible for observed changes in enzyme activity. Miteva *et al.* (1992), for example, have recently demonstrated that a decrease in activity of RubisCO in salt stressed barley leaves was due to the inhibition of synthesis of the RubisCO protein.

Metabolic energy supply

It has been suggested that the supply of metabolic energy may be the basic limitation to plant growth under conditions of a salinity stress (Nieman *et al.*, 1988; Wilson *et al.*, 1992). A plant growing in a saline environment is required to maintain a state of inequilibrium with its environment in order to survive and achieves it by maintaining intracellular ion concentrations that are lower than that of the external medium, and which support normal cellular functioning (Greenway and Munns, 1980). The accumulation and compartmentalisation of solutes required to balance the osmotic pressure of the cytoplasm has been shown to result in the expenditure of

energy that would otherwise be available for growth (Nieman, 1962; Gale, 1975). A variety of glycophytic plants grown in saline environments exhibit increased respiration in roots (Beevers, 1961) and shoots (Nieman, 1962; Livne and Levin, 1967; Gale, 1975) and have been reported to possess decreased ATP pools in roots (Roberts *et al.*, 1985; Peterson *et al.*, 1987) and leaves (Nieman *et al.*, 1988). In addition, it has been demonstrated recently that salt stress reduced the level of ATP in mature and not growing leaves of beans, suggesting that the observed decline in plant growth was due to a diminished ability of mature leaves to supply essential metabolites to the growing regions of the plant (Wilson *et al.*, 1992).

Several contradictory reports exist concerning the respiratory activity of salt stressed plants. A decrease in the respiration rate of many crop plants such as wheat (Sarin and Rao, 1958), cotton (Boyer, 1965) and glycophytes in general (Pokrovskaia, 1958) has been detected. In contrast, an increase in respiration of bean leaves (Nieman, 1962), peas (Livne and Levin, 1967) and a variety of other plants (Gale, 1975) has been also shown. Alterations in the metabolic pathways of respiration such as an increase in the pentose phosphate pathway (Goris, 1969) at the expense of glycolysis (Sokolova and Azimov, 1974) have been proposed as contributing factors to a decrease in respiration during a period of salt stress. On the other hand, an increased demand for energy, derived from the oxidation of carbohydrates, and required for the uptake and compartmentation of salt ions is believed to be the cause of the increase in respiration in salt stressed plants (Peterson *et al.*, 1987). This has been supported by a reduction in the ATP pool size in maize root tips (Roberts *et al.*, 1985; Peterson *et al.*, 1987) and bean leaves (Wilson *et al.*, 1992).

It has been known for some time that salinity depresses photosynthesis, the most obvious indication thereof being a decrease in chlorophyll content of photosynthetically active tissues (Greenway and Munns, 1980). Salinity levels of 100 mM applied to tomato plants caused a decrease in carbon assimilation which was related to a decrease in net photosynthesis and carbon reallocation to heterotrophic organs (Taleisnik, 1987). Pods of chickpea (*Cicer arietinum*) supplied with 100 mM NaCl were shown also to exhibit reductions in photosynthetic carbon assimilation (Murumkar and Chavan, 1986). The photosynthetic capacity of a plant is related to the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) and was demonstrated by Seemann and Sharkey (1986) to be reduced in bean plants upon exposure to a salt stress. Similarly, Taleisnik (1987) also indicated a decrease in RubisCO activity in salt stressed tomato. Seeman and Sharkey showed that the

reduction in RubisCO activity in salt stressed beans was related to a reduction in the pool size of its substrate, ribulose-1,5-bisphosphate. In contrast, Miteva *et al.* (1992) indicated that a decrease in RubisCO activity in salt stressed barley leaves was due to a pronounced inhibition of the synthesis of the RubisCO protein.

2.3 NITRATE ASSIMILATION IN SALT STRESSED PLANTS

As indicated in Section 2.2, salt is responsible for causing a wide range of osmotic and ionic effects on plant growth and metabolism. The processes of nitrogen metabolism, fundamental to higher plant growth and productivity, are known to be adversely affected by salinity and a substantial number of investigations relating to the effects of salt on nitrogen assimilation have been performed (Section 2.1). However, due to the difficulties often encountered during attempts to distinguish between osmotic and ionic effects, it is not always possible to determine which of the two effects is responsible for causing disturbances in nitrogen metabolism. The following discussion presents some of the evidence regarding the effects of salt stress on nitrate assimilation and where possible, indicates the contribution of osmotic and ionic effects.

2.3.1 Nitrate uptake

Controversial results have been reported for the effects of salinity on nitrate uptake and assimilation. No alteration in nitrate uptake was detected by Helal and Mengel (1979) in barley seedlings exposed to 80 mM salt. Luque and Bingham (1981) also detected little or no effect on nitrate uptake in barley supplied with NaCl, $CaCl_2$, and Na_2SO_4 at isosmotic potentials. Those authors related this response to a specific effect of Cl^- on NO_3^- transport which they proposed was connected via a decrease in malate by Cl^-. Recent work has disclosed that the addition of NaCl to a nutrient solution supplied to young ryegrass (*Lolium perenne*) did not significantly affect either uptake or reduction of nitrate and was proposed to be due to the ability of the plant to rapidly adjust osmotically to the applied stress (Ourry *et al.*, 1992). In contrast, several reports have confirmed an adverse effect of salinity on nitrate uptake. Helal *et al.* (1975) proposed that the impairment of nitrate uptake by salinized barley seedlings was not a specific effect but was related to the overall weakened metabolism of the plants on exposure to salt. Later investigations by Aslam *et al.* (1984) showed that the major effect of salt in barley was on the activity of the nitrate transporter. The induction of the nitrate

transporter by nitrate appeared to be affected very little but the uptake of nitrate was inhibited severely by salt. The absolute concentration of the salt ions (100 mM and 200 mM) also appeared to be more inhibitory to nitrate uptake than the osmolarity of the solution, suggesting a specific effect of the salt ions on the activity of the nitrate transporter. The lack of a distinct inhibitory effect of salt on the rest of the processes of nitrate assimilation led Aslam and coworkers to propose that the susceptibility to injury of the nitrate transporter, and not the nitrate uptake system, may be an important factor to plant survival during a salt stress. Experiments conducted to assess the recovery of nitrate transport after exposure of barley seedlings to a salinity stress provided evidence as to the possible mechanisms of salt injury to the nitrate transport system (Klobus *et al.*, 1988). It was demonstrated by Klobus and coworkers that recovery of nitrate uptake after a salt stress was prevented by inhibitors of protein, RNA and fatty acid synthesis, and an absence of nitrate. Those workers suggested that salt ions had caused damage to the putative nitrate transporter and a resynthesis thereof may be required for recovery of nitrate uptake.

2.3.2 Nitrate reductase activity

Evidence pertaining to the effect of salinity on NR activity has indicated a variety of responses in a number of plant species. Aslam *et al.* (1984) showed that the *in situ* rate of nitrate reduction in barley seedlings was marginally affected by the presence of salt. They suggested that NR activity was protected from salt injury in the cytoplasm by preferential injury to the nitrate transporter and the compartmentalization of salt ions in the vacuole. An increase in NR activity was observed by Luque and Bingham (1981) and appeared to be related to the chloride concentration of the nutrient medium. These authors suggested that chloride had a specific positive effect on nitrate transport, although these observations are questionable due to the known antagonism between chloride and nitrate for uptake (Kafkafi, 1982; Feigin *et al.*, 1987; Cerdá and Martínez, 1988; Shaviv, 1990).

A number of explanations have been offered for the observed decline in NR activity exhibited by many plants upon exposure to a salt stress. As discussed in Section 2.2.1, the presence of salt in the external medium may lead to an osmotic stress in plant tissues as well as a nitrogen deficiency due to antagonism of nitrate uptake by chloride. NR enzyme is highly sensitive to an osmotic stress (Larsson *et al.*, 1989) and also requires the presence of nitrate for induction of its activity (Section 2.1.2).

Investigations suggest that both effects are responsible for causing a reduction in NR activity in salinity stressed plants. Salt stressed wheat plants exhibiting a decrease in NR activity showed no accumulation of nitrate in shoots, evidence which strongly supported an inhibition of nitrate uptake by chloride (Abdul-Kadir and Paulsen, 1982). On the other hand, those workers demonstrated also the accumulation of proline in shoots, which is a typical response of osmotically stressed plants (Section 2.2.2). Abdul-Kadir and Paulsen (1982) concluded that the osmotic effects of salt and antagonism of nitrate uptake by chloride were responsible for causing a decline in NR activity in salt stressed wheat. Martínez and Cerdá (1989) disclosed an inhibition of nitrate uptake by chloride in leaves of tomato and cucumber (*Cucumis sativus*) plants and recognised that the resultant low concentration of nitrate in the cytoplasm could contribute to the low observed NR activity. However, Martínez and Cerdá found that the low leaf nitrate content was not always the main factor limiting the maintenance of NR activity under saline conditions. Those authors suggested that salt may inhibit the flux of nitrate into the cytoplasm from its storage pool in the vacuole, thus reducing the capacity of the plant to utilize stored nitrogen for growth processes. The reduction in NR activity was suggested to be due to specific ionic effects of salt and may have been a major contribution to the reduction in growth observed in those plants. Examination of the effects of salt on NR activity of a drought tolerant variety of sorghum allowed the separation of water stress (osmotic) effects from salt stress effects (Krishna Rao and Gnanam, 1990). Although the contribution of an osmotic effect could not be entirely ruled out, due to the drought tolerant nature of the plant and its reported ability to exclude sodium, Krishna Rao and Gnanam concluded that the observed reduction of NR activity was due primarily to a specific toxic effect of the chloride ion.

2.3.3 Protein synthesis

Due to the changes in nitrate uptake and NR activity observed in plants exposed to saline conditions, it is to be expected that protein synthesis, the final process of nitrogen assimilation, would exhibit also alterations under saline conditions. As discussed in Section 2.2.3.2, it is generally accepted that total protein synthesis is affected adversely by a salt stress (Helal and Mengel, 1979; Abdul-Kadir and Paulsen, 1982; Pessarakli and Tucker, 1985a) and frequently contributes to a decline in growth and productivity of a plant. A reduction in protein synthesis is associated usually with an accumulation of nitrate, ammonium and free amino acids in the

cytoplasm (Udovenko *et al.*, 1971; Pessarakli and Tucker, 1985a). Protein biosynthesis is subject, however, to complex regulation by other biochemical pathways in the plant, and is dependent also on a variety of environmental and physiological conditions. The reaction of protein synthesis to a salt stress may be influenced therefore by a variety of factors from which it may be mutually indistinguishable.

Some early work by Helal *et al.* (1975) demonstrated that an increase in protein synthesis occurred in barley plants on exposure to 60 mM and 120 mM NaCl, despite a reduction in growth rate. Those authors advocated that the negative influence of NaCl on plant growth was due to a detrimental effect of salt on metabolic pathways other than protein biosynthesis. In contrast, later work on barley by Helal and Mengel (1979) revealed that salinization (80 mM) impaired the incorporation of labelled ^{15}N into protein, and was associated with an accumulation of labelled inorganic nitrogen. Those workers attributed the contradiction in results to the dependence of the metabolic response to salinity on other factors such as growth rate, age of the plant and availability of nutrients. The response of protein synthesis to salinity was dependent in that study on the potassium status of the tissues as an enhancement of nitrogen uptake and incorporation into protein occurred with potassium supplementation. A comparison of protein synthesis of three cultivars of bean under salt stress conditions revealed that different plant cultivars may behave differently under salt stress conditions (Pessarakli *et al.*, 1989). Although the crude protein and total protein content of all cultivars was decreased significantly under stress conditions, a range of responses to the stress was observed between the various cultivars.

The effects of salt on the pattern of protein synthesis has been discussed previously in Section 2.2.3.2 where it was revealed that salt stress resulted in the increase in the net synthesis of some proteins and a decrease in the synthesis of others. This may or may not be concomitant with the induction of unique stress proteins. These alterations in protein production appear to be critical to the ability of a plant to tolerate conditions of salinity stress, and seem to be regulated coordinately throughout the duration of the stress (Ramagopal, 1986; Hurkman and Tanaka, 1987). Unique protein changes during germination of barley suggested that expression of root, shoot and embryonic tissue-specific proteins played an important role in growth and development of salt tolerant and salt sensitive barley genotypes (Ramagopal, 1988). During salt stress of maize callus it was observed that the initial

phase of stress (up to one week) was characterised by a strong inhibition of protein synthesis and amino acid uptake, representing a shock response to sudden exposure to the stress (Ramagopal, 1986). At a later stage, after a week, protein synthesis continued but at a declining rate and coincided with the specific expression of certain *de novo* proteins. Furthermore, Ramagopal demonstrated that the expression of those proteins also occurred when NaCl was replaced with isosmotic concentrations of mannitol and suggested that ionic and osmotic stresses induced by salt generate a common signal which controls the expression of these proteins. Ramagopal proposed that those proteins were important to the development of salt tolerance of the cells.

2.4 INTERACTIONS BETWEEN NITROGEN NUTRITION AND SALT STRESS EFFECTS

Changes in nitrogen metabolism induced by excess salt are accepted commonly as one of the most important factors responsible for abnormal plant metabolism under conditions of salt stress. It was proposed a number of years ago that increased nitrate fertilization to salt stressed plants may help to overcome the negative effects of salinity (Heinmann, 1958; Ravikovitch and Porath, 1967). In support of this it was noted later that when nitrate levels supplied to plants under saline conditions were greater than the optimum supply under non-saline conditions, the plants exhibited a greater tolerance to salt (Ravikovitch and Yoles, 1971). More recently, Lewis *et al.* (1989) demonstrated that nitrate supplementation to salt stressed maize resulted in vigorous growth of these plants under saline conditions.

Although nitrate is the predominant form of nitrogen nutrition available to plants, ammonium ions present in the external environment are also available for assimilation and be used directly for protein biosynthesis. Plant growth in mixed nitrate/ammonium nutrient solutions under controlled conditions is usually superior to that on nitrate alone (Hageman and Reed, 1969; Hageman, 1984; Pessarakli and Tucker, 1985b; Shaviv, 1990). Plants usually utilise nitrate preferentially to ammonium (Scherer and MacKown, 1987) and nitrate uptake from a mixed solution is often greater than from nitrate alone (Below and Gentry, 1987; Bock, 1987; Scherer and MacKown, 1987). Nitrate and ammonium nutrition studies have shown that the form of nitrogen supplied exerts a pronounced effect on the growth and chemical composition of a plant (Maynard and Baker, 1969; Maizlish *et al.*, 1980; Novoa and Loomis, 1981; Greef and Kullmann, 1992). The observed antagonism

between ions such as nitrate and chloride forms the basis for the hypothesis that nitrate fertilization can reduce the deleterious effects of salinity on plant growth but the factors involved in this apparent salinity-fertility relationship are not well understood.

2.4.1 The influence of the nitrogen source on growth and metabolism of salt stressed plants

2.4.1.1 Plant growth and productivity

The form of nitrogen supplied to salt stressed plants, either as nitrate (NO_3^--N) only, ammonium (NH_4^+-N) only, or as a combination, has a pronounced effect on the growth response of the plant. Peanut plants grown in hydroponic culture in the presence of 50 mM NaCl reacted favourably to the addition of KNO_3 up to 7 mM by exhibiting a significant enhancement in growth (Silberbush *et al.*, 1988). Shoot dry weight production was more stimulated by NO_3^- than root dry weight but the addition of NO_3^- lead to an increase in root length and root diameter such that they were similar to those in the absence of salt at the same NO_3^- concentration. Similar results were obtained when the plants were supplied with NH_4^+ as sole nitrogen source, even at NaCl concentrations up to 100 mM (Leidi *et al.*, 1992). Nitrate additions of up to 20 mM were shown also to have positive effects on the fruit yield of tomato and melon plants exposed to maximum salinity concentrations of 110 mM (Feigin *et al.*, 1987). Maize plants fed with 4 mM KNO_3 showed no reduction in dry mass and only a very slight reduction in fresh mass when exposed to 50 mM NaCl, although, their ammonium-fed counterparts were severely inhibited by salinity, even at levels as low as 20 mM (Lewis *et al.*, 1989). The greater sensitivity exhibited by maize to salinity when supplied with NH_4^+ as sole nitrogen source is reflected by other plant species. Ammonium-grown wheat (Lewis *et al.*, 1989) and cotton (Leidi *et al.*, 1991; Leidi *et al.*, 1992) supplied with NaCl were shown to produce less biomass than those supplied with NO_3^-. The larger growth inhibition caused by salinity in NH_4^+-fed plants has been proposed to be the result of a combination of several factors. As NH_4^+ is exclusively assimilated in the roots (Blacquière, 1987; Van Beusichem *et al.*, 1988; Lewis *et al.*, 1989), it is metabolically more expensive to roots than NO_3^- as it necessitates the diversion of large quantities of carbon to the roots to provide carbon skeletons for the products of nitrogen assimilation (Blacquière, 1987). As the energy supply for growth is limited in salt stressed plants (Section 2.2.3.2), such a diversion may result in insufficient energy and carbon

availability for root growth (Leidi *et al.*, 1992). The similarity in growth of salt stressed peanut plants supplied with either NO_3^- or NH_4^+ is thought to reflect a higher capacity of peanuts to assimilate NH_4^+ in the roots (Leidi *et al.*, 1992).

There is much evidence which indicates superior growth of plants under normal (unstressed) conditions when supplied with mixed NO_3^-/NH_4^+ nutrition (Hageman, 1984; Pessarakli and Tucker, 1985b; Bock, 1986; Olsen, 1986b). Little is known, however, about how the proportion of NO_3^-:NH_4^+ supplied to salt stressed plants affects growth and productivity. An increase in the supply of NO_3^- + NH_4^+ (1:2) to salinized tomato and cucumber plants resulted in an increase in the shoot and root dry weights although tomato was more positively affected by the mixed nutrition than cucumber, which grew better when the nitrogen source was NO_3^- alone (Cerdá and Martínez, 1988). Shoot dry weights of peanut plants supplied with NO_3^- + NH_4^+ in a 1:1 ratio not only did not change when the nitrogen concentration was increased from 2 to 6 mM, but were unaltered when the NaCl supply was increased from 0 to 50 mM (Silberbush and Lips, 1988). In this instance, the reproductive organs of the plant displayed a greater sensitivity to the salt stress than the vegetative structures. When salt stressed wheat plants were supplied with mixed NO_3^-/NH_4^+ nutrition in ratios of 3:1 and 1:1, larger grain yields were obtained than when plants were supplied with NO_3^- alone (Shaviv *et al.*, 1990). In addition, grain dry matter in plants supplied with the 1:1 nitrogen mixture was equal to, or greater than that in plants grown in non-salinized soil supplied with NO_3^- only.

2.4.1.2 Nitrogen assimilation

An alteration in the dry matter production in response to nitrogen supply must reflect changes in the process of assimilation of nitrogen into proteinaceous compounds. Limited information is available, however, regarding the effect of levels and forms of nitrogen on patterns of nitrogen uptake, NR activity and protein production in the presence of salt. When peanut plants grown in the presence of 50 mM NaCl were supplied with increasing KNO_3 levels up to 9 mM, an increase in the shoot and root nitrogen concentrations were detected (Silberbush *et al.*, 1988). A stimulation of NR activity by increased NO_3^- supply was also detected in the same study. Leidi *et al.* (1992) showed, however, that an increase in the NaCl supply to cotton and peanut plants caused little change to the concentration of nitrogen in all plant parts, irrespective of the nitrogen source. When the NO_3^- supply was increased to tomato plants, an associated increase in NO_3^- uptake was found to be

dependent on the level of salinity supplied to the plants (Papadopolous and Rendig, 1983). At low salinity levels (10 mM) a significant increase in NO_3^- uptake was observed with an increase in NO_3^- supply up to 1.2 mM but at salinity levels of 50 mM and 95 mM, NO_3^- uptake was inhibited significantly regardless of NO_3^- supply. Papadopoulos and Rendig observed that the NO_3^- and salinity supply had the same effect on leaf nitrogen concentrations as obtained for NO_3^- uptake.

Martínez and Cerdá (1989) compared the effect of NaCl and nitrogen source on NR activity of tomato and cucumber leaves. These workers showed that at high levels (15 mM) of nitrogen, supplied as NO_3^- only or NO_3^- + NH_4^+ (1:2), NR activity decreased when the NaCl concentration was increased, an effect which was not observed at low nitrogen concentrations. Thus, an increase in the nitrogen concentration, regardless of form, led to an increase in inhibition of NR activity by salinity. It was noted also during the above investigations that when plants were exclusively supplied with NO_3^-, the enzyme activity was 5-6 times greater than when they were supplied with a mixed source of nitrogen. Furthermore, when Martínez and Cerdá analysed the accumulation of leaf NO_3^- they demonstrated that irrespective of the salinity treatments, leaf NO_3^- contents were always higher when the plants were fed with NO_3^- than with NO_3^- + NH_4^+. Salinity treatment did cause a significant decrease in leaf NO_3^- content, but only when NO_3^- was the sole nitrogen source.

Protein synthesis in plants supplied with different forms of nitrogen has been shown to be altered under conditions of salinity stress (Abdul-Kadir and Paulsen, 1982; Pessarakli and Tucker, 1985b; Pessarakli *et al*, 1989; Shaviv, 1990). When equal quantities of NO_3^--N or NH_4^+-N were supplied individually to three bean cultivars under normal (non-saline) conditions, it was noticed that the plants absorbed more NO_3^--N than NH_4^+-N (Pessarakli *et al.*, 1989). This was associated with a higher protein content in NO_3^- fed than NH_4^+ fed beans. When the same bean cultivars supplied with varying amounts of NO_3^- or NH_4^+ were exposed to a salt stress, nitrogen metabolism and protein synthesis was significantly inhibited, regardless of the source of nitrogen (Pessarakli *et al.*, 1989). Both the crude protein and protein-N contents of all three cultivars were severely depressed by salt, with shoot protein being more adversely affected than that of the root. This result appeared to indicate that under conditions of a salt stress, NO_3^- incorporation into protein was more severely affected than NH_4^+ incorporation. Shaviv *et al.* (1990) have shown that although the total nitrogen content of grain was found to decrease with an increase

in NaCl supply to wheat, higher seed nitrogen contents were obtained when the plants were treated with mixed NO_3^-/NH_4^+ nutrition than with NO_3^- alone. An alteration in the NO_3^-/NH_4^+ ratio from 3:1 to 1:1 led to an increase in grain nitrogen content. Furthermore, the relative increase in grain nitrogen content induced by the 1:1 treatment became more pronounced as the salinity level was raised, and often reached levels greater than non-salinized plants treated with NO_3^- only. Shaviv and coworkers commented on the potential for obtaining higher grain protein yields from salt stressed wheat by controlling the proportion of NO_3^--NH_4^+ supply to these plants.

2.4.2 The effect of the nitrogen supply on uptake of Na^+ and Cl^- ions

An increase in the NO_3^- supply from 1 mM to 9 mM caused a concomitant decrease in the Na^+ and Cl^- concentration of shoots, but not roots, of peanuts salinized with 50 mM NaCl (Silberbush *et al.*, 1988). Similarly, the Cl^- content of tomato plants supplied with 10 mM, 35 mM and 70 mM Cl^- was depressed by increasing the NO_3^- concentration from 7.5 mM to 20 mM (Kafkafi *et al.*, 1982). Melon plants exposed to high concentrations of NO_3^- (20 mM) also contained low concentrations of Cl^- when supplied with NaCl up to 90 mM (Feigin *et al.*, 1987). Leaves of salt stressed cucumber and tomato plants exhibited reduced Cl^-, but not Na^+ contents when supplied with NO_3^- levels up to 15 mM (Cerdá and Martínez, 1988). In addition, this beneficial NO_3^- effect on tomato and cucumber tended to decrease as the NaCl concentration was increased from 4 to 100 mM. As reflected by growth and nitrogen metabolism, plants supplied with a mixed nitrogen nutrition often behave differently to those supplied with a single nitrogen source (Section 2.4.1). The change in Na^+ and Cl^- content of salt stressed plants supplied with NO_3^- + NH_4^+ is no exception. When NO_3^- was the predominant nitrogen source supplied to tomato and cucumber plants, leaf Cl^- contents were found to decrease with increase in NO_3^- concentration (Martínez and Cerdá, 1989). In contrast, when the NO_3^-/NH_4^+ ratio was manipulated such that NH_4^+ was the main nitrogen supply, the leaf Cl^- content was not reduced by an increase in the nitrogen level. The opposite was detected in tomato plants when Na^+ levels were monitored (Cerdá and Martínez, 1988). In this instance, tomato plants grown on NO_3^- + NH_4^+ accumulated less Na^+ than when supplied with NO_3^-. Cucumber leaf Na^+ levels showed the same trend as Cl^- with respect to nitrogen supply. Shaviv *et al.* (1990) found that the effect of additional NO_3^-/NH_4^+ nutrition supplied to salt stressed wheat to be in agreement with the hypothesis that raising the external NO_3^-

concentration should be antagonistic to chlorides but they also showed that when the NH_4^+ component of a mixed NO_3^-/NH_4^+ supply was increased, the Na^+ levels in the plant also decreased. Shaviv's group suggested therefore that an antagonistic relationship also existed between NH_4^+ and Na^+ and that this could be exploited in the use of mixed nitrogen nutrition on salt stressed plants.

2.4.3 The participation of other macronutrient ions in the relationship between nitrogen metabolism and NaCl stress

Many studies have focused on the interactions of macronutrient ions under saline conditions (Lynch and Läuchli, 1984; Ward *et al.*, 1986; Cramer *et al.*, 1987; Munns *et al.*, 1988; Cramer *et al.*, 1989, 1991). Most of these studies have investigated nutrients such as N, K, Ca and P, which are required in large quantities by plants to sustain growth. Although the action of inorganic ions other than those based on nitrogen on salt stressed plants are not emphasised in this review, the relationships between such ions contribute to an understanding of the mechanisms involved in ionic interactions under saline conditions. It is often the mutual antagonisms between ions and not their absolute concentrations which may have important consequences for growth (Cramer *et al.*, 1991). Therefore, the following discussion focuses briefly on the interactions between N and Ca^{2+} and K^+ ions during a salt stress.

Calcium supplementation to salt stressed plants supplied with various forms of nitrogen has been reported to have a positive effect on growth (Ward *et al.*, 1986; Lewis *et al.*,1989; Leidi *et al.*, 1991). The presence of Na^+ ions are reported to reduce the binding of Ca^{2+} to the plasma membrane, inhibit influx while increasing the efflux of Ca^{2+}, and deplete the internal stores of Ca^{2+} from endomembranes (Rengel, 1992). The addition of Ca^{2+} to salt stressed plants may counteract the effects of Na^+ on disturbance of the cell Ca^{2+} homeostasis by maintaining the integrity and function of the plasma membrane in roots and shoots (Läuchli and Schubert, 1989; Läuchli, 1990). This effect of Ca^{2+} on cell membranes may be responsible for the observed increase in nitrate uptake by salt stressed plants supplied with Ca^{2+}. Ward *et al.* (1986) showed that the enhancement of short-term NO_3^- uptake by Ca^{2+} in barley seedlings under saline conditions was dependent on the presence of Ca^{2+} during the imposed stress, as the effect was not detected under non-saline conditions. Ward and coworkers indicated that, in the long term, an increase in the Ca^{2+} concentration of the nutrient solution resulted in increases in

NO_3^- assimilation and seedling growth. Those authors proposed that Ca^{2+} increased the activity of the NO_3^- transporter under saline conditions, a concept which was supported by growth response studies of maize and wheat to saline conditions (Lewis *et al.*, 1989). Lewis's group ascribed the improvement in plant growth after addition of Ca^{2+} to improved NO_3^- uptake due to Ca^{2+} protection of the NO_3^- transporter. This effect was specific to NO_3^- grown plants as no benefit of Ca^{2+} addition was observed in NH_4^+ grown plants (Lewis *et al*, 1989; Leidi *et al.*, 1991).

Potassium levels in plant cells have been regularly shown to be reduced under conditions of salt stress. High levels of K^+ and low levels of Na^+ in the cytoplasm are essential for the activities of some enzymes (Greenway and Munns, 1980) and are indispensable for metabolic processes and plant growth (Kent and Läuchli, 1985). Potassium addition to salt stressed barley seedlings has been shown to enhance NO_3^- uptake and incorporation into protein (Helal *et al.*, 1975; Helal and Mengel, 1979). Associated with this was an increase in growth. Much of the recent research has focused, however, on the interaction between K^+ and Ca^{2+} ions, in particular the maintenance of high intracellular K^+ concentrations by Ca^{2+} addition to salt stressed plants (Cramer *et al.*, 1987; Nakamura *et al.*, 1990). These relationships emphasise the complex regulation of ions under conditions of salt stress and suggest that addition of ions such as NO_3^- to salt stressed plants may only serve to alleviate a portion of the intracellular ionic disruptions induced by the stress.

2.4.4 The potential for the alleviation of salt stress by nitrogen fertilization

Increasing nitrogen fertilization of salinity stressed plants appears in some cases to be limited in the extent of its positive effects on plant growth and productivity. Optimum nitrate nutrition resulted in only a favourable effect on yield of melon and tomato plants at low (5 mM) and moderate (30, 60 mM) salinity levels (Feigin *et al.*, 1987). High salinity levels of 90 mM still resulted in 50 to 60% yield reduction despite the addition of nitrate to these plants. Similarly, increasing levels of NO_3^- supplied to tomato plants could serve only to alleviate a salt stress at low salinity levels of 10 mM (Papadopoulos and Rendig, 1983). At higher salinity levels of 50 mM and 95 mM, increasing the NO_3^- concentration had no positive effect on growth. Therefore, increasing the nitrogen fertilization may be ineffective under some conditions in counteracting the adverse effects on growth associated with high

external salinity levels. As was highlighted in Section 2.4.1, application of a mixed NO_3^-/NH_4^+ nutrition to salinized plants may have certain advantages over a single NO_3^- source, but the optimal ratio between NO_3^- and NH_4^+ appears to differ between plant species. In addition, salinity effects on other essential inorganic ions such as Ca^{2+} and K^+ may influence the overall effectiveness of nitrogen application. Bernstein *et al.* (1974) concluded that an increase in the supply of nitrogen would be beneficial only if salinity induced a nutrient deficiency or seriously impaired root development, thereby reducing the ability for nutrient uptake. This concept was supported by Maas and Hoffman (1977) who stated that additional nitrogen fertilization would have little effect on plant growth unless salinity caused specific nutritional imbalances within the plant. Conditions of excess salinity, or nitrogen deficiency, may thus control plant growth and yield regardless of the levels of the other (Bernstein *et al.*, 1974).

2.5 THE USE OF AN *IN VITRO* CULTURE SYSTEM IN STUDIES OF STRESS PHYSIOLOGY

2.5.1 General principles of *in vitro* cultures

In vitro culture systems essentially involve the aseptic growth of small pieces of tissue isolated from a living organism on a semi-defined or defined nutrient medium for an indefinite period of time (Mantell *et al.*, 1985). Plant cell culture encompasses a wide range of sterile culture procedures ranging from the growth of plant protoplasts, cells and tissues, to the growth of whole organs, embryos and plantlets (Tisserat, 1985). The regeneration of whole plants from any cell, tissue or organ is intrinsically reliant on the concept of totipotency which, as defined in Mantell *et al.* (1985), is "the capacity of a single cell to regenerate the phenotype of the complete and differentiated organism from which it was derived". The micropropagation of clonal material arising via the *in vitro* culture of plant parts allows the rapid production of large numbers of identical plants, a feature which has proved useful to plant breeders, industrialists and biochemists alike (Dix, 1990).

The regeneration of plants via an *in vitro* system is mostly reliant on the composition of the nutrient medium and the culture environment (Mantell, 1985; Tisserat, 1985; Dix, 1990). The most common nutrient medium used is that developed by Murashige and Skoog (1962) which consists of a number of inorganic macro- and micronutrients, organics, vitamins and growth regulators, and may or may not be

solidified with agar. The intensity, type and duration of light, temperature, oxygen/carbon dioxide and other gas concentrations also influences the growth of plant material in culture (Tisserat, 1985). Plant regeneration from an explant is usually accomplished by the routes of somatic embryogenesis or organogenesis, and may be direct or indirect. The indirect mode of embryogenesis involves the formation of callus, a mass of cells with a low level of organisation, and the subsequent initiation of embryos which, under suitable conditions, will germinate to produce plantlets (Tisserat, 1985). The direct mode of somatic embryogenesis involves the formation of an asexual embryo from a single cell or group of cells without an intervening callus phase (Tisserat, 1985). Direct organogenesis encompasses the emergence of adventitious organs directly from the explant without a callus phase, and the production of plantlets from outgrowth of axillary buds (Mantell, 1985; Tisserat, 1985). In addition, adventitious organs may be derived indirectly via callus formation. The shoots arising by both direct and indirect organogenesis may be transferred to fresh culture medium on a regular basis by subculture to further enhance the propagation procedure (Binding and Krumbiegel-Schroeren, 1984).

2.5.2 *In vitro* cultures as a tool in physiological studies

2.5.2.1 Advantages of *in vitro* culture systems

Many physiological studies involve the analyses of plants grown either in a soil and or vemiculite based environment or in liquid (hydroponic) culture. Such studies and, in particular studies of the mechanisms of stress physiology, can benefit from the use of *in vitro* culture methodology. There are many advantages to using *in vitro* cultures for physiological studies. Tal (1984) lists a wide range of such advantages, some of which are as follows: (i) experiments may be performed the whole year round as the growth of *in vitro* cultured material is independent of seasonal fluctuations; (ii) the time between generations can be reduced; (iii) large numbers of identical plants (clones) may be produced; (iv) the environment and composition of the nutrient medium may be closely controlled; (v) *in vitro* cultures may be used as a model system for the whole plant, but only in studying those mechanisms which operate both on the cellular and whole plant level; (vi) cultured cells and tissues are more responsive to environmental stimuli than the whole plant and (vii) mechanisms which operate on the cellular level only may be distinguished from those operating at the whole plant level.

2.5.2.2 The utilisation of *in vitro* culture systems in studies of nitrogen metabolism

There are several examples of the use of *in vitro* culture systems for the study of aspects of the nitrogen assimilatory pathway. As early as 1971, Heimer and Filner were utilising tobacco cell cultures to investigate the regulation of nitrate uptake and NR activity. In some instances, the study of nitrogen assimilation pathways provides vital information about the state of developing cultures. The growth of protoplast derived-cells was strongly dependent on a proper nitrogen metabolism as demonstrated by Lenee and Chupeau (1989) during the monitoring of nitrogen assimilatory enzymes during growth of cells derived from tobacco and sunflower (*Helianthus annuus*). Those studies indicated that the poor growth of sunflower protoplasts was related to the slow development of certain nitrogen assimilatory enzymes.

The differentiation of organised structures such as roots and shoots from callus tissue is reflected by changes in the patterns of enzymes (Bonner, 1965), the enzymes of nitrogen assimilation being no exception. Comparisons between shoot-forming and non-shoot forming callus cultures of sugarcane revealed that shoot differentiation occurred concomitantly with the peak in NR activity, mobilisation of nitrate was better in shoot-forming callus, and the glutamine synthetase/glutamate synthase pathway of ammonia assimilation became operative prior to shoot differentiation (Dwivedi *et al.*, 1984). Similar studies were performed by Hardy and Thorpe (1990) in shoot-forming tobacco callus cultures. These authors found that the highest levels of total-N, protein-N, nitrate and ammonium-N, as well as nitrate and nitrite reductases, were found in shoot-forming callus in comparison to the non-shoot forming proliferating tissues. Hardy and Thorpe (1990) concluded that enhanced nitrogen assimilation occurs during shoot organogenesis. The growth of shoot-forming callus cultures of tobacco has been shown by Kavi Kishor and Mehta (1988) to be characterised by high specific activities of the enzymes involved in malate metabolism. This led them to propose that tobacco callus may utilize this pathway for deriving reducing power required for the processes of organogenesis.

The regulation of the genes for nitrogen assimilation have been studied also using *in vitro* culture systems due to the elimination of many of the complications resulting from transport, storage, and compartmentation of nitrate subsequent to its uptake (Privalle *et al.*, 1989). The patterns of induction of nitrate uptake, NR activity and

NiR mRNA by nitrate in maize cell suspension cultures were found by Privalle and coworkers (1989) to be similar to those reported for maize seedlings.

2.5.2.3 The utilisation of *in vitro* culture systems in studies of salt stress physiology

The situations where *in vitro* culture systems have been utilised to study aspects of salt stress physiology are many and varied. Plant cell cultures provide a means whereby the cellular mechanisms of salinity stress may be studied. Analyses of protein synthesis in salt stressed maize callus (Ramagopal, 1986) and sugarcane suspension cells (Ramagopal and Carr, 1991) allowed the identification of distinct polypeptide species that appeared upon exposure to the stress (Section 2.2.3.2). The expression of genes for photosynthesis in a salt tolerant line of cultured alfalfa cells allowed the evaluation of the role which the activation of genes for photosynthesis played in salt tolerance (Winicov and Seemann, 1990). Furthermore, the detection of fluctuations in the relative mRNA amounts in suspension culture cells of the halophyte *Distichlis spicata* exposed to a salt stress suggested a controlled response by the plant cells to the stress (Zhao *et al.*, 1989). By investigating a number of *Asparagus officinalis* tissues at different levels of organisation (friable callus with no organogenesis; compact callus exhibiting organogenesis; one-bud shoot segments; and plantlets), Mills (1989) was able to demonstrate that the response of *Asparagus officinalis* to a salt stress was dependent on tissue organisation.

As described in Section 2.5.2.1 above, one of the advantages of using an *in vitro* culture system is that it may be used as a model system for the whole plant. However, assessments of the homology between the responses of cultured cells and whole plants to a salt stress have often revealed discrepancies in the sensitivity to an applied stress (Smith and McComb, 1981a; Warren and Gould, 1982; Stavarek and Rains, 1984; Mills, 1989). On the other hand, Mills and Benzioni (1992) and Benzioni *et al.* (1992) demonstrated that the response to salinity of nodal segments of jojoba (*Simmondsia chinensis*) grown *in vitro* shared many similarities with the whole plant grown *ex vitro* in a greenhouse. These investigations supported earlier studies on tomato (Tal, 1984), *Distichlis spicata* (Warren and Gould, 1982), and *Spartina pectinata* (Warren *et al.*, 1985) which showed a positive correlation between salinity responses at the cellular and whole plant level.

There have been many successful reports of the use of *in vitro* culture systems in the selection of salt tolerant cell lines from salt sensitive species such as tobacco

(Nabors *et al.*, 1980), alfalfa (Smith and McComb, 1983), chickpea (Pandey and Ganapathy, 1984), sorghum (Bhaskaran *et al.*, 1986), and barley (Ye *et al.*, 1987). However, the regeneration of salt tolerant plants from salt tolerant cell lines have not always proved that effective. Examination of salt tolerant *in vitro* cultured alfalfa could not identify an *in vitro* NaCl tolerance mechanism which conferred whole plant NaCl tolerance (McCoy, 1987). Only a few cases have been reported of successful plant regeneration from salt tolerant cell lines of tobacco (Nabors *et al.*, 1980), sorghum (Bhaskaran *et al.*, 1986), and flax (*Linum usitatissimum*) (McHughen, 1987) with the result that the potential for inheritance of the trait for salt tolerance has been questioned. In another study, embryonic cultures of maize were used to produce salt tolerant somaclones which still exhibited *in vitro* tolerance to NaCl three months after being subcultured on salt free media (Lupotto *et al.*, 1989). Plant regeneration capability was maintained also in the long-term salt tolerant cultures suggesting that for maize, the acquired trait of salt tolerance was at least preserved *in vitro*. The relationship between the salt tolerance of cultured cells and whole plants is crucial to the use of cultured cells in selecting for salt tolerance but, as indicated by Dracup (1991), these relationships as well as the mechanisms of salt tolerance in cultured cells are not yet fully understood. Dracup (1991) proposes that an improvement in the knowledge of the processes involved in salt tolerance are required before specific physiological objectives may be identified and selected for in cultured cells.

3. MATERIALS AND METHODS

3.1 ESTABLISHMENT OF *in vitro* CULTURES

3.1.1 Source of plant material

Material, *Nicotiana tabacum* L. var. Samsun, was obtained from greenhouse grown plants. Plants were grown in vermiculite from seed and watered regularly with Long Ashton nutrient solution (Hewitt, 1966). For three days prior to culture initiation plants were watered with nutrient solution supplemented with 0.1 g/l Benlate and 100 μg/l penicillin + 100 μg/l streptomycin antibiotic cocktail to reduce endemic fungal and bacterial contamination.

3.1.2 *In vitro* plantlet production

3.1.2.1 Callus induction

Leaves were surface sterilised for 15 min in a solution of 1% (w/v) Na hypochlorite. Leaf segments were then placed on solid MS nutrients (Murashige and Skoog, 1962) containing 1 μg/l 6-benzylaminopurine (BAP), 20 g/l sucrose and 10 g/l agar (Associated Chemical Enterprises), pH 5.7. Cultures were placed in the dark for 27 d at 23 ± 1 °C.

3.1.2.2 Shoot regeneration

After callus production, cultures were transferred to a 14 h/10 h (light/dark) photoperiod, with PPFD at 200 μE m^{-2} s^{-1}. Regenerated shoots were excised and subcultured on to fresh MS nutrients, 10 g/l sucrose and 10 g/l agar, for plantlet establishment.

3.2 THE EXPERIMENTAL SYSTEM

Individual excised shoots (30 mm in length) were transferred to tubes containing 10 ml of medium. Medium composition was MS nutrients, 10 g/l sucrose and 10 g/l agar, NaCl and different forms and/or concentrations of N according to the treatment. The experimental design consisted of a range of NaCl concentrations (0

- 180 mM) factorially combined with a range of N concentrations (0 - 120 mM), supplied as either NO_3^--N only or NH_4^+-N only. The standard MS formulation (40 mM NO_3^--N + 20 mM NH_4^+-N) was used as a control. Nitrate-N was supplied as KNO_3 and NH_4^+-N as $(NH_4)_2SO_4$. Where necessary, total K^+ in the medium was adjusted by supplementing MS nutrients with K_2SO_4. In some experiments, NaCl was replaced with isosmotic concentrations of mannitol. Shoots were maintained *in vitro* under the above-mentioned growth conditions for a period of 35 d.

3.3 GROWTH MEASUREMENTS

Plantlets were harvested at intervals during the 35 d growth period and root number, root fresh mass, length of the longest root, stem fresh mass and stem length were measured. Dry mass measurements of root, stem and leaf components were determined by drying the material in aluminium foil packets for 48 h at 80 °C.

3.4 CHLOROPHYLL DETERMINATION

Leaf chlorophyll content was measured according to the method of Arnon (1949).

3.5 PROTEIN DETERMINATION

Leaf protein was extracted according to a modified version of the method of Wetter and Dyck (1983). The tissue was washed with 0.16 M mannitol and homogenised at 4 °C with an Ultra-Turrax for 1 min 30 sec in 350 µl extraction buffer (0.2 M Tris-HCl, 1 M sucrose, 0.056 M 2-mercaptoethanol, pH 8.5). The homogenate was centrifuged at 16 000 rpm for 1 h at 4 °C. A 100 µl sample of supernatant was mixed with 3 ml distilled water and 25 µl 2% (w/v) Na deoxycholate. After 15 min, 1 ml of 24% (w/v) trichloroacetic acid (TCA) was added. The sample was kept at room temperature for 10 min and then centrifuged at 3000 rpm for 30 min at room temperature. The supernatant was discarded and the protein precipitate allowed to dry. The protein concentration was determined by the Folin-Lowry assay (Lowry *et al.*, 1951), using BSA as a standard.

3.6 DETERMINATION OF NITRATE REDUCTASE ACTIVITY

Nitrate reductase activity of leaf tissue was assayed *in vivo* according to the method of Watt *et al.* (1986). Nitrite formation was quantified following extraction from

tissue by boiling for 20 min. Nitrite was assayed colorimetrically according to the sulphanilamide and N-(1-naphthyl)ethylenediaminedihydrochloride method of Hageman and Reed (1980).

3.7 QUANTIFICATION OF NITRATE, SODIUM AND CHLORIDE UPTAKE

3.7.1 Extraction from growth medium

After plantlet harvest, culture medium was deep-frozen for a minimum of 48 h (-20 °C) and then centrifuged at room temperature for 1 h at 18 000 rpm to pellet the agar. The resultant supernatant was assayed for ion content.

3.7.2 Assay procedures

Nitrate was determined colorimetrically by the salicylic acid method of Cataldo *et al.* (1975). Sodium was measured by flame emission spectroscopy on a Varian atomic absorption/emission spectrophotometer Series AA-575, and chloride assayed gravimetrically by precipitation with $AgNO_3$ after acidification with HNO_3 (Jeffery *et al.*, 1989).

3.8 PREPARATION OF NR DNA PROBE

3.8.1 Source of probe

The probe sequence used was a 1.6 kb Eco RI insert of cDNA complementary to NR mRNA and was in the plasmid pBMC102010 (Calza *et al.*, 1987), a pUC 9 plasmid with ampicillin resistance (Vieira and Messing, 1982). The bacterial strain *E. coli* HB101 (ampicillin sensitive), (Bolivar and Backman, 1979) was transformed with the plasmid pBMC102010 in our laboratories. Transformed cells were selected on solid Luria Bertani (LB) medium (10 g/l tryptone [Difco Laboratories,USA], 5 g/l yeast extract [Difco Laboratories, USA], 10 g/l NaCl, 15 g/l agar, pH 7.5) containing 50 μg/ml ampicillin.

3.8.2 Growth and maintenance of bacteria

Long-term storage of transformed bacterial cells was as anaerobic stab cultures in solid LB medium + 50 μg/ml ampicillin, stored at room temperature in the dark.

Bacterial cultures were grown by inoculation of LB broth medium + 50 μg/ml ampicillin with a single bacterial colony (obtained from a streak plate preparation) followed by overnight incubation on a horizontal shaker (140 rpm) at 37 °C.

3.8.3 Plasmid generation, isolation and purification

The plasmid pBMC102010 was isolated from transformed *E. coli* cells by a modification of the method for large scale plasmid preparation of Armitage *et al.*, 1988. The modifications were: growth of bacterial cultures on LB broth medium containing 50 μg/ml ampicillin; addition of 1% Triton X-100 to the CsCl gradient before centrifugation to isolate the plasmid DNA (Little, 1990); CsCl gradient centrifugation at 100 000 rpm and 18 °C for 4 h and dialysis of the plasmid solution for ±20 h against five changes of buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) (TE) buffer. The yield of plasmid DNA from 500 ml of bacterial culture (determined spectrophotometrically by absorption at 260 nm) was 459.03 μg. The concentration of purified plasmid DNA obtained was 267.5 μg/ml. The ratio of $A_{260/280} = 1.76$.

3.8.4 Restriction enzyme digestion

Restriction enzyme digestion was performed on Lambda DNA (Boehringer Mannheim, SA) or plasmid DNA with the endonucleases Eco RI, Hind III, KpN I and Sma I (Boehringer Mannheim, SA) used singly or in combination. Each reaction mixture was prepared according to the manufacturer's recommended protocol and the digestion performed at 37 °C for 2 h.

3.8.5 Agarose gel electrophoresis

Whole plasmids or restriction digests of plasmids were analysed by separation on 1% agarose mini - gels. The gel was stained with 0.5 μg/ml ethidium bromide and DNA fragments visualised with a UV transilluminator at 300 nm. Eco RI restriction digests of pBMC102010 DNA were run on 1% low melting temperature agarose mini - gels when isolation of probe sequence was required.

3.8.6 Probe labelling

3.8.6.1 Preparation of DNA probe

The NR DNA probe sequence was excised from a low melting temperature agarose gel and stored at -20 °C. For the ^{32}P-labelling reaction, 3 ml/g distilled water was added to the gel slice and the mixture boiled for 10 min (Feinberg and Vogelstein, 1984). Aliquots were removed for the labelling reaction. For the digoxigenin (DIG)-labelling reaction, the probe was prepared in two ways: 1) The probe sequence was recovered from the low melting temperature agarose according to the method of Wieslander (1979) and used directly in the labelling reaction, 2) pBMC102010 was restricted with Eco RI and the entire digest used in the labelling reaction.

3.8.6.2 Labelling of probe

Radioactive and non-radioactive labelling of the DNA probe was according to the random-primer method of Feinberg and Vogelstein (1983). The probe was radiolabelled with ^{32}P- TTP as radioactive precursor and the reaction mixture incubated at room temperature overnight. When non-radioactive labelling was used, digoxigenin-dUTP was utilised as a precursor and the DNA was labelled according to the standard procedure accompanying the DIG DNA Labelling and Detection Kit (Boehringer Mannheim, Germany). The reaction was allowed to proceed for 20 h at 37 °C.

3.8.7 Probe purification

The ^{32}P-labelled DNA was purified through a Sephadex G-50 spun column according to the method of Sambrook *et al.*, 1989. The DIG-labelled DNA was purified by ethanol precipitation according to the method outlined in the DIG DNA Labelling and Detection Kit.

3.8.7.1 Measurement of specific activity of ^{32}P-labelled DNA

Labelled DNA was assayed directly and after precipitation with trichloroacetic acid (TCA). A 2 µl subsample of labelled DNA was added to a solution of carrier DNA and incubated on ice for 15 min in the presence of 10% (w/v) TCA. The

solution was vacuum filtered and the precipitate rinsed several times with cold TCA (5% w/v) followed by cold ethanol (95% v/v). The filter was air dried and scintillation counted. The specific activity of the labelled DNA was found to be 4 x 10^8 cpm/μg DNA.

3.9 SLOT BLOT ANALYSIS FOR QUANTIFICATION OF NR mRNA

3.9.1 Extraction of total RNA from leaf tissue

At each sampling time, leaves were collected, frozen immediately and stored in liquid nitrogen (-196 °C). RNA was isolated from fresh or frozen leaf material according to a modification of the method of Verwoerd *et al.*, 1989. Modifications included the grinding of leaves in liquid nitrogen using a small porcelain mortar and pestle and the subsequent transfer of the powder to a precooled eppendorf tube. In addition, after hot (80 °C) extraction buffer was added to the powder, the sample was returned to an 80 °C waterbath for 30 sec to ensure even heating. The protocol routinely yielded between 40 - 60 μg RNA ($A_{260/280}$ = 1.7-1.9) from 200 - 210 mg tissue. Storage of extracted RNA was at -20 °C.

3.9.2 Preparation of slot blot

RNA samples were applied to nitrocellulose membranes (Hybond - extra, Amersham) using a Biorad Bio-Dot SF slot blot apparatus. Two methods of RNA denaturation were investigated: heat and alkaline denaturation. Samples to be heat denatured were mixed with a combination of formamide (100% v/v), formaldehyde (37% v/v) and 0.02 x SSC (0.3 mM NaCl, 0.03 mM Na-citrate, pH 7.0) and incubated at 68 °C for 15 min. When the alkaline procedure was used, RNA samples were denatured immediately prior to application by dissolving in ice-cold 10 mM NaOH, 1 mM EDTA. Samples were loaded into the apparatus and applied to the membrane by vacuum filtration according to the manufacturer's recommended protocol. Sample wells were then rinsed with cold 10 mM NaOH, 1 mM EDTA. The blotted membrane was washed with 2 x SSC (0.3 M NaCl, 0.03 M Na-citrate, pH 7.0), 0.1% (w/v) SDS and then baked for 2 h at 80 °C.

3.9.3 Hybridisation of DNA probe to NR mRNA

Hybridisation of the radiolabelled probe to RNA slot blots was performed under the following conditions: the prehybridisation buffer used was 0.9 M NaCl, 60 mM

sodium phosphate, 6 mM EDTA, pH 7.7; Denhardt's reagent (1 g/l Ficoll, 1 g/l polyvinylpyrrolidone, 1 g/l bovine serum albumin); 0.5% (w/v) SDS; 100 μg/ml denatured, fragmented salmon sperm DNA and 50% (v/v) formamide. Prehybridisation was for approximately 6 h at 42 °C. Dextran sulphate (10% w/v) and 100 ng ^{32}P-labelled probe were added to fresh prehybridisation buffer and hybridisation was carried out for 16-18 h at 42 °C. Hybridisation with the DIG-labelled probe (100 - 650 ng) was performed according to the instructions provided with the DIG DNA Labelling and Detection Kit. Prehybridisation ranged between 4 h and 18 h at 68 °C and hybridisation between 16 h and 48 h at the same temperature.

3.9.4 Detection methods

RNA slot blots hybridised to ^{32}P-labelled DNA probe were air-dried and exposed to X-ray film for a period of 12-72 h. The autoradiographic image was developed in Agfa G127c developer for 2 min and fixed with G334c X-ray fixer for 5 min. Quantification of the amount of NR mRNA present was performed by densitometric scanning of the image with a Hoefer GS 300 transmittance/ reflectance scanning densitometer.

3.9.5 Immunological detection

Detection of hybridised RNA blots using the DIG-labelled probe was performed using an immunological procedure provided with the DIG DNA Labelling and Detection Kit. After preparing the blots for detection according to the instructions provided with the kit, blots were incubated in anti-digoxigenin-AP conjugate for 30 min. After washing off unbound antibody-conjugate the blots were equilibrated in an alkaline buffer (0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5) and the colour reaction initiated by incubation with X-Phosphate and NBT solutions. A blue-purple precipitate was formed which was allowed to develop for up to 3 d. Results were recorded by photography and densitometric scanning of blots.

3.10 STATISTICAL ANALYSES

Where appropriate, results were analysed statistically by Duncan's Multiple Range Test with a significance level of 5%. This test performs a comparison of means, with significant differences between means being represented by letters of the alphabet.

When standard error bars were omitted on graphs due to clutter, means were compared by the Least Significant Difference (LSD) test at 5% significance level.

3.11 PHOTOGRAPHY

Photographic records were taken where appropriate. Colour photographs were taken using Kodak 100 ASA film at F-stop 11. Black and white photographs were taken with Kodak Tmax 400 ASA film at F-stop 11.

4. RESULTS

4.1 *In vitro* GROWTH RESPONSES OF *Nicotiana tabacum* SHOOT CULTURES TO VARIATIONS IN THE LEVEL OF NaCl AND NITROGEN COMPOSITION OF THE NUTRIENT MEDIA

4.1.1 The effect of NaCl on *in vitro* shoot cultures

4.1.1.1 Growth and morphology

The growth response of excised shoots of *N. tabacum*, placed on MS nutrient medium (containing 40 mM NO_3^- and 20 mM NH_4^+) (Murashige and Skoog, 1962) with or without NaCl, may be observed in Figure 4.1. Control plantlets, grown in the absence of NaCl, exhibited increases over time in all growth parameters tested. Stem fresh mass (Figure 4.1 E), for example, increased by 800% and stem length (Figure 4.1 F) by 300% over the 35 d growth period. Root number (Figure 4.1 C) increased initially over a period of 14 d during root establishment, but stabilised thereafter. Leaf chlorophyll content (Figure 4.1 D) remained approximately constant over the entire growth period. All of these trends were negatively affected by NaCl supply to plantlets, with the exception of the length of the longest root (Figure 4.1 A) which was found to be stimulated by low (45 mM and 90 mM) levels of NaCl: by 35 d the roots of plantlets grown on 45 mM and 90 mM NaCl were found to be approximately 50% longer than those of the control. The negative effect of the presence of NaCl in the growth medium was found to be directly related to the concentration of NaCl supplied. As the NaCl concentration was increased, root number (Figure 4.1 C) inhibition after 35 d in culture, as compared to the control, ranged from 21% (45 mM) to 54% (180 mM). Root fresh mass (Figure 4.1 B) was suppressed in a similar manner: recorded inhibition values were 38% at 45 mM and 83% at 180 mM NaCl. Analogous growth retardation was evident in both stem parameters measured, with length (Figure 4.1 F) and fresh mass (Figure 4.1 E) being inhibited by 62% and 70%, respectively, at the highest salt concentration supplied. The leaf chlorophyll levels (Figure 4.1 D) decreased with time in the presence of salt and reacted rapidly and drastically to 180 mM NaCl. This extreme response in leaf chlorophyll exhibited by plantlets grown under high salt conditions was particularly noticeable upon examination of the plantlets after 35 d in culture (Plates 4.1). The leaves of plantlets grown in the presence of

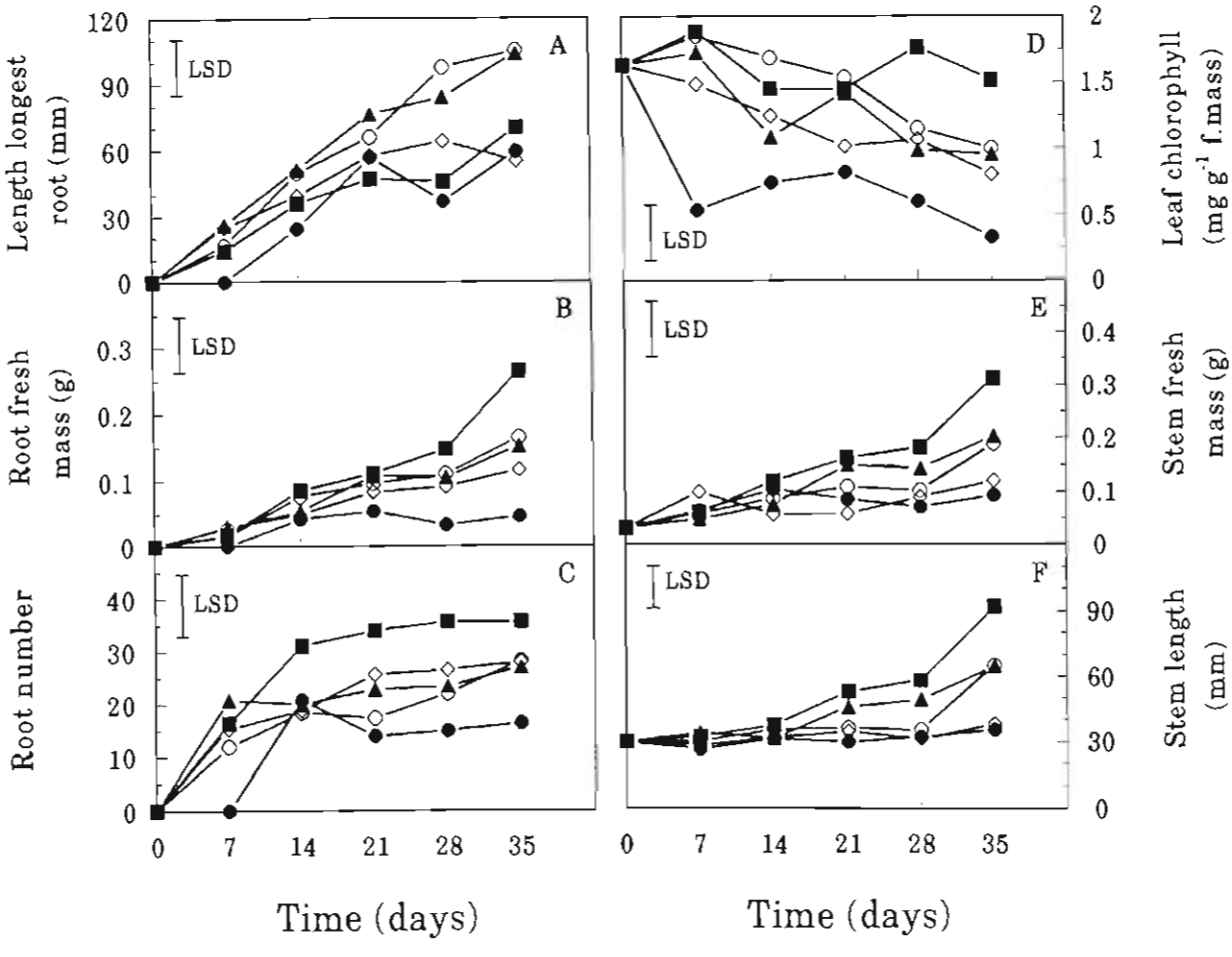


Figure 4.1: Effect of NaCl on root and stem growth of *in vitro* plantlets of *N. tabacum*.

Excised *in vitro* shoots were cultured on solid MS medium containing 60 mM N (as 40 mM NO_3^--N + 20 mM NH_4^+-N) and 0 - 180 mM NaCl for a 35 d period. Roots emerged by day 7 in all treatments except 180 mM NaCl.
Symbols: (mM NaCl) ■ (0), ○ (45), ▲ (90), ◇ (135), ● (180).

Bars represent Least Significant Difference (LSD) between treatments for each parameter. n=3.

A: 0 mM NaCl

B: 45 mM NaCl

C: 90 mM NaCl

D: 135 mM NaCl

E: 180 mM NaCl

Plate 4.1: Effect of NaCl on general morphology of *N. tabacum in vitro*.

Treatments as described in Figure 4.1. Photographs were taken after 35 d in culture. Note the chlorosis of the leaves and reduced root number in plantlets grown on 180 mM NaCl. Photographs are to scale.

A: 0 mM NaCl

B: 45 mM NaCl

C: 90 mM NaCl

D: 135 mM NaCl

E: 180 mM NaCl

180 mM NaCl (Plate 4.1 E) were visibly chlorotic on comparison to plantlets exposed to lower levels of NaCl (Plates 4.1 A-D). Differences in leaf chlorophyll content were not distinct visually for any of the salt treatments other than 180 mM NaCl. Plate 4.1 E also demonstrates the marked reduction in root number of plantlets grown on 180 mM NaCl on comparison to control plants grown in the absence of NaCl. The decline in root number was not as prevalent in plantlets grown on 45 to 135 mM NaCl as in those plantlets grown on 180 mM NaCl.

At each particular concentration tested, suppression of growth by NaCl became more pronounced with increased exposure time (Figure 4.1). Comparison of plantlet growth on 180 mM NaCl after 21 d and 35 d in culture revealed that inhibition of root fresh mass (Figure 4.1 B) increased from 51% to 83%, respectively. Similarly, stem mass inhibition (Figure 4.1 E) increased from 48% (21 d) to 70% (35 d).

4.1.1.2 Root fresh mass:shoot fresh mass ratio

Although both root and stem parameters were found to be inhibited by salt, the effects appeared to be more pronounced on root growth. This is illustrated by the root fresh mass:shoot fresh mass ratio, calculated after 35 d in culture (Table 4.1 A). A decrease in the root fresh mass:shoot fresh mass ratio from 0.838 to 0.512 was observed for plantlets grown on 0 and 180 mM NaCl, respectively. This result is in contrast to a number of other investigations which have shown that shoot growth was more negatively influenced by salt than root growth (Munns and Termaat, 1986; Sharp and Davies, 1989; Larsson *et al.*, 1989; Robertson *et al.*, 1990) (Section 2.2.2.1).

Table 4.1: Effect of NaCl and N supply on the root fresh mass:shoot fresh mass ratio of *in vitro* plantlets of *N. tabacum*.

Treatments as described in Figures 4.1, 4.2, 4.4. n=3.

Data indicates root fresh mass:shoot fresh mass ratio of plantlets.

NaCl (mM) / (MPa) / N (mM) NO_3^--N	NH_4^+-N	0 -0.00	90 -0.44	180 -0.88
A 40	20	0.838a	0.708ab	0.512b
B 60	-	1.182a	1.386a	0.291d
-	60	0.445cd	0.469cd	0.231d
C 0	-	2.757a	1.196bc	0.633de
30	-	0.842cd	1.102cd	1.148bc
60	-	1.182bc	1.386b	0.291f
120	-	1.198bc	0.525ef	0.478ef

Letters adjacent to values represent mean separation by Duncan's Multiple Range Test, 5% significance value.

4.1.2 The effect of the form of nitrogen nutrition supplied to salt stressed shoot cultures

The investigations presented in Section 4.1.1 were performed on cultures grown on MS nutrient medium which contains a 2:1 molar ratio of NO_3^--N:NH_4^+-N with a total N content of 60 mM (Murashige and Skoog, 1962). In the present study, the nitrate and ammonium content (40 and 20 mM, respectively) of the standard MS formulation was replaced with either NO_3^--N only or NH_4^+-N only to a total N content of 60 mM. The nutrient composition of standard MS medium was used as the control.

4.1.2.1 Growth and morphology

The growth response of plantlets to the form of N supplied, in the absence and presence of NaCl after 35 d, is shown in Figure 4.2. The source of nitrogen, either as NO_3^--N, NH_4^+-N, or a combination of both (MS nutrient medium), had a pronounced effect on all measured growth parameters, and leaf chlorophyll content, of plantlets grown for 35 d *in vitro*. In the absence of NaCl, plantlet growth on 60 mM NH_4^+-N only was significantly lower for all growth parameters (Figure 4.2 A,B,C,E,F) than on both NO_3^--containing media. Leaf chlorophyll content (Figure 4.2 D) in NH_4^+-grown plantlets was reduced also on comparison to plantlets supplied with NO_3^--N only and NO_3^--N + NH_4^+-N. Plantlet growth on a combination of NO_3^--N + NH_4^+-N was superior to growth on NO_3^--N only for all parameters except length of the longest root (Figure 4.2 A). Root length of plantlets grown on NO_3^--N only exhibited a 47% increase over those grown on a mixed N nutrition. Leaf chlorophyll content (Figure 4.2 D) was greater on NO_3^--N + NH_4^+-N fed plantlets than those supplied with a single N source.

The presence of NaCl altered the growth responses that were observed under control (0 mM NaCl) conditions. In addition, the differences in growth between the shoot cultures grown on the three different N regimes were altered as the NaCl concentration was increased from 90 mM to 180 mM NaCl. The supply of 90 mM NaCl to plantlets grown on NO_3^--N only inhibited growth measured by all parameters except stem fresh mass (Figure 4.2 E). Growth inhibition ranged from 20% (stem length) to 40% (root number) of the values exhibited by control plantlets. There was no significant difference in stem fresh mass (Figure 4.2 E) of plantlets grown on NO_3^--N in the absence and presence of 90 mM NaCl. The leaf chlorophyll contents (Figure 4.2 D) of NO_3^--N grown plantlets were reduced by 20% with an increase in NaCl supply from 0 mM to 90 mM. When plantlets were supplied with NO_3^--N + NH_4^+-N, exposure to 90 mM NaCl resulted in a decrease in growth for all parameters except, in this case, the length of the longest root (Figure 4.2 A). Root length of these plantlets was stimulated by 68% over those grown without NaCl. As for plantlets grown on NO_3^--N only, the leaf chlorophyll content (Figure 4.2 D) of plantlets grown on a combination of NO_3^--N + NH_4^+-N was significantly inhibited also by the presence of 90 mM NaCl. In contrast, the supply of 90 mM NaCl to plantlets growing on 60 mM NH_4^+-N did not have any significant effect on growth except for the parameter of root number (Figure 4.2 C)

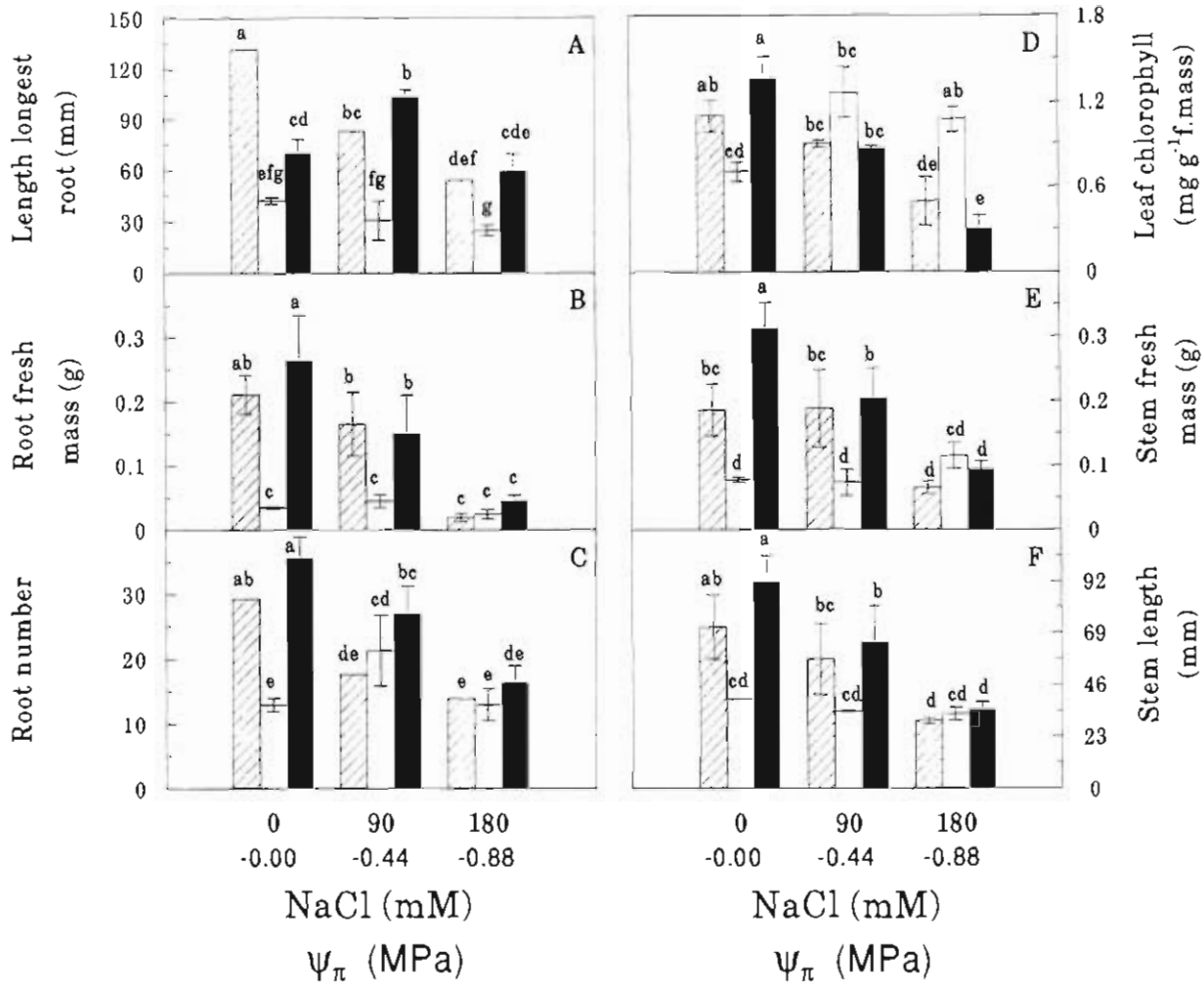


Figure 4.2: Effect of NaCl on root and stem growth of *in vitro* plantlets of *N. tabacum*, in the presence of 60 mM N supplied as NO_3^--N, NH_4^+-N, or NO_3^--N + NH_4^+-N (2:1).

Excised *in vitro* shoots were cultured on solid MS medium containing 0 - 180 mM NaCl and 60 mM N, where total N in the medium was supplied as NO_3^--N only, NH_4^+-N only, or NO_3^--N + NH_4^+-N (2:1). Measurements were obtained after 35 d in culture.

Symbols: ▨ (NO_3^--N), □ (NH_4^+-N), ■ (NO_3^--N + NH_4^+-N).

Values represent mean ± s.d.. Absence of error bars indicates a s.d. smaller than the lines on the graph. Letters associated with values represent mean separation by Duncan's Multiple Range Test, 5% significance value. n = 3.

where a positive response to NaCl addition was detected. Although the root number of these plantlets was still 20% lower than plantlets supplied with mixed N nutrition, it showed a 17% stimulation over plantlets supplied with NO_3^--N only. A similar trend was observed for the leaf chlorophyll content (Figure 4.2 D) where NH_4^+-N grown plantlets had approximately 30% more chlorophyll than both NO_3^--N grown and NO_3^--N + NH_4^+-N grown plantlets. Under non-saline conditions, NO_3^--N + NH_4^+-N nutrition was generally the most and NH_4^+-N the least favourable for growth. However, a supply of 90 mM NaCl appeared to reduce the variations in growth response such that there was little or no significant difference between the growth of plantlets on NO_3^--N or NO_3^--N + NH_4^+-N (60 mM total N).

An increase in the NaCl supply from 90 mM to 180 mM had drastic consequences for plantlet growth on all N regimes. Figure 4.2 illustrates a significant retardation of growth in all parameters. The root fresh mass (Figure 4.2 B) of NO_3^--grown plantlets was recorded to be inhibited by 91% compared to the control (0 mM NaCl). Similarly, the stem fresh mass (Figure 4.2 E) and stem length (Figure 4.2 F) of plantlets supplied with NO_3^--N + NH_4^+-N were inhibited by 70% and 62%, respectively, on comparison to the control. Furthermore, there was little or no significant difference in the growth response at 180 mM NaCl between the shoots subjected to the three N regimes for all parameters, with the exception of the length of the longest root (Figure 4.2 A). Although root length was inhibited by 180 mM NaCl, the same growth response to the various N regimes that was observed at 90 mM NaCl, applied at the highest NaCl concentration. The leaf chlorophyll content (Figure 4.2 D) of NH_4^+-N-grown plantlets was significantly greater at 180 mM NaCl than either of the two NO_3^--N-containing treatments, a response which paralleled that at 90 mM NaCl. In particular, the leaf chlorophyll content of the plantlets grown on NO_3^--N + NH_4^+-N was affected adversely by 180 mM NaCl, sustaining only 22% of the chlorophyll measured in control plantlets.

4.1.2.2 Root fresh mass:shoot fresh mass ratio

As discussed previously (Table 4.1 A), an increase in the supply of NaCl to plantlets grown on the standard MS formulation resulted in a decrease in the root fresh mass:shoot fresh mass ratio. Table 4.1 B indicates the effect of a variation in the N source on the relationship between root fresh mass and shoot fresh mass after 35 d growth in the presence and absence of NaCl. Plantlets supplied with NO_3^--N only exhibited a higher root fresh mass:shoot fresh mass ratio than NH_4^+-N fed

plantlets, at both 0 mM and 90 mM NaCl. In addition, an increase in the NaCl supply from 0 mM to 90 mM did not change the root fresh mass to shoot fresh mass ratio of these plantlets. However, at NaCl levels of 180 mM, the root fresh mass:shoot fresh mass ratio was significantly lower than at 0 mM and 90 mM. Furthermore, there was no difference in the ratio between plantlets supplied with NO_3^--N only and NH_4^+-N only. These observations suggest that an increase in the NaCl supply from 0 to 90 mM does not affect the ratio between root mass and stem mass under a specific N regime, but, a further increase to 180 mM has a more detrimental effect on root than stem growth. Moreover, at 180 mM NaCl, the effect on the ratio between root fresh mass and stem fresh mass was more drastic in plantlets supplied with NO_3^--N only than those supplied with NH_4^+-N only.

4.1.3 The effect of the level of nitrogen nutrition supplied to salt stressed shoot cultures

Having investigated the effects on growth of the form of N supply as a single source (NO_3^--N or NH_4^+-N) or a combination of NO_3^--N + NH_4^+-N in a 2:1 molar ratio with a total N concentration of 60 mM, the following analyses focused on the effect of an alteration in the level of NO_3^--N and NH_4^+-N supply. Nitrogen was supplied to shoot cultures as a single N form (either NO_3^--N or NH_4^+-N) but at different concentrations.

4.1.3.1 Nitrogen supply as NH_4^+-N only

Figure 4.3 illustrates the response of shoots after 35 d growth *in vitro* to an increase in the NH_4^+-N supply from 0 to 60 mM in both the absence and presence of NaCl. The growth response of shoot cultures provided with standard MS nutrients served as the control. Plantlets grown in the absence of N exhibited superior root fresh mass (Figure 4.3 B) and length of the longest root (Figure 4.3 A) on comparison to plantlets grown in the presence of 30 or 60 mM NH_4^+-N. An increase in the NaCl concentration did not result in any further significant change in growth response, regardless of the NH_4^+-N concentration supplied to the shoots. An elevated NH_4^+-N supply in the absence of NaCl did not favor an increase in root number (Figure 4.3 C) but in the presence of 90 and 180 mM NaCl, a positive stimulation of root production by increased NH_4^+-N levels was observed. Leaf chlorophyll contents (Figure 4.3 D) of plantlets grown without N were greater than those supplied with both NH_4^+-N levels and, furthermore, were similar to the MS control in the

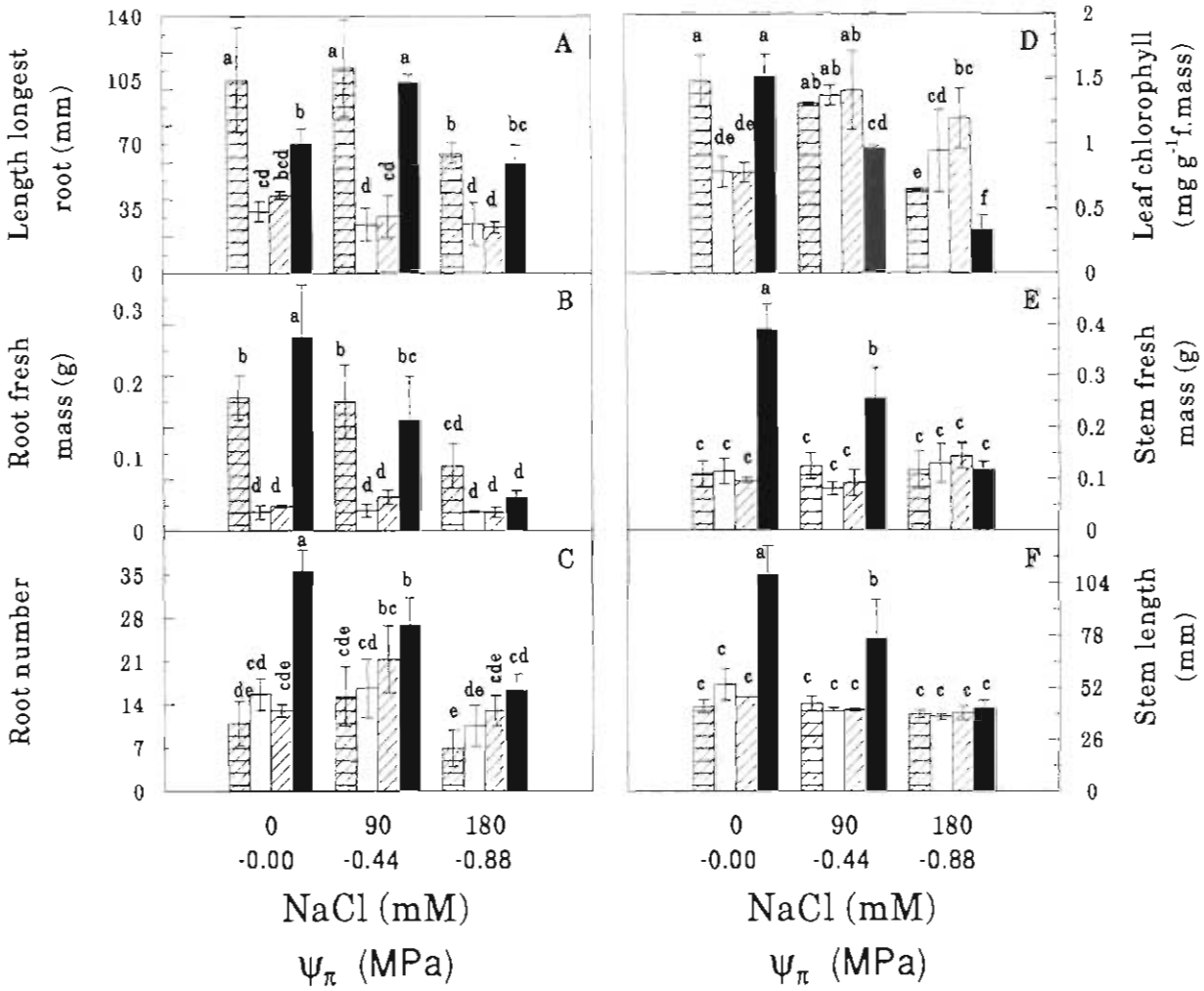


Figure 4.3: Effect of NaCl on root and stem growth of *in vitro* plantlets of *N. tabacum*, in the presence of NH_4^+-N.

Excised *in vitro* shoots were cultured on solid MS medium containing 0 - 180 mM NaCl and 0 - 60 mM NH_4^+-N, where total N in the medium was supplied as NH_4^+-N. Shoots cultured on standard MS nutrients (40 mM NO_3^--N + 20 mM NH_4^+-N) served as controls. Measurements were obtained after 35 d in culture.
Symbols: (mM N) (0), (30), (60), (60) (control).

Values represent mean ± s.d.. Letters associated with values represent mean separation by Duncan's Multiple Range Test, 5% significance value. n = 3.

absence of NaCl. Under saline conditions, leaf chlorophyll levels were enhanced in plantlets supplied with NH_4^+-N. An increase in the NH_4^+-N concentration, regardless of NaCl level, had no significant effect on growth of any of the stem parameters (Figure 4.3 E, F). The results obtained here indicate that the negative effects of NH_4^+-N on *N. tabacum* growth in culture may be more severe than those of NaCl.

4.1.3.2 Nitrogen supply as NO_3^--N only

Figure 4.2 demonstrates that plantlet growth on 60 mM N as NO_3^--N is superior to that on 60 mM NH_4^+-N. The observed growth response of shoots to NaCl on nutrient media supplied with increasing levels of NO_3^--N only (Figure 4.4) contrasted with those on NH_4^+-N only (Figure 4.3). Shoots supplied with NO_3^--N as the sole N source showed significantly improved growth at all N levels tested and for all parameters, over those supplied with NH_4^+-N as sole N source.

Growth and morphology

In the absence of NaCl, an increase in NO_3^--N up to 60 mM resulted in a significant improvement in root number (Figure 4.4 C) and length of the longest root (Figure 4.4 A), while no significant effect of NO_3^--N concentration was detected in the response of root fresh mass (Figure 4.4 B). For the stem parameters (Figure 4.4 E, F), a NO_3^--N supply of 30 mM produced the most positive response. Visual examination of the cultures (Plates 4.2 A, B) indicated a marked improvement in stem growth when the NO_3^--N supply was augmented from 0 mM (Plate 4.2 A) to 30 mM (Plate 4.2 B). Growth analyses confirmed this observation and further indicated that a supply of 30 mM NO_3^--N was the most favourable for stem production (Figure 4.4 E, F). Leaf chlorophyll content (Figure 4.4 D) was maximal in the absence of N and, in addition, was higher than that of plantlets grown on standard MS nutrients. The highest NO_3^--N concentration tested (120 mM NO_3^--N) resulted in a significant inhibition of all growth parameters and leaf chlorophyll content, except for root fresh mass (Figure 4.4 B) which showed no significant alteration when supplied with any of the concentrations of NO_3^--N tested.

An increase in the NaCl concentration from 0 mM to 180 mM resulted in a reduction in growth at all NO_3^--N levels tested. At both 90 and 180 mM NaCl, an increase in NO_3^--N up to 60 mM still resulted in slight growth improvements for

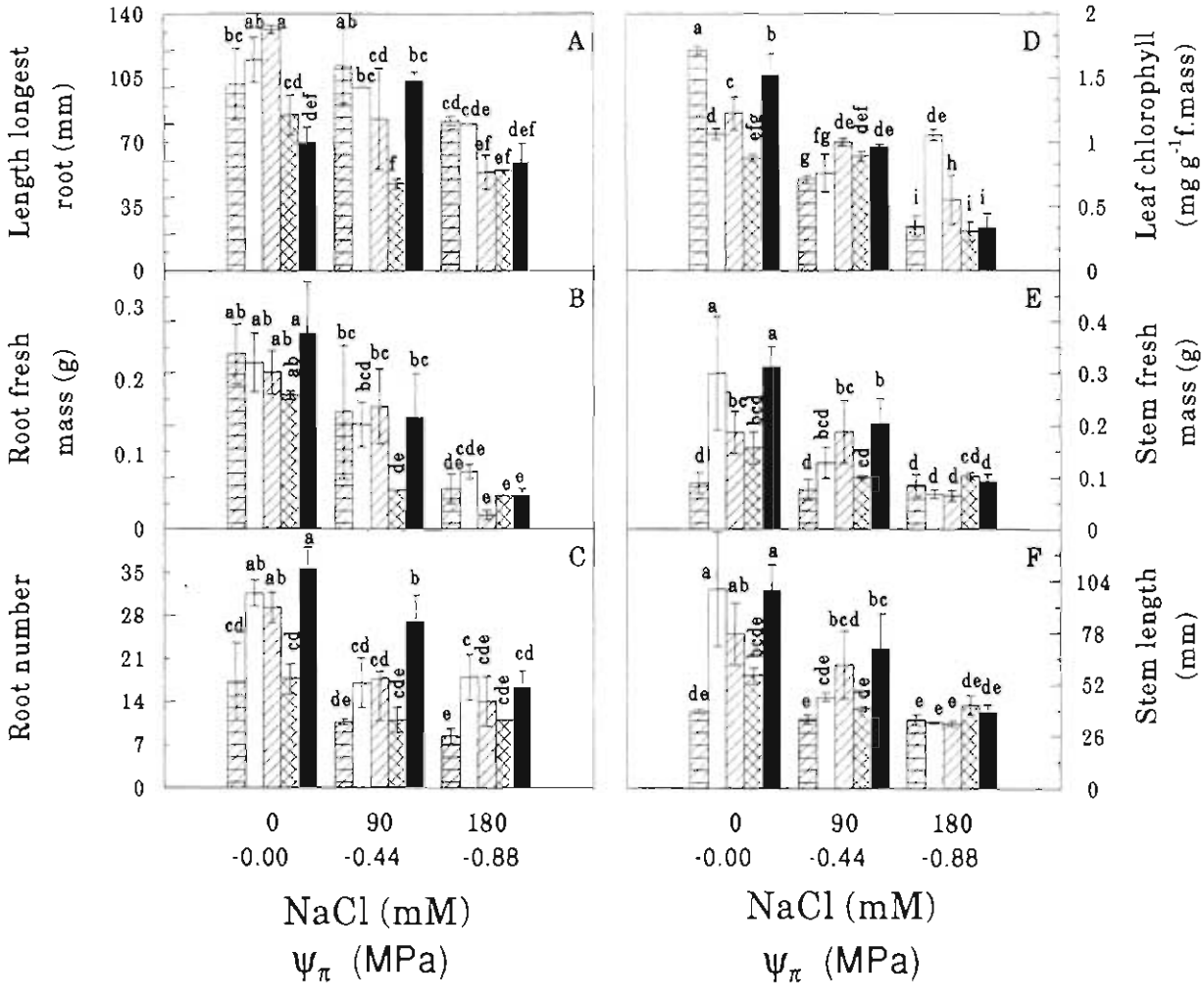


Figure 4.4: Effect of NaCl on root and stem growth of *in vitro* plantlets of *N. tabacum*, in the presence of NO_3^--N.

Excised *in vitro* shoots were cultured on solid MS medium containing 0 - 180 mM NaCl and 0 - 120 mM NO_3^--N, where total N in the medium was supplied as NO_3^--N. Shoots cultured on standard MS nutrients (40 mM NO_3^--N + 20 mM NH_4^+-N) served as controls. Measurements were obtained after 35 d in culture.
Symbols: (mM N) (0), (30), (60), (60) (control), (120).

Values represent mean ± s.d.. Absence of error bars indicates a s.d. smaller than the lines on the graph. Letters associated with values represent mean separation by Duncan's Multiple Range Test, 5% significance value. n=3.

Plate 4.2: Effect of NaCl and nitrate supply on stem growth of *in vitro* plantlets of *N. tabacum*.

Treatments as described in Figure 4.4. Photographs were taken after 35 d in culture. Photographs are to scale.

A: 0 mM NaCl, 0 mM NO_3^-

B: 0 mM NaCl, 30 mM NO

C: 180 mM NaCl, 0 mM NO_3^-

D: 180 mM NaCl, 30 mM NO_3^-

E: 180 mM NaCl, 60 mM N

most parameters, as was observed in the absence of NaCl. However, the parameters of root fresh mass (Figure 4.4 B) and stem length (Figure 4.4 F) showed no growth improvement with an increase in NO_3^--N when grown on 90 and 180 mM NaCl, respectively. The retardation in stem length at 180 mM NaCl was particularly noticeable, as demonstrated in Plates 4.2 C-E. A supply of 0 mM (Plate 4.2 C), 30 mM (Plate 4.2 D) and 60 mM (Plate 4.2 E) NO_3^--N had no positive effect on stem growth in these cultures. The length of the longest root (Figure 4.4 A) was inhibited significantly by an increase in NO_3^--N up to 120 mM at both 90 and 180 mM NaCl. A NO_3^--N supply of 120 mM resulted in an inhibition of growth at 90 and 180 mM NaCl for all parameters except stem length (Figure 4.4 F) and stem fresh mass (Figure 4.4 E) where no alteration in growth response to an increase in NO_3^--N supply at 180 mM NaCl was detected. Leaf chlorophyll content (Figure 4.4 D), although reduced by the presence of NaCl, was not affected by NO_3^--N supply at 90 mM NaCl. A NO_3^--N level of 60 mM produced the highest chlorophyll levels in plantlets exposed to 180 mM NaCl. The above data indicated that, regardless of levels of NO_3^--N supply, growth of *in vitro* shoot cultures of *N. tabacum* on NaCl-containing growth media was always inferior to growth in the absence of NaCl.

Root fresh mass:shoot fresh mass ratio

These growth studies (Figure 4.4) disclosed that stem and root growth are differentially affected by changes in the NaCl concentrations in the culture medium in the presence of NO_3^--N. This is reflected by variations in the root fresh mass:shoot fresh mass ratios (Table 4.1 C). An increase in the NaCl concentration from 0 to 180 mM resulted in a decrease in the root fresh mass:shoot fresh mass ratio for all N treatments except at 30 mM NO_3^--N, where no change was observed. Similarly, a general decline in the root fresh mass:shoot fresh mass ratio was observed with an increase in the NO_3^--N concentration from 0 to 120 mM, at any particular NaCl concentration. These ratios suggest that while an increase in the NO_3^--N supply to a plantlet has a more positive effect on stem growth than root growth, an increase in NaCl supply results in a more negative effect on root growth than stem growth.

Dry mass:fresh mass ratio

Table 4.2: Effect of NaCl and N supply on the dry mass:fresh mass ratio of *in vitro* plantlets of *N.tabacum*.

Treatments as described in Figures 4.1, 4.4. n=3.

Data indicates dry mass:fresh mass ratio of plantlets.

NaCl (mM) / (MPa) / N (mM) NO_3^--N	NH_4^+-N	0 -0.00	90 -0.44	180 -0.88
ROOTS				
40	20	0.057ef	0.057ef	0.068cdef
0	-	0.072cde	0.082abc	0.078bcd
30	-	0.057ef	0.052f	0.096a
60	-	0.053f	0.067cdef	0.079bcd
120	-	0.063def	0.091ab	0.074cd
STEMS				
40	20	0.044bc	0.058cde	0.047efg
0	-	0.072bc	0.056def	0.029h
30	-	0.039gh	0.111a	0.049efg
60	-	0.042g	0.11a	0.06cde
120	-	0.062cd	0.069bcd	0.081b
LEAVES				
40	20	0.047d	0.052cd	0.06bc
0	-	0.069b	0.064bc	0.07b
30	-	0.043d	0.051cd	0.061bc
60	-	0.044d	0.062bc	0.063bc
120	-	0.059bc	0.054cd	0.103a

Letters adjacent to values represent mean separation by Duncan's Multiple Range Test, 5% significance value.

The dry mass:fresh mass ratio of individual plantlet parts (roots, stems and leaves) was determined after plantlet harvest at the end of 35 d and is shown in Table 4.2. An incremental increase in the NaCl supply from 0 to 180 mM generally resulted in an increase in the dry mass:fresh mass ratio for both leaves and roots of plantlets grown on all concentrations and forms of N. No apparent relationship between the various N treatments with an increase in NaCl was observed for the stem component. Similarly, an increase in the NO_3^--N supply from 0 mM to 120 mM, and a N supply as 40 mM NO_3^--N + 20 mM NH_4^+-N, did not appear to affect the dry mass:fresh mass ratio in a uniform manner in plantlets exposed to a particular NaCl concentration. The increase in dry mass:fresh mass ratio of plantlets exposed to an increase in NaCl supply suggests a decrease in the water content of these plantlets under saline conditions.

4.1.4 The effect of levels and forms of nitrogen on the rate of root emergence from salt stressed shoot cultures

The time of root emergence in response to the presence of NaCl from shoot cultures grown on standard MS nutrients (40 mM NO_3^--N + 20 mM NH_4^+-N) was monitored, and results indicate that the period taken for root emergence increased with increments in NaCl supply (Table 4.3 A). For control plantlets (0 mM NaCl), root emergence occurred after 10 d. An increase in the NaCl supply up to 180 mM repressed emergence by a further 5 days. This delay is reflected in the reduction of root fresh mass and root number shown by plantlets grown under conditions of high NaCl (Figure 4.1). When the N content of the media was altered such that shoot cultures were supplied with varying levels of NO_3^--N only or NH_4^+-N only, certain differences in root emergence compared to that on MS nutrient medium were detected (Table 4.3 B). An increase in NaCl concentration from 0 mM to 180 mM resulted in a delay of root emergence for shoots grown on both NO_3^--N only and NH_4^+-N only. However, in most cases, shoots grown on nutrient media containing 0 mM and 90 mM NaCl produced roots faster when supplied with a single N source (NO_3^--N or NH_4^+-N) than when provided with a mixed NO_3^--N/NH_4^+-N source (MS nutrients). There was no difference in the time of root emergence for shoot cultures grown on 180 mM NaCl under all tested N regimes. Little overall difference between the rooting response of shoots provided with NO_3^--N only or

NH_4^+-N only was detected with an increase in the NaCl supply. In addition, an increase in the N supply had very little positive effect on root emergence in most cases.

Table 4.3: Effect of NaCl and N supply on the rate of root emergence from *in vitro* shoot cultures of *N. tabacum*.

Treatments as described in Figures 4.1, 4.3, 4.4. n=10.

Data indicates days taken for 100% of shoot explants to produce roots.

NaCl (mM) / (MPa)		0	90	180
		-0.00	-0.44	-0.88
N (mM) NO_3^--N	NH_4-N			
A 40	20	10	11	15
B 0	-	6	6	15
30	-	6	7	15
60	-	6	9	15
120	-	7	12	15
-	0	6	6	15
-	30	7	9	15
-	60	8	8	15

4.1.5 The effect of nitrogen nutrition on *in vitro* shoot cultures grown on a non-ionic osmoticum

4.1.5.1 The effect of mannitol on *in vitro* shoot cultures

Once it had been established that NaCl concentrations between 45 and 180 mM caused a decline in growth of *in vitro* cultures of *N. tabacum*, the question arose whether the growth responses observed were due primarily to osmotic or ionic

effects of NaCl. This was investigated by replacing NaCl with isosmotic concentrations of mannitol, and culturing shoots of *N. tabacum* on MS nutrient media (40 mM NO_3^--N + 20 mM NH_4^+-N) as described in Section 4.1.1 above. The osmotic potential (MPa) of the mannitol, corresponding to 0, 45, 90 and 180 mM NaCl, was calculated as -0.00, -0.22, -0.44, and -0.88 MPa. In addition to the osmotic potential imposed by NaCl, the MS nutrient media components also exerted an osmotic potential of -0.19 MPa.

Growth and morphology

The growth of shoots of *N. tabacum* on a non-ionic osmoticum was monitored over a 35 d period (Figure 4.5). There was a general increase in growth for all parameters over a 35 d period, with a subsequent inhibition as the osmotic potential was changed from -0.22 to -0.88 MPa. Root number (Figure 4.5 C) increased over a period of 21 d and stabilised thereafter, while the leaf chlorophyll content (Figure 4.5 D) showed a steady decline, at all osmotic potentials, with an increase in time. At the end of 35 d, growth of plantlets on -0.00 MPa was superior for all parameters except length of the longest root (Figure 4.5 A). Plantlets grown on -0.22 MPa had longer roots than those grown on -0.00 MPa. For the stem parameters measured after 35 d in culture (Figure 4.5 E, F), a decrease in the osmotic potential from -0.22 MPa to -0.88 MPa correlated with a progressive decline in growth. However, there was little difference in the root fresh mass (Figure 4.5 B) between plantlets grown on -0.00, -0.22 and -0.88 MPa, except for those on -0.44 MPa which exhibited the lowest root fresh mass. There was no difference in root number (Figure 4.5 C) between plantlets grown on -0.22 and -0.44 MPa, while the least number of roots were produced in plantlets exposed to -0.88 MPa. Visual comparisons of plantlets grown on mannitol for 35 d revealed that the new leaves produced by these plantlets had a high chlorophyll content (Plate 4.3 A-D), particularly noticeable at an osmotic potential of -0.44 MPa (Plate 4.3 C). Determinations of leaf chlorophyll content revealed no significant differences between plantlets grown on all tested osmotic potentials.

The growth of shoot cultures on mannitol (Figure 4.5) shared many similarities with growth on NaCl at isosmotic concentrations (Figure 4.1). For comparative purposes, the response of plantlets *in vitro* to growth on NaCl and mannitol were related after 35 d growth, and are illustrated in Figure 4.6.

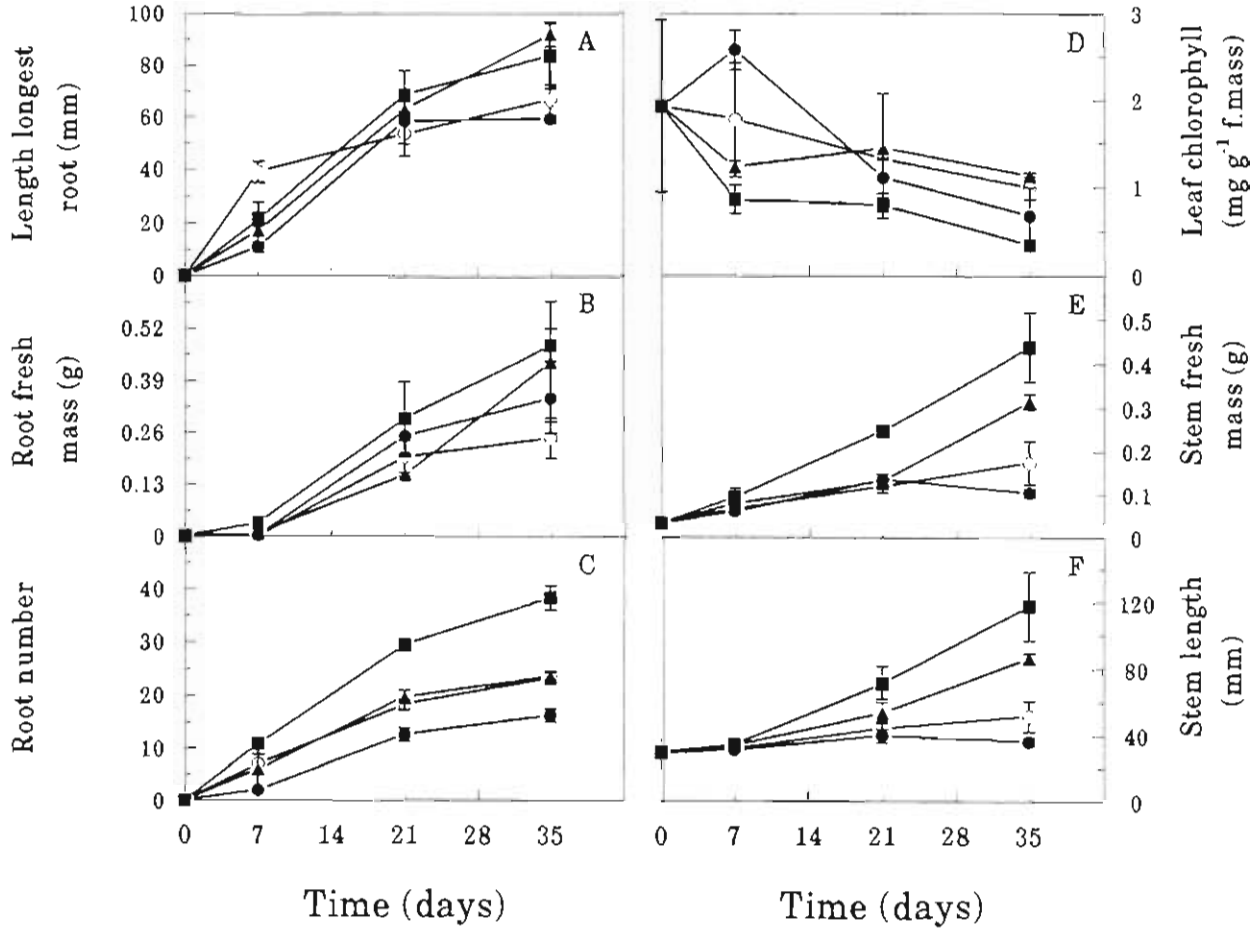


Figure 4.5: Effect of mannitol on root and stem growth of *in vitro* plantlets of *N. tabacum*.

Excised *in vitro* shoots were cultured on solid MS medium containing 60 mM N (as 40 mM NO_3^--N + 20 mM NH_4^+-N) and -0.00 to -0.88 MPa mannitol for a 35 d period.

Symbols: (MPa mannitol) ■ (-0.00), ○ (-0.22), ▲ (-0.44), ● (-0.88), corresponding to 0, 45, 90, 180 mM NaCl respectively.

Values represent mean ± s.d.. Absence of error bars indicates a s.d. smaller than the symbol. n=3.

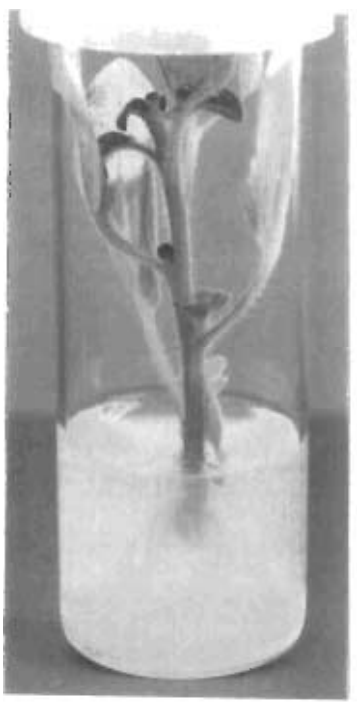
Plate 4.3: Leaf chlorophyll content of plantlets grown on mannitol.

Treatments as described in Figure 4.5. Photographs were taken after 35 d in culture. Note the particularly dark green leaves of the plantlet grown on -0.44 MPa. Photographs are to scale.

A: -0.00 MPa

B: -0.22 MPa

C: -0.44 MPa



D: -0.88 MPa

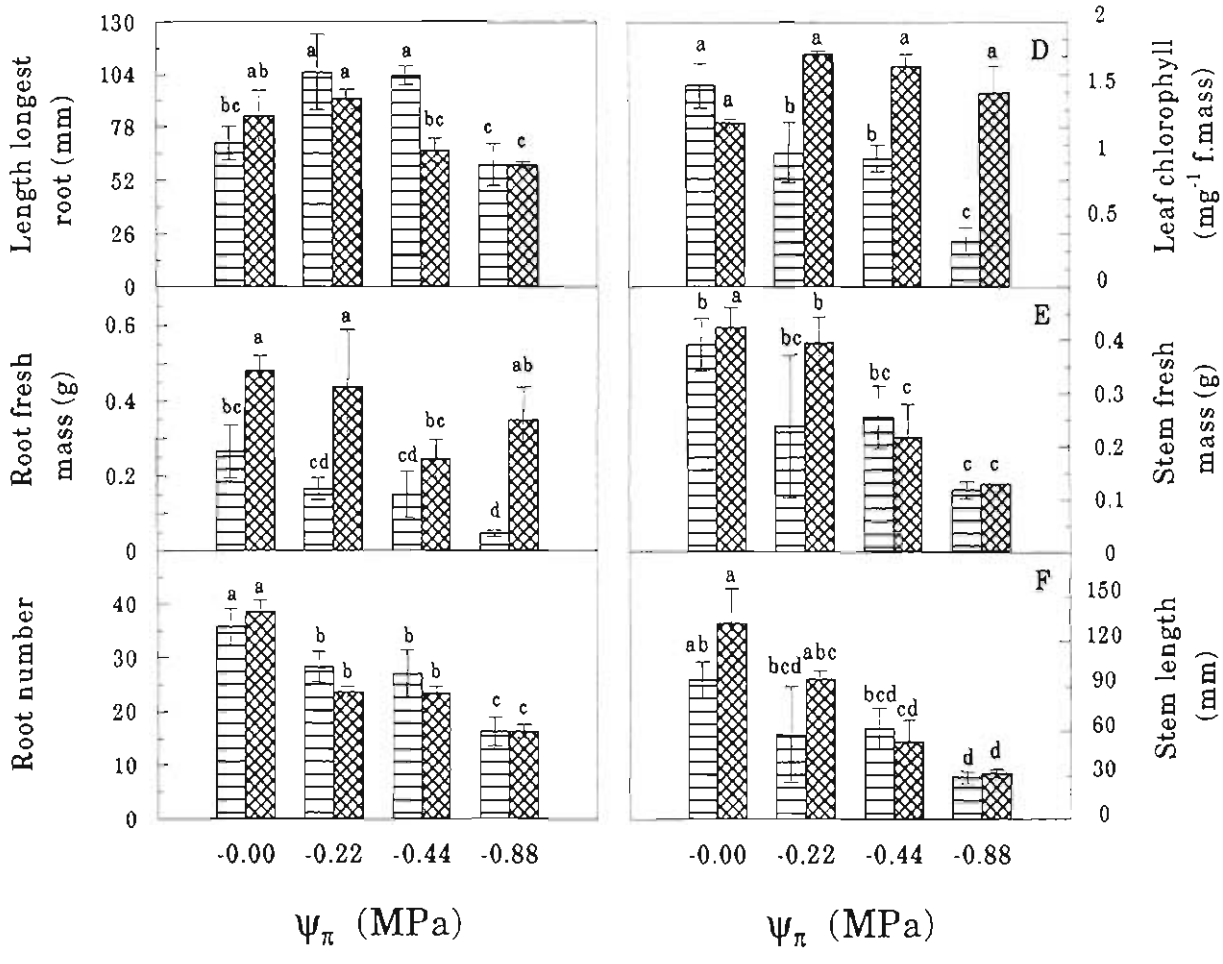


Figure 4.6: Comparative effects of isosmotic concentrations of NaCl and mannitol on root and stem growth of *N. tabacum* plantlets *in vitro*.

Excised *in vitro* shoots were cultured on solid MS medium containing 60 mM N (as 40 mM NO_3^--N + 20 mM NH_4^+-N) and 0 - 180 mM, and -0.00 - -0.88 MPa, NaCl or mannitol, respectively. Measurements were obtained after 35 d in culture.
Symbols: ▤ (NaCl), ▩ (mannitol).

Values represent mean ± s.d.. Letters associated with values represent mean separation by Duncan's Multiple Range Test, 5% significance value. n=3.

Root fresh mass:shoot fresh mass ratio

An increase in the mannitol supply isosmotic to NaCl resulted in a marked increase in the root fresh mass:shoot fresh mass ratio (Table 4.4 A). This indicates that shoot growth was more negatively affected by an increase in the osmotic potential from -0.00 MPa to -0.88 MPa, induced by a non-ionic osmoticum, than root growth.

Table 4.4: Effect of mannitol and N supply on the root fresh mass:shoot fresh mass ratio of *in vitro* plantlets of *N. tabacum*.

Treatments as described in Figures 4.6, 4.7. n=3.

Data indicates root fresh mass:shoot fresh mass ratio of plantlets.

Mannitol (MPa)		-0.00	-0.44	-0.88
N (mM) NO_3^--N	NH_4^+-N			
A 40	20	0.838a	1.536ab	3.413b
B 0	-	2.757a	1.645b	0.505d
30	-	0.842bcd	1.603b	1.240bcd
60	-	1.182bcd	0.832bcd	1.34bc
120	-	1.198bcd	1.010bcd	0.545cd

Letters adjacent to values represent mean separation by Duncan's Multiple Range Test, 5% significance value.

4.1.5.2 The effect of the level of nitrogen nutrition supplied to *in vitro* shoot cultures grown on mannitol

Previous investigations of mannitol grown plantlets (Figure 4.5) demonstrated variations in the response of the growth parameters root fresh mass, stem fresh mass

and stem length, to variations in the osmotic potential of the growth medium as induced by a non-ionic osmoticum. In contrast, leaf chlorophyll content exhibited no significant alteration with a change in the osmotic potential. As the plantlets had been cultured on standard MS nutrients containing 40 mM NO_3^--N + 20 mM NH_4^+-N, subsequent analyses of these parameters were performed on shoot cultures supplied with variable amounts of NO_3^--N only, while in the presence of mannitol (Figure 4.7).

Growth and morphology

An increase in the NO_3^--N supply from 0 to 120 mM had a negligible effect on stem fresh mass (Figure 4.7 D), stem length (Figure 4.7 C), and leaf chlorophyll content (Figure 4.7 A) at both osmotic potentials of -0.44 and -0.88 MPa. Root fresh mass (Figure 4.7 B) was inhibited by an incremental NO_3^--N supply at -0.44 MPa, while at -0.88 MPa a favourable response to NO_3^--N additions up to 60 mM was detected. In most cases, shoot cultures grown on mannitol with an increasing supply of NO_3^--N only exhibited less favourable responses to those grown on a combination of NO_3^--N + NH_4^+-N. The addition of NO_3^--N to shoot cultures supplied with a non-ionic osmoticum did not appear thus to have a very significant effect on growth.

Root fresh mass:shoot fresh mass ratio

A change in the osmotic potential from -0.00 to -0.88 MPa, as induced by mannitol, did not result in a significant change in the root fresh mass:shoot fresh mass ratio for plantlets supplied with 30 mM, 60 mM and 120 mM NO_3^--N (Table 4.4 B). A decrease in the ratio between root fresh mass and shoot fresh mass with increase in osmotic potential was detected in plantlets grown in the absence of N. An increase in the NO_3^--N supply from 0 mM to 120 mM to plantlets grown at any particular osmotic potential tested did not result in a linear relationship in the root fresh mass:shoot fresh mass ratio.

4.1.5.3 <u>The effect of levels and forms of nitrogen on the rate of root emergence from shoot cultures grown on mannitol</u>

Root emergence from shoots cultured on standard MS nutrients (40 mM NO_3^--N + 20 mM NH_4^+-N) and supplied with mannitol at concentrations isosmotic to the

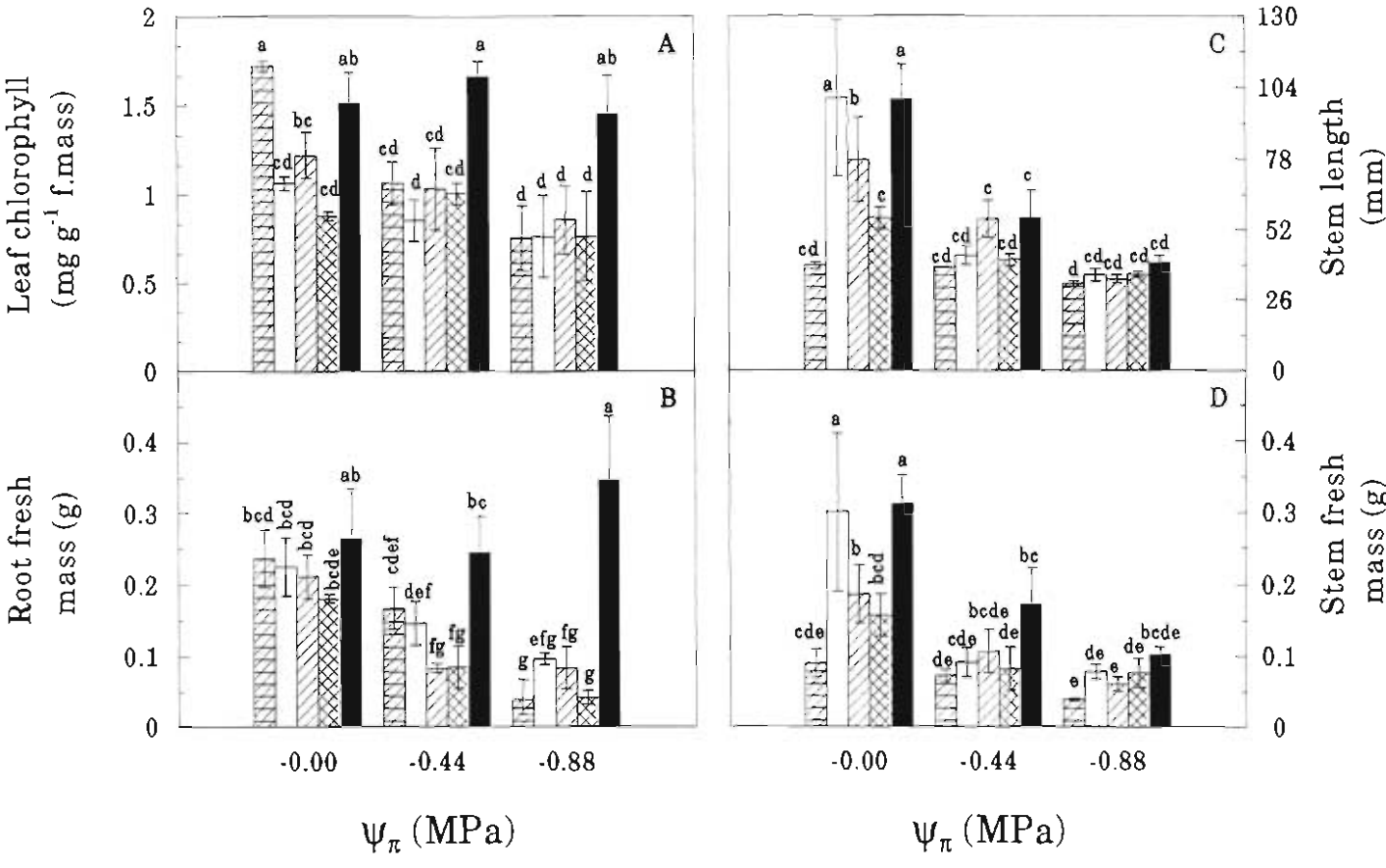


Figure 4.7: Effect of mannitol on root and stem growth of *in vitro* plantlets of *N. tabacum*, in the presence of NO_3^--N.

Excised *in vitro* shoots were cultured on solid MS medium containing -0.00 to -0.88 MPa mannitol and 0 - 120 mM NO_3^--N, where total N in the medium was supplied as NO_3^--N. Shoots cultured on standard MS nutrients (40 mM NO_3^--N + 20 mM NH_4^+-N) served as controls. Measurements were obtained after 35 d in culture. Symbols: (mM N) (0), (30), (60), (60) (control), (120).

Values represent mean ± s.d.. Letters associated with values represent mean separation by Duncan's Multiple Range Test, 5% significance value. n = 3.

NaCl supply described previously in Sections 4.1.1 to 4.1.3 are represented in Table 4.5 A. An osmotic stress applied in the form of a non-ionic osmoticum resulted in the emergence of roots from shoot cultures after 10 d at all osmotic potentials. When shoots were supplied with NO_3^--N only (0 to 120 mM), the time of root emergence contrasted with that on MS nutrient medium (Table 4.5 B). At -0.44 MPa, roots emerged four days earlier in the absence of N than when supplied with 40 mM NO_3^--N + 20 mM NH_4^+-N (Table 4.5 A), but were delayed by four days in the presence of all tested concentrations of NO_3^--N. In contrast, shoots grown on mannitol at an osmotic potential of -0.88 MPa, in the absence of N, took nine days longer to emerge than when grown at -0.44 MPa. When cultured in the presence of 30 mM to 120 mM NO_3^--N, no difference in the time of root emergence was observed between shoots grown on -0.44 MPa or -0.88 MPa.

Table 4.5: Effect of mannitol and N supply on the rate of root emergence from *in vitro* shoot cultures of *N. tabacum*.

Treatments as described in Figures 4.6, 4.7. n = 10.

Data indicates days taken for 100% of shoot explants to produce roots.

Mannitol (MPa)		-0.00	-0.44	-0.88
N (mM) NO_3^--N	NH_4-N			
A 40	20	10	10	10
B 0	-	6	6	15
30	-	6	14	14
60	-	6	14	14
120	-	7	14	14

4.2 ION UPTAKE BY *in vitro* CULTURES OF *N. tabacum* GROWN ON VARIOUS NaCl AND NITROGEN NUTRITION REGIMES

4.2.1 Nitrate uptake

4.2.1.1 The effect of the form and level of nitrogen supplied to salt stressed shoot cultures

The level and form of nitrogen supplied to shoot cultures of *N. tabacum* during a 35 d growth period in the presence and absence of NaCl had a pronounced effect on the uptake of nitrate (Figure 4.8). Nitrate uptake was determined after plantlet harvest at the end of 35 d and was expressed as the disappearance of nitrate from the culture medium. An increase in the concentration of N provided to plantlets as NO_3^--N only, in the absence of NaCl, resulted in a significant increase in the disappearance of nitrate from the culture medium as the NO_3^--N concentration was augmented from 30 mM to 120 mM. Plantlets supplied with 40 mM NO_3^--N + 20 mM NH_4^+-N (standard MS nutrients) removed similar amounts of NO_3^--N from the medium as those exposed to 60 mM NO_3^--N only (344.44 and 354.20 μmol NO_3^--N, respectively, after 35 d in culture). When nitrate uptake was expressed as the total percentage removed from the medium (Figure 4.8 insert), it indicated that plantlets grown on 30 mM NO_3^--N removed 73% of the total supply while those grown on 40 mM NO_3^--N + 20 mM NH_4^+-N, sequestered 86% of the nitrate provided in the nutrient medium. Nitrate uptake of plantlets exposed to 60 and 120 mM NO_3^--N represented only approximately 60% and 50%, respectively, of the total NO_3^--N supply.

The addition of NaCl to the culture medium resulted in a significant inhibition of nitrate uptake by plantlets grown on all NO_3^--N concentrations, and when supplied with 40 mM NO_3^--N + 20 mM NH_4^+-N. Nitrate uptake in the presence of NaCl exhibited an analogous trend in response to alterations in the NO_3^--N supply as that reported in the absence of NaCl. The differences in nitrate uptake between plantlets exposed to 90 mM and 180 mM NaCl were slight. Nitrate uptake in plantlets grown on 40 mM NO_3^--N + 20 mM NH_4^+-N was the most adversely affected by the presence of NaCl in the culture medium, being reduced by 40% upon exposure to NaCl. In addition, uptake was no different to that of plantlets supplied with 30 mM NO_3^--N in the presence of NaCl. These data indicate that the presence of NaCl has an adverse effect on nitrate uptake, but suggests that nitrate

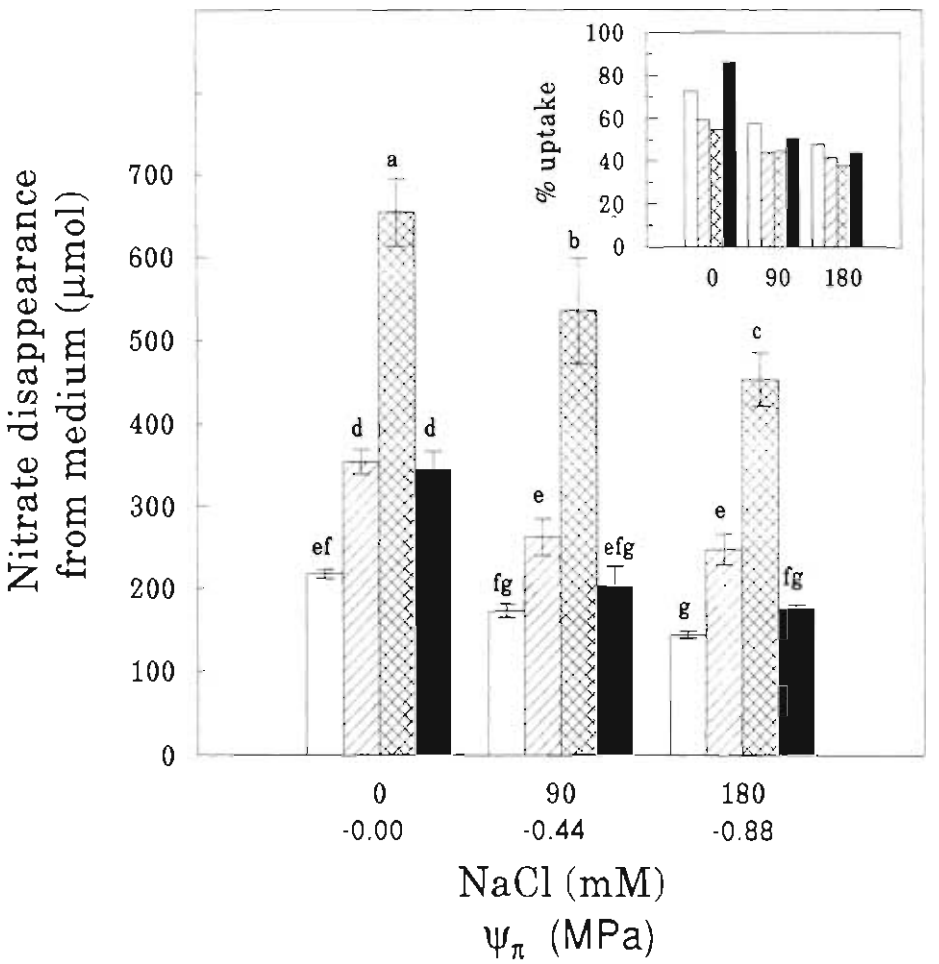


Figure 4.8: Effect of NaCl and N supply on nitrate uptake by *in vitro* plantlets of *N. tabacum*.

Treatments as described in Figure 4.4. Measurements were obtained after 35 d in culture. Nitrate uptake is expressed as disappearance from the growth medium. Symbols: (mM N) (30), (60), (60) (control), (120).

Values represent mean ± s.d.. Letters associated with values represent mean separation by Duncan's Multiple Range Test, 5% significance value. n=3.

utilisation from a mixed N supply as NO_3^--N and NH_4^+-N is more severely affected by salt than a sole N source as NO_3^--N only.

4.2.1.2 The effect of the form and level of nitrogen supplied to shoot cultures grown on a non-ionic osmoticum

The response of nitrate uptake by shoots grown under various conditions of N nutrition, to an increase in mannitol supply over a 35 d period, is shown in Figure 4.9. When mannitol was supplied at concentrations isosmotic to the NaCl supply, nitrate uptake was inhibited in plantlets grown on -0.44 and -0.88 MPa, on comparison to the control (-0.00 MPa). An increase in the NO_3^--N supply from 0 mM to 120 mM resulted in a concomitant increase in nitrate uptake. Nitrate uptake by plantlets supplied with 40 mM NO_3^--N + 20 mM NH_4^+-N (MS nutrient medium) was reduced, with values lower than those for plantlets supplied with 60 mM NO_3^--N. However, when expressed on a percentage basis, plantlets supplied with NO_3^--N + NH_4^+-N removed approximately 66% of the nitrate supplied in the growth medium at both -0.44 and -0.88 MPa, matching the percentage uptake of plantlets supplied with 30 mM NO_3^--N (Figure 4.9 insert). There was little difference in nitrate uptake between plantlets grown on -0.44 and -0.88 MPa for all NO_3^--N concentrations except 120 mM, for which nitrate uptake was greater in plantlets grown on -0.44 MPa. Furthermore, there was little significant difference between nitrate uptake by plantlets grown on an osmotic potential of -0.00, -0.44, and -0.88 MPa when supplied with either 30 mM or 60 mM NO_3^--N.

4.2.2 Sodium and chloride uptake

Competition between sodium and chloride ions and nitrate ions for uptake has been reported (Section 2.4.2). In particular, a direct antagonistic relationship between nitrate and chloride ions has been proposed. Also, it has been suggested that an additional nitrate supply to a saline environment would allow for an increase in nitrate uptake by plants, with a concomitant decrease in chloride uptake (Section 2.4.2). Similarly, there have been reports of an analogous relationship between sodium uptake and an ammonium supply (Section 2.4.2). The disappearance of sodium (Na^+) and chloride (Cl^-) ions from the nutrient medium of *in vitro* shoot cultures of *N. tabacum* supplied with varying levels of N and NaCl was measured after 35 d growth and is represented in Figure 4.10.

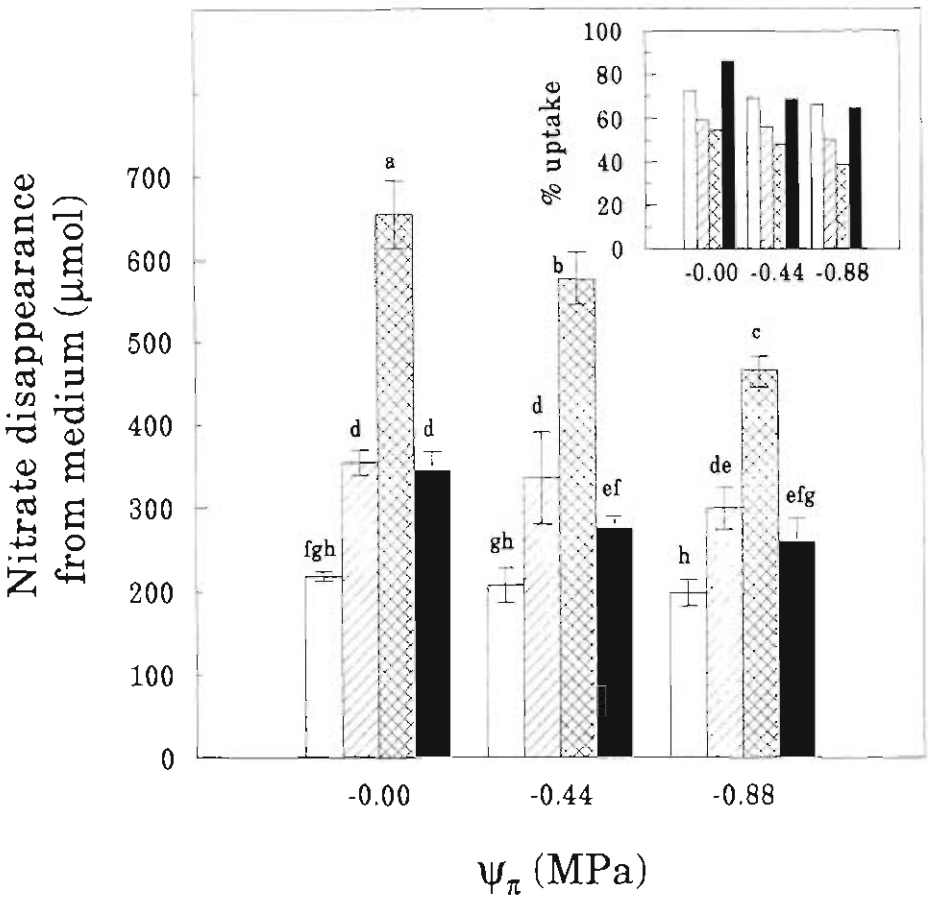


Figure 4.9: Effect of mannitol and N supply on nitrate uptake by *in vitro* plantlets of *N. tabacum*.

Treatments as described in Figure 4.7. Measurements were obtained after 35 d in culture. Nitrate uptake is expressed as disappearance from the growth medium. Symbols: (mM N) ☐ (30), ▨ (60), ■ (60) (control), ▩ (120).

Values represent mean ± s.d.. Letters associated with values represent mean separation by Duncan's Multiple Range Test, 5% significance value. n = 3.

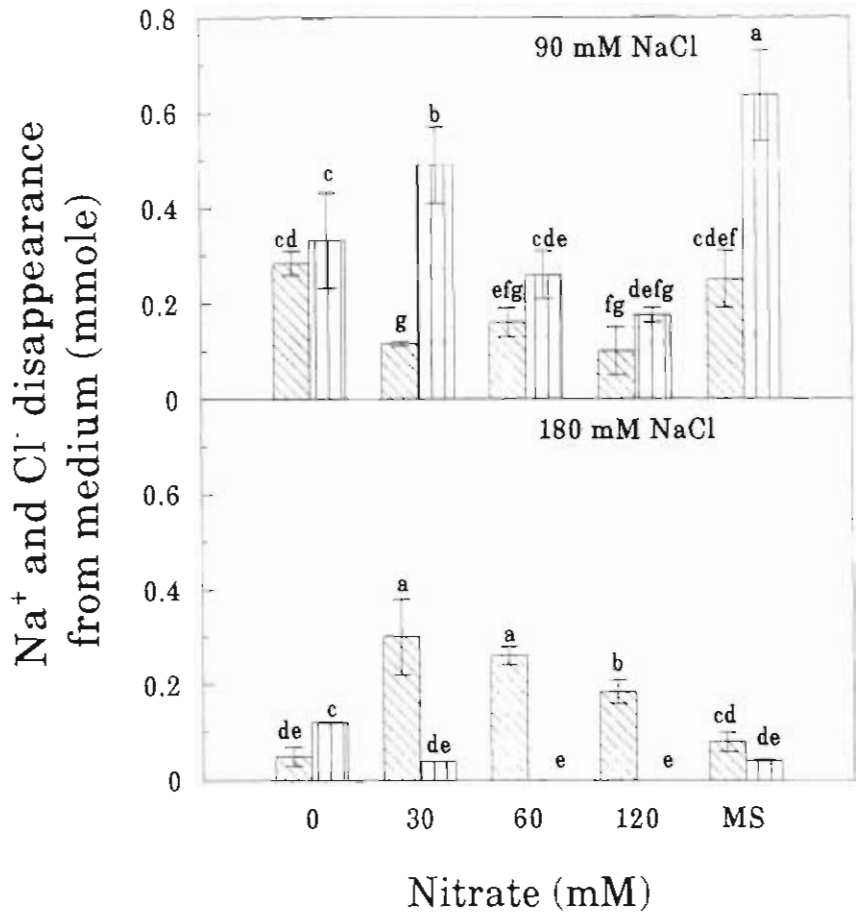


Figure 4.10: Effect of N supply on sodium and chloride uptake by *in vitro* plantlets of *N. tabacum*.

Treatments as described in Figure 4.4. Measurements were obtained after 35 d in culture. Sodium and chloride uptake is expressed as disappearance from the growth medium.

Symbols: (Na$^+$), (Cl$^-$).

Values represent mean ± s.d.. Absence of error bars indicates a s.d. smaller than the lines on the graph. Letters associated with values represent mean separation by Duncan's Multiple Range Test, 5% significance value. n=3.

When plantlets were grown on 90 mM NaCl, an alteration in the NO_3^--N supply resulted in both increases and decreases in the uptake of Na^+ ions. Sodium uptake was maximal in the absence of N (0.29 mmole) and was inhibited by a NO_3^--N supply from 30 mM to 120 mM. Little difference in Na^+ uptake between the various NO_3^--N regimes was recorded. When N was present in the nutrient medium as NO_3^--N + NH_4^+-N, Na^+ uptake was similar to that observed in the absence of N. An increase in the NaCl supply up to 180 mM resulted in a decline in Na^+ uptake. At this NaCl level, Na^+ uptake was minimal in the absence of N, while an increase in the NO_3^--N concentration from 30 mM to 120 mM caused a significant decline in Na^+ uptake. Plantlets supplied with a mixed N nutrition removed similar amounts of Na^+ from the medium as those with no N supply, a response similar to that in the presence of 90 mM NaCl.

A different trend to that of Na^+ uptake was recorded for Cl^- uptake in response to the various N regimes. In the presence of 90 mM NaCl, plantlets supplied with 40 mM NO_3^--N + 20 mM NH_4^+-N exhibited the greatest amount of Cl^- uptake (0.64 mmole). Although incremental NO_3^--N additions from 30 mM to 120 mM resulted in a significant decline in Cl^- uptake, plantlets grown in the absence of NO_3^--N removed less Cl^- from the growth medium than those in the presence of 30 mM NO_3^--N. In the case of a NaCl supply of 180 mM, Cl^- uptake was maximal for plantlets grown in the absence of N, but an increase in the availability of N, both as NO_3^--N alone and NO_3^--N + NH_4^+-N, resulted in negligible removal of Cl^- ions from the growth medium.

4.3 PROTEIN SYNTHESIS IN SHOOT CULTURES GROWN ON VARIOUS NaCl AND NITROGEN NUTRITION REGIMES

The leaf protein content of plantlets, harvested after 35 d growth on culture medium containing variable NaCl and N levels, is shown in Figure 4.11. In the absence of NaCl, leaf protein content (expressed on a fresh mass basis) (Figure 4.11 A) responded favorably to an incremental NO_3^--N supply from 0 mM to 120 mM, and was enhanced significantly when plantlets were supplied with standard MS nutrients (40 mM NO_3^--N + 20 mM NH_4^+-N). A NaCl supply at 90 mM caused a significant inhibition of leaf protein content at all N levels tested. Leaf protein content was maximal for plantlets supplied with 60 mM NO_3^--N and a mixed N supply of 40 mM NO_3^--N + 20 mM NH_4^+-N, in the presence of 90 mM NaCl. In contrast, a dramatic increase in the leaf protein content on exposure to 180 mM

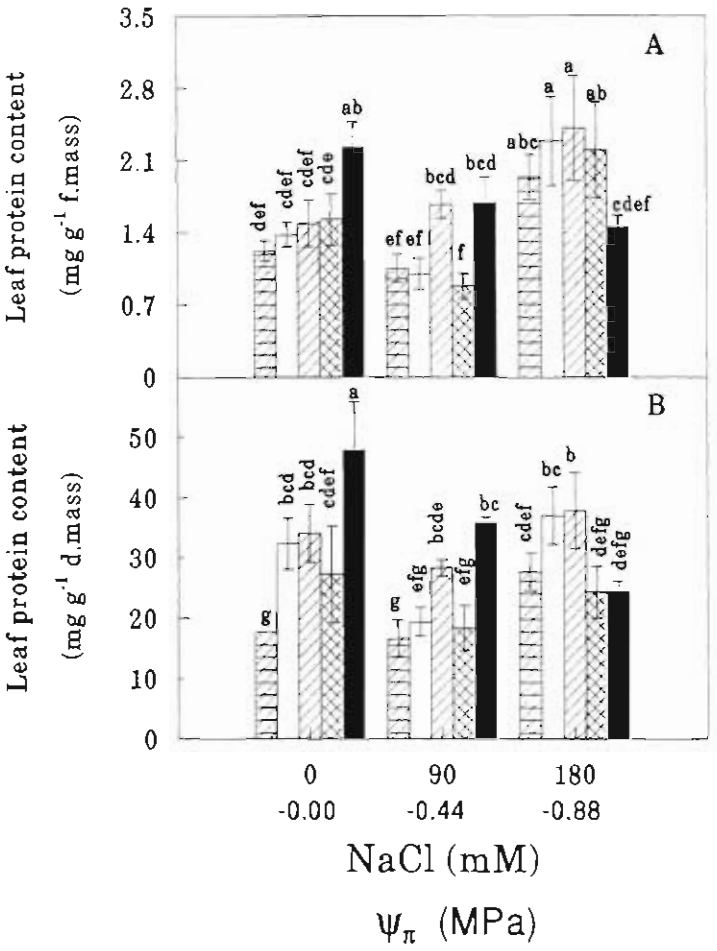


Figure 4.11: Effect of NaCl and N supply on leaf protein content of *in vitro* plantlets of *N. tabacum*.

Treatments as described in Figure 4.4. Measurements were obtained after 35 d in culture. Protein content expressed on a fresh mass (A) and a dry mass (B) basis. Symbols: (mM N) (0), (30), (60), (60) (control), (120).

Values represent mean ± s.d.. Letters associated with values represent mean separation by Duncan's Multiple Range Test, 5% significance value. n = 3.

NaCl was detected in plantlets supplied with a sole NO_3^--N source. In addition, at all NO_3^--N concentrations these protein levels were higher than those of plantlets grown without NaCl. For plantlets exposed to 180 mM NaCl, nitrate-N supplementation up to 60 mM resulted in an increase in protein content, while 120 mM NO_3^--N caused a slight inhibition of protein production. This favourable response of plantlets to a NO_3^--N source in the presence of 180 mM NaCl was not reflected by plantlets supplied with mixed NO_3^--N/NH_4^+-N nutrition. Protein levels of plantlets grown on standard MS nutrient media were reduced significantly on comparison to plantlets grown on 0 mM and 90 mM NaCl under the same N regime.

It has been indicated (Table 4.2) that an increase in the NaCl concentration resulted in an increase in the dry mass:fresh mass ratio of leaves sampled at the end of 35 d growth. When those results of leaf protein production in response to various NaCl and N regimes were expressed on a dry mass basis (Figure 4.11 B), it was noted that there was a reduction in the difference in protein content between leaves from plantlets exposed to 0 mM and 180 mM NaCl. However, even when expressed on a dry mass basis, the leaf protein content of plantlets supplied with 0 mM to 60 mM NO_3^--N was higher in the presence of 180 mM NaCl than at lower NaCl levels. A slight enhancement of leaf protein content (mg g^{-1} d.mass) was observed for plantlets grown on 40 mM NO_3^--N + 20 mM NH_4^+-N in the presence of 90 mM NaCl, while in the absence of NaCl, the leaf protein levels appeared to be reduced in 0 mM and 120 mM NO_3^--N treatments.

4.4 NITRATE REDUCTASE ACTIVITY IN SHOOT CULTURES GROWN ON VARIOUS NaCl AND NITROGEN REGIMES

4.4.1 Time-course of nitrate reductase activity, nitrate uptake and leaf protein accumulation in non-stressed shoot cultures

Nitrate reductase (NR) activity of leaves from shoot cultures exposed to various levels and forms of N nutrition was monitored over a 35 d period in order to ascertain the time of peak enzyme activity during the growth period (Figure 4.12 A). As part of this study, nitrate disappearance from the culture medium (Figure 4.12 B) and leaf protein content (Figure 4.12 C) were monitored also.

Nitrate reductase activity (Figure 4.12 A) in leaves of shoots supplied with a range of NO_3^--N concentrations (30 mM to 120 mM NO_3^--N), and 40 mM NO_3^--N + 20

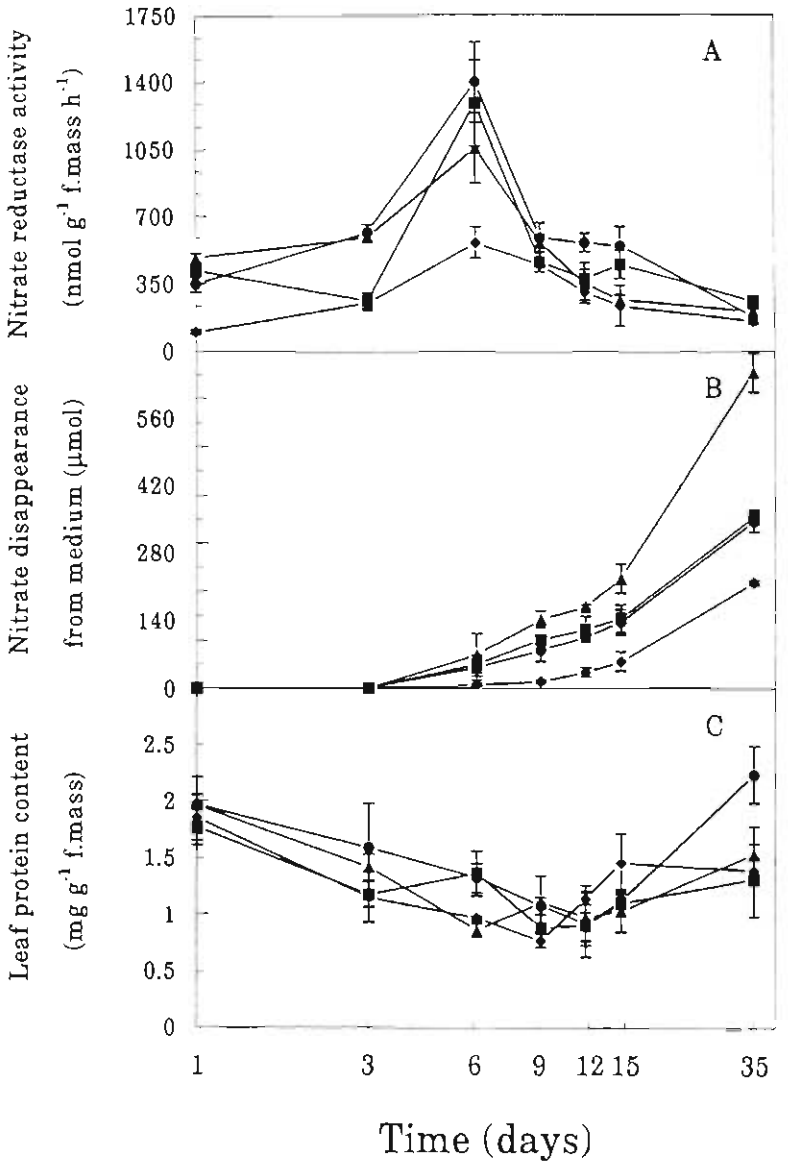


Figure 4.12: Time-course of the effect of N supply on leaf nitrate reductase activity, leaf protein content and nitrate uptake by *in vitro* plantlets grown in the absence of NaCl.

Excised *in vitro* shoots were cultured on solid MS medium containing 0 -120 mM NO_3^--N, where total N in the medium was supplied as NO_3^--N. Shoots cultured on standard MS nutrients (40 mM NO_3^--N + 20 mM NH_4^+-N) served as controls. Measurements were taken every 3 days for 15 d, and then after 35 d in culture. The x-axis represents time as a function of log base 10.

Symbols: (mM N) ◆ (30), ■ (60), ● (60) (control), ▲ (120).

Values represent mean ± s.d.. Absence of error bars indicate a s.d. smaller than the symbols. n=3.

mM NH_4^+-N, showed a dramatic increase after 6 d in culture. Shoots supplied with a mixed NO_3^--N/NH_4^+-N nutrition displayed the maximum NR activity, (1395.97 nmol NO_2^- g^{-1} f.mass h^{-1}) followed by 60 mM NO_3^--N only and 120 mM NO_3^--N. Shoots growing on 30 mM NO_3^--N exhibited the lowest recorded enzyme activity (560.19 nmol NO_2^- g^{-1} f.mass h^{-1}). Nitrate reductase activity declined between 6 d and 9 d in culture and after 15 d had stabilised to values similar to those at the start of the culture period. At all times throughout the first 15 d of investigation, NR activity remained the highest in plantlets supplied with 40 mM NO_3^--N + 20 mM NH_4^+-N, and the lowest in those grown on 30 mM NO_3^--N, as observed at the time of peak NR activity (day 6). In contrast, by the end of 35 d, no significant difference in NR activity between the respective N treatments was detected and coincided with a very low enzyme activity (± 200 nmol NO_2^- g^{-1} f.mass h^{-1}).

Shoots began to remove nitrate from the growth medium (Figure 4.12 B) after 3 days in culture only, which was at least 3 d before roots began to emerge (Table 4.2). The initiation of nitrate uptake was found to correspond with the incline in NR activity. Nitrate uptake increased with time and was maximal from day 6 to day 35 for cultures supplied with 120 mM NO_3^--N. No significant difference in nitrate uptake was detected between shoots supplied with 60 mM NO_3^--N or MS nutrients (40 mM NO_3^--N + 20 mM NH_4^+-N) throughout the whole culture period, while the lowest quantity of nitrate taken up occurred when shoots were supplied with 30 mM NO_3^--N.

After an initial decline for the first 3 d, leaf protein content (Figure 4.12 C) did not alter significantly throughout the experimental period, except in shoots grown on MS nutrients which displayed enhanced protein levels at the end of 35 d growth.

4.4.2 The effect of NaCl on nitrate reductase activity

Nitrate reductase activity was measured in leaves of salt stressed shoots supplied with 30 mM and 60 mM NO_3^--N, as well as 40 mM NO_3^--N + 20 mM NH_4^+. Determination of NR activity was performed after 6 d in culture to coincide with the peak activity observed in control (unstressed) plants (Figure 4.12), and the results are shown in Figure 4.13.

The presence of NaCl in the culture medium resulted in a drastic inhibition of NR activity at both 90 mM and 180 mM NaCl. Furthermore, there was no significant

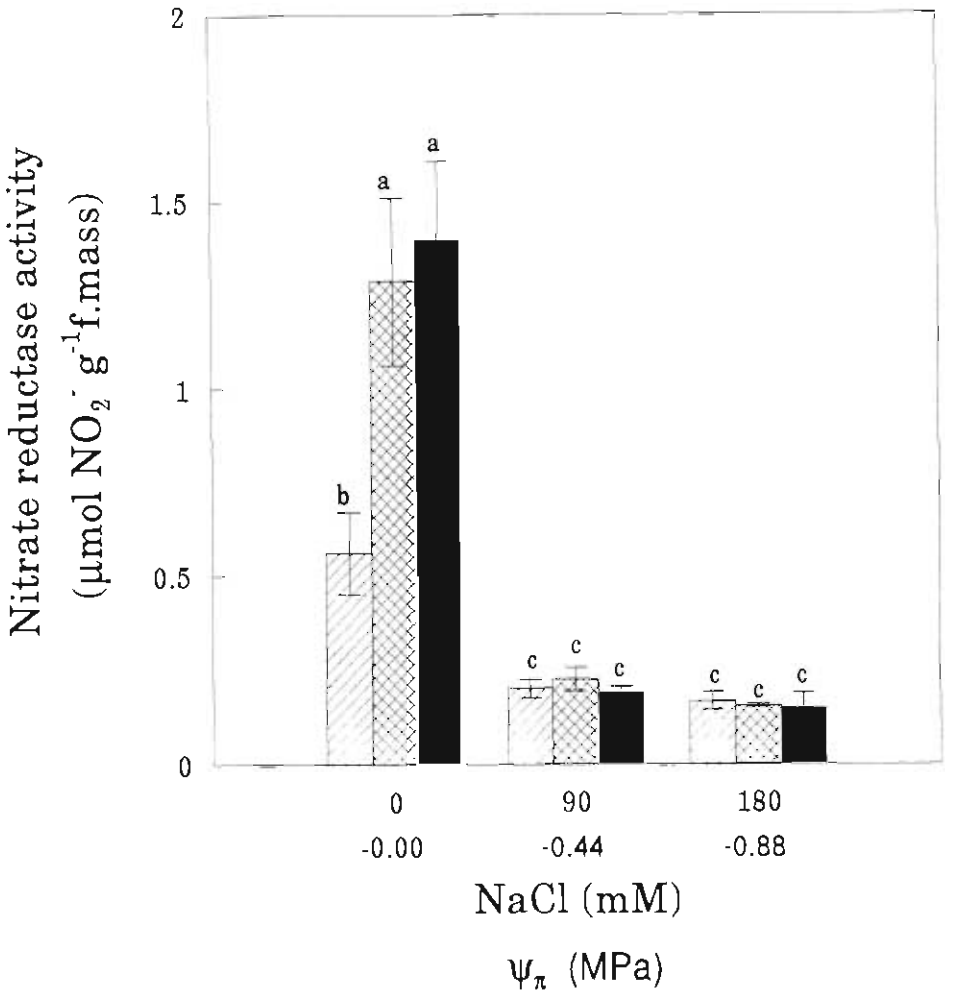


Figure 4.13: Effect of NaCl and N supply on leaf nitrate reductase activity of *in vitro* plantlets of *N. tabacum*.

Excised *in vitro* shoots were cultured on solid MS medium containing 0 - 180 mM NaCl and 30 - 60 mM NO_3^--N, where total N in the medium was supplied as NO_3^--N. Shoots cultured on standard MS nutrients (40 mM NO_3^--N + 20 mM NH_4^+-N) served as controls. Assays were performed after 6 d in culture.
Symbols: (mM N) (30), (60), (60) (control).

Values represent mean ± s.d.. Letters associated with values represent mean separation by Duncan's Multiple Range Test, 5% significance value. n=3.

difference between the two NaCl treatments or the various nitrogen regimes. These results suggest that nitrate reductase activity in the leaves of *in vitro* shoot cultures of *N. tabacum* is highly sensitive to the presence of NaCl in the external medium.

4.5 NITRATE REDUCTASE mRNA PRODUCTION IN RESPONSE TO VARIOUS NaCl AND NITROGEN REGIMES

The extreme response of leaf NR activity observed in plantlets grown under saline conditions led to speculation regarding the specific manner by which salt inhibits the NR enzyme. The question arose whether salinity had an effect at the transcriptional level, causing a decrease in the production of mRNA for NR. In addition, it was questioned whether a variation in nitrogen levels supplied to salt stressed plantlets would have an effect on NR mRNA production. The approach taken in attempts to quantify the level of mRNA for NR was the utilisation of a DNA probe, the sequence of which is complementary to that of the mRNA sequence coding for NR. The probe used was a 1.6 kb tobacco NR cDNA insert located in the plasmid pBMC102010 (a pUC 9 derivative) (Calza *et al.*, 1987). A number of procedures were required before the NR cDNA probe could be used to quantify NR mRNA production in response to various NaCl and N regimes. The plasmid first had to be isolated and purified from transformed *E. coli* cells and its structure confirmed. It was necessary then to test whether a linear relationship existed between the load of leaf total RNA applied to a slot blot and the detected signal when the probe was used in a hybridisation reaction. In addition, the probe concentration for optimal detection of signal had to be determined. Both radioactive and non-radioactive techniques were utilised to investigate the parameters required for successful hybridisation and detection of NR mRNA.

4.5.1 Total RNA content in leaves

RNA was extracted from leaf tissue of shoots cultured for 6 days on growth media containing various combinations and levels of NaCl and N. Extractions were performed after 6 d to coincide with peak NR activity (Figure 4.12), on the assumption that this would correspond with maximum NR mRNA production. Total RNA yields are shown in Figure 4.14.

Prior to subculture of shoots from standard MS nutrient media onto media containing different levels of N and NaCl, leaves were found to contain

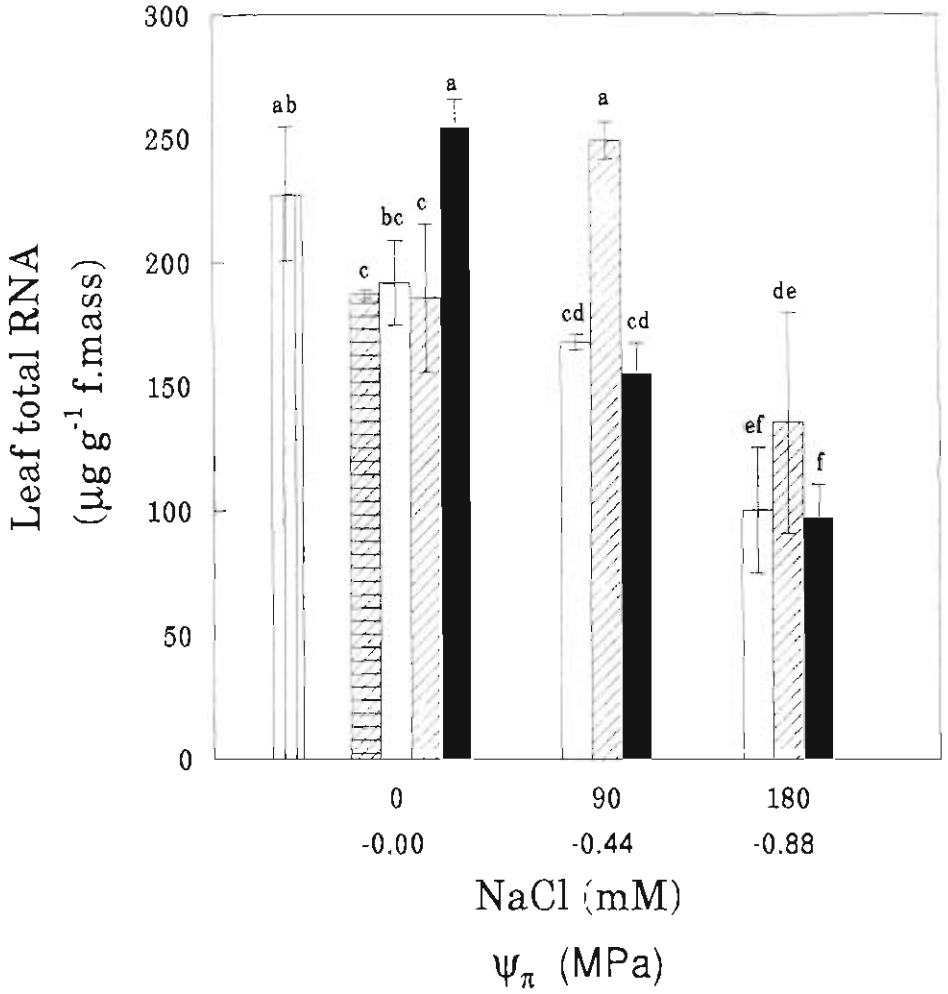


Figure 4.14: Effect of NaCl and N supply on leaf total RNA content of *in vitro* plantlets of *N. tabacum*.

Excised *in vitro* shoots were cultured on solid MS medium containing 0 - 180 mM NaCl and 0 - 60 mM NO_3^--N, where total N in the media was supplied as NO_3^--N. Shoots cultured on standard MS nutrients (40 mM NO_3^--N + 20 mM NH_4^+-N) served as controls. Total RNA was extracted after 0 d and 6 d in culture.
Symbols: (mM N) (0), (30), (60), (60) (control), (Time 0).

Values represent mean ± s.d.. Letters associated with values represent mean separation by Duncan's Multiple Range Test, 5% significance level. n = 3.

approximately 230 μg g^{-1} f.mass total RNA. After 6 d culture on 0, 30 and 60 mM NO_3^--N in the absence of salt, RNA production had declined slightly. Very little difference in total RNA levels between the various NO_3^--N treatments was detected but a N supply as 40 mM NO_3^--N + 20 mM NH_4^+-N resulted in a significant increase in RNA levels. The presence of NaCl in the culture medium caused a significant decline in total RNA levels, particularly in leaves of shoots grown on 180 mM NaCl. However, the highest RNA content measured in leaves of shoots supplied with 60 mM NO_3^--N and in the presence of 90 mM NaCl, was not significantly different from non-salinised shoots supplied with MS nutrients. The decline in leaf total RNA in response to the presence of NaCl provided a possible indication of salt effects at the general transcriptional level in shoot cultures of *N. tabacum*.

4.5.2 Confirmation of pBMC102010 structure

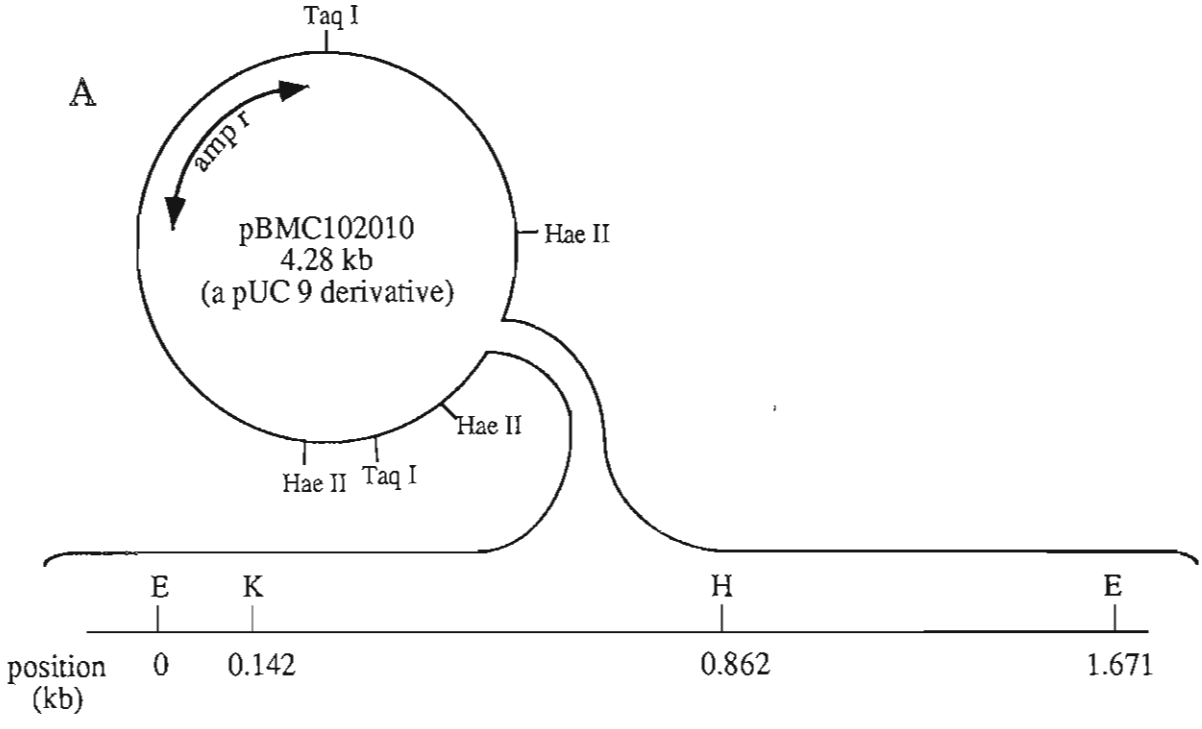
Once the plasmid pBMC102010 had been isolated and purified, it was digested with a number of restriction enzymes and the fragments analysed by agarose gel electrophoresis in order to confirm the plasmid structure and the presence of the 1.6 kb insert (Calza *et al.*, 1987). Plasmid size and positions of restriction sites were found to conform to those described by Calza and coworkers and the NR cDNA insert was identified (Figure 4.15 A and B). Whole, undigested plasmid pBMC102010 was found to yield two bands on the gel with the faster moving band representing plasmid DNA in the supercoiled form, and the slower moving band, DNA in the nicked (open) circle form. A similar result was obtained for the plasmid pBR322 (Sutcliffe, 1979) which has a known molecular weight of 4.36 kb. As the pUC plasmid, from which pBMC102010 was derived, has a molecular weight of 2.68 kb, it would be expected that pBMC102010, with its 1.6 kb insert, would have a molecular weight of approximately 4.28 kb. This was confirmed by its similar position to pBR322 in the gel. Cleavage with KpN I, Hind III and Sma I should have cut the plasmid in the polylinker region, thus linearising the plasmid. Digestion with KpN I was incomplete and produced three fragments representing supercoiled and nicked circle plasmid DNA, as for undigested plasmid, with the linear form occurring inbetween. Cleavage with Hind III should have produced the same result as KpN I, but the fragments obtained did not match those of KpN I, the reason for which is not known. Sma I successfully linearised the plasmid producing one fragment only of approximately 4.27 kb, the predicted size. Digestion with Eco RI produced two fragments, one which represented the original pUC 9 plasmid

Figure 4.15: A. A simplified map of the pBMC102010 construct indicating the 1.6 kb NR cDNA insert with restriction sites and sizes of fragments analysed in B.

B. Agarose gel electrophoresis of restriction fragments of pBMC102010.

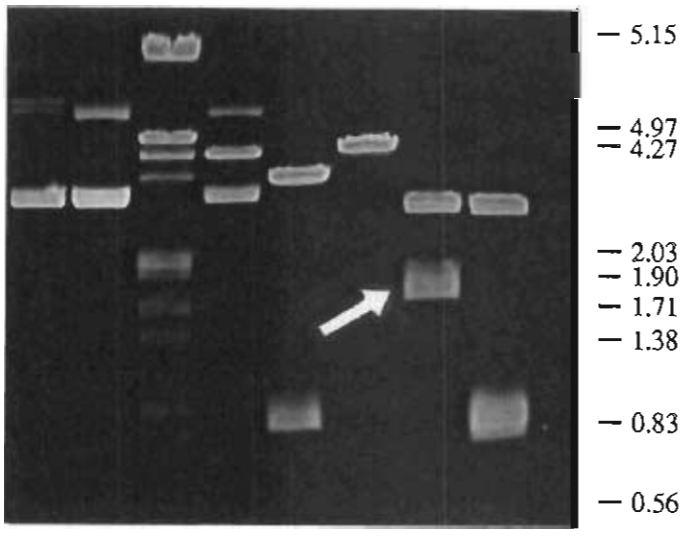
Samples were run on an agarose gel (1%) and stained with 0.5 μg/ml ethidium bromide. Lanes: 1, undigested pBMC102010 (0.5 μg); 2, undigested pBR 322 (0.5 μg); 3, Eco RI/Hind III digest of Lambda DNA molecular weight markers (0.9 μg); 4, KpN I digest of pBMC102010 (0.4 μg); 5, Hind III digest of pBMC102010 (0.5 μg); 6, Sma I digest of pBMC102010 (0.5 μg); 7, Eco RI digest of pBMC102010 (0.5 μg); 8, Eco RI/Hind III digest of pBMC102010 (0.5 μg).

A



E : Eco RI / H : Hind III / K : KpN I

B



(Viera and Messing, 1982) and the other, the NR cDNA insert (indicated by the arrow in Figure 4.14 B). The position of the NR cDNA insert on the gel confirmed that the insert size was in the region of 1.6 kb. As predicted by the map (Figure 4.15 A), plasmid digestion with Eco RI and Hind III cut the 1.6 kb insert at both its extremities and approximately in the centre, resulting in two fragments around 0.8 kb, visible as a single band. A third fragment, representing the original pUC 9 plasmid, was located, as expected, just below the 3.53 kb marker at the same position as was obtained with the Eco RI digestion alone.

4.5.3 Relationship between RNA slot blot load and detected signal

Radioactive and non-radioactive procedures were employed to determine whether a linear relationship existed between the concentration of total RNA on the slot blot and the signal obtained when the probe was hybridised to NR mRNA.

A concentration series of 0 to 16 μg total RNA was prepared and probed with 100 ng ^{32}P-labelled NR cDNA. It was found that the intensity of the signals obtained did not correlate with the RNA concentrations on the slot blot. Furthermore, despite an adequete specific activity of the probe (4 x 10^8 cpm/μg), the signal intensity obtained was extremely weak, even after 72 h exposure to X-ray film (results not shown). Non-specific binding of the ^{32}P-labelled probe to slots on the blot containing no RNA samples was detected also.

When the non-radioactive digoxigenin-dUTP (DIG) DNA labelling system was used, two preparations of NR cDNA probe were utilised for the labelling procedure. Firstly, 500 ng probe was isolated from low melting temperature agarose gel prior to DIG DNA labelling. The DNA probe was recovered from low melting temperature agarose by extraction with phenol and phenol:chloroform (1:1), followed by precipitation with ammonium acetate (10 M) and ethanol (Wieslander, 1979). An immunological detection procedure performed to determine the efficiency of the probe labelling, resulted, however, in only a very faint colour reaction after 60 h (results not shown). The process of recovery of the DNA probe from agarose was presumed to have resulted in a substantial loss of DNA, thus rendering the method unsuitable for subsequent investigations. When, secondly, an aliquot of pBMC102010 (2 μg) was digested with Eco RI and labelled with DIG-dUTP without separating the NR cDNA insert from the remainder of the plasmid, a predictable 650 ng of labelled probe was obtained. Immunological detection of the labelled

probe resulted in a strong signal in less than 24 h (results not shown), which was a marked improvement over the signal obtained for the first method of probe preparation tested. This method of probe preparation was used for the remainder of the investigations.

A duplicate concentration series of 0 to 12 μg total RNA was prepared and probed with 100 ng and 400 ng DIG-dUTP labelled NR cDNA probe respectively, the results of which are presented in Plate 4.4. A very weak signal was obtained after 40 h hybridisation with both 400 ng (Plate 4.4 A) and 100 ng (Plate 4.4 B) labelled probe, with little difference in signal intensity being detected between the two probe concentrations. Visually, no increase in signal intensity with increase in total RNA load could be perceived. Densitometric scanning of the blots was not sufficiently sensitive to pick up the signal from any one of the lanes, thus disallowing the quantification of the signal produced. A signal was detected also from lanes not containing RNA, suggesting that non-specific binding of the probe to the slot blot had occurred.

4.5.4 NR mRNA analysis

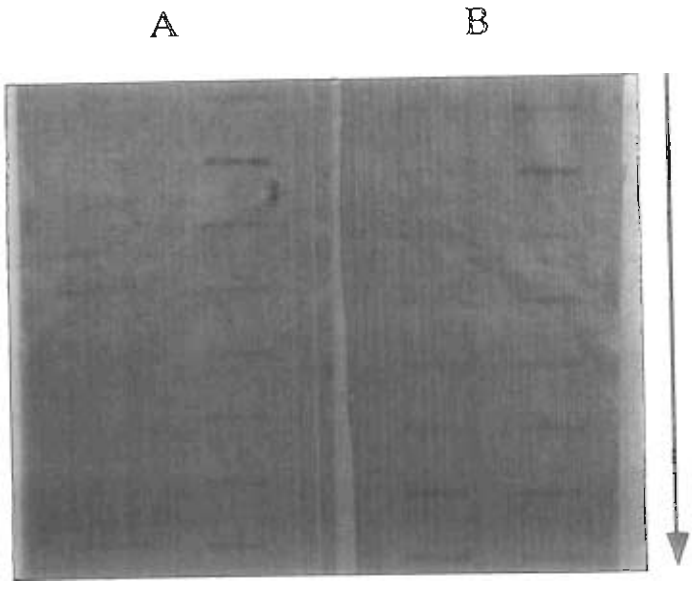
Despite unsuccessful attempts to determine the optimal probe concentration and RNA load onto slot blots, total RNA extracted from leaves of plantlets grown on various NaCl and N regimes was analysed for NR mRNA using the non-radioactive DIG-DNA method. In the previous studies described above, RNA samples to be analysed had been heat denatured prior to loading onto the slot blot. As problems with non-specific binding of the probe had been encountered when utilising this procedure, the following study employed the use of an alkaline denauration procedure which accompanied the slot blot apparatus. An RNA load of 10 μg and a labelled probe concentration of approximately 650 ng was used. Slot blots were prehybridised for 18 h and hybridised in the presence of the labelled probe for 48 h and the results are shown in Plate 4.5. Although a strong signal was detected for lanes containing the RNA samples, a strong signal was obtained also from those lanes supplied with only the alkaline buffer used to denature the RNA prior to loading. This interfered with interpretations of NR mRNA production in response to the various NaCl and N treatments. Densitometric scanning of the blot revealed that the signal intensity was not identical between those lanes containing buffer only (Figure 4.16). Furthermore, the signal intensity was greater in most cases than those lanes containing RNA samples corresponding to the various NaCl and N treatments.

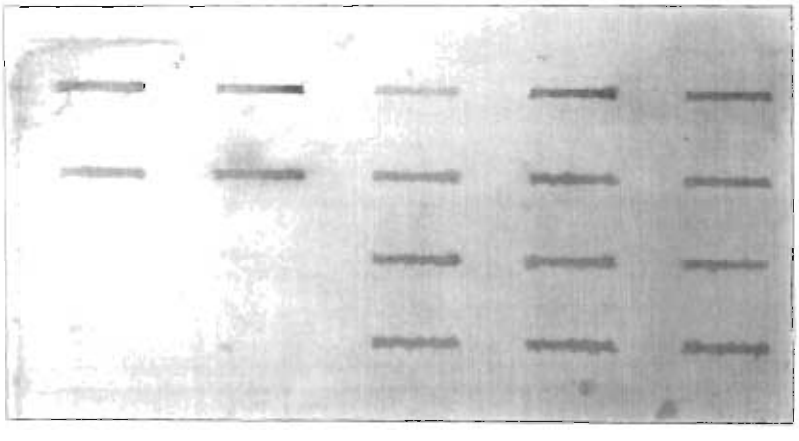
Plate 4.4: Hybridisation of DIG labelled NR cDNA to a total RNA slot blot concentration series.

Duplicate RNA samples (0 to 12 μg) were slot blotted onto nitrocellulose membranes and probed with 400 ng (A) and 100 ng (B) of DIG labelled NR cDNA. Arrow indicates increase in RNA load from 0 to 12 μg.

Plate 4.5: Slot blot hybridisation analysis of total RNA obtained from *in vitro* shoot cultures grown under different NaCl and N regimes.

RNA was extracted from leaves of *in vitro* shoot cultures grown on 0 - 180 mM NaCl and 0 - 60 mM NO_3^--N, where total N in the medium was supplied as NO_3^--N. Shoots supplied with standard MS nutrients (40 mM NO_3^--N + 20 mM NH_4^+-N) served as controls. RNA extractions were performed after 0 d and 6 d in culture. RNA load per slot, 10 μg. RNA samples were slot blotted onto nitrocellulose membranes and probed with 650 ng DIG labelled NR cDNA.





	←	No Samples	→		Row 1
T_0	0 mM NaCl 0 mM NO_3^-	0 mM NaCl 30 mM NO_3^-	90 mM NaCl 30 mM NO_3^-	180 mM NaCl 30 mM NO_3^-	Row 2
		0 mM NaCl 60 mM NO_3^-	90 mM NaCl 60 mM NO_3^-	180 mM NaCl 60 mM NO_3^-	Row 3
		0 mM NaCl 40 mM NO_3^- + 20 mM NH_4^+	90 mM NaCl 40 mM NO_3^- + 20 mM NH_4^+	180 mM NaCl 40 mM NO_3^- + 20 mM NH_4^+	Row 4

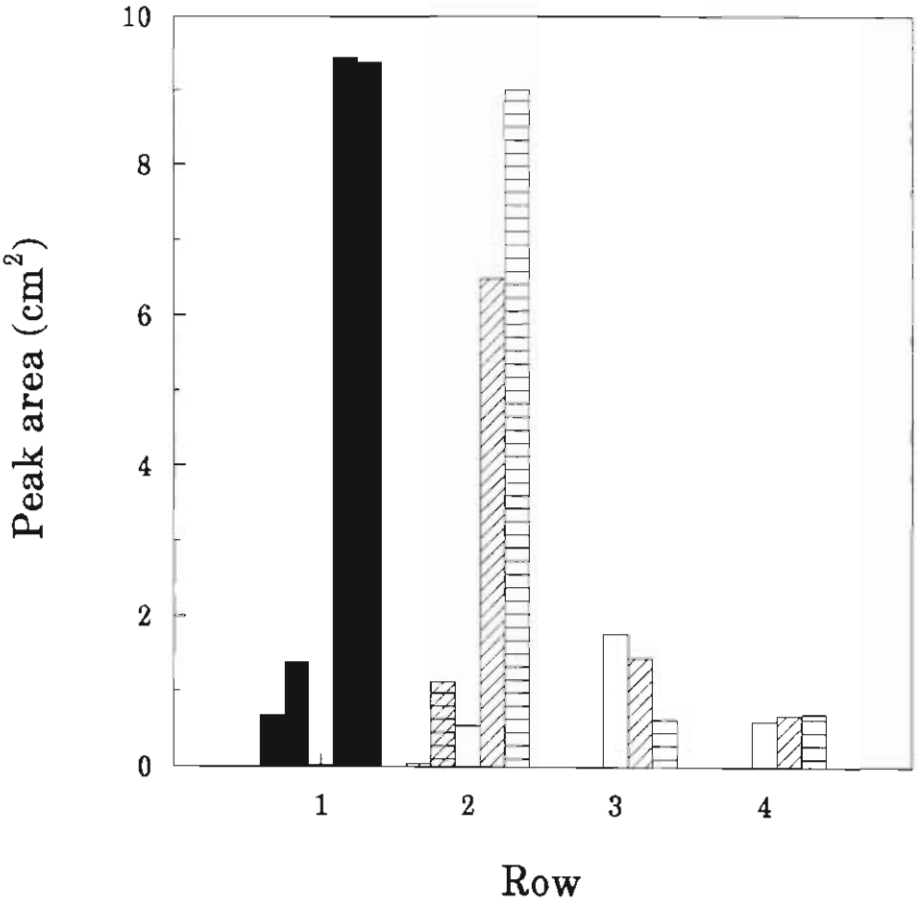


Figure 4.16: Densitometric scan of DIG labelled NR cDNA hybridisation to a total RNA slot blot.

Treatments as described in Plate 4.5.

Symbols: No Sample, Time 0, 0 mM NaCl, 0 mM NO_3^--N

0 mM NaCl and
- 30 mM NO_3^- – N if Row = 2
- 60 mM NO_3^- – N if Row = 3
- MS nutrients if Row = 4

90 mM NaCl and
- 30 mM NO_3^- – N if Row = 2
- 60 mM NO_3^- – N if Row = 3
- MS nutrients if Row = 4

180 mM NaCl and
- 30 mM NO_3^- – N if Row = 2
- 60 mM NO_3^- – N if Row = 3
- MS nutrients if Row = 4

Thus, when differences between the intensity of the buffer-containing lanes and those containing RNA were obtained by densitometric scanning, or where the signal was found to differ between the various treatments, it could not be concluded that these were due to real variations in NR mRNA production.

An additional observation made was that a brown discolouration of the slot blot lanes had occurred (Plate 4.5), which is in contrast to the blue-purple precipitate characteristic of the DIG immunological detection procedure. This raised the possibility of a chemical reaction having taken place between the alkaline buffer and the nitrocellulose membrane used for the slot blot. This further confounded any attempts to determine whether the production of mRNA for NR was altered in plantlets grown under different NaCl and N nutrition regimes.

5. DISCUSSION

5.1 THE EFFECT OF NaCl ON GROWTH, NUTRIENT UTILISATION, AND NITROGEN METABOLISM OF *in vitro* PLANTLETS OF *N. tabacum*

It is well known that the presence of NaCl in the external environment has a detrimental effect on the growth and metabolism of salt sensitive plant species (Section 2.2). When *in vitro* shoot cultures of *Nicotiana tabacum* (tobacco) were exposed to NaCl in a culture medium containing standard MS nutrients (40 mM NO_3^--N + 20 mM NH_4^+-N), growth of the plantlets was severely inhibited, with growth repression being proportional to NaCl supply (Figure 4.1). The limits of survival of tobacco under a salt stress is reported to be in the range of 30 mM to 60 mM NaCl (Table 2.1), thus the choice of a NaCl supply between 0 mM and 180 mM was expected to provide a range of stress responses by plantlets grown under these conditions. In addition, monitoring the response of plantlets to these salinity levels over a period of 5 weeks enabled analyses of long-term responses to the stress, an important consideration when investigations were conducted to assess ways in which the growth of these plantlets might be improved.

Parameters relating to growth of both roots and stems were investigated and revealed that, over an experimental period of 35 d, root growth was more adversely affected by an increasing supply of NaCl than stem growth (Figure 4.1). Roots are reported to be among the first organs affected by a salt stress and are considered also to be one of the most sensitive (Waisel, 1972; Levitt, 1980; Okusanya and Ungar, 1984). Radish (*Raphanus sativus*) roots were found to be severely stunted upon exposure to 200 mM NaCl (Waisel and Breckle, 1987) as were maize roots exposed to 100 mM NaCl (Zidan *et al.*, 1990). Several changes in the processes involved in root extension under saline conditions have been identified. Zidan and coworkers attributed the reduction in maize root growth to reductions in the length of the root tip elongation zone, the length of the epidermal cells, and to a decline in the rate of cell production. Furthermore, such reductions have been reported to be associated with changes in the cell wall extensibility (Pritchard *et al.*, 1987). Plants growing under saline conditions may develop a predominantly vertical root system in an effort to locate a less brackish water supply (Waisel and Breckle, 1987). Waisel and Breckle stated that lateral root production may be more negatively affected by the presence of salt in the external environment than tap root growth. In

this study, although growth of individual root types was not examined, a stimulation of root length was detected in plantlets exposed to 45 mM and 90 mM NaCl (Figure 4.1 A). This may be representative of the formation of a vertical root system in shoot cultures grown *in vitro*. The lack of a similar stimulation of root length in plantlets exposed to salinity levels greater than 90 mM (Figure 4.1 A) is most likely due to those NaCl levels being greater than the limits for survival of tobacco plants.

A decrease in the root fresh mass:shoot fresh mass ratio with increase in NaCl supply over a 35 d period (Table 4.1 A) confirmed further a more drastic effect of NaCl on root growth than stem growth of tobacco grown *in vitro* on standard MS nutrients. This result is in contradiction to those of Munns and Termaat (1986) who showed that for several plant species (barley, wheat, Egyptian clover (*Trifolium alexandrinum*), white clover (*T. repens*)) grown in saline soil (50 mM-100 mM NaCl), leaf growth was more sensitive to salinity than root growth. Similarly, sunflower, red kidney bean and tomato plants grown in hydroponic culture were found to have increased root:shoot ratios when exposed to 100 mM NaCl (Salim, 1989). However, *in vitro* studies of plantlet cultures of *Asparagus officinalis* indicated that root growth and production was inhibited by 170 mM NaCl, while shoot growth was affected only at 300 mM NaCl (Mills, 1989). The more severe effect of NaCl on root growth than stem growth found by Mills (1989), and as exhibited by tobacco in this study, may be characteristic of the response to salinity of plants grown under *in vitro* conditions. This is supported by a comparison between the rate of root emergence from *in vitro* shoot cultures of tobacco and hydroponically grown radish plants (Waisel and Breckle, 1987). While root emergence from *in vitro* grown tobacco shoots was delayed by 5 d with an increase in the NaCl concentration from 0 mM to 180 mM (Table 4.3 A), among all processes of root growth studied in radish plants, root initiation was found to be the least sensitive to salinity levels of 200 mM (Waisel and Breckle, 1987).

Plants growing under saline conditions most often suffer from a water deficit due to a loss of turgor, which in turn is thought to contribute to a reduction in growth (Greenway and Munns, 1980; Flowers and Yeo, 1986; Termaat and Munns, 1986). The increase in the dry mass:fresh mass ratio of roots and leaves of tobacco plantlets grown for 35 d *in vitro* with an incremental NaCl supply (Table 4.2) suggests the possibility of a reduction in the water content of these organs. There has been much debate as to whether a water deficit, particularly in the shoots, is the major contributing factor to the reduction in plant growth under saline conditions.

In the short-term (days), this has been shown to be the case (Munns and Termaat, 1986), but over a period of weeks or months the decline in growth of a number of soil grown plant species has been demonstrated to be related to the inability of the plants to produce new leaves at a faster rate than at which mature leaves were dying, due to an excessive build up of salt ions in the leaf tissues (Munns and Termaat, 1986). This led to a reduction in the photosynthetic area of the plants and consequently, insufficient carbohydrate production to support growth (Munns and Termaat, 1986). The extent to which a reduction in water content of leaves and roots of *in vitro* grown tobacco contributes to the reduction in plantlet growth, and whether salt-specific effects, or a water deficit, are primarily responsible for the deleterious effects of salt on growth of these plantlets, are issues which will be raised later in this discussion.

The growth and productivity of a plant is highly dependent on the efficient uptake of nitrate from the external medium and its assimilation into proteinaceous compounds (Section 2.1). Salinity has a profound effect on the processes of nitrogen assimilation, which may contribute significantly to the reduction in plant growth observed under saline conditions (Section 2.3). When growing *in vitro* for 35 d on a nutrient medium containing 40 mM NO_3^--N + 20 mM NH_4^+-N (MS nutrients), tobacco plantlets supplied with 90 mM and 180 mM NaCl were found to remove significantly less nitrate from the medium than those plantlets grown in the absence of NaCl (Figure 4.8). A reduction in NO_3^--N uptake under saline conditions has been demonstrated in hydroponically grown barley seedlings exposed to 120 mM (Helal *et al.*, 1975), 100 mM (Aslam *et al.*, 1984) and 200 mM NaCl (Klobus *et al.*, 1988). In this study, there was no significant difference between the uptake of nitrate by plantlets supplied with 90 mM or 180 mM NaCl suggesting that, for these NaCl levels, the presence of NaCl and not the concentration thereof, appeared to be responsible for the suppression in nitrate uptake in *in vitro* grown tobacco plantlets. The availability of cellular nitrate is required to induce the activity of the nitrate reducing enzyme, nitrate reductase (Section 2.1.2). The increase in NR activity in the leaves of non-stressed tobacco shoots after 3 d in culture was found to coincide with the removal of nitrate from the culture medium (Figure 4.12), suggesting an induction of NR activity by nitrate in these cultures.

When NR activity was determined for plantlets exposed to 90 mM and 180 mM NaCl, it was measured after 6 d in culture to coincide with peak NR activity in non-stressed plantlets (Figure 4.13). A marked reduction in leaf NR activity of salt

stressed plantlets was recorded, with NaCl supplies of 90 mM and 180 mM resulting in a similar inhibition of enzyme activity (Figure 4.13). This decline in NR activity could have been related to the reduction in nitrate uptake observed at the end of 35 d. It was found, after 35 d, that salinity had caused a significant inhibition of nitrate uptake, with an increase in the NaCl supply from 90 mM to 180 mM corresponding to a decline in the removal of nitrate from the culture medium (Figure 4.8). The effect of NaCl on NR activity was particularly severe, however, and it is thought that the reduction in nitrate uptake alone could not account for the drastic response of NR activity to a salt stress. Martínez and Cerdá (1989) have indicated that in hydroponically grown tomato and cucumber plants, a low leaf NR activity in salt stressed plants was not related to a low nitrate content in the leaves as a result of a reduction in nitrate uptake. Those workers proposed that NaCl ions present in the leaves could inhibit the efflux of nitrate ions from the storage pool in the vacuole into the cytoplasm, thus decreasing the capacity of the plants to utilise stored nitrate-N for growth. The reduction in NR activity in leaves of tobacco plantlets exposed to salinity *in vitro* may, therefore, be due to a salinity-induced derangement in cellular nitrogen metabolism. This may in turn have contributed to the reduction in growth of these plantlets.

The foregoing argument for the reduction in NR activity in salt stressed tobacco shoot cultures presupposes, however, the presence of Na^+ and Cl^- ions in the leaves of these plantlets. When plantlets were supplied with 90 mM NaCl, both Na^+ and Cl^- ions were found to be removed from the nutrient medium, with a greater proportion of Cl^- ions being taken up than Na^+ ions (Figure 4.10). Nevertheless, when supplied with 180 mM NaCl, plantlets removed negligible quantities of these ions from the medium. As similar NR activities were obtained for plantlets grown on both 90 mM and 180 mM NaCl (Figure 4.13), it is questioned to what extent the reduction in NR activity in leaves of salt stressed tobacco plantlets could have been due to the presence of NaCl ions in the leaves. It is possible that a reduction in leaf water potential as a consequence of the presence of NaCl in the external environment may have contributed to the inhibition of nitrate reductase activity. It has been shown that the NR enzyme is highly sensitive to a reduction in leaf water potential (Shaner and Boyer, 1976b; Heuer *et al.*, 1979; Larsson *et al.*, 1989). In addition, work in this laboratory has indicated that the NR activity of tobacco callus was identical when cultured in the presence of NaCl and isosmotic concentrations of mannitol (Erdey, *pers. comm.*). Although it is not always possible to extrapolate the response of callus cells to those at the level of the whole plantlet, the sensitivity of

callus NR to the osmotic potential of the culture medium suggests the possibility that, in this study, leaf NR activity may have been affected by a reduction in leaf water potential of tobacco plantlets. It is not certain, however, whether the observed reduction in NR activity in plants exposed to an osmotic stress is due to enzyme inactivation or degradation (Larsson *et al.*, 1989). Hence, the reduction in NR activity in leaves of salt stressed shoot cultures of tobacco may have been due to a disturbance in cellular metabolism caused by the presence of NaCl ions (direct effect), and/or a reduction in turgor and subsequent dehydration (indirect effect).

In addition to, or as an alternative to, an inactivation or degradation of NR, a reduction in the synthesis of the NR enzyme may have contributed to the inhibition of NR activity of salt stressed tobacco plantlets. By probing for the level of mRNA for NR, attempts were made to determine what the effect of salinity was on the production of the NR enzyme. A decrease in the production of NR mRNA would imply a reduction in the synthesis of the NR enzyme. Although the plasmid pBMC102010, containing the cDNA insert complementary to NR mRNA, was successfully isolated and its structure confirmed (Figure 4.15), slot blot hybridisations to total RNA extracts from leaves of tobacco plantlets grown in the presence of NaCl were unable to reveal differences in the level of NR mRNA (Plate 4.5). Problems were encountered with non-specific binding of the probe to lanes of the slot blot not containing RNA, and were coupled with a brown discolouration of the lanes, a phenomenon which was suspected to be due to a chemical reaction between the alkaline RNA denaturing buffer and the nitrocellulose membrane. The successful utilisation of pBMC102010 to detect the level of NR mRNA in differentiating tobacco callus has been demonstrated in our laboratories (Roberts, *pers. comm.*). This worker employed the use of Northern blots hybridised to ^{32}P-labelled NR cDNA probe. It is suggested that slot blots do not provide the most suitable means of determining the level of NR mRNA and future work should utilise Northern blotting procedures. In addition, a critical assessment of the non-radioactive DIG-DNA labelling and detection procedure should be performed to determine whether it provides a level of sensitivity sufficient to detect, quantitatively, changes in the levels of mRNA for NR. The differences in total RNA levels extracted from the leaves of plantlets grown on different NaCl regimes provide an encouraging basis for future investigations of NR mRNA (Figure 4.14). Although no inferences could be made about the level of NR mRNA from these results, the fact that total RNA was reduced on exposure to NaCl suggests that salinity had an effect on total RNA production.

The final pathway involved in the assimilation of nitrate is its integration into amino compounds, the point at which it contributes directly to plant growth and development (Section 2.1). The decline in the processes of nitrate uptake and reduction in salt stressed plantlets of tobacco grown *in vitro* is suggestive of a deleterious effect of salt on protein synthesis. Plantlets grown on MS nutrient media for 35 d in the presence of 90 mM and 180 mM NaCl were found to have significantly lower leaf protein contents than those grown in the absence of salt (Figure 4.11). This relationship remained the same when leaf protein content was expressed on both a fresh mass and dry mass basis, indicating that the observed decline in protein in response to salinity was due to a reduction in the dry matter production. A reduction in protein synthesis by a salinity stress has been indicated for several salt sensitive plant species such as barley (Helal and Mengel, 1979), wheat (Abdul-Kadir and Paulsen, 1982) and cotton (Pessarakli and Tucker, 1985a). A low protein content in the leaves of tobacco plantlets corresponded to the general reduction in plantlet growth under saline conditions (Figure 4.1). Although the effect of salinity on photosynthetic processes other than the production of leaf chlorophyll were not investigated in this study, it is important to consider briefly the relationship between nitrate metabolism and photosynthesis in order to consider the effect of salinity on protein synthesis. The incorporation of nitrate into protein in leaves is dependent on a carbohydrate supply produced during photosynthesis (Aslam *et al.*, 1979). Hence, plant productivity is highly dependent on the photosynthetic process. As *in vitro* shoot cultures are provided with a source of carbon (usually as sucrose) in the growth medium, it is probable that plantlets in culture are not photosynthetically active. However, little information is available about photosynthesis *in vitro* and the occurrence of photosynthetic carbon assimilation in shoot cultures cannot be totally excluded. A drastic reduction in the leaf chlorophyll content in tobacco shoot cultures, particularly in the presence of 180 mM NaCl, was measured (Figure 4.1, Plate 4.1 E). This reduction in chlorophyll content of salt stressed plantlets was probably related directly to the stress and could have been due to a number of factors. It has been indicated in non-stressed barley that 50% of the total soluble leaf protein was RubisCO and that RubisCO levels were reduced on exposure of the plants to salinity (Miteva *et al.*, 1992). It is expected therefore that in salt stressed tobacco shoot cultures, the reduction in total leaf protein content would include a significant reduction in the RubisCO content (Figure 4.11). A decline in the chlorophyll and RubisCO levels would result in a reduction in the photosynthetic efficiency of a plant, which would in turn exert a

control over the rate of nitrate incorporation into protein. The reduction in total leaf protein in salt stressed shoot cultures of tobacco (Figure 4.11) may have been related not only to a decrease in nitrate assimilation, but also to a deleterious effect of salt on photosynthesis. Although this study was not intended to elucidate the nature of interactions between photosynthesis and nitrate metabolism under conditions of salinity stress, any assessment of processes contributing to the growth and productivity of salt stressed plants must consider the impact that photosynthesis has on nitrate assimilation and vice-versa.

5.2 THE EFFECT OF VARIATIONS IN THE NITROGEN SUPPLY ON GROWTH OF SALT STRESSED *in vitro* PLANTLETS OF *N. tabacum*

As discussed previously, the decline in growth of *in vitro* shoot cultures of tobacco grown in the presence of NaCl on MS nutrient media (40 mM NO_3^--N + 20 mM NH_4^+-N) appeared to be related to a disruption in nitrate assimilation and a subsequent reduction in protein content. This study set out to investigate the proposal that an increase in the nitrate supply to salt stressed plants may alleviate the adverse effects of salinity on plant growth (Section 2.4.1). As both NO_3^--N and NH_4^+-N are available sources of N for plant growth it was necessary, prior to the investigation, to establish whether NO_3^--N as a sole nitrogen source was more beneficial to plantlet growth in culture than NH_4^+-N. In addition, comparisons to the MS control were made in order to determine how the growth responses of plantlets to a single N source related to those on a mixed NO_3^--N/NH_4^+-N nutrition.

Examination of plantlets grown *in vitro* for 35 d in the absence and presence of NaCl with a N supply (60 mM total N) as NO_3^--N only or NH_4^+-N only indicated that for all parameters measured, plantlet growth was significantly enhanced in the absence of NaCl and in the presence of 90 mM NaCl with a N supply as NO_3^--N only (Figure 4.2). At these NaCl levels, growth on MS nutrients was superior in most cases to growth on a single N source. At 180 mM NaCl, the lack of a distinction in plantlet growth between all the tested N regimes is suspected to be due to a severe inhibition of growth by NaCl. The root fresh mass:shoot fresh mass ratio of plantlets grown on NH_4^+-N (Table 4.1 B) was not significantly altered by an increase in salinity, indicating that root growth was inhibited to a similar extent as stem growth by salinity in the presence of NH_4^+-N. A reduction in the level of NH_4^+-N supplied to tobacco plantlets from 60 mM to 30 mM had no effect on root or stem growth, both

in the presence and absence of NaCl (Figure 4.3), suggesting that plantlet growth was highly sensitive to this form of N. It was only in the absence of N that a variation in growth responses were observed, with root fresh mass and root length being significantly improved in both the absence and presence of NaCl (Figure 4.3). Growth inhibition by NH_4^+-N under saline and non-saline conditions has been reported for several plant species grown in hydroponic culture, for example rice (Youngdahl *et al.*, 1982), maize and wheat (Lewis *et al.*, 1989), and cotton (Leidi *et al.*, 1992). Those workers attributed the reduction in growth of plants supplied with NH_4^+-N to a combination of several factors such as an alteration in the plants' water status, interference of NaCl ions with NH_4^+-N assimilation in the roots, and ion deficiencies in the plants induced by the presence of NH_4^+-N. When plants were fed with NO_3^--N only, those workers (Youngdahl *et al.*, 1982; Lewis *et al.*, 1989; Leidi *et al.*, 1992) found that plant growth was more vigorous than when the plants had been supplied with NH_4^+-N only. In addition, under saline conditions, NO_3^--N fed plants were less severely affected by salt than NH_4^+-N fed plants (Lewis *et al.*, 1989; Leidi *et al.*, 1992). The results obtained in the present study indicated that NO_3^--N as a sole N source was indeed favoured over NH_4^+-N for growth of tobacco plantlets *in vitro*, both in the absence and presence of NaCl. It was apparent from this study, however, that a mixed source of N (as NO_3^--N/NH_4^+-N, 2:1) was the most favourable for growth under both saline and non-saline conditions. This is supported by the observations of other workers who found plant growth to be superior under conditions of a mixed N nutrition, on comparison to a single N source, when grown in the absence (Hageman, 1984; Pessarakli and Tucker, 1985b; Bock, 1986) and presence (Cerdá and Martínez, 1988; Shaviv *et al.*, 1990) of salt.

The addition of nitrate to plants affects root and shoot growth in different ways. While shoot development is essential for the photosynthetic efficiency of a plant, a good root system is necessary for the acquisition of water and nutrients. It has been shown that the level of nitrate nutrition affects leaf growth and photosynthesis (Novoa and Loomis, 1981), while reports of the response of root development to the level of nitrate supply have yielded inconsistent results (Tennant, 1976; Maizlish *et al.*, 1980; Greef and Kullmann, 1992). Many workers investigating the growth responses of plants to saline conditions, either in a hydroponic or soil system, have indicated a more negative effect of salinity on shoot than root growth (Munns and Termaat, 1986; Larrson *et al.*, 1989; Robertson *et al.*, 1990). As discussed previously (Section 2.2), the presence of salt in the external environment will affect the absorption of water and nutrients by the roots. This may result in a nutrient

deficiency in the shoot and consequently, a reduction in growth. Thus, under saline conditions where shoot growth is inhibited, an increase in the nitrate supply may improve shoot development, resulting in an increase in photosynthetic efficiency. This would lead to an improvement in the growth and productivity of the plant.

A variation in the level of NO_3^--N as sole N source supplied to tobacco shoots grown *in vitro* in the absence of NaCl, had different effects on root growth and stem growth (Figure 4.4). Although the number of roots produced by plantlets appeared to be dependent on the presence of NO_3^--N in the media, root growth appeared to be unaffected by a NO_3^--N supply between 0 mM and 60 mM. The significant increase in stem growth in the presence of NO_3^--N suggested the incorporation of NO_3^--N into dry matter in the shoots. The growth inhibition by 120 mM NO_3^--N for all parameters suggested that these levels were toxic to plantlet growth, possibly due to the accumulation of NH_4^+-N as the plantlets failed to assimilate all the available NO_3^--N into protein (Figure 4.4). In most cases, plantlet growth was superior when supplied with NO_3^--N + NH_4^+-N (MS nutrients) compared to growth on all NO_3^--N levels tested.

The most noticeable feature with an increase in the NaCl supply was the lack of a significant improvement in growth when tobacco plantlets were provided with increasing levels of NO_3^--N (Figure 4.4). In the presence of NaCl, root production and growth (reflected as an increase in fresh mass) were unaffected by the presence or absence of NO_3^--N and were inhibited severely by NaCl, especially at 180 mM NaCl. The lack of a difference in the rate of root emergence between salinized shoots supplied without N and with varying levels of NO_3^--N or NH_4^+-N (Table 4.3 B) indicated that the processes of root initiation were not nitrogen dependent in salt stressed tobacco plantlets grown *in vitro*. A reduction in the length of the longest root with an increase in NO_3^--N supply, in the presence of both 90 mM and 180 mM (Figure 4.4) reinforces perhaps the lack of any positive effect of NO_3^--N nutrition on root growth in the presence of salt. The similarities between root growth of plantlets grown on 0 mM to 120 mM NO_3^--N only and those supplied with MS nutrients (40 mM NO_3^--N + 20 mM NH_4^+-N) in the presence of 90 mM and 180 mM NaCl suggests that neither the form nor the level of N affected root growth under saline conditions. A smaller root system in salt stressed tobacco plantlets most likely resulted in a reduction in the transport of water and nutrients to the shoots and probably contributed towards the inhibition of stem growth in these plantlets. The small improvement in stem growth with an increase in the NO_3^--N

supply observed at 90 mM NaCl could not be considered as an indication of an alleviation of the stress by NO_3^--N as stem growth was severely inhibited by 180 mM NaCl, regardless of NO_3^--N supply. The growth limiting factor in tobacco shoot cultures exposed to NaCl *in vitro* appeared therefore to be salinity rather than nitrate. The lack of an improvement in leaf chlorophyll content with an increase in the NO_3^--N supply, especially when exposed to 180 mM NaCl (Figure 4.4), was not thought to have contributed to the reduction in growth observed in these plantlets as carbon assimilation in *in vitro* plantlets is predominantly heterotrophic and thus not highly dependent on chlorophyll.

The lack of a significant improvement in the growth *in vitro* of salt stressed tobacco shoot cultures with an increase in the NO_3^--N supply contrasts with the findings of other authors. The addition of 6 mM to 10 mM NO_3^--N to tomato and cucumber plants grown in hydroponic culture in the presence of 100 mM NaCl enhanced the development of both shoot and root dry weights (Cerdá and Martínez, 1988). Hydroponically grown peanut plants exposed to 50 mM NaCl were affected particularly favourably by the addition of KNO_3 (1-9 mM), which resulted in a stimulation of root length and shoot dry weight (Silberbush *et al.*, 1988). Silberbush and coworkers suggested that NO_3^--N ions had inhibited the translocation of Na^+ and Cl^- ions from the roots to the shoots, thus protecting leaf metabolism against salt damage. In addition, those workers indicated an increase in nitrate uptake and NR activity in salinized plants supplemented with NO_3^--N, suggesting an improvement in the assimilation of nitrate and hence, dry matter production. However, the results obtained in this study were found also to be in agreement with those of other workers. Papadopoulos and Rendig (1983) showed that an increase in the NO_3^--N supply to hydroponically grown tomato could alleviate a salt stress only at low salinity levels of 10 mM. Furthermore, Feigin *et al.* (1987) found that the yield of tomato and melon plants in hydroponic culture was reduced by 50% in the presence of 90 mM NaCl, regardless of the NO_3^--N supply. The homology between the growth responses of those plants growing in hydroponic culture and the *in vitro* shoot cultures of this study is interesting as the general behaviour of plants growing in hydroponic culture may differ from those growing *in vitro* in a variety of ways. For example, plantlets grown *in vitro* are not as photosynthetically active as hydroponically grown plants. In addition, it has already been indicated (Section 5.1) that root and shoot growth under saline conditions differs between *in vitro* grown and hydroponically grown plants. Thus, due to such variations in the growth of plants *in vitro* and in soil/hydroponic culture, it cannot be certain whether the lack

of a positive effect of NO_3^--N on salt stressed plants in this study and in those of others is due to a similar response to the stress. The above discussion highlights the controversial nature of the issue surrounding the effects of NO_3^--N supplementation to salt stressed plants and emphasises the lack of understanding of the processes involved in salt stress and its relationship, if any, with nitrate metabolism. In order to clarify the lack of a positive effect of NO_3^--N nutrition on the growth of salt stressed tobacco *in vitro*, it was necessary to address the nature of the effect of salt on the processes of nitrate metabolism when the NO_3^--N supply was varied.

5.3 THE EFFECT OF VARIATIONS IN THE NITROGEN SUPPLY ON NUTRIENT UTILISATION AND NITRATE METABOLISM OF SALT STRESSED *in vitro* PLANTLETS OF *N. tabacum*

The lack of a positive correlation between the supply of NO_3^--N and growth of salt stressed tobacco shoot cultures *in vitro* could have been the result of two key factors: 1) the increase in the availability of NO_3^--N did not lead to a corresponding increase in NO_3^--N uptake and 2) if NO_3^--N was being removed from the medium in proportion to its supply, the observed growth responses were related to a disruption in the processes of reduction and incorporation of NO_3^--N into protein.

Although NO_3^--N uptake was reduced in the presence of NaCl, a corresponding increase in uptake with NO_3^--N availability was detected (Figure 4.8). In addition, the effect of salinity, although significant, was not particularly severe suggesting that salt did not pose a major barrier to the uptake of NO_3^--N in tobacco shoot cultures. When NO_3^--N was combined with NH_4^+-N in the growth medium (standard MS nutrients), NO_3^--N uptake from this medium appeared to be more affected by salinity than uptake from a NO_3^--N supply alone. The drastic reduction of NR activity, coupled with the absence of an effect by the NO_3^--N supply, suggests that the reduction of NO_3^--N was especially sensitive to salinity (Figure 4.13). The reason for the lack of an effect of NO_3^--N supply on NR activity could be two-fold: as NO_3^--N uptake occurs in the roots and its reduction in the leaves, the transport of NO_3^--N to the sites of reduction may have been inhibited by the presence of NaCl, and/or, salinity may have caused a severe inactivation or inhibition of synthesis of the NR enzyme, thus diminishing its capacity to reduce NO_3^--N. These two options will be discussed in more detail at a later stage in relation to Na^+ and Cl^- uptake and, the possible contribution of water stress effects to the disturbance in NR activity.

The response of leaf protein content to NO_3^--N additions of tobacco plantlets growing in the presence of NaCl provided a possible indication as to the effect of NO_3^--N nutrition under saline conditions (Figure 4.11). The positive response of leaf protein content (expressed on a fresh mass and a dry mass basis) to an increase in the NO_3^--N supply, particularly in the presence of 180 mM NaCl, initially raised the possibility of an efficient utilisation and incorporation of NO_3^--N into protein, despite the diminished availability of reduced NO_3^--N. However, the lack of a general improvement in growth of salinized plantlets supplied with increasing levels of NO_3^--N indicates that the proteins being produced were not being utilised for growth processes. If this was so, the question arises as to what function they served in the plantlets. It is proposed that the increase in protein content, particularly at 180 mM NaCl, represented the production of specific "stress" proteins that have been described by several workers as characteristic of a salt stress. It has been demonstrated that a salinity stress applied to maize callus (Ramagopal, 1986), barley roots (Hurkman and Tanaka, 1987) and barley seedlings (Ramagopal, 1987) resulted in the production of distinct polypeptide species which were not present when the stress was absent. Although these proteins are presumed to be directly involved in the plant response to the stress, their function is not known at present.

It was questioned above whether one of the causes of the lack of an effect of a NO_3^--N supply on NR activity was due to a disturbance in the transport of NO_3^--N from the roots to the leaves by salt. Thus, it is necessary to consider the relationship between NO_3^--N uptake and Na^+ and Cl^- uptake. An antagonism between NO_3^--N and Cl^- ions for uptake has been proposed as one of the major causes of a disturbance in cellular nitrogen metabolism by salt (Section 2.4.2). It has been suggested earlier that the inhibition of leaf NR activity in plantlets grown on MS nutrient media (NO_3^--N + NH_4^+-N) was not due specifically to the uptake of Na^+ and Cl^- ions, but appeared related rather to a reduction in the leaf water potential (Section 5.1). Like plantlets supplied with MS nutrients, Na^+ and Cl^- uptake from nutrient media containing NO_3^--N only did not appear to be excessive and, in addition, was not negatively correlated with the level of NO_3^--N supply. There did not appear to be a consistent relationship between a NO_3^--N supply of 0 mM to 120 mM and the removal of Na^+ and Cl^- ions from the nutrient medium. As NO_3^--N was taken up by plantlets in proportion to its supply, even under saline conditions, (Figure 4.8) the results obtained for Na^+ and Cl^- uptake were not suggestive therefore of a significant repression of NO_3^--N transport to the leaves by specific interference of Cl^- ions.

It is possible that the transport of NO_3^--N from the roots to the leaves to the sites of reduction by NR was inhibited as a result of a disturbance in the water relations of the plantlets by NaCl. It was discussed briefly in Section 5.1 that the inhibition of leaf NR activity in plantlets supplied with MS nutrients may be related to a reduction in leaf water potential as a consequence of the presence of NaCl in the external medium. If the water potential of the leaves of the plantlets were not reduced to levels lower than those of the external environment, there would be a reduction in the quantity of water and nutrients transported to the leaves. Not only would this contribute to a nutrient deficiency in the leaves, but it would result in loss of cell turgor and as a consequence, a reduction in growth (Section 2.2.2). Larsson (1992) indicated that the inhibition of nitrate uptake from the roots to shoots of osmotically stressed wheat seedlings, grown in hydroponic culture, was correlated strongly to a deterioration in the plant's water status. Although no specific studies were performed to assess the water potential of the leaves of the tobacco plantlets grown *in vitro*, an increase in the dry mass:fresh mass ratio of leaves of salt stressed plantlets was recorded with an increase in the NaCl supply, and was not affected by the NO_3^--N supply (Table 4.2). This increase could have resulted from a reduction in the leaf water content. The reduction in the transport of NO_3^--N to the leaves, despite the increase in its supply to the medium, as a result of the lowering of the external water potential by salt could have contributed significantly to the reduction in NR activity. On the other hand, Larsson *et al.* (1989) observed an increase in the NO_3^--N concentration in leaves of osmotically stressed wheat seedlings which showed a drastic decline in NR activity. It is possible thus that in the *in vitro* shoot cultures of salt stressed tobacco a significant transport of NO_3^--N to the leaves, in proportion to its supply, had occurred. However, the reduction of NO_3^--N may have been limited by a degradation of the NR enzyme or a decline in its synthesis as a result of a disturbance in the cellular water relations.

The above disussion presents alternative explanations for the effect of the NO_3^--N supply on nitrate uptake and NR activity of salt stressed tobacco plantlets grown *in vitro*. It was beyond the scope of this study to determine the relative contributions of the various mechanisms proposed. It is suggested that future investigations, such as 1) the translocation of NO_3^--N in the plantlets, 2) levels of NO_3^--N in the roots and leaves, 3) analyses of the synthesis of NR via determinations of NR mRNA and 4) studies of the water relations of plantlets exposed to salinity *in vitro*, may provide an indication as to the nature of the interactions between salinity and nitrate

metabolism. The most significant conclusion that could be made from the above analysis of NO_3^--N uptake and NR activity is that the lack of an improvement in growth of salt stressed tobacco plantlets, when provided with an increase in the NO_3^--N supply, was related to an inadequate utilisation of the available NO_3^--N for growth processes. These results appeared to indicate that the osmotic effects of salt played a large role in affecting the metabolism and ultimately the growth of tobacco plantlets *in vitro*.

5.4 OSMOTIC AND IONIC EFFECTS OF NaCl ON *in vitro* PLANTLETS OF *N. tabacum*.

The similarity in growth of plantlets grown on standard MS nutrients (40 mM NO_3^--N + 20 mM NH_4^+-N) in the presence of increasing osmotic potentials, induced by either NaCl or mannitol, indicated that the detrimental effect of NaCl on growth of these plantlets appeared to be predominantly osmotic (Figure 4.6). Root fresh mass was the only growth parameter which exhibited a more positive response to growth on mannitol than NaCl, suggesting that the inhibition of root mass was related to specific ionic effects of NaCl. This response may be due to the close proximity of the roots to NaCl in the growth media which may have decreased the root cell wall extensibility and thus resulted in a reduction in growth (Section 5.1). The lack of an ionic effect at the shoot level is suggestive of a reduction in growth due to a decline in the flow of water from roots to shoots, leading to dehydration and a loss of turgor. A reduction in water uptake may have been due both to the low water potential of the external medium and the diminished root system in plantlets exposed to NaCl.

Although root number and root length did not appear to be affected by an ionic stress, the rate of root emergence from shoot explants appeared to be sensitive to the presence of NaCl specifically. Root emergence from NaCl stressed plants was inhibited by NaCl (Table 4.3) whereas no effect on root emergence was detected with an osmotic stress imposed by mannitol (Table 4.5). An increase in root fresh mass:shoot fresh mass ratio of plantlets grown on increasing levels of mannitol was recorded (Table 4.4), which was in contrast to that of plantlets grown on NaCl (Table 4.1). For tobacco shoot cultures grown on mannitol, the increase in root fresh mass:shoot fresh mass appeared to be the result of an increase in root mass relative to stem mass. This further supports the existence of a salt specific effect on root growth. An osmotic effect on root growth cannot not be ruled out, however, as a decrease in the dry mass:fresh mass ratio of roots from plantlets grown on NaCl

was recorded (Table 4.2). This is suggestive of a reduction in the water content of these roots. Leaf chlorophyll content appeared to be very sensitive to salt-specific effects of NaCl, exhibiting a marked increase when the decrease in osmotic potential of the external medium was due to a non-ionic osmoticum only (Figure 4.6). Visual examination of osmotically stressed plantlets revealed that the elevated chlorophyll levels measured were due to the production of chlorophyll-rich new leaves (Plate 4.4). It is possible that NaCl ions in the growth medium interfered with the uptake of a nutrient ion or ions essential for chlorophyll formation in salt stressed plantlets. Cramer *et al.* (1991) indicated that the absorption of potassium, calcium and manganese ions by salt stressed barley seedlings was inhibited by a stress of 125 mM NaCl.

When NO_3^--N was supplied as the sole N source to tobacco shoot cultures exposed to an osmotic stress induced by mannitol (Figure 4.7), many similarities in growth to a NaCl-induced stress were detected (Figure 4.4). Both stem parameters of length and fresh mass exhibited identical growth patterns when plantlets were provided with NaCl or mannitol, indicating that the osmotic effects of NaCl were responsible for the observed decline in growth. Furthermore, there were no differences in stem growth between plantlets supplied with NaCl or mannitol with an incremental NO_3^--N supply from 0 mM to 120 mM. This suggests that the effect of an increase in the NO_3^--N supply on stem growth of salt stressed plantlets was largely independent of specific ionic effects of NaCl. For root fresh mass, the response to NO_3^--N was essentially the same as for the stem parameters, with an increase in the NO_3^--N supply from 0 mM to 120 mM resulting in very similar growth patterns when plantlets were supplied with NaCl or mannitol. Root growth has been shown both in this study and elsewhere (Greef and Kullmann, 1992) to be indifferent to the NO_3^--N supply under control (non-stressed) conditions and remained as such in the presence of NaCl and mannitol, despite a reduction in root growth caused by salt. The processes of root expansion under saline conditions in *in vitro* shoot cultures of tobacco were possibly limited by a reduction in the availability of a nutrient ion other than NO_3^--N. The large stimulation of root growth in plantlets supplied with a combination of NO_3^--N + NH_4^+-N (standard MS nutrients) detected in mannitol-grown but not NaCl-grown plantlets, suggests that the ionic effects of NaCl on root growth (expressed as changes in root fresh mass) were limited to plantlets grown on a mixed NO_3^--N/NH_4^+-N nutrition. This implies that a different relationship exists between salinity and plant growth when the form of nitrogen nutrition is varied. This is an area which warrants future research.

The root fresh mass:shoot fresh mass ratio of plantlets supplied with increasing amounts of NO_3^--N only was found to differ between plantlets grown on mannitol or NaCl. While a general decrease in the root fresh mass:shoot fresh mass ratio was detected for plantlets exposed to NaCl (Table 4.1), a change in the NO_3^--N concentration supplied to mannitol-grown plantlets had no effect on the ratio with an increase in the osmotic potential (Table 4.4). This suggests that a water deficit resulting from an osmotic stress affects root and stem growth of *in vitro* grown tobacco plantlets equally wheras the presence of NaCl appears to have an additional effect on root growth. In addition, the presence of varying levels of NO_3^--N in the growth medium did not appear to affect root growth relative to stem growth, when the stress was osmotic only. The rate of root emergence from tobacco shoot explants, exposed to NaCl or mannitol *in vitro* and supplied with varying levels of NO_3^--N, did not correlate with the results obtained for plantlets supplied with standard MS nutrients (Table 4.5). In the latter case, root emergence appeared to be affected by the ionic component of salt stress but when the NO_3^--N supply was varied to plantlets supplied with mannitol, root emergence appeared to be sensitive to an osmotic stress at -0.88 MPa (Table 4.5). At -0.44 MPa, root emergence appeared delayed by mannitol which suggests the possibility of a specific effect of mannitol itself on the process of root emergence. Evidence has indicated that mannitol may in some cases penetrate the cells, thus exerting more than an osmotic effect alone (Levitt, 1980; Termaat and Munns, 1986). The synthesis of leaf chlorophyll was stimulated in the presence of mannitol when plantlets were supplied with increasing levels of NO_3^--N. No significant difference between the respective NO_3^--N levels were detected at both -0.44 and -0.88 MPa, indicating that the improvement in chlorophyll production in plantlets supplied with mannitol was not due to an increase in the NO_3^--N supply (Figure 4.6). As was suggested earlier (Section 5.1), NaCl may have led to a specific inhibition at the root level of the absorption of an essential nutrient required for chlorophyll synthesis.

Nitrate uptake by salt stressed plantlets supplied with standard MS nutrients and increasing levels of NO_3^--N was enhanced when NaCl was replaced with isosmotic concentrations of mannitol (Figure 4.9). Nitrate uptake was proportional to NO_3^--N supply in plantlets grown on mannitol, as observed for plantlets grown on NaCl. No significant differences between NO_3^--N uptake were recorded for plantlets grown on 30 mM and 60 mM NO_3^--N when supplied with NaCl or mannitol. These results suggest that the absorption of NO_3^--N by the roots was affected primarily by specific

ionic effects of NaCl present in the external medium. This ionic effect of salt on NO_3^--N uptake is positively correlated to a reduction in root growth (expressed as a change in root fresh mass) which was suggested to be due to ionic effects of NaCl. The reduction in the absorption of NO_3^--N into salt stressed tobacco plantlets may thus have been related to a specific interference by NaCl ions, or a reduction in the size of the root system.

It may be concluded, therefore, that the ionic effects of salt were largely, although not exclusively, responsible for the reduction in root growth, and uptake of NO_3^--N into the roots, of salt stressed tobacco plantlets. Osmotic effects of salt appeared to be the dominant factor causing a reduction in stem growth. A reduction in the flow of water from the roots to the shoots, due to an osmotic stress, provides an explanation for the lack of a positive effect of increased NO_3^--N availability on plantlet growth. Under saline conditions, an increase in the NO_3^--N supply would be unable to improve shoot growth if growth was limited by a flow of water and nutrients up the plantlet.

There have been several contradictory reports concerning whether osmotic or ionic effects of NaCl are primarily responsible for causing injury to salt stressed plants. Termaat and Munns (1986) indicated that for several species exposed to NaCl (100 mM) in hydroponic culture, a reduction in shoot growth was due to a reduction in the transport of an essential nutrient to the shoot, a result of a salt specific effect at the root level. On the other hand, Larsson *et al.* (1989) indicated that in osmotically stressed wheat seedlings (-1.6 MPa), an inhibition of shoot growth was a result of a decline in the leaf tissue water content. The primary cause of salinity toxicity in hydroponically grown maize and wheat supplied with 60 mM to 80 mM NaCl was reported to be due to osmotic factors which resulted in a curtailment of cell growth and eventually, wilting (Lewis *et al.*, 1989). An improvement in the growth of salinized peanut plants (50 mM NaCl) fed with additional NO_3^--N was described as being the result of a reduction in the specific toxic effects of NaCl, due to the preferential uptake of NO_3^--N (Silberbush *et al.*, 1988). These examples indicate that the contribution of osmotic and ionic effects of salt differ between plant species, NaCl supply and experimental design. It is possible also that the same plant, under different conditions, may exhibit alterations in the osmotic and ionic effects of salinity. However, as all the above studies were performed using the methods of hydroponic culture, it is not certain whether a direct comparison may be made between the results of those studies and the responses determined for *in vitro* grown tobacco shoot cultures.

It is generally accepted that a plant exposed to saline conditions is required to osmotically adjust to the stress if it is to survive. Plant are reported to achieve this osmotic adjustment by either taking up NaCl ions or synthesising organic solutes such as proline, to balance the osmotic potential in the cytoplasm (Section 2.2.1). In this study, the removal of Na^+ and Cl^- ions from the growth medium by tobacco plantlets did not appear to be very significant and thus could not be considered to be the major mechanism by these plantlets in achieving osmotic adustment. The accumulation of proline in leaves of salt stressed plants has been indicated in contributing to the osmotic balance of the cytoplasm, and has also been suggested to have a protective effect on enzymes exposed to high ionic concentrations in the cells (Greenway and Munns, 1980; Levitt, 1980). However, proline was not determined in this study and thus its contribution to osmotic adjustment could not be assessed. Nevertheless, plantlets grown *in vitro* have an abundant supply of sugars in the nutrient medium and may utilise these sugars for osmotic adjustment (Dracup, 1991). Tobacco shoot cultures were supplied with 10 g/l sucrose in the MS nutrient medium and it is possible that these plantlets utilised sucrose as an osmoticum preferentially to NaCl. Reliance on sugars for osmotica would be costly, however, to plants growing in soil or in hydroponic culture. Plantlets growing in culture may thus respond to a salt stress in a different manner to when grown under alternative experimental conditions. The effect of NaCl on tobacco plantlets grown *in vitro* was found to be predominantly osmotic which was thought to explain the absence of a significant improvement in growth when provided with increasing levels of NO_3^--N. It is questioned whether a similar response would have been recorded had a similar study been performed using either soil grown or hydroponically grown plants. The MS nutrient medium has an osmotic contribution of -0.19 MPa, which is greater than that of soil or the nutrient solution used in hydroponics. The removal of this osmotic contribution may or may not have resulted in an increase in the ionic effects of NaCl in tobacco plants.

This study aimed to test the hypothesis that nitrate additions to plants growing in saline environments may alleviate the stress. During the tenure of this study, it became apparent that the responses of plants to salinity *in vitro* may differ in many aspects from those of plants grown in the field. These observations may have repercussions for the efforts of workers endeavoring to produce salt tolerant plants via *in vitro* culture systems. Some success has been achieved in the production of salt tolerant cell lines from salt sensitive species, but there have been limited reports

of the regeneration of salt tolerant plantlets from these cell lines (Section 2.5.2.3). In addition, regenerated plants that appear salt tolerant *in vitro* usually lose their tolerance when transferred to the field. This may be largely due to the selection of traits *in vitro* which are inappropriate to salt tolerance of whole plants, probably the result of assumptions that mechanisms of salt tolerance in cultured cells and whole plants are similar. The results obtained in this study emphasise the need for future in-depth investigations of the morphological and physiological responses of plants grown *in vitro* to salinity before *in vitro* culture systems may be utilised successfully to improve the growth of plants under saline conditions.

6. CONCLUSIONS

Several conclusions were arrived at while investigating the effects of nitrate nutrition on the growth of salt stressed tobacco shoot cultures *in vitro*.

1. The presence of NaCl in the growth medium had deleterious effects on the growth of *in vitro* shoot cultures of *Nicotiana tabacum*.

2. Root growth appeared to be more sensitive to salinity than shoot growth, a response which is proposed to be characteristic of salt stress *in vitro*.

3. It appeared that the reduction in root growth was predominantly the result of ionic effects of salt while the inhibition of shoot growth was suggested to be related to a reduction in the flow of water and nutrients from the roots to the leaves, due to the osmotic effects of salt.

4. Salinity had an adverse effect on nitrate assimilation which most likely contributed towards the reduction in growth of salt stressed plantlets.

5. The addition of varying levels of nitrate to salt stressed shoot cultures did not result in a significant improvement in growth.

6. Nitrate uptake was proportional to its supply in salt stressed plantlets, but NR activity did not reflect a change with nitrate supply and was drastically affected by the presence of salt.

7. The effects of salt on nitrate assimilation were proposed to be related to the following factors: a) a reduction in the uptake and transport of nitrate to the leaves, b) a reduction in the amount of cellular nitrate available for reduction, and c) a degradation of and/or reduction in the synthesis of the NR enzyme. These factors may not be mutually exclusive.

8. The issue of whether salinity had an effect at the transcriptional level on the synthesis of the NR enzyme remained unresolved.

9. The lack of a positive effect of nitrate on plantlet growth under saline conditions was proposed to represent an inadequate utilisation of the available

nitrate for growth processes. This may be related to a reduction in root growth due to ionic effects of salt and the predominance of osmotic effects of salt on the growth of shoots.

10. It is proposed that the response of plants to salinity *in vitro* may differ in several aspects from those in the field. As a consequence, more research to determine the mechanisms operating at the physiological and molecular level in *in vitro* salt stressed plants is required before *in vitro* culture systems may be successfully utilised to improve the growth of salt stressed plants.

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