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ISBN 0-315-56120-3

Effects of NaCl on Tobacco Callus Cultures

Ann Francine Greer

A Thesis

in

The Department

of

Biology

Presented in Partial Fulfillment of the Requirements
for the Degree of Master of Science at
Concordia University
Montréal, Québec, Canada

July 1989

ABSTRACT

Effects of NaCl on Tobacco Callus Cultures

Ann Francine Greer

Tobacco (*Nicotiana tabacum* L var. Wisconsin 38) calluses capable of growing on NaCl supplemented Murashige and Skoog's (MS) medium were obtained by culturing them on a stepwise gradient of NaCl (0, 26, 51, 103, 171, 257, and 342 mM). With increasing tolerance to NaCl, the calluses showed reductions in water content, cell size, and K^+ content, and increases in osmotic potential and in the levels of Na^+, Cl^-, and chlorophyll. Both non-tolerant and NaCl-tolerant calluses were capable of producing shoots on NaCl-free shoot-inducing medium containing 0.1 mg/l indoleacetic acid (IAA) and 10.0 mg/l benzylaminopurine (BAP). More shoots per callus were produced from non-tolerant than tolerant calluses, likely due to the fact that the levels of starch were lower in the latter calluses. The salt-tolerant calluses lost their tolerance to salt stress after the calluses were cultured for three passages (15 weeks) on salt-free growth medium, suggesting a phenotypic rather than a genotypic selection.

Acknowledgements

I would like to thank my supervisor, Dr. B. S. Mangat for his guidance and constructive criticism throughtout my project. Appreciation is expressed to Dr.R. K. Ibrahim and Dr. S. S. Ashtakala for their helpful advice as members of my committee.

Special thanks are extended to Dr. P. Widden, for allowing me the use of his phase contrast microscope, Dr. N. Kapoor, for the use of his microscopic and photographic equipment and for his assistance in photography, Dr. P. Anderson for the use of his atomic absorption spectrometer, Mr. P. Aysola for his expertise in the analysis of ions, and Mr. G. Selig for his direction in statistical analysis. Thanks to Mr. C. Boer and all the technical staff for their cooperation.

My gratitude is expressed to Kannan Krishnan, Barbara Pierce, and Jane Fitzgerald for reading the thesis. I am indebted to my colleagues and friends, Louise Brisson, Margaret Cooke, Suzette Dawaf, Georges and Barbara Dodds, Carole Eng, Sharon Harney, Diana Lobo, Claudine Luu, Filoteo Pasquini, Bill Pong, Janet Rokas, Mihir Roy, and Boon Tang for their valuable assistance, moral support, and encouragement.

Dedication

To my family for giving me strength

The chapter has come to an end.

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A. INTRODUCTION

A major problem affecting the use of arable and potentially arable land is the increasing accumulation of salt in the soil (Nabors, 1983; Rains *et al.*, 1980; Flowers *et al.*, 1977; Pojakoff-Mayber, 1975). Soil salinity arises as a result of arid to semi-arid climates, lowering of the water table, removal of vegetation, and/or low precipitation (Norlyn, 1980; Chapman, 1975). However, it is believed that secondary salinization caused by irrigation water is the greatest problem (Flowers *et al.*, 1986, 1977; Tyagi, 1986; Bessler, 1983; Croughan *et al.*, 1981; Norlyn, 1980).

It has been estimated that one-third of the world's irrigated land is affected by salinity (Nabors, 1982b; Norlyn, 1980). In many instances, the irrigation water has been found to be of poor quality, containing high levels of soluble salts which eventually end up in the soil. This results in reduced crop yield and the gradual degradation of once-productive agricultural land (Bessler, 1983; Nabors, 1983). Plants growing in saline environments are often faced with high levels of toxic ions, low external water potentials and deficiencies of essential nutrients (Greenway and Munns, 1980; Rains *et al.*, 1980; Flowers *et al.*, 1977; Poljakoff-Mayber, 1975; Levitt, 1972). Because of these stressful conditions, a number of biological systems are altered resulting in reductions in plant growth.

The majority of economically-important plants are glycophytes. Although

the level of salt tolerance in glycophytic plants is known to vary within species and varieties as well as with the development stage of the plant, they are not capable of completing their life-cycle in the presence of high levels of salt (Carter, 1975). Variations in morphology and physiology have been noted in glycophytes exposed to high salt concentrations (Greenway and Munns, 1980); however, the mechanisms of salt tolerance by these plant species are not fully understood. In an attempt to understand how glycophytes deal with the adverse effects of high salinity, studies have been carried out with halophytes (Poljakoff-Mayber and Gale, 1975; Waisel,1972). Since many halophytes complete their life cycle successfully under conditions of high salinity, they appear to be an excellent model for studying the effects of salinity on plants. Results of several investigations have indicated that halophytes can tolerate high salt conditions via ion accumulation and compartmentalization and through the production and accumulation of organic compounds i.e., proline, glycinebetaine (Flowers *et al.*, 1986; 1977). The occurrence of succulence, a thickened cuticle, and salt glands in these plants do aid in their adaptation to and tolerance of high salt.

Apart from using halophytes to examine the mechanisms of salt tolerance, glycophytes which are traditionally non-salt tolerant can be rendered tolerant by several means. Primarily, salt tolerant plants can be developed through conventional whole-plant breeding systems where existing germplasm is screened in order to obtain tolerant cultivars (Norlyn, 1980; Ramage, 1980). However, because of the inadequacy of the breeding systems, success rates have been limited, and only a few tolerant cultivars have been produced (Raghava Ram and Nabors, 1985; Ramage, 1980; Nabors, 1982). Owing to the immense time and expense involved, researchers have been pressed to

investigate other means of screening for tolerance.

More recently, pla · tissue and cell culture techniques have proven to be a more rapid and efficient means of screening cells exhibiting salt tolerance (Maliga, 1980; Rains *et al.*, 1980; Croughan *et al.*, 1978; Dix and Street, 1975; Nabors *et al.*, 1975). The potential utility of the salt tolerant cells and tissues is two-fold: (1) the development of salt tolerant plants; and (2) the elucidation of processes involved in salt tolerance. Tolerance induced in cells and tissues can be studied at the cellular level without the involvement of specialized anatomical and physiological processes of the whole plant (Smith and McComb, 1981). Consequently, observations at the cellular level may help explain some of the processes involved in a whole plant.

Previous investigations focussed mainly on establishing tolerant cells and regenerating plants in an attempt to determine whether or not salt tolerance was being inherited by the progeny (Nabors *et al.*, 1975, 1980; Dix and Street, 1975). More recently, researchers have examined changes in morphological, physiological and metabolic processes in salt tolerant cells and tissues. These studies have shown that tolerant cells and tissues accumulate Na^+, Cl^-, and other nutrient ions to levels which differ from those of non-tolerant cultures when both are exposed to high salt concentrations (Salgado-Garciglia *et al.*, 1985; Pandey and Ganapathy, 1984; Kochba *et al.*, 1982; Dix *et al.*, 1982; Heyser and Nabors, 1981a,1981b).

Systematic characterization of the changes that take place in cells acquiring salt tolerance may provide some useful information in the identification of biological markers. This could aid in one-step selection, efficient screening (faster detection of cells with desirable traits), and perhaps in

increasing the level of tolerance in existing plants (Chandler and Thorpe, 1986; Stavarek and Rains, 1985).

Several different approaches can be utilized to induce salt tolerance in plant cells and tissue cultures. They are: 1) genetic manipulations, obtaining and transferring genes regulating salt tolerance in order to express increased tolerance; 2) mutagenesis, a phenomenon whereby cells undergo changes in the heritable genetic material when exposed to mutagens (usually chemicals and irradiation); and 3) spontaneous producion of variants from cells and tissue cultures by exposing them to selective agents (e.g. NaCl) (Raghava Ram and Nabors,1985; Bhojwani and Razdan, 1983). At present, only the latter two procedures are being successfully employed in the production of cells with increased salt tolerance (Raghava Ram and Nabors 1985).

A.1.0. Types of culture systems available

A number of systems are available for selecting cells for salt tolerance. These include protoplast, haploid, cell suspension, and callus cultures. Protoplasts are thought to be a superior selection system since isolates consist of a population of single cells that can directly be isolated from the plant. Cultures and plants regenerated from protoplasts are likely to be genetically identical. However, protoplasts are difficult to isolate from most plant species, and in many cases the rate of division and proliferation are quite low (Maliga, 1984; Flick, 1983; Tomes and Swanson,1982). Haploid cultures provide

another alternative for selection. This system can be employed to detect recessive alleles and, therefore, plants regenerated from selected cells are likely to be true to type. Again, as with protoplasts, it is difficult to generate haploid cultures from most plant species, and successful plantlet regeneration is low.

A survey of the pertinent literature indicates that cell suspension and callus cultures have been utilized in majority of the studies designed to select salt-tolerant lines (Hassan and Wilkins, 1988; Kavi Kishor, 1988; Paul and Ghosh, 1986; Binzel *et al.*, 1985; Harms and Oertli, 1985; Pandey and Ganapathy, 1985, 1984; Salgado-Garciglia *et al.*, 1985; Chandler and Vasil, 1984; McHughen and Swartz, 1984; Bhaskaran *et al.*, 1983; Ben-Hayyim and Kochba , 1983; Nabors, 1983; Rangan and Vasil, 1983; Smith and McComb, 1983; Watad *et al.*, 1983; Ben-Hayyim and Kochba, 1982b; Handa *et al.*, 1982b; Kochba *et al.*, 1982; Warren and Gould, 1982; Dix and Pearce, 1981; Heyser and Nabors, 1981a, 1981b; Sobko *et al.*, 1981; Tyagi *et al.*, 1981;Hasegawa *et al.*, 1980; Orton ,1980; Nabors *et al.*, 1980, 1975; Croughan *et al.*, 1978; Tal *et al.*, 1978; Dix and Street, 1975). Cell suspension cultures are generally preferred over callus cultures owing to a more uniform exposure to the selective agent when cells in suspension are bathed in the medium (Bhojwani and Razden, 1983). However, very fine cell suspension cultures are difficult to obtain, grow poorly, and may sometimes have increased chromosomal abnormalities. They also show a low potential for regeneration.

Calluses (small cell aggregates), on the other hand, can be easily obtained from a wide variety of plants. The callus culture maintenance is well established and the relative loss of regenerative ability is low (Maliga, 1980).

Furthermore, stable salt-tolerant cell lines and regenerated plants have previously been obtained from callus cultures (Ben-Hayyim and Kochba , 1983, 1982b; Kochba *et al.*, 1982; Croughan *et al.*,1978). Experiments by Nabors (1983) have demonstrated that cells in callus culture can tolerate a higher concentration of NaCl than can these cells in suspension culture.

Using cell and tissue culture techniques, salt-tolerant cultures have been obtained from a variety of plants including *Nicotiana tabacum* (Binzel *et al.*, 1985; Nabors, 1983; Watad *et al.*, 1983; Hasegawa *et al.*, 1980; Nabors *et al.*, 1975), *Ipomoea batatas* (Salgado-Garciglia *et al.*, 1985), *Pennisetum typhoides* (Chandler and Vasil, 1984), *Cicer arietinum* (Pandey and Ganapathy, 1984), *Medicago sativa* (Smith and McComb, 1983; 1981), *Citrus sinensis*, *Citrus aurantium* (Kochba *et al.*, 1982), and *Capsicum annuum* and *Nicotiana sylvestris* (Dix and Street, 1975).

A.1.1. Media

a) Medium composition

Maintenance and growth of plant cells and tissues are carried out on a defined nutrient medium consisting of inorganic salts and organic nutrients. Of the many inorganic salt formulations available, the most widely used is that of Murashige and Skoog (MS) (Murashige and Skoog, 1962), which is generally employed to culture a broad variety of tissues and organs. The organic nutrients added to the culture medium include vitamins, amino acids, growth regulators, and carbohydrates. Often added as vitamin supplements are

nicotinic acid, thiamine, and pyridoxine. Inositol is also added to enhance culture growth. Though amino acids are not essential, glycine is included in most media. The growth regulators required for the initiation and growth of cell and tissue cultures are auxins and cytokinins. The most commonly used auxins are indoleacetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and 1-naphthaleneacetic acid (NAA). The two most common cytokinins are 6-furfuryl aminopurine (kinetin) and benzylaminopurine (BAP). Sucrose (2-4% w/v) is usually added as a carbon and an energy source (George and Sherrington, 1984; Thorpe, 1982).

The medium is prepared in an aqueous solution if cell suspensions are cultured. Otherwise, the nutrient medium is solidified with agar at a concentration of 0.6 to 1.0% (w/v) for callus cultures.

b) Selective agent

One or more of the salts such as KCl, Na_2SO_4, $CaCl_2$, or NaCl, representing saline and/or alkaline conditions may be added to the growth medium to select cells tolerant to a particular salt (Raghava Ram and Nabors, 1985; Tal, 1982; Maas and Nieman, 1978; Flowers *et al.*, 1977). In the majority of selection studies, NaCl is added (Paul and Ghosh, 1986; Ben-Hayyim *et al.*, 1985; Binzel *et al.*, 1985; Salgado-Garciglia *et al.*, 1985; Chandler and Vasil, 1984; Pandey and Ganapathy, 1984; Kochba *et al.*, 1982; Hasegawa *et al.*, 1980; Croughan *et al.*,1978; Dix and Street, 1975; Nabors *et al.*, 1975).

c) Other additives

Several compounds have been found to accumulate in plants, cell suspensions, and tissue cultures in response to salt stress. These include proline, abscisic acid (ABA), and betaine and its derivatives, such as, glycinebetaine, choline and trigonelline (McDonnell and Wyn Jones, 1988; Binzel *et al.*, 1987; Chandler and Thorpe, 1987, 1986; Rains *et al.*, 1986; LaRosa *et al.*, 1985; Pandey and Ganapathy, 1985; Handa *et al.*, 1983; Watad *et al.*, 1983; Storey and Wyn Jones, 1977). Investigations suggest that adding one of these metabolites to the culture medium improves salt tolerance in sensitive cell cultures (Kavi Kishor, 1988; Pandey and Ganapathy, 1985). Particularly, the application of betaines to the culture medium of cells undergoing selection may lead to cultures with enhanced salt tolerance (Kavi Kishor, 1988). In addition, polyethylene glycol, mannitol, and dextran have been used to obtain cells tolerant to a particular salt (Harms and Oertli, 1985; Handa *et al.*, 1982a; Bressan *et al.*, 1981).

A.1.2. Selection of salt tolerant cultures

Cells tolerant to salt stress can be induced by either direct or indirect selection. Direct selection entails adding the salt directly to the medium. This can be carried out by one of two methods- salt is either supplemented at near lethal concentrations (i.e., one step selection) or it is increased gradually throughout the culture period (i.e., stepwise selection) (Rains *et al.*, 1986;

Chandler and Thorpe, 1986; Chandler and Vasil, 1984; Maliga,1984; Nabors,1982; Sobko et al., 1981).

Adding inhibitory levels of salt to the culture medium represses the growth of non-tolerant cells, thus promoting the growth of tolerant cells capable of overcoming the stress. These cells eventually make up the majority of the population (Chandler and Vasil, 1984; Maliga, 1984; Nabors, 1982). Variant cells obtained through stepwise selection are slowly acclimated to the increasing concentrations of salt in the medium. It has been suggested (Harms and Oertli, 1985) that stepwise selection reduces the severity of the initial osmotic shock and promotes a gradual build-up of tolerance which may more closely parallel selection in nature. Stepwise selection is deemed a suitable method to select tolerant calluses, while one-step selection is preferred for the isolation of variants from cell suspension cultures (Tomes and Swanson, 1982).

Naturally occurring salt-tolerant variants have been found to occur in tobacco, in one out of every 10^5 to 10^6 cells (Raghava Ram and Nabors, 1985; Nabors, 1982). A typical 250 ml flask of 100 ml of a suspension culture (cells or cell aggregates grown in liquid medium) contains upwards of $5x10^6$ cells. On the other hand, a 25x70 mm vial containing a 20x23 mm piece of callus (a mass of undifferentiated cells grown on solid medium) contains upwards of $5x10^4$ cells (Raghava Ram and Nabors,1985; Nabors,1982, 1976). It is apparent that cell populations of both callus and suspension cultures are sufficiently large to produce variants.

Isolated cell lines and regenerated plants capable of growing at high salt concentrations have been termed tolerant, resistant, selected, or adapted. Used interchangeably, the terms tolerant, resistant and selected all imply that cells are

capable of surviving and growing at high salt concentrations after pre-exposure to salt conditions. Changes may or may not be genetic in origin. Salt-adapted lines are not necessarily genetically different but are able to grow under salt stressed conditions (Rains *et al.*, 1986).

The time required to select tolerant cultures is variable. Cells obtained through one-step selection under near-lethal concentrations are usually cultured between 26 and 52 weeks. Those selected by the stepwise process can be cultured within a period of 24 to 60 weeks. These cultures are maintained at each salt concentration for 4 to 24 weeks (Hassan and Wilkins, 1988; Chandler and Vasil, 1984; Nabors, 1982).

A.1.3. Assessing tolerance and stability

It is important to examine the stability of the selected trait once a desired level of salt tolerance has been achieved. Cultures assessed to be tolerant have the ability to remain viable and grow in the presence of salt. Tolerance is usually evaluated by determining the growth of selected cell cultures over a gradient of salt concentrations. Tolerant cell cultures are transferred to a medium without salt for a number of passages; subsequently, cells are returned to media with salt and their stability is then assessed on the basis of growth. In many cases, the growth of selected cells is compared to that of unselected cells over several salt concentrations. Growth of cell cultures and calluses is commonly measured by one or more of the following methods: cell fresh weight, cell dry weight , packed cell volume, total cell protein and cell

number (Chandler and Thorpe, 1987; Binzel *et al.*, 1985; Harms and Oertli, 1985; Pandey and Ganapathy, 1985; Warren *et al.*, 1985: Rangan and Vasil, 1983; Handa *et al.*, 1982b; Tyagi *et al.*, 1981, Nabors *et al.*, 1975; Dix and Street, 1975).

Tolerance can be phenotypic or genotypic in nature. A tolerant culture is considered to be stable if it retains the ability to grow in the presence of the selection agent after being cultured in the absence of that agent. Stability of the selected trait is evidence of genetic change. NaCl-tolerant calluses of *Ipomoea batatas* L. (Salgado-Garciglia *et al.*, 1985) and *Cicer arietenum* L. CV. BG-2D3 (Pandey and Ganapathy, 1984) were shown to retain their tolerance when their respective cells were grown for three passages without NaCl and then were exposed to NaCl. Similar results have also been obtained for a variety of other plant cultures (Rangan and Vasil, 1983; Ben-Hayyim and Kochba, 1982; Kochba *et al.*, 1982; Sobko *et al.*, 1982; Tyagi *et al.*, 1981). Other investigators have established that stability is lost in the absence of the selective agent. After being placed in medium without added salt for several subcultures Hasegawa *et al.* (1980) found that salt tolerance was not retained in *Nicotiana tabacum* cell suspension cultures.

Variations which occur in the morphology of cells and in regenerated plants may be used to characterize salt tolerance. The persistence of phenotypic characteristics in regenerated plants and in their progeny can thus indicate inheritability of the salt tolerant trait and possible other variations in the genotype as well (Nabors , 1982).

A.2.0. CHARACTERIZATION OF SALT-TOLERANT CULTURES

A.2.1. Osmotic adjustment and ion regulation

Several reports have indicated that halophytes adjust osmotically via ion accumulation, ion compartmentalization, and synthesis of organic osmotica (Chandler and Thorpe, 1986; Epstein, 1980; Flowers *et al.*, 1977, 1986; Maas and Nieman, 1978). It has also been suggested that non-halophytes exhibit similar ways of dealing with salt (Greenway and Munns, 1980). As yet, it is not known which of these processes are adopted by tolerant cells. By characterizing ion regulation patterns in salt tolerant and non-tolerant cells, an understanding of one of the factors involved in salt stress tolerance may be achieved.

Ion uptake, usually in the form of Na^+ and Cl^-, has been suggested by several investigators to be one of the methods by which tolerant cells undergo osmotic adjustment (Binzel *et al.*, 1988,1987 ; Heyser and Nabors, 1981a). Determinations of Na^+ and Cl^- levels in salt tolerant and non-tolerant cell lines have indicated that tolerant cells take up greater amounts of Na^+ and Cl^- as the concentration of NaCl is increased in the medium. Under the same conditions, non-tolerant cells take up Na^+ and Cl^- to a lesser degree (Binzel *et al.*, 1987; Ben-Hayyim *et al.*, 1985; Pandey and Ganapathy, 1984; Dix *et al.*, 1982; Liu and Yeh, 1982; Heyser and Nabors, 1981a ; Rains *et al.*, 1980). Binzel *et al.* (1987) found that Na^+ and Cl^- levels increased with decreases in osmotic potential. Accumulated Na^+ and Cl^- were suggested to be the principal solutes for osmotic adjustment in NaCl-tolerant tobacco cell lines (Binzel et al., 1987;

Heyser and Nabors, 1981a, 1981b). However, there have been reports of tolerant cell lines of *Medicago sativa* (Croughan *et al.*, 1978) and *Citrus sinensis* (Ben-Hayyim and Kochba, 1983) which absorb Na^+ and Cl^- ions at equal or lower levels than non-tolerant cells. In a number of other studies, when tolerant cell lines have been examined for K^+ content, it has been found that tolerant cells contained lower levels of K^+ than control cells (Binzel *et al.*, 1987; Ben-Hayyim *et al.*, 1985; Pandey and Ganapathy, 1984; Ben-Hayyim and Kochba, 1983; Liu and Yeh, 1982).

A.2.2. Organogenesis and starch content

The use of plant cells and tissue cultures as tools for tolerance selection has been useful because of its potential for the production of a whole plant from a single cell. Regenerated plants can be examined for phenotypic characteristics and inheritability of selected traits. However, one of the major problems faced by investigators involved in selecting desirable traits is that the tolerant cells lose the ability to regenerate through organogenesis and/or embryogenesis.

A number of factors, including the length of selection, the salt content of the medium and the starch content of the tissue may be responsible for the reduction or loss of organogenic capacity. Long term selection has been proposed to promote; 1) chromosomal mutations, 2) hormonal imbalances, or 3) reductions in totipotency, all of which may affect organogenesis (Bhojwani and Razdan, 1983). Normally the presence of salt inhibits organogenesis. Salt has

been suggested to affect organogenesis because of: imbalances of hormones and osmotic adjustment (Pua *et al.*, 1985). However, organogenesis in the presence of salt has been reported. One reason for the differences observed in organogenesis in salt-containing media could be the presence of starch in the tissue. Stavarek and Rains (1985) observed a decrease in the utilization of starch in NaCl-selected alfalfa cells growing in 1% NaCl. They also observed that starch utilization in unselected cells is inhibited by NaCl. Starch has been found to accumulate in calluses prior to shoot development, and shoots were formed only in regions with heavy starch deposits (Mangat *et al.*, 1989; Bhojwani and Razdan, 1983; Nabors , 1982). It has been indicated that starch is used as an energy source as well as an osmoregulator in organogenic processes (Thorpe *et al.*, 1986; George and Sherrington, 1984; Brown *et al.*, 1979).

A.3.0. Objectives

From the aforementioned, it would appear that there are a number of conflicting reports on the morphological, physiological and biochemical characteristics (tolerance, stability, solute accumulation, and organogenetic capacity) of cells and tissues selected to tolerate high salinity. Therefore, the objectives of the present study were: (1) To establish *Nicotiana tabacum* var Wisconsin 38 calluses tolerant to NaCl concentrations of up to 342 mM by a stepwise selection method; (2) To investigate the effect of NaCl tolerance on growth, cell size and shape, osmotic regulation, organogenetic capacity, and Na^+, Cl^-, K^+, chlorophyll and starch contents of calluses tolerant at the different concentrations of NaCl; (3) To evaluate the effect of trigonelline on the salt-tolerance of tobacco calluses.

B. MATERIALS AND METHODS

B.1.0. ESTABLISHMENT OF NaCl TOLERANT TOBACCO CALLUS CULTURES

B.1.1. Initiation of callus cultures

Callus cultures were initiated from stem pith explants of 8-10 week old greenhouse grown *Nicotiana tabacum* (L.) var. Wisconsin 38 plants. Plants were stripped of their leaves and their stems were wiped with 95% ethanol. A 15 cm long stem segment was cut from the plant (10 cm below the shoot tip) and subdivided into three 5 cm segments. Surface sterilization was carried out by placing the stem segments in a 3% solution of sodium hypochlorite (commercial bleach) for 4-6 minutes. Segment pieces were then thoroughly rinsed three times with sterile distilled water and transferred to pre-sterilized petri dishes. Pith parenchyma cylinders of approximately 5 cm in length and 0.5 cm in diameter were cut with a sterile cork borer. As a further precaution against contamination, approximately a 2 mm portion of the cylinder was removed from either end. Excised segments were further subdivided into 5 mm slices. Three to four pieces (explants) were transferred to a 125 ml Erlenmeyer flask containing 50 ml of Murashige and Skoog (1962) (MS) medium (Table A1 (Appendix A)). Each flask was plugged with a foam stopper and covered with a piece of aluminum foil. A total of ten flasks were used to initiate cultures. MS

basal nutrient medium was supplemented with 100 mg/l, myo-inositol; 1000 mg/l, casein hydrolyzate; 0.2 mg/l, dichlorophenoxyacetic acid (2,4-D); 2.0 mg/l, indoleacetic acid (IAA); 0.02 mg/l, kinetin; 2.4% sucrose and 0.5% agar (OXOID, Agar bacteriological No. 1). Fresh medium was prepared for each experiment. The pH of the medium was adjusted to 5.7 ± 0.1 (with 1N NaOH or 1N HCl) prior to autoclaving at 121°C for 20 minutes. All manipulations were performed under aseptic conditions with sterile instruments in a laminar flow work station (Forma Scientific).

Cultures were maintained for five weeks (1 passage) in a Conviron incubator at 23 ± 1°C under continuous light (fluorescent and incandescent) at an intensity of 320-360 ft. candles. After five weeks, greenish callus pieces of approximately equal size and weight were excised and subcultured onto fresh media. Subculturing was repeated three times. The 15 -week -old- calluses were then used to initiate tolerant calluses.

B.1.2. Selection for NaCl-tolerant callus cultures

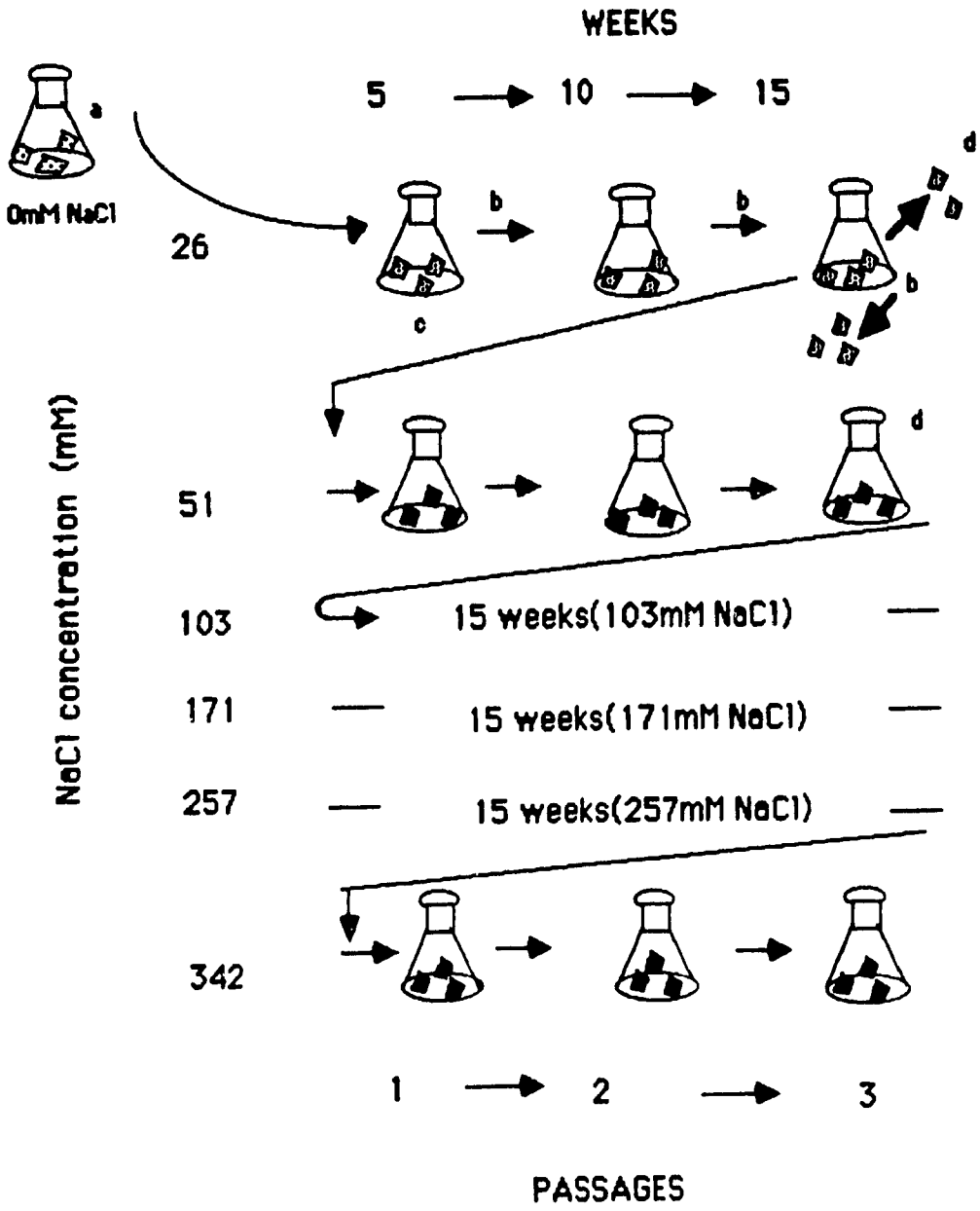
Tolerance to NaCl was achieved following the stepwise selection procedure (Chandler and Vasil , 1984; Handa *et al.*, 1982; Heyser and Nabors, 1981a). The concentration of the medium was gradually increased from 0 to 26, 51, 103, 171, 257, and 342 mM of added NaCl (Figure 1). Fifteen-week-old-calluses were first transferred to MS medium containing 26 mM NaCl. After 5

weeks, calluses were subcultured into pieces of equal size and weight onto fresh MS medium containing the identical concentration of NaCl. This step was repeated three times (15 weeks or 3 passages). Pieces of callus tolerant to 26 mM NaCl were then transferred to medium containing 51 mM NaCl. These steps were repeated for each of the following concentrations: 103, 171, 257 and 342 mM NaCl. Calluses which had been subcultured thrice (15 weeks or 3 passages) on media with the different concentrations of NaCl were subsequently used for all experimental procedures. These samples were lyophilized in a Lab. Con. Co. freeze dryer and conserved at -15°C for subsequent analysis.

B.1.3. Effects of trigonelline

Fifteen week old pieces of calluses maintained on NaCl-free medium were transferred to media containing 0, 257, and 342 mM NaCl. In addition to the salt, trigonelline (Nutritional Biochemicals Corporation) was added to the medium at three concentrations: 0, 0.146, and 0.292 mM. Cultures were maintained for a period of 5 weeks under each treatment. At the end of the culturing period, callus growth was assessed by determining changes in their fresh and dry weights.

Figure 1: Procedure for the selection of tobacco calluses tolerant to media containing increasing concentrations of NaCl. (a) Pieces of callus maintained for 15 weeks (3 passages) on NaCl-free medium were transferred to medium containing 26 mM NaCl; (b) After 5 weeks calluses were subdivided and transferred to fresh medium; (c) Callus cultures incubated for 5 weeks at 23 °C under continuous light; (d) After 15 weeks (3 passages), half of the callus pieces were freeze dried and saved for subsequent analysis, the rest were transferred to the next higher concentration of NaCl (51 mM). The stepwise procedure was continued until calluses tolerant to 342 mM NaCl were obtained.


WEEKS
5
10
15
a
0mM NaCl
26
b
b
b
c
d
d
51
103
171
257
342
15 weeks(103mM NaCl)
15 weeks(171mM NaCl)
15 weeks(257mM NaCl)
NaCl concentration (mM)
1
2
3
PASSAGES

B.2.0. ASSESSMENT OF NaCl TOLERANCE

B.2.1. Morphology of callus cultures

Visual observations of calluses cultured at each level of salt stress were made at weekly intervals. Callus samples were removed from their respective flasks and such observations as callus color, texture and consistency were made. A small amount of each of these samples was then placed on a microscopic slide, immersed in 0.1 M phosphate buffer (pH 7.20), and covered with a coverslip. Using an Olympus light microscope and a Reichert Austria phase-contrast microscope the shape and size (μm) of the callus cells were recorded.

B.2.2. Growth parameters

Growth was determined by measuring changes on the basis of increases in dry weight. Dry weights were obtained from calluses maintained on their respective NaCl-containing medium after each of the three passages. At the time of culture initiation, duplicate samples were weighed. The contents of four flasks containing 2-3 callus pieces each were weighed and the fresh weights recorded. The dry weight was determined after oven-drying the callus pieces at 80 ^{o}C until constant weights were obtained.

A number of criteria have been used to express growth ratio, usually in terms of growth in the absence of salt (Chandler and Vasil, 1984; Ben-Hayyim and Kochba, 1983). In the present studies, growth of callus cultures maintained on increasing NaCl concentrations were calculated using the following equation: $R = [(F-I)/ I_{c\ NaCl}] / [(F-I)/ I_{m\ NaCl}]$. Where R = Growth ratio; F = Final weight of sample; I = Initial weight of sample; cNaCl = Growth at current NaCl concentration; mNaCl = Growth at maintenance NaCl concentration. It was decided that determining the growth ratio as a percentage of growth at the level of maintenance would give a better indicator of tolerance.

Callus water content was also estimated from final fresh and dry weight values. Water contents were expressed as a percentage of the dry weight and calculated as: [(final fresh weight-final dry weight)/final dry weight x 100].

B.2.3. Chlorophyll determination

Chlorophyll contents of the test calluses from each salt treatment were determined following the procedure of Arnon (1949). Two grams of fresh callus tissue (4 samples per treatment) were placed in a mortar containing 8 ml of 80% aqueous acetone and homogenized for 60 seconds. The slurry was then poured into a 15 ml graduated centrifuge tube. Any residue left in the mortar was rinsed with 2 ml of acetone and added to the tube. Samples were centrifuged for 10 minutes at full speed in a bench-type clinical centrifuge. Absorbances of the decanted supernatant were read at 663 and 645 nm on a PYE UNICAM SP8-100 Ultraviolet spectrophotometer.

Total chlorophyll was estimated as follows:

$$\text{Total chlorophyll } (\mu g/g \text{ Fwt}) = \frac{\text{conc. of chlorophyll a+b} = (20.0 \times Abs_{645}) + (8.02 \times Abs_{663}) \times \text{volume}}{\text{fresh weight}}$$

B.2.4. Quantification of tolerance

Tolerance determinations were performed on callus which had been maintained for 15 weeks (3 passages) on media containing different concentrations of NaCl. Ten callus pieces of similar size and weight were used for each test. Calluses maintained at 103 mM NaCl were transferred to media containing 0, 103, 171, 257, and 342 mM of NaCl. Cultures exposed to 171, 257 and 342 mM NaCl were treated in the same manner. Calluses growing on NaCl-free medium were also transferred to medium containing each of the five different NaCl concentrations. Dry weights were obtained four weeks after culture initiation. Comparisons between tolerant and non-tolerant calluses were made through visual assessments and growth measurements (Croughan *et al.*, 1978; Pandey and Ganapathy, 1984).

B.2.5. Determination of stability

Calluses grown on media containing high sodium chloride (342 mM) and those cultured on medium containing no sodium chloride were used to

determine the stability of NaCl tolerant cells. Twenty-four pieces of calluses tolerant to 342 mM NaCl were transferred to NaCl-free medium and grown for 15 weeks. Subculturing was repeated three times. After 15 weeks, calluses were recultured on medium containing 342 mM NaCl. Stability was determined on the basis of growth after 5 weeks. As controls, callus tolerant to 342 mM NaCl and maintained continuously on medium containing the same level of salt, and callus continuously grown on NaCl-free medium and transferred to medium containing 342 mM NaCl, were also observed.

B.3.0. CHARACTERIZATION OF NaCl-TOLERANT CALLUS CULTURES

B.3.1. Measurement of osmotic potential

The osmotic potential of the cell sap and agar-solidified media was determined by the freezing point depression method. Osmotic potentials expressed in milliosmoles (mOsm) were measured from 100 µl aliquots with a Micro-Osmette osmometer.

Fresh callus samples (0.5 g) were harvested from third passage test cultures. Agar-solidified medium (0.5 g) was also obtained prior to and after growth; these constituted the fresh and stale medium samples respectively. These samples were frozen, thawed, and homogenized with 9 ml of double deionized water. Homogenized samples were centrifuged for 20 minutes at 23,500 g. The supernatants were decanted and osmotic potentials measured (Heyser and Nabors, 1981a).

B.3.2. Analysis of Na^+, K^+, and Cl^- Ions

The concentrations of Na^+ and K^+ in non-tolerant and calluses tolerant to 103, 171, 257, and 342 mM NaCl were measured by atomic absorption spectrophotometry (Perkin-Elmer 503). Chloride was estimated following the titration method of Schales and Schales (1941). Two independent experiments were carried out with each callus obtained from different mother plants.

For the determination of Na^+ and K^+ contents, calluses were extracted by two separate methods (Aysola *et al.*, 1987 ; Heyser and Nabors , 1981a,1981b). Following the procedure of Heyser and Nabors (1981a,1981b), extractions were carried out with water. Lyophilized callus samples (0.1 g) were placed in a mortar with 10 ml of double-deionized water. The mixture was homogenized for 60 seconds and the slurry was placed in a freezer overnight. Frozen samples were thawed, homogenized, and centrifuged for 20 minutes at 23,500 g in a Sorvall centrifuge. The supernatant was decanted and contents were poured into a 50 ml volumetric flask. The volume was brought up to the mark with double-deionized water. Duplicate samples were wet ashed as described by Aysola *et al.* (1987). Lyophilized samples (0.1 g) were placed in Savillex vessels and solublized with 3 ml of a mixture of H_2SO_4 and HNO_3 (1:1 v/v). Each vessel was covered with a lid, gently swirled, placed in a plastic jar and transferred to a microwave oven. Samples were heated at high power in a microwave oven for 20 seconds. Upon removal, the vessels were transferred to a tray of ice until they were cooled. The content of each vessel was then poured into a 50 ml volumeteric flask, and distilled water was added to

bring the volume up to the 50 ml mark.

Chloride analysis was performed on water extracts of lyophilized cells. Extracted samples were titrated as follows : to 0.2 ml of the extract was added 1.8 ml of double-deionized water and 0.06 ml of diphenylcarbazone (indicator solution). The solution was placed in an Erlenmeyer flask and titrated with a 0.01N mercuric nitrate solution. The endpoint was indicated by the intense violet blue color which is formed when excess mercuric ions combine with diphenylcarbazone.

B.3.3. Determination of starch content

The extraction of starch from test calluses maintained for 15 weeks was carried out as previously described (Mangat et al., 1985). To 10 mg of lyophilized callus were added 5 drops of 95% ethanol and 1 ml of 6% NaOH. The mixture was homogenized and the samples were allowed to stand overnight. Samples were subsequently placed in a boiling water bath for three minutes. They were then cooled and centrifuged for 10 minutes at full speed in a bench-type clinical centrifuge. The supernatant was transferred to a 25 ml volumetric flask and the volume adjusted with distilled water. Starch determinations were performed by means of a modified phenol-sulfuric acid method (Robyt and Bemis,1967). Five milliliters of concentrated sulfuric acid and 0.1 ml of 80% liquified phenol were added to a 0.5 ml aliquot of the diluted supernatant. The mixture was shaken and after 30 minutes the absorbance was read at 490 nm. Starch contents were expressed in glucose units.

B.3.4. Determination of organogenetic capacity

The ability to regenerate shoots was evaluated in cultures which were tolerant to 342 mM NaCl. Callus maintained on NaCl-free medium and callus tolerant to 342 mM NaCl were placed on shoot-producing media in the absence and presence of 342 mM NaCl. The shoot- producing medium consisted of MS medium supplemented with 0.1 mg/l indoleacetic acid (IAA) and 10.0 mg/l benzylaminopurine (BAP) (Cooke, 1987). The cultures were observed weekly and the time of shoot initiation noted. Organogenetic responses were scored with respect to the number of calluses producing shoots and the number of shoots per callus.

B.4.0. Statistical Analyses

Experimental results were subjected to statistical analyses using the Statistical Package for the Social Sciences (SPSSX V2.0 Northwestern University) on the Concordia University Cyber 1 (Central 830D). Data was analyzed by analysis of variance (ANOVA): when two similar experiments were carried out separately, they were considered replicates if statistical analysis revealed no significant difference.

One-way analysis of variance (one-way ANOVA) was performed to

determine the effect of NaCl tolerance on the following parameters: stability of the NaCl tolerance trait, callus cell shape and size, shooting response, tissue starch content and osmotic potential. Subsequently, when differences in the treatment means were found, a Tukey's postpriori test was executed. Multiple comparisons between NaCl treatments were performed to determine whether significant differences existed at $p < 0.05$.

Differences in growth were analyzed by a two-way ANOVA, from which the effect of both passage number and medium NaCl concentration were assessed. New variables transformed from the two independent variables. passage number and NaCl concentration, were used to evaluate comparisons between treatments at the 0.05 level of significance. Data from tolerance, water content, chlorophyll and trigonelline experiments were treated similarly.

A three way analysis of variance was carried out in order to evaluate the effects of NaCl, method, and experiment to experiment variation on the sodium and potassium content of the callus. Postpriori tests (Tukey) were employed on transformed variables to estimate if differences between treatments were prevalent at $p < 0.05$. The 95% confidence interval for the mean of each sample was calculated for all results.

C. RESULTS

C. 1.0. GROWTH OF TOBACCO CALLUSES

a. NaCl supplemented medium

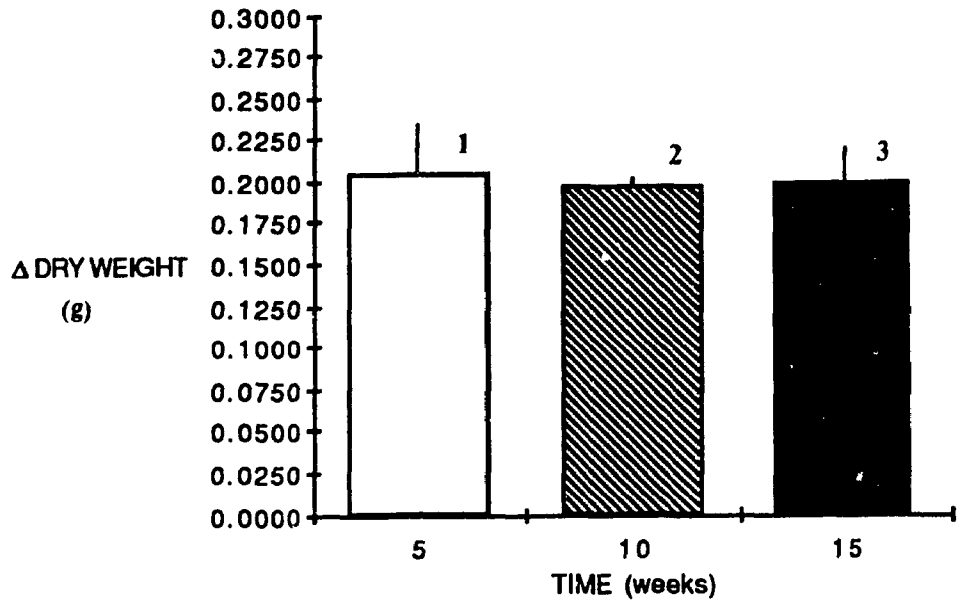
Pieces of tobacco (*Nicotiana tabacum* L.) callus cultured on NaCl-free medium showed no significant difference ($p > 0.05$) in their respective dry weights over the three passages (Figure 2A). When calluses were cultured on media containing increasing NaCl concentrations, their initial growths were slow (passage 1). However, by week 10 and 15 (passages 2 and 3) there was an increase in the dry weights of the calluses (Figures 2B-E).

Results of ANOVA indicated that at NaCl concentrations of 103, 171, 257, and 342 mM, significant increases in dry weights ($p = 0.001$) were observed between calluses cultured for 5 weeks (1 passage) and those maintained for 15 weeks (3 passages).

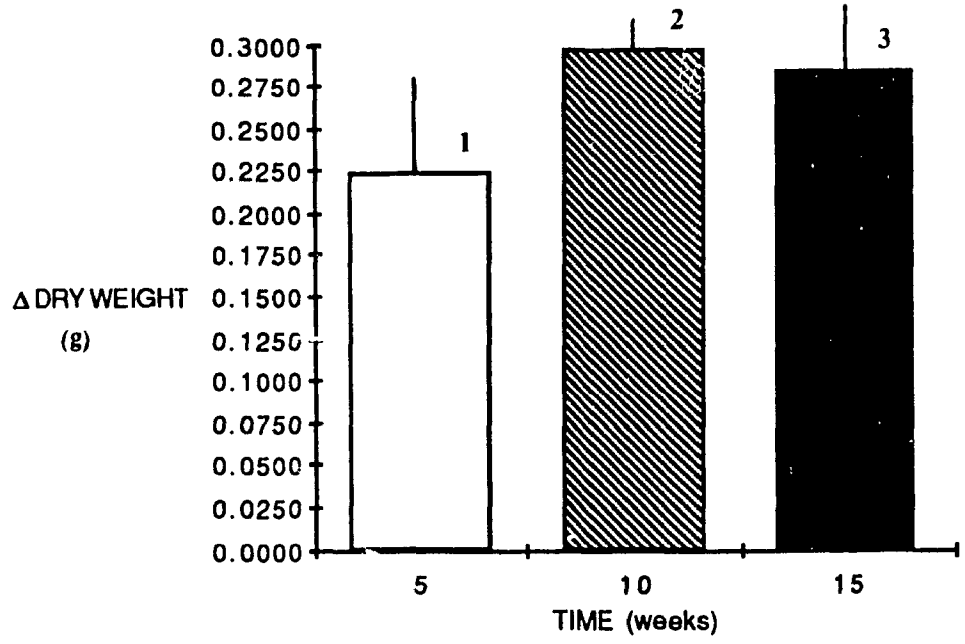
b. Trigonelline supplemented medium

Table 1 shows the effect of trigonelline on the growth of calluses maintained on NaCl-free medium and cultured on media containing varying concentrations of NaCl. Results of analysis of variance indicated that culturing

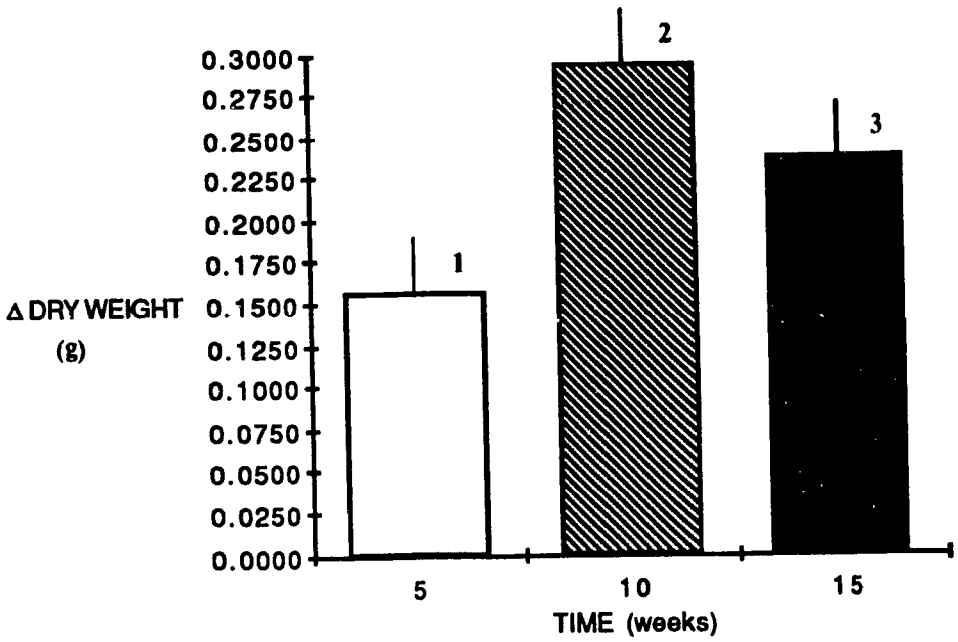
Figure 2: Growth of tobacco calluses maintained for three successive passages (1-3) on NaCl containing MS medium. Dry weights of 10 samples were determined at 5 weeks (passage 1), 10 weeks (passage 2), and 15 weeks (passage 3). Vertical lines represent 95% confidence interval of the mean.



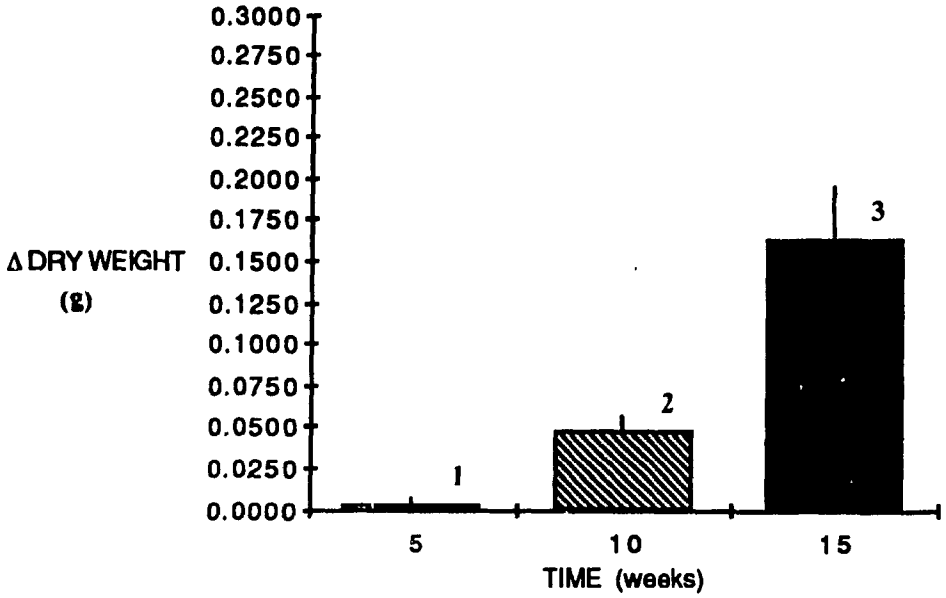
A- cultured on medium containing 0 mM NaCl



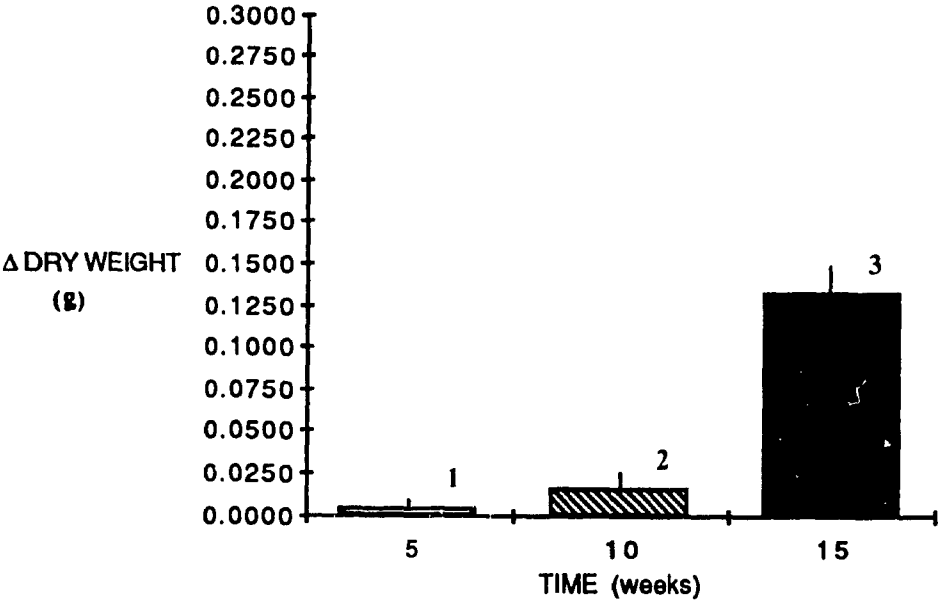
B- cultured on medium containing 103 mM NaCl



C- cultured on medium containing 171 mM NaCl



D- cultured on medium containing 257 mM NaCl



E- cultured on medium containg 342 mM NaCl

Table 1: Average final weights of tobacco calluses maintained on NaCl-free medium and cultured on medium containing varying concentrations of NaCl and trigonelline. 170 mg of fresh callus were subcultured and fresh and dry weights were determined after 5 weeks of growth. The data show the results of nine replicates ± 95% confidence interval.

Medium NaCl concentration (mM)	Trigonelline Concentration (mM)		
	0	0.146	0.292
		Fresh weight (g)	
0	3.7270±1.1615	3.9418±1.1124	4.3353±0.9873
257	0.2209±0.0470	0.2251±0.0277	0.2497±0.0314
342	0.1953±0.0466	0.1968±0.0271	0.1725±0.0303
		Dry weight (g)	
0	0.1779±0.0647	0.1500±0.0400	0.1463±0.0301
257	0.0181±0.0046	0.0184±0.0039	0.0191±0.0051
342	0.0145±0.0035	0.0145±0.0023	0.0129±0.0031

Note. Differences in mean fresh and dry weights are independent of the trigonelline concentration $p > 0.05$ and dependent on the NaCl concentration $p = 0.001$.

NaCl-free maintained calluses on NaCl-free medium containing 0, 0.146 and 0.292 mM trigonelline had no effect on their final fresh and dry weights (p >0.05). Similar results were obtained when calluses maintained on NaCl-free medium were transferred to medium containing 257 or 342 mM NaCl supplemented with varying concentrations of trigonelline.

C.2.0. VISUAL AND MICROSCOPIC OBSERVATIONS

a. Visual

The external appearances of calluses grown on NaCl-free medium and on media containing high concentrations of NaCl were found to differ. Calluses maintained on media with 0, 103, and 171 mM NaCl were similar in appearance. However, marked differences were observed when calluses were maintained on medium supplemented with 257 or 342 mM NaCl. Calluses cultured on media containing from 0 to 171 mM NaCl were transparent and beigy-green to bright green in color. Calluses grew as irregularly shaped masses composed of an outer layer of fluffy cells and a hard compact center. On the other hand, calluses maintained at 257 and 342 mM NaCl were usually dark olive green in color and consisted of soft friable tissue which broke apart easily on handling. Figure 3 shows fifteen week (third passage) calluses cultured on medium without NaCl and with 342 mM NaCl.

Figure 3: Example of 15 week (3 passages) old tobacco calluses tolerant to medium containing 0 mM (A) and (B) 342 mM NaCl.

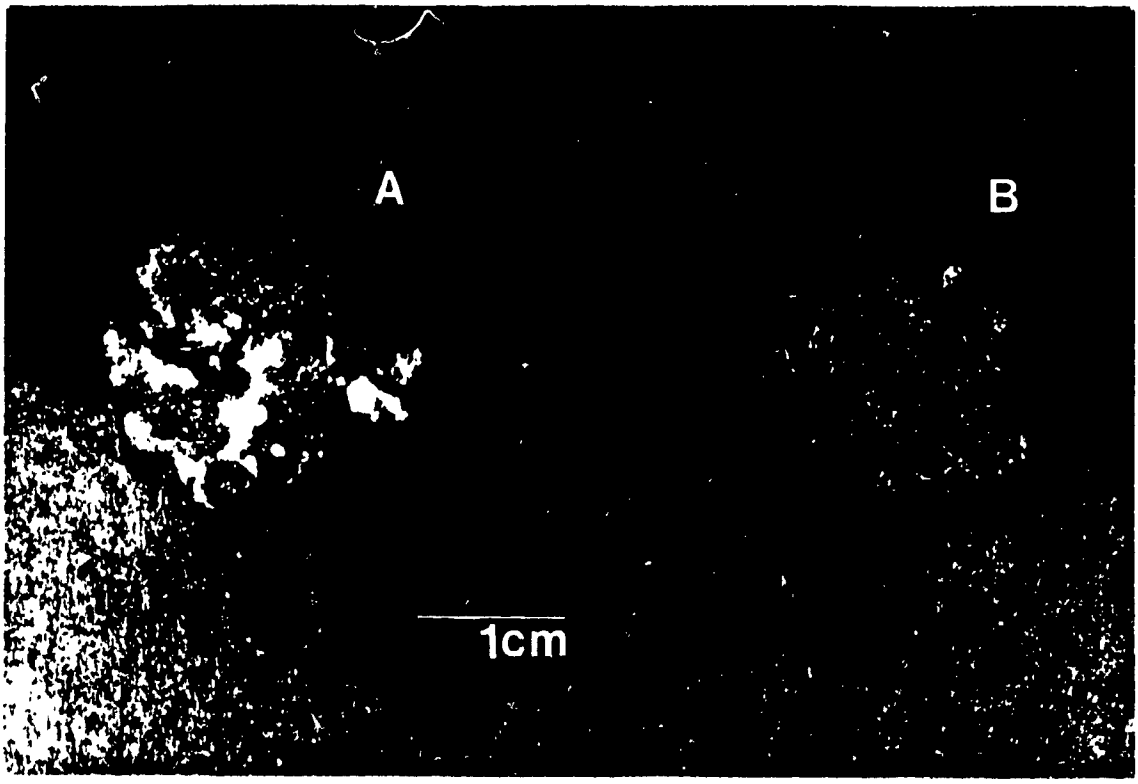
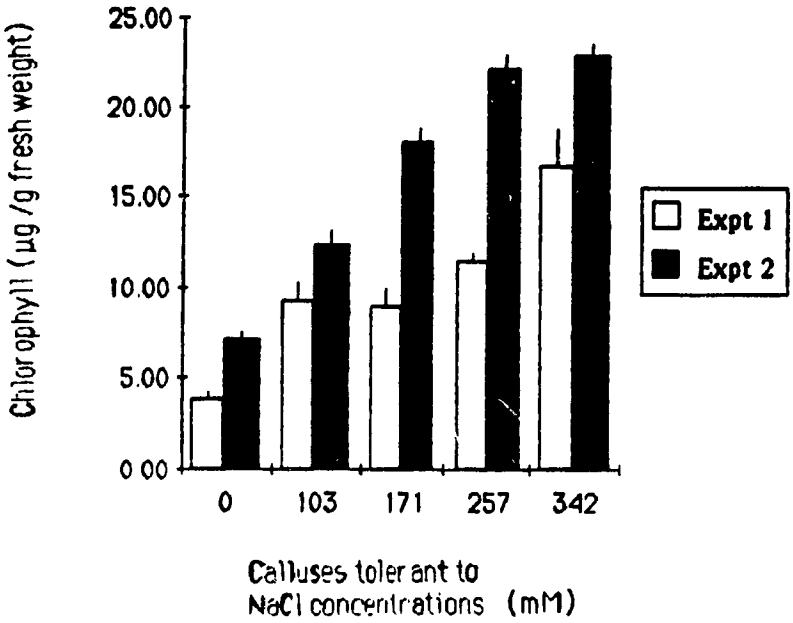

A
B
1cm

Figure 4: Chlorophyll content of calluses tolerant to different concentrations of NaCl. Tissue chlorophyll contents were determined after calluses had been maintained for 15 weeks (3 passages) on their respective NaCl-containing MS medium. The data presented are the means of six replicates from two separate experiments. Bars represent 95% confidence intervals.


Chlorophyll (µg /g fresh weight)
25.00
20.00
15.00
10.00
5.00
0 00
0
103
171
257
342
Expt 1
Expt 2
Calluses tolerant to
NaCl concentrations (mM)

Variations in the green color of calluses growing at increasing NaCl concentrations were reflected in their chlorophyll content. The total chlorophyll content of the calluses increased with increasing NaCl (Figure 4). Results from replicates of two separate experiments appear to show similar trends; however, the levels of chlorophyll determined from experiment 2 were found to be significantly higher (p =0.0001) than those of experiment 1. By the Tukey's test of multiple comparisons, it was shown that the chlorophyll concentration of the non-treated callus was significantly lower than that of any of the NaCl treated calluses(p = 0.001).

b. Microscopic

When cells were observed under the light microscope, the cells from calluses cultured on NaCl-free medium appeared to be either spherical, filamentous, or irregularly shaped. With increasing concentrations of NaCl in the growth medium, there was a noticeable decrease in the number and size of filamentous and irregularly-shaped cells (Tables 2 and 3). The population of cells from calluses maintained on media supplemented with 257 and 342 mM NaCl contained mainly small spherical cells with few large spherical cells, filamentous or irreguarly-shaped cells. Figure 5 shows a representation of some of the various types of cells observed in calluses cultured at 0, 103, 171, and 342 mM NaCl. Cells obtained from calluses cultured at 257 mM NaCl appeared to be similar to those grown on medium containing 342 mM NaCl.

Figure 5: Microscopic appearance of fifteen week (3 passages) old tobacco callus cells cultured on 0 (A), 103 (B), 171 (C), and 342 (D) mM NaCl. Magnification 400X.

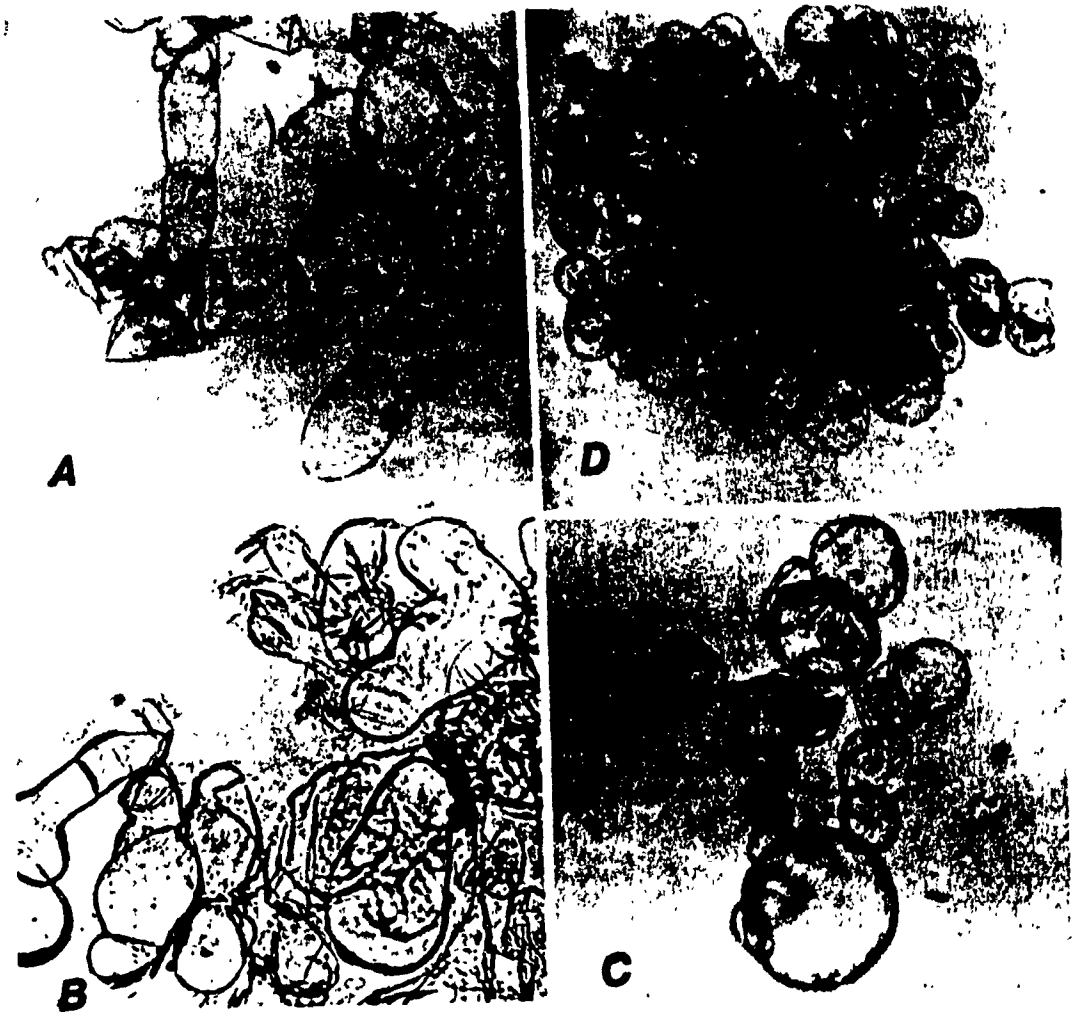

A
D
B
C

Table 2: Percentage of cells (± 95% confidence interval) from calluses showing diversity in shapes. Cells were observed after 15 weeks (3 passages) of culture on their respective NaCl-containing MS medium. The data represent observations from ten fields with an average of 76 cells per field. (Sp, spherical cells; Fil, filament cells; Irr, irregular cells).

Shape of cell	Calluses maintained at NaCl concentration (mM)				
	0	103	171	257	342
Sp[a]	79.57±3.56	96.65±1.05	92.12±4.13	77.11±1.49	98.37±1.81
Fil	9.75±2.41	1.02±0.51	0.74±0.72	0.25±0.37	0.72±1.33
Irr	10.69±3.96	2.42±0.85	7.14±3.53	2.65±1.50	0.91±1.22

[a]Large spherical cells were normally not present in cultures maintained on MS medium containing 257 and 342 mM NaCl.

Table 3: Mean cell size (µm± 95% confidence interval) of callus cells. Cells were observed after 15 weeks (3 passages) of culture on their respective NaCl-containing MS medium. Thirty cells were measured for each treatment. (Sp, spherical cells; Fil, filament cells; Irr, irregular cells).

Shape of cell	Calluses maintained at NaCl concentration (mM)				
	0	103	171	257	342
Sp	13.18±1.24	14.58±1.88	8.17±1.23	5.94±0.76	7.14±0.67
Fil	33.10±5.31	37.41±12.24	19.42±10.48	10.46±6.33	14.28±12.20
Irr	29.61±2.74	29.88±3.91	15.78±2.14	13.21±2.24	14.54±1.88

C.1.2. Quantification of tolerance and stability of callus cultures

Tolerance of tobacco calluses to NaCl was determined after callus cultures were maintained for 15 weeks (3 passages) on their respective NaCl-containing medium. As shown in Figure 6, the amount of callus was observed to decrease when calluses maintained at the various NaCl concentrations were cultured on media with increasing levels of NaCl. Calluses maintained at high NaCl concentrations (257 and 342 mM) and cultured on medium containing the different concentrations of NaCl showed a consistent pattern of growth, compared to those previously maintained on 0 and 171 mM NaCl. On medium supplemented with 257 and 342 mM NaCl, these calluses showed a brownish color. Experimental data indicated that, when calluses maintained continuously on MS medium without NaCl were subcultured on MS medium containing increasing amounts of NaCl, the dry weights of these calluses decreased. Similarly, when calluses maintained on MS medium with a concentration of 103 mM NaCl were transferred to growth media containing higher amounts of NaCl, there was a resultant decrease in their respective dry weights. The dry weight of callus cells maintained on medium supplemented with 171 mM NaCl appeared to increase when these calluses were cultured on medium with less salt (0 and 103 mM NaCl) than the maintenance NaCl concentration, and decrease at higher salt concentrations (257 and 342 mM NaCl) (Figure 7). Similar results were observed for calluses growing on 257 mM NaCl; however,

Figure 6: Tobacco calluses continually tolerant to MS medium containing 0, 103, 171, 257, and 342 mM NaCl and cultured for 4 weeks on medium containing different concentrations of NaCl.

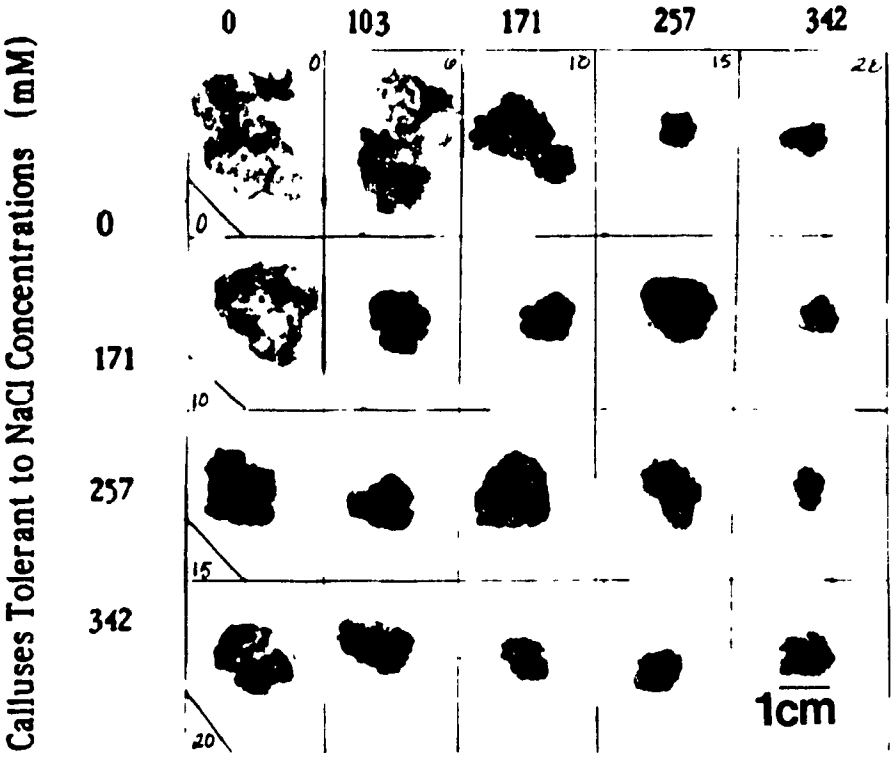
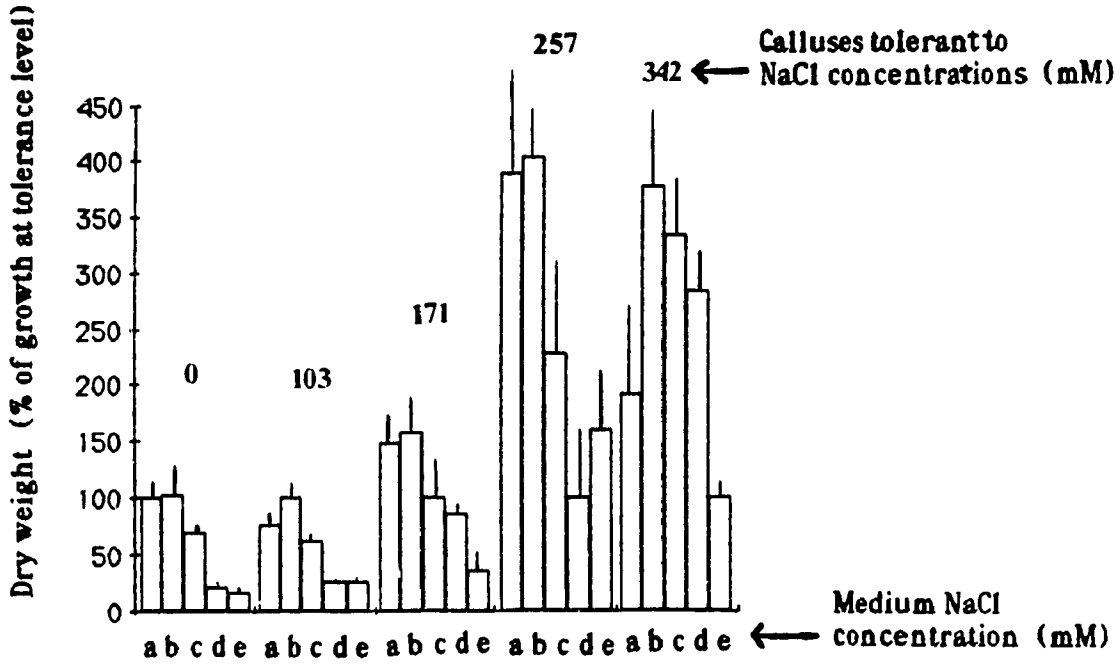

Medium NaCl Concentration (mM)
0
103
171
257
342
Calluses Tolerant to NaCl Concentrations (mM)
0
171
257
342
1cm

Figure 7: Relative tolerance to NaCl of calluses tolerant to media supplemented with 0, 103, 171, 257, and 342 mM NaCl and cultured on medium containing increasing concentrations of NaCl, (a-e= 0,103,171,257, and 342mM NaCl respectvely). Dry weights of ten pieces of callus were determined after 4 weeks of growth.100% growth of calluses at tolerance level 0, 103, 171, 257, and 342 NaCl was 0.1082, 0.1759, 0.0913, 0.0491, and 0.0331 g respectively. Error bars represent 95% confidence interval.


Dry weight (% of growth at tolerance level)
450
400
350
300
250
200
150
100
50
0
0
103
171
257
342
Calluses tolerant to NaCl concentrations (mM)
a b c d e a b c d e a b c d e a b c d e a b c d e
Medium NaCl concentration (mM)

changes were more pronounced. The growth patterns of calluses maintained at 342 mM NaCl and cultured at various NaCl concentrations were found to differ from those of the other callus lines. Initially when these calluses were cultured on NaCl-free medium they grew poorly. When the medium NaCl concentration was increased, growth also increased; however, no significant differences (p >0.05) were observed between calluses cultured on medium NaCl concentrations of 103 to 257 mM.

With regard to stability, Table 4 shows that when calluses maintained on a medium containing 342mM NaCl were cultured for 15 weeks (3 passages) on NaCl-free medium and then transferred back to medium containing 342 mM NaCl, showed poor growth compared to those cultured only on medium supplemented with 342 mM NaCl . However, their growth was found to be similar to that obtained for calluses maintained continuously on NaCl-free medium.

Table 4: Growth of calluses cultured on MS medium containing 342 mM NaCl. Dry weights of 16 pieces of callus were obtained after a 5 week culture period.

Calluses tolerant to NaCl concentration (mM)	Concentration of NaCl in medium (mM)	Dry weight (% increase±95% confidence interval)
0[a]	342	329.052±85.407
342[b]	342	656.408±119.393[d]
342[c]	342	388.526±37.678

[a]Callus maintained on NaCl-free medium.
[b]Callus cultures tolerant to 342 mM NaCl and cultured on medium containing 342 mM NaCl.
[c]Callus tolerant to 342 mM NaCl and maintained for 15 weeks (3 passages) on NaCl-free medium.
[d]Mean is significantly different at $p < 0.05$.

C.2.0. OSMOTIC POTENTIAL AND WATER CONTENT

a. Osmotic potential

Increasing the concentration of NaCl in the medium resulted in increases in the medium osmotic concentrations. Similarly, as the tobacco callus cells became more tolerant to increasing concentrations of NaCl in the growth medium, their osmotic concentrations were also observed to increase (Table 5). The osmotic concentrations of calluses tolerant to medium NaCl concentrations of up to 171 mM were found to be noticeably higher than those of the stale medium. However, for calluses tolerant to 257 and 342 mM NaCl, no significant differences (p >0.05) were found between the osmotic concentration of the callus and the medium in which they were cultured.

b. Water content

As shown in Table 6, after 15 weeks (3 passages) the water content of NaCl-tolerant calluses was found to decrease with increasing tolerance to NaCl. The response of callus tolerant to 103 mM NaCl to medium containing 103 mM NaCl was found to be similar to that of the non-tolerant callus on NaCl-free medium (p >0.05). However, calluses growing on medium containing 171, 257, and 342 mM NaCl showed significant reductions (p =0.001) in their tissue water contents.

Table 5: Osmotic potential of growth medium and NaCl-tolerant and non-tolerant calluses cultured on their respective NaCl containing MS medium for 15 weeks (3 passages). The data shows the means from twelve replicates ±95% confidence interval .

Calluses tolerant to NaCl concentration (mM)	Osmotic potential (mOsm)		
	Medium		Callus
	fresh	stale	
0	148.000±14.740[a]	25.455±4.619[a]	54.546±9.194 [a]
103	292.500±17.371[b]	185.000±31.136[b]	289.091±19.805[b]
171	401.250±23.830[c]	373.636±26.364[c]	460.909±33.382[c]
257	590.000±36.304[d]	720.909±91.940[d]	716.364±46.279[d]
342	717.750±69.525[e]	812.727±80.173[d]	817.273±14.747[e]

Note. Means having the same superscript are not significantly different at p =0.0001.

Table 6: Water content of NaCl-tolerant and non-tolerant callus tissues maintained on their respective NaCl containing MS medium for 15 weeks (3 passages). The data shown are the means of ten samples.

Calluses tolerant to NaCl concentration (mM)	Water content
	% of dry weight±95% confidence interval
0	3810.362±318.196[a]
103	3721.007±110.214[a]
171	2268.261±88.232[b]
257	1565.604±54.790[c]
342	1363.574±133.685[c]

Note. Means having the same superscript are not significantly different at p =0.001.

C.2.1. Inorganic and organic content of calluses

a. Inorganic

Na^+, K^+ and Cl^- ion contents of tobacco calluses maintained on their respective NaCl-containing medium for 15 weeks (3 passages) are presented in Tables 7, 8, and 9. The Na^+ content in the callus tissues was found to increase significantly (p =0.0001) with increasing tolerance to NaCl (Table 7). There was a marked difference between the levels of Na^+ measured in calluses tolerant to 103 mM NaCl and those maintained at 257 mM NaCl. However, in the case of calluses tolerant to 342 mM NaCl, the Na^+ content appeared to reach a plateau. Results similar to those obtained for sodium were observed for chloride (Table 8). The level of Cl^- was found to be most elevated when the medium NaCl concentration was 257 mM.

The concentration of K^+ in the tissues, on the other hand, decreased with increasing tolerance to NaCl (Table 8). Results of analysis of variance show that the K^+ content of calluses tolerant to 342 mM NaCl was significantly lower (p =0.0001) than that of NaCl-free cultured calluses.

Variations were noted when ions were extracted by two seperate methods (water extraction and wet ashing) and measured by atomic absorption spectrophotometry from tobacco callus tissues maintained on increasing NaCl concentrations (Aysola *et al.*, 1987; Heyser and Nabors, 1981a, 1981b). The pattern of change in Na^+ and K^+ content of the tissue in response to increasing

Table 7: Sodium ion content of tobacco callus after maintenance for 15 weeks (3 passages) on their respective NaCl containing MS medium. The data shows the results from two separate experiments.Fifteen samples were measured for each treatment.

Calluses tolerant to NaCl concentration (mM)	$^{a}Na^{+}$ mmol\g dry weight±95% confidence interval			
	M1[b]		M2[c]	
	Expt1	Expt2	Expt1	Expt2
0	0.132±0.085	0.208±0.063	0.236±0.095	0.487±0.045
103	3.073±0.138	4.110±0.244	3.805±0.069	4.470±0.067
171	4.793±0.082	3.854±0.137	5.614±0.266	4.836±0.279
257	5.742±0.229	3.421±0.358	6.292±0.392	5.503±0.203
342	4.819±0.215	4.067±0.085	5.744±0.192	5.288±0.114

[a]Na^{+} ions were determined by atomic absorption spectrophotometry.
[b]Ions extracted with water (Heyser and Nabors, 1981a,b).
[c]Ions extracted by wet ashing (Aysola et al., 1987).

Table 8: Potassium ion content of tobacco callus after maintenance for 15 weeks (3 passages) on their respective NaCl containing MS medium. The data shows the results from two separate experiments. Fifteen samples were measured per treatment.

Calluses tolerant to NaCl concentration (mM)	$^{a}K^{+}$ mmol/g dry weight±95% confidence interval			
	M1[b]		M2[c]	
	Expt1	Expt2	Expt1	Expt2
0	0.875±0.062	1.029±0.078	0.875±0.051	1.179±0.031
103	0.579±0.033	0.800±0.043	0.598±0.034	0.951±0.054
171	0.329±0.025	0.367±0.020	0.336±0.020	0.566±0.026
257	0.382±0.027	0.279±0.018	0.299±0.020	0.406±0.016
342	0.120±0.029	0.242±0.023	0.064±0.023	0.386±0.019

[a]K^{+} ions were determined by atomic absorption spectrophotometry.
[b]Ions extracted with water (Heyser and Nabors, 1981a,b).
[c]Ions extracted by wet ashing (Aysola et al., 1987).

Table 9: Chloride ion content of tobacco callus after maintenance for 15 weeks (3 passages) on their respective NaCl containing MS medium. The data shows the results from two separate experiments.Fifteen samples were measured for each treatment.

Calluses tolerant to NaCl concentration	$^{a}Cl^{-}$ mmol/g dry weight±95% confidence interval (mM)	
	M1[b]	
	Expt 1	Expt 2
0	0.050±0.036	0.125±0.066
103	2.970±0.313	3.413±0.269
171	2.579±0.262	4.446±0.302
257	2.781±0.213	4.957±0.175
342	2.786±0.280	4.331±0.341

[a]Cl^{-} ions were determined by the titration method described by Schales and Schales(1941).
[b]Ions extracted with water (Heyser and Nabors,1981a,b)

NaCl concentrations was similar when extractions were carried out with either water or by wet ashing. However, upon measuring the levels of Na^+ and K^+ in the calluses, higher concentrations of the ions were found when calluses were extracted using the wet ashing technique (p =0.001). In some cases, the sodium content was two times that measured after extraction with water. There were also slight variations between the two experimental cultures (p =0.001). Nonetheless, the general pattern observed appear to be similar. These results suggests that the extraction of ions by the wet ash method may be more efficient than that of the water extraction method; in addition to extraction of free ions, bound ions may be involved.

b. Organic

The starch content of the calluses was found to remain relatively constant over medium NaCl concentrations of up to 171 mM (Table 10). No significant differences (p >0.05) were evident between calluses maintained under low levels of NaCl stress and those cultured on medium without salt. However, when the level of tolerance to NaCl was increased to 257 mM, a reduction of approximately 12% over that of calluses maintained on NaCl-free growth medium was observed. A further decrease of 14% was seen when calluses were cultured on medium containing 342 mM NaCl.

Table 10: Starch content of NaCl-tolerant and non-tolerant callus tissues. Starch content was determined after 15 weeks (3 passages) of maintaining the calluses on their respective NaCl containing MS medium. The data shows the means of eighteen replicates ±95% confidence interval.

Calluses tolerant to NaCl concentration (mM)	Starch content (glucose equivalents) mg/mg dry weight
0	0.192±0.016[a]
103	0.213±0.018[a]
171	0199±0.020[a]
257	0.169±0.011[b]
342	0.142±0.011[b]

Note. Means having the same superscript are not significantly different at p =0.0001.

C.2.2. Organogenic response of non-tolerant and NaCl tolerant callus

On observing calluses maintained on shoot-inducing medium free of NaCl and supplemented with 342 mM NaCl, different responses were observed. Shoots were produced on calluses cultured on NaCl-free regeneration medium and not on medium containing 342 mM NaCl. Non-tolerant callus pieces subcultured onto medium containing 342 mM NaCl turned brown, whereas the same calluses placed on NaCl-free medium produced bright green calluses (Figure 8A,C). Shoot initiation was inhibited when non-tolerant calluses were incubated on shooting medium supplemented with 342 mM NaCl, and the number of calluses producing callus was decreased by 80%. Of the fifteen pieces of non-tolerant callus transferred to salt-free shoot inducing medium thirteen of them shooted and all produced callus readily (Table 11).

New callus produced from NaCl-tolerant calluses cultured on salt-containing medium and on NaCl-free medium were olive green to brownish olive green in color (Figure 8B,D). When callus tolerant to 342 mM NaCl was cultured on shoot-producing regeneration medium containing 342 mM NaCl, new callus was produced but no shoots were observed by the eighth week. On placing tolerant calluses on NaCl-free shooting medium, shoots were produced by eleven of the twelve test calluses. The shoots formed from calluses tolerant to 342 mM NaCl were comparable to those established in cultures which had never been exposed to salt. Data also indicated that non-tolerant calluses produced twice as many shoots as did those calluses tolerant to 342 mM NaCl ($p < 0.05$).

Figure 8: Organogenetic response of non-tolerant and callus tolerant to 342 mM NaCl. Responses were observed after 8 weeks on shoot inducing MS medium with 0 mM and 342 mM NaCl containing 0.1 mg/l IAA and 10.0 mg/l BAP. (A) non-tolerant callus cultured on medium supplemented with 342 mM NaCl; (B) callus tolerant to 342 mM NaCl cultured on medium supplemented with 342 mM NaCl; (C) non-tolerant callus cultured on NaCl-free medium ; (D) callus tolerant to 342 mM NaCl cultured on NaCl-free medium.

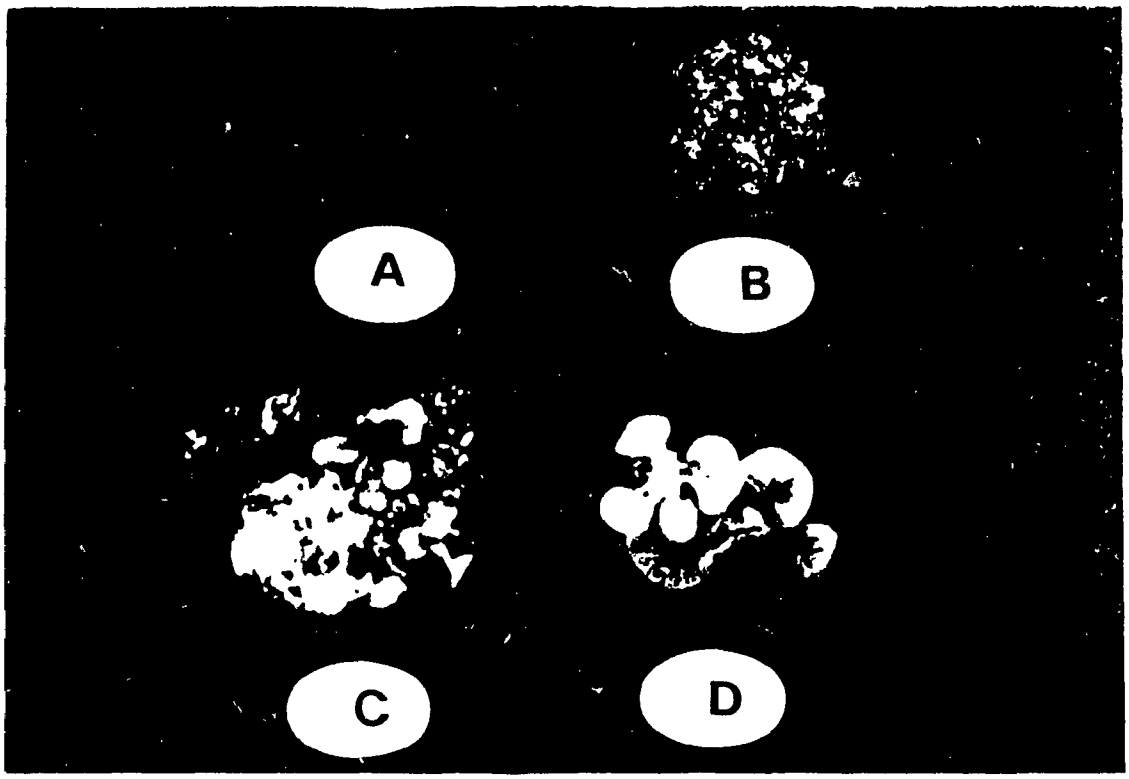

A
B
C
D

Table 11: Organogenic response of NaCl-tolerant and non-tolerant callus tissue. Morphogenetic responses were observed after calluses were cultured for 8 weeks on shoot producing MS medium with 0 mM and 342 mM NaCl supplemented with 0.1 mg/l IAA and 10 mg/l BAP.

Calluses tolerant to NaCl concentration (mM)	Concentration of NaCl in medium (mM)	Percentage of calluses showing a response		Average number of shoots per callus (±95% CI)[a]
		callus	shoots	
0	0[b]	100.00(15)	86.67(13)	10.23±3.73
	342[b]	20.00(3)	0	0
342	0[c]	100.00(12)	91.67(11)	4.91±2.32
	342[b]	100.00(15)	0	0

Note. Figures in parentheses are the numbers of calluses showing a response.
[a] 95% CI= 95% confidence interval.
[b] Fifteen calluses.
[c] Twelve calluses.

D. DISCUSSION

The selection and characterization of salt-tolerant cultures using tobacco cell suspensions have frequently been reported in the literature (Binzel *et al.*, 1988, 1987, 1985; Watad *et al.*, 1983; Dix and Pearce, 1981; Heyser and Nabors, 1981a, 1981b; Hasegawa *et al.*, 1980; Nabors *et al.*, 1980, 1975; Dix and Street, 1975). However, very few reports have appeared on the selection and characterization of tobacco callus tissues in salt tolerance studies (Pua *et al.*, 1985; Nabors, 1983; Chen *et al.*, 1980; Umiel *et al.*, 1980). In the present study, tobacco callus cultures were established which were tolerant to 342 mM NaCl after 90 weeks (18 passages). The tolerant calluses were obtained by stepwise increases in the NaCl concentration in the medium; calluses growing under control conditions (0 mM NaCl) were cultured sequentially on growth media containing 26, 51, 103, 171, 257, and 342 mM NaCl. Noticeable increases in the dry weights of tobacco calluses after a 15 week culture period was evidence that these calluses had become tolerant to a particular concentration of NaCl. Other investigators have also used the increase in dry weight as a criterion for salt tolerance (Hassan and Wilkins, 1988; Binzel *et al.*, 1985; Chandler and Vasil, 1984; Sobko *et al.*, 1981). Using the stepwise selection method calluses of various plants, in most cases, show a significant stimulation of growth after exposure to a particular salt concentration after 4 to

28 weeks i.e. after one to seven passages of subculturing every fourth week (Hassan and Wilkins, 1988; Chandler and Vasil, 1984; Tomes and Swanson, 1982; Sobko *et al.*, 1981). The studies of Binzel *et al.* (1985) with tobacco suspension cultures showed enhanced gains in both fresh and dry weights of the cultures at 342 mM NaCl, following a minimum of 10 passages (50 generations) in each of 171, 240, and 342 mM NaCl containing growth medium. Interestingly, in the present study with tobacco callus, a significant increase in fresh and dry weights were observed even after three passages at NaCl concentrations of 171 mM and higher.

Apart from determining changes in weight at a particular NaCl tolerance level, tolerance can also be assessed by culturing calluses at different NaCl concentrations (Hassan and Wilkins.,1988; Binzel *et al.*,1985; Pandey and Ganapathy, 1984; Ben-Hayyim and Kochba, 1982; Heyser and Nabors, 1981b; Tyagi *et al.* ,1981; Hasegawa *et al.*,1980). Calluses continuously maintained on NaCl-free media showed a decrease in dry weight when cultured on media containing increasing NaCl concentrations. This decrease in dry weight indicates the inability of these calluses to tolerate NaCl. When salt-tolerant calluses were placed on media containing different concentrations of NaCl, variations in their growth were observed. The general trend showed an increase in growth at low NaCl concentrations (i.e., those below the selected tolerance level of the calluses), whereas higher NaCl concentrations depressed their growth. But, a concentration of 103 mM NaCl under the present experimental conditions was found to stimulate growth of non-tolerant and NaCl-tolerant calluses. The stimulation in growth could be due to several factors, namely osmotic influences, rapid turnover of amino acids, and

decreases in reducing sugar concentrations (Brown *et al.*, 1979; Kimball *et al.* ;1975). This observation is in agreement with those reported by Kimball *et al.* (1975) with soybean tissues and Strogonov (1973) with tissues of grape, willow, tobacco and carrot exposed to mild osmotic and salt stresses. However, in the present studies, when calluses tolerant to 342 mM NaCl were transferred to a medium without NaCl, growth was reduced. This diminished growth may indicate that calluses tolerant to 342 mM NaCl are being subjected to osmotic shock when placed on NaCl-free medium and consequently they must adjust osmotically (Chandler and Vasil, 1984). A similar pattern has been reported with *Oryza sativa* callus (Rains *et al.*, 1980).

In comparison to calluses maintained on NaCl-free medium, the tolerant tobacco calluses were characterized by reductions in cell size and potassium content, and increases in chlorophyll levels, callus friability, osmotic potential, and cellular content of sodium and chloride. Noticeable differences in the green color of whole plants and callus tissues exposed to salt and other stresses have been reported (Pandey and Ganapathy, 1984; Goldner *et al.*, 1977; Strogonov, 1973). Goldner *et al.*(1977) and Pandey and Ganapathy (1984) found reductions in green pigment formation in calluses of carrot and *Cicer arietinum* at high salt concentrations. They concluded that the decrease in chlorophyll resulted from the toxicity of salt. However, in other studies with various plants, Strogonov (1973) reported that under saline conditions salt-tolerant plants showed marked increases in their levels of chlorophyll, whereas lower levels were recorded in more sensitive plants. He suggested that the higher amounts of chlorophyll in tolerant plants were due to an accumulation of chlorophyll a and/or b, whereas a rapid breakdown of chlorophyll a occurs in less tolerant

plants in the presence of salt. In the present work, it was observed that calluses maintained on media without salt and those containing low levels of salt appeared beigy-green to bright green but, the calluses tolerant to 257 and 342mM NaCl were dark olive green in color. The dark green color could have resulted from the elevated levels of chlorophyll. It would appear that the increase in total chlorophyll observed in this study and others may be a sign of chlorophyll accumulation which occurs in salt-tolerant plants. Strogonov (1973) has suggested that this accumulation is caused by the enhanced activity of oxidizing enzymes and increases in compounds necessary for chlorophyll synthesis. On the other hand, direct exposure of sensitive tissues to high salt concentrations would result in the loss of chlorophyll (Goldner *et al.*, 1977; Pandey and Ganapathy, 1984).

When tobacco callus cells were examined under the light microscope, three distinct cell shapes were observed. In the control calluses (cultures grown in the salt-free medium) large filamentous (9.75%), irregular (10.69%), and spherical shaped (79.57%) cells were noted (Table 2). With increases in the salt concentration in the growth medium, there was a gradual decrease in the cell size and a predominance of smaller spherical cells. The cells of calluses tolerant to 342 mM were approximately one-half the size of non-tolerant cells (Table 3). In a number of other studies with tobacco cells cultured in high NaCl-media, similar reductions in cell size have been reported (Binzel *et al.*, 1985; Hasegawa *et al.*, 1980; Dix and Street, 1975). Binzel et al. (1985) reported that tobacco cells adapted to grow in medium with 428 mM NaCl were 4 to 5 times smaller than cells growing in medium without added NaCl. They suggested that the decrease in size of salt-adapted cells may be a result of reduced cell

expansion, the inhibition of cell elongation and changes in the properties of the cell wall such as decreased elasticity.

Reductions in cell size under salt stress conditions are reflected in differences in growth (Binzel et al.,1985; Salgado-Garciglia *et al.*, 1985). In the present work, it was shown that with increasing tolerance to high NaCl concentrations, the total water content of the tissue decreased. The fresh weight of the salt-tolerant calluses was also decreased when the media NaCl concentrations were 171 mM and higher (Table A2). Microscopic examination of tolerant calluses indicated that their cells were smaller, but they were not plasmolyzed (Figure 5). These observations are consistent with Binzel *et al.* (1985) and Dix and Street (1975) who observed decreases in fresh weight and in packed cell volume of tobacco cells that were correlated with reduced cell size. It has therefore been suggested that the decrease in fresh weight, at high salt concentrations, is not the result of fewer number of cells, but of cells with reduced cell volume (Binzel *et al.*, 1985; and Dix and Street, 1975).

Two different methods (water extaction and wet ashing) were used to extract Na^+ and K^+ from tobacco callus tissues (Aysola *et al.*, 1987; Heyser and Nabors, 1981a, 1981b). When the levels of Na^+ and K^+ were measured, the two different methods resulted in similar patterns of change with the various concentrations of NaCl in the growth media. However, slightly higher concentrations of Na^+ and K^+ were determined when the the wet ashing technique was used. These findings suggest that the method using water may result in inadequate extraction of Na^+ and K^+.

With increasing concentrations of NaCl in the growth media, the K^+ content of the tissues decreased whereas the concentration of Na^+ increased.

Comparisons of third passage calluses also indicated a reduction in the gains in fresh and dry weight of calluses growing at high NaCl concentrations. Similar decreases in K^+ content and growth with increasing tolerance to salt have been reported by several investigators (Binzel *et al.*,1987; Pandey and Ganapathy, 1984; Watad *et al.*,1983; Croughan *et al.*,1978; Tal *et al.*, 1978). Watad *et al.* (1983) have suggested that the loss of K^+ in wild type cells (nonselected cells) growing in medium with increasing concentrations of NaCl may have resulted in a decrease in growth. From the present study, the accumulation of Na^+ and decrease in K^+ in the tissues at high salt concentrations suggests a deficiency in potassium may cause growth to be reduced (Poljakoff-Mayber and Gale, 1975; Strogonov, 1973).

Ion accumulation, specifically Na^+ and Cl^-, may facilitate salt tolerance in plant cells by aiding in osmotic adjustment (Binzel *et al.*, 1987; Heyser and Nabors, 1981a). Binzel *et al.* (1987) have indicated that the reduction in cell size may contribute to the higher cellular levels of Na^+ and Cl^-. In the present studies, the levels of Na^+ and Cl^- increased with increasing tolerance to NaCl. Changes in Na^+ and Cl^- contents did not however increase proportionally to medium NaCl concentration. Maximum Na^+ and Cl^- concentrations were found when the medium NaCl concentration was 257mM. However, at concentrations higher than 257mM NaCl, the Na^+ and Cl^- contents appeared to plateau. The chloride concentrations of the callus tissues were found to be lower than those detected for sodium. In most investigations, it has been shown that levels of Na^+ and Cl^- increase with increasing salt tolerance (Binzel *et al.*, 1987; Ben-Hayyim *et al.*, 1985; Pandey and Ganapathy, 1984; Dix *et al.*, 1982; Liu and Yeh, 1982; Heyser and Nabors, 1981a; Rains *et al.*, 1980; Croughan *et al.*

,1978). The increase in the content of Na^+ and Cl^- suggests that the uptake of these solutes relieves the osmotic stress (Heyser and Nabors, 1981b).

A number of reports indicated that salt and water stress tolerant cells are able to osmoregulate by reducing their cell volumes, accumulating Na^+ and Cl^- and maintaining their turgor (Binzel *et al.*, 1987,1985; Handa *et al.*, 1983; Hasagawa *et al.*, 1980). In the present work, it was shown that with increasing tolerance to NaCl the cellular osmotic potentials measured by freezing point depression also increased. The osmotic potentials of tolerant tobacco calluses were found to be greater than that of the osmotic potentials of the medium that they were maintained on. In related studies, Binzel *et al.* (1985) found that with tobacco cell suspensions tolerant to media NaCl concentrations of up to 428 mM, maximal osmotic adjustment occurred at the higher NaCl concentrations. Heyser and Nabors (1981b) reported that cellular osmotic potentials were 1 to 2 bars more negative than those of the medium. The results of the present experiments and those obtained in other studies, suggest that the reduction in cell size may play a role in osmoregulation in saline environments (Binzel *et al.*, 1985; Hasegawa *et al.*, 1980). Oertili (1986) has implied that smaller cells may be more resistant against collapse and that they reduce the effects of negative turgor pressure.

Other observations in the present experiment indicated that with increased salt concentration in the growth medium, there was a gradual increase in the friability of the callus tissue. Similar observations by Chandler and Vasil (1984) have been reported with embryogenic callus cultures of *Pennisetum purpureum* . These calluses became more friable and less organized when cultured on media containing more than137 mM NaCl. The

increased friability which occurs with increasing salt tolerance in callus tissues may be attributed to less cellular contact between the smaller cells.

In most studies where tolerance to stress is induced over long periods, cells have been shown to lose their regenerative ability. In the present work, when calluses tolerant to 342 mM NaCl and non-tolerant calluses were transferred to shoot producing NaCl-free medium, shoots were produced. However, shoot-inducing medium containing 257 and 342 mM NaCl inhibited shoot initiation (Table A3). Twice as many shoots were produced from non-tolerant calluses than from salt-tolerant ones. The average number of shoots formed by non-tolerant calluses was 10.23, whereas an average of 4.91 shoots was produced from tolerant calluses. These findings are in agreement with those of Pua *et al.* (1985,1986) who reported that the number of shoots per callus produced by salt-tolerant tobacco calluses decreased with increasing concentrations of Na_2SO_4. Other studies also showed the stability of organogenetic capacity of salt tolerant cultures in shooting media with no added salt. Organogenesis was shown to occur from salt-tolerant lines of flax (McHughen and Swartz, 1984) and tomato (Kurtz ,1982) on medium free of salt whereas the presence of salt inhibited organogenesis. Smith and McComb (1983) reported that NaCl concentrations greater than 62.5 mM inhibited regenerative capacity. In studies where regeneration was successful on medium with added salt, the medium concentration was usually no higher than 171 mM NaCl. Nabors (1983) and Pua *et al.*, (1985) have suggested that the time required for long-term selection and the addition of salt to the induction medium are factors which may be responsible for the reduction in organogenesis.

Organogenesis is a high energy requiring process involving prior osmoregulation (Hammersley-Straw and Thorpe, 1988; Pua *et al.*, 1985; Rains *et al.*, 1980; Brown *et al.*, 1979). The energy requirement for osmotic adjustment and organogenesis is usually met by carbohydrates, sugars and starch, from the media. However, carbohydrates previously stored by the cells can also be utilized (Stavarek and Rains, 1985). In addition to its role as a source of energy, starch has been suggested to act as an osmoticum (Brown *et al.*, 1979; Thorpe *et al.*, 1986). Reports have indicated that starch accumulates in areas which eventually give rise to shoots (Mangat *et al.*, 1989; Thorpe *et al.*, 1986). In the present study, the callus starch content was found to be lowered in calluses maintained on media containing high NaCl concentrations (257 and 342 mM). Hence, it is reasonable to speculate from our findings that the slow differentiation and the reduction in the number of shoots from NaCl-tolerant calluses, may be due to osmotic shock which occurs when calluses are transferred to NaCl-free shooting medium and to the lower levels of starch found to be present in calluses maintained on salt-containing medium respectively.

Salt tolerance might be permanent in some cases and temporary in others. In the former case, when tolerant cells are exposed to non-stress conditions over several weeks and then re-exposed to the stress, the cells retain their ability to grow well (Hassan and Wilkins, 1988; Pandey and Ganapathy, 1984; Nabors, 1983; Watad *et al.*, 1983; Sobko *et al.*, 1981; Dix and Street, 1975; Nabors *et al.*, 1975). However, in the latter case, the cells might lose their capacity to tolerate salt stress conditions once the salt is removed from the medium, resulting in poor growth (Hasegawa *et al.*, 1980). In the present study, when tobacco calluses tolerant to 342 mM NaCl were cultured for 15 weeks (3

passages) on NaCl-free medium and then transferred back to medium containing 342 mM NaCl, growth was repressed. The dry weights of the tolerant calluses were similar to those of calluses continuously maintained on NaCl-free medium, when they were cultured on medium containing 342 mM NaCl. It is inferred from our study that the salt tolerance is temporary. In agreement with the findings of Hasegawa *et al.* (1980) who reported that NaCl-tolerant *Nicotiana tabacum* suspension cultures lost their ability to tolerate salt when cultured for 5 cell doublings (1 passage) on medium without NaCl. Sensitivity to salt of these tolerant cells was similar to that observed in the control population. Other studies with polyethylene glycol tolerant *Lycopersicon esculentum* cells, showed that these cells lost the ability to grow, and closely resembled non-selected cells when they were placed in medium not containing PEG (Bressan *et al.*, 1981). The findings from the present study as well as that from Hasegawa *et al.* (1980) suggest that callus tissues revert back to their original state once the stress is removed. Therefore, the observed salt tolerance may be more of a phenotypic than a genotypic change.

A number of studies have shown an increase in the endogenous levels of proline and betaines (glycinebetaine, choline and trigonelline) with increased salt tolerance (McDonnell and Wyn Jones, 1988; Binzel *et al.*, 1987; Pandey and Ganapathy, 1985; Watad *et al.*, 1983; Dix and Pearce, 1981; Storey and Wyn Jones, 1977; Strogonov, 1973). Further, when proline and betaines are added to the culture medium, salt tolerance is enhanced (Kavi Kishor, 1988; Pandey and Ganapathy, 1985; Wyn Jones and Gorham, 1984). The role of these compounds has not been determined although it has been suggested that they may act as cytoplasmic osmoticums and/or protective agents for

cytoplasmic enzymes (Dix and Pearce, 1981). Trigonelline, a methylbetaine of nicotinic acid, is naturally occurring and has been found in seeds, tubers, roots, leaves, and seedlings of many plant species (Tramontano *et al.*, 1986; Joshi and Handler, 1960) and in callus cultures of *Trigonella foenum-gracecum* L. (Khanna and Jain, 1972). It has been suggested to play the role of a hormone, in promoting cell arrest in G2 of the cell cycle in cells of root and shoot meristems of *Pisum sativum* (Evans and Tramontano, 1981) and it is important as a precursor of nicotinamide adenine dinucleotide (NAD) (Neuhann *et al.*, 1979). The levels of trigonelline have been shown to increase in plants under certain stress conditions, particularly in injury stress (Mangat, 1968). It was therefore of interest to examine if the addition of trigonelline to the culture medium would increase salt tolerance. Preliminary studies indicated that the addition of trigonelline to the growth media does not enhance salt tolerance in tobacco calluses. This may suggest that the effect of this methylbetaine in salt-tolerance of tobacco calluses is minimal. These results endorse a previous observation that no significant change in the amount of trigonelline occurs in salt stressed plants (Storey and Wyn Jones, 1977). Further studies under more controlled experimental conditions are required to determine if trigonelline behaves like other betaines.

From the present studies, it would appear that tobacco calluses tolerant to different concentrations of NaCl in the growth medium resemble salt-tolerant cell suspensions. A comparison of characteristics identified in the tolerant calluses suggests that the effects of NaCl occur gradually as the level of tolerance is increased.

SUMMARY

Tobacco calluses were induced to tolerate up to 342 mM NaCl in their growth medium by the stepwise selction method. With increasing tolerance to NaCl, the callus cells were found to be smaller in size, contain lower amounts of potassium, and have increasing levels of chlorophyll, sodium and chloride. Shoots were produced from tolerant and non-tolerant calluses only on medium without added NaCl, but twice as many shoots were formed from the non-tolerant calluses. Reductions in the levels of starch in the tissues at high NaCl concentrations could be correlated with reductions in the number of shoots produced. The observed tolerance to NaCl of tobacco calluses was inferred to be temporary. Tolerant calluses could not retain their ability to grow after being cultured on medium without NaCl. This tolerance could not be further enhanced by the addition of trigonelline to the growth medium. Under the present experimental conditions the growth of non-tolerant and tolerant calluses were stimulated at a concentration of103 mM NaCl.

The selection and characterization of tobacco calluses tolerant to high NaCl concentrations indicated that NaCl stress symptoms appear to be similar in calluses and cell suspensions of *Nicotiana tabacum*. Further studies are required to resolve why salt tolerance is sometimes not stable in selected cultures and in their regenerated progeny. Additional work is also needed to determine the relationship among reductions in cell size, ion accumulation and osmotic adjustment in tolerant calluses.

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APPENDIX A

Table A1 : Nutrient Medium Composition (Murashige and Skoog,1962).

Ingredients	Amounts
Macronutrients	**mg/l**
$MgSO_4.7H_2O$	370.00
KH_2PO_4	170.00
KNO_3	1900.00
NH_4NO_3	1650.00
$CaCl_2.2H_2O$	440.00
Micronutrients	**mg/l**
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
$NaMoO_4.2H_2O$	0.25
$CuSo_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
KI	0.83
$FeSO_4.7H_2O$	27.8
Na_2EDTA	37.3
Vitamins	**mg/l**
Thiamine.HCl	1.0
Pyridoxine.HCl	0.5
Nicotinic acid	0.5
Glycine	2.0
Growth hormones	**µM**
2,4-D	1.0
IAA	2.0
Kinetin	0.1
Others	**mg/l**
Myo-Inositol	0.1
Casein-hydrolysate	1.0
Sucrose	24.0
Agar	5.0
pH	5.7±0.1

Table A2: Growth of tobacco calluses maintained for three successive passages on NaCl containing MS medium. Fresh weights of 10 samples were determined at 5 weeks (passage 1), 10 weeks (passage 2), and 15 weeks (passage 3).

Medium NaCl concentration (mM)	Time (weeks)		
	5	10	15
	Δ Fresh weight (g ± 95% confidence interval)		
0	8.786±1.215	8.552±0.643	7.672±1.083
103	6.524±1.750	10.948±1.258	10.852±1.191
171	3.581±0.755	7.729±0.836	5.608±0.713
257	-	0.727±0.159	2.632±0.498
342	-	0.128±0.061	1.842±0.296

- No change in fresh weight

Table A3 : Organogenetic response of NaCl-tolerant and non-tolerant callus tissue.Organogenetic responses were observed after calluses were cultured for 8 weeks on shoot producing MS mediumwith 0 mM and 342 mM NaCl supplemented with 0.1 mg/l IAA and 10 mg/l BAP.

Calluses tolerant to NaCl concentration (mM)	Concentration of NaCl in medium (mM)	Percentage of calluses showing a response — shoots	Average number of shoots per callus (±95% CI)[a]
0	0[b]	100.00(16)	15.19±5.90
	257[b]	0	0
257	0[c]	85.00(17)	2.30±1.89
	257[c]	0	0

Note. Figures in parentheses are the numbers of calluses showing a response.
[a] 95% CI= 95% confidence interval.
[b] Sixteen calluses.
[c] Twenty calluses.