Selection and Characterization of NaCl Tolerant Callus Cultures of Mungbean [*Vigna radiata* (L.) Wilczek]

By

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To my loving family...

CERTIFICATE I

This is to certify that this thesis entitled, "Selection and characterization of NaCl tolerant callus cultures of Mungbean [*Vigna radiata* (L.) Wilczek]", submitted for the degree of **Master of Science** in the subject of **Botany** of CCS Haryana Agricultural University, Hisar, is a bonafide research work carried out by **Anju Lalit** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE II

This is to certify that this thesis entitled, "Selection and characterization of NaCl tolerant callus cultures of Mungbean [*Vigna radiata* (L.) Wilczek]", submitted by **Anju Lalit** to the Chaudhary Charan Singh Haryana Agricultural University, Hisar in partial fulfilment of the requirements for the degree of **Master of Science** in the subject of **Botany** has been approved by the Student's Advisory Committee after an oral examination on the same.

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Anju

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Dated:

Chapter 1

Introduction

There is an ever increasing demand to produce more food due to increasing human and livestock population. High productivity potentials are limited by various abiotic stresses. Among an array of stresses, soil salinity is of serious concern to all those involved in maximizing food production. The adverse effects of salinity are more prominent when crop plants inhabitat arid and semi-arid regions. Among different strategies adopted to combat salinity effects, amendment of salt affected soils by using curative and preventive measures like scraping, leaching or by using gypsum etc., is one of the most potent remedy. But, it is very expensive and sometimes not practical. Such a predicament logically leads to the idea of evolving salt tolerant genotypes by manipulating the heritable variations present in the germplasm through plant breeding methods (Bhumbla *et al.*, 1968; Epstein and Norly, 1977; Poonamperuma, 1978; Ashraf, 1994). Concerted efforts have been directed towards identification of crop species/varieties that are tolerant to salinity and recurrent selection of fundamental importance in plant breeding. This conventional approach is cumbersome as well as a time consuming process (Reddy and Vaidyanath, 1986). Moreover, direct selection under field conditions for quantitative traits like salt tolerance is difficult because variable environmental factors affect the precision and repeatability of such traits (Jones and Qualset, 1984). In this respect *in vitro* approach has been quite beneficial as complex organ and plant environment interactions can be avoided. Moreover, salt tolerant callus cultures serve as an ideal systems to assess the physiological effects of salt at cellular level (Chandler and Thorpe, 1987).

Regeneration of plantlets from these salt tolerant calli is anticipated to produce salt tolerant plants (Winicov, 1996). Presence of excess of NaCl in the culture medium proves lethal in sensitive cell cultures through ionic and osmotic effects. Repeated culturing of calli on salt supplemented medium will enable nontolerant cells with labile metabolism to adapt to imposed stress (Gosal and Bajaj, 1984; McHughen and Swartz, 1984; Chandler and Thorpe, 1987). Comparison of metabolite profile and mineral make up of these selected salt tolerant calli with nontolerant callus cultures isolated from the same initial explants enables to investigate plant metabolism and the mechanism of acquisition of salt tolerance at cellular level (Chandler and Thorpe, 1987; Piqueras and Hellin, 1992).

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Leguminous plants are economically very important for their nutritional value as food and fodder and their role in nitrogen fixation and as industrial raw material. Among grain legumes, mungbean [*Vigna radiata* (L.) Wilczek] is one of the most important crop of tropics and sensitive to salinity. It is a monocarpic plant serving as an excellent source of high quality dietary-proteins ranging form 22-30 per cent (Teli *et al.*, 2000). It ranks fourth among pulses after chickpea, pigeonpea and urdbean in area and production in India (Anonymous, 1994). Mungbean being autogamous and diploid, can be effectively used for screening of naturally occurring variations in cell cultures and exploited for isolating salt resistant lines. Present study thus taken up with following objectives.

1. To compare the callusing potential of different explants under NaCl stress.
2. To attempt raising of salt adapted lines and compare the growth and biochemical profile of adapted and unadapted calli.

Chapter 2

Review of Literature

Soil salinity is one of the widespread constraint in the agriculture restricting the plant growth and productivity (Kuznetsov and Shevyakova, 1997). Adverse effects of salinity on crop performance are attributable to the osmotic effect, ionic toxicity, ion deficiency, diversion of photosynthates from growth functions to osmotic adjustment and for tissue repairation damaged due to excess of salt ions (Greenway and Munns, 1980; Munns and Termatt, 1986; Sharma, 1997). This mini-review is a compilation of available literature on the selection of NaCl tolerant callus cultures and their characterization in terms of biochemical changes and mineral composition which bear relevance to the present investigation

Haberlandt (1902) for the first time conceived the idea of culturing plant cells in artificial nutritive medium in controlled environment conditions Though he failed to get the desirable results, his pioneering work had paved the way for future research workers to explore further the possibility of regenerating plants through tissue culture. The fascinating outcome of these endeavours has been the developments in fields such as cell suspension cultures and eventually protoplasts culture (Vasil and Hilderbrandt, 1965; Takebe *et al.*, 1971; Hammatt and Davey, 1990). Successful establishment of callus cultures depends upon genetic make up of the plant, explant type, media regime, culture environment and their interaction (Pierik, 1987). Composition of the medium is one of the most crucial factor in successful establishment of cultures.

2.1 CALLUS INDUCTION IN LEGUMINOUS PLANTS

A perusal of literature indicates that a basal medium alone is not sufficient for callus induction and growth or regeneration. It requires certain stimulatory additives like plant growth regulators. A proper balance of auxin(s) and cytokinin(s) is known to regulate the *in vitro* response of explant tissue and development of organs. The requirement for auxin/cytokinin vary with the type of explants and the plant species. In general, legumes require a high cytokinin/auxin ratio in the medium for callus induction. This has been reported for a number of plant species, viz. *Phaseolus vulgaris* (Saam *et al.*, 1987), *Pisum sativum* (Gamborg *et al.*, 1974), *Vigna radiata* (Kumar, 1987; Kumar and Sharma, 1989), *Vigna acontifolia* (Bhargava and Chandra, 1989). Callus induction was evident from 3-4 day old macerated shoot apices of *Pisum sativum* on B_5 medium containing 0.2 to 5.0 μM BAP and 1 μM NAA (Gamborg *et al.*, 1974).

Singh *et al.* (1982) succeeded in raising callus cultures from hypocotyl segments of chickpea on B_5 medium adjuncted with 2,4-D (0.5 mg l^{-1}) and kinetin (0.1 mg l^{-1}). Malmberg (1979) initiated callus cultures in pea from epicotyl segments placed on MS medium containing 2 mg l^{-1} NAA and 1 mg l^{-1} BAP. Puonti-Kaerlas *et al.* (1990) obtained best callusing from epicotyls of pea by using B_5 medium supplemented with 0.5 mg l^{-1} of both BAP and 2,4-D and solidified with 0.4 per cent agarose. In chickpea, Atlaf and Ahmad (1986) reported callusing response from cotyledonary nodes, root and young leaves on MS medium supplemented with growth regulators 2,4-D (1-2 mg l^{-1}), BAP (1-2 mg l^{-1}) and NAA (0.5 mg l^{-1}). Gosal and Bajaj (1984) raised callus cultures from hypocotyl segment of aseptically grown one week old seedlings of chickpea, pea and greengram on MS medium containing 2,4-D (2 mg l^{-1}) alone. Callus cultures of *Cicer arietinum* were initiated from embryos of seeds on B_5 medium containing 2.0 mg dm^{-3} 2,4-D. However, the callus was maintained on MS medium without adenine sulphate but having 1.0 mg dm^{-3} NAA and 1.0 mg dm^{-3} BAP (Pandey and Ganapathy, 1984). Callus was initiated successfully from 3-5 day old seedling hypocotyl segments of *Vigna acontifolia* on MS medium supplemented with 0.2 mg l^{-1} each of 2,4-D and kinetin (Bhargava and Chandra, 1989). Eapen and George (1990) succeeded in getting callus induction from immature cotyledons of *Vigna acontifolia*, *V. mungo* and *V. radiata* on media supplemented with high concentrations of auxins like NAA (10 mg l^{-1}), 2,4-D (5 mg l^{-1}) or picloram (5 mg l^{-1}). Light green callus was obtained within 3-4 days if cultured on NAA supplemented medium. Development of callus was better when the abaxial side of the cotyledon was in contact with medium compared to the adaxial side. Olmos and Hellin (1996); Olmos *et al.* (1994) reported callus responses form young leaves of *Pisum sativum* on MS medium adjuncted with 2,4-D (1 mg l^{-1}). Recently, Arockiasami *et al.* (2000) succeeded in callusing leaflets of two cultivars of chickpea (K850 and ICC12426) on MS medium adjuncted with 5 or 10 μM NAA and 1 or 5 μM BAP. The calli obtained on MS + NAA were mostly colourless and friable but those obtained on MS+ NAA+ BAP were slightly greenish.

The PC-L_2 (Phillips and Collins, 1979) medium has been used extensively for raising callus in mungbean (Kumar, 1987; Kumar and Sharma, 1989; Gulati and Jaiwal, 1992) and best callus responses has been obtained with 0.5 mg l^{-1} NAA, 0.5 mg l^{-1} 2,4-D and 1.0 mg l^{-1} BAP (Kumar and Sharma, 1989). Kumar (1987) reported callusing responses from root tips and root segments of 2-3 days old aseptically grown seedlings. Gulati and Jaiwal (1992) successfully cultured leaf explants of aseptically grown 7-days old seedlings. Gosal and Bajaj (1984). reported callusing from hypocotyl segment on MS medium containing 2 mg l^{-1} 2,4-D. B_5 medium (Gamborg *et al.*, 1968) has also been employed for mungbean callus cultures. Singh *et al.* (1985) observed initiation and growth of callus from root, shoot and hypocotyl explants on B_5 medium containing 0.1 μg ml^{-1} kinetin and 1.0 μg ml^{-1} 2,4-D. Callus was initiated from young roots of mungbean on modified MS medium in which thiamine was increased 10 fold plus 3 per cent sucrose, 0.5 per cent glucose, 0.1 mg l^{-1} 2,4-D and 0.05 mg l^{-1} kinetin (Yoshida *et* *al.*, 1993). Recently, Teli *et al.* (2000) reported callus initiation from leaf explants of mungbean within 8 days of culturing on MS basal medium supplemented with 2,4-D (3-9 µM) or NAA (5-10 µM) alone or in combination with kinetin (2-9 µM) or BAP (5 µM). The frequency of callusing was 100 per cent on MS medium with 2,4-D and kinetin higher than 7 µM inhibited callus growth.

2.2 SELECTION FOR SALT TOLERANT CALLUS LINES

The salt tolerant variants have been isolated by exposing callus to a sublethal concentration of salts, once or few times (one step/direct selection)or by gradual stepwise increase in salts at each subculture (stepwise selection). Salt tolerance of these variants is determined on the basis of fresh or dry weights of callus, packed volume or cell number in a cell suspension. According to McHughen and Swartz, 1984) the first strategy is more effective than stepwise selection because of its close resemblance with the situation in the field where the seeds are planted- directly and therefore immediately encounter the saline environment. Stepwise imposition of stress is inefficient as it more readily induces physiological adaptations. Non-tolerant cells with a labile metabolism will have enough time to adapt to the gradually imposed stress and will therefore be positively selected. Bowman (1987) pointed out the genetic differentiation with respect to growth and survival is expressed better under a rapidly induced short term treatment rather than gradually imposed salinity. Harms and Oertli (1985) however, advocated for stepwise selection method since one step selection primarily checks the ability of cells to withstand osmotic shock and to recover from it rather than their ability to tolerate a certain level of salt. Direct shift to stress of high magnitude does not leave sufficient time for the cells to express their inherent capacities for stress response and adaptation to salt stress may enable a distinction between adapted cells and mutant and thus may help to improve the efficiency of selection of salt tolerant mutant cells (Tal, 1993).

Melchers (1972) was the first to discuss the advantages of tissue culture technique for selecting salt resistant mutants. Zenk (1974) employed this approach successfully by selecting a cell line from the haploid cultures of *Nicotiana sylvestris* that was able to grow on a medium containing 170 mM NaCl on which unselected cells did not grow. Dix and Street (1975) selected a number of cell lines of *Nicotiana sylvestris* and *Capsicum annuum* which were capable of growing in liquid medium containing NaCl up to a level of 340 mM. Some of these lines retained the resistance to salt after several subcultures in media lacking NaCl. Dix (1980) succeeded to regenerate plants from these resistant cultures previously grown for 2-6 passages in media supplemented with 340 mM NaCl. Hasegawa *et al.* (1980) observed that *N. tabacum* cells resistant to 1 per cent NaCl lost their tolerance once they had been grown away from salt for 5 cell mass doublings. Similarly, Smith and McComb (1983) observed that alfalfa regenerants from NaCl resistant cultures did not reveal any improved resistance. However, Croughan *et al.* (1978) isolated stable, NaCl resistant cell lines, which could grow on a medium containing 170 mM NaCl, from a cell culture of alfalfa. Nabors *et al.* (1980) also isolated NaCl-resistant cell line form suspension cultures of *Nicotiana tabacum* that could tolerate 0.88 per cent NaCl. Plants regenerated form these calli also inherited this trait and the level of resistance in the progeny of regenerated plants was higher than the original cells in the culture. Watad *et al.* (1983) developed NaCl resistant cell line from the suspension cultures of *N. tabacum gossi* by stepwise increase in NaCl concentration in the medium. Resistance showed stability through at least 24 generations in the absence of added NaCl. Stavarek *et al.* (1980) were able to regenerate plants form long term maintained cell culture of alfalfa, by sequential transfer on a new medium.

Gosal and Bajaj (1984) in chickpea and Zhou jia Ping *et al.* (1981) in soyabean reported the isolation of stable NaCl resistant cell lines. However, Gosal and Bajaj (1984) also reported the isolation of unstable NaCl resistant cell lines of *Vigna radiata* and *Pisum sativum*. Tyagi *et al.* (1981) reported the isolation of stable NaCl-tolerant cell lines from haploid callus cultures of *Datura innoxia*.

Kochba *et al.* (1980, 1982) isolated 'Shamouti' orange callus lines with increased resistance to NaCl. The resistance was maintained in embryos obtained from these lines. These observations were further supported by Ben-Hayyim and Kochba (1982, 1983) in citrus. Spiegel-Ray and Ben-Hayyim (1985) observed that the regenerated plantlets from these salt resistance cell lines of *Citrus sinensis* retained the tolerance character. In *Medicago sativa*, McCoy (1987) regenerated salt tolerant plants from stable salt tolerant callus lines selected by step up selection method but the regenerants were morphologically abnormal and showed poor growth than the parents and the only plant that flowered was both male and female sterile. Selection of NaCl tolerant tomato cell lines was effectively accomplished by using a protocol in which the callus tissue was subjected to increasing NaCl concentration at the rate of 20 mM per passage (Rahman and Kaul, 1989). Sumaryati *et al.* (1992) were able to regenerate stable salt tolerant *Nicotiana plumbaginifolia* plants form NaCl and PEG-adapted cells. Ibrahim *et al.* (1992) selected fertile salt tolerant plants of *Coleus blumei* by exposing leaf disc directly to regeneration medium adjuncted with NaCl. Seed progeny of selected plants showed more vigorous growth in the presence and absence of NaCl than progeny from unselected plants. Winicov (1996) selected salt tolerant cell lines from callus cultures of rice by a single step. There is a report in which batch suspension cultures of lucerne established directly from surface sterilized leaflet explants were subcultured into media of progressively higher salinity to produce a cell line tolerant to 400 mol m^{-3} NaCl. These cells were grown for eight passages of 28 days each on NaCl free medium, the cells were still more salt tolerant than nonselected cells when subcultured to salt supplemented media (Chaudhary *et al.*, 1997). Chen *et al.* (1998) induced salt tolerance in *Eucalyptus microcorys* shoot cultures by a stepwise increase in NaCl over 12 months to tolerate 100 and 150 m mol L^{-1} NaCl, which were otherwise unable to survive 50 mM NaCl. The *in vitro* production of a stable salt tolerant cell line of potato by direct recurrent selection was achieved by Ochatt *et al.* (1999). Recently, Santos *et al.* (2000) reported the selection of stable salt tolerant cell lines of *Helianthus annuus* by subculturing cotyledon derived calli on medium containing 0, 50, 100, 200 and 300 mmol L^{-1}

NaCl. These calli were compact and developed a dark green colour. They succeeded in regenerating plant but these were stunted and did not survive to acclimation.

Rus *et al.* (2000) studied the salt responses induced by long term callus culture in leaf callus tissue of the wild type salt tolerant *Lypopersicon pennellii* and in cultivated tomato species *L. esculentum*. They observed that in salt tolerant wild species, the callus relative growth rate (RGR) were higher in the salt medium than in control after only three subcultures in salt medium. But in cultivated species callus RGR tended to decrease with the subculture both in control and saline media. In the 7th subculture, callus RGR increased as subculture advanced and in 8th subculture RGR became near to that obtained in 1st subculture. They concluded that the salt tolerance of the wild species was much higher than that of cultivated species.

2.3 GROWTH CHARACTERISTICS OF CULTURED CELLS UNDER SALT STRESS

There are a large number of reports which indicate that the cultured cells either grow poorly or exhibit growth inhibition kinetics upon exposure to increasing level of salt stress (Croughan *et al.*, 1978; Starvarek and Rains, 1984; Gulati and Jaiwal, 1992). Effect of Na_2SO_4 and NaCl on callus culture in *Brassica campestris* was studied by Paek *et al.* (1988). They reported that Na_2SO_4 was more than twice as inhibitory in comparison to same concentration of NaCl when growth and fresh : dry weights ratios of established callus were compared.

Salt tolerance manifested in callus cultures of several species, such as tomato, barley and sugarbeet is also reflected in the whole plant (Tal *et al.*, 1978; Orton, 1980; Smith and McComb, 1981). Smith and McComb (1981) have compared plants and their corresponding callus cultures of a salt-tolerant glycophyte (*Beta vulgaris*), a salt-sensitive glycophyte (*Phaseolus vulgaris*) and two halophytes (*Atriplex undulata* and *Suaeda australis*) to increasing levels of NaCl (0-250 mM). *Beta vulgaris* (both callus and plant) showed an increase in growth at intermediate salt levels and a decrease at higher levels, whereas, *P. vulgaris* (both callus and plant) revealed a decrease in growth with increasing levels of salt. But for both the halophytes, the callus growth decreased with increasing levels of salt, while the plants showed increased growth under identical conditions. They concluded that *Beta vulgaris* seem to have a cellular mechanism of salt tolerance and the two halophytes have whole plant tolerance mechanisms (salt glands, succulence) for dealing with salt and hence tolerance is not expressed in their calli. The degree of growth inhibition in cultured cells grown under salt stress varies from species to species, cultivar to cultivar as has also been observed at whole plant level (Tal, 1985). While comparing the salt tolerance of *Hordeum vulgare* and *H. jubatum* in whole plants and callus cultures, Orton (1980) observed that callus response paralleled the whole plant response as the level of NaCl was increased to 0.17 M. Moreover, the callus cultures of the two species did not show growth inhibition at 0.17 M NaCl but did exhibit differential response towards their recovery after 30 days on the medium supplemented with 0.17 M NaCl i.e.

H. jubatum callus cultures did recover after 30 days, whereas, *H. vulgare* failed to do so.

Bourgeais-chaillou (1992) observed that relative growth rates of calli from roots increased on 25-50 mM NaCl media, while *in vitro* plant root biomass decreased, conversely plant shoot biomass increased on 25-50 mM NaCl media, while growth rate of corresponding calli decreased. Stimulatory effect of low concentration of salts on the growth of calli has been reported in a number of plants. Pandey and Ganapathy (1984) observed a slight stimulation of growth at 25 and 50 mM NaCl in callus cultures of *Cicer arietinum*. However, further increase in salt concentration was deleterious to the growth. Similarly, improvement in relative growth at low salt concentration have been reported in callus cultures of mungbean (Kumar, 1987). Gulati and Jaiwal (1992) studied the comparative salt responses of callus cultures of *Vigna radiata* to various concentration of NaCl, KCl, Na_2SO_4 alone or their mixture and mannitol. Callus growth was inhibited more severely with Na_2SO_4 followed by KCl, salt mixture NaCl and mannitol. They established a correlation between growth inhibition and the concentration of inorganic solutes in callus cultures.

Gangopadhyay *et al.* (1997) reported that all the adapted tissues grown either on NaCl or mannitol containing media showed a lower growth rate in comparison to the unadapted one grown on stress free media. The growth of selected and nonselected cell lines was significantly decreased in response to various sodium and potassium salts but the selected cell line was significantly more tolerant than the nonselected cell lines (Chaudhary *et al.*, 1997). Chen *et al.* (1998) observed that growth was initially inhibited during salt adaptation, but it recovered over time, even though NaCl concentrations were being increased. The NaCl adapted calli of *Helianthus annuus* to 200 mM and to 300 mM NaCl had maximum growth rates at respectively 50 and 100 mM NaCl (Santos *et al.*, 2000. Rus *et al.* (2000) reported higher callus RGR in salt tolerant (*Lycopersicon pennellii*) in salt medium than in control. They further observed that callus RGR of the adapted line of *L. esculentum* was similar or even lower than those of the unadapted line.

The growth cycle stage of the cells influenced the response to salinity of unadapted cells and cells adapted to 10g l^{-1} NaCl in tobacco. Cells in the exponential phase exhibited highest degree of tolerance to NaCl. The response was correlated with cell turgor, cells with highest turgor were the most tolerant (Binzel *et al.*, 1985).

2.4 ACCUMULATION OF ORGANIC OSMOTICA

A class of low molecular weight organic molecules like glycine-betaine, proline-betaine, proline, soluble sugars and polyamines found in scores of higher plants grown under saline conditions are thought to help in osmotic adjustments.

2.4.1 Proline accumulation in salt stressed culture cells

The most commonly observed effect of salinity on nitrogen metabolism is accumulation of proline in both glycophytes and halophytes (Sheoran and Nainawatee, 1990). Lerner (1985) observed that salt selected cell lines accumulated more proline than the sensitive ones upon exposure to NaCl. Proline accumulation was found to be a reversible phenomenon because its content decreased upon transfer to low salt or salt free medium. Pandey and Ganapathy (1985) reported accumulation of free proline in NaCl tolerant callus line (R-100) of *Cicer arietinum* in response to increasing NaCl concentration but sensitive line (C-0) did not. Further the tolerant callus line grown on NaCl free medium accumulated proline when recultured on NaCl medium. Chandler and Thorpe (1987) observed that proline accumulation was correlated with the inhibition of growth and there was a negative correlation between proline concentration and culture age for tolerant *Brassica napus* callus. Kumar and Sharma (1989) reported that NaCl resistant calli of *Vigna radiata* showed a slight increase in proline content as compared to sensitive callus on a salt free medium. On the other hand, NaCl resistant calli when grown under NaCl stress showed greater accumulation of proline than either sensitive or NaCl resistant callus lines grown on normal medium. This is suggestive of an osmoregulatory role of proline. Yang *et al.* (1990) observed that salt tolerant genotype (*Sorghum halepense*) accumulated more proline as compared to sensitive genotype (*Sorghum bicolor*). The accumulation of proline in *S. halepense* was seven times that of control as compared to *S. bicolor* where it was only twice that of the control. Safarnejad *et al.* (1996) indicated that there is a positive correlation between proline accumulation and adaptation to salt or osmotic stress in calli of *Medicago sativa*. Olmos and Hellin (1996) observed that content of proline was much higher in adapted than unadapted calli of *Pisum sativum*. High proline accumulation was also favoured in salt adapted calli of tobacco (Gangopadhyay *et al.*, 1997). Chaudhary *et al.* (1997) reported that proline content in *Hordeum vulgare* increased linearly with increasing salinity both in case of salt selected and unselected callus lines. Observations on proline accumulation in NaCl selected and nonselected cell lines of *Medicago sativa* cultured on medium supplemented with sodium and potassium salt revealed that proline accumulation was much higher in the salt selected over unselected cell line under NaCl or Na_2SO_4 stress. K_2SO_4 or KCl stress, however, did not favour proline accumulation in either selected or nonselected cell lines, suggesting that this is a response to Na^+ rather than Cl^- or K^+ (Chaudhary *et al.*, 1997). Proline level were significantly elevated at the salt conditioning period in shoot cultures of *Eucalyptus microcorys* (Chen *et al.*, 1998). Proline level was found to increase 14.2 times in leaf tissue treated with salt than in tap water treated tissue (Boscherini *et al.*, 1999) contrary to what happened in salt adapted cells of sunflower. The maintenance of soluble proteins associated with the observed increase in amino acids other than proline suggest that some of these amino acids may substitute some functions traditionally attributed to proline such as osmoregulation or protection (Santos *et al.*, 2000).

2.4.2 Ion regulation of salt stressed culture cells

The involvement of inorganic ions in osmoregulation has been reviewed by Jeschke (1984) and Pitman (1984). Like low molecular weight organic molecules, ions have also been found to be equally important in cell volume and cell turgor regulations. Potassium is nutritionally important and high Na^+/K^+ ratio account for metabolic damage (Winter and Preston, 1982). Salt tolerant varieties of wheat, rice are reported to accumulate lower Na^+ and Cl^- and higher K^+ than the sensitive ones (Joshi *et al.*, 1985; Sharma, 1986). These studies along with others suggested that K^+/Na^+ could be taken as suitable criterion for testing salt tolerance. Contrary to this generalisation, Greenway and Munns (1980) reported high Na^+ content in one of the tolerant variety of rice. Another mechanism of salt tolerance is exclusion of specific ions from plant cells so that their cytoplasm are protected from the ion toxicity (Levitt, 1980). For instance, Na^+ is excluded from leaves of many plant species including legumes (Winter and Lauchli, 1982), but Van Steveninck *et al.* (1982) observed opposite report in two species of lupin. *Lupinus luteus* appeared more salt tolerant and less effective in excluding NaCl than *L. angustifolius*. Thus, some legumes can adapt to moderate salinity by exclusion of Na^+ and / or Cl^- from the leaves whereas the salt resistant *L. luteus* may be termed as salt accumulator and thus features a halophytic response.

In vitro responses of cultured cells to salt stress could be useful in two ways-to evaluate the tolerance or sensitivity to salt and to identify cellular responses which would indicate the behaviour of the whole plant to salt stress Croughan *et al.* (1978) reported that salt tolerant alfalfa cell lines selected on NaCl medium maintained a higher K^+ content at all levels of NaCl than the non-selected cell lines. Increase in Na^+ and Cl^- accumulation in salt selected calli of chickpea coupled with decrease in K^+ in both salt selected and salt sensitive calli has also been observed under saline conditions (Pandey and Ganapathy, 1984). However, salt selected calli maintained higher concentration of K^+ than did the cells of salt sensitive line despite high Na^+ in the system. They concluded that maintenance of an appreciable level of K^+ in the selected calli was responsible for their increased tolerance than the sensitive calli. Watad *et al.* (1983) observed that both salt selected and wild type cell lines of *N. tabacum gossii* showed steady rise in internal Na^+ as a function of external Na^+ concentration.

The wild type cells lost a considerable part of their K^+ and extent of loss increased with rise in external NaCl. The selected cells on the other hand, lost no K^+ below 50 mM external NaCl and loss at higher NaCl was less over the wild type. This suggested that selectivity for K^+ accumulation in cytosol of salt selected cell lines was the possible mechanism of the increased tolerance of NaCl stress. Gulati and Jaiwal (1992) reported that accumulation of Na^+ in callus was accompanied by loss of K^+ at all levels of Na_2SO_4, NaCl and salt mixture. Olmos and Hellin (1996) observed that in response to external NaCl, the intracellular levels of Na^+ and Cl^- increased in adapted calli. Kumar and Sharma (1989) reported lower K^+ content of the NaCl selected callus line of mungbean in case of NaCl medium than on the normal medium. There was an increased accumulation of Na^+ as well as Cl^- ions in all cell lines, irrespective of their sensitiveness to salinity. Ben-Hayyim and Kochba (1983) noticed that in citrus there was increased accumulation of Na^+ and Cl^- ions in sensitive cell lines as compared to the resistant ones while K^+ was not affected.

Ion exclusion mechanism for salt tolerance does not seem to operate at the cellular level. Dix *et al.* (1983) did not observe any difference in the kinetics of Na^+ uptake in the salt tolerant and salt sensitive cell lines of *Nicotiana sylvestris*. Stavarek and Rains (1981) similarly observed that NaCl selected alfalfa cells accumulated approximately the same quantity of Na^+ and Cl^- as the unselected tissue when grown on different levels of NaCl. They opined that the salt selected cells did not exclude salts as a mechanism of tolerance towards salinity but were more efficient in the compartmentation of toxic ions than the nonselected ones. Hamada *et al.* (1994) reported that the contents of sodium in shoots and roots of broad bean and pea plants generally increased with increasing salinity. The concentration of potassium in broad bean shoots decreased significantly with the rise of NaCl salinization. On the contrary, the roots exhibited a marked and progressive increase in potassium content with increasing salinity. Olmos *et al.* (1994) reported uptake of Na^+ from the culture media was higher in tolerant cells of *Pisum sativum* than in control calli. Levels of Na^+ and Cl^- ions were elevated during the adaptation process and K^+ was reduced in shoot cultures of *Eucalyptus microcorys* (Chen *et al.*, 1998). Chaudhary *et al.* (1997) reported that salt tolerant cell line of lucerne showed enhanced Na^+ exclusion and was able to maintain higher K^+ and much lower Na^+:K^+ ratios than the nonselected cell line in response to NaCl or Na_2SO_4 stress. Higher concentration of Na^+ ion was detected in leaf tissue of wild type plants of *Lycopersicon esculentum* by salt treatment (Boscherini *et al.*, 1999). Santos *et al.* (2000) reported an increase in Na^+, Cl^-

content whereas K^+ levels decreased in salt selected cotyledon derived calli of *H. annuus*.

2.5 EFFECT OF SALT STRESS ON METABOLIC PATHWAYS

2.5.1 Carbohydrate metabolism

Carbohydrate metabolism is generally found to be adversely affected by salinity (Malik *et al.*, 1983 and Polonenko *et al.*, 1983). Munns *et al.* (1982) reported accumulation of sucrose in response to salt stress which is considered to be one of the effective mechanism of physiological adaptation to salinity. It is suggested that accumulation could be either due to the allocation of assimilates including sugars to a turgor regulation unavailable for metabolism or to inhibition of growth leading to the availability of metabolites for turgor regulation (Aspinall. 1980). Reducing and non-reducing sugars play a critical role in adjustment to osmotic potential. In tomato, cell osmotic potential was reduced mainly by accumulation of reducing sugars (Handa *et al.*, 1983). Reddy and Das (1978) reported that reducing sugars decreased while those of non-reducing sugars increased upon the exposure to salt stress. Reports of Binzel *et al.* (1987) indicated an increase in intracellular concentration of soluble sugars and free amino acids which were responsible for adaptation to NaCl stress. Chandler *et al.* (1988) reported a decrease in reducing sugars in control and sensitive callus cultures of *Nicotiana tabacum* when exposed to Na_2SO_4 but converse was time for salt selected callus cultures.

The increase in sugars may or may not be associated with the corresponding changes in the starch content. Kuznetsov and Shevyakova (1997) observed an increased level of soluble sugars and decreased level of starch in salt stressed tobacco plants. Rathert (1983) however, reported an increase in the both sugar and starch content in response to salt stress. On the other hand, Sharma *et al.* (1997) observed a decrease in starch and soluble sugar content with increasing salinity in leaves of chickpea. Similarly, Aggarwal and Gupta (1995) observed decrease in soluble sugars in callus of cowpea under saline conditions. On contrary, Olmos and Hellin (1996) observed that average concentration of reducing sugars increased in callus of *Pisum sativum* under salt stress. Other organic compounds as sucrose, amino acids and organic acids were less significant in the osmotic adjustment. Chen *et al.* (1998) observed that in salt adapted shoot callus cultures of *Eucalyptus microcorys* accumulated proline, glycine betaine, sucrose and glucose but had lower amounts of choline and fructose. Recently, an increase in glucose, fructose and sucrose levels in NaCl adapted calli of *Helianthus annuus* is reported by Santos *et al.* (2000).

2.5.2 Protein metabolism

Enormous literature is available regarding the effect of salinity on protein metabolism, yet the information is quite controversial. Reddy and Vora (1985) reported that protein and RNA content decreased with salinity in bajra leaves concomitant with an increase in protease activity and accumulation of free amino acids. Ashraf (1989) found that increasing concentration of NaCl significantly increased protein in the leaves of blackgram and this increase in protein content was ascribed to enhanced protein synthesis. An enhanced protein content under saline conditions was reported in mungbean (Upadhaya *et al.*, 1981). Salama and Ahmed (1987) on the other hand reported a decrease in protein content in the leaves of mungbean under saline conditions. Olmos and Hellin (1996) observed an accumulation of amino acids in NaCl adapted calli. The increase in amino acids in NaCl adapted calli could be the result of protein hydrolysis. However, the accumulation of amino acids may be due to other sources (Igoriyen *et al.*, 1992). On the one hand, as salt stress induces a secondary water stress that disturbs the metabolism, the elevated level of amino acids might indicate the susceptibility of callus tissue to osmotic stress (Delauney and Verma, 1993). Likewise, the depletion of organic acids may be as an outcome of blockage of oxidative respiration causing a flux of organic acids to the pool of amino acids in adapted calli. Accumulation of sugar and amino acids provides supporting evidences (Perez-Alfocea *et al.*, 1995). Total amino acid levels did not change with increase in salinity in leaf callus cultures of sorghum species (Yang *et al.*, 1990). Helal and Mengel (1979) observed that salinization of young barley plants impaired growth and incorporation of labelled N into the protein coupled with accumulation of inorganic N. Addition of K enhanced N uptake and incorporation into protein, reduced accumulation of inorganic N and improved the growth of salinized plants. It evidently suggests that impairment of protein metabolism is probably induced by the disturbance of K^+/Na^+ balance of tissue under saline conditions. Total amino acid contents in wild type leaf tissues treated with salt were 1.5 times greater than C_4 under the same treatment and 3.5 times than wild type treated with tap water (Boscherini *et al.*, 1999). The contents of free amino acid increased significantly in adapted calli of *Helianthus annuus* (Santos *et al.*, 2000).

Chapter 3

Materials and Methods

3.1 MATERIALS

3.1.1 Plant material

Mungbean [*Vigna radiata* (L.) Wilczek], one of the main dry beans cultivated in India and well known for its salt sensitivity, was selected for the present study. The genotype used was MH85-111. The seeds were procured from Department of Plant Breeding of CCS Haryana Agricultural University, Hisar.

3.1.2 Chemicals and glassware

The chemicals used were of high purity and were obtained from Hi-media, BDH, Glaxo, Sisco Research Laboratories, India or Sigma Chemicals Company (USA).

All the glassware used were of the borosilicate quality and obtained from either Borosil India or Corning Glass Company.

3.2 METHODS

3.2.1 Sterilization of glassware and instruments

Glassware was thoroughly washed with teepol and rinsed several times with distilled water. Then were subjected to dry heat sterilization by drying them in oven at 180°C for 2-3 hours. Other instruments such as scalpel, forceps etc. were flame sterilized prior to use.

3.2.2 Preparation of the culture medium

Each medium was prepared by adding the appropriate amount of different ingredients. All stock solutions were stored in a refrigerator. Growth regulators used for callus induction and proliferation were added before testing the pH of the medium. The pH of the medium was adjusted with the help of either 1 N NaOH or 1 N HCl and then agar was added. The medium was melted at a pressure of 0.726 kg cm^{-2} for ten minutes in an autoclave. Nearly fifty millilitres of molten medium was dispensed into 100 cm^3 Erlenmeyer flasks. The flasks were plugged with non-absorbent cotton and sterilized at 1.08 kg cm^{-2} pressure for fifteen minutes. After sterilization, these flasks were kept at room temperature and used for incubation within a week of their preparation. Schematic presentation of medium preparation is presented in Fig. 3. 1.

3.2.3 Raising of seedlings under aseptic conditions

The seed surfaces were wetted by dipping these in a dilute teepol solution for 5-7 minutes followed by washings in running tap water. These were then surface sterilized by immersing in 0.2 per cent mercuric chloride solution for 5-6

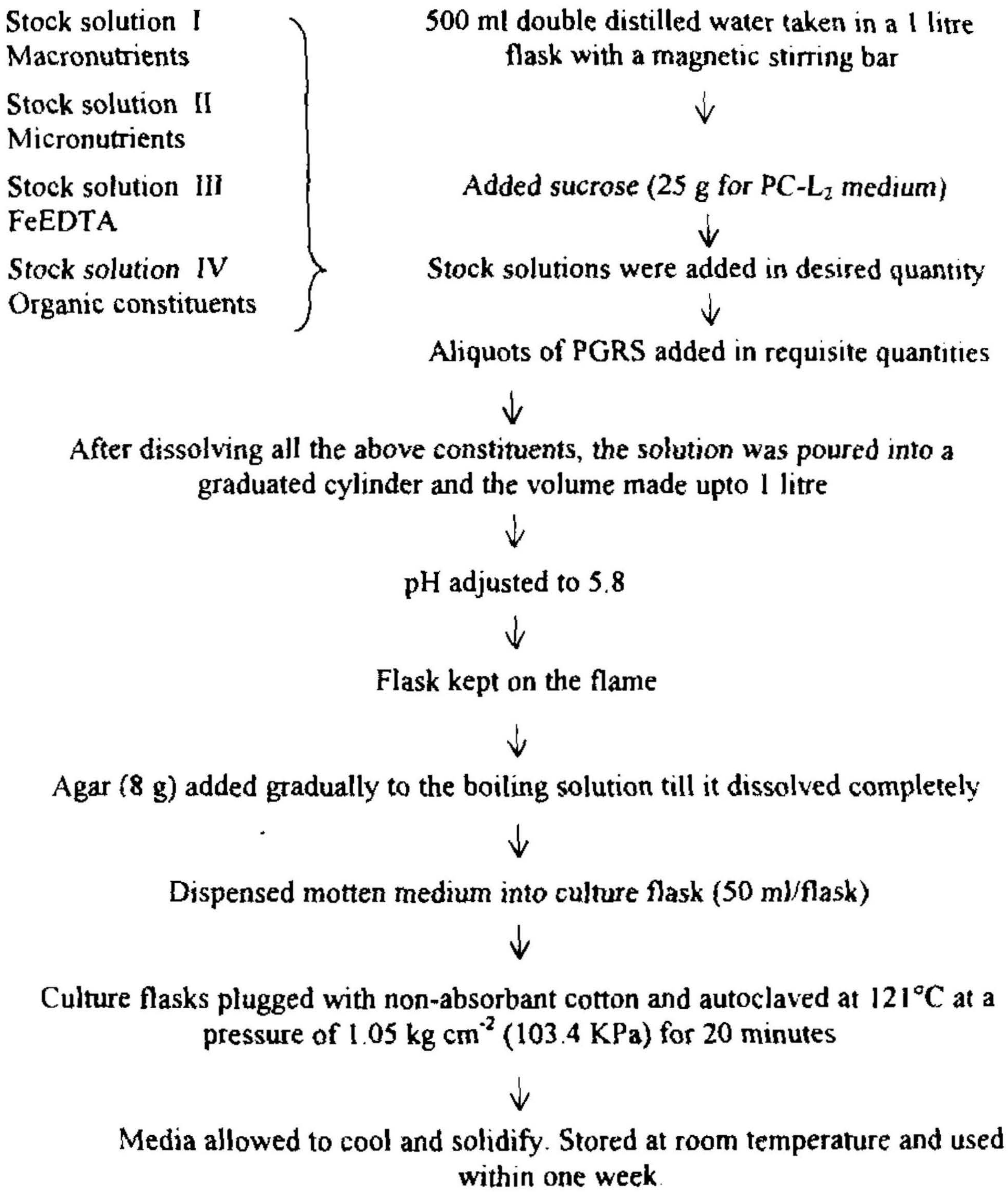


Fig.3. 1 **Schematic presentation of medium preparation**

minutes and were subsequently rinsed 4-5 times with sterilized double distilled water to remove the traces of $HgCl_2$. Sterilized seeds were then transferred on MS basal medium (Table 3.1) supplemented with 3 per cent sucrose and 0.8 per cent agar and incubated in a culture room under a photoperiod of 16 hours light and 8 hours dark at a temperature of 25±2°C.

Table 3.1 Composition of Murashige and Skoog medium (1962)

	Constituents	Concentration (mg l^{-1})
	Inorganic constituents:	
A	**Major nutrients**	
	NH_4NO_3	1650
	KNO_3	1900
	$CaCl_2.2H_2O$	440
	$MgSO_4.7H_2O$	370
	KH_2PO_4	170
	Na_2EDTA	37.3
	$FeSO_4.7H_2O$	27.8
B	**Minor nutrients**	
	H_3BO_3	6.2
	$MnSO_4.4H_2O$	22.3
	$ZnSO_4.7H_2O$	8.6
	KI	0.83
	$CuSO_4.5H_2O$	0.025
	$CoCl_2.6H_2O$	0.025
	$Na_2MoO_4.2H_2O$	0.25
	Organic additives	
	Myoinositol	100
	Glycine	2.0
	Thiamine-HCl	0.1
	Pyridoxine	0.5
	Nicotinic acid	0.5
	Sucrose	30,000
	Agar powder	8,000
	pH	5.8

Table 3.2 Composition of modified PC-L2 (Phillips and Collins, 1979) medium

	Inorganic Constituents	Concentration (mg l^{-1})
A	**Major nutrients**	
	NH_4NO_3	1000.0
	KNO_3	2100.0
	KH_2PO_4	325.0
	$NaH_2PO_4.H_2O$	85.0
	$CaCl_2.2H_2O$	600.0
	$MgSO_4.7H_2O$	435.0
	$FeSO_4.7H_2O$ (EDTA)	25.0
B	**Minor nutrients**	
	H_3BO_3	5.0
	KI	1.0
	$MnSO_4.H_2O$	15.0
	$ZnSO_4.7H_2O$	5.0
	$CuSO_4.5H_2O$	0.1
	$Na_2MoO_4.2H_2O$	0.4
	$CoCl_2.6H_2O$	0.1
	Organic additives	
	Thiamine-HCl	2.0
	Pyridoxine HCl	0.5
	Myoinositol	250
	Sucrose	25000.0
	Growth regulators	
	α-Napthaleneacetic acid (NAA)	0.5
	2,4-Dichlorophenoxyacetic acid (2,4-D)	0.5
	6-Benzyl aminopurine (BAP)	1
	Agar powder	8,000
	pH	5.8

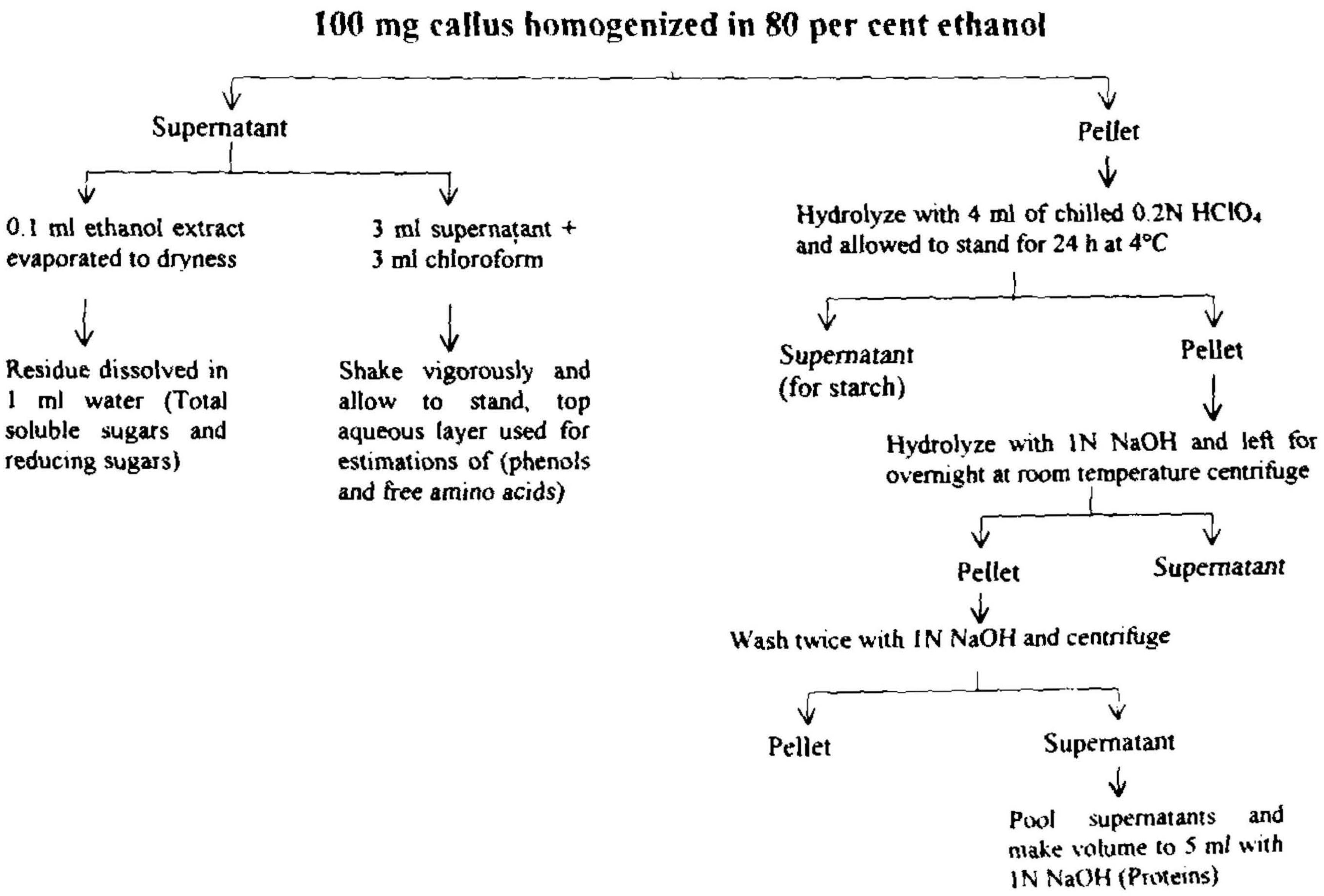
3.2.4 Experiment 1: Callus induction and its growth on NaCl supplemented medium

To assess callogenic and growth potential, different explants viz. leaf, hypocotyl and basal internode from 7-8 days old seedlings were inoculated separately on PC-L_2 medium (Phillips and Collins, 1979; Table 3.2) containing 0.5 mg/l 2,4-D, 0.5 mg/l NAA and 1.0 mg/l BAP (PC-DNB_1 medium, Kumar and Sharma, 1989) alone and with different concentrations of NaCl (0, 25, 50, 100, 150, 200 and 250 mM). Inoculated flasks were incubated in the culture room with already defined cultural conditions. Per cent callus induction in different explants was recorded. Fresh and dry weights of the calli were also recorded after three weeks of incubation.

3.2.5 Experiment 2: Selection and characterization of NaCl tolerant calli

For selection of NaCl tolerant calli, nearly 150 mg of actively growing calli raised in experiment 1 were inoculated on PC-DNB_1 medium adjuncted with different concentrations of NaCl (0, 50, 100, 150, 200, 250 and 300 mM) and incubated for three weeks in the culture room. Selection for salt tolerance was carried over continuous four subcultures (passages) of three weeks each on respective salt supplemented fresh medium. Callus growth was measured in terms of fresh and dry weights after every subculture.

After fourth subculture, one set of salt adapted calli (SAC^s) was employed for estimation of metabolite profile and mineral composition and another for testing the stability for salt tolerance. For this, calli were subcultured on PC-DNB_1



Flow diagram **Fractionation of sample for starch, total soluble sugars, reducing sugars, phenols and free amino acids** medium without NaCl (SAC^{n}) for three weeks. Fresh and dry weights of these calli were recorded and also subjected to biochemical estimations and mineral makeup by the standard methods. The callus lines obtained from the above NaCl free medium were recultured on a fresh medium of corresponding salt concentration for three weeks. Fresh and dry weights of these calli along with their mineral composition and proline content were recorded. Tolerance index (Ti) of SS, SAC^{s} and SAC$^{n\rightarrow s}$ was calculated by the following formula:

$$\mathrm{Ti} = \frac{\text{RGR of callus on NaCl medium}}{\text{RGR of callus on control medium}} \times 100$$

3.2.6 Biochemical estimations

Various metabolites viz. starch, total soluble sugars, reducing sugars, total soluble proteins, total free amino acids, proline and phenols were analysed in calli subcultured on PC-DNB$_1$ medium with and without NaCl. Three replicates were used for each estimation.

One hundred mg fresh weight of callus was homogenized in 80 per cent ethanol (v/v). The homogenate was refluxed for 15 minutes on a water bath at 60°C and centrifuged. The residue was further refluxed thrice with 80 % ethanol. The supernatants were pooled together and volume was made to 5 ml. The left over pellet was further partitioned according to procedure shown in flow diagram.

3.2.6.1 Estimation of starch

Estimation of starch was done using the method McCreddy *et al.* (1958).

Reagents

Anthrone reagent: 0.4 per cent anthrone in concentrated sulphuric acid.

Procedure

To 0.5 ml of the aliquot prepared for starch estimation, 4 ml of distilled water was added. Tubes were placed in an ice bath and to it 10 ml of anthrone reagent was added. The mixture was heated for 10 minutes at 100°C and then cooled to room temperature. Optical density was recorded at a wave length of 630 nm against the reagent blank. For standard 200 μg/ml D-glucose was used. Starch content was calculated by multiplying equivalent by 0.9.

3.2.6.2 Estimation of total soluble sugars

Total soluble sugars were estimated by the method of Yemm and Willis (1954).

Reagents used

Anthrone reagent: 0.4% anthrone in concentrated sulphuric acid

Procedure

Ethanolic extract measuring 0.1 ml was evaporated to dryness in a test tube After cooling, the residue was dissolved in 1 ml distilled water. These tubes were placed in an ice bath and 4 ml of anthrone reagent was added in to each tube The mixture was then heated in a water bath for 10 minutes. After cooling, optical density (OD) was recorded at a wavelength of 620 nm against reagent blank on spectronic-21 UVD. For standard, 100 µg/ml D-glucose was used.

3.2.6.3 Estimation of reducing sugars

Estimation was done by method of Honda *et al.* (1982).

Reagents used

(i) 1% aqueous cyanoacetamide

(ii) 0.1 M borate buffer (pH 9.0)

(a) 0.2 M boric acid (12.4 g of boric acid dissolved in distilled water and volume made to 1000 ml).

(b) 0.05 M borax (19.05 g borax dissolved in distilled water and volume made to 1000 ml.

To 50 ml of solution (a) 59 ml of solution (b) was added and volume made to 200 ml with distilled water.

Procedure

To 1.0 ml of aliquot taken in a test tube, 1 ml of 1% aqueous cyanoacetamide and 2.0 ml of 0.1 M borate buffer were added. The contents were mixed thoroughly on cyclomixer and heated on a waterbath for 10 minutes. After cooling, final volume was made to 10 ml by the addition of distilled water. Optical density of the solution was recorded at 276 nm on spectronic-21 UVD within two hours against reagent blank. For standard, 100 µg/ml D-glucose was used.

3.2.6.4 Total soluble proteins

Protein content was determined by the method of Lowry *et al.* (1951).

Reagents used

(1) 2% aqueous Na_2CO_3

(2) 0.5% $CuSO_4.5H_2O$ in 1% sodium citrate

(3) Fresh alkaline $CuSO_4$ (Mix 50 ml of reagent 1 with 1.0 ml of reagent 2)

(4) Folin-Ciocalteau reagent (1 N)

Procedure

To 1 ml of aliquot taken from protein extract prepared in 1 N NaOH, 5 ml of alkaline $CuSO_4$ was added and mixed thoroughly. The mixture was allowed to stand for 10 minutes at room temperature. Then 0.5 ml of 1 N Folin's reagent was added and contents were mixed vigorously. After 30 minutes, optical density was recorded at 750 nm against reagent blank. For standard, 1000 µg/ml bovine serum albumin (BSA) was used.

3.2.6.5 Estimation of free amino acids

Estimation of free amino acids was done by following method of Yemm and Cocking (1955).

Reagents

(1) 0.01M KCN: 0.1682 g of KCN was dissolved in 250 ml of 60 % ethanol.

(2) KCN-acetone: Five ml of 0.01 M KCN was diluted to 250 ml with acetone.

(3) Acetone-ninhydrin: 5 % solution (w/v) of ninhydrin was prepared in acetone.

(4) KCN-acetone-ninhydrin solution: 50 ml of acetone-ninhydrin solution was mixed with 250 ml of KCN-acetone solution just before use.

(5) 0.2 M citrate buffer pH 5.0: 21.008 g of citric acid ($C_6H_8O_7.{}_2O$) was dissolved in 200 ml of distilled water mixed with 200 ml of 1 N NaOH and volume was made to 500 ml.

Procedure

To 0.5 ml of the ethanol extract, 0.5 ml of citrate buffer and 1 ml of KCN-acetone ninhydrin solution were added. This was diluted with 5 ml of water and the mixture was then heated for 20 minutes in a boiling water bath. The mixture was cooled and volume was made to 10 ml with distilled water. Optical density was recorded at 570 nm using reagent blank on spectronic-21 UVD. For standard. 100 μg/ml glycine was used.

3.2.6.6 Estimation of phenols

These were estimated by method of Swain and Hillis (1959).

Reagent used

(1) Folin Cio-Calteaù Reagent (1 N).

(2) Saturated sodium carbonate solution. It was prepared by dissolving 17.50 g of anhydrous sodium carbonate was dissolved in 50 ml of distilled water by heating on a water bath (70-80°C). The content was cooled over night and then filtered.

Procedure

To 1.0 ml of aliquot taken in a test tube, 5.0 ml of distilled H_2O was added To each tube 0.5 ml of 1 N Folin Cio-Calteau reagent was added. Exactly after 3 minutes, 0.5 ml of saturated sodium carbonate was added. mixed vigorously and allowed to stand for 60 minutes. Optical density was recorded at 750 nm against reagent blank on spectronic-21 UVD. For standard, pyrogallol (100 µg/ml) was used.

3.2.6.7 Estimation of free proline

Free proline was estimated spectrophotometrically according to Bates *et al.* (1973). One hundred mg of callus was homogenised in a pestle mortor with 3 ml of 3 per cent sulphosalicylic acid and centrifuged at 5000 rpm for 15 minutes. The supernatant was used for proline estimation.

Reagents

1) 3% aqueous sulphosalicylic acid
2) Acid ninhydrin: It was prepared by warming 1.25 g of ninhydrin in 30 ml concentrated acetic acid and 20 ml of 6.0 M orthophosphoric acid until dissolved.

Procedure

To 0.5 ml aliquot taken in a test tube 2.0 ml of acid ninydrin, 2.0 ml of acetic acid were added and the mixture was heated in water-bath for 1 h at 100°C. The reaction was terminated in an ice bath. The reaction mixture was shaken vigorously with 3.0 ml of toluene on cyclomixer. The coloured organic phase was separated from the aqueous phase. On attaining room temperature. O.D. of the organic phase was recorded at 520 nm using toluene as a blank on spectrophotometer. For standard, 100 µg/ml proline was used.

3.2.7 Mineral analysis

The sodium, potassium and chloride contents were determined from the oven dried calli.

Extraction

Fifty mg of dried and well ground callus was extracted by adding ten ml of double distilled water and heated on a water bath for two hours. Supernatant was decanted and residue was resuspended in double distilled water and boiled thrice on water bath for at least one hour each to ensure total extraction of the salts. Supernatants were pooled together and final volume was made to 15 ml.

Sodium and potassium

Sodium and potassium content in aqueous extracts were determined on digital flame photometer (Model Elico) using standard solution of NaCl and KCl. respectively and values expressed as m mol g^{-1} dry weight.

Estimation of chloride

Reagents

(1) Potassium chromate: 5% solution was prepared by dissolving 5 g of potassium chromate in 50 ml of double distilled water and to it 1 N $AgNO_3$ was added dropwise until a slight permanent red precipitate was produced. It was filtered and volume was made to 100 ml with double distilled water.

(2) N/50 $AgNO_3$ (3.39 g of $AgNO_3$ dissolved in double distilled water and volume made to 1 litre by adding double distilled water).

Procedure

To 1 ml of aliquot, one drop of potassium chromate were added. It was titrated under bright light against N/50 silver nitrate dropwise while stirring until permanent brick red colour was produced. A blank using double distilled water was titrated in a similar way.

meq.l^{-} of chloride = (ml. of $AgNO_3$ used for titration of sample – ml of $AgNO_3$ used for titration of blank) X 1000 X Normality of $AgNO_3$.

Chapter 4

Experimental Results

Present investigation was carried out to select and characterize NaCl tolerant callus cultures of mungbean [*Vigna radiata* (L.) Wilczek] genotype MH85-111. Different explants viz. leaf, hypocotyl and basal internode form eight days old seedlings were cultured on PC-L_2 medium containing 0.5 mg l^{-1} NAA, 0.5 mg l^{-1} 2,4-D and 1.0 mg l^{-1} BAP with different concentrations of NaCl. Hormone supplemented PC-L_2 medium without NaCl served as control. The results obtained in the present investigation are detailed below.

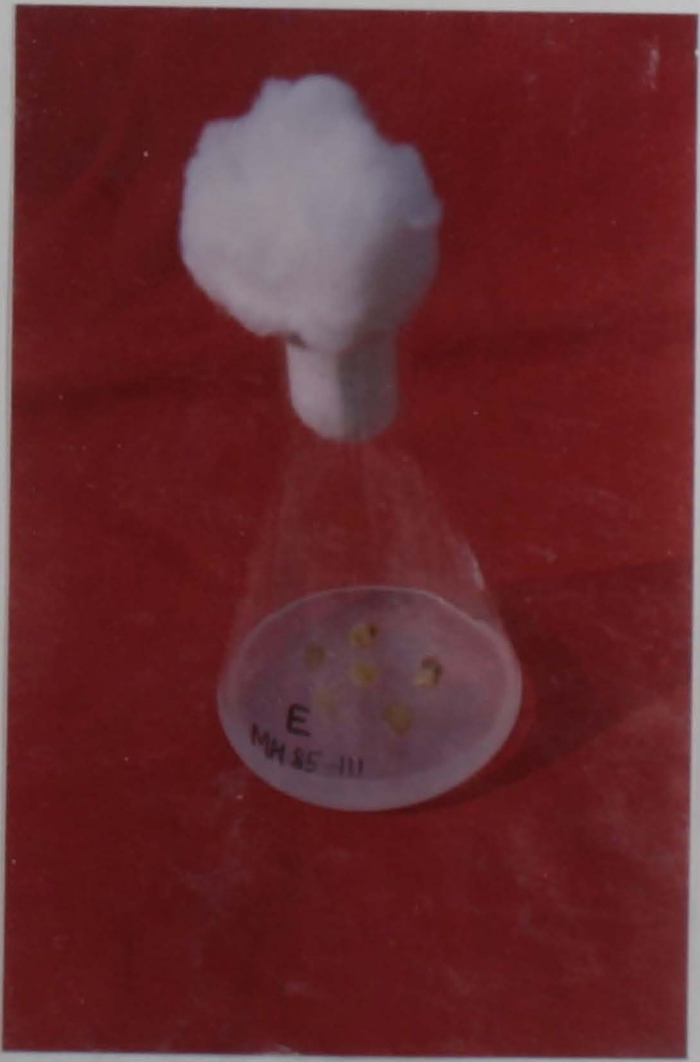
4.1 PER CENT CALLUS INDUCTION

The visible callus induction started within 5-7 days in salt free medium or low salt (up to 100 mM NaCl) medium while it was delayed by another 5-7 days at NaCl concentrations above 150 mM. In leaf explant, callus induction was from cut ends which were in direct contact with medium whereas in basal internode and hypocotyl segments, callus induction was by generalized swelling (Plate 1). Mean

PLATE 1 Callus induction from cut ends of the leaf segments of mungbean

PLATE 2 Callus induction from generalised surface of basal internodes of mungbean


L
MH 85-111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MH 85-111

callus induction was hundred per cent in hypocotyl, leaf and internode segments on salt free medium. Leaf and basal internode segments also showed hundred per cent callusing at NaCl concentration up to 150 mM. But at 200 mM NaCl, the response was nearly 90 per cent (Table 1). On the other hand, supplementation of NaCl up to a concentration of 200 mM did not change per cent callus induction from hypocotyl segments. Higher concentrations of NaCl (>200 mM) inhibiting callus induction completely from all the tested explants.

Callus raised on salt free medium was compact and green in colour irrespective of its explant origin, but with the increasing salt concentration, it turned light brown and necrosis was evident in calli raised on NaCl above 100 mM concentration. The callus obtained form basal internode and hypocotyl was more sensitive to salt as compared to leaf derived callus, because necrosis was more prevalent in calli of former explants. Leaf derived callus was more compact than calli derived from hypocotyl and basal internode segments.

4.2 CALLUS GROWTH

Perusal of data on fresh and dry weights of callus cultures established on PCDNB$_1$ medium with and without NaCl revealed that NaCl caused a general decrease in both fresh and dry weights of calli and reduction increased with increasing levels of NaCl (Table 2). Callus growth from leaf and basal internode explant was not affected appreciably up to 100 mM NaCl while higher concentration decreased it significantly. Low level of NaCl (25 mM) stimulated dry matter accumulation in callus of basal internode origin. On the other hand,

Table 1 **Effect of NaCl on per cent callus induction in different explants of 8 days old seedlings in mungbean (NaCl was supplemented to PC-L_2 medium containing 0.5 mg l^{-1} 2,4-D, 0.5 mg l^{-1} NAA and 1 mg l^{-1} BAP)**

Explant	Control	Concentration of NaCl (mM)				
		25	50	100	150	200
Leaf	100	100	100	100	100	87.5±3.1
Hypocotyl	100	100	100	100	100	100
Basal internode	100	100	100	100	100	93.75±1.5

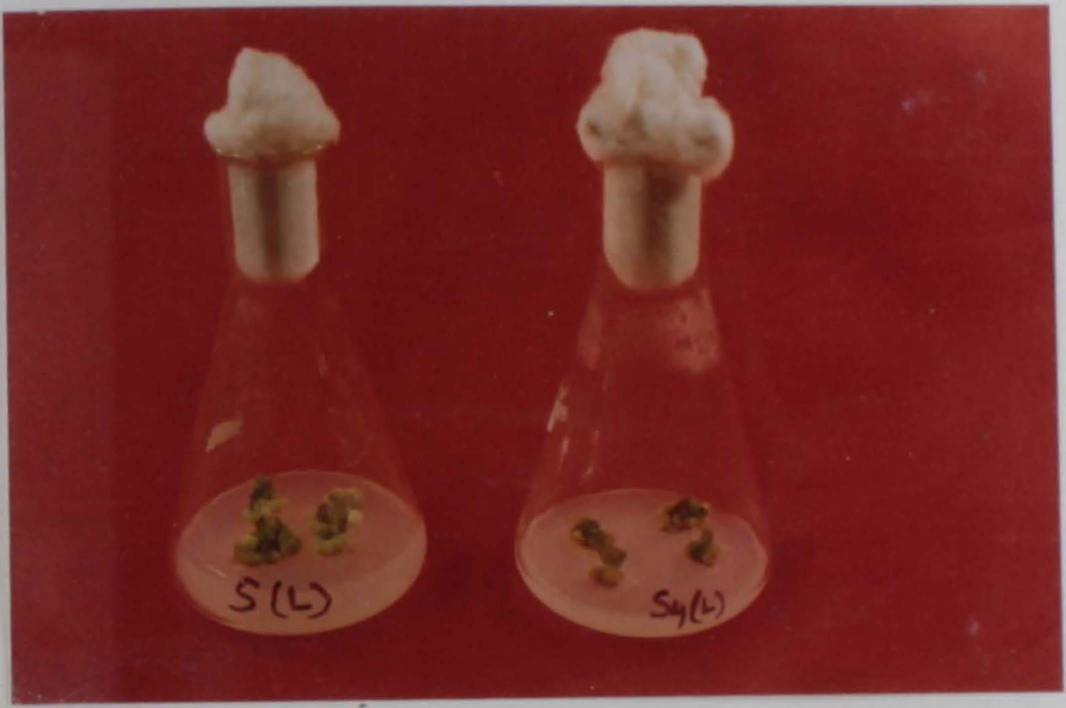
Each value represents mean of 20 replicates±S.E.

Table 2 **Effect of NaCl on fresh and dry weights (mg) of calli derived from different explants of 8 days old seedlings in mungbean (NaCl was supplemented to PC-L_2 medium containing 0.5 mg l^{-1} 2,4-D, 0.5 mgl^{-1} NAA and 1 mg l^{-1} BAP)**

Explant	Control	Concentration of NaCl (mM)				
		25	50	100	150	200
Leaf	676.6±16.3	679.0±7.4	627.6±19.7	732.0±17.4	373.0±9.8	105.4±14.0
	(78.2±7.7)	(76.8±1.9)	(71.9±5.2)	(76.8±2.4)	(48.9±1.5)	(16.82±2.5)
Hypocotyl	254.3±30.0	280.0±29.0	277.0±9.1	146.3±16.6	117.5±6.4	93.6±7.5
	(19.8±1.0)	(32.6±2.7)	(27.1±0.7)	(15.6±1.6)	(13.6±0.4)	(11.19±1.7)
Basal internode	261.6±8.1	272.0±45.0	276.0±13.3	237.9±22.6	111.2±3.4	47.46±4.0
	(24.9±0.8)	(25.1±2.2)	(25.2±2.3)	(28.7±1.5)	(15.2±2.0)	(8.20±0.7)

Each value represents mean of 20 calli ±S.E.
Figures in parentheses indicate respective values for dry weight.

PLATE 3 Callus growth from leaf explant of [*Vigna radiata* (L.) Wilczek] genotype MH85-111 grown on salt free and NaCl (200 mM) supplemented medium


S(L)
S4(L)

NaCl upto 50 mM concentration did not change fresh weights of calli of hypocotyl origin while higher concentrations were inhibitory. Lower levels of NaCl (25 mM) also increased dry weights of calli over control. Perusal of data on water content of calli (Table 3) vividly shows the highest value for hypocotyl callus and least for leaf callus. It remained nearly unchanged for calli of leaf and basal internode origin on medium supplemented with 50 mM NaCl. The water content of calli increased on a medium containing 100 mM NaCl and decreased thereafter. On the other hand, it decreased gradually with the increasing level of NaCl in calli of hypocotyl origin.

4.3 SELECTION OF NaCl TOLERANT CALLUS CULTURES

Three week old calli of leaf explant raised on $PCDNB_1$ medium without NaCl were cut in to small pieces weighing nearly 150 mg and subcultured on $PCDNB_1$ medium alone and in the presence of NaCl for four passages on respective salt supplemented medium to get salt adapted calli. Data on fresh and dry weights recorded after every subculture clearly evinces that fresh and dry weights of calli decreased with every subculture up to 4th passage on both salt free and salt supplemented medium (Table 4). With the increasing concentration of NaCl up to 200 mM, a gradual decrease in fresh and dry weights of calli was observed as the subculturing advanced. Higher NaCl level (250 and 300 mM) did not support callus growth. Lower level (50 mM) of NaCl stimulated growth both in terms of fresh and dry weights of callis in the first subculture.

PLATE 4 Browning of the leaf callus of *mungbean* on *NaCl* (150 mM) supplemented medium

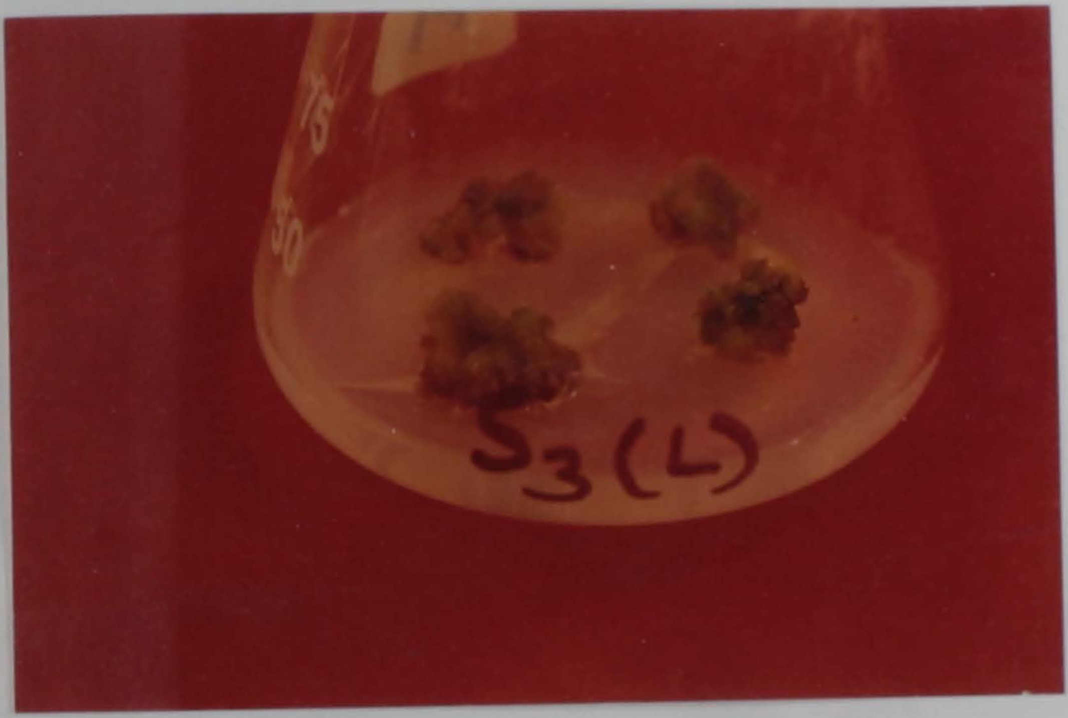

75
50
S_3(L)

Table 3 **Water content (g g^{-1}DW) of calli derived from different explants of 8 days old seedlings in mungbean cultured on PCDNB$_1$ medium supplemented with different levels of NaCl**

Explants	Control	Concentrations of NaCl (mM)				
		25	50	100	150	200
Leaf	7.84	7.10	7.73	8.53	6.63	5.27
Hypocotyl	11.84	7.59	9.22	8.38	7.64	7.36
Basal internode	9.47	9.83	9.95	7.24	6.32	4.79

Table 4 Effect of different concentrations of NaCl supplemented to $PCDNB_1$ medium on fresh and dry weights (mg) of calli of leaf origin in mungbean at various passages

Passages	Control	Concentration of NaCl (mM)			
		50	100	150	200
1st	2883±8.1	3450±7.0	2250±6.4	1473±15.1	762±2.7
	(183±1)	(216±4.6)	(177±3.5)	(141±4.0)	(117±5.1)
2nd	2646±5.3	1719±4.0	1914±6.2	789±7.4	594±4.6
	(210±6.5)	(129±2.4)	(99±3.4)	(72±1.5)	(54±4.0)
3rd	1983±10.0	1506±5.2	1290±6.8	1215±9.1	273±3.4
	(138±1.5)	(120±8.0)	(111±1.0)	(102±6.0)	(23±0.5)
4th	1260±4.5	1134±13.0	1047±8.1	960±12.6	234±1.9
	(92±6)	(78±4.0)	(90±1.0)	(69±1.0)	(24±0.5)
5th	2773±7.5	2385±16.7	2363±22.6	1749±3.5	1332±2.1
	(227±12)	(173±5.5)	(189±0.5)	(152±0.6)	(133±7.5)
6th	2637±6.4	2687±7.8	1555±5.2	742±3.2	294±0.7
	(158±10)	(151±9.0)	(87±1.5)	(56±0.5)	(37±4.0)

$PCDNB_1$ = $PC\text{-}L_2$ medium adjuncted with 0.5 mg l^{-1} 2,4-D. 0.5 mgl^{-1} NAA and 1 mg l^{-1} BAP)
Figures in parentheses indicate respective values for dry weight
Each value represents mean of 3 replicates ±S.E.

PLATE 5 Effect of different concentrations of NaCl (0, 50, 100, 150, 200 mM) on callus growth after four passages on respective salt medium


S
S_1
S_2
S_3
S_4
MH85-111
MH85-111
MH85-111
MH85-111
MH85-111

Shifting of the salt adapted calli after 4th subculture to a salt free medium for a single passage of three weeks revealed a significant increase in callus growth as compared to that on salt supplemented medium. During 6th subculture, when the calli were recultured on respective salt supplemented medium, growth of calli both in terms of fresh and dry weights was significantly higher over those after 4th subculture.

Relative growth rate values as presented in Table 5 and 6 revealed that there was a continuous decrease in relative growth rates of calli up to fourth subculture both in control and the salt supplemented media except at 50 mM NaCl during first subculture where an increase in RGR was evident. In 5th subculture, the RGR of calli both in terms of fresh and dry weights increased remarkably in control as well as in salt supplemented media. In next subculture, the RGR of the calli in salt free medium become almost equal to first subculture, while those of salt adapted calli was higher over those after 4th subculture when compared on fresh weight basis which was not true when compared over dry weight basis. Perusal of the data on water content as presented in Table 7 clearly shows that this ratio decreased with the increasing level of NaCl in the medium during first passage. During 2nd passage the ratio decreased in salt free medium, however, an increase in water content was evident on medium supplemented with NaCl up to 100 mM. Higher concentrations were inhibitory but still the value was appreciably more at 200 mM Nacl as compared to value at passage 1. After 4' subculture, the water content was relatively lower in salt free and salt added (up to100mM) medium as compared to first subculture. Callus lines adapted at 3rd and 4th passage

PLATE 6 S - Normal callus on salt free medium after 5th passage

$S_4 \rightarrow$ S - SAC^s line on salt free medium after subculturing medium supplemented with 200 mM NaCl

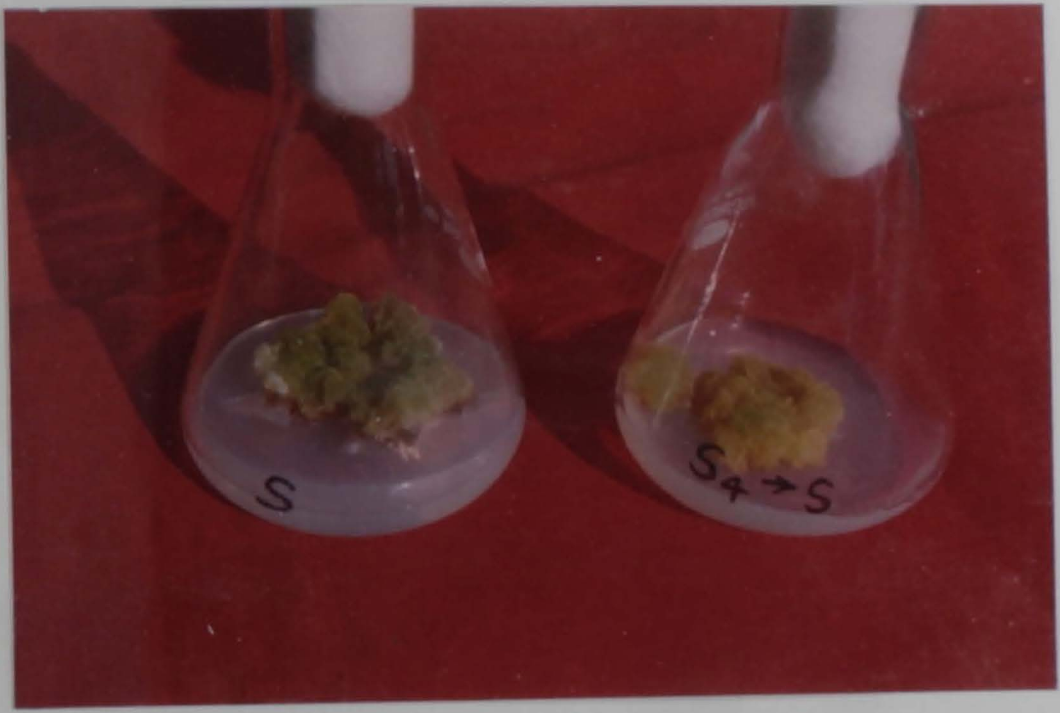

S
S4→S

Table 5 **Relative growth rates expressed on fresh weight basis of different callus lines of leaf origin in mungbean at various passages in the presence of different concentrations of NaCl**

Passages	Control	Concentration of NaCl (mM)			
		50	100	150	200
1st	0.915	1.095	0.714	0.467	0.241
2nd	0.840	0.545	0.607	0.250	0.188
3rd	0.631	0.478	0.409	0.386	0.086
4th	0.400	0.360	0.332	0.304	0.074
5th	0.867	0.757	0.750	0.555	0.422
6th	0.887	0.853	0.493	0.235	0.123

Table 6 **Relative growth rates expressed on dry weight basis of different callus lines of leaf origin in mungbean at various passages in the presence of different concentrations of NaCl**

Passages	Control	Concentration of NaCl (mM)			
		50	100	150	200
1st	0.968	1.142	0.936	0.746	0.619
2nd	1.112	0.682	0.523	0.380	0.285
3rd	0.730	0.634	0.587	0.539	0.121
4th	0.449	0.412	0.476	0.365	0.126
5th	1.201	0.945	1.000	0.804	0.703
6th	0.835	0.798	0.460	0.296	0.100

Table 7 **Water content (g g^{-1} DW) of callus lines of leaf origin in mungbean at various passages in the presence of NaCl**

Passages	Control	Concentrations of NaCl (mM)			
		50	100	150	200
1st	14.75	14.79	11.71	9.45	5.51
2nd	11.60	12.33	18.33	9.96	10.00
3rd	13.37	11.55	10.62	10.91	10.86
4th	12.70	13.54	10.63	9.78	8.75
5th	11.22	12.79	11.50	10.51	9.01
6th	15.69	16.79	16.87	12.25	6.95

Table 8 **Tolerance index on dry weight basis of callus lines of leaf origin on PCDNB$_1$ medium supplemented with different concentrations of NaCl in mungbean at different passages**

Concentration of NaCl	SS	SAC^{s}	SAC$^{n \rightarrow s}$
50 mM	117.9	118.0	63.9
100mM	96.6	136.3	36.0
150mM	77.0	104.5	35.4
200 mM	63.9	36.1	11.9

PCDNB$_1$ = PC-L$_2$ medium adjuncted with 0.5 mg l^{-1} 2,4-D, 0.5 mgl^{-1} NAA and 1 mg l^{-1} BAP)
SS = Salt sensitive lines on different levels of salt after one subculture
SAC^{s} = Salt adapted callus lines after 4 subcultures on respective salt medium
SAC$^{n \rightarrow s}$ = SAC^{s} callus lines shifted to salt free medium for 1 passage of 21 days then back to respective salt medium

for higher levels of NaCl had relatively more water content as compared to those of first passage. Shifting of these calli to salt free medium (5th passage) did not improve water content on both salt free and salt added medium as compared to 4th subculture. Reculturing of these calli on respective salt medium improved water content both on salt free and salt added medium except at 200 mM NaCl.

4.4 TOLERANCE INDEX

Tolerance index was calculated in SS, SAC^{s} and $SAC^{n \rightarrow s}$ lines. Tolerance index in SS lines decreased with increasing salt concentrations SAC^{s} lines adapted for 4 passages in 100 mM NaCl had higher tolerance index than those adapted in 50 mM NaCl. Tolerance index decreased thereafter with increasing NaCl concentration in the culture medium. Values of tolerance index in SAC^{s} lines were higher as compared to SS lines up to 150 mM, but at 200 mM NaCl, the value was lower than that of corresponding SS lines. There was a general decrease in tolerance index of calli when recultured on respective salt medium after a passage of growth on salt free medium (Table 8).

4.5 BIOCHEMICAL ESTIMATIONS

Important metabolites viz. total soluble sugars, reducing sugars, starch, proteins, free amino acids, proline and phenols were analysed in two callus lines i.e. salt adapted callus lines (SAC^{s}) after four subcultures on respective salt medium and $SAC^{s \rightarrow n}$ lines (SAC^{s} lines shifted for one passage to salt free medium).

4.5.1 Total starch content

Starch content decreased sharply in SAC^{s} lines adapted at 50 mM NaCl for four passages over the control unselected calli. Starch content was nearly identical in SAC^{s} lines irrespective of NaCl level at which these were adapted. An appreciable reduction in starch content was evident at 5th subculture in unselected calli on salt free medium over the calli of 4th passage on the same medium but starch content of salt adapted calli was nearly identical to control unadapted calli.

4.5.2 Total soluble sugars

It is evident from Fig. 1(b) that SAC^{s} lines raised on different concentration of NaCl, had a lower total soluble sugar content over salt sensitive unselected ones. The decrease was relatively more in SAC^{s} lines raised on 50 mM NaCl over those raised on higher levels of NaCl. In SAC$^{s\rightarrow n}$ lines, the levels of total soluble sugars in salt sensitive unselected lines was much lower as compared to respective callus at 4th subculture. Soluble sugar content of SAC$^{s\rightarrow n}$ calli was nearly identical to that of SAC^{s} lines.

4.5.3 Reducing sugars

Reducing sugar content decreased significantly in SAC^{s} lines adapted on 50 mM NaCl for four passages which remained nearly unchanged in SAC^{s} lines raised on 100 and 150 mM NaCl and then increased marginally in those adapted on 200 mM NaCl. A decrease in reducing sugars was evident at 5th subculture in unselected calli raised on salt free medium over the calli of 4th passage on the same medium. Shifting of SAC^{s} lines to the salt free medium increased reducing sugars

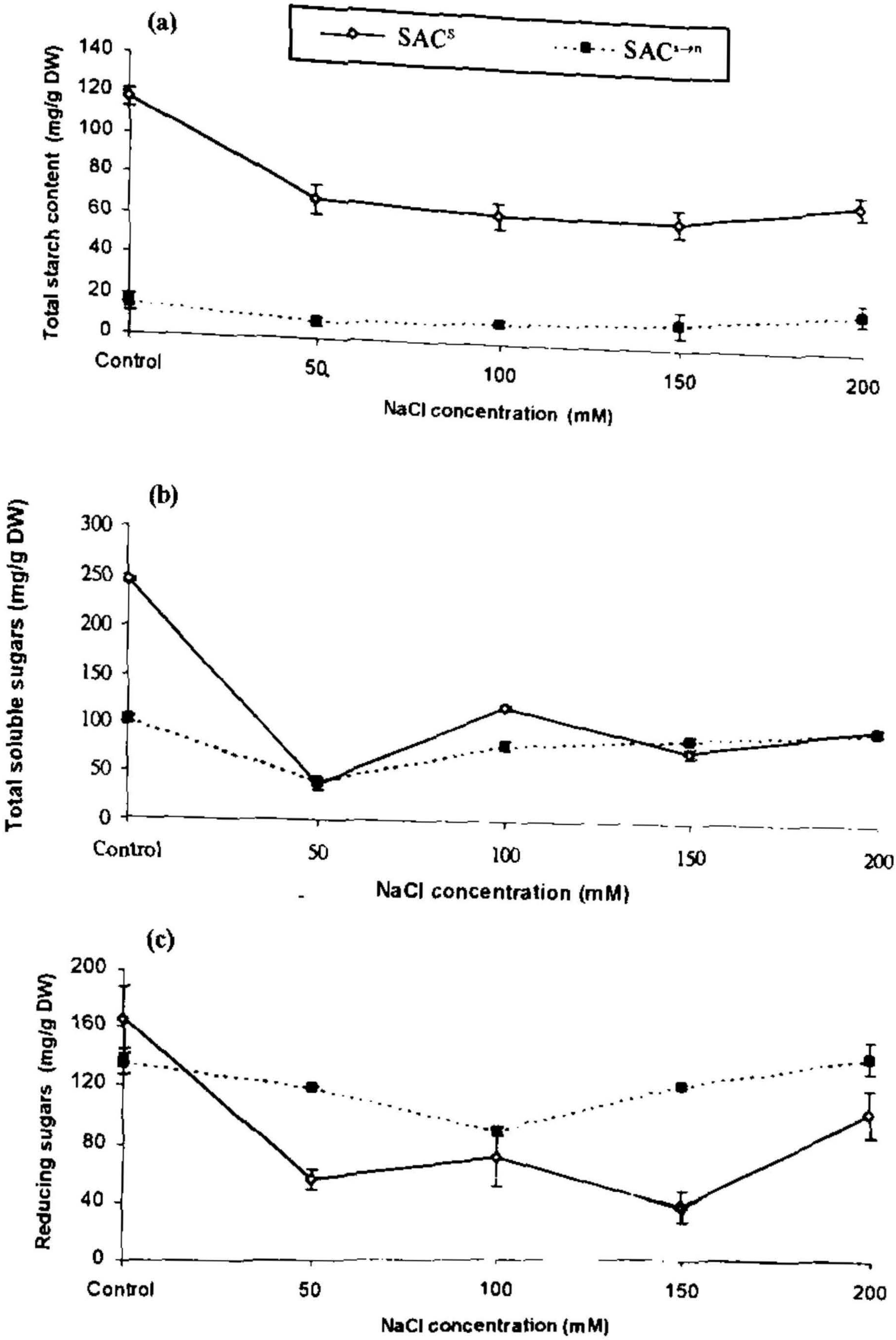


Fig. 1 : Total starch content, soluble sugars and reducing sugars in callus lines of leaf origin on PCDNB$_1$ medium supplemented with different concentrations of NaCl in mungbean at different passages

content as compared to SAC^{s} lines on the respective medium and the values were comparable to those of unselected ones of fifth passage.

4.5.4 Total proteins

Total protein content decreased significantly in salt adapted callus lines (SAC^{s}) adapted in 50 mM NaCl for four passages which steadily increased SAC^{s} adapted for higher NaCl stress [Fig. 2(a)]. At 200 mM NaCl stress, the total protein levels of SAC^{s} were comparable with unselected callus lines (control). A significant decrease in total protein content was evident at 5th subculture in unselected calli on salt free medium over the calli of 4th subculture on the same medium. Shifting of SAC-^{s} to salt free medium in general decreased total protein content over respective SAC^{s} lines. Protein content did not change appreciably upon shifting of SAC^{s} adapted for NaCl up to 100 mM but increased in those adapted for 150 and 200 mM NaCl.

4.5.5 Free amino acids

A perusal of the data presented in Fig. 2(b) clearly evinces that free amino acids content in salt adapted callus lines (SAC^{s}) gradually decreased with the increasing level of NaCl up to 150 mM in the culture medium and marginally increased in those adapted to 200 mM NaCl. Shifting of these SAC^{s} to salt free medium decreased free amino acid content significantly and the content was nearly identical, irrespective of callus source [Fig. 2(b)].

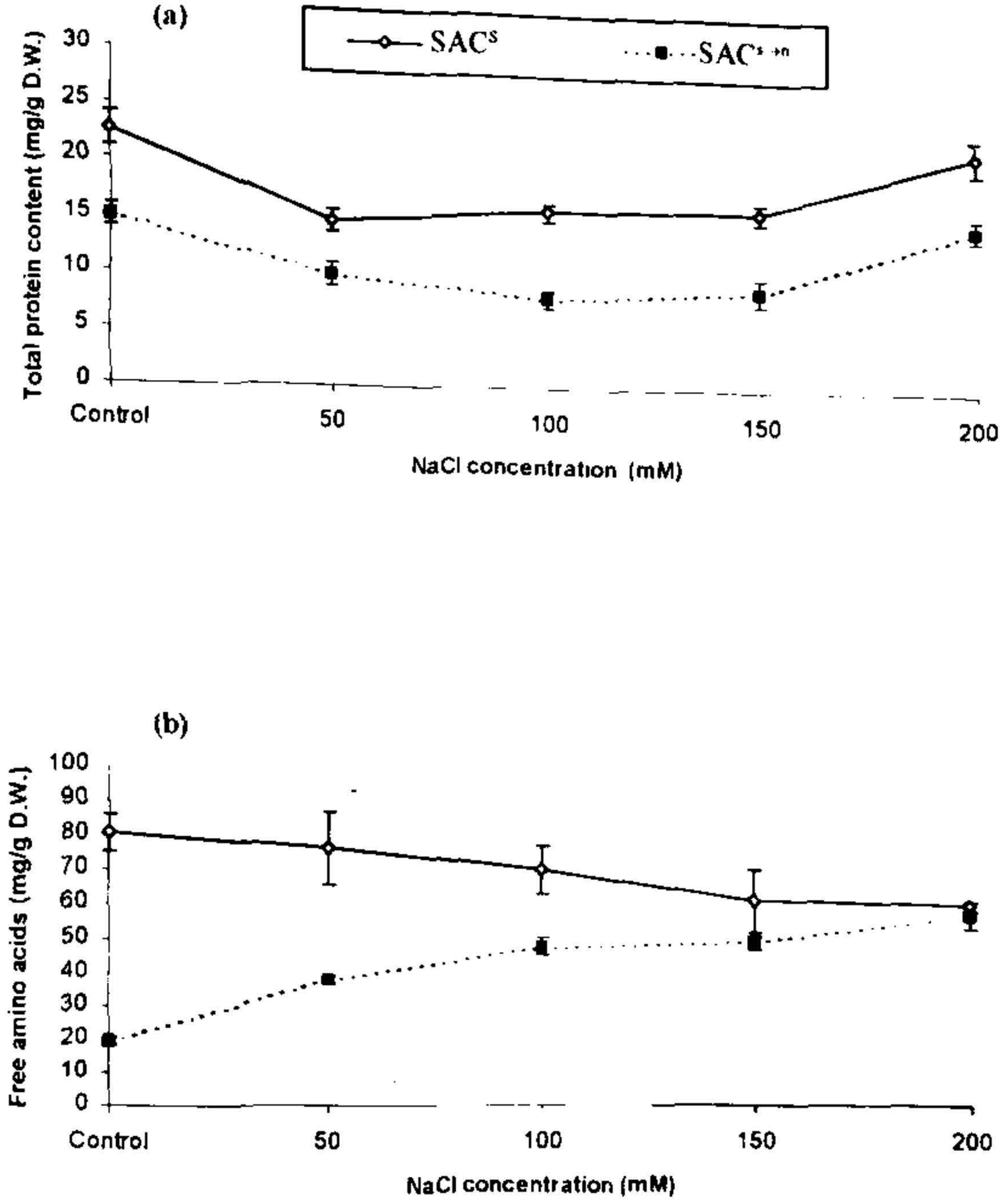


Fig. 2 : Total protein content and free amino acid content in callus lines of leaf origin on $PCDNB_1$ medium supplemented with different concentrations of NaCl in mung bean at different passages

4.5.6 Free proline

Free proline content in SAC^{s} lines raised on 150 and 200 mM was considerably higher than the unadapted calli (control). Shifting of these adapted calli to salt free medium showed sharply decrease in accumulation of proline and the values were closer to unadapted control calli. But the values at 150 and 200 mM NaCl were lower as compared to those at 4th passage. Reculturing of SAC$^{n\rightarrow s}$ lines on respective salt supplemented medium although increased proline content over unadapted callus but the values were lower than those of SAC^{s} lines.

4.5.7 Phenol content

The phenolic content showed a significant decrease in calli adapted on 50 mM NaCl for four passages which showed an increase in calli adapted in NaCl at higher concentration (200 mM) but the value was comparatively less than that of unadapted calli. Shifting of SAC^{s} calli to salt free medium led to an increase in phenolic content. Interestingly, phenolic content increased significantly at 5th subculture in unselected calli in salt free medium over the calli of 4th subculture on the same medium (Fig. 4).

4.6 MINERAL COMPOSITION

Mineral content were analysed in three callus lines i.e. salt adapted callus lines after fourth subcultures on respective salt medium (SAC^{s}), SAC$^{s\rightarrow n}$ lines (SAC^{s} lines shifted for one passage of 21 days to salt free medium) and SAC$^{n\rightarrow s}$ lines (SAC$^{s\rightarrow n}$ lines recultured back to respective salt supplemented medium).

4.5.6 Free proline

Free proline content in SAC^{s} lines raised on 150 and 200 mM was considerably higher than the unadapted calli (control). Shifting of these adapted calli to salt free medium showed sharply decrease in accumulation of proline and the values were closer to unadapted control calli. But the values at 150 and 200 mM NaCl were lower as compared to those at 4th passage. Reculturing of $SAC^{n\rightarrow s}$ lines on respective salt supplemented medium although increased proline content over unadapted callus but the values were lower than those of SAC^{s} lines.

4.5.7 Phenol content

The phenolic content showed a significant decrease in calli adapted on 50 mM NaCl for four passages which showed an increase in calli adapted in NaCl at higher concentration (200 mM) but the value was comparatively less than that of unadapted calli. Shifting of SAC^{s} calli to salt free medium led to an increase in phenolic content. Interestingly, phenolic content increased significantly at 5th subculture in unselected calli in salt free medium over the calli of 4th subculture on the same medium (Fig. 4).

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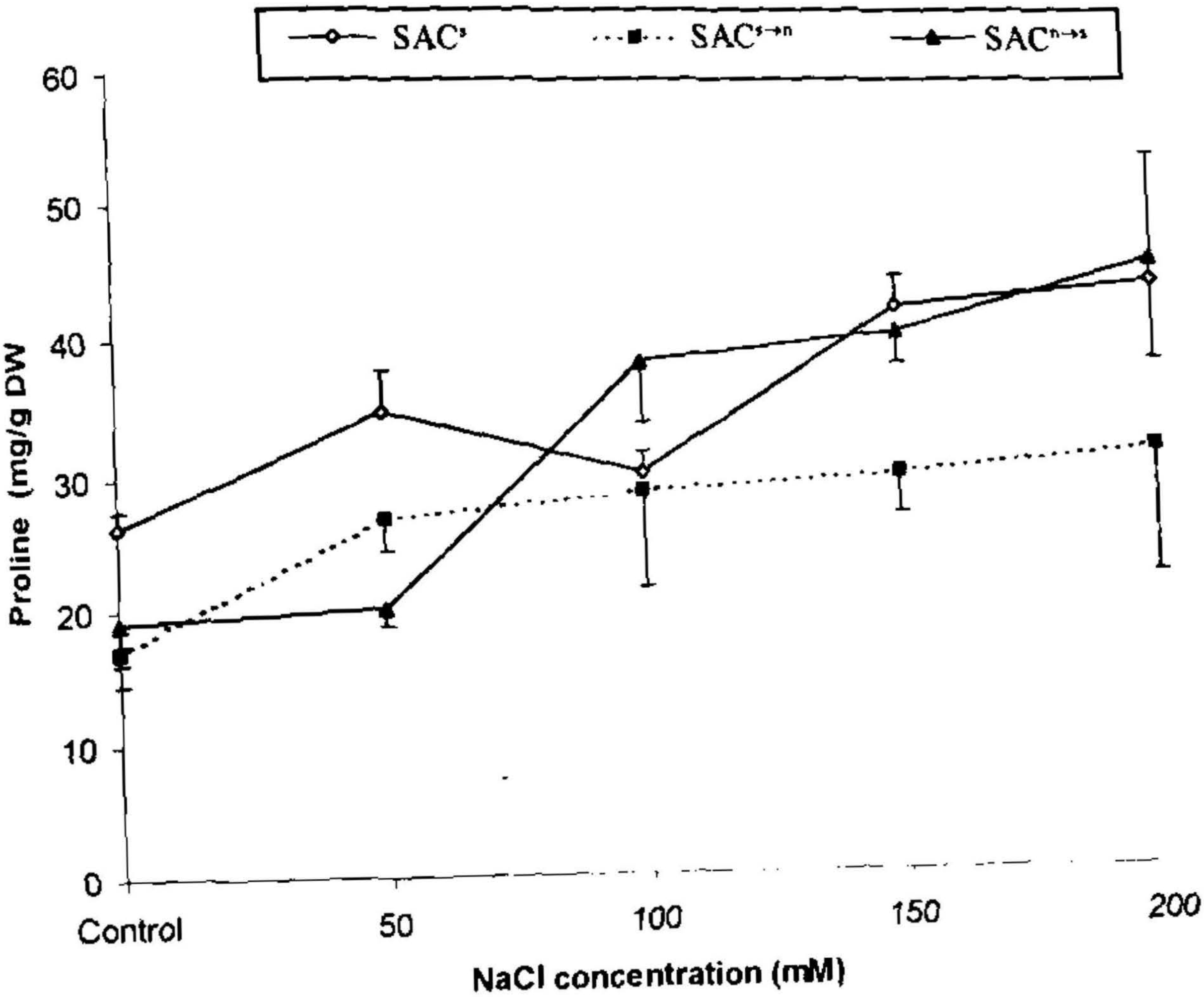

Fig. 3 : Free proline content in callus lines of leaf origin on PCDNB$_1$ medium supplemented with different concentrations of NaCl in mungbean at different passages

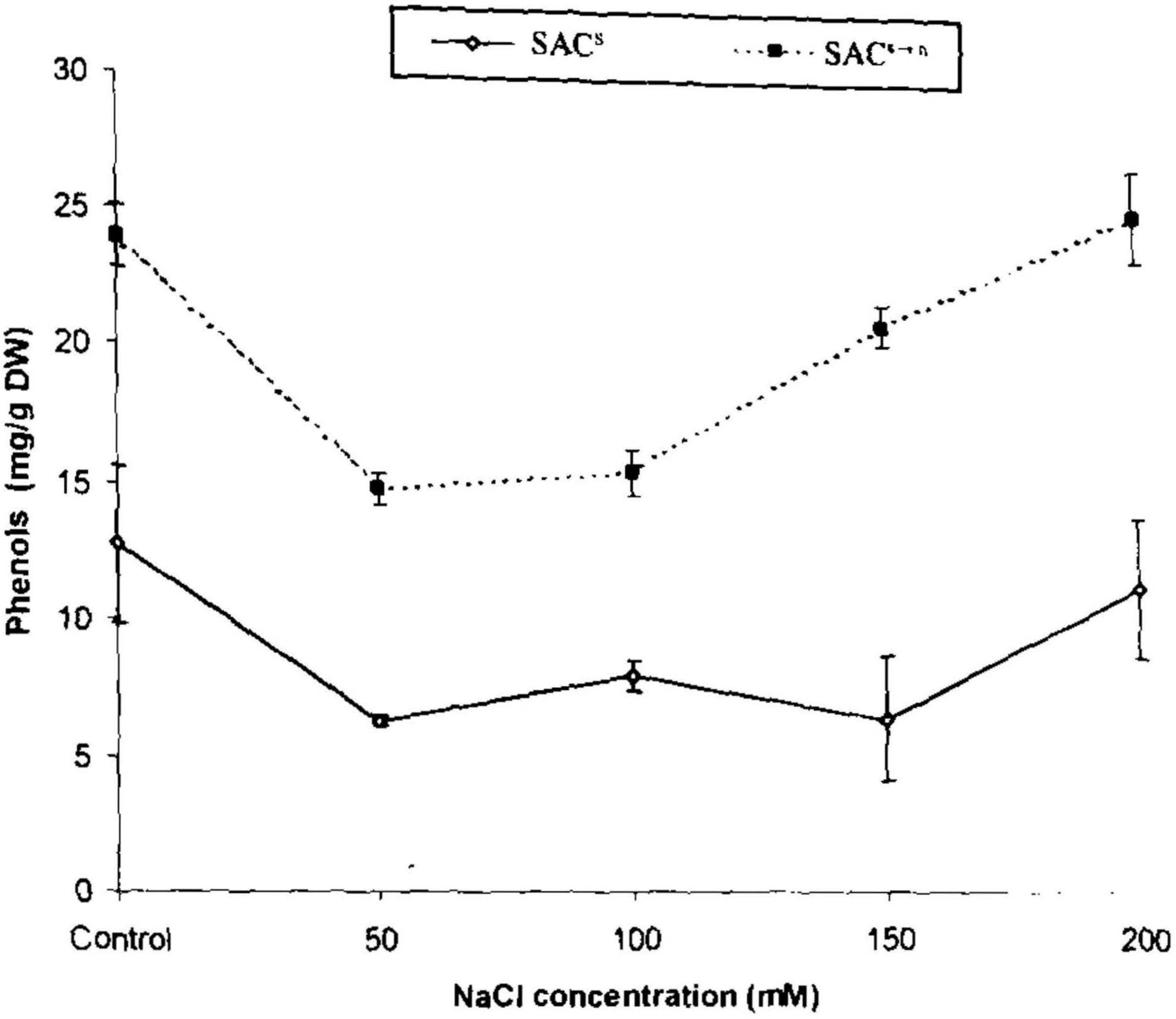

Fig. 4 : Phenol content in callus lines of leaf origin on PCDNB$_1$ medium supplemented withdifferent concentrations of NaCl in mungbean at different passages

4.6.1 Sodium content

A perusal of data presented in Table 9 clearly shows salt adapted callus lines (SAC^s showed an appreciable increase in Na$^+$ concentration. Shifting of these calli to salt free medium decreased sodium content over the SAC^s lines. Reculturing of these calli on respective salt supplemented medium again increased the sodium content and values were nearly identical to those of SAC^s lines.

4.6.2 Potassium content

Potassium content of salt adapted callus lines (SAC^s) was higher than the unadapted lines raised on salt free medium. Transfer of these calli adapted up to 100 mM NaCl to salt free medium did not change their potassium content but those adapted for higher level of NaCl showed a decrease in K$^-$ content. Reculturing of these calli in respective salt supplemented medium decreased their K$^+$ content at higher concentration (Table 10).

4.6.3 Chloride content

It is evident from Table 11 that salt adapted callus lines (SAC^s) accumulated chloride which was a 2-3 times higher than unadapted callus lines raised on salt free medium. Shifting of SAC^s lines to salt free medium lowered the chloride content close to values of unadapted callus lines grown on salt free medium. Reculturing of these calli on respective salt medium although increased Cl$^-$ content but the values were lower than the SAC^s lines.

Table 9 **Sodium content (μ moles g^{-1} dry weight) in callus lines of leaf origin on $PCDNB_1$ medium supplemented with different concentrations of NaCl in mungbean at different passages**

Concentration of NaCl	SAC^{s}	$SAC^{s\rightarrow n}$	$SAC^{n\rightarrow s}$
Control	26.0±10.0	22.0±12.1	21.0±6.4
50 mM	158.2±5.6	39.6±10.6	154.4±7.1
100mM	170.0±8.1	49.3±7.2	209.5±5.4
150mM	185.8±4.9	55.1±7.0	188.4±4.6
200 mM	242.5±9.4	69.6±5.7	228.5±10.0

SAC^{s} = Salt adapted callus lines after 4 subcultures on respective salt medium

$SAC^{s\rightarrow n}$ = SAC^{s} callus lines shifted to salt free medium for one passage of 21 days.

$SAC^{n\rightarrow s}$ = $SAC^{s\rightarrow n}$ callus lines shifted back to respective salt medium.

Each value represents mean of 3 replicates

Table 10 Potassium content (μ moles g^{-1} dry weight) in callus lines of leaf origin on $PCDNB_1$ medium supplemented with different concentrations of NaCl in mungbean at different passages

Concentration of NaCl	SAC^{s}	$SAC^{s \rightarrow n}$	$SAC^{n \rightarrow s}$
Control	273±4.0	203±2.1	264±5.6
50 mM	321±2.7	320±4.5	381±3.5
100mM	291±4.6	277±3.0	435±4.7
150mM	293±3.4	228±3.9	255±3.0
200 mM	259±7.5	264±4.7	201±6.4

SAC^{s} = Salt adapted callus lines after 4 subcultures on respective salt medium

$SAC^{s \rightarrow n}$ = SAC^{s} callus lines shifted to salt free medium for one passage of 21 days.

$SAC^{n \rightarrow s}$ = $SAC^{s \rightarrow n}$ callus lines shifted back to respective salt medium.

Each value represents mean of 3 replicates

Table 11 Chloride content (μ moles g^{-1} dry weight) in callus lines of leaf origin on PCDNB$_1$ medium supplemented with different concentrations of NaCl in *mungbean at different passages*

Concentration of NaCl	SAC^s	SAC$^{s\rightarrow n}$	SAC$^{n\rightarrow s}$
Control	65±6.3	63±7.7	60±5.7
50 mM	165±7.0	63±9.1	66±6.8
100mM	130±8.4	66±8.0	90±11.4
150mM	195±12.8	72±6.6	126±6.9
200 mM	150±7.1	57±10.4	144±7.4

SAC^s = Salt adapted callus lines after 4 subcultures on respective salt medium

SAC$^{s\rightarrow n}$ = SAC^s callus lines shifted to salt free medium for one passage of 21 days.

SAC$^{n\rightarrow s}$ = SAC$^{s\rightarrow n}$ callus lines shifted back to respective salt medium

Each value represents mean of 3 replicates

Chapter 5

Discussion

Salinity is an increasing global problem more particularly in arid and semi-arid regions. The problem is further exacerbated by use of poor quality of irrigation water. Plant tissue culture technique has been successfully exploited to develop new cell lines with valuable characters for agricultural use, particularly in relation to resistance to stress and herbicides (Chaleff, 1981). Such tolerant cell lines are of great value in elucidating the physiological and molecular mechanism of salt tolerance in angiospermous species. Furthermore, these lines have been employed for regeneration of salt tolerant plants though with limited success.

In the present study attempt was made to select salt tolerant callus lines of mungbean by culturing the sensitive callus lines for four passages on different concentrations of NaCl. Stability of these salt tolerant callus lines was evaluated by transferring these to salt free medium for one passage and reculturing them on respective salt supplemented medium. Salt tolerance was assessed in terms of growth, biochemical profile and ion content.

Callus induction was evident in 5-7 days of inoculation from leaf, basal internode and hypocotyl segments. Addition of NaCl above 100 mM level delayed callusing by another 5-7 days. NaCl concentration above 200 mM inhibited callusing completely in all tested explants. Callusing was 100 per cent in all the three explants on salt free medium. NaCl did not change per cent callus induction in hypocotyl but 200 mM NaCl reduced callusing to nearly 90 per cent in leaf segments and basal internodes. It evinces that NaCl above 200 mM is lethal to callus induction.

NaCl caused a general decrease in fresh as well as dry weights of calli and the effect was pronounced at highest level of NaCl. Similar NaCl mediated reduction in growth of calli has been reported for calli in chickpea (Pandey and Ganapathy, 1984) and other plants (Tal *et al.*, 1978; Chen *et al.*, 1980; Chaudhary *et al.*, 1997 and Rus *et al.*, 2000). Low level of NaCl (25 mM) stimulated dry matter accumulation in calli of basal internode and hypocotyl origin. Similar stimulation of growth in bean suspension culture (Gale and Boll, 1979), chickpea (Pandey and Ganapathy, 1984) and pea (Gosal and Bajaj, 1984) has been reported. Such a stimulation may be due to osmoregulatory role of Na^+, thereby suggesting that osmotic strength of the medium was suboptimum (Kumar and Sharma, 1989). Water content was highest in calli of hypocotyl and least in those of leaf origin. NaCl, in general, decreased water content. Lower levels of NaCl (up to 50 mM) however, did not affect water content in leaf and basal internode calli. Reduction in water content of calli raised on salt supplemented medium is obviously due to less absorption of water by tissue. Possibly these calli are not fully adjusted osmotically with the culture medium. Alternatively, this could be due to the inability of some of the cells to retain water in the presence of increasing level of salt stress and these cells, tended to accumulate high levels of NaCl and eventually died. The accumulation of high levels of NaCl possibility contributed towards dry matter increment (Kumar, 1987).

Three week old calli of leaf origin raised on salt free $PCDNB_1$ medium were subcultured for four passages on above medium supplemented with a range of NaCl concentrations. Fresh and dry weights of these calli decreased with every subculture on both salt free and salt supplemented medium. A gradual decrease in fresh and dry weights of calli with the increasing concentration of NaCl upto 200 mM was also evident. Higher levels of NaCl did not support callus growth. Similar reduction in callus growth has been reported in *Lycopersicon pennellii* and *L. esculentum* which recovered after 3rd and 8th passage, respectively (Rus *et al.*, 2000). Decrease in callus growth up to 4th subculture can be assigned to high osmotic pressure of the basal medium which may be inducing an osmotic stress (Dracup, 1991) which recuperated upon fifth subculture resulting the same level of growth as that obtained in the first subculture on control medium. The number of passages required for recuperation seem to depend upon inherent salt tolerance status of plant species in question. Subculturing of these calli on salt free $PCDNB_1$

medium for a single passage of 21 days revealed a significant increase in callus growth, irrespective of their source. Reculturing of salt adapted callus lines from salt free medium to respective salt supplemented medium, resulted in significantly higher growth both in terms of fresh and dry weights over those after fourth subculture. This evidently suggests that the calli have got osmotically adjusted to the respective culture medium.

Tolerance index of calli cultured on salt supplemented medium (first subculture) decreased with the increasing NaCl concentration in salt medium. Culturing of these calli on respective salt medium for four passages improved tolerance index in those calli adapted up to 150 mM NaCl. Such an improved salt tolerance was further confirmed by the fact that these salt adapted calli particularly those adapted on 50 and 100 mM NaCl could grow on media supplemented with 250 and 300 mM NaCl which were otherwise lethal to unadapted callus lines. Santos *et al.* (2000) also succeeded in raising calli of *Helianthus annuus* which acquired capacity to grow at extreme concentrations of NaCl that were lethal to calli. Shifting of SAC^s calli to salt free medium for just one passage and reculturing on the respective salt medium led to a sharp decline in tolerance index. This vividly shows the salt tolerance acquired during four passages on salt medium is not a stable trait and is rather indicative of physiological adaptation. A further increase in the number of passages (longer than critical time period) on salt supplemented medium may help in stabilizing trait of salt tolerance. Watad *et al.*, (1983) succeeded in obtaining NaCl resistant line in tobacco by stepwise increase in NaCl concentration in the medium and resistance showed stability through at least 24 generations in the absence of added NaCl. Osmotic adjustment is a fundamental adaptive response of plant cell exposed to salt stress and is necessary for survival and growth. Osmotic adjustment in response to salt stress is achieved through accumulation of organic solutes and inorganic ions.

Analysis of metabolite profile of SAC^s and $SAC^{s\rightarrow n}$ lines revealed a lower starch content in SAC^s over the unadapted control calli. Such a reduction in starch content was not accompanied by an accumulation of soluble sugars including reducing sugars. It possibly suggests that activity of starch synthesizing enzyme (ADP-glucose pyrophosphorylase) is adversely affected by NaCl and soluble sugars are being used up as respiratory substrate for maintenance in a stressful environment. Unlike the reports of Goyal *et al.* (1987). Soluble sugars are not serving as osmolyte in mungbean calli raised on salt supplemented medium. Kumar (1987) also reported that NaCl resistant callus lines accumulated low levels of soluble carbohydrate when grown under NaCl stress conditions.

Shifting of SAC^s callus lines to salt free medium decreased starch content appreciably and values were nearly identical to unadapted control calli. This decrease in starch content was however, unaccompanied by accumulation of soluble sugars. It evidently suggests that starch hydrolysing enzymes are either synthesized or activated upon shifting to salt free medium and the resulting sugars are used up in growth as evident from a substantial improvement in growth of calli. Strogonov *et al.* (1970) also reported a decrease in soluble sugars in horsebean upon transfer to non-saline conditions.

Protein and free amino acid contents of SAC^s lines raised on 150 and 200 mM NaCl were considerably higher than the unadapted control calli. A large number of reports are available which show many fold increase in proline content in calli upon exposure to salt stress (Watad *et al.*, 1983; Jain *et al.*, 1987; Binzel *et al.*, 1987; Kumar and Sharma, 1989; Gulati and Jaiwal, 1993). High proline content in these calli may either be due to an increased rate of synthesis or decreased rate of oxidation of this compound (Wyn Jones and Gorham, 1984). Rus-Alvarez and Guerrier (1994) observed accumulation of proline only in salt sensitive tomato thus suggesting that its accumulation is a symptom of metabolic dysfunction and resulted from inactivation by water stress of normal control mechanism. Perusal of tolerance index coupled with observations on the ability of SAC^s line adapted on 50 and 100 mM NaCl to grow at lethal concentrations of NaCl (>200 mM) supports the idea of proline accumulation as a symptom of metabolic dysfunction and thus injury. Aggarwal (1986) failed to establish any correlation between salt tolerance and proline accumulation. Ashraf (1989) also observed a negative correlation between proline level and salt tolerance. Chandler and Thorpe (1987) suggested that proline accumulation may be correlated to growth inhibition rather than salt tolerance. Contrarily, steep rise in proline content in salt adapted callus which serve as an osmoprotectant has been reported by Watad *et al.* (1983), Jain *et al.* (1987), Binzel *et al.* (1987). Lerner (1985) opined that it is not necessary that proline was directly involved in osmotic adjustment when accumulated. All these contradictory findings suggest that additional studies are required to elucidate conclusively the role of proline in plant adaptation to stress. Gzik (1996) opined that some other functions besides osmoticum can be attributed to this amino acid.

Shifting of SAC^s lines to salt free medium sharply decreased accumulation of proline. Reculturing of these calli on the respective salt supplemented medium resulted in elevated level of proline in the calli. It vividly suggests that proline accumulation is a fully reversible process and accumulation of proline is a stable character. These results adduce support to the observations of Watad *et al.* (1983).

Mineral analysis of SAC^s line revealed an appreciable accumulation of Na^+ and Cl^- over the unadapted control ones. K^+ content of these calli was also higher than the unadapted lines raised on salt free medium. Kumar (1987) also reported that salt resistant callus lines in mungbean maintained higher levels of K^+ than sensitive ones when grown under increasing levels of salt stress. Shifting of these calli to salt free medium lowered both Na^+ and Cl^- content. On the other hand, K^+ content did not change in SAC^s lines adapted up to 100 mM NaCl and decreased in those subcultured on medium with higher level of NaCl. Tal *et al.* (1979) and Siddique (1980) also reported dramatic reduction in Na^+ and Cl^- content within a day upon desalinization of pea plants. Reculturing of above calli ($SAC^{s \to n}$) on the respective salt medium again increased Na^+, K^+ and Cl^-. Accumulation of Na^+ and Cl^- in calli of different plant species with an increase in external NaCl has been observed by Watad *et al.* (1983), Pandey and Ganapathy (1984), Binzel *et al.* (1987), Olmos *et al.* (1994), Chen *et al.* (1998). Kumar and Sharma (1989) reported that K^+ content in root segment callus of mungbean adapted over NaCl adjuncted medium was lower on salt medium over normal medium. Accumulation of Na^+ and Cl^- has been suggested to be associated with salt tolerance trait since it helps in osmotic adjustment. Ashraff (1997) opined that salt tolerance in mungbean, lentil and chickpea is closely related to accumulation of high concentration of Na^+ and/or Cl^-. However, He and Cramer (1993) failed to observe any correlation between salinity tolerance and $Na^+ : K^+$ ratio in Brassica (Chen *et al.*, 1980; Piqueras, 1992; Chaudhury *et al.*, 1997). Gulati and Jaiwal (1992) observed a negative correlation between growth inhibition and concentration of inorganic solutes of leaf callus of mungbean. They reported that accumulation of Na^+ was accompanied by loss of K^+. It may be concluded that elevated levels of Na^+ and Cl^- are helping for osmotic adjustment but both of these are toxic as evident from decreased callus growth. This toxicity is evident from the observed decline in K^+/Na^+ and K^+/Cl^- ratio as also suggested by Chen *et al.* (1998).

An overview of the present study vividly evinces that repeated subculturing of calli upto four cycles on salt supplemented medium induced some degree of salt tolerance in SAC^s lines of mungbean. Acquisition of salt tolerance is possibly an outcome of osmotic adjustment with the media by accumulating proline and inorganic ions like Na^+ and Cl^-. Although K^+ content also increased with increasing level of NaCl but per cent increase was much lower than Na^+. Soluble sugars including reducing sugars and free amino acids do not seem to contribute towards osmotic adjustment. Observed reduction in the growth of the SAC^s lines further indicates Na^+ and Cl^- are not sequestered into the vacuole from the cytoplasm and hence affect the metabolic machinery adversely. Observed decrease in K^+/Na^+ and K^+/Cl^- ratio is also indicative of disturbed metabolism. Reculturing of SAC^s lines on respective salt supplemented medium (6th passage) after a passage of 21 days on salt free medium resulted in decrease of tolerance index indicating that trait of salt tolerance is not stably acquired in four passages on salt supplemented medium. An increase in the number of passages on salt supplemented media is anticipated to yield positive results. It is worthwhile to mention that salt tolerance attained during four passages enabled the calli to grow on media supplemented with lethal doses of NaCl on which unadapted calli fail to grow.

Amzallag (1997) has opined that the primary cause of growth reduction under sublethal level of salt stress is not the direct inhibitory effect of Na^+ or Cl^- on metabolic process; rather it is due to changes in hormonal balance. It suggests that metabolic perturbations are consequent to hormonal changes induced by NaCl. It seems better growth of calli can be achieved on salt supplemented medium by changing ratio of plant growth regulators.

Chapter 6

Summary and Conclusion

Present study carried out on mungbean [*Vigna radiata* (L.) Wilczek] genotype MH85-111 was aimed at selecting salt adapted callus lines and to work out biochemical basis of salt tolerance. Hypocotyl, leaf and basal internode segments of eight days old seedlings showed 100 per cent callus induction on both salt free and salt supplemented PC-L_2 medium containing 0.5 mg l^{-1} 2,4-D, 0.5 mg l^{-1} NAA and 1.0 mg l^{-1} BAP but leaf and basal internode explants showed nearly 90 per cent callus induction on medium containing 200 mM NaCl. Growth of calli in terms of both fresh and dry weights was maximum in the leaf. Low concentration stimulated growth of calli while higher concentrations of NaCl inhibited growth. Water content was highest in hypocotyl callus and least in leaf callus. Culturing of calli raised on NaCl free PCDNB$_1$ medium supplemented with NaCl for salt adaptation resulted in a decrease in both fresh and dry weights of calli with every subculture up to 4th passage in control as well as NaCl supplemented medium. Transferring of these salt adapted calli on salt free medium for one passage relieved calli from inhibitory effect of salt and growth was improved. Reculturing, these calli on respective salt medium showed higher callus growth over those after 4th subculture on salt. During the 5th subculture, the RGR of calli in salt free medium becomes almost equal to first subculture while those of salt adapted calli was higher over those after 4th subculture when compared on fresh weight basis. Tolerance index in salt adapted callus lines was higher as compared to sensitive lines up to 150 mM but after single passage on salt free medium, tolerance index was decreased indicating salt tolerance trait was not a stable character and number of passages needed to be increased. Calli adapted over NaCl for passages were able to grow on media adjuncted with 250 and 300 mM NaCl which were otherwise lethal to runadapted control calli.

Biochemical estimations revealed that there was a decrease in starch, total soluble sugars and reducing sugars . Protein content decreased in both SAC^s and $SAC^{s\rightarrow n}$ lines, free amino acids also decreased but at higher concentration, value was near to unselected callus lines of 5th passage. Salinity resulted in accumulation of free proline in SAC^s lines but decreased to control value upon NaCl free medium, It was again accumulated when salt adapted callus lines were recultured on the respective NaCl supplemented medium. Mineral analysis indicated an accumulation of sodium, potassium and chloride in salt adapted calli but the per cent increase in Na^+ content was much higher over K^+ content.

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