CHAPTER IV

Selection of NaCl tolerant cell lines
5.1. Introduction

Each year more and more land becomes non-productive owing to salt accumulation. At least 25% of currently cultivated land throughout the world suffers from excess salinity, principally from NaCl (Carter, 1975; Nabors, 1990). Excess of salt in the soil, especially in the coastal regions of South and Southeast Asia (Akbar and Ponnampuruma, 1982) limits the yield potential of many cultivars (Binh et al., 1992). In recent years, it is possible to expose plant cells to a salt stress, select tolerant cell lines and regenerate salt-tolerant plants through tissue culture technique (Croughan et al., 1978; Nabors et al., 1980; Rains et al., 1980; Heszky et al., 1991; Winicov, 1996; Patnaik and Debata, 1997a; Basu et al., 1997; Ochatt et al., 1999).

The induction of salt-tolerant crops in hitherto sensitive plant genotypes has been studied in many species (Maas, 1985; Epstein and Rains, 1987; Tal, 1996). The mechanisms responsible for in vitro adaptation and tolerance and/or resistance of plant cells to salt stress have also been examined (Hasegawa et al., 1980; Hurkmans, 1992; Bray, 1993). Salinity is regarded as one of the most important stress factors in agriculture (Caldiz, 1994), but the number of in vitro studies that have actually resulted in the production of stable salt-tolerant plants is scanty (Tal, 1996). An alternate strategy for salt tolerance improvement has included the regeneration of plants with improved salt tolerance after selection of salt tolerant cells in culture (Winicov, 1991, 1996). Previous attempts at the direct and successful isolation of vigorous salt tolerant plants via cell culture have been
inconclusive. In several cases, cellular salt tolerance has not always correlated with whole
plant salt tolerance (Bingham and McCoy, 1986). A major roadblock in using this
technique has been that, although salt tolerant cell lines have been selected in a variety of
plant species (Stavarek and Rains, 1984), only limited number of plants could be
regenerated from these lines (Winicov, 1991).

One step selection strategy is an more effective method for the selection of salt tolerant
mutants (McHughen and Swartz, 1984). The gradual imposition of stress is inefficient,
as it more readily induces physiological adaptations. Chandler and Vasil, (1984) and
Bowman (1987) had pointed out that genetic differentiation with respect to growth and
survival is expressed better under a rapidly induced short-term salinity treatment rather
than gradually imposed salinity. Another possibility is that adaptation is an epigenetic
phenomenon, depends on changes in the pattern of DNA methylation which affects gene
expression (Sabbah et al., 1995). The adaptation can be prevented by exposing the cells
directly to sublethal salt concentration to select salt tolerant mutant in one step
(McHughen and Swartz, 1984; Blum, 1988; Sumaryati et al., 1992). An additional
problem with long-term selection is that more and more genetic abnormalities seem to
occur and be retained in cell populations. Increase in unsuitable chromosome changes
result in the decline of regenerability (Gulati and Jaiwal, 1997).

*In vitro* selection for cells exhibiting increased tolerance to salt stress is considered a
suitable system to investigate plant metabolism and the selective accumulation of
inorganic and organic compounds that contribute to turgor maintenance (Heyser and
Nabors, 1981; Piqueras et al., 1996).

Under field cultivation, mungbean is known to be sensitive to salinity. It is widely grown
in the subcontinent, because of its importance as pulse crop (Ashraf, 1997). So this crop
is suitable for cultivation in non-saline soils (Ashraf, 1997). If grown on saline soils the
yield losses are considerable. Therefore, development of salt tolerance in this crop would
be of great economic value, because yield increase of only 5 percent in this crop on these
saline areas would be worth of million rupees (Ashraf, 1997).
Although salt tolerant callus lines of mungbean were selected under a high saline conditions, on few occasions, in absence of mutagen (Kumar and Sharma, 1989; Gulati and Jaiwal, 1994b), in presence of EMS (Gosal and Bajaj, 1984), the attempts to regenerate plants from salt selected line were unsuccessful (Jaiwal and Gulati, 1995). The salt tolerant variants/ mutants have been isolated by exposing mainly mutagen untreated cells/ tissues such as various explants, callus cultures, cell suspensions, protoplasts and even microspores to a sublethal concentration of salts, one or few times. (in one-step or short term/direct selection) or by gradual stepwise increase in salt(s) at each subcultures (stepwise selection). The tolerant cells are identified on otherwise inhibitory levels of salt(s) (Gulati and Jaiwal, 1997).

In the light of these consideration, in the present study, an attempt has been made to standardize a suitable protocol for in vitro recurrent selection of NaCl tolerant cell line and subsequent plant regeneration from the selected callus. The ions (Na⁺, K⁺, Ca²⁺, Cl⁻) and proline contents were analysed among the selected and non-selected callus.
5.2. Materials and Methods

5.2.1. Initiation of callus cultures

The callus cultures of *Vigna radiata* (L.) Wilczek) (cv. V1) were initiated from distal end of the cotyledons from 12 hours imbibed seeds on MS medium containing 3% sucrose, 0.7% agar (Hi-Media, Mumbai), NAA (2.0mg/l) and BAP (2.0mg/l). The pH of the medium was adjusted to 5.8 before autoclaving at 1.06kg cm\(^{-2}\) (121\(^\circ\)C) for 15 minutes. The callus cultures were grown under a 16 hr photoperiod 40\(\mu\)mol m\(^{-2}\) s\(^{-1}\), provided by cool-white fluorescent lamps at 25±2\(^\circ\)C.

5.2.2. Screening of NaCl – tolerance in callus culture

After one subculture (30 days) on the same medium, 200 mg/l of actively-growing fresh calli were transferred to MS medium containing NAA (2.0mg/l) + BAP (2.0mg/l) along with increasing concentrations of NaCl (25, 50, 150, 200, 250 and 300mM) in order to determine the concentration at which the growth of normal callus was completely inhibited. The pH of the medium was adjusted to 5.8 before autoclaving at 1.06kg cm\(^{-2}\) (121\(^\circ\)C) for 15 minutes. The cultures were incubated in the above mentioned photoperiod. The inhibitory (lethal) concentration thus determined was used for the selection of cell lines resistant to NaCl. The calli (after 10, 20 and 30 days) from each test tubes was removed and its fresh and dry weights (oven dried at 80\(^\circ\)C for 48 hr) were determined for each treatment. 20 replicates were maintained for each level of NaCl treatment.

5.2.3. Selection of NaCl tolerant cell lines

For the selection of NaCl tolerant cell line, the fresh callus pieces of approximately 200 mg/l were transferred to MS medium containing NAA (2.0mg/l) + BAP (2.0mg/l) along with 250 mM of NaCl. After one month, the selected clone which remained as green was selected and subcultured for four more additional passages (30 days each) on
fresh medium containing the same concentration of NaCl in order to remove the escapes. The cell line (callus) which showed tolerance to NaCl (250mM) was named as selected calli. On the otherhand, the calli continuously maintained on NaCl free medium for same passages or durations were referred as non-selected calli.

5.2.4 Stability of the selected cell line

The selected clones were tested for the stability of the altered response by allowing them to grow away from NaCl for two transfer over a period of 60 days. Then the tolerant clones were again grown on the medium containing inhibitory concentration (250mM) of NaCl. Such callus line was designated as NaCl tolerant line. Then the selected and non-selected calli were subcultured in the medium containing various concentrations (0.0 – 300mM) of NaCl.

Biochemical studies

The selected and non-selected calli were chosen for biochemical studies. Sodium, potassium, calcium, chloride and proline contents were estimated.

5.2.5 Estimation of Na⁺, K⁺ and Cl⁻ ions

500 mg of calli were digested in a conical flask by triple acid method ie, nitric acid, sulphuric acid and perchloric acid (10: 4: 1 v/v). The initial digestion was done in cold state. The triple acid extract was neutralized with ammonium hydroxide and kept in a water bath, till the solution became colourless. Each digested sample was made upto 100ml with de-ionized water. Sodium, potassium and chloride contents were estimated with the help of flame photometer. The readings were converted into ppm by referring standard graph of ionic content and were expressed in percentage per gram of sample.
5.2.6. **Estimation of Calcium**

10 ml of triple acid extract was taken in a china dish and 0.1 g Murexide indicator was added. This was titrated against versanate solution of known strength. The change of colour to violet was taken as the end point. From the titrate value, the calcium content was calculated by using the factor 0.0004. The result was expressed as percentage of calcium content per gram of sample.

5.2.7. **Estimation of proline**

The proline was estimated by the method of Bates et al. (1973). 500 mg sample was homogenized with 3% sulphosalicylic acid and centrifuged at 3000 rpm for 10 minutes. The supernatant was made up to 10 ml with sulphosalicylic acid. To 2 ml of this extract, 2 ml of glacial acetic acid, 2 ml of 6 M orthophosphoric acid, 2 ml of acid ninhydrin mixture (16ml of orthophosphoric acid + 60 ml glacial acetic acid + 24 ml of distilled water + 2.5 g ninhydrin) were added. The contents were boiled in water bath for 1 hour and cooled to room temperature to stop the reaction. The contents were taken in a separating funnel along with 10 ml of toluene. After thorough shaking, the coloured upper layer of toluene was collected and the intensity was read at 520 nm in Beckman Du-26 spectrophotometer. Proline content was expressed as $\mu$g/ 100 mg dry weight of tissue.

All experiments were repeated at least six times

5.2.8. **Regeneration of NaCl tolerant cell lines**

For regeneration, the calli from selected, non-selected and control cell lines were transferred to MS basal medium with NaCl (0 –300mM) and NaCl free medium supplemented with NAA (0.5mg/l) and BAP (2.0 mg/l) with casein hydrolysate (200mg/l) individually. Cultures were kept under 16 hr photoperiod (40$\mu$ mol m$^{-2}$s$^{-1}$) at 25 ± 2 °C . The plantlet regenerated from both selected and non-
selected calli were maintained as separate lines in MS medium containing BAP (1.0mg/l). Roots were produced when the regenerated shoots were transferred to half strength MS medium fortified with IBA (1.0mg/l).

5.2.9. Hardening

The well developed plants were removed from the culture vessels and washed thoroughly and transferred to plastic cups containing sand, soil, vermiculite mixture (1:1:2) and subsequently transferred to field after 20 days.

5.2.10. Retention of NaCl tolerant

To confirm the retention of NaCl tolerance in the regenerated shoots of selected callus, the leaf explants were excised from the regenerated shoots of both selected and control callus and inoculated on MS medium containing NAA (2.0mg/l) + BAP (2.0mg/l) for callus induction. After one month of culture, 50 mg (Fresh weight) of callus pieces were transferred to MS medium containing NAA (2.0mg/l) + BAP (2.0mg/l) and further supplemented with various concentrations (0 –300mM) of NaCl and their fresh weight were noted after one month.
5.3. Results and Discussion

The aim of the work was to evolve NaCl tolerant cell line in mungbean using *in vitro* selection method. The NaCl tolerant cell lines were selected from callus derived from cotyledon of mature seeds.

5.3.1. Screening of NaCl tolerance in callus culture

In the present study, when the callus was exposed to increasing concentrations of NaCl (25mM - 300mM), fresh and dry weight of callus tissues decreased with increase in the salinity level of the medium except at 25mM, where there was a slight increase in the fresh and dry weight of the callus (Table-21) thereby indicating that the lower concentrations of NaCl stimulated callus growth. Similar results were also observed by many workers (Kumar and Sharma, 1989; Belouly and Bouharmant, 1992; Rao and Krupanidhi, 1996). The reduction of growth was relatively small during first and second subcultures. From the third week onwards there was marked reduction in the growth of callus particularly at 100mM, 150mM, 200mM and 250mM NaCl which did not support the growth of callus as the callus turned brown and death of the cells occurred after 25 days of culture (Table-21). This inhibitory concentration (250mM) was chosen for the selection of NaCl tolerant cell line, because at this concentration most of the cells of the calli died after 25 days of culture except few cell clumps (about 10%).

5.3.2. Selection of NaCl tolerant cell lines

The exposure of cells to lethal concentration of NaCl permitted selection of cell line with increased resistance to NaCl toxicity compared to non-selected cells. (Rao and Krupanidhi, 1996). Similar procedures have been followed to select NaCl resistant cell lines in *Nicotiana tabacum* and *N. sylvestris* (Nabors *et al.*, 1975) and in *Capsicum annum* (Dix and Street, 1975), *Medicago sativa* (Croughan *et al.*, 1978), *Oryza sativa* (Rains *et al.*, 1980), *Solanum melongena* (Jain *et al.*, 1987), *Cajanus cajan* (Rao and Krupanidhi, 1996) and *Citrus limon* (Piqueras *et al.*, 1996).
Table- 21

Effect of NaCl on fresh and dry weight of cotyledon derived callus of *Vigna radiata*

<table>
<thead>
<tr>
<th>Conc. of NaCl (mM)</th>
<th>10 days</th>
<th>20 days</th>
<th>30 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FW</td>
<td>DW</td>
<td>FW</td>
</tr>
<tr>
<td>Control</td>
<td>550.4b</td>
<td>51.3b</td>
<td>850.2b</td>
</tr>
<tr>
<td>25</td>
<td>635.6a</td>
<td>59.6a</td>
<td>990.3a</td>
</tr>
<tr>
<td>50</td>
<td>608.3ab</td>
<td>56.2ab</td>
<td>892.5</td>
</tr>
<tr>
<td>100</td>
<td>424.2c</td>
<td>40.4c</td>
<td>510.4c</td>
</tr>
<tr>
<td>150</td>
<td>310.3d</td>
<td>27.6d</td>
<td>284.5d</td>
</tr>
<tr>
<td>200</td>
<td>250.4e</td>
<td>22.3e</td>
<td>220.3e</td>
</tr>
<tr>
<td>250</td>
<td>225.3f</td>
<td>16.2f</td>
<td>186.2f</td>
</tr>
<tr>
<td>300</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
</tbody>
</table>

Data were analysed using Duncan's multiple range test to separate the means, the values having the same letters are not significantly different at 5% level.
Each experiment consists of 20 replicates and was repeated thrice.
FW-Fresh weight in mg, DW- Dry weight in mg.
Nil response due to death of callus.
In the present study, for the selection of NaCl tolerant cell lines, callus was initiated from cotyledon explants on MS medium supplemented with NAA (2.0 mg/l) + BAP (2.0 mg/l). One month old calli when transferred to 250mM NaCl amended medium, most of the calli died after 25 days of transfer and only 10% of cultures showed surviving pockets of cells (Fig.68). These surviving pockets of cells were again transferred to media containing 250mM NaCl and maintained on this media for 4 more subcultures by subculturing to fresh NaCl (250mM) containing media every 30 days to remove the escapes. After completion of four subcultures in the NaCl (250mM) medium, the calli were selected and named as selected calli (Fig.69). On the other hand, the callus continuously maintained on the NaCl free medium for four subcultures were referred as non-selected calli.

For comparison, the wild type callus (non-selected) and the selected calli were grown on MS medium supplemented with 0.0 - 300mM of NaCl. From the data on the fresh and dry weight of the callus (Table-22), it is suggested that the non-selected cell lines showed decreasing fresh and dry weight with increase in NaCl concentrations. At 250mM complete death of the cells occurred after 30 days, whereas the selected calli were able to grow on 300mM NaCl (Table-22). Comparatively, selected calli showed better growth rate than non-selected calli even up to 300mM NaCl (Table-22). This observation suggested that the salinity tolerance was stable. Similar type of growth pattern in NaCl selected cell lines has been reported in *N. sylvestris* (Dix and Street, 1975) and in *Citrus aurantium* (Ben-Hayyim *et al.*, 1985) *Cajanus cajan* (Rao and Krupanidhi, 1996) and *Solanum tuberosum* (Ochatt *et al.*, 1999). However, the overall growth pattern of selected and non-selected calli showed inverse relation with NaCl concentration as already observed in *Vigna radiata* (Kumar and Sharma, 1989) in which they have selected that the NaCl tolerant cell lines at 300mM from the root tip explants as callus source, but were not documented about the plant regeneration.
Table-22

Effect of NaCl on average fresh and dry weight of selected, non-selected calli in *Vigna radiata* (L.) Wilczek.

<table>
<thead>
<tr>
<th>Conc. Of NaCl(mM)</th>
<th>Selected calli</th>
<th></th>
<th></th>
<th>Non - selected calli</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 days</td>
<td>20 days</td>
<td>30 days</td>
<td>10 days</td>
<td>20 days</td>
<td>30 days</td>
</tr>
<tr>
<td></td>
<td>FW</td>
<td>DW</td>
<td>FW</td>
<td>DW</td>
<td>FW</td>
<td>DW</td>
</tr>
<tr>
<td>Control</td>
<td>674.8cd</td>
<td>68.8cd</td>
<td>1010.6b</td>
<td>100.8b</td>
<td>1150.5b</td>
<td>116bc</td>
</tr>
<tr>
<td>25</td>
<td>740.6a</td>
<td>75a</td>
<td>1030a</td>
<td>110.3a</td>
<td>1166.3a</td>
<td>125.5a</td>
</tr>
<tr>
<td>50</td>
<td>704.3b</td>
<td>71.3b</td>
<td>968.2c</td>
<td>97.4bc</td>
<td>1053.4c</td>
<td>119.8b</td>
</tr>
<tr>
<td>100</td>
<td>680.5c</td>
<td>70.6c</td>
<td>875.5d</td>
<td>90.6d</td>
<td>978.4d</td>
<td>95.6d</td>
</tr>
<tr>
<td>150</td>
<td>495.3c</td>
<td>55.3e</td>
<td>574.4c</td>
<td>62.2e</td>
<td>730.2e</td>
<td>78.8e</td>
</tr>
<tr>
<td>200</td>
<td>447.2f</td>
<td>50.2f</td>
<td>520.2f</td>
<td>53f</td>
<td>556.3f</td>
<td>69.2f</td>
</tr>
<tr>
<td>250</td>
<td>435.6g</td>
<td>48.1g</td>
<td>498.2g</td>
<td>48.3g</td>
<td>520.6g</td>
<td>53.3g</td>
</tr>
<tr>
<td>300</td>
<td>425.1h</td>
<td>39.2h</td>
<td>485.1h</td>
<td>45.6h</td>
<td>500.8h</td>
<td>49.2h</td>
</tr>
</tbody>
</table>

Data were analysed using Duncan's multiple range test to separate the means, the values having the same letters are not significantly different at 5% level.

Each experiment consists of 20 replicates and was repeated thrice.

FW-Fresh weight in mg, DW- Dry weight in mg.

- Nil response due to death of callus.
5.3.3. Stability of selected cell line

The NaCl resistant calli were subcultured for one generation on NaCl free medium and then the NaCl containing medium. It was noticed that even after alternate transfer to NaCl free and NaCl containing medium, the resistance developed was not lost. NaCl resistance of selected cell line which was retained even after one alternate transfer to NaCl free and NaCl containing medium indicated that the selected cell lines were stable. The NaCl – resistant clone exhibited a greater increase in fresh and dry weight on normal medium (Table –22). These observations indicated that growth and metabolic activity of NaCl – resistant callus line under stress conditions exceeded that of the sensitive callus line and increased still further when grown on normal medium. This indicated that change induced in the NaCl-selected callus line was a stable one. Similar changes in growth pattern of stress- selected cell line have been reported in Nicotiana sylvestris (Dix and Street, 1975), Cajanus cajan (Rao and Krupanidhi, 1996), Medicago media (Chaudhary et al., 1997) and Cymbopogon martini (Patnaik and Debata, 1997a).

5.3.4. Callus morphology

Regeneration potential could easily be identified on the basis of callus morphology. Nabors and Dykes (1985) had morphologically distinguished the embryogenic and non-embryogenic rice calli. In the present study, higher regeneration frequency was observed in green- compact callus. In high concentration of NaCl, the calli colour changed in to dark - brown.

Biochemical studies

5.3.5. Sodium and chloride ions accumulation

Sodium ion

The Na⁺ content of the callus samples at different concentrations is given in Table - 23. The accumulation of Na⁺ content increased with an increase in NaCl concentration and
more amount of Na⁺ accumulation was noticed in the selected calli than the non-selected calli at higher concentrations of NaCl. In the case of selected calli four fold increase was observed at 250mM NaCl than control. The non-selected calli showed three fold increase of sodium at 250mM NaCl. In many plants resistance to salinity was correlated with accumulation of Na⁺ in their tissue for osmotic adjustment (Greenway and Munns, 1980; Levitt, 1980). Similar observations were made previously in Vigna radiata (Gulati and Jaiwal, 1992) and Oryza sativa (Yeo and Flowers, 1984), Cymbopogon martini (Patnaik and Debata, 1997a).

Chloride ion

Selected calli showed highest amount of Cl⁻ accumulation. Minimum Cl⁻ content was observed in non-selected calli. In the case of non-selected calli, higher amount of Cl⁻ accumulation was noticed at 250mM NaCl. However, the Cl⁻ content was four fold higher in selected calli at high concentration of NaCl (250mM) than the selected and control (Table-23). Whereas in the non-selected calli, the accumulation was only two fold higher at 250mM NaCl than the control. There was a two fold increase in Cl⁻ in selected calli when compared to non-selected calli. Similar observations had been made by number of workers (Belouly and Boharmant, 1992; Sabbah and Tal, 1990).

Ben-Hayyim and Kochaba (1983), reported in Citrus sinensis that the Na⁺ and Cl⁻ uptake was considerably lower in salt tolerant cells than in salt sensitive cells. There was no difference in the Na⁺ and Cl⁻ accumulation at low levels of salt in both selected and non-selected cells in Cicer arietinum. But with increasing concentrations of NaCl more accumulation of Na⁺ and Cl⁻ was recorded in selected cells (Pandey and Ganapathy, 1984). In the calli continuously subcultured at higher concentration of NaCl in the medium, the Na⁺ and Cl⁻ uptake increased and Ca²⁺ uptake decreased (Chae et al., 1988; Kingsbury et al., 1984).
5.3.6. Potassium and Calcium ions accumulation

Potassium ion

Generally K⁺ content decreased with increasing NaCl concentrations in the medium. In the case of selected calli, the K⁺ accumulation slightly increased at 50mM and a decreasing trend of K⁺ was observed at and above 150mM (Table-23). The decreasing trend was observed more in selected calli than non-selected calli whereas K⁺ decreased continuously in the non selected callus. The decline in K⁺ levels was more pronounced in non-selected than observed in the selected lines. In the present study although a decreasing K⁺ was observed in selected calli, Na⁺/K⁺ ratio was maintained in the selected calli. The levels of K⁺ present in the NaCl medium are sufficient to meet the demands of cellular metabolic processes and the additional role of K⁺ as an osmoregulatory monovalent cation to some extent by elevated levels of Na⁺ in the selected cells in response to high levels of NaCl in the medium (Kumar and Sharma, 1989).

Calcium ion

Generally, Ca²⁺ content decreased with increasing concentrations of NaCl in both selected and non-selected calli, but the decreasing trend was more in non-selected calli. In the selected calli, at low concentration of NaCl (50mM) the Ca²⁺ content was increased than the control. Further, increase in NaCl led to sharp decrease of Ca²⁺. Maximum Ca²⁺ accumulation was observed (63.6µg) at 100mM NaCl in selected calli. Minimum calcium content (31.2µg) was recorded in non-selected calli at 250mM NaCl.

In NaCl tolerant cells K⁺ and Ca²⁺ were significantly reduced by exposure to a high external NaCl concentration and this was in agreement with that reported at the cellular level by other authors (Paek et al., 1988; Muralitharan et al., 1993; Piqueras et al., 1996). In the present study, the accumulation of K⁺ and Ca²⁺ content decreased with an increase in concentration of NaCl in both selected and non-selected cell lines. But in the selected
Table -23

Accumulation of Na⁺, K⁺, Ca²⁺ and Cl⁻ in the selected and non-selected calli derived from cotyledon explants of Vigna radiata at different NaCl concentrations (µg/g)

<table>
<thead>
<tr>
<th>NaCl conc. (mM)</th>
<th>Na⁺</th>
<th></th>
<th>K⁺</th>
<th></th>
<th>Ca²⁺</th>
<th></th>
<th>Cl⁻</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Selected</td>
<td>Non selected</td>
<td>Selected</td>
<td>Non selected</td>
<td>Selected</td>
<td>Non selected</td>
<td>Selected</td>
<td>Non selected</td>
</tr>
<tr>
<td>0.0</td>
<td>75.5g</td>
<td>58.5g</td>
<td>85.4cd</td>
<td>75.5a</td>
<td>41.3d</td>
<td>53.4a</td>
<td>77.5gh</td>
<td>65.8gh</td>
</tr>
<tr>
<td>25</td>
<td>86.3fg</td>
<td>64.1fg</td>
<td>90.3ab</td>
<td>73.3ab</td>
<td>53.7cd</td>
<td>51.7ab</td>
<td>78.3g</td>
<td>67.3g</td>
</tr>
<tr>
<td>50</td>
<td>98.3f</td>
<td>82.3f</td>
<td>92.3a</td>
<td>69.7c</td>
<td>55.2bc</td>
<td>45.6c</td>
<td>94.6f</td>
<td>83.5f</td>
</tr>
<tr>
<td>100</td>
<td>178.1e</td>
<td>131.2e</td>
<td>87.3c</td>
<td>65.2d</td>
<td>63.6a</td>
<td>34.2d</td>
<td>159.5e</td>
<td>126.5e</td>
</tr>
<tr>
<td>150</td>
<td>220.2d</td>
<td>152.3d</td>
<td>83.2d</td>
<td>58.2e</td>
<td>57.4b</td>
<td>29.1e</td>
<td>209.7d</td>
<td>142.6d</td>
</tr>
<tr>
<td>200</td>
<td>236.3e</td>
<td>173.6c</td>
<td>71.3e</td>
<td>51.5f</td>
<td>39.1d</td>
<td>24.4f</td>
<td>231.5c</td>
<td>154.3c</td>
</tr>
<tr>
<td>250</td>
<td>265.4ab</td>
<td>184.1ab</td>
<td>69.4ef</td>
<td>47.1g</td>
<td>32.3f</td>
<td>15.6g</td>
<td>244.5ab</td>
<td>163.3ab</td>
</tr>
<tr>
<td>300</td>
<td>268.3a</td>
<td>186.2a</td>
<td>68.3f</td>
<td>46.2gh</td>
<td>31.2fg</td>
<td>15.2gh</td>
<td>248.6a</td>
<td>164.2a</td>
</tr>
</tbody>
</table>

Data represent average of six replicates and repeated thrice.

Data were analysed using Duncan’s multiple range test to separate the means, the values having the same letters are not significantly different at 5% level.
PLATE 16

Selection of NaCl tolerant cell line

Fig. 68. Cotyledon derived callus cultured on MS medium containing NAA and BAP (each at mg/l) and combined with NaCl (250mM).

69. Selected callus from 250mM NaCl containing medium.

70. Regeneration of shoots from selected callus on MS medium containing NAA (0.5mg/l) and BAP (2.0mg/l) and casein hydrolysate (200mg/l).

71. Further development of shoots on same composition of medium after three weeks of culture.

72. Hardening of regenerated plantlets on sand, soil, vermiculite (1:1:2) mixture.
cell lines, such a decreasing trend started at higher NaCl concentration. But the accumulation of K⁺ was low in selected calli and non-selected cells according to Pandey and Ganapathy (1984). Comparatively, K⁺ level was high in the selected cells than in the non-selected cells with an increasing concentration of NaCl in the medium (Groughan et al., 1978; Watad, et al., 1983; Stavarek and Rains, 1984), which corroborated the present observation.

5.3.7. Proline accumulation

In many plants, resistance to salinity has been correlated with the accumulation of proline in their tissues for osmotic adjustment (Stewart and Lee, 1974). Proline is known to play a crucial role in osmoregulation in stress environment in many plant systems (Rudulier et al., 1984). Dix and Pearce (1981) found accumulation of proline both in selected and non-selected cells of N. sylvestris cell lines on transfer from salt free medium to salt medium. Watad et al. (1983) found that proline content increased in the selected cells of N. tabaccum and N. gossil with increase in the levels of salt. The non-selected cells accumulate higher amount of proline. In the present study, the selected cell lines showed higher amount of proline than the non-selected cell line. Similar observations have been reported in Cicer arietinum (Pandey and Ganapathy, 1985) and Brassica campestris (Paek et al., 1988). These reports confirmed the fact that selected cell lines acclimatized to salt stress. In the present study, 250mM concentration of both selected and non-selected calli accumulated high levels of proline. But the selected calli showed more accumulation of proline than non-selected calli (Fig.73).

Proline may serve as intercellular osmotic soluble for the maintenance of osmotic balance between cytoplasm and vacuole (Flowers et al., 1977; Brown and Hallebust, 1978). The induction of proline accumulation may be due to an activation of the glutamate pathway involving glutamyl kinase, glutamyl phosphate reductase and Pyrroline 5-carboxylate reductase (Bellinger and Larher, 1987; Brayan, 1990).
Fig. 73

Proline content in the selected and non selected calli at various concentrations of NaCl

Each value is the mean of six replicates. Bars bearing the different letters are significantly different at 5% level according to Duncan's multiple range test.
Sivaprakash and Ballasarin (1993) reported accumulation of organic molecules like proline and glycine betaine in *Cajanus cajan* in response to salt stress. Similar result has been reported in *Cicer arietinum* (Panday and Ganapathy, 1985) *Oryza sativa* (Reddy and Vaidyanath, 1986) *Brassica campestris* (Paek et al., 1988) and in mulberry (Kathiravan et al., 1995).

Most of the suggestions regarding involvement of proline in salinity tolerance in plants was based on its enhanced accumulation in response to stress (Jains et al., 1987; Thomas et al., 1992; Martinez et al., 1996; Petrusa and Winicov, 1997). The protective function of proline against stress has also been advocated from the findings that the cell and callus lines selected for NaCl tolerance and the plants derived from them accumulated proline in excess over the non-selected counterparts (Petrusa and Winicov, 1997; Patnaik and Debata, 1997b).

In the present study, the endogenous free proline content of the plants regenerated from NaCl adapted callus was significantly higher than those obtained from unadapted calli.

### 5.3.8. Regeneration of NaCl selected cell lines

Salt tolerant cell lines have been selected in a variety of plant species (Tal, 1990). However, the regeneration of plants via either organogenesis or somatic embryogenesis from salt tolerant (treated) cells have been exceedingly difficult in most of the cases, due to the loss of regeneration potential during long periods required for selection or due to the use of non-embryogenic cells or due to the presence of high concentration of NaCl in the regeneration medium (Gulati and Jaiwal, 1997). The salt tolerance of regenerants and their seed progenies have been demonstrated in only a few cases (Tal, 1996). Since the presence of salt (NaCl) is inhibitory to regeneration, the salt tolerant cell lines (even when verified for salt tolerance) have been regenerated on salt free medium in most of the cases (Li and Heszky, 1986; Ben-Hayyim and Goffer, 1989). It is difficult to induce regeneration in the medium with high levels of salt. The excess of salt may be inhibitory to regeneration and organogenesis. Presence of salt in regeneration medium also results in lowering the rate of regeneration (Binh et al., 1992). In the present study, the selected
and non-selected and control calli were transferred to NaCl free regeneration medium. Within 4 to 5 weeks, plantlets were regenerated from selected, non-selected and control calli. But regeneration frequency varied with types of callus. In the control, 16-17 shoots were developed per culture. 7-8 shoots were obtained from non-selected callus and only 5-6 shoots (Fig. 70-71) were developed from selected callus (Table-24). The decline in regeneration potential may be due aging and prolonged subculture of callus in salt stress.

Decline in regeneration frequency was also observed in carrot (Jones, 1974), Orange (Kochba et al., 1978), Hevea (Ferriere and Casson, 1989) and potato (Mribu and Veilleux, 1990). Thus, it is established that the regeneration ability was severely inhibited by aging of callus. Reduced regeneration frequency may even be due to callus aging, accumulation of inhibitory substances or may be due to decreased metabolism and transport and interaction between growth regulators (Halperin, 1986, Hansen et al., 1987, Palani et al., 1988). Rice cells selected from sea water (Yano et al., 1982), when cultured in medium with sea water did not show regeneration and only in the absence of sea water in the regeneration medium produced plantlets. However, the plantlets died after 10 days in salinized solution. Kavi kishore and Reddy (1984) observed only 2-3% percentage of plantlet regeneration from selected calli. Chant prem et al. (1984) produced anther callus that could not grow on media containing 1.5% NaCl and not able to regenerate into plants. Li and Heszky (1986) have reported that the ability for redifferentiation of the salt selected calli lines was strongly inhibited by the presence of NaCl in the regeneration medium in rice. In Medicago sativa, McCoy (1987) regenerated salt-tolerant plants from stable salt-tolerant callus lines selected by step-up selection procedure, but the regenerants were morphologically abnormal and showed poor growth than the parent type, and the only plant that flowered was sterile (male and female) and precluding further genetic analysis. Recently, healthy and fertile salt tolerant plants that transmitted salt tolerance as dominant trait through seeds to R1 generation were obtained from salt tolerant callus lines of Medicago sativa selected by a single-step selection process (Winicov, 1991). In the present study, about 70% of plantlet regenerated were albino with pale green colour leaves which showed poor survival during hardening, whereas the remaining cultures produced healthy normal plantlets which survived well during hardening. After rooting of regenerated shoots on MS medium with IBA
Table -24

Regeneration of plants from control, selected and non selected calli of cotyledon explants of *Vigna radiata* on MS medium with NAA (0.5mg/l) + BAP (2mg/l) and supplemented with casein hydrolysate (200mg/l).

<table>
<thead>
<tr>
<th>Nature of callus</th>
<th>Culture response (%)</th>
<th>Mean shoot/ culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>65a</td>
<td>17.5a</td>
</tr>
<tr>
<td>Selected</td>
<td>15bc</td>
<td>5.6bc</td>
</tr>
<tr>
<td>Non selected</td>
<td>25b</td>
<td>7.3b</td>
</tr>
</tbody>
</table>

Data were collected after four weeks of culture
Data were analysed using Duncan’s multiple range test to separate the means, the values having the same letters are not significantly different at 5% level.
Each experiment consists of 20 replicates and was repeated thrice.
Effect of different concentrations of NaCl on fresh weight of callus derived from leaves of NaCl resistant shoot and control shoots after one month of culture of mungbean.

Each value represents the mean of three experiments with 20 replicates per treatment. Graph bearing the same letters are not significantly different at 5% level according to Duncan's multiple range test.
(1.0mg/l), the plants were transferred to plastic cups containing soil, sand, vermiculite (1:1:2) (Fig. 72.) for hardening.

5.3.10. Retention of NaCl tolerance

When the leaf callus of the regenerated shoots of control and selected cell line were transferred to MS medium containing various concentrations (25mM – 300mM) of NaCl, about 70% the growth of the leaf callus from the shoots of control callus was inhibited. On the other hand, the callus raised from leaves of NaCl selected tolerant shoots showed stimulation in growth at 100 and 150mM, while its fresh weight remained unaffected upto 300mM (Fig. 74.). It showed the persistence even after alternate transfer of NaCl free medium and then to NaCl (250mM) containing medium. The cellular salt tolerance characteristic can be passaged through the regenerated plants, since callus cultures initiated from immature ovaries of the salt tolerant regenerated plants of *Medicago sativa* without additional selection in NaCl (Winicov, 1991). Chaudhary et al. (1994) observed that the retention of increased NaCl tolerance of leaflets derived from the plants regenerated from NaCl when they were recultured on NaCl medium without prior selection in *Medicago media*.

The last century has seen enormous gains in plant productivity and resistance to a variety of pests and diseases through much innovative plant breeding and more recently molecular engineering to prevent plant damage by insects. In contrast, improvements to salt and drought tolerance in crop and ornamental plants has been elusive, partially because they are quantitative traits and part of mutagenic responses detectable under salt/drought stress conditions (Winicov, 1998). The cell culture approach has been proven very effective in obtaining salt resistant cell lines in many species (Sabbah and Tal, 1990). Recently, successful tolerant plants were regenerated through tissue culture techniques. In the present study, a successful selection of NaCl tolerant cell line and subsequent regeneration protocol were standardized for *Vigna radiata* using the cotyledon derived callus. This would have a tremendous application in the improvement of salt sensitive mungbean.