Discussion
The cultivated Tomato, \textit{(Lycopersicon esculentum} Mill) ranks foremost among the important vegetable crops in numerous countries of the World (Sink and Reynolds, 1986). The conventional method of propagation of the plant is through seeds. Because of changing market, consumer demands and the need to induce resistance to disease and environmental stresses, new characteristics must be produced by exploiting both somaclonal as well as induced variations. In the present investigation, I have attempted to micropropagate \textit{Lycopersicon esculentum} cv. Sakthi through shoot tip culture, direct and indirect \textit{de novo} organogenesis and anther culture. Successful protocols have been established for most of the attempted routes of regeneration except for anther culture, wherein plantlets could not be raised. The protocols precipitated from these experiments were utilised for selection for NaCl tolerance, as well as to understand the physiological basis of the tolerance mechanism through biochemical analyses.

1. General tissue culture programmes  
Culture establishment

The successful establishment of explants in culture is limited by several factors. According to Pierik (1987), the most important source of contamination is the plant itself and a properly selected and sterilized explant form the basis of successful culture establishment. The surface sterilisation of explants or seeds in \textit{Lycopersicon} is achieved with relative ease, a uniform method did not work for the other explants. For leaf, stem pieces, shoot tips, petiole and seeds, a 6-8 min sterilization with 0.1\% HgCl$_2$ (w/v) was sufficient to get 90\% contaminate free cultures. For flower buds, in addition to HgCl$_2$ sterilization, a 30 sec dip in 70\% ethanol
was also essential. Eventhough prolonged exposure to ethanol might lead to excessive dehydration (Pierik, 1987) exposure to few seconds was proved fruitful and advised by several authors (Sink and Reynolds, 1986, Gamborg and Phillips, 1996).

**Callus induction, proliferation and organogenesis**

In tomato, the most important factor in establishing and maintaining callus culture, has been the genotype (Sink and Reynolds, 1986). This was recognised in the earlier studies by Ulrich and Mackinney (1969) where genotypes responded differentially to levels of nutrient factors in medium. De Langhe and De Brujine (1976) observed that *L. peruvianum* varieties were much more vigorous in growth compared to *L. esculentum* varieties. In *L. esculentum* cv Sakthi, callus could be readily induced on various explants (both juvenile and mature phase) when cultured in the induction medium. However, juvenile explants developed callus early while those from mature phase plants took 13-15 days to show similar response. It has been well accepted that explants from juvenile plant tissue which are rich in nutrients and possible endogenous hormones are the best choice for the induction of cell division (Dodds and Roberts, 1995). This observation in *L. esculentum* cv Sakthi, is consistent with findings in *Lactuca sativa* (Brown *et al.*, 1986), *Beta vulgaris* (Yu, 1989) and *Solanum tuberosum* (Webb and Henshaw, 1982) where cotyledonary explants showed more rapid callus induction than those from the mature phase explants. However in plants like *Hedera helix* (Banks, 1979) and *Parthenium hysteriophorus* (Wikham *et al.*, 1980) explants from mature plants were more responsive than seedlings.

In *L. esculentum* cv. Sakthi, though callus was induced from various explants under the influence of hormones IAA, NAA, 2,4-D
alone or in combination with cytokinins, callus obtained in presence of IAA/NAA in combination with BA/KIN alone was organogenetic. A perusal of the literature pertaining to callus culture clearly substantiate this observation (Kut et al., 1984; Sink and Reynolds, 1986; George et al., 1987 and George 1996). Callusing was optimum in presence of 2,4-D. Incorporation of low concentration of KIN to 2,4-D containing medium resulted in profuse rooting. In *Artemisa absinthium* callus induced from leaf explants in presence of 2 mg l\(^{-1}\) 2,4-D was a dark friable tissue mass which grew well during subsequent subcultures and developed roots (Nin et al., 1996). Similar root formation in presence of 2,4-D was reported in *Aristidia oligantha* (Lowe and Kurl, 1981), *Bupleurum falcatum* (Wang and Huang, 1982) and in presence of KIN in *Medicago sativa* (George, 1996).

Eventhough callus could be initiated at moderate concentration of Auxins (NAA/IAA/2,4-D) increase in the concentrations of these hormones was detrimental to the explanted tissue as it either resulted in rapid loss of explant viability or reduction in callusing rate. This is in agreement with the fact that at higher concentrations most of the auxins are phytotoxic to plants (Wareing and Phillips, 1981).

For inducing shoot formation from callus, transfer to a regeneration medium with changed hormones was essential. Callus transferred to medium containing 3-5 mg l\(^{-1}\) BA/KIN along with 0.5-2 mg l\(^{-1}\) NAA/IAA resulted in the formation of green spots/compact regions in the subcultured callus. Such regions in the callus are considered to be meristematic centers which later develop into shoot or root (Nabors et al., 1982, Ishi, 1982). The callus developed maximum shoots in 2 mg l\(^{-1}\) BA
and KIN containing medium irrespective of the callus source (Explant). This points to the high requirement of cytokinins for shoot formation in tomato. When used together, the synergistic action of the hormones might have enhanced the shoot proliferation. The earlier reports in Tomato have shown that BA was superior to Zeatin since shoot development occurred earlier (Kartha et al., 1976). However, none of the previous reports on callus regeneration in different tomato cultivars viz ‘Rutgers’ (Padmanabhan et al., 1974), ‘Cantella’ (De Langhe and De Bruijne, 1976), ‘Starfire’ (Kartha et al., 1976), ‘Prophyre’ (Ohki et al., 1978), ‘Pixle’ (Herman and Hass, 1978) and ‘Xa-ag’ (Bekhi and Lesli, 1980) have used two cytokinins together for regeneration.

In Tomato, cv Sakthi, regeneration from callus was also obtained in presence of BA and GA$_3$. The stimulatory effect of GA$_3$ on development is not surprising because it is known to promote cell division and elongation in the sub apical zone of the shoots (Sacchs, 1965). Additionally, Sekoika and Tanaka (1981) have reported that GA$_3$ can act as a replacement for auxin in the induction of shoots. However, in tomato a combination of BA and GA$_3$ is seldom used for callus regeneration (Kut et al., 1984; Sink and Reynolds, 1986). Nevertheless, in solanaceous plants like Solanum tuberosum (Roca et al., 1979), Solanum brevidens (Nelson et al., 1983) and in some wild Solanaceous species (Gleadle et al., 1990) successful caulogenesis was observed in presence of BA and GA$_3$.

Coconut water used at 10-15% level was the most effective organic supplement in the present study as it promoted shoot organogenesis in all the 4 hormonal combinations selected. The beneficial effect of CW is well discussed in literature and is related to its growth regulator content, especially cytokinins (Dodds and Roberts, 1995).
Eventhough successful use of coconut water has been reported in species like *Solanum tuberosum* (Behnke, 1975), *Solanum dulcamara* (Binding and Nehls, 1977) and *Solanum nigrum* (Mandal and Gadgil, 1979) and several other solanaœeous species the recent trend is to eliminate the use of crude natural extracts and define all the constituents of a given medium (Dodds and Roberts, 1995).

Adenine sulphate and casein hydrolysate moderately enhanced the shoot formation in tomato. Adenine is known to promote or reinforce responses that are normally attributed to cytokinin action (George and Sherrington, 1984). Earlier reports in tomato, however, show only sporadic uses of Adenine. Vunchkova (1977) used 70 mg/l AdS in the callus regeneration medium which resulted only in few shoots. However, successful use of Adenine has been reported in species like *Hyocyamus niger* (Raghavan, 1978), *Solanum melongena* (Matsuoka and Hinata, 1979), *Solanum tuberosum* (Gavintertvatana and Li, 1980) and *Solanum brevidens* (Nelson et al., 1983).

Casein hydrolysate also influenced shoot organogenesis however, its impact was less compared to adenine sulphate. The hydrolysed form of casein yields an ill defined mixture of 18 different amino acids which forms a source for the organic nitrogen in the medium (Klein and Kelin, 1970). However, in medium like MS where the nitrogen content is very high (George et al., 1988) addition of this organic supplement may not be beneficial as is observed in the present work.

**Isozyme analysis in the different stages of callus culture**

Research to elucidate basis for growth and development has correlated organogenesis with limited number of biochemical makers.
Peroxidase isozyme analysis is one among them (Murashige and Huang, 1985). However only a few investigations have focused on isozyme variation during in vitro differentiation (Chawla, 1988; Kavikishore and Mehta, 1988; Jain et al., 1990; Yuernkova, et al., 1995). Peroxidases are involved in several biological functions and therefore can exhibit a degree of versatility not surpassed by any other single enzyme (Cortelazzo et al., 1996). Peroxidase isozyme variation during the different stages of development is studied as a marker in the present investigation.

Isoperoxidase pattern of Tomato cv. Sakthi were characteristic of different morphogenetic events. It was observed that isoform of Rf 0.07 was present in all the stages of development. This indicates that the basic isozyme pattern is retained in both in vivo and in vitro systems, similar to suggestion of Khavikin and Sukhorvheskia (1979). Comparable observations were made in barley and wheat where some of the isoforms were common to all the stages irrespective of the difference in the stages/age (Chawla, 1988). Secondly new isoforms appeared with the onset of shoot and root organogenesis. Since these isoforms were absent in the undifferentiated tissue it is presumed that they are involved only in the cellular differentiation.

Finally, a highly conspicuous isoform of Rf 0.59 was noticed always in association with rhizogenesis. This substantiate the contention that individual isoperoxidases carry out specialized functions (Conroy, 1986). Moreover, it was also reported that rhizogenesis occur concomitant with increase in peroxidase activity. (Gasper et al., 1982; Zheng and Van Huystee, 1992).
Regeneration from long-term callus cultures

Loss of morphogenetic potential of callus tissues in prolonged cultures in many plant species is a great disadvantage of utilizing them for in vitro production of plants, especially in vitro selection studies. In the present study, consistent loss of regeneration potential was observed with the increase in serial subcultures, notably beyond 8 weeks. A more or less similar observation was made by Chandler et al. (1982) in Solanum lacinatum where callus cultures lost their initial ability to undergo organogenesis as a result of the culture age. In tomato, this loss of morphogenetic potential was attributed to the lack of cellular organization (Herman and Hass, 1978; Morgan and Cocking, 1982).

The major problem encountered in the present investigation on prolonged callus cultures was the accumulation of phenolics with the increase in the number of subcultures. As a result of this, the calluses became non-morphogenetic and necrotic. Phenolic browning was considered as a major problem associated with tomato callus cultures in as early as 1969 by Ulrich and Mackinny. Lam and Street (1977) and Patel et al. (1984) have speculated that the cessation of morphogentic potential is the consequence of phenolic accumulation. A reduction in cytoplasmic volume and expansion of vacuoles as a result of phenolic accumulation was reported in Picea abies (Kovarik et al., 1973). The phenolic toxicity is due to phenolases which reversibly attach to the proteins by hydrogen bonding and there by oxidising them to highly active quinones (Harms et al., 1983).

To prevent the problem of phenolic exudation and to facilitate normal growth of the cultures, I have used certain antioxidants
and adsorbents like PVP, PG, ascorbic acid and activated charcoal. 500 mg/l PVP used along with 2 mg/l BA and KIN in MS medium was successful in preventing browning and hence revitalising the old callus (up to 12 month old). PVP is considered as a strong antioxidant polymeric agent and is a very strong proton acceptor (Rhodes, 1977). Phenolic hydroxyl groups act as very strong proton donor in hydrogen bonding which is readily accepted by PVP. For this reason, PVP appears to be a stronger and more specific phenol binding agent than any other available material (Salame and Zieslin, 1994). In addition to this, there are reports of the activity of PVP at the enzyme levels. Peroxidase activity was enhanced in cultures of *Syngonium podophyllum* and Rose through the removal of phenolic substance. (Salame and Zieslin, 1994; Zieslin and Ben-Zaken, 1993). Successful PVP induced prevention of phenolic browning and enhanced organogenesis was documented in *Casteana sativa* (Vestri. *et al.*, 1991), *Datura metal* (Babber and Gupta, 1982), *Malus* (Mc Comb, 1978), *Piper nigrum* (Sarasan, 1992) and *Aristolochia indica* (Anita, 1994).

Phloroglucinol, another additive used, was moderately effective in preventing phenolic browning. 150 mg/l PG incorporated into a cytokinin rich (BA and KIN) regeneration medium differentiated a maximum of 20-24 shoots from 3 month old callus. Simultaneous rhizogenesis was also observed. Phloroglucinol (1,3,5-trihydroxyl benzene), one of the degradation products of phloridizin, was originally used for rooting of apple shoots (Jones and Hatfield, 1976; Welander and Huntriezer, 1981). However, it also has been successfully used to reduce tissue blackening, revival of growth and induce morphogenesis in some species (Sarasan and Nair, 1991; Sarasan, 1992; Anita, 1994). In a report
in *Cucumis sativus*, Lazarte and Sassor (1982) have reported greening of callus derived from anthers in presence of PG and BA, suggesting its effectiveness in preventing tissue blackening in presence of a cytokinin. However, the effectiveness of PG in terms of revival of growth and regeneration in tomato cv. Sakthi was limited only to 7 month old callus as compared to the regeneration achieved in 12 months old callus in presence of PVP.

Ascorbic acid and activated charcoal were less effective in this regard. The ineffectiveness of the ascorbic acid probably can be correlated with the fact that in tissue culture media ascorbic acid is oxidised rapidly to dehydro-ascorbic acid (Elmore *et al.*, 1990) which is an ineffective form. However, in *Solanum melongena*, regeneration from callus was achieved in presence of 0.1 μM ascorbic acid (Fassuliotis *et al.*, 1981). Activated charcoal on the other hand, though was reported to absorb phenols/secondary products released by the cultured tissues (Wang and Huang, 1976; Fridborg, *et al.*, 1978), it is reported to absorb medium components such as vitamins (Weatherhead *et al.*, 1978) thereby inhibiting growth and morphogenesis (Constantin *et al.*, 1977). Probably because of this reason charcoal was not found to be a good additive for revival of growth from blackened callus in the present investigation.

**Direct regeneration**

In direct adventitious bud formation, organogenesis is induced without an intervening callus phase on explants devoid of any pre-existing buds. Eventhough the number of plant species that can regenerate adventitious buds on excised explant is relatively small (Brown and Thorpe, 1995), this method of *in vitro* propagation can serve as an excellent
regeneration system in gene transfer technique for a particular species of interest (Iapichino et al., 1991).

In *Lycopersicum esculentum* cv. Sakthi, direct regeneration was achieved from leaf segments, internodal segments and petioles of greenhouse grown plants as well as leaves and hypocotyl segments of axenic seedlings. However, a considerable difference exist between various explants with respect to the number of shoots formed. Both mature phase and cotyledonary leaf segments cultured in 3 mg/l BA resulted in maximum number of shoots than the other explants. Between mature phase and seedlings explants, the number of shoots developed was high in the former than the latter. This is in quite contrast with most of the earlier reports in tomato, wherein cotyledonary explants were found to be more responsive (Crompton and Veilleux 1988, 1991; Branca et al., 1990; Fari et al., 1991).

As regards the hormonal requirement for direct regeneration in tomato, BA was found to be essential and irreplaceable since it induced shoot formation in concentrations ranging from 1-3 mg/l. In Solanaceous plants like *Physalis minima* (Chaturvedi and Sinha, 1979) and *Solanum tuberosum* (Thomas, 1981) this cytokinin was successfully utilized for shoot regeneration. However, none of the earlier reports in tomato have used BA alone for de novo organogenesis, instead, a low dose of either IAA or NAA along with BA was necessary in all these experiments, and in most of the cases the explants developed callus. The argument for the incorporation of auxin was that low level of auxin is necessary to prevent the loss of explant vigour (Meridith, 1979). A similar situation was noticed in *Picrorhiza kurroa* where Lal et al. (1988) reported
increased shoot growth and development in presence of IAA, eventhough either BA or KIN alone was sufficient for shoot morphogenesis. In tomato cultivar Sakthi, all low dose treatments of IAA (0.1-0.5mg/l) increased the callusing. However, instead of IAA, when GA3 was incorporated to the BA containing medium, it readily induced shoots without callusing. This is because, GA3 can act as a substitute of auxin without causing any ill effects on the shoot development. GA3 was successfully utilized for shoot regeneration in Begonia (Simmonds, 1984), Solanum melongena var. insanum (John et al., 1997), Naregamia alata (John et al., 1997).

Some of the shoots regenerated in presence of BA and KIN when transferred to BA and GA3 containing medium for elongation, developed flower buds. Both GA3 and BA are reported to have properties of floral induction. GA3 induced flowering was reported in plants like Lolium temulentum (Evans et al., 1990) and Pharbitis nil (King et al., 1987), where as BA was found useful in Helianthus annus (Grecco et al., 1984), Torena (Tanimotto and Harada, 1990) and Capsicum annuum (Soniya, 1995). However, the floral induction noticed in the present study closely resembled with that of Chrysanthemum cultivar where a combination of BA and GA3 induced flowering (Salisbury and Ross, 1992). Similar to the observation in Helianthus annus (Finer, 1987) and Hordeum sativum (Ruiz et al., 1991) flowers developed were premature and did not develop fruits.

Additives like Ads and coconut water added to BA containing medium developed callus from the explant with simultaneous shoot formation. Though Ads has been found to enhance shoot formation denovo in several species (Start and cumming, 1975; Nickerson, 1978) it induced profuse callusing in the present study. Coconut water(10%)
though induced a few shoots, it was always accompanied by callusing. The promotory role of CW in induction of de novo bud development was earlier reported in tomato (Sree Ramlu et al., 1976; Cappadocia and Sree Ramulu; 1980, De Langhe and De Brujine, 1976). However in these reports also, shoot formation occurred in accompaniment with callus development.

**Shoot tip and nodal segment cultures**

Shoot tip culture is the most widely practiced in vitro technique because of the high multiplication rate that can be achieved in a short span of time. In tomato cv. Sakthi, shoot tips from both axenic seedlings as well as from green house grown plants were cultured. It was observed that shoot tips from green house grown mature phase plants were more responsive than those from axenic seedlings. This is in contrast with the earlier reports in tomato where Frankenberger et al., (1981) observed high frequency shoot formation from shoot apices of axenic seedlings, while Gunay and Rao (1980), working with a Karnatic hybrid, observed that explants from green house grown plants produced more shoots than in vitro raised seedlings. These contrasting observations recorded in the same species confirm the role of the genotype, environment and growth conditions in regulating the in vitro responses.

 Shoots were initially established in a low concentration of BA containing medium. However there was no multiplication even after increasing its concentration in Stage II. For shoot proliferation, it was mandatory to add a low concentration of IAA (0.1-0.5mg l\(^{-1}\)) to (6mg l\(^{-1}\)) BA containing medium. This may be because the resting buds and meristems sometimes do not produce (or retain) sufficient endogenous
auxins and therefore it becomes necessary to supplement them exogenously for shoot growth (Hu and Wang, 1983). In tomato, the growth of unbranched shoots from shoot tip explants has been reported to be enhanced by the addition of 0.5-1.75 mg l\(^{-1}\) IAA (Kartha et al., 1977; Turner et al., 1987). In cultivar ‘Fantastic’, shoot multiplication was promoted by incorporating 5 mg l\(^{-1}\) BA alone (without auxins) into the multiplication medium (Schnapp and Preece, 1986). This emphasizes that among the different cultivars, the requirements of hormones for in vitro responses may be different. Nevertheless, it is presumed from the present work that exogenous auxin may promote axillary shoot proliferation in conjunction with cytokinin and improve culture growth.

It may also be noted that BA concentration as high as 6 mg l\(^{-1}\) was essential for a better shoot multiplication. This may be because, a high cytokinin concentration is necessary to combat the strong apical dominance in the plant. Similar requirement of high levels of BA was reported in shoot multiplication protocols of Solanaceous species like Atropa belladonna (Lorz and Potrykus, 1979), Capsicum annuum (Soniya, 1995), Nicotiana tabacum (Gamborg et al., 1979), Solanum brevidens (Nelson et al., 1983) and Solanum dulcamara (Binding and Nehls, 1977). However, kinetin used at this concentration and above with IAA did not influence the shoot proliferation. Thus it is obvious that IAA-BA combination was superior than IAA-KIN which is in line with the report in tomato by Kartha et al. (1976).

Nodal segment culture is another in vitro method of micropropagation which was proven to be successful in many shrubby plants. This is an ideal method for shoot multiplication in tomato where the
prevalent apical dominance can affect axillary proliferation (Pierek, 1987). In tomato c.v. Sakthi, though shoot regeneration occurred through this mode of regeneration, the number of shoot developed was less (1-2) in comparison with shoot tip cultures. However, single node method had a good measure of success in plants like *Solanum tuberosum* (Hussey and Stancey, 1981), rose (Elliot, 1970) and *Hedera helix* (Hackett, 1970).

In tomato, unlike shoot tip culture, where high levels of BA was essential for organogenesis, nodal segment culture required BA only in lesser amounts. There are instances in literature where shoot growth was obtained even without any hormones. In *Solanum tuberosum*, formation of single shoot was reported from cultured nodal segment without the use of any hormones (Hussey and Stancey, 1981). This is in agreement with the suggestions of Pierek (1987) that for single node culture, cytokinins are not required or are required only in lesser quantity. In tomato, nodal cultures also required a low dose of auxin for shoot proliferation. Though the role of IAA to promote shoot growth in conjunction with cytokinin is fairly understood, according to Ludergan and Janick, (1980), IAA in the medium probably masks the suppressive effect of cytokinin in the shoot elongation and hence a normal growth of shoot is achieved.

**Anther culture**

Culture of pre-treated and untreated anthers containing uninucleate microspores of *Lycopersicon esculentum* c.v. Sakthi in a range of hormones and different media failed to develop haploid plants. Even though, callusing could be induced in some of the combinations of hormones used, the subsequent regeneration was not possible. There are few reports on androgenesis either directly or indirectly in tomato (Sink
and Reynolds, 1986). A perusal of recent literature on anther culture of tomato (Chlyah et al., 1990) has shown only 4 instances of haploid callus production (Gresshoff and Doy, 1972; Sharp et al., 1972; Gulshan and Sharma, 1981; Gao et al., 1980) which was subsequently used in cell culture studies. There are only two instances where haploid plantlets were regenerated (Gresshoff and Doy, 1972; Gao et al., 1980). The difficulty in achieving haploid plantlet development is reflected in the fact that only anthers of 3 races cultured in vitro gave rise to haploid plants through callus out of the 43 available races of tomato (Gresshoff and Doy, 1972). Thus a strong genotype effect is envisaged in tomato androgenesis which is evident from the present study that even after transferring the callus to GD III medium (regeneration medium of Gresshoff and Doy, 1972) none of the subcultured calluses showed meristemoid induction. Several authors have reported the ineffectiveness of changing in vitro culture conditions in improving the androgenic responses and suggested dependence for androgenic responses on genotype (Lazar et al., 1984; Henry and de Buyser, 1985; Tuveson, et al., 1989).

In the present study, maximum callus induction occurred on anthers pre-treated at 0°C for 12 hrs upon incubation in GDI medium. Chlyah et al. (1990) observed that pretreatment of tomato flower buds to 4°C stimulated callus formation. Low temperature pre-treatment has been found to be effective in callus induction from cultured anthers also in Solanaceous species like Nicotiana, Datura and Petuina (Maheswari, et al., 1980). In Capsicum annuum on the contrary a high temperature was beneficial (Dumas de Valuex et al., 1981).
Rooting, hardening and planting out

In the present investigation, rooting was best in 1/4th MS medium supplemented with 2 mg l⁻¹ IBA. The roots formed were highly branched and healthy. NAA at similar concentrations induced only few roots which were fragile. However, NAA was most frequently used in tomato cultures for rooting (George et al., 1987). In present work, survival rate of plantlets rooted in IBA, during subsequent stages also was high.

The plantlets developed in agar medium were transferred to liquid medium, but supported by filter paper slants. This not only enabled the hardening, it also induced the formation of new roots as well as lateral roots within 15-20 days. About 90.37% of plantlets survived this hardening phase. Profuse root hair formation on shoots supported above liquid medium on filter paper bridges was reported by Mascarenhas et al. (1978) and Attree et al. (1990).

A comparison of shoots rooted in presence and absence of sucrose has indicated that the maximum percentage of shoots rooted were in presence of the sugar supplement. However, during hardening, sucrose was eliminated from the media which facilitated their better survival in the field. This is because, under standard conditions, micropropagated plants are not photo-autotrophic and produce only a little amount of their carbohydrate requirement through CO₂ fixation. Therefore, factors affecting photosynthesis may play an important role in their acclimatisation and survival (Kozai, 1991; Deberg, 1991). Moreover, omitting sugar from the medium benefits the plantlets not only in promoting photoautotrophy but also in reducing the loss of plantlets due to biological contamination of the culture medium (Kozai, 1991).
Hardened plantlets were transferred to vermiculite moistened with Hoagland's solution. 91.27% of the transferred plantlets survived at this stage. Acclimatisation in vitro involves the exposure of plantlets to reduced relative humidity and an environment which will affect the shoot system without disturbing or injuring the rather delicate root system (Ziv, 1986). In the present work, humidity around the plantlets was adjusted by keeping them in plastic bags (punctured at places) and through controlled illumination. Plants grown in highly humid conditions especially in flasks or test tubes do not have mechanism to prevent excessive transpiration owing to the lack of cuticle or of a poorly deposited epicuticular wax (Sutter and Langhans 1982). Acclimatisation of cultured plum plants under reduced relative humidity in the greenhouse, induced wax formation on the abaxial leaf surface and reduced water loss (Fuchigami, et al., 1981). In apple plants, stomatal function was improved after 4-5 days of exposure to low humidity (Brainard and Fuchigami, 1981). Survival rate of similarly acclimatised carnation plants increased from 72% to 90% after nine days of exposure to 50% RH (Ziv, 1986). In the present investigation also, a high survival rate (93%) was noticed at the field transfer stage, suggesting the effectiveness of the hardening/acclimatisation methods adopted.

2. In vitro selection for NaCl salinity tolerance

Eventhough cell culture techniques are considered as supplementary procedures to conventional plant breeding systems to enhance the response of crops to saline stress (Epstein et al., 1980), consistent success eluded this field because of several factors. Almost all cases of cell culture selection of NaCl tolerance, cell lines have been selected from spontaneously occurring variations (Mc Coy, 1987a). Where
mutagenesis was employed, it was not found to increase the recovery rate of NaCl tolerant cell lines, except the report by Gosal and Bajaj (1984) in certain grain legumes. Secondly, even though tolerant cell lines were selected in many plants, the capacity for regeneration diminished rapidly with time in culture. Finally, very little is known about the factors (physiological, biochemical and molecular) that are responsible for the variations developed. As these aspects are equally and crucially important, special emphasis has been given in the present investigation to study them in detail through neatly designed experimentation.

**In vitro mutagenesis**

As one of the primary goals of this study was to enhance the recovery rate of tolerant lines, various mutagenesis techniques were used to create additional variability. For this, gamma irradiation as well as treatment with acridine orange and EMS were employed. A comparative assessment of the response obtained in terms of tolerance to the salinity stress after mutagenesis is being discussed here.

Calluses after irradiation/mutagen treatments were transferred to fresh media and their sensitivity to different doses/concentration was tested. The treated cultures were transferred to fresh media, immediately after exposure for their better survival. It is well established that radiation can produce chemical changes in culture media, especially alterations of the sugar components (Ammirato and Steward, 1969; Bajaj, 1971). It was observed that calluses treated with lower doses of radiation viz. 10 Gy showed an increase in fresh weight in comparison with control, though further increase in the radiation doses arrested the growth. With EMS/acridine orange treatments though no such increase in
F.wt was observed at lower doses the deviation from control was also not significantly less. It is apparent that the marked increase in F.wt at 10 Gy and other exposures was the direct consequence of growth stimulation at the lower doses. Stimulation of growth by lower dose of mutagen has been reported. Exposure to low dose of gamma radiation stimulated callus growth of Phaseolus vulgaris (Bajaj, 1970). Low dose gamma irradiation of ovular callus of Citrus sinensis stimulated embryoid formation whereas doses at 28-32 Kr were proved to be lethal (Speigal-Roy and Kochba 1973). Similar observations were also reported in tissue cultures of Nicotiana tabacum (Hell et al., 1978), Datura innoxia (Jain et al., 1984) and Starria italica (Reddy and Vidyanath, 1990). In tomato irradiation doses between 500-1000 r caused significant increase in the weight of fruit (Sidrak and Suess, 1973)

Higher doses of exposure consistently decreased the F.wt. F.wt drop due to mutagen treatment comparatively was maximum in the EMS treated calluses. This is not surprising since EMS is considered as one of the most powerful mutagens known. It is classified as a DNA reactive chemical which add methyl or ethyl groups to the ring of DNA, thereby altering the genetic code directly (Watson et al., 1987).

At sublethal levels of exposures, only very small percentage of cells survived. They were in general characterised by slow growth and appeared as patches among black necrotic cells. These cells were made to proliferate in a fresh medium. This not only enhanced the number of variants but also helped the cells to recover from the initial shock. Lindsey and Jones (1989) postulated that a period of several days between mutagenesis and selection would enhance the induced mutation to proliferate and become fully expressed. As a result of this,
enough tolerant cells may be present in the population to meet the minimum density requirement (Dix, 1986). Thus, in the present work, the enhancement in tolerance level observed among the mutagenised calluses probably is due to this reason.

**Selection of NaCl tolerant callus lines**

The selection process used in the present investigation was the 'short-term' one step procedure of Mc Hughan and Swartz (1984). Here the cells were plunged directly into the lethal concentration of salt instead of gradually imposing the stress. This method closely resembles the situation in the field, where seeds are planted directly into soil and therefore immediately encounter the saline environment. According to Mc Hughan and Swartz (1984) and Chandler and Vasil (1984), the gradual imposition of the stress will be ineffective because it will more readily select for physiological adaptations.

The response of treated (mutagen) and untreated calluses to NaCl stress was assessed both qualitatively and quantitatively. Qualitatively the growth, compactness and browning of the calluses were highly affected by the concentration of the added NaCl. With an increase of NaCl concentration from 00-470 mM, there was a decrease in fresh weight followed by an increase in the browning and necrosis of the callus. The cells growing in high levels of NaCl were isolated and subcultured for 3 passages in the absence of selection pressure (NaCl). Quantitatively, the T.I% of the various lines indicates that in all the experiments, the ST line grew better than the non-selected (SO) line in presence of NaCl. Though the biomass as well as T.I% were extremely less in SO line, a high T.I of 95-110% was recorded in the mutagen treated calluses.
From these data, several conclusions can be drawn. Firstly, unlike the previous reports, the variation induced through induced mutagenesis enhanced the NaCl tolerance capacity of the cells. Kochba et al., (1982) observed that for selecting stable salt-tolerant callus cell lines in *Citrus sinensis* and *C. auranntium*, irradiation prior to exposure to salt gave no selective advantage. However, the untreated cells retained increased tolerance for salt even after 3 consecutive transfers in medium without salt. Similar ineffectiveness of mutagenesis was recorded in *Nicotiana tabacum* (Nabors et al., 1975) and *Solanum tuberosum* (Van Swaaij et al., 1986). However, when the cell suspensions of *Cicer arietinum*, *Pisum sativum* and *Vigna radiata* were treated with EMS (0.25%) for 2-4 hrs, it increased the efficiency of the salt tolerant colonies (Gosal and Bajaj, 1984). Tolerance of NaCl levels as high as 3% was recorded in such cells whereas in untreated cells, NaCl concentrations beyond 0.5% caused browning and necrosis. This, and the observation of higher NaCl tolerance through mutagenesis in the present investigation, suggests that NaCl tolerance capacity of cells can be effectively increased through various mutagenesis techniques.

Secondly, a tolerance index of 95 and above 100 is a good indication that the selected line is better adapted and can grow better than the SO cells exposed to NaCl. Additionally, this may also be an indication of the selected line shifting towards a halophytic behaviour as the growth of ST callus was stimulated by concentrations of NaCl up to 230 mM. Similar observation were also made in species like *Citrus auranntium* (Ben-Hayyim and Kochba, 1983), *Lycopersicon peruvianum* (Hassan and Wilkins, 1988), *Medicago sativa* (Rains et al., 1980) and *Catharanthus roseus* (Vazques-Flota and Loyla-Vargas, 1994).
Lastly, on re-exposure to the sublethal level of NaCl (after 3 passages in a NaCl free media) the selected line exhibited good growth and survival, indicating that the variation achieved is stable. Several workers have reported loss of tolerance after passages in stress free media. For instance, no stability was found when similar test was applied to a selected NaCl-tolerant tobacco cell lines or selected polyethylene glycol introduced water stress resistant tomato cells (Hasegawa et al., 1980; Bressan et al., 1981). It is presumed that the stability observed in the present investigation might be due to the enrichment of cell population with pre-existing mutated cells (induced or natural) having stable tolerance to enhanced levels of NaCl.

According to Kochba et al. (1982), the salt tolerant selected lines are of agronomic value only if the tolerance is maintained in all the stages of development. Thus the ultimate proof for isolation of salt tolerant variant is the regeneration of salt tolerant plants from the tolerant cell lines and the demonstration of the sexual inheritance of the tolerance (Tal, 1990). Plants were regenerated from the tolerant callus in 3 of the experiments (untreated, gamma and acridine orange). For this, salt tolerant calluses were transferred to a regeneration medium containing 2 mg/l each of BA and KIN supplemented with 500 mg/l PVP. The subcultured calluses sprouted 2-4 shoots over a period of 25-30 days in this medium. The shoots thus raised were transferred to a normal rooting medium (1/4th MS+ 2mg/l IBA) for rooting. However, the shoots regenerated from the gamma treated and acridine orange treated calluses exhibited better rhizogenesis only in presence of NaCl. The rooted plantlets after hardening in sugar free MS basal medium were transferred to vermiculite. Similar regeneration of plantlets from stable callus was achieved only in a few
species like *N. tabacum* (Nabors *et al.*, 1975), *Oryza sativa* (Yamada *et al.*, 1983), *Kichxia ramosissima* (Mathur *et al.*, 1980), Colt cherry (Ochatt and Power 1989). The practical difficulty in achieving regeneration from selected callus as pointed out by several workers is the long duration/passages involved in the selection process, owing to which regeneration potential gets gradually diminished (Tal, 1990; Buiatti and Morpurgo, 1990). In addition to this, mutation treatments also reduce the morphogenetic potential (Cocking, 1989). However in the present study, this problem was overcome through the effective usage of PVP. The role of antioxidant PVP in preventing phenolic browning and thereby enhancing the morphogenic potential of the calluses is discussed elsewhere in the thesis.

Though plants could be regenerated in 3 of the experiments it was not possible to demonstrate the sexual transmittance of the trait as the plant could not be grown to maturity in the gamma and acridine orange treatment experiments. The plants regenerated in the first experiments were sterile and did not develop flowers. However, some of the features in the shoots regenerated from gamma and acridine orange treated calluses were typical. Both the lines showed better rooting in presence of NaCl. This closely resembles the reports in *Vigna radiata* where shoots rooted on medium containing 25 and 50 mM NaCl was greater than control shoots rooted in NaCl free-rooting medium (Gulati and Jaiwal, 1993). Salt extrusion was also noticed from the basal portion of the shoot regenerated from the gamma treated callus. Salt extrusion from leaf and stem is a feature of certain halophytes to maintain a constant salt concentration within the tissue (Salisbury and Ross, 1992). Thus, even though it was not possible to show the genetic inheritance of the trait,
these features are good indication of possible shift to halophytic nature by the selected line.

3. Biochemical analysis

Despite the wealth of literature documenting the occurrence of somaclonal variation in salt stress, few experiments have rigorously explored the factors which might regulate the variations produced. The main physiological and biochemical traits contributing to the acquisition of tolerance to salt stress are associated with different levels of organization. Hence resistance is not conferred by a single factor (Yeo and Flowers, 1986). The intent of this investigation was to provide a comparative idea of interactions of various such factors in the tolerant lines so as to suggest the mechanism of tolerance to the NaCl stress in an important cultivar of tomato.

In the ensuing discussion, ‘SO’ line refers to the stock callus while ‘ST’ line to the callus selected after 3 passages in NaCl free medium. Both calluses were exposed to different levels of NaCl and the biochemical parameters were assessed in them. ‘SO line in the in vitro mutagenesis experiments refers to the respective calluses which survived the sublethal dose of mutation. This was also exposed to different levels of NaCl and compared with respective ST lines.

1. Total proteins, amino acids and proline

Protein synthesis is an essential metabolic event required for cell survival and growth. In tomato c.v. Sakthi, both S0 as well as ST lines showed decreasing levels of proline content with increase in NaCl in all the 4 experiments. However amino acid content increased with stress in both the lines, with maximum levels shown by the ST line
selected through gamma treatment. According to these data the increase of amino acid content could be the result of proline hydrolysis. Increase in AA content with concomitant decrease in proline level was earlier observed in *Lycopersicon* calluses exposed to NaCl (Perez-Alfocea *et al.*, 1994). Similar observations were made in plants like *Medicago sativa* (Irigoyen *et al.*, 1992), *Pennisetum americanum* (Das *et al.*, 1990) and *Zea mays* (Thakur and Rai, 1982). NaCl, on the other hand was reported to suppress the synthesis of RNA and proline and reduced the number of cells replicating DNA (Nieman and Poulsen, 1971). According to Ram and Nabors (1985), such suppression of essential metabolites subsequently leads to growth inhibition. However the suppressive effect probably was overcome by the rise in amino acid contents in the calluses of the present cultivar.

The marked increase in amino acid pool is thought to be the result of the selective accumulation of certain intra-cellular amino acids especially the proline (Stewart and Lee, 1974). However, recently increase in other amino acids like aspargine, leucine and valine also are reported (Lehle *et al.*, 1992). Proline accumulation in SO and ST lines exposed to graded concentration of NaCl shared one or two common features. In all the 4 experiments ST lines always accumulated higher levels of proline. Maximum value is recorded in the ST line selected through gamma treatment. However SO line in this experiment also accumulated some amount of proline. Free proline accumulation in response to salt or osmotic stress was reported in several higher plants (Chandler and Thorpe, 1986, Delauney and Verma, 1993, Kavikishore *et al.*, 1995). Accumulation of proline is due primarily to *de novo* synthesis (Rhodes *et al.*, 1986; Voetberg and Sharp, 1991). In addition to acting as an osmoprotectent (Kavikishore, *et al.*, 1995), proline also serves as a sink for energy to
regulate redox potentials (Saradhi and Saradhi, 1991) and also to provide a store of nitrogen/respiratory substrate to facilitate post stress recovery (Aspinall and Paleg, 1981). However, it is still a matter of controversy whether proline accumulation reflects a regulatory process or it indicates damage (Vartanian et al., 1992). In the present work, ST line showed satisfactory growth and accumulated maximum amount of proline on exposure to sublethal levels of NaCl. This indicates that proline accumulation is not symptomatic of stress injury, but accompanies survival and growth in saline environment. A similar conclusion also was derived by Panday and Ganapathy (1985) in *Cicer arietinum*.

The accumulation of proline in SO line is consistent with the reports in *Nicotiana sylvestris* (Dix and Pearse, 1981) and *Vigna radiata* (Gulati and Jainwal, 1993). One explanation for this could be that eventhough growth was inhibited in this line, production of osmoticum still proceeded and that proline was far more important here than it was in the tolerant (ST) callus. In the ST line, other solutes or ions might be utilized in addition to proline (Handa et al., 1983). Whatever the cause of enhanced levels of proline in the SO line, it is clear that proline accumulation was not confined to tolerant callus alone.

**Carbohydrates**

A comparative analysis of the impact of NaCl on the various carbohydrate reserves has shown that soluble sugars increased where as reducing sugars and starch decreased with increase in stress. Generally, the total carbohydrate levels decreased with stress in both S0 and ST lines. The decrease was more prominent in the S0 line.
Soluble sugar content of the ST line was maximum in the 1 experiment, where as the least was recorded in the ST line selected through EMS treatment. It was suggested that this accumulation of soluble sugars is due to the decreased respiration rate which accompanies high salinity levels (Lamberts, 1985). The high accumulation of S.S noticed in the ST line is also connected with the proline accumulation in that line. It is well established that high sugar levels inhibit proline oxidation (Stewart, 1981) and carbohydrates are necessary to supply hydrogen or reducing power for proline synthesis (Stewart, 1978). It is presumed that the great difference noticed between the sugar contents in regenerated plants and ST lines was due to the abundant supply of sugars in the culture medium (Dracup, 1991; Perez- Alfocea et al., 1994).

S.S accumulation in the ST callus lines of tomato c.v. Sakthi is in contrast with the report of Handa et al., (1983). They observed an increase in reducing sugars in tomato cells subjected to osmotic stress. In the present work, higher levels of R.S was noticed in the ST lines of acridine orange and EMS treatment experiments. Handa et al. (1983) have suggested that reducing sugars contribute most of the changes in the osmotic potential and osmotic adjustments in cultured cells adapted to low water potential. Nevertheless, it is speculated that accumulation of these solutes provides advantage to the dividing cells through turgor maintenance and there by sustained growth (Morgan, 1984).

The reduced accumulation of starch content was more prominent in the ST lines. Much higher values were recorded in the SO lines. A similar situation was noticed in the salt sensitive and salt tolerant calluses of Citrus sinensis (Libal-weksler et al., 1994). Here, salt sensitive cells were characterised by slower rate of growth and high starch
accumulation than the salt tolerant lines. A possible reason for the reduced carbohydrate accumulation in ST line is the feedback effect caused by the salt stress (Hall and Milthorpe, 1978).

Pigments

In the present investigation, a considerable reduction in the photosynthetic pigments was induced by increasing levels of NaCl in both SO and ST lines in all the experiments. At high NaCl levels, the reduction in chlorophyll content was lower in the ST line than the SO line. Chlorophyll ‘a’ content was mostly affected, whereas chlorophyll ‘b’ and carotenoid levels, though less, did not show much variation among the treatments. The difference between chlorophyll ‘a’ and ‘b’ is consistent with the possibility that chlorophyll ‘b’ synthesis proceeds independently of chlorophyll ‘a’ synthesis (Khon et al., 1967).

Among the ST lines, maximum chlorophyll ‘a’ content was recorded in Exp I and least in acridine orange treatments (Exp III). The inhibitory effect of NaCl on pigments is reported in plants like Oryza sativa (Shimose, 1973), Phaseolus vulgaris (Heikal et al., 1979), and Hordeum vulgare (Kalaji and Nalborezyk, 1991). The reduction in pigment biosynthesis due to salt stress could be attributed to structural changes in photosynthetic apparatus (Hamada and El-Enany, 1994). Strognove (1973) opined that salinity may affect the forces binding the pigment-protein-lipid complex in chloroplast structures. Salama et al., (1994) correlated the low chlorophyll degradation in tolerant lines with its high capability to maintain intra-cellular Mg²⁺ concentrations. They observed that on exposure to 200 mM NaCl, several sensitive lines in wheat showed swelling of chloroplast membranes while it had little effect on tolerant ones. Further investigation carried out by Murota et al. (1994) revealed
that thylakoid membrane of the NaCl adapted cells had higher oxygen evolving activities and were more tolerant to high concentrations of NaCl than the unadapted cells.

Ascorbic Acid, Phenol and Phenylalanine ammonia lyase

Ascorbic acid level was significantly higher in ST lines selected through mutagen treatments. Maximum value was recorded in the EMS treated ST line. This is in consistence with the report that ascorbic acid is a radioprotectant (Conger, 1975) and it reduces the potentially harmful oxidized products formed by the radiolysis of water molecule in the cell wall (Levitt, 1980). Increased levels of ascorbic acid was recorded in pearl millet calluses exposed to 1% NaCl (Das et al., 1990). They presumed that ascorbic acid provide the callus cells with enhanced osmotic value as well as reductants for sustenance of growth under salt stress conditions.

Total phenol content, though increased with stress in both SO and ST lines, the levels were much higher in the SO lines. The comparative low level of phenols noticed in the ST lines is probably due to the increased growth rate (Tolerance index) in those lines, as phenolic accumulation is always noticed in association with cellular damage. It was reported that phenols constitute a part of cellular solutes and provide a reducing environment to the system (Das et al., 1990). It was also reported that salt stress exerts its effects through membrane peroxidation, which indicate that oxygen free radicals are formed (Halliwel, 1978). The rise in the phenol content therefore is due to an increase in the oxidative atmosphere of the callus lines (SO and ST) exposed to NaCl. Since oxidative damage is more observed in SO line, phenol content was also
maximum in that line. It is presumed that phenol accumulation could be a cellular adaptive mechanism for scavenging oxygen free radicals during stress. The compounds like phenols and ascorbic acid could readily get oxidised in the cellular system of the callus tissue, preventing subcellular damage (Das et al., 1990).

The PAL enzyme activity is often correlated with biotic stress (Nagarathna et al., 1993). In the present work, PAL activity showed a strong correlation with that of phenolic content. Similar to phenols, maximum PAL activity was also noticed in the S0 lines. PAL, the first enzyme of phenyl propanoid metabolism, plays a significant role in plant as a response to external stimuli (Lawton et al., 1980). When the PAL activity was compared with the accumulation of phenolic materials it became apparent, that maximum rate of phenol synthesis generally followed the development of maximum enzyme activity. It may be that formation of free oxygen radicals as a result of membrane peroxidation due to salt stress might trigger the biosynthesis of PAL enzyme, which eventually results in the phenolic accumulation.

Antioxidant enzymes

The data from the present study indicates a strong correlation between antioxidant activity and ability of tomato calluses to grow on media amended with NaCl. The high superoxide dismutase activity in the ST lines selected through gamma and EMS treatments is consistent with speculations of Gossett et al. (1994) that the more salt tolerant callus tissue has a higher superoxide ($O_2^-$) scavenging and dismutating capacity than the unselected lines. It is well established that superoxide diamutases are a group of metallic enzymes that catalyse the disproportionation of
superoxide (O$$_2^\cdot$$) to Hydrogen peroxide (H$$_2$$O$$_2$$) and O$$_2$$ (Fridovich, 1986). In the present work simultaneous with the increase in SOD activity, catalase, peroxidase and ascorbate oxidase enzyme activities also increased in the ST lines when grown in higher levels of NaCl. On the other hand, none of the enzyme activities increased significantly when the SO line from these experiments were grown in increasing levels of NaCl. At the whole plant level (regenerated plant), the enzyme activities were higher than the SO line. The NaCl induced enhancement of peroxidase, catalase and ascorbate oxidase in the salt tolerant lines indicates that the more salt tolerant cultures has higher capacity for the decomposition of H$$_2$$O$$_2$$ generated by SOD. This is because catalase, ascorbate oxidase (Chen and Asada, 1989) and variety of general peroxidases (Chang et al., 1984) are known to catalyse the breakdown of H$$_2$$O$$_2$$ generated by SOD (Salin, 1991). This induction of SOD and the subsequent rise in PRX, CAT and AO in the NaCl tolerant cells as well as in the regenerated plants are in agreement with the findings in *Gossypium hirsutum* (Gosset et al., 1994), *Pisum sativum* (Hernandez et al., 1995) and *Citrus limon* (Piqueras et al., 1996). Siegal (1993) correlated the putative role of these enzymes in preventing the oxidative damage as a result of the stress. According to him, SOD may function as the first line of defense against oxidation at membrane boundaries, but its end product is H$$_2$$O$$_2$_. Catalase, though rapidly destroys the peroxide, is a large molecule which limits its diffusional mobility. However, peroxidase provides an efficient mechanism for removing the peroxide. Thus the enzyme together sets up an effective defensive barrier against the reactive oxygen species and thereby preventing the oxidative damage. This would ultimately lead to the enhanced NaCl tolerance.
Amylase, invertase and protease

A comparative analysis of the activity of starch degrading enzymes like amylases and invertases have shown a considerable variation among the salt tolerant (ST) and the S0 lines. It was observed that amylolytic as well as invertase activities were maximum in the salt tolerant line selected through gamma treatment, whereas comparatively lower values were recorded in the S0 line. Invertase activity was substantially low in the regenerated plant. The high activity of amylase in the ST line, probably is one of the reasons for the low accumulation of starch in that line. This is because mobilisation of carbohydrate reserves are essential for the adaptation process.

The S0 lines, on the other hand, accumulated starch at high concentration of NaCl and showed a lesser enzyme activity. This is in agreement with the results obtained in salt tolerant and salt sensitive calluses of *Citrus sinensis* where maximum amylolytic activity was noticed in salt tolerant line exposed to 01M NaCl (Libal-weksler et al., 1994). The decline in invertase activity noticed in the S0 lines is consistent with the observations in bush beans and maize (both salt sensitive plants) where the enzyme activity decreased with salinity (Rathert, 1982, Hawker and Walker, 1978).

Another enzyme which showed increased activity in the ST lines was protease. In the present work, maximum protease activity was noticed in the salt tolerant lines selected through EMS treatments. The activity of enzyme was low in the S0 lines. Enhancement of protease activity under salanisation has also been noticed in mung bean (Sheoran and Garg, 1978) and rice (Dubey and Rani, 1990). It has been suggested that
salinity affects the activity of hydrolytic enzymes and increases the hydrolysis of macromolecules like proteins (Reddy and Vora, 1985).

Ions

One of the reasons accorded for salt damage in plant is the toxic effect caused by specific ions (Heyser and Nabors, 1981). In tomato cultivar Sakthi, SO lines in all the experiments significantly accumulated Na\(^+\) and Cl\(^-\) where as in salt tolerant lines (ST), reduced values were recorded. The reduced growth rate noticed in the SO lines can probably be related with the accumulation of Na\(^+\) and Cl\(^-\) as a consequence of the competition with nutrient ions (Levitt, 1980). Increase in Na\(^+\) and Cl\(^-\) as a response to NaCl salinity was reported in Triticum aestivum (Begum et al., 1992) Sapindus trifoliatus (Unnikrishan et al., 1991) and several species (Ram and Nabors, 1985).

In the present work, however, K\(^+\) content was maximum in the ST lines, especially those selected through gamma treatment. It was observed that at low concentrations, Na\(^+\) may activate an increase in uptake of K\(^+\) (Nimbalker and Joshi, 1975). It may be that salt tolerant lines have an inherent capacity to exclude Na\(^+\) to a considerable extent whilst maintaining high K\(^+\) levels as observed in Solanum melongena (Jain et al., 1988) and Vigna radiata (Gulati and Jaiwal, 1993). An enhanced K\(^+\) accumulation with increase in salinity can be regarded as trait of adaptive value under salt stress (Kawasaki et al., 1983; Walker, 1986; Trivedi et al., 1991). Levitt,(1980) reported that in Phaseolus vulgaris, Pisum sativum and Citrus aurantiun growth inhibition due to salt stress was overcome by K\(^+\). Similarly in the leaves of Vitis vinifera, the preferential accumulation of K\(^+\) over Na\(^+\) was reported to inhibit the toxic effects caused by Na\(^+\) (Downton and Loveys, 1981). Gulati and Jaiwal (1993)
speculated that K⁺ apart from involvement in various biochemical aspects may contribute to osmotic adjustments of *Vigna radiata* cells.

The ability of leaf segments (from the regenerated plant) to maintain high levels of Cl⁻ in presence of moderate levels of K⁺ was earlier observed in tomato (Tai *et al.*, 1978). This indicates a considerable difference in the ion accumulation pattern of the organised tissue and that of in calluses. Nevertheless, the ability of the selected tolerant cell lines to maintain high K⁺ in the presence of Na⁺ is a characteristic which suggest the tolerance was the consequence of a shift towards a halophyte nature similar to the apprehensions of Croughan *et al.* (1978) and Jain *et al.* (1988).

**Isozyme studies**

Peroxidase catalyses the oxidation of various substrates in the cell at the expense of H₂O₂ (Dange and Reddy, 1984). Under stress conditions, plants produce large amount of peroxidase and is often the first enzyme to alter its activity after stimulation (Gasper *et al.*, 1985, Siegal, 1993). In the present investigation, a considerable increase in peroxidase activity was noticed in the ST line in all the experiments in presence of increasing levels of NaCl. Isozyme phenotypes of the salt tolerant lines were considerably different from the control cell lines.

Although many functions have been postulated for peroxidases, identifying physiologically relevant roles in plant metabolism for specific peroxidase isozyme has been difficult (Van Huystee, 1987; Bronner, *et al.*, 1991). The role frequently cited for peroxidase is the catalysis of terminal steps in lignin biosynthesis (Grisebach, 1981, Lagrimini, *et al.*, 1987). Salt stress tends to stunt growth presumably
through early lignification (Chang et al., 1984, Subashini and Reddy, 1990). Increase in peroxidase activity parallel to an increase of lignin like compounds in the cell wall was earlier observed in NaCl adapted tomato suspension cells (Sancho et al., 1996). As peroxidases are responsible for synthesis of polymers like lignin in cell wall as well as prevent peroxidation, the increased activity of peroxidase in salt tolerant lines might be a useful adaptation under saline conditions. The significant alterations noticed in mutagen treated calluses in the present study, may also be due to a high concentration of toxic peroxy radical produced by mutagens as suggested by Jain et al (1990).

In the present study, salt treatment resulted in the appearance of new isoenzymes and increased the intensities of some of the pre-existing bands in the ST lines and in the regenerated plants. This is consistent with the high peroxidase activity (total enzyme activity) noticed in these lines. As opined by Gossett et al. (1994), it is not clear or proved whether the increase in peroxidase activity was due to an upregulation of genes controlling enzyme or increased activation of the enzyme pool. Studies in Pisum sativum showed the development of new superoxide dismutase isoenzymes in the salt stressed NaCl tolerant calluses whereas in the NaCl sensitive cells, this enhancement was not observed (Olmos et al., 1994). Hence it is quite possible that the salt induced isozyme phenotype observed in ST lines of tomato c.v. Sakthi may also be due to changes in the activity of the levels or changes in the activity of the enzyme through modifications.

**Esterases**

Esterases include a group of enzymes consisting of a host of ester hydrolyases. Cubbada and Quattrucie, (1974) reported that
esterase isozymes that act up on α and β-napthyl acetate (the substrate used in the present work) are carboxyl esteras. Very little information is available on its behaviour and role in NaCl stress or any other stress conditions. However, due to the degree of multiplicity and diversity of the physiological functions in the cell, they are convenient models for studying changes in their isoforms (Yurenkova et al., 1995). The result of the present work indicated a significant difference among the isoesterase patterns of SO and ST lines. The hydrolytic enzyme like esterases are considered to be lysosomal in nature (Sharma et al., 1979). Lysosomal enzymes also hydrolyse the stored reserves like starch, proteins and sugars; peptides and amino acids thus derived are utilized in the metabolism of the cell (Chaffey and Harris, 1985). The high activity of isoesterases in the ST line can be correlated to the increased hydrolysis of macromolecules which probably help the cells to tolerate the high levels of NaCl in the medium. The banding pattern of the regenerated plants in the acridine orange and gamma treated materials showed a close similarity with the ST calluses from which they were regenerated, except for the absence of 1-2 bands in the former. This may be due to the inactivation of genes involved in the synthesis of these isozymes during regeneration which is similar to the report in Plantago ovata (Parmanik et al., 1996).