SUMMARY, CONCLUSIONS AND FUTURE LINES OF WORK

Investigations on *in vitro* selection and characterization of salt-tolerant variant callus line were carried out in an economically important grain-legume - *Vigna radiata*. The lack of large scale plant regeneration ability from cultured cells of this plant has been considered as one of the major problems in assessing the utility of tissue culture for the recovery of resistant variants. Therefore, simple and reproducible protocols for efficient plant regeneration from different seedling explants, i.e. cotyledon, shoot tip and cotyledonary node with or without cotyledons of *Vigna radiata* cv. K-851 have been developed. These protocols were found applicable to other mungbean cultivars. Complete plants were regenerated directly, without an intervening callus phase from cotyledons, shoot tips and cotyledonary nodes on basal medium. Regeneration potential of these explants, however, varied with genotype, size, orientation and age of the explants and the different plant growth regulators combination in the medium. Cotyledon and shoot tip explants excised from 2- and 7-d old seedlings, on 'C' (MS salts + B_5 vitamins) basal medium + BAP (5×10^{-6} M) produced 9 shoots per explant in 60% and 100% of the cultures, respectively. Cotyledonary node with both cotyledon, excised from 4-d-old seedlings produced the highest number of shoots (6) in 100% of the cultures on B_5 basal medium containing 5×10^{-7} M BAP. Regenerated shoots from all the explants developed into flowering plants. Formation of multiple shoots from the studied seedling explants could be of practical application for raising hybrid seedlings of difficult crosses and mutagenesis *in vitro*. Histological examination of cotyledonary node explants suggests the shoots to be of *de novo* origin, which would make the system suitable for transformation experiments. Conditions were also standardized for induction, establishment and maintenance of callus derived from different vegetative and reproductive explants in different basal media supplemented with auxin and cytokinin at various concentrations. Leaf derived calli produced shoots in only 10% of the cultures. Further studies are needed to increase the efficiency of regeneration from truely dividing cells.

A comparison of the effect of NaCl on the growth and ion accumulation in the whole plants and in cells isolated from them shows that cells have the same tolerance to salts as the whole plants, suggesting that *V. radiata* appears to have a mechanism(s) for salt tolerance which operates at cellular level. Such a positive correlation between growth response of whole plant

and callus of *V. radiata* is a pre requisite not only for the successful application of callus tissue for screening the germplasm for salt tolerance, but also important for selection of salt-tolerant cell lines.

In vitro evaluation of NaCl-tolerance in wild and cultivated species of *Vigna* and 12 different cultivars of *V. radiata* shows that wild species, *V. vexillata* followed by *V. setulosa* are the most tolerant, compared even to the most tolerant cultivar G-65 of the most salt-tolerant cultivated species, *V. radiata*. The wild species accumulate more Na^+ and K^+ than the cultivated species.

For the selection of salt-resistant callus lines of *V. radiata*, two selection strategies, i.e. direct and indirect selection, were used. Salt-resistant callus line was isolated as a spontaneous variant by exposing 25 $\pm$ 2 mg fresh weight callus pieces (obtained after one subculure from leaf explant) on agar solidified PC-L2 medium supplemented with growth regulators and 300 mol m^{-3} NaCl (EC = 22 ds m^{-1}, ψ = -19 bars), a concentration inhibitory to wild type cells (direct selection). This line is a true variant as it retains its tolerance after subculture for 3 passages of one month each (3 month), on NaCl- free medium. The salt-resistant line grew significantly better than sensitive line at all the levels of NaCl. However, the growth of selected line on 300 mol m^{-3} NaCl was not comparable to that of sensitive line growing in the absence of NaCl.

Cross tolerance of selected line was tested by exposing it to various ionic and osmotic stresses. The growth of NaCl-resistant line was significantly higher than sensitive line under KCl stress. However, both the lines responded similarly to Na_2SO_4 and salt mixture at concentrations higher than 100 mol m^{-3}.

The growth of calli of both the cells lines was less severely inhibited by osmotic stress induced either by mannitol (iso osmotic to NaCl) or PEG (0-20%) than salt(s) suggesting that in latter case(s) it was affected by ion(s) toxicity. The growth (in terms of dry weight) of NaCl-resistant line in mannitol was similar to that of sensitive line, suggesting that the superiority of NaCl-resistant line under NaCl stress was not due to osmotic stress tolerance. However, resistant line showed no advantage to osmotic stress induced by PEG. It seems that during prolonged selection for NaCl resistance, cells acquired resistance to specific ion effect and an adaptation to osmotic stress has not occurred.

In indirect selection strategy, cultured cells of *V. radiata* selected for resistance to osmotic stress induced by absorbable (mannitol 540 mol m^{-3}, iso- osmotic to 300 mol m^{-3} NaCl) and non-absorbable (PEG, 20%, ψ = -12 bar) osmoticans have shown incerased resistance to salt (upto 300 mol m^{-3}) (-19 bar) and 100 mol m^{-3} (-9 bar), respectively)) than unselected cells. Similarly, proline over-producing cells selected on 10 mol m^{-3} hydroxyproline (Hyp) were also found to be more tolerant upto 250 mol m^{-3} salt stress than unselected cells. High endogenous levels of K^+ and free proline of osmotically (PEG or mannitol) and Hyp resistant cell line appear to impart dual resistance to their respective selected agents and salt stress. However, a salt mixture resistant cell line isolated at salt mixture ($NaCl:KCl:Na_2SO_4$, 8:1:1) concentration equimolar to 300 mol m^{-3} NaCl was found to be more sensitive to NaCl than non-selected cells.

At present, we have no evidence whether the resistant cell lines isolated in the present study are real mutants or epigenetic variants. The ultimate proof of a true genetic variant lies in the regeneration of resistant plants and genetic analysis of their R_1 progeny. Unfortunately, our attempts to regenerates plants from selected cells were unsuccessful. However, NaCl-resistant calli produced only roots on modified PC-L2 medium with or without NaCl.

A third selection method employing cotyledon explants was based on their high potential for regenerating multiple shoots. Out of 850 explants cultured on medium containing 200 mol m^{-3} NaCl, three survived and each regenerated one shoot. The shoot selected on salt showed persistance of salt tolerance not only during their *in-vitro* multiplication through axillary buds in the absence of NaCl upto 3 months, but even their calli maintained on normal medium for 2 months also retained salt tolerance. The salt selected shoots were rooted on MS basal medium supplemented with IAA (5 x 10^{-6} M) and NaCl (200 mol m^{-3}). The regenerants flowered precociously but produced normal pods and viable seeds.

Both salt resistant and sensitive lines showed increase in internal Na^+ and Cl^- concentrations as the external NaCl concentration was increased. However, salt resistant line accumulated more Na^+ and Cl^- than sensitive line. The former line also maintained higher levels of K^+ despite high Na^+ in the medium and tissue, than the latter on all external NaCl levels. This trait is known to be highly correlated with salt-resistance in both halophytic bacteria

and halophytic plants. The sensitive line, on the other hand, showed a considerable decline in K^+ as NaCl was increased in the medium. It appears that salt-selected line do not exclude salts as the mechanism of tolerance, but it utilizes uptake of these ions for osmotic adjustment and enhancing turgor. Thus, the accumulation of Na^+ and maintaining K^+ levels could be the mechanism allowing better growth in salt-resistant line at all salinity levels.

Both the callus lines were analysed for various metabolites which accumulate during adaptation to salt stress. Both the lines showed increased in soluble proteins under NaCl stress. However, at similar levels of salt treatments, the salt-sensitive calli had non-significantly higher levels of protein than salt-resistant calli. The amount of soluble amino-nitrogen was also higher in sensitive than resistant calli. Thus, the increased accumulation of soluble amino-nitrogen under salinization can not be correlated with salt tolerance in *Vigna radiata*.

The resistant callus line showed satisfactory growth and accumulated greater proline on a medium containing 250 to 300 mol m^{-3} NaCl. These results indicate that proline accumulation is not symptomatic to stress injury, but accompanies survival and growth in a saline environment. On the other hand, sensitive calli whose growth was inhibited by high salt concentrations (250 to 300 mol m^{-3} NaCl) produced non-significantly higher amount of proline than resistant calli. One explanation for this could be that even though growth was inhibited in unselected callus, production of osmotica still proceeded and that proline was far more important here than it was in salt-resistance callus. An alternative explanation for enhanced proline level in sensitive callus could be that excess production was a symptom of stress damage. Whatever the cause of enhanced levels of proline in the sensitive callus, it is clear that proline accumulation was not confined to resistant callus, therefore, it can not be the sole mechanism of salt stress tolerance.

The reducing sugars accumulated in both the callus lines at all the salt levels after 14-d of growth, but this accumulation being higher in resistant than sensitive calli. On the other hand, sucrose content also increased in resistant calli during 4th week of growth. The amount of reducing sugars in both the callus line in the presence or absence of NaCl was higher than their sucrose contents. Thus, the accumulation of soluble sugars provide an advantage to the dividing cells through turgor maintenance and, thereby, sustained growth. The sugars are likely

to be the major osmoregulants in *Vigna radiata* under stress as the concentration of these compounds was much higher than the measured intracellular concentration of other metabolites.

The resistant callus line at inhibitory salt concentration maintained higher NR activity than sensitive line. the maintenance of high levels of enzymes can be a method for coping with salt stress. Salt stress also affect aminating and deaminating activities of GDH. The NADH-GDH activity (aminating) in the presence of NaCl was higher in resistant than sensitive calli. On the other hand, NAD-GDH activity in both the lines was completely inhibited after 7-d of growth. Thus, increased activity of NADH-GDH in resistant calli may play a vital role in protecting the cells from stress effect. NADH-GOGAT activity was found to decrease under salt stress in both the calli. Nitrogen assimilation in salt-resistant calli under salt stress was found to be characterized by high level of NR and NADH-GDH activity. Concomitantly with low GOGAT activity. During salt stress, when NO^-_3 reduction is occurring very efficiently and GOGAT is inefficient, NH^+_4 is probably assimilated by GDH pathway. However, whether the shift in enzyme level would also imply a shift in ammonia assimilation from GS-GOGAT to GDH pathyway during salt stress, cannot be predicted with certainty at this stage of study.

A comparison of one-dimensional SDS-PAGE polypeptide profile of sensitive and resistant callus lines in the presence or absence of NaCl shows that three polypeptides of 34.6, 45.7 and 79.4 kD are salt induced of which former two are detected in salt stressed calli of both the lines, while the latter is unique to salt stressed resistance calli. The synthesis five other polypeptides of 158.5, 138.0, 131, 69.2 and 41.6 kD appeared to be inhibited, while the amount of others of 12 to 31.6, 100 to 125.8, 57.4, 54.9, 44.6 and 43.6 kD decreased in resistant line under salt stress. Thus, the newly induced proteins as well as the ones whose synthesis is depressed by salt treatment, may be particularly important for salt-resistance.

The present work has been able to bring a few lines of investigations which need to be persued. These are:

(i) Characterization of Na^+-, K^+-stimulated ATP-ases;

(ii) Changes in membrane permeability and its composition.

(iii) The cause and effect relationship between the salt resistance and proline concentrations.

(iv) Identification, localization and characterization of salt induced proteins.

(v) Evaluation of salt resistance in R_1 progeny.