Discussion

DISCUSSION

Under the NaCl stress, the callus cultures of both rice varieties showed a reduction in their dry weights as compared to control. Similar observations have been made by Janardhan Reddy and Vaidyanath (1986) with embryo derived rice callus growing on increasing concentrations of NaCl. From the results it is clear that the callus of tolerant rice variety Bhoora rata showed less reduction in its dry weight as compared to the callus of susceptible GR_{11}. Guerrier and Bourgeais Chaillou (1994) reported that under NaCl stress relative growth rate of calluses derived from *Lycopersicon penellii* (tolerant) was higher than that of *Lycopersicon esculentum* (susceptible). Relative fresh weight growth of calluses of *Sorghum halepense* in response to increased salinity was greater as compared to *Sorghum bicolor* (Yang *et al.*, 1990). Growth inhibition by saline stress may be due to an increase in osmotic pressure in the culture medium due to the presence of increasing salt concentration (Chen *et al.*, 1980). Also cells exposed to saline stress encounter reduced water availability due to lowered water potential of growth media. In a saline medium, the cells have to acquire essential nutrients from a milieu with a preponderance of ions that are potentially toxic. In this ionic environment the good performance of a plant cell will require intracellular tolerance and/or specific acquisition of nutrients essential for normal metabolic functioning. The better growth of Bhoora rata may be due to its ability to maintain better internal osmotic regulation that favours uptake of water into the cell. It is observed that hydroxyproline ameliorated to some

extent the deleterious effect of NaCl and favour better growth of callus (of both rice varieties).

It is generally envisaged that a primary consequence of exposing plants to a high NaCl environment is the rapid accumulation of Na^+ and Cl^- in the cytoplasm of cells that are directly in contact with the saline environment. Since Na^+ and Cl^- lower the water potential of the external environment of plants salt adaptation must involve cellular mechanisms that facilitate osmotic adjustment, in order to re-establish turgor and growth. This is accomplished by utilization of these ions as osmotic solutes(Flowers *et al.*, 1977; Binzel *et al.*, 1987) or by compartmentalization of these ions in the vacvole (Binzel *et al.*, 1988).

In callus cultures of both rice varieties exposed to NaCl, a significant increase in the level of Na^+ was observed (a 20 fold and 23 fold rise in BR and GR_{11} respectively). In contrast to Na^+ the levels of K^+ registered a reduction in the salinized cultures. BR salinized callus exhibited 1.4 fold decrease while GR_{11} salinized callus exhibited 1.9 fold decrease in their K^+ content as compared to their controls at the end of fourth week. Increased accumulation of Na^+ with concomitant drop in the level of K^+ under saline condition has been reported in callus cultures of *Brassica campestris* (Paek *et al.*, 1988); *Vigna radiata* (Gulati and Jaiwal, 1992) and in cell suspension cultures of *Kosteletzkya virginica* (Blits *et al.*, 1993).

Na^+ is known to cause maximum salt toxicity out of all the inorganic cations. Toxic levels of Na^+ disturb normal physiological and biochemical processes

associated with growth. Na^+ is also known to control the uptake of K^+ ions. At low intercellular concentrations Na^+ is shown to enhance and at high concentrations block the uptake of K^+ in rice. The observed decline in the growth of callus cultures (exposed to NaCl) may be due to high Na^+ concentrations. It is seen that the tolerant variety as compared to the susceptible variety accumulated less of Na^+ and maintained better levels of K^+.

This indicates that BR variety has either a special mechanism for preventing excess uptake of Na^+ ions during stress or a mechanism for preferential removal of excess intracellular Na^+ ions. This may be one of the reasons for the better growth of BR callus under NaCl. Krishnamurthy *et al.* (1987) while studying the growth and yield performance of different rice cultivars under NaCl salinity in pot culture experiments found that sensitive cultivars accumulated higher level of Na^+ ions when compared with control. They also observed that the increase in Na^+ concentration was significantly reduced with a concomitant rise in K^+, Ca^{2+} and Mg^{2+} levels in tolerant varieties.

Hydroxyproline helped the cells of both rice cultivars in maintaining higher levels of K^+ (nearly the same as that of their controls) and lower levels of Na^+ under saline conditions. Apart from its role as an osmotic component K^+ is essential for the formation of starch, protein, photosynthate partitioning, and as an activator of a number of monovalent cation requiring enzymes (Epstein, 1972). Thus, it seems that by affecting the intracellular Na^+ and K^+ contents of the cells, hydroxyproline helps in better growth of the cells under NaCl stress.

In response to salinization the level of Cl^- increased in cultures of both rice varieties. However, at the end of fourth week, as compared to GR_{11}, BR callus showed less accumulation of Cl^-. Ben-Hayyim and Kochba (1983), while working with citrus cell lines observed that at a given external concentration of NaCl, tolerant cells accumulated less Na^+ and Cl^- than the salt sensitive cells. They concluded that salt selected citrus cell line probably tolerates elevated levels of NaCl by its partial avoidance. In the present study it is seen that hydroxyproline checks the accumulation of Cl^- ions in the cells to some extent; thereby protecting the cells against the deleterious effects of Cl^-.

Analysis of Mg^{2+} and Ca^{2+} content in the callus cultures of both varieties of rice revealed that the levels of both these ions were highest during the period of maximum growth. Under all treatments and throughout the growth periods, the tolerant BR callus contained higher levels of Mg^{2+} and Ca^{2+} than the susceptible GR_{11}. It was observed that under saline conditions, there was increase in the levels of both these ions. Pretreatment of the calli with hydroxyproline further increased the contents of these ions in calli exposed to both NaCl and hydroxyproline.

Calcium has been demonstrated to ameliorate the inhibitory effects of high salinity on nutrient transport in plants (Lauchi and Schuber , 1989). Calcium also plays an important role in preservation of plasma membrane integrity (Cramer *et al.*, 1987). An increase in the level of intracellular calcium in response to salinity stress has been reported in corn root protoplasts by Lynch and Lauchli (1988).

The relatively low content of Na^+ in the tolerant BR callus may be partly due to the high Ca^{2+} content. Inhibition of Na^+ uptake by a high Ca^{2+} concentration has been reported in a wide range of plant species and experimental conditions (Huq and Larher, 1984 : in cowpea ; Cramer *et al.*, 1989 in barley plants).

It has been observed that hydroxyproline favourably affects the Ca^{2+} contents of the cells. Calcium partly reduces vacuolar alkalinization associated with Na^+ uptake, by restoring the salt induced breakdown of the pH gradient across the tonoplast. A qualitative relationship between accumulation of Na^+ and vacuolar pH changes as affected by Ca^{2+} has been shown by Martinez and Lauchi (1993) in barley roots.

The increased calcium content, thus may be ameliorating to some extent, the inhibitory effect of NaCl on nutrient transport. This may be one of the reasons for better growth of the calli (pre-treated with hydroxyproline) under NaCl conditions.

An increase in proline level from second week onwards was observed under all treatments. As compared to control cells, salinized cells showed more content of free proline. An increase in proline level in response to NaCl has been reported by many workers in many plant species. Li (1990) reported that rice calluses growing under increasing NaCl concentrations in the medium exhibited more proline content. Janardhan Reddy and Vaidyanath (1986) also observed several fold increase in the proline content of salt-stressed callus of rice. Salt tolerant cell lines of *Cajanus cajan* maintained on NaCl showed 6 fold increase in

their proline levels (Prakash and Sarin, 1993). Proline accumulation is a characteristic feature of salt tolerant lines of citrus (Deng *et al.*, 1993), *Catharanthus roseus* (Vazquez Flota and Loyola Vargas, 1994) and tobacco (Zhou *et al.*, 1993).

Proline has been implicated in osmoregulation of cytoplasmic enzymes (Pollard and Wyn Jones, 1979) stabilization of proteins (Schobert and Tschesche, 1978) and membrane (Jolivet *et al.*, 1982) and also to provide a store of nitrogen or respiratory substrate to facilitate post stress recovery (Aspinall and Paleg, 1981). These unusual properties of proline may be rendering enhanced salt tolerance to the stressed cells. Proline overproducing cell lines have been reported to show better salt tolerance. Ricardi *et al* .(1983) showed better tolerance of proline overproducing mutants of *Spirulina platensis* and *Daucus carota*. Dix *et al.* (1984) succeeded in regenerating plants with increased salt tolerance from proline accumulating *Nicotiana sylvestris* cell lines. Moreover, other workers have found high proline contents in cell lines selected for better salt tolerance in *Nicotiana* (Watad *et al.*, 1983) and *Cicer arietinum* (Pandey and Ganapathy, 1985). In contrast to the wild type cells those of NaCl resistant strain were distinguished by NaCl induced proline overproduction (Shevyakova *et al.*, 1994).

Proline also acts as a osmoprotectant so as to prevent damage from cellular dehydration by balancing the osmotic strength of the cytoplasm with that of environment. Hydroxyproline exposed cells growing on medium containing both hydroxyproline and NaCl showed maximum proline production and better

growth with the BR callus showing nearly 5 fold increase in its proline content at the end of sixth week while GR_{11} callus showed 6 fold increase (as compared to their controls) . This higher endogenous level of proline in the cells may be imparting dual resistance to hydroxyproline and NaCl to the hydroxyproline selected cell line. Similar observation has been made by Gulati and Jaiwal (1993a) with hydroxyproline resistant callus lines of *Vigna radiata* wherein, the hydroxyproline resistant callus line showed a greater tolerance of NaCl than the wild type. Hydroxyproline resistant cell lines of *Solanum tuberosum* (Van Swaaij *et al.*, 1986) and sugarcane (Chen and Wang, 1991) also exhibited better salt tolerance .

Investigation by Boggess *et al.* (1978) and Sells and Koeppe (1981) revealed that oxidation of proline could be a mechanism in the regulation of cellular proline pool. Further under stress conditions, a decrease in proline oxidation has been shown to contribute to proline accumulation in spinach, barley and many other plants (Steward *et al.*, 1977; Steward and Boggess, 1978; Huang and Cavalieri, 1979; Naik and Joshi, 1986). Thus the elevated proline content discerned in salt stressed plants could be a reflection of NaCl induced inhibition of proline oxidase activity as well. In the present investigation, a decrease in the activity of proline oxidase was observed in callus cultures of both rice varieties under NaCl stress. A decrease in proline oxidase activity under salt stress has been reported by Rus Alvarez and Guerrier (1994) in callus cultures of *Lycopersicon*.

Hydroxyproline administration further reduced the activity of proline oxidase in the salt-stressed calli of both cultivars of rice. At the moment, no convincing

evidence is available to state that hydroxyproline regulates proline oxidase activity. However, from the present results it can be suggested that the increase in proline levels in hydroxyproline treated salt stressed calli could be, at least partly, due to reduced activity of proline oxidase.

The protein contents of the tissues of both rice cultivars rose sharply during the growth period. Treatment of tissues with hydroxyproline resulted in a 7.1 fold and 6.6 fold increase in the protein contents in case of susceptible and tolerant varieties respectively at the end of sixth week (as compared to 0 day). However, NaCl caused a significant reduction in the protein level of salinized BR and GR_{11} callus (35% and 75% respectively). According to Croughan *et al.* (1981), cells grown under stress may have to spend more metabolic energy than those grown in the absence of stress. This extra energy is most probably used up in regulating osmotic adjustment. The observed decline in dry weight and soluble protein content in presence of NaCl may be due to diversion of some quantum of energy from growth and metabolism (Janardhan Reddy and Vaidyanath, 1986). It is observed that BR callus when compared to GR_{11} showed less reduction in its protein content. This may be one of the reasons for its increased salt tolerance. Prior exposure of the callus to hydroxyproline resulted in nearly 45% and 58% increase in protein levels of BR and GR_{11} callus respectively, growing on salt and hydroxyproline containing medium (as compared to their salt controls at the end of the sixth week). This increase in protein level may be partly responsible for the observed better dry weight accumulation under stressed conditions.

Administration of NaCl into the medium reduced the titers of polyamines, namely

spermidine and spermine in the callus cultures of both rice cultivars. However, the callus of BR registered a higher level of polyamines compared to that of GR_{11}. In contrast to the level of spermidine and spermine, the putrescine level of salinized tissues showed a sharp increase. Accumulation of putrescine has been shown in plants under potassium deficiency (Klein *et al.*, 1979), magnesium deficiency (Smith, 1973), low pH conditions (Young and Galston, 1983), atmospheric pollutants (Priebe *et al.*, 1978; Weinstein *et al.*, 1986), salt stress (Shevyakova *et al.*, 1985; Friedman *et al.*, 1989), osmotic stress (Flores and Galston, 1984a,b; Turner and Stewart, 1988; Foster and Walters, 1991) and under low oxygen concentrations (Reggiani *et al.*, 1989). The observed increase in putrescine level may be due to its increased synthesis or decreased conversion to spermidine and spermine. Putrescine can be synthesized directly from L-ornithine by ornithine decarboxylase or indirectly from L-arginine by arginine decarboxylase via agmatine, which is, in turn, stoichiometrically converted to putrescine (Pegg, 1986; Slocum *et al.*, 1984; Tabor and Tabor, 1984). Spermidine and spermine are formed by the transfer of an aminopropyl moiety from decarboxylated S-adenosylmethionine to putrescine and spermidine, respectively.

Data on putrescine level show more accumulation of putrescine in salinized GR_{11} callus as compared to BR. A report by Di Tomaso *et al.* (1989) demonstrated that high putrescine levels can lead to membrane damage, probably as a result of the free radicals generated following the action of diamine oxidase on putrescine. During loss of viability either of cereal protoplasts due to osmotic

shock or during dark-induced senescence in leaves, a rapid increase in arginine decarboxylase and a massive accumulation of putrescine has been observed (Flores and Galston, 1984a). Higher levels of putrescine has also been found detrimental to thylaKoid membrane of osmotically stressed oat leaves (Besford *et al.*, 1993).

The salinized callus cultures of BR maintained higher levels of spermidine and spermine coupled with a low level of putrescine i.e. lower ratio of putrescine to polyamines (spermidine + spermine) as compared to salinized cultures of GR_{11}. It is suggested that high content of spermidine stabilizes membrane systems and prevents passive membrane transport in many plants (Smith, 1985). It is also observed that spermidine stimulates various processes associated with nucleic acid and protein synthesis and is present in high concentration in actively growing tissues (Slocum *et al.*, 1984).

Many of the biological functions of polyamines appear to be attributable to their cationic nature. They are highly protonated at physiological pHs which should favour electrostatic binding of polyamine to nucleic acid and negatively charged functional groups of membranes and proteins (Slocum *et al.*, 1984). Thus polyamines could bind to the negatively charged phospholipid head groups on membranes, thereby conferring greater stability to membrane bilayers and protecting it from disruption under stress condition (Slocum *et al.*, 1984; Guye *et al.*, 1986). Support for the binding mechanisms is that addition of Ca^{2+} counteracts the ability of polyamines to prevent chlorophyll loss from thylakoid membranes in senescing leaves (Kaur Sawhney and Galston, 1979; Srivastava

and Smith, 1981). It has been shown by Sechi *et al.* (1978) that polyamines inhibit the activity of phospholipases, the inhibitory effect being highest with spermine and lowest with putrescine. The suggested mechanism of inhibition was that of steric hindrance of enzyme substrate interaction as a consequence of binding of the polycations to the membranes. Phospholipase action may result in the dislodging of proteins from the membrane making them more vulnerable to proteolytic attack. Manipulation of polyamine levels has been shown to inhibit the rise in protease activity (Altman, 1982; Kaur-Sawhney and Galston, 1979), thereby preserving membrane integrity.

Exposure of cells to hydroxyproline resulted in higher polyamine titers (spermidine and spermine) and lower level of putrescine in salinized calli of both rice cultivars. Thus, it appears that spermidine and spermine play an important role in preserving the integrity of membranes under NaCl stress. The mechanism probably involves direct binding of the polyamines to the membranes, thereby preventing lipid peroxidation and proteolytic attack, or inhibition of ethylene synthesis through inhibition of 1-aminocyclopropane-1-carboxylic acid synthase (Fuhrer *et al.*, 1982; Drolet *et al.*, 1986; Winer and Apelbaum, 1986). In addition, it also appears that enhanced K^+ content (as affected by hydroxyproline) may also be responsible for less accumulation of putrescine under NaCl stress, as potassium ions have been shown to modulate the level of putrescine in plants (Suresh *et al.*, 1978; Reggiani *et al.*, 1993; Aurisano *et al.*, 1993). It also appears that the low levels of Na^+ and Cl^- ions (as compared to salt control) and higher levels of Ca^{2+} and Mg^{2+} in the

hydroxyproline treated cells subjected to NaCl stress may be due to the preservation of integrity of membranes by spermidine and spermine.

It is argued that the disruption of plant growth and development by salinity is mainly due to the hormonal imbalance (Wright, 1978; Levitt, 1980). Exogenous application of IAA could counteract the negative effects of salinization (Sarin. 1962; Darra and Saxena, 1973). It was observed that rice calli when subjected to NaCl stress registered an increase in their IAA oxidase activities. The salinized susceptible variety exhibited more IAA oxidase activity as compared to the salinized tolerant variety. The ability of BR callus to check its activity of IAA oxidase might be responsible for its better salt tolerance. Cells exposed to hydroxyproline, growing on NaCl and hydroxyproline containing medium exhibited a decrease in the IAA oxidase activity (as compared to corresponding salt control values at the end of the fourth week). It is well recognised that IAA oxidase regulates growth by limiting the concentration of IAA (Scott, 1984). An inverse relationship between endogenous IAA levels and IAA oxidase activity has been demonstrated by Jasrai *et al.* (1988). Thus it appears that hydroxyproline favourably affects the level of IAA (as evidenced by better growth) by lowering the activity of IAA oxidase enzyme.

Contrary to the IAA oxidase activity, the activity of amylase, invertase and cellulase was found to be decreased upon salinization. There was a reduction in the protein content of salt stressed calli. Hence considering the changes in the protein content, the decreased activity of the enzyme upon salinization could be due to the low synthesis of enzyme protein or its degradation. NaCl has been

reported to increase or decrease the production of some enzymes by either increasing or decreasing the rate of transcription or translation (Filho *et al.*, 1983; Ostrem *et al.*, 1987) as well as the turn over rates of enzymes (Cooke *et al.*, 1973; Karlekar *et al.*, 1985). It is now well established that during salt stress some proteins are produced which play an important role in the adaptation of cells to stress (Ericson and Alfinito, 1984; Singh *et al.*, 1985). Ramani and Apte (1994) have reported the denovo synthesis of a set of specific proteins called salt stress proteins (SSPs) in rice cultivars.

It is again suggested that the ionic disturbance and cell dehydration which is caused by NaCl may possibly be altering the conformation of enzyme protein either at the active site or changing the tertiary and quarternary structure of the protein, so as to make the enzyme in a more active or inactive form (Heuer and Plaut, 1982; Filho *et al.*, 1983; Kalir *et al.*, 1984). An alternate explanation for the reduced activity of these enzymes could be the reduced level of carbohydrates under salt stress (as observed by less growth). Thus the changes in the enzyme activity observed in the present study could be due to some or all of the above factors induced by salinity. It is also interesting to note that as compared to BR, the GR_{11} callus exhibited more pronounced reduction in their levels of amylases, invertases and cellulases. Thus it suffices that these enzymes may be playing an important role in salt tolerance.

An important observation in the present investigation is the modulation of enzyme activity by hydroxyproline under NaCl stress. An enhanced activity of amylase, cellulase and invertase (as compared to salt control) was discerned in

both rice cultivars (exposed to hydroxyproline) growing on medium containing both hydroxyproline and NaCl. Thus it appears that the relatively higher activity of amylase, cellulase and invertase may be responsible for better growth of hydroxyproline treated tissues under saline conditions.

There have been several reports on *in vitro* production of salt tolerant plants (discussed by Dix, 1993; Tal, 1993). In rice also, salt tolerant plants have been produced employing tissue culture techniques (Kavi Kishor and Reddy, 1985; Janardhan Reddy and Vaidyanath, 1986; Subhashini and Reddy, 1991; Vajrabhaya *et al.*, 1989).

In the present study, an attempt was made to regenerate hydroxyproline resistant calli of both rice cultivars. Callus cultures growing under various treatments were tried for regeneration. The regeneration medium employed was MS basal medium supplemented with BA (13.32 μM) and IBA (2.46 μM). The callus cultures gave rise to both roots and shoots when incubated on the regeneration medium (by end of 3-4 weeks). Thus it appears that a low ratio of auxin to cytokinin is necessary when both roots and shoots are being formed from the callus of rice. Similar observation has been made by Kavi Kishor(1989).

Incorporation of NaCl into the regeneration medium resulted in poor growth of the regenerants. However, it is interesting to note that callus cultures which were pretreated with hydroxyproline and then grown on regeneration medium containing NaCl showed comparatively better growth of the regenerated plantlets. A possible explanation for this seems to be that hydroxyproline

favours proline accumulation in stressed cells. It has been reported that proline enhances the formation of somatic embryoids in embryogenic callus of maize (Armstrong and Green, 1985) and alfalfa (Stuart and Strickland, 1984). Subhashini and Reddy (1991) have also observed an increase in frequency of plant regeneration from salt adapted callus growing on medium supplemented with 5 mM proline.

An alternate explanation for better growth of the regenerants under stressed conditions seems to be due to the increased levels of polyamines in hydroxyproline treated stressed cells. Increased polyamine biosynthesis has been shown to precede or accompany organogenesis and somatic embryogenesis in a number of plant cell cultures (Desai and Mehta, 1985; Fobert and Webb, 1988; Meijer and Simmonds, 1988). Torrigiani *et al.* (1987) have shown that polyamines are important in formation of floral buds from thin layer explants of *Nicotiana tabacum.* The promotive effect of exogenous polyamines on *de novo* shoot regeneration from cotyledons of *Brassica campestris* has been demonstrated by Chi *et al.* (1994).

Thus it appears that better growth of the regenerants under the influence of hydroxyproline may be due to either enhanced proline levels or increased polyamine titers or both.